ABSTRACT

BARRINGTON, WILLIAM THOMAS. Individual Variation in Diet Response: Health Effects of Dietary Interventions on Mice with Diverse Genetic Backgrounds (Under the direction of David Threadgill).

Population-level studies have shown that dietary patterns are strongly correlated with the health of a population. However, a major limitation of population-level dietary studies is the absence of information on the relationship between individual and population-level responses. Several recent studies have suggested that individuals have large variation in their response to diet and question the efficacy of universal dietary guidelines. This study investigated how individuals’ genetic background impacts their response to dietary modifications. Diet-related conditions including obesity, metabolic syndrome, and colorectal cancer were analyzed in four inbred mouse strains (A/J, C57BL/6J, FVB/NJ, NOD/ShiLtJ) fed four human-based diets (Western, Mediterranean, Japanese, ketogenic) and a standard laboratory mouse chow. The health outcomes were associated with physiological changes, including shifts in gut microbiome composition. We identified that the impact of each diet was dependent on the genetic background of the individual. In some cases, the same diet could produce opposite results in different mouse strains. C57BL/6J mice had mostly positive health markers on the ketogenic diet, while the FVB/NJ strain had high adiposity and increased incidence of colorectal cancer on the ketogenic diet. Alternatively, the Western and Mediterranean diets produced particularly negative health effects in the C57BL/6J strain, while the FVB/NJ strain faired much better. Some strains such as NOD/ShiLtJ exhibited negative health effects on any high-fat diet, including Western, Mediterranean, and ketogenic diets. The A/J strain was largely resistant to the effects of diet, but suffered liver-specific negative effects from the Japanese diet. The A/J strain also exhibited a large increase in
resting metabolic rate when fed the ketogenic diet, although this had no measured effect on overall health of the mice. We determined that diet effects on both cardiometabolic health and colorectal cancer incidence varied by strain. In the C57BL6/J and FVB/NJ strains, we identified shifts in gut microbiome composition that may play a role in colorectal cancer susceptibility. Ultimately, this study demonstrates that individuals can have drastically different responses to identical dietary interventions and promotes the identification of personalized nutritional approaches based on an individual’s genetic makeup.
Individual Variation in Diet Response: Health Effects of Dietary Interventions on Mice with Diverse Genetic Backgrounds

by
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BIOGRAPHY

William Thomas Barrington was born on December 3, 1987 in Ohio’s Akron City Hospital, the birthplace of basketball greats LeBron James and Stephen Curry. William attended the College of Wooster in Wooster, Ohio where he gained appreciation for undergraduate research opportunities. He studied hormones and behavior in zebra finches with Dr. Sharon Lynn and gene evolution in Paramecium with Dr. Dean Fraga. William received an Honors award for his thesis work studying the evolution of the calcineurin gene family. In 2010, he graduated with a B.A. in Biochemistry and Molecular Biology.

Following college, William worked as a Research Aide at The Ohio State University’s Ohio Agricultural Research and Development Center in Wooster, Ohio. He worked with Dr. Daniel Herms studying the invasive Emerald Ash Borer and co-authored two papers.

William Barrington chose to pursue a PhD at North Carolina State University in 2011 because of the historical strength, reputation and communal feeling of the Genetics Department. Upon disbanding of the department in 2014, William followed his professor, David Threadgill, to Texas A&M to finish his PhD research. During his graduate research, William emphasized inclusion of undergraduates in his research and mentored more than a dozen students.

William plans to continue researching diet, metabolism, and individual variation as a postdoctoral researcher.
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A number of individuals have helped make this dissertation a reality. My parents and sisters have provided a great deal of support and encouragement throughout my academic career. My uncle took a long road trip with me to visit prospective graduate schools and has continued to aid in my success as a graduate student.

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# TABLE OF CONTENTS

LIST OF TABLES ........................................................................................................... vi
LIST OF FIGURES ......................................................................................................... vii

CHAPTER 1 ....................................................................................................................... 1
MODELING DIETARY RESPONSE IN MICE: IMPACT OF INDIVIDUAL VARIABILITY ............................................................... 2
  ABSTRACT ................................................................................................................... 3
  INTRODUCTION ........................................................................................................... 4
  HISTORICAL PERSPECTIVE ON DIETARY RECOMMENDATIONS ....................... 5
  INDIVIDUAL VARIATION IN DIET RESPONSE ..................................................... 8
  IMPACT OF GUT MICROBIOME ON DIET RESPONSE ....................................... 13
  MOUSE MODELS AS A TOOL TO STUDY INDIVIDUAL VARIATION IN DIET RESPONSE ..................................................................................... 15
  DIETS OF INTEREST FOR STUDYING INDIVIDUAL DIET RESPONSE .................. 18

CHAPTER 2 ....................................................................................................................... 23
PRECISION NUTRITION TO OPTIMIZE METABOLIC HEALTH IN MICE ............. 24
  ABSTRACT ................................................................................................................... 25
  INTRODUCTION ........................................................................................................... 27
  MATERIALS AND METHODS .................................................................................. 30
  RESULTS .................................................................................................................... 37
  DISCUSSION ............................................................................................................... 58
  ACKNOWLEDGEMENTS ........................................................................................... 62

CHAPTER 3 ....................................................................................................................... 63
INVESTIGATION OF DIETARY EFFECTS ON COLORECTAL CANCER IN GENETICALLY DIVERSE MOUSE STRAINS .......................... 64
  ABSTRACT ................................................................................................................... 65
  MATERIALS AND METHODS .................................................................................. 71
  RESULTS .................................................................................................................... 73
  DISCUSSION ............................................................................................................... 81

CHAPTER 4 ....................................................................................................................... 84
GUT MICROBIOME COMPOSITION DIFFERS WITH DIET AND GENETIC BACKGROUND ........................................................................... 85
  ABSTRACT ................................................................................................................... 86
  INTRODUCTION ........................................................................................................... 92
  MATERIALS AND METHODS .................................................................................. 93
  RESULTS .................................................................................................................... 95
LIST OF TABLES

Table 1. Comparison of abundance (% of total) for B6 mice on standard (Stand) vs. ketogenic (Keto) or Western (West) diet. ................................................................. 103
Table 2. Comparison of abundance (% of total) for FVB mice on standard (Stand) vs. ketogenic (Keto) or Western (West) diet. ................................................................. 103
Table 3. Comparison of species abundance (% of total) for B6 mice on standard (stand) vs. ketogenic (keto) or Western (west) diet. ................................................................. 105
Table 4. Comparison of species abundance (% of total) for FVB mice on standard (stand) vs. ketogenic (keto) or Western (west) diet. ................................................................. 106
Appendix Table 5. Diet ingredients. ......................................................................................... 142
Appendix Table 6. Macronutrient ratios of diets and relative contribution of ingredients... 143
Appendix Table 7. Comparison of beta-hydroxybutyrate (BHB) concentrations by diet and strain........................................................................................................... 147
Appendix Table 8. Primers sequences used for qPCR assays. ...................................................... 150
Appendix Table 9. Expression comparison of thermogenesis-related genes in A strain mice on ketogenic vs. standard diet ................................................................. 151
Appendix Table 10. Survival rate in Cancer Study NCSU Cohort 1 .............................................. 152
Appendix Table 11. Survival rate in Cancer Study NCSU Cohort 2 .............................................. 152
Appendix Table 12. Survival rate in Cancer Study TAMU Cohort .............................................. 152
Appendix Table 13. Effects of variables influencing microbiota composition at the phylum level ................................................................................................................. 153
Appendix Table 14. Effects of variables influencing microbiota composition at the species level ................................................................................................................. 153
LIST OF FIGURES

Figure 1. Effects of diets on adiposity compared to standard diet (baseline) in each mouse strain. .......................................................................................................................................................................................................................... 38
Figure 2. Effects of diets on glucose tolerance compared to standard diet (baseline) in each mouse strain. ............................................................................................................................................................................................................. 40
Figure 3. Effects of diets on insulin tolerance compared to standard diet (baseline) in each mouse strain. ......................................................................................................................................................................................................... 41
Figure 4. Effects of diets on total cholesterol compared to standard diet (baseline) in each mouse strain. ........................................................................................................................................................................................................... 42
Figure 5. Effects of diets on LDL cholesterol compared to standard diet (baseline) in each mouse strain. ........................................................................................................................................................................................................ 43
Figure 6. Effects of diets on HDL cholesterol compared to standard diet (baseline) in each mouse strain. ......................................................................................................................................................................................................... 44
Figure 7. Effects of diets on mean arterial pressure compared to standard diet (baseline) in each mouse strain. ........................................................................................................................................................................................................ 46
Figure 8. Effects of diets on liver triglyceride concentration compared to standard diet (baseline) in each mouse strain. ........................................................................................................................................................................................................ 47
Figure 9. Effects of diets on ALT concentration compared to standard diet (baseline) in each mouse strain. .......................................................................................................................................................................................................... 49
Figure 10. Effects of diets on oxygen consumption compared to standard diet (baseline) in each mouse strain. ...................................................................................................................................................................................................... 50
Figure 11. Effects of diets on heat expenditure compared to standard diet (baseline) in each mouse strain. ........................................................................................................................................................................................................ 51
Figure 12. Effects of diets on activity compared to standard diet (baseline) in each mouse strain. .......................................................................................................................................................................................................... 51
Figure 13. Effects of diets on food consumption (calories/per) compared to standard diet (baseline) in each mouse strain. ........................................................................................................................................................................................................ 54
Figure 14. Effects of diets on feed efficiency (calories per gram of weight gain) compared to standard diet (baseline) in each mouse strain. ...................................................................................................................................................................................................... 55
Figure 15. Effects of diets on respiratory control ratio (RCR) compared to standard diet (baseline) in each mouse strain. ...................................................................................................................................................................................................... 57
Figure 16. Colorectal cancer incidence in the United States compared to Japan from 1955-1995. Figure adapted from [109]. ........................................................................................................................................................................................................... 68
Figure 17. Effect of diets on tumor penetrance in each inbred strain. ................................................................................................................................................................................................................. 74
Figure 18. Likelihood ratio of colorectal cancer compared to standard diet (baseline) in each inbred strain. ....................................................................................................................................................................................................... 75
Figure 19. Effect of diets on average tumor size in each inbred strain. ................................................................................................................................................................................................................. 76
Figure 20. Effect of diets on tumor multiplicity in each inbred strain. ................................................................................................................................................................................................................. 78
Figure 21. Effect of diets on average tumor load in each inbred strain. ................................................................................................................................................................................................................. 79
Figure 22. Phylum-level diet effects in B6 mice. ................................................................. 97
Figure 23. Effects of diets on species richness. ................................................................... 100
Figure 24. Relative abundance of *Parabacteroides distasonis* in B6 and FVB mice........ 107
Appendix Figure 25. Macronutrient compositions of diets. ..................................................... 144
Appendix Figure 26. Change in body fat after two months by feeder type, diet, and strain. 148
Appendix Figure 27. Feeder Comparison. ............................................................................. 149
CHAPTER 1

INTRODUCTION
MODELING DIETARY RESPONSE IN MICE: IMPACT OF INDIVIDUAL VARIABILITY

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ABSTRACT

Diet has been long known to play an important role in human health and disease with national dietary guidelines changing as new knowledge is gained on the impact of dietary nutrients. However, dietary guidelines are written with the assumption that individuals have similar diet responses and that a particular type of diet is optimal for all individuals. Recent studies have begun to provide evidence that physiological effects of diet can vary among individuals. While few studies have examined how individuals respond differently to diet depending on their genetic makeup, most dietary studies have evaluated effects of diet across populations. Consequently, most research has sought to identify average response to diet, but average response may not be indicative of individual response. We review the history of US nutritional guidelines, evidence that average population responses are not predictive of individual response, and how mouse models can be used to study diet effects on individuals.
INTRODUCTION

Diet has been long known to play a critical role in human health and disease. Preventable diseases that are profoundly influenced by diet, including cardiovascular disease, cancer, and diabetes, are responsible for the majority of disease-related deaths in the United States [1]. Rates of overweight, obesity and associated metabolic syndrome, important diet-dependent risk factors for these diseases, have greatly increased over the last half-century. Today, more than two-thirds of Americans are overweight or obese with the trend of excess weight starting earlier in life than previous generations [2]. While the consequences of poor diet are apparent, defining what type of diet people should eat in order to avoid these maladies is less clear. A major obstacle is that most diet studies have evaluated effects on populations, but few have studied individual responses to diet. Herein we review the historical basis of dietary research and discuss how mice can be utilized to accurately model variable diet responses in genetically diverse individuals.
HISTORICAL PERSPECTIVE ON DIETARY RECOMMENDATIONS

United States Dietary Guidelines

Dietary recommendations have existed in the United States for more than a century. In 1894, the United States Department of Agriculture’s (USDA) first dietary guidelines were published in a Farmers’ Bulletin. Though specific vitamins and minerals had not been identified, W.O. Atwater suggested diets based on content of protein, carbohydrate and fat [3]. In 1916 the USDA released its first food guide which grouped food into specific categories [4]. Recommendations on appropriate consumption of these food groups was released the following year [5]. Dietary recommendations were updated sporadically with the goal of preventing malnutrition.

In the 1950s, a shift was made from prescribing dietary recommendations to avoid malnutrition to suggestion avoidance of certain food components based on associations with disease. During that time, American researcher Ancel Keys originated the theory that people’s consumption of saturated fat and cholesterol, two dietary components that have received disproportionate attention over the last half-century, led to increased serum cholesterol and eventually heart disease. The theory was, in part, driven by his Seven Countries Study which found that countries in which people ate more fat and cholesterol had higher rates of heart disease [6]. The American Heart Association (AHA) adopted Key’s theory and in 1961 it recommended that Americans lower their intake of saturated fat [7]. In 1977, the United States government followed suit with their recommendations in the Dietary Goals report [8].
The federal dietary guidelines remained largely unchanged for decades even as obesity rates increased and health status of Americans decreased. Over time, there was growing skepticism about the role of saturated fat and cholesterol in heart disease because an emerging body of evidence began to suggest that dietary cholesterol is only moderately associated with heart disease risk [9]. In fact, certain types of saturated fats may have neutral or even protective effects against heart disease. Furthermore, increasing sugar intake to replace saturated fat appeared to increase the risk of developing heart disease [10].

The latest Dietary Recommendations for Americans has started to shift to account for our changing understanding of the effects of diet. The 2015 Dietary Recommendations for Americans recommended reduction of sugar intake to less than 10% of calories. While low saturated fat intake is still recommended, restrictions have been lifted on total fat and cholesterol consumption, as dietary cholesterol is no longer thought to be a major risk factor for increased serum cholesterol [11].

The USDA has attempted to provide the best diet advice available based on the current scientific understanding, but the practice of prescribing Dietary Recommendations for Americans in itself is flawed. Making such recommendations assumes that all individuals respond similarly to diet. However, the multitude of genetic and physiological differences between individuals necessitates unique dietary requirements.

Confounding the elucidation of dietary effects on health, the Dietary Recommendations for Americans rely heavily on reductionist science that attempts to identify the effect of single variables on health outcomes. This approach has been useful in many areas of science, such as biochemistry to identify how two proteins interact. However,
the same approach used in nutrition to identify how single food constituents impacts health is largely uninformative because people do not eat single food constituents, they eat food. The same food constituent may have different effects under different dietary contexts. For example, a high fat diet may be deleterious when combined with high sugar but innocuous when eaten the context of a low carbohydrate diet.
INDIVIDUAL VARIATION IN DIET RESPONSE

Discrepancies Between Population and Individual Responses

For decades there has been data suggesting that identical nutritional interventions can cause diverse responses in individuals. Correlations between nutritional interventions and a response can be significant when analyzed at a group level, but the correlations may disappear when individuals were assessed independently. Researchers encountered this problem when trying to interpret the relationship between dietary fat composition and serum cholesterol. Several population studies showed that when group averages were investigated, saturated fat and/or dietary cholesterol intake were positively associated with serum cholesterol [6, 12, 13]. Controlled studies in metabolic wards found a similar result when comparing group means. However, studies of individuals repeatedly showed weak or no correlation between dietary fat composition and serum cholesterol [14-20]. Individuals given the identical dietary intervention failed to produce the same response, indicating that diet alone could not account for changes in serum cholesterol [21-26].

This paradox was largely viewed as an inconvenience. It was thought that the association between dietary fat and serum cholesterol identified at a group level must be representative of the response in most individuals. Statistical methods were created to “fix” the problem of the individual data not agreeing with the group data [27]. Discrepancies between group and individual data were attributed to sample size, study duration, participant cooperation, contribution of peripheral dietary components, measurement error, and statistical analysis. Almost every component of the study designs were considered, except
that the paradox may instead be indicative of an underlying reality. Individuals might actually have a heterogeneous response and group means may be of no predictive value when considering individual response to diet.

