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OPTIMIZATION AND APPLICATION OF METABOLOMIC ASSAYS FOR

ANALYZING DIET-INDUCED AND GUT MICROBIOTA-DERIVED SHORT-

CHAIN FATTY ACIDS IN MICE AND HUMANS

BY

ROBERT M. JUENEMANN

A thesis submitted in partial fulfillment of the requirement for the

Master of Science

Major in Nutrition, Exercise, and Food Science

Specialization in Nutrition

South Dakota State University

2016

OPTIMIZATION AND APPLICATION OF METABOLOMIC ASSAYS FOR

ANALYZING DIET-INDUCED AND GUT MICROBIOTA-DERIVED SHORT-

CHAIN FATTY ACIDS IN MICE AND HUMANS

This thesis is approved as a creditable and independent investigation by a

candidate for the Master of Science in Nutrition, Exercise, and Food Science and is

acceptable for meeting the thesis requirements for this degree. Acceptance of this does

not imply that the conclusions reached by the candidates are necessarily the conclusions

of the major department.

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CONTENTS

ABBREVIATIONS

AB: Antibody

BFC: Bioactive Food Componant

BF3-B: Boron trifluoride 1-butanol

BF3-M: Boron trifluoride 1-methanol

BL: Baseline

BMI: Body Mass Index

BP: Blood Pressure

CF: Control Flour

CO_2:   Carbon Dioxide

DI: Deionized Water

DNA: Deoxyribonucleicacid

DXA: Dual-Energy X-ray Absorptiometry

ELISA: Enzyme Linked Immunosorbant Assay

FDR: False Discovery Rate

GC-MS: Gas chromatography-mass spectrometry

GI: Gasterointestinal

HbA1C: Glycated Hemoglobin

HCl-B: Hydrogen chloride 1-butanol

HDL: High-density lipoprotein cholesterol

H_3PO_4:   Phosphoric Acid

IDF: International Diabetes Federation

IL-6:   Interleuken-6

IS: Internal Standard

LDL: Low-density lipoprotein cholesterol

MCFS: Medium-chain fatty acids

MetS: Metabolic Syndrome

miRNA: Micro Ribonucleicacid

NaOH: Sodium hydroxide

NGS: Next generation sequencing

NSB: Non-Specific Binding

OTU: Operational Taxonomic Unit

PCF: Propylchloroformate

PCR: Polymerase Chain Reaction

PPAR-γ: Peroxisome proliferator-activated receptor-gamma

QC: Quality Control

QIIME: Quantitative Insights Into Microbial Ecology

RIA: Radioimmunoassay

RNA: Ribonucleicacid

RS2:   Resistant starch type 2

RS4:   Resistant starch type-4

SCFA: Short-chain fatty acids

SDSU: South Dakota State University

S.E.M: Standard Error of Mean

STD: Standard

TC: Total cholesterol

TGC: Triglycerides

TNF-α: Tumor neucrosis factor-alpha

US: United States

WC: Waist Circumference

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ABSTRACT

OPTIMIZATION AND APPLICATION OF METABOLOMIC ASSAYS FOR
ANALYZING DIET-INDUCED AND GUT MICROBIOTA-DERIVED SHORT-
CHAIN FATTY ACIDS IN MICE AND HUMANS

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Introduction: In recent decades, the obesity epidemic worldwide has prompted the need for research targeting disease prevention, treatment, and maintenance. Dietary interventions are one of the primary methods to instill positive nutrition habits into one's lifestyle. Thus, resistant starch type 4 (RS4), a prebiotic dietary fiber, has been proposed to induce beneficial immunometabolic health outcomes. Currently there is a lack of knowledge on the health outcomes of RS4 in adults with metabolic syndrome (MetS). **Goal:** The goal of this research was to optimize a metabolomic assay to quantify fecal short chain fatty acids (SCFAs), a byproduct of microbial fermentation in the gut, and to apply this assay to health outcomes of RS4 intervention in an adult population with MetS as well as genetically induced obese mice. **Methods:** An assay was optimized to extract and derivatize fecal SCFA from human stool samples followed by quantification using gas chromatography – mass spectrometry (GC-MS). Retrospective analysis of fecal samples from adults including both men (n=4) and women (n=12) with signs of MetS, collected at four time points throughout an *ad libitum* dietary intervention of RS4[2], were processed and quantified. This method was also retrospectively applied to cecum samples

of KK.Cg-A^y/a, genetically induced obese mouse model, to quantify the effects of RS4 on cecum SCFA concentrations. 16S rRNA sequencing was performed to study the effect of RS4 on gut microbial composition. Blood biomarkers, glycemic, and lipid viariables, anthropometric measurements, and diet nutrient composition were also studied. **Results:** GC-MS analysis revealed significantly increased SCFAs following RS4 consumption including butyrate, propionate, valerate, isovallerate, and hexanoate. 16S-rRNA gene sequencing revealed a differential abundance of 71 bacterial operational taxonomic units, including the enrichment of three *Bacteroides* species and one each of *Parabacteroides*, *Oscillospira*, *Blautia*, *Ruminococcus, Eubacterium*, and *Christensenella* species in the RS4 group. RS4-specific associations were found between gut microbial composition and SCFA concentrations. Cholesterols, fasting glucose, glycosylated haemoglobin, and proinflammatory markers in the blood as well as waist circumference and % body fat were lower post intervention in the RS4 group compared with the control group. In KK.Cg-A^y/a mice, butyrate was significantly enriched in RS4 fed mice intestinal tissue. **Discussion:** An optimized method to quantify intestinal and fecal SCFA was created. The biological function of RS4 on gut microbiota in inidividuals with MetS was also identified. Larger studies are needed to fully understand the mechanistic action of RS4 in individuals with metabolic dysfunction for future implications on dietary guidelines.

CHAPTER 1. REVIEW OF LITERATURE

1.1   Nutrigenomics and Health

In recent years, technology has advanced allowing for the development of many different, but related -omics technologies including genetics and disease/condition risk - genomics, protein expression -proteomics, gene transcription -transcriptomics, and metabolism -metabolomics. These –omics technologies in general are a consortium of modalities used to investigate the roles, interations, and mechanisms of cells within an organism. The creation of these new –omics technologies has led to a dramatic increase in the amount of data available to study factors that influence disease susceptibility, occurrence, progression and cessation. Dating back to the Greek physician Hippocrates, the significant association between an individual's diet and their health has been cited. Bringing together past theories and current technological advancements, interest has arisen between bioactive food componants BFCs and its relationship with ones genes.

Nutrigenomics was first mentioned in 2001 as the new frontier in the science of nutrition[3,4]. The term Nutrigenetics was earlier described however, while similar, these two disciplines are not interchangeable. Nutrigenomics is the science of how bioactive food compounds effect gene expression through epigenetic modifications, miRNA and RNA modifications, changes in protein expression and metabolite changes[3,5]. Nutrigenetics is the study of genetic variation and dietary response in an individual or population[5-7]. The main goals of these sciences include both personalized and preventative nutrition[4]. The future of Nutrigenomics applied to real life has been an exciting endeavor however; significant ethical debate around its current application exists. A recent review by Pavlidis *et al.,* 2015 outlined the current status of

Nutrigenomics science and its future applications. Its applications on personalized

nutrition for a subset of responders vs. overall public health brings scrutiny to the overall

costs and rigor of the newly formed –omics sciences[4]. Bioactive food componants may

influence gene expression in a variety of mechanisms including via signal transduction

molecules, their metabolites, or directly[6]. It is widely known that individual's respond

differently to dietary consumption, hence the interest in nutrigenomics. Humans are

predisposed to many different health conditions including obesity, cancers and

cardiovascular disease based off not only their genetics but also their environment. With

that being said BFCs may have either a positive or a negative effect on an individual's

health. Moving forward, the complexity of this science requires a significant amount of

research to effectively be applied into today's dietetic and healthcare settings.

1.2   Metabolic Syndrome

The rise in obesity in recent decades has quickly become one of the largest

burdens on healthcare globally[8] as it may elevate the risk for development of more

serious conditions including diabetes mellitus, certain cancers and cardiovascular

disease[9], the leading cause of mortality in the US[10]. Obesity in adults can be defined as

having a BMI at or above 30. In children and adolescents', obesity is defined as a BMI at

or above the 95th percentile for their age range and gender. In 2011-2012 it was estimated

that more than one-third or 34.9% of adults were obese and two-thirds of adults were

overweight in the US[11], similar to rates in 2003-2004, however the prevalence of obesity

has been predicted to rise in coming years[12]. Thompson *et al., 2001* found that

overweight individuals (BMI 25-29) and obese individuals (BMI ≥30) had 37% and

105% higher annual prescription costs and 13% and 39% higher primary care costs compared to their health counter parts respectively[13].

Abdominal obesity, presenting comorbid with some of the most dangerous risk factors for developing a heart attack including hypertension, dyslipidemia and insulin resistance encompass an even more serious condition, metabolic syndrome. Globally it is estimated that 20-25% of the adult population has metabolic syndrome[14]. Between 2003-2012 in the United States it was estimated that 33% of the adult population and over 50% of the population of over 60 years of age had metabolic syndrome causing concern with the rapidly growing geriatric population in the US[15]. The International Diabetes Federation (IDF) defines metabolic syndrome as follows in Table 1-1.

Table 1-1: The IDF Definition of Metabolic Syndrome

Criteria	Male	Female
I. Criteria A		
Body Mass Index	>30 kg/m^2	>30 kg/m^2
Waist Circumference	>94 cm	>80 cm
II. Criteria B		
Elevated Triglycerides	≥150 mg/dL	≥150 mg/dL
Reduced HDL Cholesterol (HDL)	<40 mg/dL	<50 mg/dL
High Blood Pressure	Systolic > 130 mm Hg	Systolic > 130 mm Hg

	Diastolic >85 mm Hg	Diastolic >85 mm Hg
Elevated Fasting Plasma Glucose	>100 mg/dL	>100 mg/dL

Table 1-1: Criteria used to define MetS based off the (IDF)[14]. An individual is considered to have MetS if they present with one item from criteria A and any two from criteria B above.

Obesity and metabolic syndrome are caused by several environmental, genetic, dietary and lifestyle factors including but not limited to, sedentary lifestyle, higher energy intake than output, insulin resistance and certain genetic predispositions. Metabolic syndrome and its co-morbidities can be treated through lifestyle and/or dietary interventions as well as other therapeutics including pharmaceuticals. Despite availability of treatment and prevention options, the prevalence of MetS is rising worldwide at an alarming rate[10,14].

There are numerous treatment options for metabolic syndrome and its comorbidities including medications, dietary interventions/changes, behavioral modifications and exercise. A basic understanding of weight loss reflects the first law of thermodynamics which says energy can neither be created nor destroyed; energy intake may be neutral (expenditure=consumption), positive (expenditure<consumption), or negative (expenditure>consumption). The most obvious influence of energy consumption is food consumption, whereas there are three main categories of energy expenditure. These categories are: exercise, adaptive thermogenesis and resting metabolic rates[16]. The clearest and simplest way to increase energy expenditure is via increased exercise since the latter are both mechanisms of survival. Both weight loss medications and reducing energy intake are effective interventions in 5-9% weight loss, however weight typically

plateaus after six months[17]. A significant struggle with weight loss is long-term success and maintenance. Numerous publications identify initial success of weight loss is not predictive of long-term success[17,18]. Summerbell *et al.,* 2008 described low-energy and low-fat consumption to inverse excess energy intake are not suitable interventions for long-term weight loss[19].

1.3   Resistant Starch

In recent decades, growing evidence has been produced supporting the influence of diet on the etiology, prevention and treatment of many health conditions. Rapidly digested starchy foods such as white pastas, white bread and cakes have long been known to influence chronic diseases such as type-2 diabetes mellitus (T2DM) and obesity. Normal dietary starch begins its digestion in the mouth as it is broken down by the enzyme amylase where it travels further down into the digestive tract and is rapidly absorbed as glucose which may potentially result in a hyperglycemic environment in the blood. This hyperglycemic condition triggers the release of insulin which cascades into tissue-specific intracellular uptake of glucose ultimately leaving a hypoglycemic environment in the blood. This cascade of events may result in insulin resistance which further may contribute to T2DM and ultimately more serious conditions such as metabolic syndrome and cardiovascular disease.

To combat this rapid glycemic response growing evidence has supported the addition of resistant starches (RS) into the diet for disease prevention, maintenance and treatment[20,21]. Resistant starches are found both naturally in foods as well as mechanically engineered and are classified based on the characteristics that render them indigestible (Table 1-2)[22]. Resistant starch is capable of avoiding enzymatic digestion in

the mouth and gastral systems however it is susceptible to microbial fermentation thereby a slower, more modulated glucose response[20]. Also of interest, is the production of short chain fatty acids (SCFA) in the lower gastrointestinal intestinal system (GI) through fermentation.[21] The ability of a RS to be digested by the microbes of our gut renders them a prebiotic fiber. A prebiotic fiber is a non-digestible food component which selectively serves as a substrate for beneficial microbial species which undergoes microbial fermentation[23]. Resistant starch is a prebiotic friber as it selectively promotes growth of beneficial microbes in the gut; however, not all fibers are considered prebiotics, cohersely not all prebiotics are resistant starch. A probiotic on the other hand is the healthy, commensal bacteria that occupy our gut which through research have contributed to beneficial health outcomes[23].

Table 1-2: Types of resistant starch[20,22]

Designation	Description	Example	Reference
RSI	**Physically inaccessible starch**	**Coarsely ground or whole-kernel grains**	**Englyst *et al.*, 1992[22]**
RSII	**Granular starch with the B- or C- polymorph**	**High amylose maize starch, raw potato, raw banana**	**Englyst *et al.*, 1992[22]**
RSIII	**Retrograded Starch**	**Cooked and cooled starchy foods**	**Woo et al., 2002[24]**

| RSIV | Chemically modified starch | Crosslinked starch and octenyl succinate starch | Han, BeMiller, 2007[25] |
| RSV | Amyloid-lipid complex | Stearic acid-complexed high-amylose starch | Seneviratne, Biliaderis, 1991[26] |

1.4   Microbiome

The etiopathology of obesity and metabolic syndrome have been of interest worldwide due to their complexity and rapidly increased occurrence. More recently, the relationship between the microbiome and health has been of growing interest. The microbiome encompasses all of the microbes that occupy our body space. It is estimated that these microbes make up over ten times higher the number of cells in the body, with the majority residing in the large intestine. The gut microbiome plays a crucial role in nutritional sciences as it increases the metabolic potential of the human host thereby digesting food components we would not otherwise be able to.

The composition of the gut microbiome is influenced by many factors including age, diet, disease, medications and host genetics[27,28]. The gut microbiome is primarily made up of six bacterial phyla: Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia. Though complex, research has produced common trends associated with certain disease states including obesity, cancers and T2DM. Gut microbiome plays a crucial role in interacting with our mucosal immune system. The

interaction between the human host, gut microbiome and diet is complex as noted in Figure 1-1. It has been found that microbial fermentation may increase energy production from the diet, contribute to low-grade inflammation and influence fatty acid tissue composition[27]. Despite the rapid increase of publications regarding the gut microbiome and obesity, there is a significant variation in microbial composition from person to person however, trends suggest a healthy and more disease-like state. A significant number of studies describe an obese-like microbiome as having an increased Firmicutes: Bacteriodetes ratio[27,29,30].
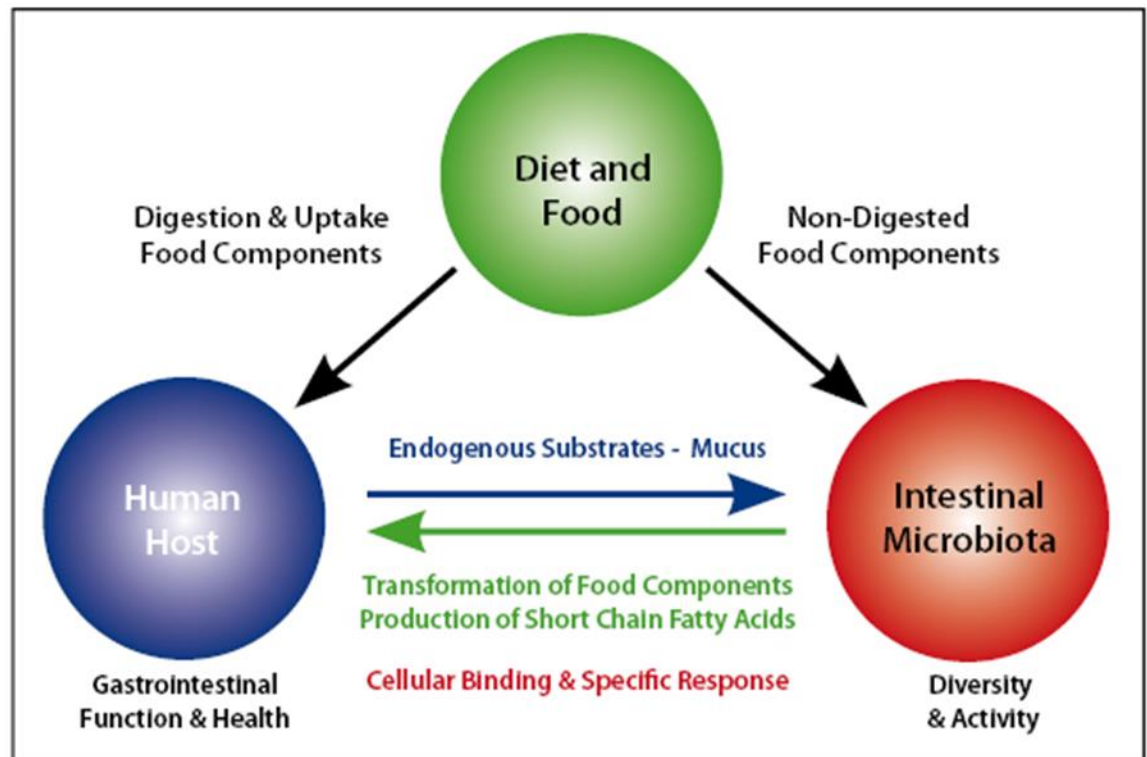

Figure 1-1: Relationship of the gut microbiome, diet, and human host[1].

The addition of RS to the diet has been shown to have beneficial influences on the gut microbiome and immunological functions in both mouse models and human interventions. RS in the diet has been shown to increase the abundance of beneficial microbes including *Bifidobacterium adolescentis, Ruminococcus bromi, Eubacterium rectale*, and *Parabacterium distasonis* as well as improving the Firmicutes: Bacteriodetes[30,31]. It is important to note that the results previously mentioned resulted from the consumption of different types of RS concluding that microbial growth and colonization are substrate dependent[32].

1.5   Hutterite Brethren Population of South Dakota

South Dakota is home to a large population of Hutterites, one of the oldest and most successful Anabaptist Brethren societies, the Hutterites. Their culture dates back to 1528 from South Tyrol, once part of Austria. Their population has endured significant hardship however, their population has expanded to over 40,000 individuals across Canada and the USA, primarily in the Midwestern states including North Dakota, South Dakota and Canada[33]. Their communal lifestyle has led to a genetically isolated population resulting in a founder effect resulting from the genetic flow from the original 89 community founders which can be associated to the Mendelian disorders present within this community[33,34]. The inclusion of the Hutterite population in genetic studies has increased significantly in recent decades positively impacting the ability to identify specific genes related to disorders that are unique to this population. Their inclusion as a study population provides a significant opportunity to avoid potential genetic factors due to their genetic isolation.

In the context of nutrition and gut microbiome research, the Hutterites restrict the potential environmental factors that may impact study results. Their communal lifestyle within their colonies involves centralized preparation and consumption of their three main meals. This communal dining limits the potential variation amongst their diet. A major cause of variation is seasonal availability of fruits and vegetables (fresh fruits and vegetables being available for consumption in summer and fall months, preserved, canned, or frozen fruits and vegetables in winter and spring months). This seasonal variability has lead to the microbial shifts across seasons including the abundance of particular taxa including Firmicutes and Bacteroidetes as well as overall microbial diversity[35].

1.6   Intervention Study

In order to further understand the effects of RS4, Nichenametla *et al.,* 2014 performed a double-blind, placebo-controlled, cluster crossover intervention in two Eastern South Dakota Hutterite colonies[2]. The two Hutterite colonies (n=86) were randomized to alternatively receive either the test flour or control flour (Table 1-3) in two consecutive 12-week treatment periods with an appropriate two-week washout period (control flour consumed) in between as shown in Figure 1-2. Preexisting colony flours were removed and replaced with test flours which were fed *ad libitum* in a domestic, free-living, communal environment. Participants in this study were ≥18 years old and provided written informed consent to participate. Exclusion criteria for this study include lactating or pregnant females, long-term antibiotic therapy, immune compromised, cancer and other chronic conditions inhibiting one's ability to provide informed consent and abide by study protocol.
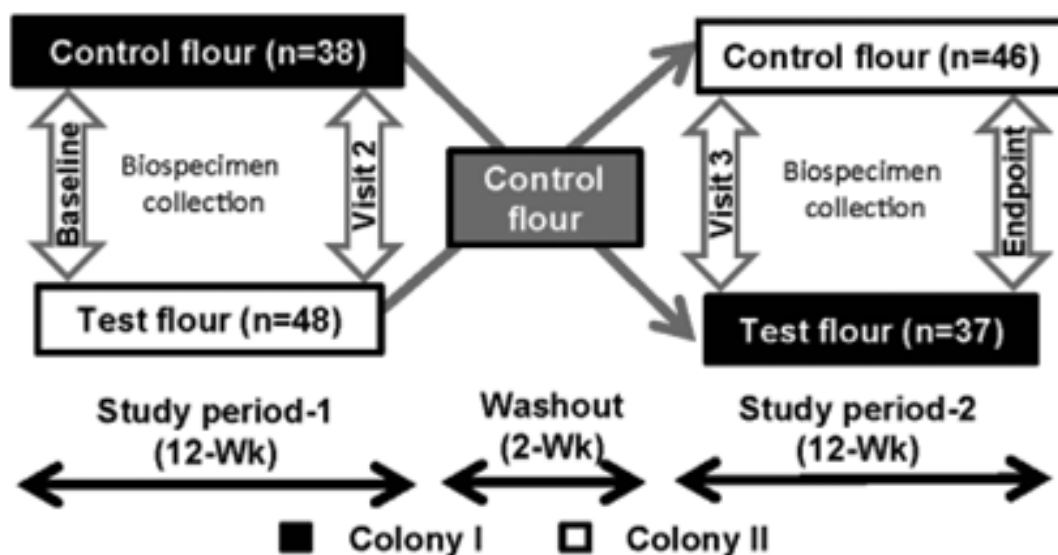
Figure 1-2: Crossover design timeline of 26 week long study period. Flours consumed in two 12 week treatment periods were randomized by colony following initial baseline (BL). The two treatments were divided by a two week washout period (WO) followed by consumption of the opposite treatment flour. Arrows depict time of data and specimen collection.

Table 1-3: Nutrient composition of treatment flours

Nutrient (g/100g)	Control Flour	RS4 Flour
Water	13.4	12.5
Protein	11.0	7.9
Carbohydrates	73.5	77.8
Total fat	1.7	1.3
Saturated fat	0.2	0.2
Monounsaturated fat	0.1	0.1

.

Polyunsaturated fat	0.7	0.6
Trans-fat	0.0	0.0
Fiber (RS4)	2.4 (0.0)	25.7 (24.0)
Sugars	0.3	0.2
Ash	0.5	0.6
Calcium (mg/100g)	24.0	50.4
Sodium (mg/100g)	2.0	91.4
Vitamin C (mg/100g)	0.0	0.4
Calories (kcal)	361.0	266.8

The results of this study show that RS4 consumption improved dyslipidemia by significantly decreasing mean total cholesterol, lower non-HDL cholesterol and HDL cholesterol in individuals with metabolic syndrome. In conclusion the aims of this thesis was to mechanistically explore the effects of RS4 on individuals with metabolic syndrome and the gut microbiome.

Initially collaboration was sought out within various departments at SDSU to investigate fecal SCFA however no optimized method was found. Protocols were found for volatile fatty acids but of larger molecular structure and concentration. Due to the low concentration of SCFAs anticipated in the potential samples, their high volatility, and low molecular weights, these GC-MS machines were incapable of reaching the lower detection limit desired. Outside collaboration was sought out to local laboratories.

Outside collaboration attempts found high cost, inadequate amount of sample, and low detection ranges. At this point the research need within SDSU for current and future studies to optimize a method for SCFA extraction and analysis via GC-MS was identified. Two professors within SDSU's Pharmacy and Chemistry with GC-MS capacity initially assisted with previously published protocol replication (outcomes of protocol replication in section 2.4). Unfortunately, retirement, machine use, and lack of laboratory assistance/support limited the success of these collaborations. Since recourses at SDSU were exhausted, Dr. Dey sought out collaboration with Dr. Ali Reza Fardin-Kia with this US Food and Drug Administration which ultimately lead to the successful protocol developement.

CHAPTER 2. OPTIMIZATION OF A METABOLOMIC ASSAY TO ANALYZE

SHORT CHAIN FATTY ACIDS IN HUMAN FECES

2.1   Introduction

Upon reaching the lower GI tract, prebiotic fibers such as RS, undergo microbial

fermentation which produces SCFAs. SCFAs of particular interest include acetate,

propionate, and butyrate which have numerous physiological impacts on the body such as

inducing satiety, hepatic lipogenesis, fat deposition, and thermogenesis[36,37]. Short-chain

fatty acid production directly correlates with the type of substrate in the diet and

microbial availability in the gut. The majority of SCFA produced are absorbed by the

intestinal epithelium and used in other metabolic processes or are used by other intestinal

microbiota. The study of the gut microbiota and fecal SCFA production is controversial

as only 5-10 % of SCFA produced are excreted in feces (the primary method of study)[38]

however this is the most practical method of SCFA study in clinical trials. It has been

estimated that SCFA production can account for 5-15% of daily human caloric intake[39].

The molar ratio of SCFA is estimated 60:20:20, acetate, propionate and butyrate

respectively[38,40].

Butyrate is of particular interest in research as it has been found to have many

effects on energy metabolism, inflammation, host immunity and cancer. There are two

proposed mechanisms of butyrate production 1) Butyrate kinase and 2) Butyryl-

CoA:acetate-CoA transferase[41]. Commonly cited butyrogenic bacteria include:

Eubacterium rectale, Eubacterium ramulus, Roseburia cecicola and members of

Clostridium cluster IV[42]. It is well known that butyrate is the main energy source for

colonocytes once absorbed. Inflammation is commonly associated with both obesity and

cancer. Butyrate plays a crucial role in inflammatory response in the gut by inhibiting pro-inflammatory markers such as IL-6 and inhibiting NF-kBactivation as well as upregulating PPARγ expression[43,44].

Propionate and acetate play fundamental roles with the liver where they both may be metabolized, acetate forming acetyl-CoA and contribute to gluconeogenesis. Propionate has been shown to increase HDL cholesterol production in humans as well as reduce blood cholesterol in animal models[45]. Acetate and propionate have also been shown to play a role in adipocyte formation through interaction with G-protein coupled receptors and also influence adipokine release which may induce satiety[46]. Acetate has been noted to induce satiety signaling through hypothalamic reaction after administration[47].

Relating this to obesity and metabolic syndrome, the addition of RS to the diet reduces the energy density by which intestinal gluconeogenesis is initiated and satiety is induced through activation of these SCFA[48]. The complex relationship between the gut microbiome, SCFA and obesity has yet to be fully understood. In today's western diet the amount of fiber in the diet is limited, drawing concern over health outcomes/trends, especially the obesity epidemic. On average in the US the daily fiber intake is 16 grams for individuals 2 and older[49]. The dietary reference intake for total fiber for adult male and females is between 21-38 grams per day[50]. The addition of fiber into the diet has been of growing interest for health organizations in recent decades due to the increasing evidence of health promotion via SCFA production. While there is no recommended dietary reference intake values for RS consumption in the US, it is estimated that the average daily intake is 3-8 grams per day in the US[51].

The increased biological relevance of these compounds has increased the need to develop rapid and accurate detection platforms. Detecting SCFA in fecal samples is particularly difficult due to the complex matrices into which they reside[52]. There are several pretreatment protocols available in which the SCFAs are extracted using an aqueous or organic solvent and often times followed by a derivatization reaction to increase the volatility and compatibility with the GC-MS analysis. In section 2.4 two methods are described which were attempted one with a simple extraction and one with an additional derivatization reaction.

The objectives of this project were 1) to develop and optimize a protocol to analyze SCFA in human fecal samples and 2) to investigate the impact of RS4 on fecal short chain fatty acid (SCFA) concentrations in adults with metabolic syndrome.

2.2   Materials and Methods

2.2.1   Participants

Participants in this study included a subset of the parent cohort (section 1.6) consisting of 20 individuals who originally participated in a dietary intervention with RS4[2]. The parent trial is registered in clinicaltrials.gov (NCT01887964). From 40 participants who had signs of metabolic syndrome at baseline, 26 participants submitted stool samples at all four data collection time points. Out of 26, 20 participants (10 from each of the two colonies) were included in the current investigation as their fecal samples were adequate to carry out both sequencing and short chain fatty acid (SCFA) analyses. Exclusion criteria included pregnancy, lactation, long-term antibiotic therapy, immune compromised state, cancer, and other conditions that would affect the ability to provide informed consent or comply with the protocol.

2.2.2 Sample preparation.

Performed at South Dakota State University, 1.5mg/mL internal standard (IS, 2-ethylbutyric acid, Thermo Fisher Scientific, Waltham, MA) was prepared in 1-butanol (Thermo Fisher Scientific, Waltham, MA). Exact weight of IS was recorded. 800-1000mg ($\pm$0.1mg) fecal sample was homogenized and added to a 30mL glass tube with a Teflon cap (Thermo Fisher Scientific, Waltham, MA) containing 1mL IS. 500µL hexane (Thermo Fisher Scientific, Waltham, MA) and 2mL of the appropriate catalyst were then added with one minute of vortexing between each addition. Boron trifluoride 1-butanol (BF3-B, Sigma Aldrich, St. Louis, Missouri) and hydrogen chloride 1-butanol (HCl-B, Sigma Aldrich, St. Louis, Missouri) were used to create butyl- esters for SCFA detection. Boron trifluoride 1-methanol (BF3-M) was used to create methyl- esters for MCFA detection. Samples were then sonicated for five minutes at 40 KHz, purged with nitrogen or helium gas (Matheson, Sioux Falls, SD), and placed in a water bath ($90\text{-}100^{oC}$) for 20 minutes. Once cooled to room temperature, 15mL of water and an additional 1.5mL of hexane were added to the samples and centrifuged at $3{,}000\text{g*min}^{-1}$ for two minutes. The organic layer (top) was then transferred into a sampling vial (Agilent Technologies, Wilmington, DE) with a final addition of ~100mg anhydrous sodium sulfate (Thermo Fisher Scientific, Waltham, MA). Fecal SCFA extracts were stored at $-20^{o}C$. A schematic view of the protocol is indicated in Figure 2-1.
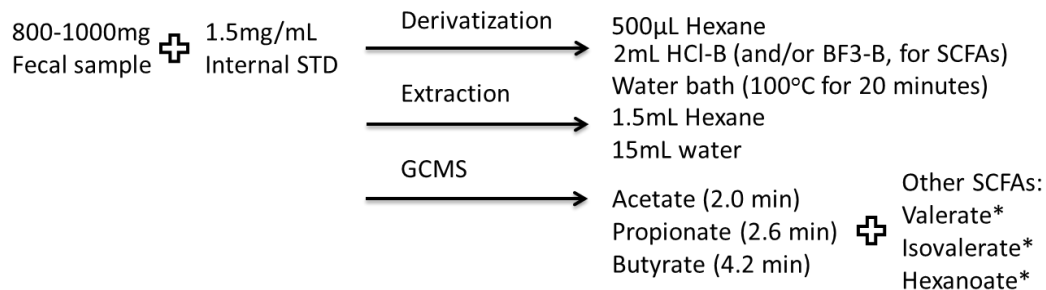
Figure 2-1: Optimization of a metabolomic assay to analyze SCFA in human feces.