“Normal” Phenotypic Response

One can envision a scenario in which the group mean response is not indicative of the response of most individuals in a group. For example, a stimulus could cause no response in the majority of a population. But, if a small subset in the population has a very strong, positive response, then the group mean would show a moderate positive association for the group. The group mean would not be informative when predicting the response of individuals in this group. Ultimately, group mean responses are only informative on an individual level when members of the group have similar responses, such as mice within one inbred strain. Even then, epigenetics is known to underlie differential responses in otherwise genetically identical individuals [28].

Many nutritional and medical studies focus on the group mean to ascertain the effect of a treatment. The underlying assumption is made that by testing a large number of individuals and determining the average or “normal” response to a treatment, we will be able to predict how the vast majority of individuals (i.e. the normal individuals) will respond to the treatment. This approach has been slowly evolving in the therapeutic field with the appreciation that individuals can have varying responses to pharmaceuticals, but the one-size-fits-all model is still widely adopted in nutritional studies.
No individuals are truly “normal” or “average.” A fitting example of this is outlined by Dr. Roger J. Williams in *Biochemical Individuality* [29]. If 95% of a population is normal with regard to one measurable phenotype, only 90.2% (.95²) of the population would be normal when considering two measurable phenotypes. Only 60% of the population would be normal with regard to 10, and less than 1% would be normal with regard to 100. Therefore, when considering the endless comparisons that can be made between individuals, there is no meaningful definition of “normal.”

It is important to remember what group means tells us about a population. Dr. Williams provides examples of two types of groups and compares the consequences of study each group [29]. Group I is a population of ten men, all with the same height, same amount of hair, same tendency to put on gain body fat, and same tendency to drink alcohol. Each member of the group has an exactly average phenotype. Group II is another population of ten men that has the same group average, but individuals vary in their response to each phenotype. One man is 6’6”; one man is bald; another is rotund and has difficulty losing weight; and one man has a high desire to drink alcohol. For Group I, the problem of finding a hotel bed long enough does not exist; and baldness, obesity, and alcoholism are not serious problems. In Group II, all of these problems exist.

Human populations are composed of phenotypically diverse individuals, much more similar to Group II. In order to address and treat the problems present in Group II, we cannot solely investigate the average response of the population. The problems in Group II “cannot be solved until we become conversant with the nature, magnitude, and distribution of the underlying deviations [29].”
When nutrition researchers analyze a wide range of diverse individuals based on group mean response or “normal” response, they are treating the population as a Group I population. Their discoveries are designed to benefit “normal” individuals, who may not actually exist. When considering nutritional requirements, people are interested in their individual needs, not the needs of the statistical mean of a population. It is important to uncover the mechanisms underlying individual differences so that individuals can be treated as such.
EVIDENCE FOR VARIATION IN INDIVIDUAL DIET RESPONSE

New lines of evidence further support evaluation of diet response on an individual basis rather than by population means. A recent study by Zeevi et al. sought to identify algorithms to predict postprandial glucose response (PPGR), an important measure for impaired glucose tolerance, which is a risk factor for development of type II diabetes mellitus [30]. While a number of assays had been developed to estimate the effects of specific foods on blood glucose levels, previous smaller studies had indicated the existence of large inter-individual variation in PPGR [30]. Thus, techniques that predicted glucose response based on population averages were largely uninformative when attempting to predict response in individuals. Zeevi et al. evaluated PPGRs in 800 individuals after eating several types of meals and found “high variability in the response to identical meals, suggesting that universal dietary recommendations may have limited utility.” The study showed that it was not the food alone that determined PPGR but the interaction of the food with the individual.

Recent studies on amylase copy number variation have highlighted how genetic differences can impact response to diet. Fachi et al. found that decreased copy numbers of the salivary amylase gene (AMY1) led to lower serum enzyme levels and increased risk of developing obesity [31]. Those individuals with the lowest copy number (n < 4) had an eightfold increased obesity risk versus those with the highest copy number (n > 9). This was the first study to provide a genetic link between carbohydrate metabolism and obesity. Future studies will inevitably identify additional genes that modify response to diet, but of perhaps greater importance is an understanding of how genes interact to modify response to diet.
IMPACT OF GUT MICROBIOME ON DIET RESPONSE

Gut microbiome varies among individuals based on their diet and genetics and has been implicated in a variety of health conditions including obesity, colonic inflammation, and colorectal cancer [32]. Studies in mice have demonstrated that genetic background strongly influences the composition of gut microbiota. A study in BXD mouse lines (recombinant inbred strains derived from a C57BL/6J and DBA/2J cross) found strong associations between genetic background and microbiota composition and identified several QTL with candidate genes underlying the strain effects [33]. A similar study found strong associations between genetic background in mice and identified 18 QTL linking genetic factors with microbiota composition [34].

Diet can strongly impact gut microbiota composition. A study examined gut microbiota shifts in 100 inbred mouse strains after feeding mice a high-fat, high-sugar (HF/HS) diet [35]. There were 17 genera overall that shifted between mice HF/HS diet and mice eating a chow diet. While some strains had large shifts in microbiota composition due to diet effects, others had minimal fluctuation. This indicates the plasticity of microbiota composition after HF/HS feeding was strongly correlated with genetic background.

There is strong evidence supporting the role of gut microbiome in obesity. In 2005, researchers inoculated germ-free mice with gut microbiota from conventionally raised, obese mice. The mice had a 60% increase in body fat despite a 29% decrease in food consumption and 27% increase in activity compared to unaltered germ-free mice [36]. Another group confirmed the finding and found the trait was hereditarily transmissible [37].
Dietary shifts influence gut microbiota in people, and these shifts alter certain CRC risk factors. A study compared the diet of rural South Africans to African Americans, who have a 13 times higher CRC incidence rate, to investigate the role of the Western diet [38]. Microbiota composition was analyzed and colon biopsies were taken to measure inflammation and proliferation before and after the groups swapped diets for two weeks. Dietary changes caused shifts in microbiota functional gene abundance, including down-regulation of the butyrogenesis pathway associated with Western diet. This is significant as butyrate, a short chain fatty acid produced by gut bacteria, is protective against CRC [39]. The detrimental microbiota effects of the Western diet were associated with increased markers proliferation and inflammation, which are risk factors for CRC. A number of other studies have correlated changes in the gut microbiome with colorectal cancer [40-42].
MOUSE MODELS AS A TOOL TO STUDY INDIVIDUAL VARIATION IN DIET RESPONSE

Human studies often compare populations rather than individuals out of necessity. People, excluding identical twins, have diverse genetic compositions. Comparing multiple diets in the same individual simultaneously is not practical. When different diets are compared consecutively on the same individual, it is difficult to determine the effect of timing differences or the order of diet exposure. As such, studies in humans have typically compared average treatment responses in groups composed of genetically diverse individuals.

It is possible to study multiple treatments simultaneously in genetically identical individuals by using inbred mouse models. In addition to comparing effects of multiple treatments in a single strain, the effects of those treatments can be compared across mouse strains that differ genetically. Dr. William’s analogy of two groups is applicable to approaches using inbred mouse models. A single inbred mouse strain would be comparable to Group I. All individuals have the same genetic makeup, thus their phenotypes are highly similar and the group mean response is indicative of the true response of most or all individuals in the strain. Conversely, a panel of mouse strains is comparable to Group II. Each strain in the panel has a distinct genetic makeup, thus their phenotypes are highly divergent (at least in some instances). The group mean response may not be indicative of the true response for any individual mouse strain. Analyzing responses of individuals (within
strain) and groups (between strains) of mice can provide a powerful tool to understand the variability in response to diet present in human populations.

Using inbred mouse strains, the effects of diet both within and between strains can be used to identify how genetic background of an individual modulates response to diet. The genetic factors underlying differential responses to diet can be identified using mouse models, which can inform human diet responses. Ninety-nine percent of mouse genes have a functional ortholog in humans, and humans and mice have similar physiology and digestive systems [43].

**Examples of Individual Variation in Mouse Studies**

Animal studies further highlight the importance of considering the individual when comparing effects of diet. One such study compared the effects of high fat, high sugar diets on A/J and C57BL/6J inbred mouse strains. A/J mice are less susceptible to development of diet-induced obesity and diabetes than C57BL/6J mice [44]. A later study investigated the propensity of mice to develop obesity on high-fat, high-sucrose diets [35]. Strain specific differences in propensity to develop obesity were reported, and polymorphisms at 11 loci were associated with the effect. These loci were shown to have significant overlap with those identified in human genome-wide association studies, further validating the overlap of genetic factors involved in diet response in humans and mice.

Dietary restriction (DR) has been shown to have life prolonging effects in a variety of species. However, a study examining dietary restriction in recombinant inbred mouse lines found that while some strains experienced increased lifespan, other strains had no effect or
suffered decreased longevity [45]. Opposing results from caloric restriction in rhesus monkeys have also been reported [46, 47]. Genetic differences between the populations of rhesus monkeys were likely not considered. While some studies have shown that, on average, individuals have a longer lifespan under DR, the benefit seems to be dependent on genetic background with some individuals experiencing negative effects from the same treatment.

These studies conclusively demonstrate that genetic background of an individual is critical when evaluating the effects of dietary interventions. However, there is a dearth of studies investigating how an individual’s genetic background influences their response to a variety of diets reported to have different health effects in humans. To address this gap in knowledge, my dissertation is centered on identifying physiological diet responses in four inbred mouse strains using diets extensively studies in humans. By feeding human relevant diets to inbred mouse strains and analyzing phenotypes including cancer and those related to metabolic syndrome, we can identify the degree to which genetic background influences diet responses and begin identifying mechanisms and genetic factors underlying variation in diet response.
DIETS OF INTEREST FOR STUDYING INDIVIDUAL DIET RESPONSE

Rationale for Diets in this Study

In order to best model how humans respond to a diet, it is worthwhile to construct diets that are based on human diets. In this section, we outline the historical basis for several diets that have been used for studies in our lab.

*Mediterranean Diet*

Mortality data from the World Health Organization Database provides evidence that people living in the Mediterranean region between 1960 and 1990 had longer life spans and lower rates of chronic disease than would be expected given the area’s high rates of smoking and inferior health care compared to North America and northern Europe [48]. A key component that seems to be driving the good health in the region is diet. The traditional Mediterranean diet is comprised of high monounsaturated-to-saturated fat ratio, moderate alcohol consumption, high consumption of legumes, cereals, fruit, and vegetables, low consumption of meat, and moderate consumption of milk and dairy [49].

A recent prospective study supported previous epidemiological evidence of the benefits of a Mediterranean diet. In a study of over 7,000 adults in Spain, those with greatest adherence to a Mediterranean-style diet had a 30% reduction in cardiovascular disease [50]. Other prospective studies have consistently found 8-25% reductions in all-cause mortality for individuals strictly adhering to a Mediterranean diet [51-53].

*Japanese Diet*
For several decades Japan has had the longest life expectancies and among the lowest rates of chronic disease. The Japanese diet has been suggested to promote these beneficial health effects. The traditional Japanese diet is low in fat and protein, and high in carbohydrates. Rice is a staple of the diet and fish is a main protein source. There is evidence that compounds found in green tea and soy used in the Japanese diet also have beneficial health effects.

Rates of coronary heart disease (CHD) in Japan have historically been among the lowest in the world. In contrast, the United States has had among the highest in the world. Migrant studies indicate that environmental differences cause the disparity between CHD rates between the countries. A gradient of CHD risk exists between Japanese living in Japan, Hawaii, and mainland United States with Hawaii having an intermediate rate of CHD between Japan (low) and the United States (high) [54]. A follow-up study found that another gradient exists within the Hawaiian population, with greater adherence to the traditional Japanese diet yielding lower rates of CHD [55].

Similar patterns are observed for cancer incidence among Japanese and Japanese-Americans. Japanese migrating to the United States experience increased rates of breast, colon, uterine, ovary, and prostate cancers within one generation [56-58]. Similarly, regular consumption of Western-style meals increased the risk of developing large bowel cancer in Japanese-Hawaiian populations [59]. The opposite pattern is observed for gastric cancer, which has higher rates in Japan than the United States [60]. It has been speculated this may be due to the high salt content of the Japanese diet [61].

*Western Diet*
Advances in healthcare have increased life expectancy and reduced deaths from communicable diseases in the United States. However, rates of non-communicable diseases such as heart disease, diabetes, and cancer have increased due to changes in lifestyle and dietary patterns. Changes in the American lifestyle and technological innovations have greatly altered the American diet over the last 100 years. Food manufacturers have responded to Americans’ increasingly fast-paced lifestyle by designing highly palatable foods that require little or no preparation and can be eaten on the go. These changes have resulted in a calorically dense diet that is high in fat and sugar and low in fiber. Adoption of the Western-style diet by other countries is associated with increased rates of non-communicable diseases, as seen in the United States [62]. As previously discussed, migration studies indicate that the Western lifestyle and particularly diet is associated with increased risk of cancer and CHD [55-58]. Together, these data indicate that at a population level, the Western diet induces negative health consequences.

Ketogenic Diet

The ketogenic diet is a high-fat, low- or no-carbohydrate diet that induces nutritional ketosis. Ketosis is a state in which the body’s energy demands are largely supplied by ketones released from the oxidation of fats. In a normal nutritional state, the body’s energy demands are supplied by glucose via glycolysis. Ketogenic diets have been used for over 100 years in the prevention of seizures and are also used as a treatment for type II diabetics [63]. Ketosis induces a physiological state that is similar to fasting. Fasting has been recognized as a treatment for epilepsy dating back to the fifth century BC when Hippocrates reported a man had cured his epileptic convulsions by abstaining from food and drink [63,
A story from the Bible detailed Jesus curing an epileptic boy with fasting [65, 66]. When his disciples inquired why they could not cure the boy, he stated “this kind can come out by nothing but prayer and fasting.” In the early 20\textsuperscript{th} century, scientific evidence began to support fasting as a treatment for epilepsy [67-69].

While fasting was an effective treatment, it is obviously not a long-term solution. In the 1920s, researchers found that individuals eating low-carbohydrate, high-fat diets had elevated urine ketone levels just like individuals undergoing a fast [70]. The diet, which became known as the ketogenic diet, was as successful at preventing seizures as fasting but allowed individuals to maintain adequate nutrition [68, 71-73]. The ketogenic diet has remained a viable treatment for a subset of epileptic patients, particularly for children whose seizures do not respond adequately to typical anticonvulsant medications.

As the obesity epidemic has grown, the ketogenic diet has been investigated for its effects on weight loss. A 24-week study compared 120 people following a ketogenic or low-fat diet [74]. The ketogenic diet group had better diet adherence and lost twice as much weight as the low-fat group. A controlled, clinic study found that short-term administration of a high-protein ketogenic diet reduced hunger and food consumption versus a medium-carbohydrate diet [75]. A recent meta-analysis found that individuals following very low-carbohydrate ketogenic diets achieved greater weight loss than low-fat dieters in the long-term [76]. Other research indicates the ketogenic diet can have beneficial effects on cardiovascular health [74].

The ketogenic diet is also being investigated as a potential cancer therapy. Otto Warburg identified that cancer cells depend heavily on glucose for energy, as they have
glycolytic rates up to 200 times higher than normal cells [77, 78]. Cells with normal mitochondrial function are able to use ketones for fuel, whereas cancer cells cannot [79-81]. It is plausible that ketosis, which increases usage of ketones for fuel while decreasing glucose usage, could induce a state unfavorable to tumor growth. There is evidence that ketogenic diets can reduce tumor size in mice [82, 83], with studies suggesting that ketogenic diets can reduce tumor progression in brain and gastric cancers [84-87]. However, the effect of ketogenic diet on cancer is still in its infancy.
CHAPTER 2

DIET, GENETIC BACKGROUND, AND METABOLIC SYNDROME
PRECISION NUTRITION TO OPTIMIZE METABOLIC HEALTH IN MICE

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\textsuperscript{1} Liver triglyceride analysis

\textsuperscript{2} Cholesterol analysis

\textsuperscript{3} Insulin and leptin analysis

\textsuperscript{4} Histological analysis

\textsuperscript{5} UNC cohort phenotyping
ABSTRACT

Epidemiological studies have associated dietary patterns of populations with various health outcomes but when diet is studied at an individual level, highly variable responses are usually observed that often do not match the average response of the population. The increase in diet-associated diseases in industrialized countries necessitates dietary strategies that are effective for diverse individuals. To explore the degree of variability of diet response in genetically distinct individuals and whether different diets are healthiest for different individuals, four inbred mouse strains (A/J, C57BL/6J, FVB/NJ, and NOD/ShiltJ) were fed one of five diets (Western diet, traditional Mediterranean diet, traditional Japanese diet, a ketogenic diet, and a standard laboratory mouse chow) for six months while monitoring metabolic syndrome-associated phenotypes. We found that diet response was highly variable and dependent upon the genetic background of the individual. When fed the same diet, different strains had positive, negative, or neutral responses for a given phenotype compared to standard mouse chow. Obesity was caused by either increased food consumption or increased feed efficiency depending on the diet and strain, while some strains were resistant to diet-induced obesity. As seen in human studies, we observed that the average diet response when combining all individuals (all strains) was often not indicative of the response in any individual strain. This study demonstrates the highly individual nature of diet response. It underscores the need to examine the effect of diet with respect to individuals rather than
populations in order to identify effective interventions for diet-related maladies, such as metabolic syndrome.
INTRODUCTION

Epidemiological studies have shown that dietary patterns across populations are strongly correlated with the spectra of diseases [88, 89], and as dietary patterns change, so do disease spectra [90]. However, a major limitation of population-level dietary studies is the absence of information on the relationship between individual and population-level responses. Public interest in precision medicine has not extended into dietary or ‘precision nutrition’ considerations despite recent studies suggesting that people can differ dramatically in their response to identical diets, questioning the utility of universal dietary guidelines [30]. The one-size-fits-all approach has been pervasive since the United States Department of Agriculture (USDA) published the first guidelines in 1985 [91], and continues today with the MyPlate nutrition guide [92].