2.2.3 Gas chromatography- mass spectrometry analysis.

Performed at the US Food and Drug Administration, (College Park, MD) fatty acid analysis was executed using GC-MS 5977A (Agilent Technologies, Wilmington, DE). Analytes were run on a HP-5MS UI capillary column (30 m x 0.25mm, 0.2 μm thickness, Agilent, Wilmington, DE). Sample volume of 1μL was injected in the split mode (1:10). The carrier gas in this system was hydrogen with a constant flow rate of 2mL/min. Oven temperature ramp was as follows: $55\,^{oC}$ for 4 minutes, then to $120\,^{oC}$ at 5 ^{oC}/min, then to $220\,^{oC}$ at $20\,^{oC}$/min for 10 minutes. Selective mass detector was utilized in the "single ion monitoring and scan" (SIM/Scan) mode with its source being maintained at $150\,^{oC}$ and the electron energy set to 70eV. A schematic view of the GC-MS protocol is shown in Figure 2-2.
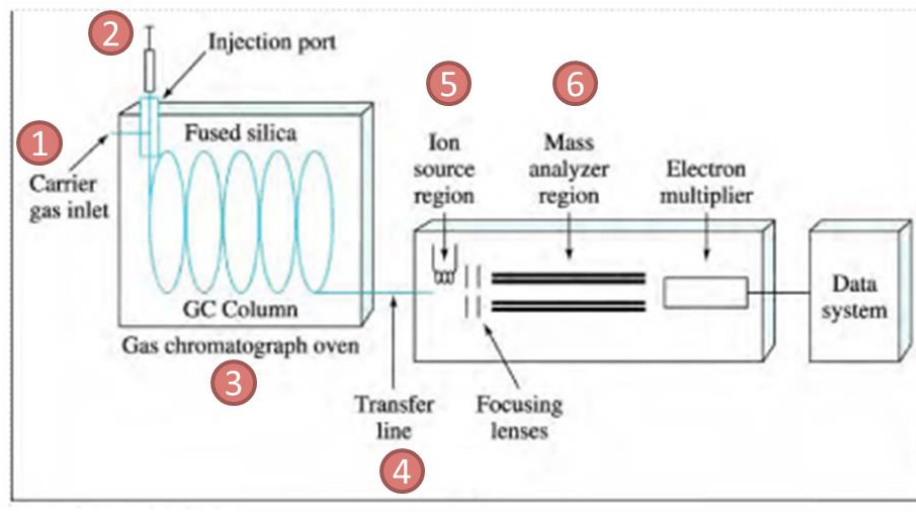
Figure 2-2: GC-MS method for SCFA analysis. 1. Hydrogen used as carrier gas at 1.9mL/min. 2. Injection volume 1uL in split mode (1:10). 3. Initial elution temperature was 55°C for 4 min, then 5°C/min to 120°C and then 20°C/min to 220°C for 10 min. 4. Mass detector operated in SIM/Scan mode. 5. Ion source 150°C. 6. Electron energy 70eV.

2.2.4   Statistical analysis

Descriptive statistics were performed on demographic information. Data was analyzed comparing end-points for outcome variables in CF and RS4 groups, or pre- and post-intervention measures. For pre- and post-intervention comparisons paired *t*-test (Wilcoxon signed-rank test for non-normal data) was used, while student's *t*-test (Mann-Whitney signed-rank test for non-normal data) was used to compare the two different diet groups. Where necessary, data was logarithmically transformed to achieve normality. A *p* value of 0.05 or less was considered significant, while *p* value of 0.05 to 0.09 was considered trend or approaching significance.

2.3   Results and discussion.

Descriptive characteristics of this study cohort are summarized in Table 2-1. Of the original cohort, at baseline, 40 individuals met the IDF definition of MetS[14]. 26 participants of these subjects submitted stool samples at all four visits. Out of these 26 individuals (ages 32-77), 20 had fecal microbial DNA at sufficient concentration for next generation sequencing (NGS). These 20 individuals were from two colonies, ten from each colony, two being men and eight being women. Medication for comorbid conditions and prophylaxis are reported in Table 2-1 below.

Table 2-1: Baseline characteristics of 20 participants.

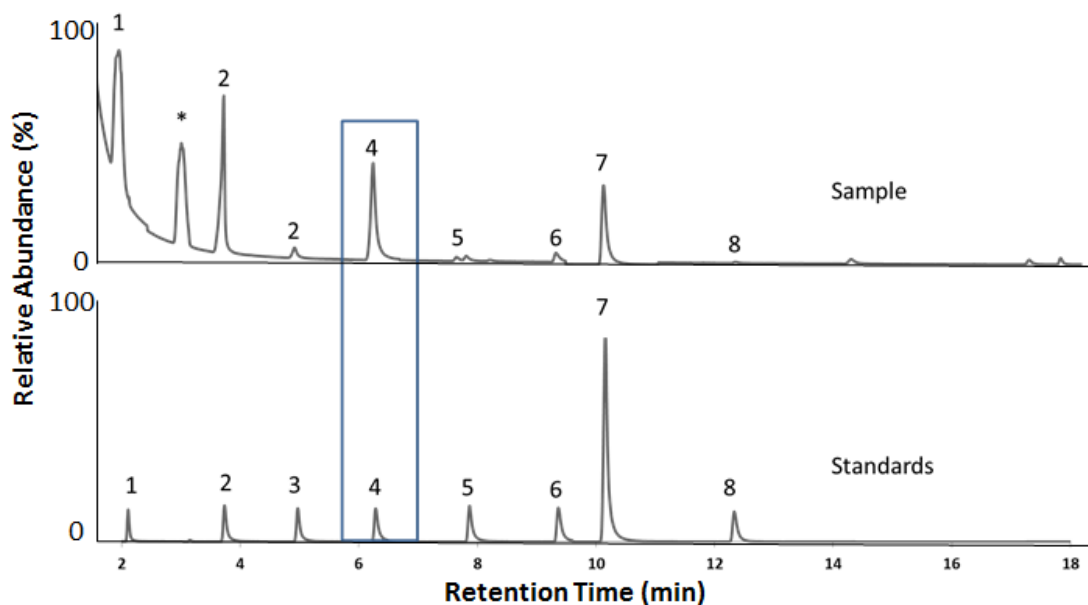| Criteria	n
Age (years)	
<50	5
≥50	15
BMI	
<30	5
≥30	15
Gender	
Male	8
Female	12
Medication for	
No medication	8
Type 2 diabetes	4
Heart diseases	2
Blood pressure	11
Fibre supplement	2
Probiotic supplement	1
Digestive support	3
Cholesterol lowering	5

n: number of individuals.[53]

Figure 2-3: Representative chromatogram showing the overlay of retention times of butyl esters of SCFA fragment from standard and biological sample. Butyl esters of acetic acid (1), propionic acid (2), iso-butyric acid (3), butyric acid (4), iso-valeric acid (5), valeric acid (6), internal standard (7), and hexanoic acid (8) represents the di-butyl ether as a side product of butylation that did not co-elute with the sample analytes.

A representative chromatogram of the SCFA butyl esters is shown in Figure 2-1. The total SCFAs were increased after RS4 intervention as expected (Figure 2-2). Acetate made up the majority of SCFA present at 60% of the total SCFAs before and after both CF and RS4 interventions (data unshown). Propionate (50.2%), butyrate (69.5%), valerate (44.1%), isovalerate (20.3) and hexanoate (19.2%) were significantly increased post RS4 intervention from baseline as expected. Interestingly a 25.6% decrease in isobutyrate was observed following RS4 diet.
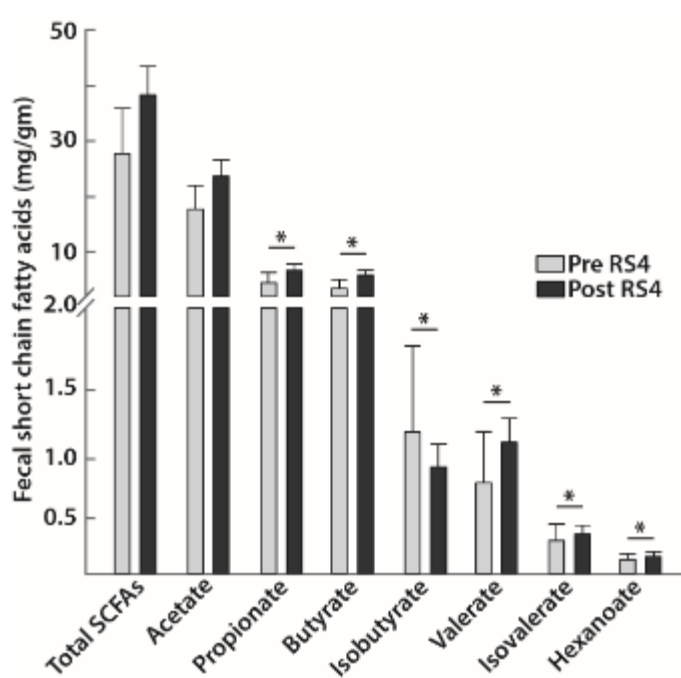
Figure 2-4: Effects of RS4 on fecal SCFAs abundance before and after RS4 intervention (* p ≤ 0.05,)[53]

2.4   Supplementary protocols attempted and Results.

Prior to optimizing the methods in section 2.2.2 and 2.2.3 we attempted to replicate two previously published methods to extract and analyze SCFA Garcia-Villalba *et al.,* 2012[52] and Zheng *et al.,* 2013[54]. The working protocol according to Zheng *et al.,* 2013[54] is briefly as follows: 50mg (25-150 is acceptable) was weighed in a 30mL test tube. 1,000μL of 0.005 M aqueous NaOH was added to the fecal sample and homogenized for ten minutes, sonicated for ten minutes, and finally centrifuged at 13,200rpm for 20 minutes. A 500μL aliquot of supernatant fecal water was transferred into a 10mL glass tube where10μL of internal standard (200ug/ml propionic2,2-D2), 250μL of water are added. Sample derivatization occurred by adding 500μL of propanol/pyridine (3:2), and 100μL of Propyl Chloroformate (PCF) to the 10 mL glass tube with prepared sample. The sample was then vortexed for ten seconds and sonicated

for one minute. 300μL of hexane was added into the glass tube, vortexed for one minute, let stand for ten minutes and centrifuged at 2,000rpm for five minutes. 200μL of the upper hexane layer was transferred to a vial as the first extraction. Another 200μL of hexane was added into the glass tube, vortexed for one minute, let stand for ten minutes and centrifuged at 2,000 rpm for five minutes. An additional 200μL of upper hexane layer was transferred to the vial with the first extraction in it followed by adding a small amount of anhydrous sodium sulfate for water absorption and vortexed for 15 seconds.

Standards of each SCFA were prepared and initially ran per protocol on the GC-MS which resulted in no visible peaks on the chromatogram. The heating scheme of the GC-MS analysis was then altered by gradually raising the temperatures however the temperature limit was eventually reached. The flow rate was increased and peak began to appear, however they exhibited "tailing" which exhibits a wide tail-end of the peak typically caused by a dirty column. This led to questioning the capabilities of the column that was used regarding polarity, solute, or solvent conflict however the DB-5ms (5% phenyl) methyl polysiloxane should behave the same as the column indicated in the protocol. The program was run several times with only hexane in an attempt to clean the potentially dirty column which eventually eluted cleaner peaks. Figure 2-3 shows an example chromatogram with acetic acid laid on top of the blank. There are a couple of early peaks (< 4.00 minutes) and then a group of peaks at approximately 18 minutes. Per protocol acetic acid should elute < 3.00 minutes concluding that the peaks present are false. Figure 2-4 shows propionic acid laid on top of the blank. The only peaks that differ between blank and sample are the two peaks shown at 18 min. Again, the reference peaks per protocol eluted < 5.00 minutes concluding that these peaks were also false. Figure 2-5

shows butyric acid laid upon the blank. The only two peaks that deviate from the blank appear very early, before the expected acetic acid and propionic acid which also conclude these peaks as false. Through further adjusting of the temperature scheme, peaks were identified for acetate, propionate, and butyrate standards all of which were at the same concentration by calculation however appeared different levels on the chromatogram, pipetting technique, multiple pipettes, and again column cleanliness were investigated. Peaks began to show at 2.00 minutes (acetate), 2.60 minutes (propionate), and 4.20 minutes (butyrate) however these peaks exhibited fronting so further concern grew regarding a more polar column, ramp speed and gas flow. The GC-MS was also changed from split to splitless mode which exhibited no change in chromatogram. Further adjustments with this system resulted in ultimately losing the peaks on the chromatogram.
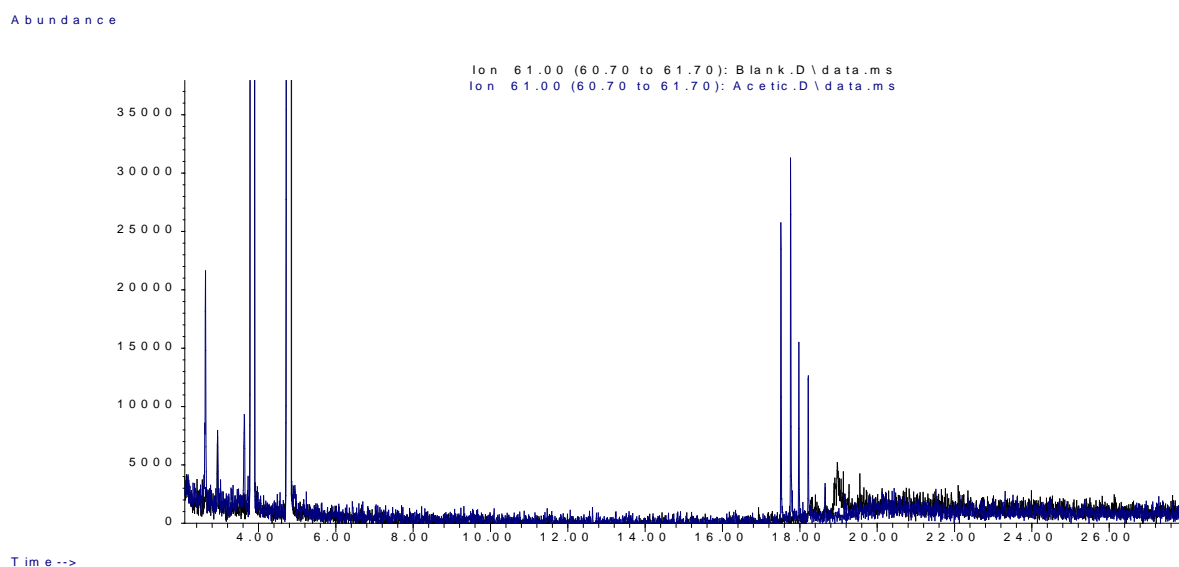

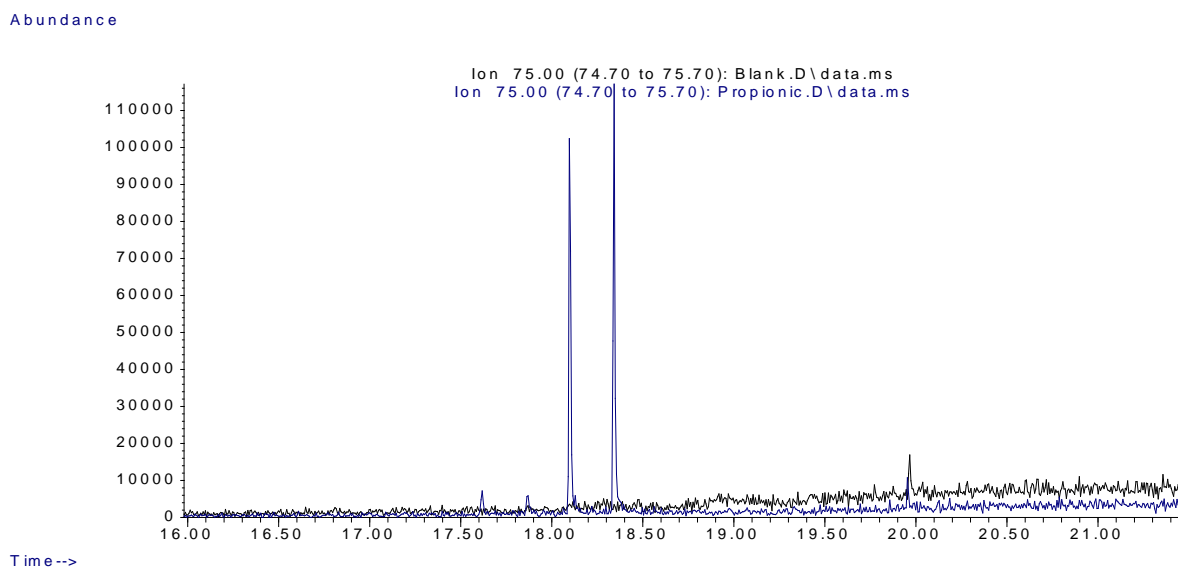**Figure 2-5: Acetic acid vs. blank chromatogram**