Efforts to identify macronutrient compositions that support optimal health continue. In one study, C57BL/6J (B6) mice were fed one of 25 diets that had identical ingredients but varied widely in macronutrient ratios and concluded that B6 mice fed high-carbohydrate, low-protein diets generally displayed healthy cardiometabolic phenotypes and long life spans [93]. Evaluating the effect of diet in a single inbred strain is comparable to studying the response in a single person. While the result is informative for one individual, it cannot be generalized to a population. Furthermore, it is unclear how the large variation in the source of ingredients, as seen in the diversity of human diets, further complicates the physiological effects. A host of research suggests that solely altering macronutrient ratios is insufficient to accurately model the broad range of diets human populations consume. Given the same
macronutrient ratio, factors including ingredient source, fiber content, and fatty acid profile can greatly impact the physiological effects of a diet [94-98].

Researchers attempting to interpret the relationship between dietary fat composition and serum cholesterol encountered this problem. Several population studies had shown that when group averages were investigated, saturated fat and/or dietary cholesterol intake were positively associated with serum cholesterol [6, 12, 13]. However, studies at the individual level repeatedly showed a weak or no correlation between dietary fat composition and serum cholesterol [14-20]. In controlled studies, individuals given an identical dietary intervention failed to produce the same response, indicating that diet alone was not reasonable for changes in serum cholesterol [21-26]. Discrepancies between population and individual data were mainly attributed to limitations of the study design including sample size, study duration, participant cooperation, contribution of peripheral dietary components, measurement error, and statistical analysis [27]. Seemly everything was considered, except that the paradox may be indicative of an underlying reality; genetic differences may be the major contributor to individual dietary responses and group means are not indicative of the true response of any individual. Thus, dietary recommendations aimed to benefit the hypothetical person who matches the group mean, as is currently done by the USDA recommendation, may not be the healthiest diet for any individual.

To elucidate the relationship between the average metabolic health response to diet of a population to the response of individuals within that population, we compared responses in four genetically diverse mouse strains to four human relevant diets for which extensive epidemiological health outcome data is available. The diets represent current Western diet,
traditional Mediterranean diet, traditional Japanese diet, and a ketogenic diet. At the population level in humans, the high-fat, high-sugar Western diet is associated with poor health outcomes, while the traditional high-fat Mediterranean and low-fat Japanese diets are associated with healthy metabolic effects. The ketogenic diet, which is high in fat with little or no carbohydrate, creates a unique metabolic state and has been utilized for weight loss and to reduce the severity of diseases like epilepsy and diabetes [63, 75, 79]. When constructing diets for these studies, macronutrient ratios but also ingredients, fatty acid profiles, and fiber content were accurately matched to recapitulate the average human diet as closely as possible. The results encourage an approach to ‘precision’ nutritional studies that embrace individual differences rather than attempting to fit individuals to an average that is reflective of few individuals.
MATERIALS AND METHODS

Animals and Husbandry

Four-week old A/J, C57BL/6J, FVB/NJ, and NOD/ShiltJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Two equally sized and identical cohorts of five mice per diet, sex and strain were studied in facilities at two different locations, North Carolina State University and Texas A&M University. Timelines for each study are listed in the appendix (Appendix Figure 26). Mice were acclimated to the research facilities during a two-week acclimation period prior to entering the study. At both facilities mice were housed five per cage, and maintained at 25°C under a 12-hour light cycle with lights on at 0700. Mice were maintained and protocols followed in accordance with North Carolina State and Texas A&M IUCAC guidelines. Mice were given ad libitum food access for the six-month duration of the study.

Diets

Powdered diets were custom designed with assistance of scientists at Research Diets, Inc. (New Brunswick, NJ). Traditional Mediterranean and Japanese diets were based on the Food and Agriculture Organization’s Food Balance Sheets from Greece and Japan in 1961 (APPENDIX B. DIET PREPARATION AND COMPOSITION) [99]. The current Western diet was based on USDA’s 2008 Dietary Assessment of Major Food Trends [100]. The ketogenic diet was based on a previous study, with menhaden oil added to provide omega-3 fats [101]. Standard mouse chow (Research Diet’s D12052701) was a typical maintenance
laboratory mouse diet composed of casein protein, cornstarch, and soybean oil. All diets were formulated in a powdered form, as it was impossible to pellet some of the diets while maintaining the desired nutrient composition.

**Body Composition**

Body composition was assessed in the NCSU and TAMU cohorts at baseline, and at the two-, three-month time points using an EchoMRI™-130 Body Composition Analyzer. A six-month time point was also conducted in the TAMU cohort.

**Blood Pressure**

Blood pressure was measured in the TAMU cohort with a MC4000 automated four-channel tail-cuff system (Hatteras Instruments). Mice were trained for three days prior to three days of testing. Each measurement graph was analyzed on MC4000 software and those with signs of animal movement were discarded. Quality control was performed by removing measurements with diastolic values less than 50 and measurements in which the systolic reading was more than 170% of the diastolic reading.

**Food Consumption**

Twenty-four hour food consumption was measured in the TAMU cohort when mice had been on diets for three months by singly housing in wire bottom cages with a paper filter at the bottom of the cages to collect spilled food (Appendix B). Starting, ending, and spilled food weights were collected. Mice were acclimated for two separate 24-hour periods prior to
two 24-hour testing periods. Mice were group housed for three days between testing periods to minimize stress.

**Glucose Tolerance**

Basal glucose levels were measured after a six-hour fast in mice at in the NCSU and TAMU cohorts after 4 months on diet. Glucose (2 g/kg) was administered via oral gavage. Blood glucose levels were measured with a Bionome GM100 glucose monitor (Bionome USA Corp.) at 30, 60, 90, and 120 minutes. The area under the curve was calculated for each mouse.

**Insulin Tolerance**

Insulin tolerance tests were performed on mice in the TAMU cohort at after 4.5 months on diet. Basal glucose levels were measured after a four-hour fast. Insulin (0.5 U/kg) was administered via intraperitoneal injection. Blood glucose levels were measured again at 15, 30, 60, 90, and 120 minutes. The area under the curve was calculated for each mouse.

**Insulin and Leptin**

Serum was collected following a six-hour fast after mice were fed diets for three months in the TAMU cohort. Serum insulin and leptin concentrations were quantified using a Mouse Serum Adipokine Immunoassay ELISA kit (Millipore).

**Liver Triglycerides**
Following necropsy, liver triglycerides were quantified in the NCSU and TAMU cohorts. Liver triglyceride isolation was based on a previous protocol [102]. Briefly, 50 mg pieces of liver were homogenized in a 2:1 choloform-methanol solution. After 30 minutes of incubation, a sodium chloride solution was added to the solution and vortexed. The lower phase was decanted and evaporated under nitrogen steam. Each sample was resuspended in a 0.5% Triton X-100/PBS solution. After sonication, samples were incubated at 55°C for 5 minutes. Infinity Triglyceride reagent (Thermo Scientific) was added and samples were incubated for 5 minutes at 37°C. Absorbance at 500 nm was measured and compared to a standard curve to quantify triglyceride concentration.

**Metabolic Rate and Activity**

Mice in the NCSU cohort were singly housed in Phenomaster metabolic chambers (TSE Systems) for 48 hours after 3 months on diet. Metabolic rate was measured by indirect calorimetry and activity level was assessed by laser detection.

**Liver Histology**

Formalin-fixed, paraffin-embedded right lobe liver samples were sectioned at 5 μm and stained with hematoxylin and eosin. The extent of steatosis was assessed in a blinded fashion by a board-certified veterinarian pathologist using a previously reported scoring system for non-alcoholic fatty liver disease [103]. Briefly, the scoring system for macrovesicular steatosis, microvesicular steatosis, and cellular hypertrophy was based on the percentage of hepatocytes within the stained section. These parameters utilized the following
categories: 0 (<5% of hepatocytes), 1 (5-33%), 2 (34-66%), and 3 (>66%). Inflammation was evaluated by counting the number of inflammatory foci per field, averaged across of 5 fields of view at 100X magnification. The level of inflammation was assigned using the following categories: 0 (normal, <0.5), 1 (slight, 0.5-1.0), 2 (moderate, 1.0-2.0), and 3 (severe, >2).

Blood Lipids and Biochemistry

Alanine aminotransferase (ALT) activity was quantified using a fluorometric ALT Activity Assay Kit (Sigma-Aldrich), and aspartate aminotransferase (AST) activity was quantified using an AST Activity Assay Kit (Sigma-Aldrich). After mice had been on a standard diet or ketogenic diet for three months, beta-hydroxybutyrate levels were tested from fresh blood samples using a Precision Xtra blood ketone meter (Abbott Diabetics Care). Serum triglyceride concentrations were analyzed using a colorimetric Serum Triglyceride Determination Kit (Sigma-Aldich). Total, LDL, and HDL cholesterol concentrations were analyzed using a colorimetric Cholesterol Quantification Kit (Sigma-Aldrich).

Mitochondrial Function

Liver mitochondrial function was evaluated using a Seahorse XF Extracellular Flux Analyzer (Seahorse Bioscience), which measures basal respiration, ATP production, proton leak, maximal respiration, glycolysis, and spare respiratory capacity in freshly isolated mitochondria from homogenized tissue. Fresh liver mitochondria were isolated using centrifugation as previously described [104]. Mitochondria concentration was quantified
using a Bradford Assay (Sigma Aldrich). 25 µg was added to each well of the Seahorse XF plate and centrifuged at 2000g for 20 minutes. The Seahorse XF Analyzer was setup to administer 0.25 mM ADP, 2.0 µM rotenone, 2.0 µM oligomycin, 4.0 µM FCCP, and 4.0 µM antimycin A. Samples were run in triplicate and compared to two blanks containing no mitochondria. Results were analyzed visually using Seahorse Wave software (Seahorse Bioscience) and outliers were removed. Samples across plates were normalized to basal measurements and respiratory control ratios under basal conditions were calculated.

**Real Time PCR**

Real time PCR was performed using cDNA generated from brown fat and liver RNA isolated using a Maxwell 16 LEV simplyRNA kit (Promega). Primers for uncoupling protein genes (*Ucp1, Ucp2, Ucp3, Ucp5*) and genes involved in mitochondrial biosynthesis (*Cox1* and *PGC1a*) were used (Appendix Table 8). Analysis was performed on a LightCycler 96 thermocycler (Roche). All samples were run in duplicate. Blank samples using water were included on each plate. PCR reactions were setup on an EpMotion 5075 liquid handling workstation (Eppendorf) to minimize pipetting error. Conditions were 95°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, 55°C for 15 seconds, and 72°C for 60 seconds. A high-resolution melting curve was produced by heating to 95°C for 10 seconds, cooling to 65°C for 60 seconds and 97°C for 1 second, followed by a cooling step of 37°C for 30 seconds. *Cq* values were averaged per sample and normalized to *Hprt* levels before statistical analysis.
Statistical Analysis

Two-way ANOVA was performed for each strain with diet, sex and cohort as factors. Strains were evaluated individually for statistical significance using Dunnett’s corrected $p$-values with standard diet as the control.
RESULTS

Genetic background by diet interactions impact adiposity

A diet’s impact on fat retention is critically important as obesity is a central component of metabolic syndrome and increases the risk of diseases including cardiovascular disease and several types of cancer [105]. Adiposity was evaluated 90 days after beginning the diet.

It is well established that C57BL/6J (B6) mice exhibit diet-induced obesity when fed a high-fat, high-sugar diet [106-108]. As expected, B6 mice fed a Western diet showed greatly elevated adiposity, gaining 86% more fat than mice fed standard chow (Figure 1). B6 mice also had a 72% increase in adiposity when fed a Mediterranean diet. Previous studies reported that fat content of a diet is the main contributor to diet-induced obesity in B6 mice [44]. However, our study and others show that B6 mice fed diets that are high in fat, but limited in carbohydrate content, have less adiposity gain than those diets that are high in fat and carbohydrate such as Western and Mediterranean diets [109, 110]. In our study, B6 mice eating a high fat ketogenic diet had a non-significant trend for increase in adiposity ($p = 0.077$).

FVB/NJ (FVB) mice were unique in that they were susceptible to adiposity gain only when fed the low carbohydrate, high fat ketogenic diet, with a 54% increase in adiposity compared to standard chow. Adiposity in FVB mice did not significantly vary when fed Western, Mediterranean, or Japanese diets.
NOD/ShiLtJ (NOD) mice had a significant increase in adiposity on most diets when compared to standard chow. Increases in adiposity above standard chow were 86% for Mediterranean, 51% for Western, and 48% for Ketogenic. NOD mice showed a non-significant trend towards increased adiposity on the Japanese diet ($p=0.059$).

Unlike the other strains, A/J mice (A) only showed modest increases in adiposity on any of the diets. Previous studies have shown that the A strain is resistant to fat gain on high fat, high sugar diets [44, 106]. We found A mice had a significant increase in adiposity when fed a ketogenic (22% increase) or Western diet (24% increase). However, the adiposity gain was much less extreme than other strains. Significant differences were not observed in A mice eating Mediterranean or Japanese diets.

![Figure 1. Effects of diets on adiposity compared to standard diet (baseline) in each mouse strain. Significance $p<0.05$ indicated by *, $p<0.01$ indicated by **.](image-url)
High fat diets negatively impacted glucose regulation in a strain-dependent manner

A glucose challenge was performed to determine the impact of diet on glucose tolerance, a key component of metabolic syndrome. Previous studies have shown that B6 mice on a Western-style diet have impaired glucose tolerance [111]. In this study, B6 mice on a Western diet showed a 46% increase in the glucose tolerance test (GTT) area under the curve (AUC) compared to B6 mice eating a standard chow (Figure 2). B6 mice eating a ketogenic diet had a similar effect of 56% increased AUC. The Mediterranean diet had a less severe, but still significant 27% increase. The Japanese diet did not significantly alter glucose tolerance in B6 mice compared to standard chow.

FVB mice also experienced detrimental effects on glucose tolerance when eating diets with high fat content. The Western, Mediterranean, and ketogenic diets had similar effects, increasing glucose AUC by 47%, 40%, and 38%, respectively, compared to standard chow. The low fat Japanese diet did not significantly impact glucose tolerance in FVB mice.

Similar to their resistance to adiposity, A mice only had mild effects on glucose tolerance irrespective of diet. Compared to a standard diet, the ketogenic and Western diet produced small, but significant increases of 12% and 10%, respectively.

Despite significant diet-induced adiposity in NOD mice, no significant differences were observed for glucose tolerance. However, this may have been anticipated since NOD mice are genetically predisposed to developing Type I diabetes even on standard chow. Higher standard error values, due to variability in diabetic state, reduced our power to detect significant differences between diets.
Insulin tolerance tests were performed to determine whether mice were insulin-resistant since reduced insulin sensitivity is another indicator of metabolic syndrome. No significant differences were observed between any of the diet/strain combinations (Figure 3).
Figure 3. Effects of diets on insulin tolerance compared to standard diet (baseline) in each mouse strain. Significance p<0.05 indicated by *, p<0.01 indicated by **.

Western and ketogenic diets generally increased total cholesterol but effects on HDL and LDL cholesterol differed

Total, HDL, and LDL cholesterol were measured from plasma in a random sampling of three mice per diet, strain, and sex. Perturbed blood lipid profiles, such as increased LDL cholesterol or decreased HDL cholesterol, are characteristics of metabolic syndrome.

Total cholesterol significantly increased in B6, FVB and NOD fed Western and ketogenic diets (Figure 4). Relative to standard chow, total cholesterol in B6 mice increased by 79% on Western diet and 58% on ketogenic diet. NOD mice experienced a 38% increase on Western diet and 66% increase on ketogenic diet, while FVB mice had increases of 36%
and 38% Western and ketogenic diets, respectively. Although both Western and ketogenic diets tended to increase total cholesterol levels, they had different effects on LDL and HDL cholesterol (discussed in the following sections). Mediterranean and Japanese diets did not cause significant changes in total cholesterol in B6, FVB, or NOD strains. The only significant effect of the Mediterranean diet on total serum cholesterol was in the A strain, which experienced a 31% reduction.

![Figure 4](image_url)

**Figure 4. Effects of diets on total cholesterol compared to standard diet (baseline) in each mouse strain.** Significance p<0.05 indicated by *, p<0.01 indicated by **.

Increased LDL cholesterol concentrations are of particular concern in metabolic syndrome. When fed a Western diet, all strains had LDL cholesterol elevations of 75-176% (Figure 5). A mice had a slight, but significant 31% elevation in LDL on a Japanese diet and
decrease of 37% on the ketogenic diet. The only other change that trended toward significance was an increase in LDL cholesterol in B6 mice fed a Mediterranean diet \((p = 0.053)\), consistent with Mediterranean diet-induced increases in adiposity and glucose tolerance in B6 mice.

**Figure 5. Effects of diets on LDL cholesterol compared to standard diet (baseline) in each mouse strain.** Significance \(p<0.05\) indicated by *, \(p<0.01\) indicated by **.

Increased HDL cholesterol provides protective effects against metabolic syndrome and comorbidities. The ketogenic diet increased HDL cholesterol in all strains (Figure 6). Particularly large increases in HDL cholesterol were observed in B6 (83% increase) and NOD (119% increase) mice, while more modest increases were observed in FVB (41%
increase) and A (29% increase) mice. The ketogenic diet’s positive effect on HDL cholesterol has also been reported in NZO mice, despite causing increased weight gain [112].

The Western diet increased HDL cholesterol only in B6 mice (86% increase), although this was accompanied by an even greater increase in LDL cholesterol noted above. The Mediterranean diet decreased HDL cholesterol by 38% in A mice, accounting for the decrease in total cholesterol observed in A mice on the Mediterranean diet.

Figure 6. Effects of diets on HDL cholesterol compared to standard diet (baseline) in each mouse strain. Significance $p <0.05$ indicated by *, $p <0.01$ indicated by **.
**Effect of diet on blood pressure varies by diet and strain**

Hypertension is a cardiovascular component of metabolic syndrome that can be measured using resting blood pressure, with human studies demonstrated strong effects of diet on blood pressure [113, 114]. Blood pressure was measured three times on five mice per strain, diet, and sex. Mean arterial pressure (MAP) was calculated and though there was high variability in blood pressure measurements, several significant results were observed. The impact of diet on MAP varied considerably depending on genetic background.

Ketogenic diet significantly decreased MAP in NOD (24%) and A (12%) mice (Figure 7). Western diet also decreased MAP in A mice by 16%. In B6 mice, Japanese diet increased MAP by 13% and Mediterranean diet increased MAP by 8%. FVB mice had a 10% decrease in MAP when fed a Mediterranean diet.
Increased liver triglyceride storage was not always indicative of liver damage

Increased triglyceride storage in the liver, known as fatty liver disease in humans, is a sign of perturbed metabolism often associated with metabolic syndrome. Triglyceride concentration was quantified with a colorimetric assay and confirmed by histological assessment.

Mice on Western diet had a consistently large increase in liver triglyceride concentrations across all strains (182-230% increase, Figure 8), while the equally high fat ketogenic diet showed strain specificity in liver triglyceride response. The ketogenic diet
increased liver triglyceride concentrations by 192% in FVB mice and 142% in NOD mice. A smaller increase of 82% was observed in A mice in response to the ketogenic diet, while no significant increase was observed in B6 mice.

Mediterranean diet increased liver triglycerides in B6 and NOD mice, and Japanese diet increased liver triglycerides in A mice. However, the diet-associated changes in triglyceride concentrations were far lower than mice fed Western diet.

Figure 8. Effects of diets on liver triglyceride concentration compared to standard diet (baseline) in each mouse strain. Significance p <0.05 indicated by *, p <0.01 indicated by **.
Increased liver triglyceride storage was not always indicative of liver damage

To determine the impact of increased triglyceride storage on liver function, alanine transaminase (ALT) and aspartate transaminase (AST) levels in the serum were analyzed. All strains experienced a strong increase in liver triglyceride storage when fed Western diet, but only NOD mice had an accompanying increase in ALT levels (Figure 9). AST concentrations were not significantly altered in NOD mice fed Western diet.

Mice of the A strain showed increased markers of liver damage compared to the other strains. Interestingly, their liver triglycerides increased when fed Japanese diet and this increase was accompanied by significant rises in both AST and ALT levels. On a Mediterranean diet, A strain mice experienced increased AST and ALT levels without significantly increased liver triglyceride concentration.
Figure 9. Effects of diets on ALT concentration compared to standard diet (baseline) in each mouse strain. Significance $p < 0.05$ indicated by *, $p < 0.01$ indicated by **.

**Metabolic rate was increased by ketogenic diet independent of activity level**

To determine if body composition changes were driven by changes in metabolic rate or activity level, mice were monitored in metabolic chambers for 48 hours. Metabolic rate was evaluated by indirect calorimetry via oxygen consumption analysis. Activity monitoring was performed simultaneously.

All mice fed ketogenic diet exhibited increased metabolic rate. The A mice had a striking 57% increase in metabolic rate compared to mice fed standard chow (Figure 10). B6, FVB, and NOD mice had more modest 9-15% increases. B6 and FVB mice fed Japanese diet had a modest decrease of 9-11% in metabolism, though adiposity was not significantly
impacted. As expected, changes in oxygen consumption were consistent with changes in heat expenditure, although significance thresholds were not met in some conditions (Figure 11).

No significant differences in activity level were observed indicating that changes in metabolic rate were not caused by altered activity levels (Figure 12).

Figure 10. Effects of diets on oxygen consumption compared to standard diet (baseline) in each mouse strain. Significance $p<0.05$ indicated by *, $p<0.01$ indicated by **.
Figure 11. Effects of diets on heat expenditure compared to standard diet (baseline) in each mouse strain. Significance $p<0.05$ indicated by *, $p<0.01$ indicated by **.

Figure 12. Effects of diets on activity compared to standard diet (baseline) in each mouse strain. Significance $p<0.05$ indicated by *, $p<0.01$ indicated by **.
Increased metabolic rate in A mice is correlated with slightly increased expression of uncoupling proteins 1 and 2

Increased metabolic rate on ketogenic diet has been correlated with increased uncoupling protein 1 (Ucp1) expression in brown fat of B6 mice [110]. To determine whether increased Ucp1 expression was associated with the highly increased metabolic rate of A mice fed ketogenic diet, we compared Ucp1 expression in brown fat of A mice eating standard or ketogenic diet. Gene expression was normalized to Hprt. Ucp1 was significantly increased by about 4% in the group fed ketogenic diet (Appendix Table 9). However, this increase is not likely to be large enough to account for the greatly increased metabolic rate in A strain mice. Ucp2 was also up-regulated by a similar degree, although expression of Ucp2 is much less than Ucp1 in brown fat.

Expression of Ucp1 and Ucp2 were also evaluated in gonadal fat, muscle and liver. Ucp2 expression was increased in the liver of mice fed ketogenic diet by about 6%. Other results in each tissue were either not significant or did not match expected directionality in gene expression change to account for changes in energy use. Cox1 and Pcg1a expression were evaluated in gonadal fat and muscle to examine ketogenic diet effects on mitochondrial biosynthesis. Cox1 was increased by about 4% in the leg muscle of mice fed ketogenic diet. Other results showed either no significant effect.

Beta-hydroxybutyrate levels were consistently elevated in all mice fed ketogenic diet
An effective ketogenic diet induces a unique physiological state, ketosis, which results in increased blood concentrations of the ketone beta-hydroxybutyrate (BHB). To verify that all strains were in a state of nutritional ketosis when fed a ketogenic diet, BHB levels in mice fed ketogenic diet were compared to mice eating a standard chow. All strains had consistently low concentrations of (0.3-0.4 mM) when fed a standard chow (Appendix Table 7). Ketogenic diet feeding consistently increased BHB concentrations (4.6-5.1 mM), but no significant differences were detected between strains.

**Adiposity differences were driven by either changes in food consumption or feed efficiency depending on strain and diet**

To determine if changes in food consumption or feed efficiency affected the observed phenotypes, food consumption was quantified during two 24-hour trials.

B6 mice consumed significantly fewer calories from Western and Mediterranean diets than standard chow, but still developed significantly higher levels of adiposity (Figure 13). NOD mice also had significantly decreased food consumption on the Western diet with higher levels of adiposity.

FVB mice ate significantly more calories of Western and ketogenic diets than standard chow. However, only ketogenic diet fed mice had significantly increased adiposity. Food consumption was not significantly affected by diet in A mice.
Figure 13. Effects of diets on food consumption (calories/per) compared to standard diet (baseline) in each mouse strain. Significance \( p < 0.05 \) indicated by *, \( p < 0.01 \) indicated by **.

Feed efficiency was evaluated by dividing weight gain over 90 days by average calorie consumption. The data indicated that mice experienced increased adiposity due to either increased feed efficiency or increased food consumption depending on diet and strain.

Adiposity gain in B6 and NOD mice fed Western and Mediterranean diets was correlated with increased feed efficiency, indicating that these strains have a predisposition for adiposity gain on these despite consuming fewer calories (Figure 14).
Adiposity gain in FVB mice fed a ketogenic diet was caused by increased caloric consumption. Feed efficiency was not significantly altered in FVB mice, but increased food consumption led to increased adiposity.

A trend of reduced feed efficiency ($p = 0.062$) was observed in A mice eating ketogenic diet. The 38% reduction in feed efficiency corresponded with a 48% increase in metabolic rate measured by indirect calorimetry, suggesting that A mice adapted by increasing metabolic rate to decrease weight gain.

Figure 14. Effects of diets on feed efficiency (calories per gram of weight gain) compared to standard diet (baseline) in each mouse strain. Significance $p <0.05$ indicated by *, $p <0.01$ indicated by **.
Effects on Mitochondrial Respiratory Control Ratio

Mitochondrial dysfunction has emerged as a possible factor underlying metabolic syndrome, particularly for glucose and insulin tolerance phenotypes [115, 116]. Isolated mitochondria from liver, the central metabolic organ [104], were used to quantify respiratory control ratio (RCR) since it is one of the best general measures of mitochondrial function [117]. RCR is the respiration rate in the presence of ADP (state 3) divided by respiration rate in the absence of ADP (state 4), which is a measure of mitochondrial ability to increase respiration rates to replenish ATP stores.

Western diet significantly decreased RCR in A (-25%), B6 (-33%) and FVB (-44%) mice, indicative of the presence of mitochondrial dysfunction (Figure 15). When fed ketogenic diet, only A mice experienced a significant decrease in RCR (-41%), which could not explain their significant increase in heat expenditure. Strong increases in RCR were observed in NOD mice eating Japanese (+142%) or ketogenic diet (+95%), suggestive of a high capacity for substrate oxidation and ATP generation with low proton leakage [117].
Figure 15. Effects of diets on respiratory control ratio (RCR) compared to standard diet (baseline) in each mouse strain. Significance $p < 0.05$ indicated by *, $p < 0.01$ indicated by **.
DISCUSSION

We have investigated metabolic syndrome-related phenotypes in response to five types of diets using four inbred mouse strains, and identified that individuals can have vastly different reactions to diet depending on their genetic background. This study demonstrates that the health effects of a diet are heavily dependent on the individual eating the diet. Some individuals are able to buffer the effect of a diet so that physiological changes either do not occur or are compensated for in a way that prevents detectable health changes. Other diets have particularly negative effects for certain individuals, demonstrating the need to evaluate diet response in genetically diverse individuals. Lastly, we find that diets with similar macronutrient ratios but different ingredients can have greatly different effects. Overall, our results, even using a small number of genetically diverse individuals (mouse strains), demonstrate a wide range of diet responses and question the efficacy of dietary recommendations based on population means.

Although most diet responses were dependent on genetic background, some phenotypes were consistent across the study population. While all mice fed a Western diet had increased liver triglyceride storage, only NOD mice also had increased markers of liver damage. Effects of Western and ketogenic diets on LDL and HDL cholesterol were consistent in directionality across all strains, though the effects varied in magnitude. Western diet increased LDL cholesterol while ketogenic diet increased HDL cholesterol. Impaired glucose tolerance was observed for all mice fed Western or ketogenic diets, except in NOD mice whose results were complicated by variability in onset of diabetes. Taken together, our
results indicate that the Western diet yields poor cardiometabolic effects across strains, but the severity ranged from mild to severe depending on genetic background.

The high-fat, high-sugar Western diet is typically viewed as the “unhealthy” diet in many rodent studies. The negative effects of the Western diet on B6 mice, the most extensively used lab strain, have been robustly demonstrated. While A and FVB mice suffered a few negative effects from the Western diet, they were much less severe than those observed in the B6 strain. Our results indicate that B6 mice are particularly susceptible to detrimental effects of a Western diet and may not be representative of most strains.

The high-fat ketogenic diet caused different effects than the Western diet, and the response was highly variable across strains. Our study, in addition to others, demonstrates that high-fat diets are not always problematic in B6 mice [109, 110]. In the context of a high-fat diet without carbohydrates, B6 mice maintain normal health. However, the diet had severe, negative health effects in FVB mice including increased adiposity. NZO mice have recently been shown to have a negative response to the ketogenic diet including a significant increase in weight gain compared to mice fed a standard chow [112]. Again, these contradictory responses between strains demonstrate the importance of evaluating diet effects in individuals with diverse genetic backgrounds.

Our results exemplify how a reductionist approach can lead to an incomplete or inaccurate understanding when applied to diet studies. For instance, many papers have attributed negative health effects to a high-fat diet. As we have shown, a high-fat content diet can be associated with negative or positive health outcomes depending on both the genetics of the individual and the other components in the diet. Even diets with similar macronutrient
compositions, such as the Western and Mediterranean diets, can cause different health effects. Thus, it is important to consider the impact of food constituents in relation to the other components of the diet and the individual eating the diet.

In addition to the Western and ketogenic diets having different effects on adiposity in B6 and FVB mice, they also had different mechanisms underlying the adiposity gain. B6 mice were highly efficient at converting Western and Mediterranean diets into body fat despite eating fewer calories than on standard chow. Similar mechanisms seem to be at work in NOD mice eating Western and Mediterranean diets. These results indicate that, in terms of adiposity gain, diet composition outranks the impact of caloric intake for some individuals. This result is consistent with previous work that found a lack of correlation between food intake and obesity in some mouse strains [35].

Whereas increased feed efficiency drove obesity in B6 and NOD mice, increased food consumption drove obesity in FVB mice. FVB mice on a ketogenic diet, and to lesser degree on Western diet, experienced increased adiposity without increased feed efficiency. Adiposity gain in FVB mice can be largely attributed to increased food consumption. These results demonstrate that some individuals gain weight on specific diets simply by eating more food, whereas others gain weight due to a predisposition to high feed efficiency on specific diets.

Mice of the A strain had a uniquely negative response to the Japanese diet including increased LDL cholesterol, increased liver triglyceride concentrations, and increased ALT concentrations. Aside from the response to the Japanese diet, A mice are largely resistant to diet-induced changes in cardiometabolic health parameters. Although significant changes
were observed for a few metabolic syndrome-related characteristics, the scale of the changes were typically much less than those seen in other strains. One exception is the ketogenic diet, which dramatically increased metabolic rate and caused mitochondrial dysfunction in A mice, although these physiological effects did not manifest in altered health outcomes. This suggests A mice have a tightly regulated metabolic process capable of buffering underlying physiological effects of diet.

The Japanese diet yielded positive health outcomes in all but A mice. While this type of diet may be beneficial for most individuals, the result could also indicate that some individuals may not reach optimal health on an otherwise healthy Japanese diet, which is most similar to standard chow.

Our study has demonstrated that the effect of a diet is largely dependent on the genetic background of the individual consuming the diet. Historically, diet studies have attempted to elucidate health effects by averaging the response of many genetically distinct individuals without accounting for individual variation. Our results suggest a new paradigm should be examined when evaluating diet responses that accounts for diet by genetic interactions in order to truly understand the effects of a diet on an individual.
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CHAPTER 3

DIET, GENETIC BACKGROUND, AND COLORECTAL CANCER
INVESTIGATION OF DIETARY EFFECTS ON COLORECTAL CANCER IN GENETICALLY DIVERSE MOUSE STRAINS

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ABSTRACT

Association studies on populations’ eating patterns and incidence of colorectal cancer (CRC) have identified strong correlations. Migration studies following people moving from areas of low CRC incidence to areas of high CRC incidence have shown increased CRC risk, particularly for those who adapt dietary patterns of the high risk population. These studies indicate dietary patterns can have a strong impact on CRC risk. However, few studies have examined how the genetic background of an individual influences their risk of CRC on different diets. To address this gap in knowledge, we examined incidence and severity of carcinogen-induced colon cancer in four inbred mouse strains (A/J, C57BL/6J, FVB/NJ, and NOD/ShiltJ) fed one of five diets (Western, Japanese, Mediterranean, ketogenic, standard mouse diet). Relative to mice fed standard diet, we found tumor penetrance, tumor multiplicity, or both to be significantly altered in B6 mice fed Mediterranean diet, FVB mice fed ketogenic diet, and FVB mice fed Japanese diet. This study indicates that the impact of diet on tumor penetrance and multiplicity is dependent on an individual’s genetic background.
BACKGROUND

Colorectal Cancer Incidence and Etiology

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States. Each year 133,000 Americans are diagnosed with the disease and nearly 50,000 die [118]. CRC is becoming a serious concern in several less developed countries. Adoption of an unhealthy Western lifestyle, including eat a calorically dense diet, is correlated with a marked increase in CRC rates in these countries [119].