Abundance

Ion 75.00 (74.70 to 75.70): Blank.D\data.ms
Ion 75.00 (74.70 to 75.70): Propionic.D\data.ms

110000
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0

16.00   16.50   17.00   17.50   18.00   18.50   19.00   19.50   20.00   20.50   21.00

Time-->

Figure 2-6: Propionic acid vs. blank chromatogram

Abundance

Ion 71.00 (70.70 to 71.70): Blank.D\data.ms
Ion 71.00 (70.70 to 71.70): Butyric.D\data.ms

8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0

2.20   2.40   2.60   2.80   3.00   3.20   3.40   3.60   3.80   4.00   4.20   4.40   4.60   4.80   5.00

Time-->
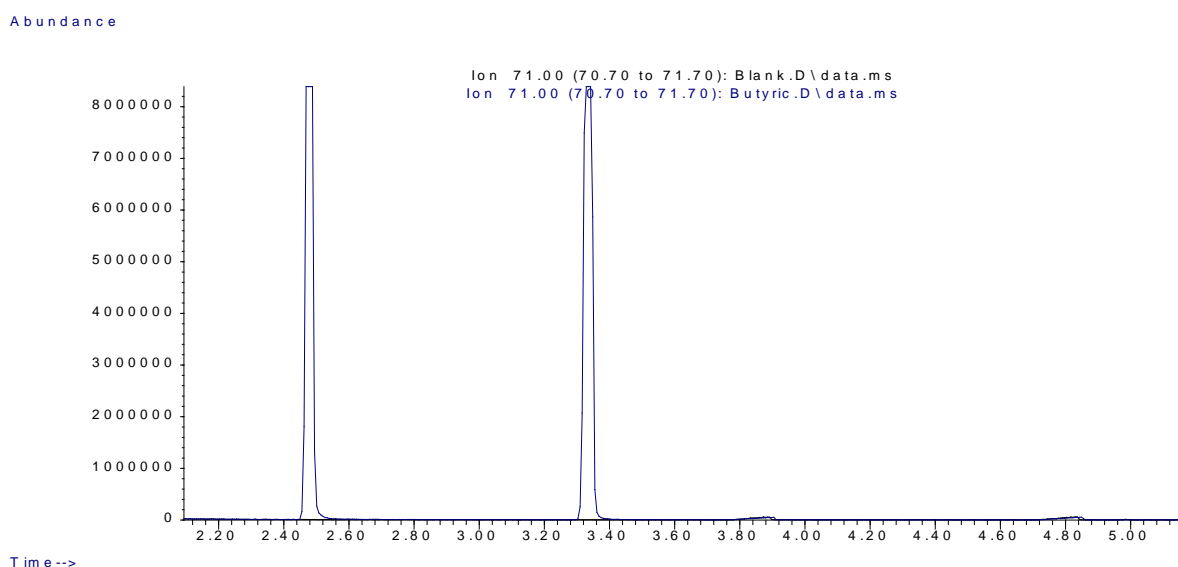
Figure 2-7: Butyric acid vs. blank chromatogram

At this point with concern over the success of the derivatization reaction so a
second protocol that was simply an extraction, void of any derivatization was attempted.
The working protocol used for Garcia-Villalba *et al.,* 2012[52] is as follows: fecal samples
were weighed to the nearest 100mg in a 30mL glass tube. The samples were suspended in
1mL of water with 0.5% phosphoric acid per 0.1g of sample. Samples were then vortexed
for two minutes and centrifuged at 17,949xg for ten minutes. The supernatant was then

pulled off and the SCFAs were extracted with 1mL ethyl acetate per 1mL of supernatant. Samples were then let stand for two minutes and vortexed followed by another centrifugation at 17,949xg for ten minutes. 600µL of supernatant was then aliquoted into a sampling vial where the internal standard propionic2,2-D2 was added at 500µM. The samples were stored at -20°C until use. Upon GC-MS analysis, no peaks eluted onto the chromatograph.

2.5   Limitations and Future Research.

The conclusion of this chapter resulted in an optimized method for fecal SCFA extraction, derivatization and GC-MS analysis. As expected we observed increased SCFA following RS diet[31,55]. The health consequences of butyrate and propionate, both of which were increased following RS4 consumption are noted above. Valerate is also of interest as it and propionate have been found to influence insulin-sensitivity in adipocytes[55]. Interestingly isovalerate (commonly associated with protein creation) was increased following RS4 consumption; this finding is unique as prior research found a decrease in isovalerate following RS consumption[56]. Further research is needed to understand the full biological effect of RS4 on SCFA production via microbial fermentation in the gut.

The optimization of this protocol leaves a resource for future investigators and elsewhere (we have demonstrated usefulness of this protocol for human subject research through our publication) to study fecal SCFA levels. The derivatization method in the protocol above produces –butyl esters which are of optimum size to study SCFA[57]. Of important note this method was also attempted using BF3-M which resulted in the formation –methyl esters which are ideal to analyze medium and long chain fatty acids.

Experimentation with BF3-M ran short due to lack of time. To move forward an optimal internal standard (IS) needs to be determined to quantify these fatty acids in samples. An optimal IS would fall into the same range on the chromatogram that MCFAs would elute. It is also important to investigate the expected fecal concentrations, elusion times, and machine capabilities.

A limitation to this method is that once analyzed via GC-MS, roughly a quarter of the duplicate samples had concentrations that were not closely related. When the samples are being homogenized it is difficult to ensure a homogenous slurry as the consistency and content of the sample is different throughout. Rotating the homogenizer in a circular motion broke down the frozen sample more rapidly than direct pressure to the sample.

CHAPTER 3. APPLICATIONS IN MATABOLOMICS RESEARCH

3.1   Introduction

The obesity epidemic over the past several decades has significantly impacted the

need for effective prevention, treatment and maintenance strategies for obesity and its

comorbidities. One mechanism to impact MetS is dietary intervention. In a previous

report, Nichenametla *et al.,* 2014 found that an RS4 diet lead to 7.2% lower mean

cholesterol (TC), 5.5% lower non-HDL cholesterol and a 12.8% reduction in HDL in

individuals with MetS[52]. Many studies report the benefit of incorporating fiber and other

nutrient dense food items to the diet to combat MetS[21,58].

Diet has been found to variably impact the composition of the microbiome in both

animal models and human dietary interventions. Physiological response and level of

change varied by the individual however are attributable to type of RS consumption[32].

Response of the microbiome is also substrate specific. RS4 consumption increased

Bacteroidetes, Actinobacteria, *Bifidobacterium adolescentis,* and, *Parabacterium*

distasonis while decreasing Firmicutes in healthy individuals, whereas RS2 increased

Ruminococcus bromii and *Eubacterium rectale*[32]. This study raises the question if RS4

will improve the Firmicutes:Bacteroidetes ratio in individuals with MetS as it did in

healthy individuals. In healthy adults RS2 was also found to increase *Bifidobacterium*

adolescentis and *Ruminococcus bromii* which supports the findings above[31].

Obesity and MetS are both associated with inflammatory conditions throughout

the body. IL-6 and TNF-α are proinflammatory cytokines secreted by adipose tissue that

are commonly linked to obesity and insulin resistance[59,60]. RS2 consumption in

individuals with T2DM was found to significantly decrease TNF-α but had no effect on

systemic IL-6 or adiponectin[61]. Gargari *et al.,* 2015 found RS2 consumption significantly

decreased TNF-α, HbA1C and triglycerides while also increasing HDL-cholesterol[62].

Adoponectin is a protein derived from adipocytes that is negatively associated with waist

circumference and MetS[63]. In diabetic rats RS consumption increased the concentration

of circulating adiponectin[64]. In mice, cecum samples are commonly used to investigate

SCFA production as this is where SCFAs are most abundant. Mice research gives

investigators a base for human intervention studies.

The objective of this project were to investigate the immunometabolic effects of

RS4 and apply the method from Chapter 2 to metabolomics research. The specific aims

of this project were: 1) to investigate how RS4 modulates the gut microbiome in

individuals with MetS; 2) to determine if RS4 is altering lipid, glucose and

anthropometric measurements in individuals with MetS; 3) to investigate if there is an

association between changes in gut microbiome and lipid, glucose, anthropometrics and

fecal SCFA levels post-RS4 intervention; and, 4) to investigate the effect of RS4 diet on

cecum concentrations of SCFAs in mice.

3.2   Materials and Methods

The methods listed directly coincide with our already published data by Liu *et al.,* 2016[65]

(3.2.1) and Nichenametla *et al.,* 2014[2] and Upadhyaya *et al.,* 2016[53] (3.2.2-3.2.7).

3.2.1   Mice and Participants

Mice data used in this study were a part of another study[65] whose objective was to

investigate the effects of RS4 in a genetically induced obese model of mice, KK.Cg-A^y/a

(Jackson Laboratories, Bar Harbor, ME). In brief, Six-week old mice were randomly

grouped for feeding with RS4 and standard (control) diets. During the first three weeks,

mice were allowed to acclimatize and fed standard rodent chow, LabDiet® 5001 (Purina,

Saint Louis, MO). Animals were then switched to experimental diets formulated based on

AIN 93 either with (23.5% - RS4 diet) or without (0% - regular/control diet) RS4.

Animals were fed experimental diets for 13 weeks followed by termination (CO_2

asphyxiation).

For human data, participants are the same cohort as indicated in section 2.2.1.

3.2.2   Fecal Genomic DNA extraction

Genomic stool DNA was extracted from human fecal samples using the QIAamp

DNA Stool Mini Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's

instructions. Initially, 0.3-0.5g of each fecal sample was weighed into an appropriately

labeled 15mL tube. ASL buffer was then added to the specimen at 10mL/g feces

followed by sufficient vortexing to achieve homogenous distribution of the sample. The

sample was then placed in a water bath at $95^{o}C$ for three minutes and subsequently

vortexed. Next, the samples were centrifuged for five minutes at 1500xg followed

removing a 1.6mL aliquot into a new 2mL tube. The samples were then heated at $95^{o}C$

for five minutes, vortexed for 15 seconds, and subsequently centrifuged for one minute at

20,000xg. 1.2mL of supernatant was then transferred into a new 2mL tube containing

InhibitEX and vortexed immediately until complete suspension of the tablet. The

suspension was then centrifuged down for three minutes at 20,000xg. All supernatant was

removed and again centrifuged down for three minutes at 20,000xg. 200µL of

supernatant was transferred to a new 2mL tube following the addition of 15µL of

proteinase K and 200μL of AL buffer. Samples were vortexed for 15 seconds and incubated at 70°C for ten minutes followed by an additional 2,000xg centrifugation for one minute. 200μL of absolute ethanol was then added, vortexed, and centrifuged for one minute at 2,000xg. The lysate was then transferred into a new spin column and centrifuged at 20,000xg for one minute. The spin column was then transferred into a new collection tube and 500μL of AW1 buffer was added. The sample was then centrifuged at 20,000xg for one minute. The transfer column was again transferred to a new collection tube, 500μL of AW2 buffer was added, followed by a three minute centrifugation at 20,000xg. The column was then transferred into a new 1.5mL tube where 200μL of AE buffer was added onto the column membrane. The sample was then incubated for three minutes at room temperature and subsequently centrifuged for one minute at 15,000xg. The concluding DNA sample was then kept on ice and quantified using the NanoDrop system (NanoDrop Technologies, Wilmington, DE) and/or the quantified via the Qubit® Quant-iT dsDNA Broad-Range Kit (Invitrogen, Life Technologies, Grand Island, NY).

3.2.3 Gut microbiome and community structure analysis

Sample DNAs were sent to Second Genome (South San Francisco, CA) for metagenome sequencing and operational taxonomic unit (OTU) identification. Briefly, samples were enriched for bacterial 16S V4 rDNA region, DNA was amplified utilizing fusion primer designed against the Illumina (San Diego, CA) flow cell adapters and indexing barcodes. Each sample was PCR-amplified with two differently bar coded V4 fusion primers. For each sample, amplified products were concentrated using a solid-phase reversible immobilization method for the purification of PCR products and quantified by electrophoresis using an Agilent 2100 Bioanalyzer® (Agilent

Technologies, Santa Clara, CA). Sequencing was carried out using Illumina MiSeq platform following standard protocols for 250 cycles with custom primers designed for paired-end sequencing. Using Quantitative Insights Into Microbial Ecology or QIIME 5 and generated custom scripts (Second Genome), sequences were quality-checked and demultiplexed to determine exact matches to the supplied DNA barcodes. Resulting sequences were then searched against the Greengenes reference database of 16S rRNA gene sequences, clustered at 97% by uclust (closed reference OTU picking). The longest sequence from each OTU was then assigned taxonomic classification via Mothur's Bayesian classifier, trained against the Greengenes database clustered at 98%. For unidentified Greengenes OTUs, we cross referenced with closest hits from NCBI 16S rRNA database with query cover (>90%), identity (>87%), and E value (<0.01).

3.2.4   Anthropometric measurements (Data previously collected and retrospectively analyzed here)

Height and waist circumference were obtained, to the nearest 0.5cm by stadiometer and Gulick tape respectively. Weight was determined by electronic scale (Seca Gmbh & Co., Hamburg Germany) to the nearest 0.1kg. Dual-energy x-ray absorptiometry (DXA) (Hologic QDR Discovery A, Waltham, MA) was used to analyze body composition at visit 1, 2 and visit 4. Digital sphygmomanometers were used to measure blood pressure.

3.2.5   Blood glycemic and lipid variables (Data previously collected and retrospectively analyzed here)

Overnight fasting blood was collected at each visit (Figure 2-1) by venipuncture.

10mL of peripheral blood was collected in heparin-coated Vacutainer tubes (BD

Biosciences, Franklin Lakes, NJ). Immediately following collection 40µL of blood was

analyzed using the Cholostech LDX point-of-care analyzer (Alere Inc, Waltham, MA)

with lipid profile and glucose cassettes (Lipid Profile GLU, Alere Inc, Waltham, MA)

according to the manufacturer's instructions to analyze blood glucose, total cholesterol

(TC), low-density lipoprotein cholesterol (LDL), high0density lipoprotein cholesterol

(HDL), non-HDL cholesterol (non-HDL), triglycerides (TGC) and TC/HDL levels.

Postprandial glucose was analyzed two hours after breakfast or lunch using a FreeStyle

Freedom Lite blood glucose meter (Abbott Diabetes Care Inc, Alameda, CA) according

to the manufacturer's instructions. Hemoglobin A1C (HbA1C) was determined in

duplicate using the Human HbA1C kit (Crystal Chem, Downers Grove, IL) according to

the manufacturer's instructions and expressed as percentage of total Hb.

3.2.6 Blood biomarkers

Interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) were determined in

serum using Human Il-6 ELISA Ready-SET-Go!® (Catalog Number: 88-7066) and

Human TNF alpha ELISA Ready-SET-Go!® (Catalog Number: 88-7346) kits

respectively, according to the manufacturer's instructions (eBioscience, San Diego, CA).

Initially, each well of the Corning Costar 9018 ELISA plate was coated with 100µL of

capture antibody in 1X Coating Buffer, followed by an overnight incubation at 4°C.