Available evidence suggests that 65-90% of the risk of developing CRC is derived from environmental factors [120, 121]. Diet is presumably the greatest environmental factor, as food passing through the colon provides a direct link between the colon and the external environment. Recent evidence suggests that diet can also impact colorectal cancer risk indirectly by altering the gut microbiome [38]. It is clear that diet, genetics, and the gut microbiome act in concert to influence CRC risk, but little is known about how these factors interact to modify disease risk especially on an individual basis.

Impact of Individual Foods on Colorectal Cancer Risk

While epidemiological evidence indicates that diet plays an important role in CRC risk, determining which foods modify risk has been largely unsuccessful. This is likely due to the complexities of nutrient differences between diets.

The strongest associations between foods and CRC incidence are those of red meat or processed meat [122-124]. The World Health Organization’s International Agency for Research on Cancer (IARC) recently classified red meat as probably carcinogenic to humans.
after reviewing over 800 studies on the subject. This was based on “limited evidence that the consumption of red meat causes cancer in humans and strong mechanistic evidence supporting a carcinogenic effect” [125]. The IARC gave stronger warnings for processed meat, classifying it as *carcinogenic to humans*, which was based on “sufficient evidence in humans that the consumption of processed meats causes colorectal cancer” [125]. They concluded that each 50 gram serving of processed meat increases colorectal cancer risk by 18% [125]. It is thought that heterocyclic amines present in well done and processed meats could contribute to the carcinogenic effects [126].

It is difficult to determine the effects of individual foods on colorectal cancer risk. People eat whole diets, not nutrients or foods in isolation. Furthermore, combinations of food components may have different effects than foods in isolation. Genetic diversity furthers compounds the problem because foods have different effects in different individuals. Ultimately, while certain components of a diet, in isolation, may modify the risk of developing CRC, it is the collective effect of an entire diet that dictates overall CRC risk and this risk must be evaluated in the context of diverse genetic backgrounds.

**Diet and Colorectal Cancer Risk in Populations**

Japanese populations have provided valuable data for epidemiological studies of CRC. The traditional Japanese diet, consumed in the mid-twentieth century, yielded much lower rates of CRC than those in the United States. However, as the Japanese population has adapted to a Western-style diet the rates of CRC have increased. Today, the Japanese eat a
diet that more closely resembles an American diet than a traditional Japanese diet, and the rates of CRC in Japan are similar to those in the United States (Figure 16) [127].

Studies of Japanese migrants to Hawaii provide further evidence for diet’s role in CRC etiology. Both Japanese and Caucasians in Hawaii have similar incidences of CRC. The rates in Hawaii, where a Western diet is eaten, are higher than the rates of those individuals consuming a traditional Japanese diet in Japan [128]. This suggests that dietary factors play a large role in CRC risk, and genetic differences alone between Japanese and American populations do not explain the difference in rates between Japan and the United States.

Some studies suggest the Mediterranean diet might be protective against CRC, while the Western diet seems to increase risk. One study of more than five million people found that populations with the greatest adherence to the Mediterranean diet experienced an 8-11% reduction in CRC incidence [129]. A similar study found that close adherence to a Mediterranean diet was associated with reduced incidence of CRC risk in smokers [130].
Conversely, epidemiological evidence suggests that the Western diet increases CRC risk [33].

**Models of Colon Cancer in Mice**

Mouse models provide a powerful tool to investigate mechanisms underlying colon tumorigenesis. Two main strategies are used to induce colon tumor formation in mice, genetic manipulation and carcinogen administration.

Genetic manipulation of the adenomatous polyposis coli (Apc) gene is commonly used model a familial form of CRC, familial adenomatous polyposis [131]. Apc mouse models have been used to discover tumorigenesis mechanisms and demonstrate the effect of modifier genes in CRC [132].

Azoxymethane (AOM) is a carcinogen used to model sporadic cases of CRC in mice. AOM causes colon-specific tumor formation after metabolism by the liver [133]. Susceptibility to AOM is widely variable and dependent upon genetic background [134]. AOM is 100% penetrant in some strains, such as A/J. Other strains are more resistant, such as C57BL6/J. Data indicates that differences in susceptibility are not due to metabolism, as aberrant crypt foci lesions were present at equal frequencies in inbred strains resistant and susceptible to AOM [135].

**Dietary Effects on AOM-Induced Colon Cancer**

A few studies have compared the effects of several dietary fats on AOM-induced colon cancer in laboratory rodents. A long-term AOM study in Sprague-Dawley rats found
diets with added corn oil or beef tallow increased tumor multiplicity while diets with added fish oil reduced tumor multiplicity, relative to a standard chow [136]. There were alterations in the Wnt signaling pathway consistent with the increased tumor load in corn oil or beef tallow fed animals. A similar study found reduced tumor multiplicity for F344 rats fed diets high in omega-3 fish and increased tumor multiplicity in rats fed omega-6 corn oil [137].

One study examined the impact of black tea, green tea, and red wine extracts on AOM-induced CRC in F344 rats. The study found a significant reduction in tumor multiplicity in mice fed black tea or red wine extract, but no effect in mice fed green tea extract [138].

In the present study, we investigated the impact of five diets on CRC in four inbred mouse strains. A standard mouse chow was compared to four human diets thought to impact CRC risk, based on epidemiologic or mechanistic data. The human diets included a current Western diet, a traditional Mediterranean diet, a traditional Japanese diet, and a ketogenic diet. Azoxymethane (AOM) was used to induce colon tumors. Four mouse strains (A/J, C57BL6/J, FVB/NJ, and NOD/ShiLtJ) were selected based on their variable susceptibility to AOM [134].
MATERIALS AND METHODS

Animals and Husbandry

Three cohorts of mice were analyzed, two at North Carolina State University (NCSU) and one at Texas A&M University (TAMU). A total of 11 mice per strain, sex, and diet (6 at NCSU, 5 at TAMU) were analyzed. In both cohorts, four-week old mice were obtained from Jackson Laboratory. Mice were acclimated to the research facilities during a two-week acclimation period prior to the start of the study. Mice were housed three per cage at NCSU and five per cage at TAMU. At both facilities, mice were given ad libitum food access and maintained at 25°C under a 12-hour light cycle with lights on at 0700. Mice were maintained and protocols followed in accordance with University of North Carolina and Texas A&M IUCAC guidelines.

Study Timeline

Mice were fed diets for ten weeks, followed by four weekly injections of azoxymethane (AOM, 10 mg/kg). NCSU mice remained on diets for 10 weeks following the final injection and then were necropsied. TAMU mice remained on diets for 20 weeks following the final injection to allow additional time for tumor development. Upon necropsy, mice colons were dissected and tumor multiplicity and size were assessed. Total tumor load per mouse was determined by summing the length of all tumors.

Statistical Analysis
\( p \)-values were calculated for diet’s effect on tumor penetrance in each strain using Pearson’s chi-square-test. Firth’s bias-correction was performed to adjust for quasi-separation of the data. The likelihood ratio (LR) was calculated to estimate the risk for colon cancer compared to mice fed standard diet. \( p \)-values were calculated for tumor number, multiplicity, and load using two-way ANOVA for each strain with diet and sex as factors. Strains were evaluated individually for statistical significance using Dunnett’s corrected \( p \)-values with standard diet as the baseline. Threshold for significance \( p < 0.05 \) for all tests.
RESULTS

Survival Rates

Survival rates were higher in the NCSU cohort than the TAMU cohort. NCSU cohorts had survival rates of 112/120 (93%) and 103/120 (85%) (Appendix Table 10, Appendix Table 11). The TAMU cohort had a survival rate of 153/200 (76.5%, Appendix Table 12). Deaths in the NCSU cohorts were evenly spread across diet and strain combinations, although NOD/ShiLtJ mice experienced slightly lower survival rates than other strains, which was likely due to this strain's predisposition to develop diabetes. Deaths at TAMU were not evenly distributed. All A/J mice on the Japanese diet died following the first AOM injection. The A/J ketogenic and Western diet groups also had low survival rates (50%) and all NOD ketogenic mice died, though those deaths were not an immediate consequence of AOM injection. Survival rate in NOD mice was 58% for all diets, except for those on the Western diet in which no mice died. Interestingly, no deaths were observed in NOD mice eating Western diet in any cohort.

Tumor Penetrance

To determine the effect of diet on tumor penetrance, we calculated the percent of mice with at least one tumor, determined significance using Pearson’s chi-square-test and estimated likelihood ratios of colon tumors for each diet compared to standard diet.

Significant effects of diet on tumor penetrance were identified in the C57Bl/6J (B6) and FVB/NJ (FVB) strains (Figure 17, Figure 18). Relative to mice fed standard diet, B6
mice fed Mediterranean diet had increased tumor penetrance (estimate=1.15 +/- 0.51, LR=6.69, \( p=0.0089 \)). FVB mice fed ketogenic diet had increased tumor penetrance (estimate=0.82 +/- 0.44, LR=4.04, \( p=0.0444 \)). A non-significant trend of increased tumor penetrance was also observed in FVB mice fed Japanese diet (estimate=0.72 +/- 0.43, LR=3.30, \( p=0.0691 \)). The models for diet’s effect on tumor penetrance were not statistically significant for the A and NOD strains due to high penetrance under all diets.

![Figure 17. Effect of diets on tumor penetrance in each inbred strain.](image)

Significance compared to standard diet of \( p<0.05 \) indicated by *, \( p<0.01 \) indicated by **.
Figure 18. Likelihood ratio of colon cancer compared to standard diet (baseline) in each inbred strain. Significance compared to standard diet of $p<0.05$ indicated by *, $p<0.01$ indicated by **. Estimates not available for diet by strain combinations with 100% penetrance.

Tumor Size

Average colon tumor size was significantly larger in the NCSU cohorts (1.84mm +/- .06 and 1.86mm +/- .06) than the TAMU cohort (1.54mm +/- .05, p<0.0001). This is surprising as the NCSU cohorts were kept for ten fewer weeks post injection than the TAMU cohort.

Average tumor size was significantly different between strains. Compared to B6 mice (1.48 mm +/- 0.06), the A strain and NOD strain had significantly higher tumor average
tumor size (2.05 mm, p<0.0001 and 1.82 mm, p=0.0002, respectively). FVB mice did not significantly vary from B6 (1.61 mm, p=0.3462).

Diet did not significantly affect average tumor size in any mouse strain (Figure 19). Low survival rates in the A/J mice on Japanese and ketogenic diets and NOD mice on ketogenic diet limited sample sizes and reduced power to detect effects.

![Average Tumor Size (% Change)](image)

**Figure 19. Effect of diets on average tumor size in each inbred strain.** Experimental diets are compared to standard diet (baseline) in each mouse strain and results are shown as percent change. No significant effects were detected.

**Tumor Multiplicity**

Average tumor multiplicity was significantly decreased in the NCSU cohorts (2.15 tumors +/- 0.27 and 2.12 tumors +/- 0.28) compared to the TAMU cohort (3.18 tumors +/- 0.23).
When combing data from all cohorts, average tumor multiplicity varied significantly in all strains. Compared to B6 mice (1.64 tumors +/- 0.23), A and NOD strains had significantly more tumors (2.92 tumors, $p = 0.0009$ and 4.92 tumors, $p < 0.0001$, respectively). FVB mice had significantly fewer tumors than B6 mice (1.18 tumors, $p < 0.0001$).

No significant diet effects for tumor multiplicity were detected in the A, B6, or NOD strains (Figure 20). However, a significant sex effect was observed for NOD mice with females having more tumors than males (6.58 vs. 3.26, $p = 0.0001$).

In FVB mice, tumor multiplicity significantly increased in mice fed the ketogenic diet (2.15 vs. 0.44, $p = 0.0062$). We also observed increased tumor multiplicity in FVB mice fed Japanese diet (1.68 vs. 0.44, $p = 0.049$). No significant effects were detected in the model for sex alone or the interaction of diet by sex.
Figure 20. Effect of diets on tumor multiplicity in each inbred strain. Experimental diets are compared to standard diet (baseline) in each mouse strain and results are shown as percent change. Significance $p<0.05$ indicated by *, $p<0.01$ indicated by **.

Tumor Load

There was a non-significant trend of decreased tumor load in the NCSU cohorts (3.97 mm +/- 0.44 and 3.82 mm +/- 0.46, compared to the TAMU cohort (4.80 +/- 0.38, $p=0.0670$).

Tumor load varied by strain. Compared to the B6 strain (2.32 mm +/- 0.39), A and NOD strains had significant increases in tumor load (6.00, $p<0.0001$ and 7.72, $p<0.0001$, respectively). FVB tumor load did not significantly differ from B6 (1.78 mm, $p=0.6590$).
No significant diet effects for tumor load were detected the A, B6 or NOD strains (Figure 21). A significant sex effect was identified in the A strain with increased average tumor multiplicity in females (6.58 vs. 3.27, \( p = .009 \)). A significant sex effect was also observed in NOD mice with females having greater tumor load (10.08 mm vs. 6.18 mm, \( p = 0.0001 \)). No significant sex effects were observed in B6 mice.

![Figure 21](image)

**Figure 21.** Effect of diets on average tumor load in each inbred strain. Tumor load is the sum of the length of all tumors. Experimental diets are compared to standard diet (baseline) in each mouse strain and results are shown as percent change. Significance \( p < 0.05 \) indicated by *, \( p < 0.01 \) indicated by **.

In FVB mice, tumor load significantly increased in mice fed the ketogenic diet (3.45 mm vs. 0.75 mm, \( p = 0.0015 \)). The effect of Japanese diet on tumor load failed to reach the
significance threshold (2.02 mm vs. 0.75 mm, $p = 0.2341$). No significant effects were detected in the model for sex alone or the interaction of diet by sex.
DISCUSSION

This study examined the impact of five diets on AOM-induced colon cancer in four inbred mouse strains. We found significant effects for tumor penetrance in the B6 and FVB strains, and significant effects for tumor multiplicity and tumor load in the FVB strain.

There was a striking difference in survival rates of A strain mice on Japanese diet between the NCSU and TAMU cohorts. All A strain mice fed Japanese diet in the NCSU cohort survived the duration of the study, whereas all in the mice died shortly after AOM injection in the TAMU cohort. Toxicity appears to have been the cause of death in the TAMU cohort due to the immediate death after AOM injection. We did not anticipate toxicity effects at the 10 mg/kg dosage. A previous study has shown toxic effects in the A strain at a dose of 20 mg/kg [134]. In that study, all 26 mice died immediately after injection of 20 mg/kg of AOM but none of the 55 total mice were lost in the groups administered either 10 mg/kg or 5 mg/kg of AOM. Higher doses (100 mg/kg) have been used to induce fulminant hepatic in B6 mice [139].

The reason the A strain had toxic effects in the TAMU but not NCSU cohorts is unclear, but gut microbiome differences could have played a role. The same AOM batch was used in both of our cohorts. While the diet batch differed between the cohorts, the manufacturer, formulation and purified ingredient sources were identical. Gut microbiome differences could likely have contributed to toxicity differences between cohorts. Other experiments have shown strong cohort effects between mice house at NC State and Texas A&M in the B6 and FVB strains. We are not aware of studies examining the effect of the gut
microbiome on AOM toxicity. However, studies in both mice and rats show profound effects of gut microbiota composition on tumor development in AOM, which demonstrate interactions between gut bacteria and AOM [42, 140].

Significant effects of diet on tumor penetrance were identified in both the B6 and FVB strains, although the strains differed in diet effects on tumor multiplicity and tumor load. Tumor penetrance was increased in B6 mice fed Mediterranean diet relative to B6 mice fed standard diet, which indicates that the Mediterranean diet in B6 mice increases tumor initiation. However, no significant effects for B6 mice fed Mediterranean diet were identified for tumor size, tumor multiplicity, or tumor load. The reason that significant effects were identified for penetrance but not tumor multiplicity is likely due to overall low tumor multiplicity overall in the B6 strain. Multiplicity ranged from 1.20 +/- 0.51 tumors for mice on standard diet to 2.41 +/- 0.49 for mice on the Mediterranean diet. If the sample size of the current study was doubled and results were consistent, a significant p-value < 0.05 would be expected for tumor multiplicity of mice fed Mediterranean versus standard diet.

In the FVB strain, tumor penetrance increased on the ketogenic diet. This was accompanied by increased tumor multiplicity and tumor load, but no significant effect on average tumor size. This indicates that the ketogenic diet in FVB mice influences tumor initiation rate but does not affect tumor growth.

FVB mice fed Japanese diet showed a significant increase in tumor multiplicity and a non-significant trend of increased penetrance (p =0.069). This finding was unexpected given that the Japanese diet is associated with a reduction in CRC risk in human populations. Studies on cardiometabolic health in our lab have shown beneficial effects of the Japanese
diet in FVB mice. Only the FVB strain experienced increased tumor multiplicity when fed a Japanese diet. It is feasible be that a subset of human individuals have experience similar negative effects, but that they are rare enough to not be detected in population-based studies.

Our results are consistent with a previous study that found significant effects of dietary modification on tumor penetrance, but not tumor multiplicity in C57BL/6J mice [134]. Our results are also consistent with the previous study’s finding that effects of dietary modification on tumor multiplicity are strain-dependent, as the DBA/2J mice were susceptible to dietary effects on tumor multiplicity[134].

No significant effects of diet were observed on any tumor parameters for the A or NOD strains. Low survival rates of some groups in these strains reduced power to determine significant effects. Larger sample sizes would be needed to accurately calculate penetrance in these strains, as several of the groups did not have tumor-free mice. It is likely that diet effects would be detected if a larger number of mice were sampled.

Overall these results highlight the interaction between diet and genetic background and colon tumor initiation and provide a foundation for investigating the complex relationship between human diet and colorectal cancer within the context of an individual’s genetic makeup.
CHAPTER 4

DIET, GENETIC BACKGROUND, AND GUT MICROBIOME
GUT MICROBIOME COMPOSITION DIFFERS WITH DIET AND GENETIC BACKGROUND

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ABSTRACT

There is a growing body of evidence highlighting the impact of the gut microbiome on health, especially with obesity and colorectal cancer incidence. Studies have shown that both diet and an individual’s genetic background can influence gut microbiota composition. However, little is known about how these factors interact to influence health. To address this gap in knowledge, we correlated gut microbiota composition of C57BL/6J and FVB/NJ mice fed Western, ketogenic, or standard diet with their physiological and cancer effects. We found that environmental, genetic, and dietary factors can alter microbiota composition. We found the impact of a diet on gut microbiota composition is dependent on the genetic background of an individual. We also identified several bacterial orders and species associated with colorectal cancer incidence.
BACKGROUND

Gut Microbiome Research

Advances in DNA sequencing and bioinformatics have allowed exploration of the gut microbiome and its implications for health. Bacteria and host organisms have co-evolved for at least 500 million years [141]. Bacteria have cooperative interactions with their host to carry out a variety of functions including defense, metabolism, and reproduction [34]. Shifts in gut microbiota composition are correlated with a number of conditions including colorectal cancer, inflammatory bowel disease, obesity, non-alcoholic fatty liver disease, and rheumatoid arthritis [32]. Metabolites from gut bacteria likely influence host physiology to alter disease risk. However, it is difficult to demonstrate a causal relationship, and more difficult to determine which specific species cause the effect. There are likely interactions between many bacteria that are responsible for disease-related physiological shifts. Unraveling the relationships between gut microbiota composition, host genetics, diet, and disease risk presents a great challenge with great potential benefits for human health.

Influence of Gut Microbiome on Colorectal Cancer Risk

The gut microbiome has emerged as a prominent area of colorectal cancer (CRC) research. In humans, associations have been made between the diversity and types of gut bacteria and incidence of colorectal cancer. A study in 47 CRC patients and 94 controls identified an overall reduction in gut microbiota diversity, decreased relative abundance of Clostridia, and increased presence of Fusobacterium and Porphyromonas [41]. Another study demonstrated the complexity of identifying effects of specific bacterial genera on
human health. *Coriobacteria*, a type of bacteria commonly used in probiotic supplements, was consistently associated with the presence of colon tumors, whereas the potentially pathogenic *Enterobacteria* was underrepresented in colonic tumor samples [40]. A study in which gut bacteria was eliminated with antibiotics in mice demonstrated that gut microbiota influences expression of cell cycle control genes and can alter colonic proliferation rates [142].

Genetically modified mouse models provide valuable insights into the complexity of gene by gut microbiome interactions. *Il10*-deficient mice are resistant to colitis-associated CRC when raised in a germ-free setting but develop CRC when exposed to normal mouse gut microbiota [143]. However, *Apc*<sup>Min</sup> mice, which are genetically predisposed to CRC under normal conditions, have only a small reduction in tumor load when raised in a germ-free environment [144]. Genetically modified mice with altered transforming growth factor beta (*Tgfb*) signaling show increased cancer development accompanied by increased pro-inflammatory bacterial species including *H. pylori* and *Helicobacter hepaticus* [145, 146]. These findings demonstrate that complex interactions exist between the genetic factors and gut microbiota in the development of colorectal cancer.

Dietary shifts influence gut microbiota in people, and these shifts alter certain CRC risk factors. A study compared the diet of rural South Africans to African Americans, who have a 13 times higher CRC incidence rate, to investigate the role of the Western diet [38]. Microbiota composition was analyzed and colon biopsies were taken to measure inflammation and proliferation before and after the groups swapped diets for two weeks. Dietary changes caused shifts in microbiota functional gene abundance, including down-
regulation of the butyrogenesis pathway associated with Western diet. This is significant as butyrate, a short chain fatty acid produced by gut bacteria, is protective against CRC [39]. The detrimental microbiota effects of the Western diet were associated with increased markers proliferation and inflammation, which are risk factors for CRC. This research demonstrates that dietary modifications can quickly cause microbiome changes and increase CRC risk factors.

**Influence of Gut Microbiome on Obesity**

There is strong evidence supporting the role of gut microbiome in obesity. In 2005, researchers inoculated germ-free mice with gut microbiota from conventionally raised, obese mice. The mice had a 60% increase in body fat despite a 29% decrease in food consumption and 27% increase in activity compared to unaltered germ-free mice [36]. Another group confirmed the finding and found the trait was hereditarily transmissible [37].

Transplantation of gut bacteria from monozygotic twins discordant for obesity into germ-free mice has revealed functional differences in microbiota composition. In inoculated mice, bacterial communities from obese individuals were correlated with decreased fermentation of short-chain fatty acids, increased metabolism of branched chain amino acids, and decreased formation of bile acids. Mice inoculated with gut bacteria from an obese twin had higher adiposity than those inoculated with bacteria from the corresponding lean twin [147].

The gut microbiome can influence obesity through a variety of mechanisms. The gut microbiome can influence energy extraction from the diet so that individuals with different
microbiota compositions can harvest more or less calories from the same diet [148]. Carbohydrate fermentation, resulting in the production of short chain fatty acids, is increased in obese individuals [149]. Suppression of adenosine monophosphate-activated protein kinase (AMPK) by gut bacteria has been shown to alter fat oxidation and impact obesity in mice [150]. Gut bacteria can affect production of gut hormones, such as glucagon-like peptide and peptide YY, which can alter obesity through appetite regulation [151].

**Influence of Host Genetics on Microbiota Composition**

Studies in mice have demonstrated that genetic background strongly influences the composition of gut microbiota. A study in BXD mouse lines (recombinant inbred strains derived from a C57BL/6J and DBA/2J cross) found strong associations between genetic background and microbiota composition and identified several QTL with candidate genes underlying the strain effects [33]. A similar study found strong associations between genetic background in mice and identified 18 QTL linking genetic factors with microbiota composition [34].

**Influence of Diet on Microbiota Composition**

Diet can strongly impact gut microbiota composition. A study examined gut microbiota shifts in 100 inbred mouse strains after feeding mice a high-fat, high-sugar (HF/HS) diet [35]. Compared to mice eating a chow diet, HF/HS feeding increased abundance of bacteria of the order *Clostridiales* and decreased abundance of *Bacteroidetes*. There were 17 genera overall that showed significant changes in abundance. While some
strains had large shifts in microbiota composition due to diet effects, others had minimal fluctuation. This indicates the plasticity of microbiota composition after HF/HS feeding was strongly correlated with genetic background.
INTRODUCTION

FVB and B6 had discordant responses to ketogenic and Western diets. As described in Chapter 2, B6 mice maintain normal weight and had good cardiovascular health on a ketogenic diet but become obese and show signs of metabolic syndrome on a Western diet. The opposite response is observed in FVB mice. Chapter 3 described the divergent effects of these diets on colon tumor load in the two strains. FVB mice have significantly elevated tumor load on a ketogenic diet but not on a Western diet. No significant effect of diet on tumor load was observed in B6 mice fed either diet. Given the impact of gut microbiome on colorectal cancer and obesity, we sought to determine the differences in gut microbiota composition in FVB and B6 mice eating Western, ketogenic or standard diets.

Whole genome sequencing was performed to allow for comparison of both phylogenetic and functional metagenomic analysis. However, heavy demand on metagenome analysis servers has delayed analysis of the whole genome. Alternatively, we have performed phylogenetic analysis on 16S ribosomal genes. Here, we discuss phylogenetic changes in the gut microbiota and their implications for disease.
MATERIALS AND METHODS

Sample Collection

Fecal samples were collected in both cohorts of the diet-physiology studies described in Chapter 2. While only post-diet samples were available from the NCSU cohort (Cohort 1), pre-diet (initial) and post-diet (final) samples were available from the TAMU cohort (Cohort 2). Samples were immediately placed on dry ice upon collection and then transferred to -80°C for long-term storage.

DNA Extraction and Sequencing

DNA was extracted from 3 individuals per diet, sex, and strain using FastDNA Spin Kit for Soil (MP Bio). Sequencing libraries were prepared using the TruSeq Nano Library Preparation Kit (Illumina). Whole genome sequencing was performed on the NextSeq platform (Illumina).

Data Analysis

16S ribosomal phylogenetic analysis was performed using 16S Metagenomics software (Illumina). Rarefaction curves were generated in R software using the “vegan: Community Ecology Package” [152]. Functional and non-16S ribosomal-based phylogenetic metagenome analysis is on-going using the MG-RAST software [153].
**Statistical Analysis**

Percent abundance for bacteria at the phylum, order and species levels was calculated by dividing the number of sequencing hits for the genera by total hits. $p$-values were calculated for differences in abundance using two-way ANOVA for each strain with diet and sex as factors. Strains were evaluated individually for statistical significance using Dunnett’s corrected $p$-values with standard diet as the baseline. Threshold for significance $p < 0.05$ for all tests.
RESULTS

Effects Determining Microbiota Composition at the Phylum Level

To determine the effect strength of the variables influencing microbiota composition at the phylum level, a least square means fit model was generated for post-diet microbiota composition at the level of phylum with cohort, diet, strain, gender, and their interactions as variables.

Environmental effects strongly impacted microbiota composition at the phylum level, cohort had the strongest effect on microbiota composition (Appendix Table 13). Differences between cohorts included location, feeder type, and adiposity of mice (higher in cohort two for all diets). The interaction of cohort by diet had the second largest effect, while diet alone had the fourth largest effect.

Genetic background had weaker effects than environmental (cohort) factors on microbiota composition at the phylum level. The effect of strain was stronger than cohort and diet effects. The interaction of strain by diet, while significant, had the weakest effect on microbiota composition. These data show that environmental factors are the main predictors of gut microbiota composition at the phylum level and genetic background plays a less role.

Effects Determining Microbiota Composition at the Species Level

We determined the effect strength of variables influencing microbiota composition at the species level using the same analysis was performed as for phylum except species was
used as the response (Appendix Table 14). All variables and interactions had significant effects.

Both environmental factors and genetic background had powerful effects on microbiota composition at the species level. The interaction of cohort by strain had the strongest effect, followed by cohort alone, the interaction of cohort by strain by diet, diet alone, and strain alone. These data indicate that there is a complex relationship between environmental and host genetic factors that influence the gut microbiota composition at the species level.

**Comparison of Cohort Effects on Microbiota Composition**

Gut microbiota composition varied greatly at the phylum level by cohort. This is likely due to difference in location as cohort one was studied at UNC and NC State while cohort two was studied at Texas A&M. However, we have observed that differences in the type of feeder used in the cohorts strongly impacted adiposity between cohorts and could contribute to differences in microbiota composition. Here, we discuss the cohort differences in microbiota composition. Future sections will only include analysis from cohort two because the phylogenetic composition is quite different, the cancer studies were performed on the same type of feeders used in cohort two, and cohort two has both pre-diet and post-diet samples which allowed for diet-specific changes to be evaluated.

On standard diet, B6 mice in cohort one had more bacteria of phylum *Bacteroidetes* than in cohort two (83% vs. 58%, Figure 22). This was also observed in FVB mice (80% vs. 56%). The decrease in *Bacteroidetes* in cohort two was mostly compensated for by an
increase in *Firmicutes*. In B6 mice, there was a significant decrease in *Firmicutes* in cohort one compared to cohort two (7% vs. 25%). A similar pattern was observed in FVB mice (10% vs. 27%, Figure 23). There were no significant differences in *Proteobacteria* abundance in either strain.

![Phylum-level diet effects in B6 mice.](image)

**Figure 22. Phylum-level diet effects in B6 mice.** Effects of diets on six major phylum classes are compared in cohort 1 (NSCU) vs. cohort 2 (TAMU). Cohort had a strong impact on gut microbiota composition. Differences between cohorts include location, feeder type, and level of adiposity.
Figure 23. Phylum-level diet effects in FVB mice. Effects of diets on six major phylum classes are compared in cohort 1 (NSCU) vs. cohort 2 (TAMU). Cohort had a strong impact on gut microbiota composition. Differences between cohorts include location, feeder type, and level of adiposity.

On ketogenic diet, B6 mice in cohort one had less bacteria of phylum *Bacteroidetes* than in cohort two (50% vs. 73%). This was also observed in FVB mice, although the difference was smaller (68% vs. 79%). The increase in *Bacteroidetes* in cohort two was mostly compensated for by a decrease in *Firmicutes*. In B6 mice, there was a significant increase in *Firmicutes* in cohort one compared to cohort two (35% vs. 17%). However, in FVB mice abundance of *Firmicutes* did not significantly vary between cohorts (16% vs. 12%). In B6 mice, *Proteobacteria* were more abundant in cohort one than in cohort two (9% vs. 2%). In FVB mice, a similar pattern was observed (11% vs. 3%).
On Western diet, B6 mice in cohort one had less bacteria of phylum *Bacteroidetes* than in cohort two (64% vs. 75%). There was no significant difference in FVB mice between cohorts (83% vs. 81%). The increase in *Bacteroidetes* in cohort two was mostly compensated for by a decrease in *Firmicutes*. In B6 mice, there was a significant increase in *Firmicutes* in cohort one compared to cohort two (22% vs. 15%). However, in FVB mice abundance of *Firmicutes* did not significantly vary between cohorts (7% vs. 10%). In B6 mice, *Proteobacteria* were more abundant in cohort one than in cohort two (8% vs. 4%). In FVB mice, there was less magnitude of difference in *Proteobacteria* abundance, but the difference was still significant (7% vs. 5%).

**Comparison of Diet Effects on Microbiome Diversity**

The diversity of microbial species in the gut is associated with health status of the host. Studies have found that reduction in diversity is correlated with inflammatory bowel disease, colorectal cancer, and obesity[154-156]. We generated rarefaction curves to normalize for differences in sequencing depth between samples. This allowed us to determine species richness by estimating the expected number of species observed if 1,000 individual microbes were mapped in each mouse.

In B6 mice, there were no significant changes in species richness by diet (Figure 24). The variability in species richness between samples of the same condition was higher in B6 mice than FVB mice, which reduced power to detect significant effects.

In FVB mice, there was a significant reduction species richness for mice fed ketogenic or Western diets compared to standard diet. The reduction in species richness was
more pronounced in mice fed ketogenic diet. The strong reduction in species richness corresponds with our previous observation of increased in tumor load in FVB mice fed a ketogenic diet.

![Image of bar chart](image.png)

**Figure 24. Effects of diets on species richness.** Estimated by generating rarefaction curves in B6 and FVB mice. Values indicate number of species expected if 1,000 individuals were sequenced. Significance $p<0.05$ indicated by *, $p<0.01$ indicated by **.

**Comparison of Diet Effects on Microbiota Composition at Phylum Level**

To determine diet effects on gut microbiota at the phylum level, we performed a response screen analysis, which plotted the false discovery rate logworth values against effect size for each strain using diet and gender as variables. This allowed us to identify significant phylum level changes with large effect sizes.
The major phylum in the gut microbiome of mice is *Bacteroidetes*. In B6 mice, this phylum accounted for 58% of bacterial species in mice fed a standard diet. *Bacteroidetes* increased significantly to 80% in mice fed Western diet and 73% in mice fed ketogenic diet. Abundance of *Bacteroidetes* was nearly identical in FVB mice. Phylum *Bacteroidetes* accounted for 56% of bacterial species in FVB mice fed a standard diet. This increased significantly to 81% in mice fed Western diet and 72% in mice fed ketogenic diet.