Using 250µL/well Wash Buffer and a multichannel pipette, wells were aspirated and

washed three times following a one-minute soaking to increase washing efficiency. Wells

were then blocked with 200 µL of 1X ELISA/ELISPOT Diluent and incubated for one

hour at room temperature followed by another aspiration and washing. Lyophilized standards were reconstituted with deionized water (DI) and allowed to set for 15 minutes with gentle agitation. 1X ELISA/ELISPOT Diluent was used to dilute the reconstituted standards from 1.5625 to 200pg/mL and 3.90325 to 500pg/mL and IL-6 and TNFα respectively; 100µL of this solution was also used to serve as blanks. 100 µL of each serum sample and standard was added to its appropriate well in duplicate. The plates were then sealed and allowed to incubate at room temperature for two hours. The wells were subsequently aspirated and washed five times following the previously mentioned protocol. 100µL of detection antibody diluted in 1X ELISA/ELISPOT Diluent was added to each well and then incubated for an hour at room temperature. As previously stated plates were then aspirated and washed five times. 100µL of Avidin-HRP was added to each well and incubated at room temperature for thirty minutes. Aspiration and washing of the plate then followed as previously stated five times. 100µL of TMB solution was then added to each well and incubated at room temperature for fifteen minutes. Stop solution (1M H_3PO_4) was then added at 50µL to each well and read at 450nm and 570nm on the plate reader.

Plasma adiponectin levels were detected by Human Adiponectin radioimmunoassay (RIA) according to the manufacturer's instructions (Linco Research, St. Charles, MO) in the Swine Reproductive Physiology Laboratory at South Dakota State University. Dr. Jeffrey Clapper conducted portions of this experiment where radioactive materials were used. This protocol was performed twice; first to optimize the assay and secondly to run the unknowns. This RIA was performed across two days: day one for initial set-up and day two for running the assay.

Optimization: Initially tubes were labeled and organized per sample including the serial dilutions of the human adiponectin standard (STD). The STD was diluted from 200-0.78 ng/mL. Next, 300μL of Assay Buffer was added to tubes 3-4 representing the Non-Specific Binding (NSB) tubes, 200μL was added to tubes 5-6 representing the reference tubes or 0ng/mL, 175μL to tubes 25-27, 150μL to tubes 28-30, and 100μL was added to the remaining tubes in the assay. Next, 100μL STDs were added in tubes 7-24 from lowest to highest, in duplicate. Quality Controls (QCs) were added to tubes 34-39 representing QC Low 3.125, DC Low 25, and QCLow 200 in duplicate respectively. Next, 25 µL of plasma sample "SNA 4-9-13" of test sample was added to tubes 25-27, 50μL to tubes 28-30, and 100μL to tubes 31-33. Next, 100μL of $_{125}$I-Human Adiponectin was added to all tubes followed by the addition of 100μL of Human Adiponectin antibody (AB) to all tubes except for tubes 1-4. All tubes were then vortexed, covered, and stored overnight at 4°C. The next day, 10μL of Rabbit Carrier was added to all tubes except tubes 1-2 followed by the addition of 1mL of cold Precipitating reagent (2nd AB). All tubes were then vortexed and incubated for 20 minutes at 4°C followed by a 20 minute centrifugation at 2,000 xg also at 4°C. Immediately following, the supernate was decanted into a waste jug and patted onto absorbent paper. Tubes were then counted on the gamma counter, one minute per set of tubes.

Unknowns: Once optimized, the experiment was then repeated using the same protocol as previously stated for the unknown samples with the following modifications. Initially tubes were labeled and organized per sample including the serial dilutions of the human adiponectin standard (STD). The STD was diluted from 200-0.78 ng/mL. Next, 300μL of Assay Buffer was added to tubes 3-4 representing the Non-Specific Binding (NSB) tubes,

200μL was added to tubes 5-6 representing the reference tubes or 0ng/mL, and 100μL was added to the remaining tubes in the assay. Next, 100μL STDs were added in tubes 7-24 from lowest to highest, in duplicate. Quality Controls (QCs) were added to tubes 25-30 representing QC Low 3.125, DC Low 25, and QCLow 200 in duplicate respectively. Next, 100μL of each unknown plasma sample was added to their respective tubes in duplicate. Then 100μL of 125 I-Human Adiponectin was added to all tubes followed by the addition of 100μL of Human Adiponectin antibody (AB) to all tubes except for tubes 1-4. All tubes were then vortexed, covered, and stored overnight at 4^oC. The next day, 10μL of Rabbit Carrier was added to all tubes except tubes 1-2 followed by the addition of 1mL of cold Precipitating reagent (2nd AB). All tubes were then vortexed and incubated for 20 minutes at 4^oC followed by a 20 minute centrifugation at 2,000 xg also at 4^oC. Immediately following the supernate was decanted into a waste jug and patted onto absorbent paper. Tubes were then counted on the gamma counter, one minute per set of tubes. Samples were then rerun if the values obtained for each duplicate didn't match within 10% of each other. RIA sensitivity for this assay was 1085 ng/mL. Inter- and Intra-assay coefficients of variance were 5.0% and 11.7% respectively.

3.2.7 Dietary assessment (Data previously collected and retrospectively analyzed here)

A previously validated 3-day food-frequency questionnaire (Appendix 1) based on common items in the Hutterite diet was used to assess dietary intake over two weekdays and one weekend day for each timepoint. Nutritionist Pro^{TM} (Axxya, Woodinville, WA) was used to for diet analysis and nutrient break down of all items listed on the food-frequency questionnaires.

3.2.8   Mouse cecum sample SCFA analysis

Previously collected cecal tissues, from mice fed an RS4 diet, which were snap-frozen in liquid nitrogen immediately after sacrifice and stored at -80 ^{0}C. For each pooled sample, three cecal tissues were pooled together (>500mg in total weight) and mixed with 1mL of internal standard (2-ethylbutyric acid in 1-butanol, 0.25 mg/ml). Samples were mixed by one minute vortexing followed by the addition of 0.5mL of hexane and 2mL of HCl-B. Each sample was sonicated for five minutes before purging with an inert helium gas and immediately sealed. Each sealed container was incubated in a water bath at 60 ^{0}C overnight in order to catalyze the derivatization of SCFA analytes. Upon returning to room temperature, 1.5mL of additional hexane and 15mL of deionized water were added to each sample, vortexed for 1 min each and then centrifuged at 3000xg for two minutes. The top organic layer (~2mL) was transferred into a 5mL graduated vial before blowing down with helium to one-fourth of the volume, increasing the final concentration of internal standard from 0.25mg/ml to 1mg/ml. Finally, each sample was transferred into 150μL insert inside the sampling vial and ~100mg of anhydrous sodium sulfate was added prior to being frozen at -20°C. GC-MS analysis was carried according to the protocol above in section 2.2.3.

3.2.9   Statistical analysis

Data was analyzed comparing end-points for outcome variables in CF and RS4 groups, or pre- and post-intervention measures. For mouse SCFA cecum samples intergroup comparisons were performed using Student's t-test. Linear mixed effects models (SAS MIXED procedure) were used to compare the effects of RS4 and CF on physiologic parameters. All models included variables for colony and season, where

colony was a surrogate for randomization sequence and season was a surrogate for

crossover treatment period. General linear mixed models were also used to compare the

effects of RS4 and CF on microbial abundance using R software package[66]. To correct

for multiple comparisons, a false discovery rate (FDR or Benjamini Hochberg method)

correction was used to adjust *p* values (adjusted *p* is represented as *q*). For pre- and post-

intervention comparisons paired *t*-test (Wilcoxon signed-rank test for non-normal data)

was used, while student's *t*-test (Mann-Whitney signed-rank test for non-normal data)

was used to compare two different diet groups. Where necessary, data was

logarithmically transformed to achieve normality. Intra-relationships among parameters

or bacterial species and inter-relationships between parameters and microbiota were

carried out using Pearson's linear correlation coefficient (r). Correlation matrices and

heat maps were generated using various R-packages. The data were presented as means ±

S.E.M, unless otherwise noted. A *p* value of 0.05 or less was considered significant,

while *p* value of 0.05 to 0.09 was considered trend or approaching significance.

3.3 Results

RS4 was found to improve body composition, dyslipidemia, as well as glucose

metabolism in this cohort with MetS. Percent body fat, TC, HDL, and nonHDL were

significant lower after RS4 consumption compared to CF (Table 3-1). A trend towards

lower waist circumference, fasting glucose, glycated hemoglobin, LDL cholesterol, and

TNF-α was also observed following RS4 diet compared to CF. Adiponectin was

significantly followed the same trend as previously mentioned however it was

significantly increased between baseline and RS4 consumption. Waist circumference,

TC, HDL, nonHDL, and IL-6 were significantly reduced from baseline to post RS4, while LDL trended towards significance.

Table 3-1. Means of biological parameters at baseline and at the end of intervention periods[a53]

	BL	Post CF	Post RS4	*p:* Post CF vs Post RS4	*p:* BL vs post RS4
Anthropometrics					
Weight (kg)	90.9±3.4	91.0±0.4	91.6±0.4	NS	NS
BMI (kg/m^2)	32.8±1.1	32.8±0.1	32.7±0.1	NS	NS
Waist (cm)	109.0±2.8	108.8±0.9	106.6±0.9	**0.06**	**0.02**
Systolic BP (mm Hg)	135.0±3.9	134.6±3.5	137.5±3.5	NS	NS
Diastolic BP (mm Hg)	73.7±2.2	68.6±2.0	73.3±2.0	NS	NS
% Body Fat	37.0±1.8	37.7±0.3	37.3±0.3	**0.05**	NS
Fat-free mass (kg)	58.8±3.0	58.8±0.3	58.9±0.3	NS	NS
Glycemic Variables (mg/dL)					
Fasting glucose	106.5±4.1	111.5±4.2	101.9±4.3	**0.09**	NS

Postprandial glucose	113.5±11.8	124.3±7.3	114.3±7.5	NS	NS
HbA1C (% of total Hb)	5.89±0.3	5.81±0.1	5.75±0.1	**0.08**	NS

Lipid Variables (mg/dL)

Total cholesterol	196.6±11.6	192.8±4.6	187.8± .9	**<0.001**	**0.01**
HDL cholesterol	43.6±3.3	44.1±1.3	39.8±1.3	**<0.01**	**0.001**
LDL cholesterol	122.7±10.1	117.4±5.6	118.0±6.1	**0.06**	**0.06**
NonHDL cholesterol	153.1±11.8	148.4±4.6	147.5±4.9	**<0.01**	**0.03**
TC/HDL (ratio)	5.0±0.5	4.7±0.2	5.1±0.2	NS	NS
Triglycerides[b]	161.5±19.9	144 (119-176)	138 (110-173)	NS	NS

Blood Biomarkers

IL6 (pg/mL)	1.3±0.2	1.0±0.2	0.8±0.2	NS	**0.04**
TNF-α (pg/mL)	7.9±4.2	9.9±1.2	6.0±1.3	**0.08**	NS
Adiponectin (μg/mL)	8.3±1.5	10.8±0.4	10.0±0.4	**0.02**	**<0.01**

[a] Data are Least Square Means±SEM adjusting for age, sex, season, colony and baseline values.
[b] Geometric mean and confidence interval are given for log-transformed triglyceride endpoints.

p-value ≤0.05 were considered significant, between 0.05 and 0.09 considered as approaching significant (trend), when greater than 0.09 is shown as NS (non-significant); Linear mixed model analysis was used to determine significance between post-CF and post-RS4, paired t-test for baseline vs post-RS4, *n*=18 to 20 due to missing data points. BL: baseline; CF: control flour; RS4: resistant starch type 4; BMI: body mass index; BP: blood pressure; Hb: haemoglobin; HDL: high density lipoprotein; LDL: low density lipoprotein; TC: total cholesterol; IL6: interleukin 6; TNF-α: tissue necrotic factor alpha.

Table 3-2. Estimated nutrients intake at baseline and at the end of intervention periods[a53]

Nutrients	BL	Post CF	Post RS4	*p*: post CF vs post RS4
Caloric intake (kcal/d)	1774±154	1528±121	1716±128	NS
Protein (g/d)	76±7	72±5	62±4	NS
Carbohydrate (g/d)	218±21	220±19	212±18	NS
Total fat (g/d)	68±7	60±5	53±5	NS
Cholesterol (mg/d)	415±40	442±38	407±35	NS
Saturated fat (g/d)	25±3	22±2	21±2	NS
Monounsaturated fat (g/d)	26±3	22±2	19±2	NS
Polyunsaturated fat (g/d)	10±1	9±1	7±1	NS
Total dietary fibre (g/d)	18±2	16±2	27±2	**<0.001**

[a] Data are Least Square Means±SEM adjusting for age, sex, season, colony and baseline value analysed by linear mixed model; *n*=18-20 due to missing data points.
BL: baseline; CF: control flour; RS4: resistant starch type 4

16S rRNA sequencing of the gut microbiome resulted in 5,949 OTUs the two major phyla present being ~78% Firmicutes and ~9% bacteroidetes. To avoid carry over affects between the two week washout period between diets a permutational multivariate analysis of variance was performed resulting in no significance which concludes all observations observed are directly related to diet. A principle coordinate analysis (Figure 3-1) shows 26% and 13% variation between post CF and post RS4 consumption on axes

1 and 2 respectively. RS4 consumption differentially modulated 71 OTUs, 65 Firmicutes, 3 Bacteroidetes, 1 Actinobacteria, 1 Tenericutes, and 1 Proteobacteria. Interestingly, all 3 Bacteroidetes were increased by RS4 consumption whereas the Firmicutes had a mixed response.
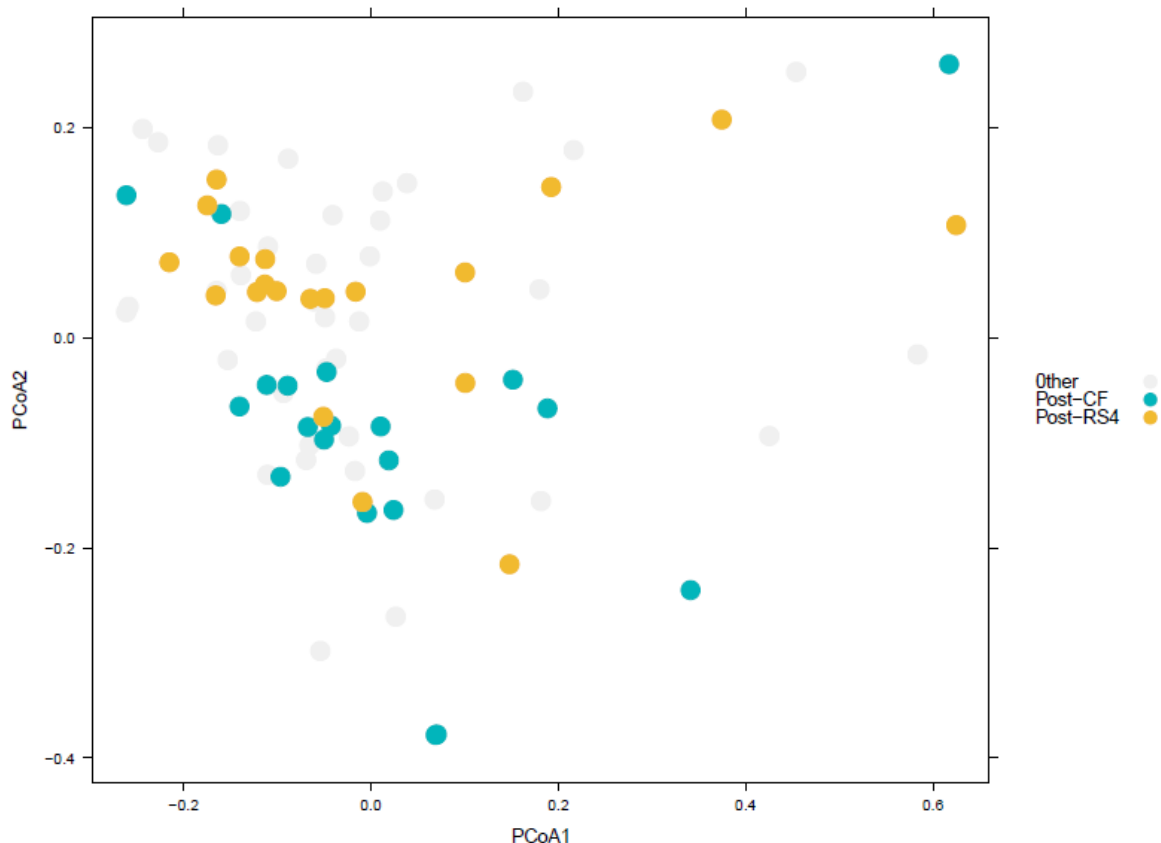
Figure 3-1: Separation of the microbiome post intervention in RS4 and CF groups. Two-dimensional principal coordinate analyses (PCoA) based on the weighted UniFrac distance between samples, given the abundance pf 5,831 taxa present in at least one sample (n=19). Axes 1 and 2 explain 26% and 13% of the variation, respectively (p=0.01)[53]