The second major phylum in the gut microbiome is *Firmicutes*. When mice of either strain were fed a Western or ketogenic rather than a standard diet, a decrease in *Firmicutes* compensated for the increase in *Bacteroidetes*. In B6 mice, *Firmicutes* accounted for 25% of bacterial species in mice fed a standard diet. *Firmicutes* decreased significantly to 11% in mice fed Western diet and 17% in mice fed ketogenic diet. Again, abundance of *Firmicutes* was nearly identical in FVB mice. *Firmicutes* accounted for 28% of bacterial species in FVB mice fed a standard diet. This decreased significantly to 10% in mice fed Western diet and 17% in mice fed ketogenic diet.

*Proteobacteria* is normally the third most prevalent phylum present in the mouse gut microbiome, although its abundance is much less than *Bacteroidetes* or *Firmicutes*. In B6 mice, *Proteobacteria* accounted for 6.0% of bacterial species in mice fed a standard diet. *Proteobacteria* decreased significantly to 3.8% in mice fed Western diet and 2.8% in mice fed ketogenic diet. Once again, abundance of *Proteobacteria* was very similar in FVB mice. Phylum *Proteobacteria* accounted for 6.7% of bacterial species in FVB mice fed a standard diet. This decreased significantly to 4.6% in mice fed Western diet and 3.9% in mice fed ketogenic diet.
A minor component of the mouse gut microbiome is of phylum *Verrucomicrobia*. In B6 mice, *Verrucomicrobia* accounted for 3.7% of bacterial species in mice fed a standard diet. *Verrucomicrobia* was totally absent in mice fed Western diet but significantly increased to 6.2% in mice fed ketogenic diet. In FVB mice, phylum *Verrucomicrobia* accounted for 3.7% of bacterial species in FVB mice fed a standard diet. *Verrucomicrobia* were not eliminated but did decrease significantly to 1.2% in mice fed Western diet, while no significant difference was observed in mice eating ketogenic diet.

**Comparison of Diet Effects on Microbiota Composition at Order Level**

To determine the diet effects on microbiota composition at the level of order in each strain, the same screening and statistical approach was used as for the phylum level but order was used as the response.

Five bacterial orders were identified in the screening report for the B6 strain. *Sphingobacteriales, Chthoniobacterales, Desulfovibrionales, Nostocales* were significantly impacted in both diets, whereas *Caldilineales* were only significantly impacted by the ketogenic diet (Table 1).
<table>
<thead>
<tr>
<th>Order</th>
<th>Abundance</th>
<th>Standard Error</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Keto</td>
<td>Stand</td>
<td>West</td>
</tr>
<tr>
<td>Sphingobacteriales</td>
<td>0.233</td>
<td>2.823</td>
<td>1.763</td>
</tr>
<tr>
<td>Caldilineales</td>
<td>0.019</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Chthoniobacteriales</td>
<td>0.492</td>
<td>0.256</td>
<td>0.000</td>
</tr>
<tr>
<td>Desulfovibrionales</td>
<td>0.047</td>
<td>0.415</td>
<td>0.031</td>
</tr>
<tr>
<td>Nostocales</td>
<td>0.003</td>
<td>0.540</td>
<td>0.316</td>
</tr>
</tbody>
</table>

Table 1. Comparison of abundance (% of total) for B6 mice on standard (Stand) vs. ketogenic (Keto) or Western (West) diet. Order is indicative of significance found in screening report. Significant results vs. standard diet shown in green.

 Twelve bacterial orders were identified in the screening report the FVB strain, five of which (Flavobacteriales, Bacillales, Verrucomicrobiales, Fibrobacterales, Chrysiogenales) had significant effects for ketogenic and/or Western diet after applying a Dunnett’s test for multiple comparisons (Table 2).

<table>
<thead>
<tr>
<th>Order</th>
<th>Abundance</th>
<th>Standard Error</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Keto</td>
<td>Stand</td>
<td>West</td>
</tr>
<tr>
<td>Flavobacteriales</td>
<td>0.426</td>
<td>1.173</td>
<td>2.389</td>
</tr>
<tr>
<td>Enterobacteriales</td>
<td>0.002</td>
<td>0.013</td>
<td>0.013</td>
</tr>
<tr>
<td>Mycoplasmaetates</td>
<td>0.006</td>
<td>0.075</td>
<td>0.071</td>
</tr>
<tr>
<td>Bacillales</td>
<td>1.273</td>
<td>0.572</td>
<td>0.823</td>
</tr>
<tr>
<td>Actinomycetales</td>
<td>0.584</td>
<td>0.497</td>
<td>0.382</td>
</tr>
<tr>
<td>Spirochaetales</td>
<td>0.002</td>
<td>0.019</td>
<td>0.010</td>
</tr>
<tr>
<td>Chthoniobacteriales</td>
<td>0.325</td>
<td>0.306</td>
<td>0.106</td>
</tr>
<tr>
<td>Verrucomicrobiales</td>
<td>4.291</td>
<td>3.558</td>
<td>1.189</td>
</tr>
<tr>
<td>Syntrophobacteriales</td>
<td>0.041</td>
<td>0.082</td>
<td>0.005</td>
</tr>
<tr>
<td>Puniceicoccales</td>
<td>0.020</td>
<td>0.016</td>
<td>0.000</td>
</tr>
<tr>
<td>Fibrobacteriales</td>
<td>0.064</td>
<td>0.002</td>
<td>0.047</td>
</tr>
<tr>
<td>Chrysiogenales</td>
<td>0.000</td>
<td>1.079</td>
<td>0.042</td>
</tr>
</tbody>
</table>

Table 2. Comparison of abundance (% of total) for FVB mice on standard (Stand) vs. ketogenic (Keto) or Western (West) diet. Order is indicative of significance found in screening report. Significant results vs. standard diet shown in green.
Several bacterial orders that were significantly affected by diet in FVB mice have been implicated in colorectal cancer or inflammation. *Bacillales* were strongly increased on the ketogenic and were moderately on the Western diet relative to standard diet (1.27% +/- 0.07, 0.82% +/- 0.50 vs. 0.57% +/- 0.50). *Bacillales* are increased in the colon of people with CRC compared to healthy controls [157]. *Verrucomicrobiales* were not significantly affected by ketogenic diet by were reduced by 300% in the Western diet group relative to standard diet (4.29% +/- 1.09, 1.19% +/- 0.77 vs. 3.58% +/- 0.77). *Verrucomicrobiales* are positively associated with the inflammatory response in a murine dextran sodium sulfate (DSS)-induced colitis model [158].

An increase in the order *Fibrobacterales* is associated with a diabetic state in mice [159]. Previous research in our lab showed that FVB mice fed either a ketogenic or Western diet have an exaggerated glucose response in glucose tolerance tests, indicative of a diabetic or pre-diabetic state. Accordingly, we found a highly significant increase 32- and 23- fold increase in *Fibrobacterales* for FVB mice fed ketogenic and Western diet, respectively, relative to mice fed a standard diet (0.064 +/- 0.012, 0.47 +/- 0.008 vs. 0.002 +/- 0.008).

**Comparison of Diet Effects on Microbiota Composition at Species Level**

To determine the diet effects on microbiota composition at the level of species in each strain, the same screening and statistical approach was used as for the phylum and order level but species was used as the response.

Only one species, *Pedobacter kwangyangensis*, was identified in the screening report for B6 mice (Table 3). It was significantly decreased in mice fed Western diet.
Table 3. Comparison of species abundance (% of total) for B6 mice on standard (stand) vs. ketogenic (keto) or Western (west) diet. Order is indicative of significance found in screening report. Significant results vs. standard diet shown in green.

<table>
<thead>
<tr>
<th>Species</th>
<th>Abundance</th>
<th>Standard Error</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Keto</td>
<td>Stand</td>
<td>West</td>
</tr>
<tr>
<td><em>Pedobacter kwangyangensis</em></td>
<td>0.0017</td>
<td>0.0025</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

Nineteen species were identified in the screening report for FVB mice (Table 4). All species showed a significant change in abundance from standard diet except for *Bacteroides uniformis*, which had a nearly significant trend.
<table>
<thead>
<tr>
<th>Species</th>
<th>Abundance</th>
<th>Standard Error</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Keto</td>
<td>Stand</td>
<td>West</td>
</tr>
<tr>
<td>Bacteroides coprocola</td>
<td>0.0000</td>
<td>0.0202</td>
<td>0.0160</td>
</tr>
<tr>
<td>Parabacteroides distasonis</td>
<td>0.0002</td>
<td>0.0027</td>
<td>0.0022</td>
</tr>
<tr>
<td>Dysgonomonas hofstadii</td>
<td>0.0027</td>
<td>0.0163</td>
<td>0.0120</td>
</tr>
<tr>
<td>Allocaulum stercoricanis</td>
<td>0.0038</td>
<td>0.0096</td>
<td>0.0004</td>
</tr>
<tr>
<td>Bacteroides acidificiens</td>
<td>0.0776</td>
<td>0.0505</td>
<td>0.1241</td>
</tr>
<tr>
<td>Akkermansia muciniphila</td>
<td>0.0688</td>
<td>0.0563</td>
<td>0.0189</td>
</tr>
<tr>
<td>Chthoniobacter flavus</td>
<td>0.0052</td>
<td>0.0048</td>
<td>0.0017</td>
</tr>
<tr>
<td>Bacteroides herarinolyticus</td>
<td>0.0383</td>
<td>0.0234</td>
<td>0.0201</td>
</tr>
<tr>
<td>Bacteroides stercoris</td>
<td>0.0224</td>
<td>0.0144</td>
<td>0.0095</td>
</tr>
<tr>
<td>Blautia hydrogenotrophica</td>
<td>0.0023</td>
<td>0.0151</td>
<td>0.0006</td>
</tr>
<tr>
<td>Blautia obeum</td>
<td>0.0004</td>
<td>0.0178</td>
<td>0.0024</td>
</tr>
<tr>
<td>Clostridium frigoris</td>
<td>0.0000</td>
<td>0.0063</td>
<td>0.0007</td>
</tr>
<tr>
<td>Catonella morbi</td>
<td>0.0004</td>
<td>0.0162</td>
<td>0.0007</td>
</tr>
<tr>
<td>Oribacterium sinus</td>
<td>0.0003</td>
<td>0.0116</td>
<td>0.0004</td>
</tr>
<tr>
<td>Gemella cunicula</td>
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<td>0.0086</td>
<td>0.0001</td>
</tr>
<tr>
<td>Bacteroides uniformis</td>
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<td>0.0392</td>
<td>0.0281</td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>0.0049</td>
<td>0.0045</td>
<td>0.0023</td>
</tr>
<tr>
<td>Prevotella buccae</td>
<td>0.0000</td>
<td>0.0031</td>
<td>0.0022</td>
</tr>
<tr>
<td>Thermobacillus xylanilyticus</td>
<td>0.0049</td>
<td>0.0002</td>
<td>0.0023</td>
</tr>
</tbody>
</table>

Table 4: Comparison of species abundance (% of total) for FVB mice on standard (stand) vs. ketogenic (keto) or Western (west) diet. Order is indicative of significance found in screening report. Significant results vs. standard diet shown in green. Trends shown in yellow.

Several species identified in the screening report have known associations with colorectal cancer. Parabacteroides distasonis abundance was reduced by more than 1000% in FVB mice fed ketogenic diet compared to those fed standard or Western diet (Figure 25). Depletion of this species has previously been implicated in colorectal cancer [160].
"Bacteroides stercoris" has been associated with increased risk of colorectal cancer in a Japanese migration study [161]. This species was significantly increased by 155% in FVB mice fed ketogenic diet relative to standard diet, and it was decreased in the Western diet group by 66%. *Blautia hydrogenotrophica* was greatly decreased in FVB mice fed ketogenic or Western diets. This species is known to play a role in healthy colon function[162]. *Blautia obeum* (previously classified as *Ruminococcus obeum*) was reduced by and 740% in FVB mice fed Western diet and by 4,400% in FVB mice fed ketogenic diet. This species is involved in propionate synthesis, a short-chain fatty acid that acts as a fuel source and HDAC inhibitor in the colononcytes [163].

![Figure 25. Relative abundance of *Parabacteroides distasonis* in B6 and FVB mice.](image)

Relative abundance is the percent abundance of the species compared to all species in the gut microbiome. No significant changes from initial (pre-diet) to final (four months on diet) samples were detected in B6 mice. Significance in FVB mice of $p<0.01$ indicated by **.
Bacteroides acidifaciens was significantly increased in by 153% in FVB mice fed ketogenic diet and by 246% in FVB mice fed Western diet. Studies suggest this species is protective against obesity and insulin resistance [164, 165].
DISCUSSION

We examined microbiota abundance in two cohorts of mice. While the same mouse strains and diets were used in each cohort, the gut microbiota composition varied greatly between cohorts. The variable “cohort” had the largest effect at phylum-level microbiome change, while the interaction of cohort by strain had the largest effect at the species-level. The cohorts were studied at different locations, NC State University and Texas A&M University, different feeders were used, and level of obesity differed. We have found that the feeder type is the major factor accounting for the differences in adiposity between the cohorts. After examining differences between cohorts, we further investigated cohort two for correlations with phenotypic changes because our cancer studies were performed on the same type of feeders used in cohort two, and cohort two has both pre-diet and post-diet samples which allows for diet-specific changes to be more accurately evaluated by controlling for starting bacterial abundance.

We identified that the effect of diet on gut microbiota composition is dependent upon the genetic background of an individual. Diet effects on phylum-level abundance were consistent across strains. However, diet effects on order abundance, species abundance, and species diversity differed by strain. When compared within strain, the abundance of more species was affected by diet in the FVB strain than in the B6 strain. There were 19 species identified in the screening report for diet effects in FVB but only one species for B6. This agrees with the previous findings that gut microbiome plasticity differs by strain [35].
Our finding in the TAMU cohort of increased *Bacteroidetes* to *Firmicutes* ratio in mice fed high-fat diets agrees with previous work [166], but also contradicts other studies [35, 155]. There is disagreement in both humans and mice about the influence of high-fat diets and obesity on the directionality of the *Bacteroidetes* to *Firmicutes* shift. It has been suggested that subject heterogeneity including differences in age, or sample processing or analyzing techniques may account for the differences [167]. However, results from the current study disagree with that assessment, as we observed opposite *Bacteroidetes* to *Firmicutes* ratio shifts in the TAMU and NCSU cohorts. These cohorts used aged-matched, genetically identical mice fed identical diets. Sample collection was identical between cohorts and analysis for both cohorts was performed concurrently. Given that similar physiological responses were observed between cohorts and the disagreement in literature of the significance of *Bacteroides* to *Firmicutes* shifts, it is likely that phylum-level shifts are an overly simplistic characterization of gut microbiome composition. Examination of specific genera within the phyla or examination of functional gene changes in the metagenome would likely serve as better predictors of the physiological relevance of microbiome composition shifts.

We correlated diet-dependent shifts in abundance of specific species implicated in colonic health with incidence of colorectal cancer in FVB mice. This study was performed on mice that had not been exposed to the carcinogen azoxymethane (AOM), which allowed us to identify diet-dependent microbiota composition changes independent of tumorigenesis, thereby improving our ability to identify diet effects that alter CRC risk.
Strain by diet interactions were evident when evaluating effects on species richness. Species richness was decreased in FVB mice fed ketogenic and to a lesser degree those fed Western diet. No significant effects were observed in B6 mice. This agrees with the finding that a greater number of species significantly varied by diet in the FVB strain than the B6 strain. A reduction in species diversity has been associated with colorectal cancer incidence and obesity [154-156]. It is unclear whether this effect is causative. Loss of diversity may increase abundance of undesirable bacterial species or eliminate certain protective bacterial species. The reduction in diversity in the FVB mice may partially explain their increase in tumor load. However, no significant effect on tumor load was observed in FVB fed Western diet mice despite a reduction in species diversity. It is likely that the specific species altered were different between the diets and caused different effects on colonic health.

Changes in *Parabacteroides distasonis* abundance are of potential importance because of the species proposed protective effects against colonic inflammation and tumorigenesis. A previous study of the gut microflora of obese Apc^{1638N} mice found that the mice harboring tumors were depleted of *P. distasonis*, and this depletion was associated with a reduction of the inflammatory cytokine Il1b [160]. *P. distasonis* has been inversely associated with presence of Crohn’s disease [168]. In individuals afflicted with Crohn’s disease, depletion of *P. distasonis* is correlated with increased severity of inflammation [168]. Oral administration of the membrane fraction of *P. distasonis* decreased colitis in a dextran sodium sulfate (DSS) mouse model [169]. In that study, the *P. distasonis* membrane fraction reduced the release of Tnf, Il6, Ccl2 (MCP-1), and Ccl12 (MCP-5) by macrophages following a lipopolysaccharide challenge, which strengthens the idea that *P. distasonis* has
anti-inflammatory effects. Evidence suggests the putative protective effects of \textit{P. distasonis} against inflammation are related to an immune-modulatory capacity of some membrane components rather than any metabolites [160].