Firmicutes and Bacteroidetes are commonly studied in relationship to the obese microbiome. Both phyla contain both beneficial and less desired microbes. Figure 3-2 shows that overall the RS4 intervention reduced the Firmicutes:Bacteroidetes ratio, a

desired attribute of prebiotic fibers, whereas CF increased this ration. Of important note

alterations of this ration are highly individual specific, grouped together these trends were

observed. Variation of the gut microbiome is dependent on substrate availability for

microbial fermentation as well as total caloric intake[36]. Seasonal variations of the

microbiome have been reported in previously, attributed to fresh fruit and vegetable

availability in the Hutterite diet[35]; this was ruled out in this case as the study period did

not overlap with winter months. Table 3-2 indicates macronutrient intake did not vary

across study periods based on the self-reported 3-day diet questionnaire. Total dietary

fiber was however significantly greater after RS4, however caloric intake was not

significant.
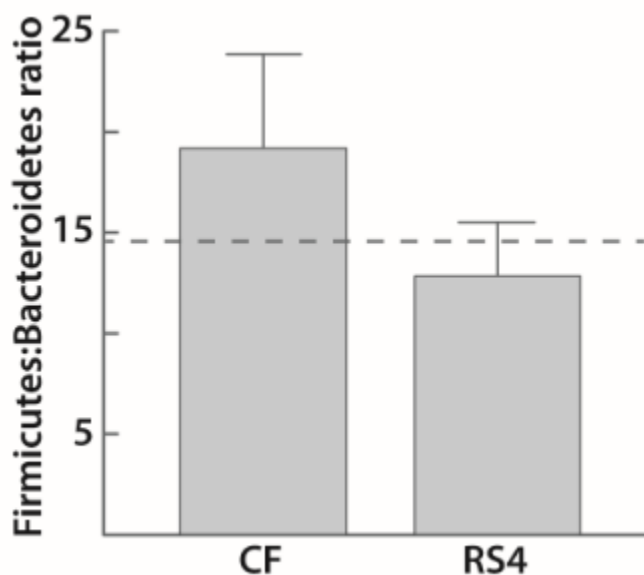
Figure 3-2: The Firmicutes/Bacteroidetes ratio after intervention (n = 14). The dotted line represents this ratio at baseline.[53]

A Pearson correlation analysis showed a potential link between significant

changes in the gut microbiota composition induced by RS4 and altered SCFA levels

shown in Figure 3-3. Acetate and butyrate levels were correlated ($p < 0.05$) with

Ruminococcus lactaris (r = 0.54) and *Oscillospira* spp. (r = 0.41). Total SCFAs were correlated with the abundance of two species: *Methanobrevibacter* spp. (r = 0.43) and *Ruminococcus lactaris* (r = 0.52). Propionate and isobutyrate levels were linked to *Methanobrevibacter* spp. (r = 0.65 and r = 0.79, respectively), *Eubacterium dolichum* (r = 0.42 and r = 0.43, respectively), *Christensenella minuta* (r = 0.39 and r = 0.59, respectively), and *Ruminococcus lactaris* (r = 0.59 and r = 0.40, respectively), of which the latter two were increased by RS4. These associations with specific microbes and fiber were not found in the CF group.
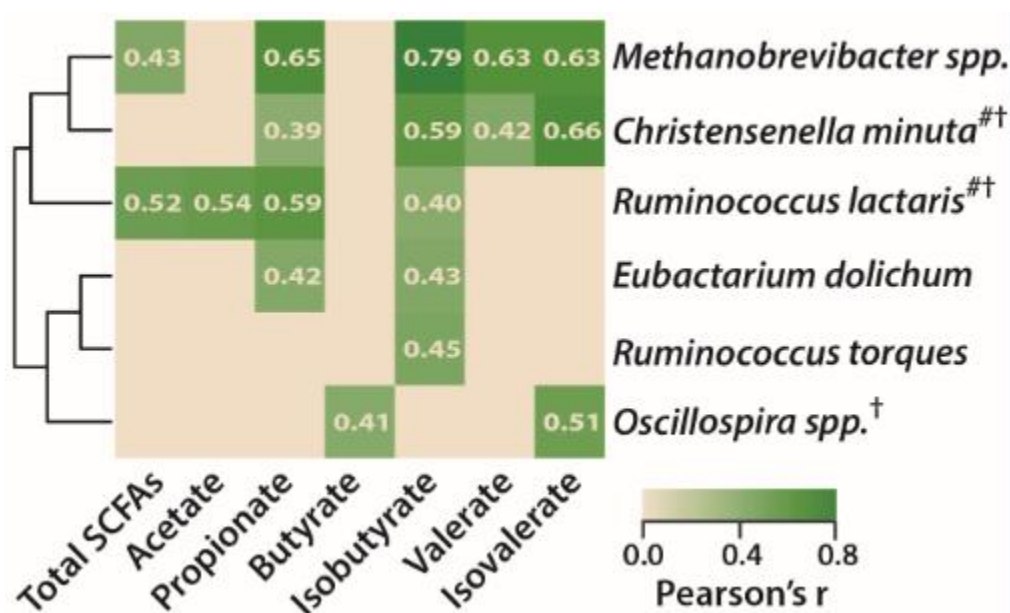
Figure 3-3: Positive correlation of six bacterial species with increased SCFA levels in an RS4-specific manner (all, *p* <0.05). Pearson coefficients are shown on heat map. #, the closest hit from the NCBI 16S rRNA database cross referenced with the OTU from the Greengenes database. †, species either significantly enriched or approached significance in the RS4 group.[53]

In the KK.Cg-A^y/a mouse model, cecal butyrate was found to be significantly enriched via RS4 consumption compared to control diet as shown in Figure 3-4. SCFAs acetate and propionate were virtually undetected in these cecum tissues. Neither RS4 nor

the control diet significantly enriched isobutyrate, valerate, isovalerate, and heaxanoate concentrations.
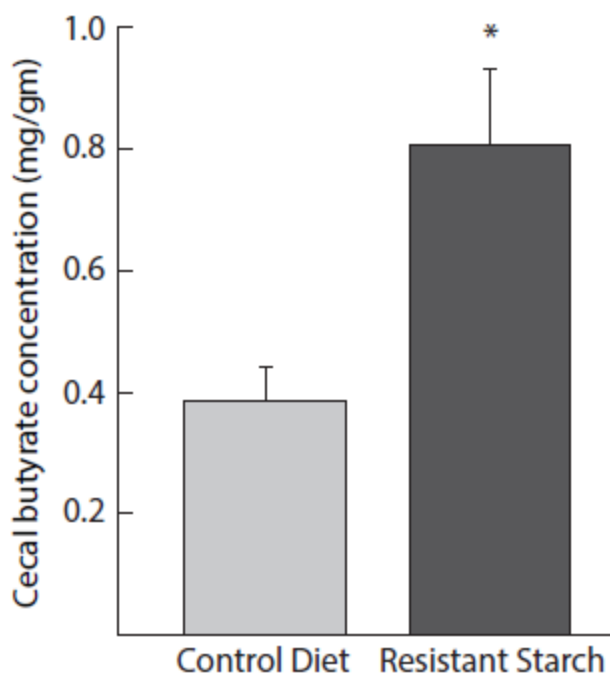
Figure 3-4: Cecal butyrate concentration in KK.Cg-Ay/a mice fed with RS4 and control diets. Data points represent the mean ± SEM (n = 3). *p<0.05, **p<0.01, ***p<0.001 compared with control diet group.[65]

3.4   Discussion

The results of this chapter further support the use of RS4 as a dietary supplement relative to MetS symptoms. Waist circumference and % body fat were significantly reduced by RS4 compared to CF, both of these variables may contribute to reduced central obesity, the number one diagnostic criteria of MetS. Adiponectin was significantly lower after RS4 compared to CF, however it was higher after RS4 compared to baseline. The plasma reduction in TC, HDL, and nonHDL cholesterol was also

observed in the parent cohort of this study[2] This is undesirable adiponectin is inversely related with MetS.

It has been well established that type and quantity of fiber in the diet differentially modify the gut microbiome[36]. Here we found that 71 OTUs were differentially affected by the supplementation of RS4, this finding was highly variable by each individual. The Firmicutes:Bacteroidetes ration has been well established as a predictor of the obese microbiome[29,30].

The ability to correlate the outcome of the method optimized in chapter two with results in this chapter was a significant outcome of this overall research study.

The mouse model studied in this chapter support the increased butyrate production in this human population as found in chapter 2. Applying the SCFA extraction method to intestinal tissue proved to be a challenge at first. The study protocol was initially attempted following the same guidelines as described in section 2.2 using one cecum sample; however, GC-MS resulted in undetectable concentrations of SCFA. In an effort to save the remainder of these delicate samples, pools of three samples were established to increase SCFA concentrations as well as using HCl-B overnight as a derivatization reagent instead of BF3-B for 20 minutes. Of note, after the overnight incubation with derivatization reagent, the sample completely dissolved into solution whereas before it appeared to be washed clean. This resulted in detectable and quantifiable SCFA concentrations.

CHAPTER 4 . SUMMARY

The goal of this research was to optimize a matabolomic assay to quantify fecal short chain fatty acids (SCFAs), a byproduct of microbial fermentation in the gut, and to apply this assay to assess associations of various phenotypic outcomes of RS4 intervention in an adult population with MetS as well as in genetically induced obese mice. This protocol was created for scientists at SDSU to extract, derivativatize, and quantify short chain fatty acids from stool samples and may in future be extended to quantify medium chain fatty acids from stool samples. Overall, metabolite profiles related to the gut microbiota can offer deep insights on the impact of lifestyle and dietary factors on chronic diseases, which is why metabolomics studies in gut microbiota related research have increased in last few years. The protocol may be utilized for other complex matrices such as intestinal samples, as described in Chapter 3. The resulting method contains a one-step derivatization followed by a simple extraction. BF3-B and HCl-B were chosen as derivatization reagents because the combination of these powerful acids with methanol and/or butanol are efficient catalysts for the esterification of fatty acids[67]. Naturally fatty acids are difficult to analyze due to their high polarity and volatility which causes sample loss either in the air or by surface absorption. The addition of a derivatization step causes esterification which attaches a functional group to the fatty acid structure creating a larger more stable molecule which are easier to detect. The final working protocol is as follows:

Chemicals:

- Boron trifluoride-1-butanol (BF3-B)
- Hexane (>97.0%)
- 1-butanol

- Sodium sulfate (granular, anhydrous, >99.0%)
- Internal standard (2-Ethylbutyric acid 99%)
- Helium (gas)
- Deionized water

Instruments:

- Vortex
- Centrifuge
- Pipettes
- Balance
- Water bath
- 30mL tube with Teflon cap
- GC sampling vial
- 50mL centrifuge tube
- Sonicator

IS prep:

(Prepare IS in butanol if no exact balance available and proceed with sample prep by adding exactly 1mL of IS solution to the sample)

1. Prepare a 1.5mg/mL IS in 1-butanol (record the exact weight of 2-ethylbutyric acid)
2. Pipette 1mL into 30mL tube
3. Record weight of 30mL tube + IS

Sample Prep:

1. Weigh at room temperature 800-1000mg (±0.1mg) in the tube with the IS
2. Add 500 μL hexane
3. Vortex for 1 min.
4. Add 2 mL HCl-B reagent
5. Vortex for 1 min.
6. Sonicate for five minutes
7. Purge the container with an inert gas (Helium)
8. Close and seal the container
9. The reaction is conducted at $100\,^\circ$C for 20 minutes
10. Cool it down to room temperature
11. Add 1.5 mL hexane
12. Vortex for 1min.
13. Add 15 mL H2O and vortex for 1 min.
14. Transfer to 50mL centrifuge tube

15. Centrifuge for 2 min at 3,000g*min^{-1}.
16. Transfer the upper organic layer into a 2 mL GC vial with or without 150μL insert
17. Add ~100mg sodium sulfate
18. Seal the vial and keep it at -20C

Stepping through this protocol one should note the importance of using a glass vial to prevent any surface absorption of the volatile fatty acids. Teflon caps are used to ensure an adequate and material/solvent compatible seal. Initially the IS was prepared at 2mg/ml in acetone, the solution was evaporated off via purging with in inert gas (either nitrogen or helium pending availability) and the exact weight of the IS was obtained. This method was found to be too variable due to solvent splashing and spilling pending air flow rate. The concentration of IS was then reduced to 1.5mg/ml and directly prepared in 1-butanol. Upon the addition of hexane and the derivatization reagent, adequate mixing must be performed as the sample tends to stick to the glass and/or lid. Proper mixing was achieved via sample vortexing both upright and upside down for complete wash of the sample tube. Also important to note is that the derivatization reagent must be closed immediately after use as it reacts with water in the air. Sonicating the sample produces a significant amount of heat, caution should be taken as to how the tubes are handled. Once samples are purged with helium, the tube should be capped immediately. Heat is applied in the water bath to help drive the derivatization reaction forward. After the water bath, samples must be returned to room temperature before opening to prevent any loss of the volatile acids.

The results of this research also support supplementation of RS4 into the diet of individuals with MetS; however, a significant amount of research is still needed to establish dietary reference intake values. We found that RS4 supplementation significantly increased fecal SCFA (butyrate, propionate, hexanoate, valerate, and

isovalerate) which directly correlated with beneficial gut microbiota. We also found that RS4 supplementation significantly reduced waist circumference, TC, HDL, nonHDL, and IL-6 which may provide alleviation of MetS and its comorbid diagnoses. Compared with the CF, RS4 significantly decreased % body fat, TC, HDL, nonHDL cholesterol, as well as adiponectin. RS4 supplementation in this cohort also reduced the Firmicutes:Bacteroidetes ratio as well as promoted growth of several beneficial microbial species. Taken together we found that RS4 in association with the gut microbiome elicits beneficial immunometabolic health outcomes on adults with metabolic syndrome.

The results of this study lead to many future implications and study ideas such as confirming the results by humanized gnotobiotic mouse model such as that described in the drafted protocol in Appendix 2. This gnotobiotic mouse model would be used to confirm that the results of the findings above were directly associated with gut microbial change. In brief, stool from human RS4 responder would be orally gavaged into gnotobiotic mice that would then be placed on RS4 or CF diet. The same parameters as above would be studied on these mice. This mouse model was originally the plan of this graduate study however due to university restrictions and lack of collaboration with outside facilities in the local area; we were unable to conduct the experiment and the protocol was left in draft form.

In conclusion, with the national trend of obesity and chronic diseases associated with obesity continue to rise at an alarming rate; it is evident that research needs to be completed to assist in reversing this epidemic trend. Overall, the research presented within this paper were beneficial in understanding the effect of an *ad libitum* RS4 dietary intervention on various health parameters in humans and mice. Even though some data is

inconclusive within this experiment, a solid foundation has been established for future research studies. The tools have been created and are ready for use to continue on with this intriguing research on dietary interventions and their influence on microbial-derived SCFA production.Implementing prebiotic and higher amounts of dietary fiber within the diet suggest improved health outcomes. The outcomes of this research may contribute in the development of future dietary guidelines of RS in the diet. With further research and testing, I am hopeful for breaking evidence suggestive of creating a healthier nation. This research should also help future clinical and mechanistic research.

Appendix 1. Gnotobiotic IACUC Protocol

This form will be updated yearly and expires December 31, 2013.

Please destroy all old forms.

For IACUC Committee Use Only	
Proposal Number	

SOUTH DAKOTA STATE UNIVERSITY

ANIMAL USAGE FORM

EXPLANATIONS AND INSTRUCTIONS

Individuals from academic institutions that receive funding from federal granting agencies such as NIH, NSF and USDA are required, by federal regulations, to follow specific guidelines concerning the care and use of vertebrate animals (mostly mammals) used in their research. Part of these requirements include the completion of an animal usage form which must be reviewed and approved by the university animal care committee before grants utilizing vertebrate animals can be submitted, and before experiments utilizing vertebrate animals can be initiated. Therefore, complete the form as completely and as clearly as possible and send to University Veterinarian, Department of Veterinary Science, SAR 106, Box 2175, SDSU. If you have any questions, call the University Veterinarian at 688-6528 or 688-6649.

SOUTH DAKOTA STATE UNIVERSITY

ANIMAL USAGE INFORMATION FORM

Instructions: Complete all items. If items do not apply, write "N/A" on the appropriate line. Send form to Veterinarian, Animal Care and Use Committee, SAR 106, Box 2175, South Dakota State Univ. (688-6649).

1. Principal Investigator/Instructor: Moul Dey   Date: 11/15/2013

2. Department: Health and Nutritional Sciences

   Campus Address: Bldg/Room Wagner Hall/ 449   Box 2203

   E-mail: Moul.Dey@sdstate.edu

3. Phones: Office 605 688 4050, Laboratory 605 688 6169, Emergency

4.   Funding Agency or Department: AES

5.   a) Proposal Submission Deadline: 11/15/2013

       b) IACUC Approval Period (not to exceed 3 years):   11/26/2013-11/25/2016

6.   Project Title: Study of metabolic status and gut microbial profiles associated with human fecal transplantation in a mouse model

7.   Animals to be used in the project:

A. Species	B. Number of animals to be acquired from outside SDSU	C. Number of animals to be utilized involving no pain* or distress	D. Number of animals subjected to pain or distress for which appropriate drugs will be used.†	E. Number of animals subjected to pain or distress for which appropriate drugs will adversely affect the results**†	F. Total number of animals (Cols. C + D + E)
Mouse	48		48		48

* Pain = pain induced for reasons other than normal animal husbandry practices (other than single or routine venipuncture & non-irritating injections)

** An explanation of the procedures producing pain or distress in these animals and the reasons such drugs were not used must be included in this submission

† Must complete question 14

       Source of Animals   Taconic Inc., Rensselaer, NY

       Housing location: Building   ARW   Room   TBD

       If the animals will be housed & cared for in ARW, you must contact the ARW facility manager (diane.baker@sdstate.edu, phone 605-688-6028) two weeks before the anticipated arrival date.

              Other

8.   For agricultural animals, will accepted management procedures and routine practices such as castration and dehorning as described in the GUIDE FOR THE CARE AND USE OF AGRICULTURAL ANIMALS IN RESEARCH AND TEACHING (Third Ed., 2010) be used in the care of these animals?

       (Available from http://www.fass.org/docs/agguide3rd/Ag_Guide_3rd_ed.pdf)

              Yes   No. If NO, then describe exceptions.

9.   Provide a complete description of the proposed use of animals. You must refer to the specific   sections and page numbers in your proposal or include on an attachment with a full description.

The principal objective of the proposed use of animals is to validate and extend our previous findings in humans that showed resistant starch-type 4 (RS4), a chemically modified resistant starch present in the wheat flour, promotes the metabolic health (Nichenametla et al., 2014). Using human stool samples from this study; we would like to investigate the direct interaction of RS4 with gut microbiota in a pre-clinical mouse model where we can customize the gut microbiota profile unlike in humans. Hence, gnotobiotic mice model with a defined gut microflora is the optimum model for human-to-mouse fecal-transplantation because these mice associated with Altered Schaedler Flora (**Table 1**) show the potential to mimic human gut microbial profile in mice . We are also able to directly reference a large database of prior work using such humanized mice, especially for gut-microbiota associated obesity and metabolic syndrome supporting the use of gnotobiotic mice.

Table 5: Altered Schaedler Flora [commensal, non-pathogenic?] also need original reference

Taxon	Oxygen sensitivity	Identity
Fusiform EOS bacteria	Yes	*Clostridium* sp **ASF356**
Fusiform EOS bacteria	Yes	*E. plexicaudatum* **ASF492**
Fusiform EOS bacteria	Yes	*Clostridium* sp. **ASF502**
Fusiform EOS bacteria	Yes	*Clostridium* sp. **ASF500**
Lactobacillus acidophilus	No	*Lactobacillus* sp. **ASF360**
Lactobacillus salivarius	No	*L. animalis & L. murinus* **ASF361**
Bacteroides distasonis	No	*Bacteroides* sp. **ASF519**
Spiral shaped organism	No	*Flexistipes* phylum **ASF457**

Dietary interventions with RS-2 and RS-4 have been shown to attenuate various risk factors for metabolic syndrome (MetS) characterized by several conditions such as insulin-resistance, dyslipidemia, abdominal obesity and hypertension that ultimately lead to atherosclerosis and cardiovascular disease (CVD) (Martinez et al., 2010; Brunner et al, 2002). As RS4 is chemically modified and therefore can evade chemical digestion, it is however susceptible to microbial fermentation in the colon, hence we are interested in showing how the interaction between RS4

and gut microbiota renders a healthy outcome. Accumulating evidence suggests that gut microflora play a central role in maintaining a balance between health and disease, including MetS [29]. Moreover, it has been postulated that there are different sets of gut microbiota based on abundance in healthy individuals versus patients with MetS [68]. Hence, we hypothesized that dietary RS4 would prevent MetS by shifting the blood, lipid and gut microbial profiles towards a favorable health-outcome after transplanting human fecal samples from MetS patients to gnotobiotic mice. This gnotobiotic mouse model, once optimized, can be utilized for future research concerning the microbiome which is the field of growing interest.

Prior to animal experiment, we will obtain the formulated control and RS4 feeds (Test Diet, Richmond, IN) (**Table 2**). These isocaloric control and RS4 diets will eliminate the possible effects of energy restriction in metabolic health of mice.

Table 2: Composition of experimental diets

Ingredients	Control Diet (%)	RS4 Diet (%)
Wheat starch[1]	31.77	27.07
Resistant wheat starch type-4[2]	0.00	23.5
Powdered cellulose	18.80	0.00
Casein – vitamin free	14.00	14.00
Dextrin	13.67	13.67
Sucrose	8.82	8.82
Soybean oil	8.00	8.00
AIN 93M mineral mix	3.50	3.50
AIN 93M vitamin mix	1.00	1.00
Choline Bitartrate	0.25	0.25
L-Cystine	0.18	0.18

t-Butylhyrdoquinone	0.0008	0.0008
Energy content (Kcal/g)	3.3	3.3

[1] Wheat starch is Midsol TM 50 with energy content of 359.5 Kcal/100 g.

[2] Resistant starch is Fibersym RW with energy content 56.5 Kcal/100g.

Overall mice experiment will be carried out in two phases:

Phase I: Microbial effects of animal health status

To know how the microbiome of mice transplanted with human feces responds to RS4 dietary intervention, we will use a gnotobiotic mouse model consisting 5-8 week old female heterozygous Swiss nude mice randomly divided into four groups (12 mice per group, two sacrificed for baseline values, and five mice per final two cages) for human fecal transplantation (**Table 3**). We chose this strain because of its availability with a defined gut-microbiota. All mice will be kept in more stringent specific pathogenic free housing to prevent contamination with any unknown bacteria

Table 3: Mouse groups and treatments

Group	Diet	No. of animals	Dose/treatment
A (A1+A2), 2 donors	Control	12 (-2 sacrifice, 5+5: final)	200μL preRS4
B (B1+B2)	Control	12 (-2 sacrifice, 5+5: final)	200μL post-RS4
C (C1+C2)	RS4	12 (-2 sacrifice, 5+5: final)	200μL preRS4
D (D1+D2)	RS4	12 (-2 sacrifice, 5+5: final)	200μL post-RS4

After one week of acclimatization, mice will undergo a fecal microbial transfer (FMT) from an individual whose response to RS4 was greatest compared to other participants. Response to RS4 was analyzed on several parameters including change in body fat, weight, total cholesterol, fasting blood glucose, metabolic status, and specific bacterial abundance. One pre-RS4 and one

post-RS4 fecal sample will be homogenized and added to 5mL of PBS to create the solution for subsequent gavage. 200μL of prepared fecal solution will be gavaged into the stomach of the recipient mice through plastic oral tubing. After fecal transplantation four groups each with 11 mice (**Table 3**) will be housed separately to avoid possible cross-contamination. One mouse from each of the four groups as indicated in Table 3 will be sacrificed prior to FMT to obtain cecal short chain fatty acids analysis. After three weeks, the blood samples will be collected from saphenous vein to take the basal readings of blood glucose and lipid profiles before dietary intervention. This method of blood collection does not involve anesthesia and is less stressful to mice. Collection of fecal pellets followed by fecal culture will be performed every week for analysis of gnotobiotic status throughout the entire experiment. At this point one additional mouse will be sacrificed from each group for basal body composition prior to dietary intervention, leaving the final total of ten mice per group.

Phase II: Impact of dietary intervention with RS4 on animal health status

Phase II of this experiment includes the addition of RS4 chow to animal groups C and D as indicated in Table 3. Mice will be observed daily and monitored for any abnormal signs of pain and distress, appearance, appetence and behavior. All mice will be weighed weekly to monitor the feed intake and growth curve. We will consult to attending veterinarian for appropriate care, treatment or euthanasia, if and when any mouse develops visible signs of distress. All mice will be euthanatized with CO_2 asphyxiation 21 days post-dietary intervention (week 7), and blood will be collected through cardiac punctuation immediately after the sacrifice. Body composition analysis will be performed at the Materials Evaluation and Testing Laboratory (METLAB) in the College of Engineering at SDSU. Biological specimens including the entire gut, mesenteric lymph node, mesenteric fat, spleen, and liver will be collected, and aliquots of tissue samples will be snap-frozen in liquid nitrogen or kept in 10% formalin for subsequent gene expression experiments or histopathological examinations, respectively. The remainder of the mouse will be disposed and incinerated later. The summarized timeline of the proposed experiment is shown in **Table 4**.

Table 4: Schedule of the proposed experiment

Week	Treatment	Comments
0	Acclimatization	Daily observation; regular chow diet
1	Fecal Microbial Transplant	**Phase I**: Blood collection through saphenous vein, 200μL oral gavage of either preRS4 or post-RS4 human stool samples. One animal from each of the four groups will be sacrificed for baseline values
4	Introduction of control or RS4 chow to	**Phase II**: Blood collection through

	designated groups	saphenous vein, one animal from each of the four groups will be sacrificed for analysis of microbial effects pre-diet intervention
7	Mice sacrifice and tissues collection	CO_2 euthanasia Body composition; cardiac puncture; snap-frozen tissues in liquid N_2; fixed-tissues in 10% formalin

Routine monitoring of animal health includes daily observation of any signs of pain and distress, weekly measurement of body weight, and weekly microbial culture of stool samples.

10. Provide specific design information that supports your rationale for involving animals and appropriateness of species and numbers to be used. (Note: This proposal may be referred to a statistician to verify appropriateness of animal numbers.)

The Swiss nude mice model has been well established for human fecal transplantation. The design of this project is based on previous literature reports on human-to-mouse fecal transplant models. As similar kind of fecal transplantation studies are not feasible in humans, appropriate mouse model is. The numbers of animals in this study are consistent with literature reports using mouse model for human fecal transplantation. The study reporting human-to-mouse fecal transplant model is given below:

We are also able to directly reference a large database of prior work using such humanized mice, especially for gut-microbiota associated obesity and metabolic syndrome supporting the use of gnotobiotic mice.

Ridaura, V. K., Faith, J. J., Rey, F. E., Cheng, J., Duncan, A. E., Kau, A. L., . . . Gordon, J. I. (2013). Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science, 341(6150), 1241214. doi: 10.1126/science.1241214

11. Alternatives to Painful Procedures:

If painful or slightly painful procedures are to be used, provide a written narrative description of the specific electronic database/information search (in addition to reading journals/periodicals) which was performed (for example, the Animal Welfare Information Center) along with the specific key words to help determine that alternatives are not available.

Date search was conducted: 11/04/2013

Key words used: Gnotobiotic, Gut microbiota, Metabolic syndrome, Resistant Starch, Mouse model

Databases searched: __Pubmed__

Period of time covered by search: __All years__

Narrative description describing the results of the alternatives search and the impact on the use of animals in this project:

Oral gavage is the straight-forward and less painful than other techniques for the fecal transplantation. Blood collection through saphenous vein is less painful because it is less invasive than retro-orbital sinus, which may have more complication.

12.   Check if proposal involves (refer to IACUC page on InsideState for more information)

    _____   Invasive Procedures (e.g. multiple injections, multiple blood sampling, surgery, catheterization)

    __X__   Pain or other Stress (e.g. injections, disease, surgery, Freund's adj., ascites fluid production)

    _____   Deprivation (e.g. limiting food, water, light)

    _____   Other (e.g. fright, noise, physiological stresses, etc.)

Briefly describe any items checked above and explain the need for these in the project.

Only one-time oral gavage will be used during the entire study. We will also collect the blood form saphenous vein twice three weeks apart.

13.   A.   For any of the procedures described above in question #13, identify the pharmaceuticals used to minimize discomfort to the animals:

	Type	Dosage	Route of Administration
Anesthesia			
Analgesia			
Tranquilizer			
Euthanasia	CO_2	To affect	Inhalation

B.   If no pain relieving measures are used, please justify:

C.   If controlled substances will be used in the protocol, whose DEA and State of South Dakota controlled substances registration will the agents be purchased and used under?

NA

14. Describe any additional approaches taken to minimize pain or discomfort, to control infections, or otherwise insure the humane treatment of animals in this project. Also describe how the animal care personnel will be trained.

Any animal suffering undue pain or distress will be humanely euthanized during the study period. Animals will be maintained at the SPF level (autoclaved food and bedding, acidified water). If signs of discomfort, injury or infections are observed, the veterinarian will be consulted, and appropriate care will be provided and recorded. If the animals develop untreatable diseases, the veterinarian will determine if euthanasia should be applied before the ending of the study. All personnel are trained in how to handle mice in a way that minimizes stress to the animals.

15. If animals will be euthanized, describe the procedures to be used and method and site of disposal. Unless a deviation is justified for scientific or medical reasons, methods should be consistent with the most current version of the AMVA Guidelines on Euthanasia (http://www.avma.org/issues/animal_welfare/euthanasia.pdf). If animals will not be euthanized as part of the research project, describe their further use or plans for their disposition; include euthanasia method for emergency situations.

All animals will be euthanized using CO_2 at the ending of study or whenever required. Blood of euthanized animals will be drawn via cardiac puncture. Biological specimens of euthanized animals that die or euthanized will be harvested, snap-frozen in liquid nitrogen and stored in a -80ºC freezer, and used for this study. The rest of the carcasses will be incinerated.

NOTE: Animals are subject to post-mortem examination if unexplained death occurs which is not a part of research protocol. Necropsy will be performed under the direction of the attending veterinarian and/or the ADRDL. Laboratory confirmation will be provided by the ADRDL. Researchers will be charged the standard fee for these services.

16. Evaluate the effect on this project of using alternative procedures not including animals.

In vitro cell-culture models are not adequate to represent the entire process of gut microbial interaction and ecology in health and diseased status. We cannot use conventional mice model because they contain a vast variety of gut microbiota, which may potentially conflict the outcomes. Hence, mice model with defined flora is the optimum model for our intent, where we can control the confounding variables unlike in humans.

17. Human health risks (Note: If this project is deemed to have significant human health risk, it may be referred to the Institutional Biosafety Committee.)

A. What are the human health risks to which investigators and other contact personnel will be subjected in working with animals in this project? Include physical, zoonotic, and allergic risks.

Bites, scratches and allergies are potential human health risks for the personnel.

B. What approach will be taken to minimize those risks, and how will laboratory and animal care personnel be trained with regard to the risks?

The personnel on this project are trained in handling laboratory mice and in the procedures taken in the protocol. The training and previous experience will minimize the above risks. The mice will arrive here as SPF with defined flora and will be kept in strict SPF environment. Personnel will wear gloves and lab coats while handling mice. These will effectively prevent the potential human health risks.

18. Personnel

A. Appropriate medical care for all animals will be available and provided as necessary by a licensed and accredited veterinarian. Standard husbandry practices as defined in the Guide for the Care and Use of Agricultural Animals in Research and Teaching Chapter 3 may be carried out by other trained individuals, as appropriate. Specify whether veterinary care will be provided by the SDSU veterinarian or a consulting veterinarian (provide the name of the individual involved with the project):

   Dr. Michele Mucciante   (name of SDSU veterinarian or consulting veterinarian) will provide the veterinary care and has been notified by me.

B. Education and Training Programs. Public Laws 99-158 and 99-198 require the University to establish education and training programs to scientists and animal technicians who handle and care for laboratory animals. Personnel must complete training from the Collaborative Institutional Training Initiative (www.citiprogram.org) read the appropriate SDSU training materials and/or view the appropriate tapes/CDs and then sign a certification statement. Animal Usage Forms cannot be processed until all persons handling and caring for the animals have signed certification statement on file. Contact Janice Kampmann, ADR 106, phone 688-6649.

C. Additional training in specific techniques (restraint, anesthesia, euthanasia, injections, etc.) are available through the University Veterinarian. The principal investigator's signature on the bottom of this form is taken to indicate that training and education requirements have been fulfilled.

D. List **RESEARCH PERSONNEL** who will have direct animal contact. Include their position (principal investigator, graduate student, etc), their specific role in the project, years of experience working with the species proposed to be used in the project, and years of experience conducting the procedures they will be doing in the project.

Name	Position	Role in Project	Experience (years) working with the proposed species	Experience (years) with procedures

Moul Dey	Principal Investigator	Oversight of the entire project	9	9
Robert Juenemann	Graduate Student	Preparation of the reagents for fecal transplantation, Animal care Collection of biological specimens CO_2 euthanasia	1	1
Bijaya Upadhyaya	Graduate Student	Animal care, Blood collection through saphenous vein, Cardiac puncture, Collection of biological specimens,	3	3
David Knudsen	Professor	Oversight of animal housing and health, histopathology	>20 years	>20 years
TBD	Undergraduate Student	Animal care and management	Will be trained	

E. For research personnel that have not worked with animals before and/or have not previously conducted the procedures described in this protocol, describe how training for these individuals will be conducted.

19. **ASSURANCE BY INVESTIGATOR:**

I assure that these activities do not unnecessarily duplicate previous experiments conducted here or elsewhere. I agree to conduct this project in accordance with applicable provisions of the Animal Welfare Act, the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, the Guide for the Care and Use of Laboratory Animals, other applicable federal laws, the laws of the State of South Dakota, and the policies of South Dakota State University. All necessary State and Federal permits have been obtained, as appropriate. I agree to conduct this project in accordance with the protocol submitted to the Animal Care and Use Committee, to ensure that all research personnel are aware of and follow the approved protocol, and to obtain prior approval from the committee before modifying the protocol.

Signature of the Investigator _____ Date _____

* *

For Committee Use Only:

Date received _____; Date Routed _____; Deadline for Review _____

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Appendix 2: 3-Day Food Checklist

3-Day Food Checklist

Name:_____   **ID**:_____
       Date:_____

Instructions

- Fill out one Food Checklist throughout the day on the three days marked below:
  - ☐ Sunday   ☐ Monday   ☐ Tuesday   ☐ Wednesday   ☐ Thursday   ☐ Friday   ☐ Saturday
- Complete each Food Checklist by marking an 'x' in the box each time *you ate* a food on that day (not what was available.
- For example, if you drank a cup of milk at breakfast and one at lunch, you would mark 2 boxes. If you had 2 slices of bread at breakfast and 2 at dinner you would mark 4 boxes.
- If you had soup, be sure to mark it under the type of broth and also mark the vegetables or meat that was in the soup.

Day 1
Mark how many servings of each of the following you ate.

Dairy – include flavored milks (serving = 1 liquid cup or 1 slice or oz for cheeses)	
Milk – whole	☐☐☐☐☐☐
Milk – 2%	☐☐☐☐☐☐
Milk – 1% or skim	☐☐☐☐☐☐
Margarine or butter (pat)	☐☐☐☐☐☐
Yogurt, all kinds	☐☐☐☐☐☐
Cheese, all kinds	☐☐☐☐☐☐
Ice Cream or sherbert (1/2 c)	☐☐☐☐☐☐

Beverages (serving = 1 cup)	
Fruit juice (orange, grape, etc.)	☐☐☐☐☐☐☐
Vegetable juice (tomato, etc.)	☐☐☐☐☐☐☐
Wine or beer	☐☐☐☐☐☐☐
Coffee	☐☐☐☐☐☐☐
Tea	☐☐☐☐☐☐☐
Pop, kool-aid, or punch	☐☐☐☐☐☐☐
Pop, kool-aid, etc. *DIET*	☐☐☐☐☐☐☐
Water	☐☐☐☐☐☐☐

How many tsp of sugar do you put in your coffee and/or tea? _____ teaspoons

Breads, Cereals & Grains	
Bread, wheat (1 slice)	☐☐☐☐☐☐
Bread, white (also biscuits, buns)	☐☐☐☐☐☐
Noodles, dumplings (also knödel, spätzel) (1/2 c)	☐☐☐☐☐☐
Maultauschen, cottage cheese pockets, etc. (1 item)	☐☐☐☐☐☐
Oatmeal or oat cereal (1 c)	☐☐☐☐☐☐
Rice or rice cereal (1 c)	☐☐☐☐☐☐
Other grains (1 c)	☐☐☐☐☐☐

Eggs, Fish, Poultry & Meat
(1 serving = 3 oz = 1 deck of cards)

Eggs (1)	☐☐☐☐☐☐
Turkey or chicken	☐☐☐☐☐☐
Sausage (include Thanksgiving sausage)	☐☐☐☐☐☐
Pork	☐☐☐☐☐☐
Beef	☐☐☐☐☐☐
Lunch meat (also chopped ham)	☐☐☐☐☐☐

Did you eat the skin on your turkey or chicken?
       ☐ No   ☐ Yes   ☐ Sometimes, not always

Fruits (serving=1 item or 1 cup)	
Apples	☐☐☐☐☐☐
Oranges	☐☐☐☐☐☐
Banana	☐☐☐☐☐☐
Melons	☐☐☐☐☐☐
Other fruit	☐☐☐☐☐☐

Vegetables (serving=1 item or 1/2 c)	
Potatoes – fried (1 med)	☐☐☐☐☐☐
Potatoes – baked (1 med)	☐☐☐☐☐☐
Potatoes – boiled (1 med)	☐☐☐☐☐☐
Lettuce	☐☐☐☐☐☐
Tomatoes (1)	☐☐☐☐☐☐
Beans, green (1/2 c)	☐☐☐☐☐☐
Carrots, peas, corn	☐☐☐☐☐☐
Beets	☐☐☐☐☐☐
Squash	☐☐☐☐☐☐
Cauliflower & broccoli	☐☐☐☐☐☐
Cabbage (not sauerkraut)	☐☐☐☐☐☐
Other vegetables	☐☐☐☐☐☐

Sweets, Crackers, Pickles & Soups	
Cake or cookies (2)	☐☐☐☐☐☐
Pie (slice) (also kuchen&strudel)	☐☐☐☐☐☐

Candy (also chocolates, bars, hard candy, etc.)	☐ ☐ ☐ ☐ ☐ ☐
Crackers (2) (include saltines)	☐ ☐ ☐ ☐ ☐ ☐
Pickled foods (1 Tblsp) (ex. pickles, watermelon, tomatoes, corn relish)	☐ ☐ ☐ ☐ ☐ ☐
Sauerkraut	☐ ☐ ☐ ☐ ☐ ☐
Soup – milk- based broth	☐ ☐ ☐ ☐ ☐ ☐
Soup – clear broth	☐ ☐ ☐ ☐ ☐ ☐

Thinking about the meat you had today. About how many of these servings were fried?
☐ All   ☐ Most   ☐ Some   ☐ None   ☐ Did not have any

Did you eat anywhere not on the colony today?
☐ Yes   ☐ No   If yes, describe_____

Any other foods?
_____

Day 2
Mark how many servings of each of the following you ate.

Dairy – include flavored milks (serving = 1 liquid cup or 1 slice or oz for cheeses)

Milk – whole	☐☐☐☐☐☐
Milk – 2%	☐☐☐☐☐☐
Milk – 1% or skim	☐☐☐☐☐☐
Margarine or butter (pat)	☐☐☐☐☐☐
Yogurt, all kinds	☐☐☐☐☐☐
Cheese, all kinds	☐☐☐☐☐☐
Ice Cream or sherbert (1/2 c)	☐☐☐☐☐☐

Beverages (serving = 1 cup)

Fruit juice (orange, grape, etc.)	☐☐☐☐☐☐☐
Vegetable juice (tomato, etc.)	☐☐☐☐☐☐☐
Wine or beer	☐☐☐☐☐☐☐
Coffee	☐☐☐☐☐☐☐
Tea	☐☐☐☐☐☐☐
Pop, kool-aid, or punch	☐☐☐☐☐☐☐
Pop, kool-aid, etc. *DIET*	☐☐☐☐☐☐☐
Water	☐☐☐☐☐☐☐

How many tsp of sugar do you put in your coffee and/or tea? _____ teaspoons

Breads, Cereals & Grains

Bread, wheat (1 slice)	☐☐☐☐☐☐
Bread, white (also biscuits, buns)	☐☐☐☐☐☐
Noodles, dumplings (also knödel, spätzel) (1/2 c)	☐☐☐☐☐☐
Maultauschen, cottage cheese pockets, etc. (1 item)	☐☐☐☐☐☐
Oatmeal or oat cereal (1 c)	☐☐☐☐☐☐
Rice or rice cereal (1 c)	☐☐☐☐☐☐
Other grains (1 c)	☐☐☐☐☐☐

Eggs, Fish, Poultry & Meat (1 serving = 3 oz = 1 deck of cards)

Eggs (1)	☐☐☐☐☐☐
Turkey or chicken	☐☐☐☐☐☐
Sausage (include Thanksgiving sausage)	☐☐☐☐☐☐
Pork	☐☐☐☐☐☐
Beef	☐☐☐☐☐☐
Lunch meat (also chopped ham)	☐☐☐☐☐☐

Did you eat the skin on your turkey or chicken?
☐ No ☐ Yes ☐ Sometimes, not always

Fruits (serving=1 item or 1 cup)

Apples	☐☐☐☐☐☐
Oranges	☐☐☐☐☐☐
Banana	☐☐☐☐☐☐
Melons	☐☐☐☐☐☐
Other fruit	☐☐☐☐☐☐

Vegetables (serving=1 item or 1/2 c)

Potatoes – fried (1 med)	☐☐☐☐☐☐
Potatoes – baked (1 med)	☐☐☐☐☐☐
Potatoes – boiled (1 med)	☐☐☐☐☐☐
Lettuce	☐☐☐☐☐☐
Tomatoes (1)	☐☐☐☐☐☐
Beans, green (1/2 c)	☐☐☐☐☐☐
Carrots, peas, corn	☐☐☐☐☐☐
Beets	☐☐☐☐☐☐
Squash	☐☐☐☐☐☐
Cauliflower & broccoli	☐☐☐☐☐☐
Cabbage (not sauerkraut)	☐☐☐☐☐☐
Other vegetables	☐☐☐☐☐☐

Sweets, Crackers, Pickles & Soups

Cake or cookies (2)	☐☐☐☐☐☐
Pie (slice) (also kuchen&strudel)	☐☐☐☐☐☐
Candy (also chocolates, bars, hard candy, etc.)	☐☐☐☐☐☐
Crackers (2) (include saltines)	☐☐☐☐☐☐
Pickled foods (1 Tblsp) (ex. pickles, watermelon, tomatoes, corn relish)	☐☐☐☐☐☐
Sauerkraut	☐☐☐☐☐☐
Soup – milk- based broth	☐☐☐☐☐☐
Soup – clear broth	☐☐☐☐☐☐

Thinking about the meat you had today. About how many of these servings were fried?
☐ All ☐ Most ☐ Some ☐ None ☐ Did not have any

Did you eat anywhere not on the colony today?
☐ Yes ☐ No If yes, describe_____

Any other foods?
_____
_____
_____

Day 3
Mark how many servings of each of the following you ate.

Dairy – include flavored milks (serving = 1 liquid cup or 1 slice or oz for cheeses)	
Milk – whole	☐☐☐☐☐☐
Milk – 2%	☐☐☐☐☐☐
Milk – 1% or skim	☐☐☐☐☐☐
Margarine or butter (pat)	☐☐☐☐☐☐
Yogurt, all kinds	☐☐☐☐☐☐
Cheese, all kinds	☐☐☐☐☐☐
Ice Cream or sherbert (1/2 c)	☐☐☐☐☐☐

Beverages (serving = 1 cup)	
Fruit juice (orange, grape, etc.)	☐☐☐☐☐☐☐
Vegetable juice (tomato, etc.)	☐☐☐☐☐☐☐
Wine or beer	☐☐☐☐☐☐☐
Coffee	☐☐☐☐☐☐☐
Tea	☐☐☐☐☐☐☐
Pop, kool-aid, or punch	☐☐☐☐☐☐☐
Pop, kool-aid, etc. *DIET*	☐☐☐☐☐☐☐
Water	☐☐☐☐☐☐☐

How many tsp of sugar do you put in your coffee and/or tea? _____ teaspoons

Breads, Cereals & Grains	
Bread, wheat (1 slice)	☐☐☐☐☐☐
Bread, white (also biscuits, buns)	☐☐☐☐☐☐
Noodles, dumplings (also knödel, spätzel) (1/2 c)	☐☐☐☐☐☐
Maultauschen, cottage cheese pockets, etc. (1 item)	☐☐☐☐☐☐
Oatmeal or oat cereal (1 c)	☐☐☐☐☐☐
Rice or rice cereal (1 c)	☐☐☐☐☐☐
Other grains (1 c)	☐☐☐☐☐☐

Eggs, Fish, Poultry & Meat (1 serving = 3 oz = 1 deck of cards)	
Eggs (1)	☐☐☐☐☐☐
Turkey or chicken	☐☐☐☐☐☐
Sausage (include Thanksgiving sausage)	☐☐☐☐☐☐
Pork	☐☐☐☐☐☐
Beef	☐☐☐☐☐☐
Lunch meat (also chopped ham)	☐☐☐☐☐☐

Did you eat the skin on your turkey or chicken?

☐ No   ☐ Yes   ☐ Sometimes, not always

Fruits (serving=1 item or 1 cup)	
Apples	☐☐☐☐☐☐
Oranges	☐☐☐☐☐☐
Banana	☐☐☐☐☐☐
Melons	☐☐☐☐☐☐
Other fruit	☐☐☐☐☐☐

Vegetables (serving=1 item or 1/2 c)	
Potatoes – fried (1 med)	☐☐☐☐☐☐
Potatoes – baked (1 med)	☐☐☐☐☐☐
Potatoes – boiled (1 med)	☐☐☐☐☐☐
Lettuce	☐☐☐☐☐☐
Tomatoes (1)	☐☐☐☐☐☐
Beans, green (1/2 c)	☐☐☐☐☐☐
Carrots, peas, corn	☐☐☐☐☐☐
Beets	☐☐☐☐☐☐
Squash	☐☐☐☐☐☐
Cauliflower & broccoli	☐☐☐☐☐☐
Cabbage (not sauerkraut)	☐☐☐☐☐☐
Other vegetables	☐☐☐☐☐☐

Sweets, Crackers, Pickles & Soups	
Cake or cookies (2)	☐☐☐☐☐☐
Pie (slice) (also kuchen&strudel)	☐☐☐☐☐☐
Candy (also chocolates, bars, hard candy, etc.)	☐☐☐☐☐☐
Crackers (2) (include saltines)	☐☐☐☐☐☐
Pickled foods (1 Tblsp) (ex. pickles, watermelon, tomatoes, corn relish)	☐☐☐☐☐☐
Sauerkraut	☐☐☐☐☐☐
Soup – milk- based broth	☐☐☐☐☐☐
Soup – clear broth	☐☐☐☐☐☐

Thinking about the meat you had today. About how many of these servings were fried?
☐ All   ☐ Most   ☐ Some   ☐ None   ☐ Did not have any

Did you eat anywhere not on the colony today?
☐ Yes   ☐ No   If yes, describe_____

Any other foods?
_____
_____
_____

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