\textit{Blautia hydrogenotrophica} is an acetogenic species, which produces acetate from carbon dioxide and hydrogen. \textit{B. hydrogenotrophica} abundance was reduced in FVB mice fed Western or ketogenic diets. Acetogenic species are important for gut health, as acetate is converted to butyrate. Butyrate an important fuel source and histone deactylase inhibitor (HDAC) in colonocytes [162]. Butyrate has been associated with decreased risk of colorectal cancer [162]. Similarly, \textit{Blautia obeum} is involved in production of the short-chain fatty acid propionate, which acts as a fuel source and an HDAC inhibitor in colonocytes [163]. This species was decreased in FVB mice fed Western diet and further decrease in FVB mice fed ketogenic diet. Reductions in these species could have a detrimental impact on health of the colon by decreasing abundance of short-chain fatty acids in the colon and altering gene expression through loss of HDAC inhibitors.

The absence of \textit{Bacteroides coprocola} in FVB but not B6 mice fed ketogenic diet was striking. The PATRIC database lists carbohydrate and lipid metabolism as pathways in which \textit{Bacteroides coprocola} is involved [170]. We did not find any previous links to colorectal cancer, but given its unique absence in FVB ketogenic diet mice, it is a potentially interesting target for further investigation.

Overall these data indicate that diet and genetic background are important in the composition of the gut microbiome. However, investigate of phylum level gut microbiome composition is not sufficient to reveal these changes. We suggest that the
Firmicutes:Bacteroidetes ratio may be more dependent on the study location than diet or genetic background effects. Lastly, we identified several bacterial species that may be involved in increased tumor multiplicity observed in FVB mice fed ketogenic diet.
CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS
CONCLUSIONS

Strong associations exist between the dietary patterns and disease spectra of populations. However, there is an absence of information on the relationship between individual and population-level responses. Recently, studies in mice and humans have suggested that individuals have highly variable diet responses, which questions the efficacy of universal dietary recommendations. Rather than determining the average diet response of a population, examining how individuals differ in diet response can lead to the discovery of the genetic factors underlying the response. The long-term goal of these studies is to develop precision nutrition, in which optimal dietary patterns are identified on an individual basis to maximize health of each person in a population.

To determine how individuals vary in response to diet, we examined a range of dietary responses in four genetically diverse inbred mouse strains (A/J, C57BL/6J, FVB/NJ, and NOD/ShLtJ mice) fed five types of diets. Four experimental diets (Western, Mediterranean, Japanese, and ketogenic) were selected due to their previous associations with health effects in people and compared to a standard laboratory mouse diet. Phenotypes studied included those related to cardiometabolic health, colonic tumors, and gut microbiome.

In the cardiometabolic studies, we examined the health status each strain of mice while fed one of the five diets for six months. Phenotypes studied included weight gain, adiposity, cholesterol, insulin, leptin, serum triglycerides, liver triglycerides, alanine...
aminotransferase (ALT), asparate aminotransferase (AST), glucose tolerance, insulin tolerance, food consumption, activity, metabolic rate, and liver mitochondrial function.

The cardiometabolic studies demonstrated that the effects of a diet are complex and dependent upon the genetic background of the individual. We found that mice can have opposite responses to a diet with one strain showing positive health effects and another showing negative effects.

One of the most striking differences was C57BL/6J (B6) and FVB/NJ (FVB) mice fed a ketogenic diet. B6 mice maintained a normal level of adiposity and had few signs of metabolic distress. FVB mice had increased adiposity accompanied by negative glucose tolerance effects and greatly increased fat deposition in the liver.

B6 and FVB mice also had divergent responses to the Western diet. B6 mice fed Western diet had increased adiposity despite reduced caloric intake, strongly increased LDL cholesterol, negative effects on glucose tolerance, and increased liver fat deposition. FVB mice also experienced detrimental effects on glucose tolerance and increased liver fat deposition, but they maintained a normal weight and appeared otherwise healthy.

B6 mice had the most detrimental effects from the Western diet of any strain. A multitude of studies have evaluated effects of high fat, high sucrose (HF/HS) diets on various phenotypes. These studies are generally performed in B6 mice, given the depth of previous data on the strain. However, caution should be used when interpreting results of HF/HS feeding in B6 mice because the response is likely not be generalized to other strains.

We found that some individuals are largely resistant to the effects of diet. It has been reported previously that A/J (A) strain mice are resistant to the effects of high fat, high
sucrose diets [44, 106]. In our study, the A strain showed few physiological shifts on either a Western or Mediterranean diet. When physiological changes were observed, they were typically milder than those observed in other strains. The ketogenic diet strongly increases metabolic rate in the A strain. But, this did not result in significal health changes as the mice maintained a normal weight and were otherwise unaffected. Cardiometabolic health effects were nearly identical in A strain mice fed standard, Western, Mediterranean, or ketogenic diets, with only the Japanese diet altering the health status of the A strain. When fed Japanese diet, the A strain has increased liver triglycerides, decreased HDL cholesterol, and elevated levels of ALT, a marker of liver damage. These responses are unique to A strain. The other strains fair better on the Japanese diet, showing positive physiological changes compared to mice fed a standard diet.

Responses in the NOD/ShltJ (NOD) strain were more variable, likely due to the strain’s predisposition to develop diabetes and the variable onset of the disease. However, it is clear that all high fat diets (Western, Mediterranean, ketogenic) produced negative health effects in NOD mice.

The ketogenic diet increased metabolic rate in all mouse strains without affecting activity. The increase in basal metabolic rate was particularly strong in the A strain. Expression of uncoupling protein (Ucp) was not significantly different between A strain mice fed standard or ketogenic diet in brown fat, white fat, muscle, or liver. There was no indication of increased mitochondrial biosynthesis based on gene expression analysis. The cause of the increased metabolic rate is currently unknown.
In human studies, the Japanese and Mediterranean diets have been repeatedly correlated with beneficial health effects. In our study, the Japanese diet showed beneficial cardiometabolic effects for all strains except the A strain. Mediterranean diets produced mixed results. The diet had detrimental effects in the B6 and NOD strains, but the A and FVB strains responded more favorably. Given that we have only tested the response in four individuals, it is feasible that other strains would show a more favorable response. The cause of the poor health effects in B6 and NOD strains could be attributed to genetic factors or to differences between the human Mediterranean diet and the mouse formulation of a Mediterranean diet. The mouse Mediterranean diet accurately recapitulates the macronutrient ratios, fatty acid profiles, types of ingredients, and fiber content found in the human Mediterranean diet. However, differences such the absence of fresh ingredients in the mouse diet could have impacted the results. Lastly, there could be genetic or lifestyle effects unrelated to diet that protect individuals in the Mediterranean region from chronic diseases.

We determined that increased adiposity was caused by either increased caloric intake or by increased feed efficiency depending on diet and strain. FVB mice fed ketogenic diet had increased adiposity due to increased caloric intake. B6 and NOD mice on Western and Mediterranean diet had increased adiposity due to increased feed efficiency. These mice ate fewer calories but gained more adiposity. This indicates that caloric intake is an important consideration for maintaining a healthy weight, but some individuals can gain weight on specific diets despite eating fewer calories.

We studied effects of diet on cancer using a carcinogen-induced mouse model of colorectal cancer in the same strains fed the same diets. Mice were acclimated to diets for ten
weeks before carcinogen exposure. Six months after carcinogen administration, the mice were necropsied and colons analyzed for tumor penetrance, multiplicity, size, and overall tumor load were analyzed.

The strongest increase in penetrance was observed in B6 mice fed the Mediterranean diet. No significant effect on tumor multiplicity was detected, likely due to the overall low tumor multiplicity in the B6 strain and limited sample size. Obtaining the same results in another, equally sized cohort is predicted to yield a significant increase in tumor multiplicity. Penetrance also increased in FVB mice fed ketogenic diet. This was accompanied by increases tumor multiplicity and tumor load but there was no effect on tumor size. A trend towards increased penetrance (p=0.069) was observed in FVB mice fed ketogenic diet. In this case, tumor multiplicity increased significantly but the increase in tumor load did not reach significance.

Average tumor size was significantly affected for any groups. The increased penetrance and tumor multiplicity without effects on size indicates that diet impacts the initiation rate of tumors but does not affect growth. Some have suggested that the ketogenic diet may reduce tumor growth by limiting glucose availability [171]. The current findings do not support this assertion in the case of colorectal tumors.

The relationship between the metabolic health of mice on a specific diet and their colorectal cancer risk on that diet varied. B6 mice on the Mediterranean diet and FVB mice on the ketogenic diet showed poor metabolic health and increased tumor penetrance. However, metabolic health in B6 mice on the Western diet was as bad or worse than those on Mediterranean diet, but there was no significant impact on colorectal cancer. Additionally,
while FVB mice on Japanese diet showed normal metabolic health, they had increased tumor multiplicity and a trend towards increased penetrance. These findings reveal that metabolic health is sometimes, but not always associated with colorectal cancer risk. Certain diets may increase cancer risk in some individuals without overt signs of metabolic distress.

Our ability to identify significant diet effects on colorectal cancer was limited in A and NOD mice due to low survival rates on some diets and high penetrance in these strains on all diets. Larger sample sizes are necessary to determine significant diet effects in these strains. Examining strains with lower penetrance may be preferable.

Given that FVB and B6 mice had such varied responses to the Western and ketogenic diets, we compared the gut microbiota composition in the two strains fed Western, ketogenic, or standard diet. Samples were isolated from mice in the cardiometabolic studies. This allowed us to determine diet effects on gut microbiota composition without the presence of tumors. We analyzed changes in bacteria genera based on 16S ribosomal mapping and correlated the changes with incidence of colorectal cancer and obesity.

We identified that the diversity of the gut microbiome is reduced in FVB mice fed ketogenic and, to a less degree, Western diet. However, no significant effect was observed in FVB mice. Loss of species diversity has been implicated in colorectal cancer and obesity [154-156]. The finding that species diversity was lowest in FVB ketogenic diet fed mice is correlated with the increased tumor load in these mice and agrees with the previous literature.

Gut microbiota composition was analyzed in two independent cohorts of mice, the UNC cohort and TAMU cohort of the metabolic study (Chapter 2). There were notable differences between cohorts in microbiota composition at the phylum level. We observed
opposite directionality of the *Firmicutes* to *Bacteroidetes* shifts in the two cohorts. The utility of the *Firmicutes* to *Bacteroidetes* shift in response to high-fat diet feeding and obesity has been under a subject of controversy in gut microbiome research, and some have suggested that age or sample handling and processing techniques may account for the differences [167]. Our study refutes that notion, as both cohorts yielded similar physiological effects, mice were age-matched and samples were processed concurrently, yet opposite directionally persisted. Our results question the utility analyzing the *Firmicutes* to *Bacteroides* shift to draw broad conclusions about the impact of the microbiome on health.

Differences between the UNC and TAMU cohorts of the metabolic study included location, feeder type, and level of adiposity. Follow-up studies have demonstrated that the feeder type may be largely responsible for the observed difference in adiposity between the cohorts. (Appendix C). We used data from the TAMU cohort of the metabolic study in this analysis of the microbiome because it more closely matches the conditions of the AOM cancer studies (e.g. feeder type) and we have pre-diet microbiome samples from the TAMU cohort to compare starting bacterial abundances.

We found an interesting relationship between abundance of *Parabacteroides distasonis* and severity of colorectal cancer in FVB mice fed ketogenic diet. *P. distasonis* was nearly eliminated in FVB mice fed a ketogenic diet but no diet effect was detected in B6 mice. The Western diet had no significant effect on *P. distasonis* in either FVB or B6 mice. Thus, the abundance of *P. distasonis* was only depleted in FVB mice fed ketogenic, which is the same group that experienced high tumor load. Previous studies indicate *P. distasonis* is protective against colonic inflammation in mice and humans [168, 169], and it has been
inversely associated with the presence of tumors in obese mice in an $Apc^{Min}$ model of colorectal cancer [160]. Evidence suggests the putative protective effects of $P.\ distasonis$ against inflammation are related to an immune-modulatory capacity of some membrane components rather than any metabolites [160]. The loss of protective species like $P.\ distasonis$ in FVB ketogenic diet fed mice but not Western diet sheds light on why loss of species diversity could have different effects in mice fed the two diets.

While $P.\ distasonis$ seems to directly impact colon health through interactions with cell membrane components, other species act indirectly through enzymatic actions that alter abundance of metabolites. $Blautia\ hydrogenotrophica$ is involved in the butyrate synthesis pathway. Butyrate is a short chain fatty acid, which has protective effects against colorectal cancer. Butyrate is a histone deactylase (HDAC) inhibitor that causes gene expression changes in colonocytes[162]. $B.\ hydrogenotrophica$ was reduced in FVB mice fed Western or ketogenic diets. Another related bacteria, $Blautia\ obeum$, is involved in synthesis of another short-chain fatty acid, propionate, which also acts as a HDAC inhibitor in colonocytes [163]. This species was reduced by over 700% in FVB mice fed western diet and by over 4,400% in FVB mice fed ketogenic diet. Reduction of these species could decrease abundance of short-chain fatty acids that are important for colonic health.
FUTURE DIRECTIONS

Follow-up studies are underway to better understand the observed diet by strain interactions and the genetic factors causing them. B6 and FVB mice have divergent responses to ketogenic and Western diets, particularly in terms of adiposity. An F1 cross was generated and adiposity gain on each diet was determined. We found that F1 mice had high levels of adiposity on both diets. We then generated an F2 population of 482 individuals and mice were placed on Western or ketogenic diets. After four months on the diets, adiposity greatly varied on both diets. There was a normal distribution of body fat percentages that ranged from 5% to over 50%. QTL mapping will be performed to identify regions of interest using adiposity, serum triglycerides, liver triglycerides, and cholesterol as phenotypes.

The A and B6 strains also had different dietary responses, particularly on Western and ketogenic diets. B6 mice had poor cardiometabolic effects on a Western diet, whereas mice of the A strain showed few detrimental effects. The A strain had a much higher metabolic rate on the ketogenic diet than B6 mice. We are currently generating F1 and F2 populations from the A and B6 parental strains to identify genetic factors influencing the resistance or susceptibility to negative effects from the Western diet, as well as those factors controlling metabolic rate in mice fed ketogenic diet.

The large increase in metabolic rate in the A strain mice fed ketogenic diet is important in understanding factors influencing metabolism and energy balance and merits further exploration. Ucp expression changes are not associated with the increased metabolic
rate. We are currently investigating the rate of protein turnover in these mice to determine its potential impact on metabolic rate.

Our study has demonstrated that dietary patterns can cause vastly different health effects in four genetically diverse individuals. Repeating this type of experiment in a large panel of mice would allow a better understanding of the range of possible outcomes form various dietary modifications and allow more precise mapping the genetic factors the underlying the varied diet responses. A panel of mice, such as the Collaborative Cross (CC), could be used to further investigation strain by diet effects. The CC is a panel of inbred mouse strains derived from eight found strains, three of which are wild derived. The level of genetic diversity in the CC panel is comparable to that of human populations. This would allow mapping of specific genetic factors that could ultimately be used to predict diet responses in people.

This study has indentified interesting correlations between gut microbiota composition in mice eating specific diets and carcinogen-induced colonic tumor incidence in mice fed those diets. *P. distasonis* is of particular interest due to the strong evidence that the ketogenic diet affects its abundance specifically in FVB mice, and its previously document impacts on colonic inflammation and tumorigenesis. Further studies will be performed to determine whether loss of *P. distasonis* in the gut microbiome of FVB mice fed a ketogenic diet causes an increase in tumor load. To do so, an AOM study could be performed in which *P. distasonis* is administered to a group of FVB mice fed a ketogenic diet and compared to a group of mice fed ketogenic diet without *P. distasonis* administration. A decrease in tumor load in mice administered *P. distasonis* would indicate a causal relationship. Similar
experiments could be performed on other bacterial species of interest including *B. hydrogenotrophica* and *B. obeum*.

Since several bacterial species of interest have been identified from mice in the cardiometabolic study, these species should be examined in mice with tumors. This could provide further evidence of a causal relationship and allow for direct correlations of cancer incidence and severity with bacterial abundance.

Additional dissection of gut microbiome data will be performed. Currently, the bioinformatic platforms that allow for whole-genome phylogenetic and function gene analysis operate inefficiently due to high demand. Therefore, only 16S ribosomal analysis has been completed, but this approach makes use of less than 5% of our collected data. Whole-genome analysis will allow for higher resolution phylogenetic mapping. Functional gene analysis can provide a plethora of information about how diets affect abundance of various bacterial genes related to obesity and cancer and how pathways in the metagenome are impacted.

The current study highlights the importance of considering the genetic background of an individual when evaluating diet response. The data demonstrate the need to analyze individual variation to diet response, rather than simply identifying population average responses. Future studies will be necessary to determine the genetic factors underlying the differential diet responses and to predict how human individuals will respond to various diets.
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172. Shirai N, Suzuki H: Effects of Western, Vegetarian, and Japanese dietary fat model diets with or without green tea extract on the plasma lipids and glucose,

## Appendix A. Timelines of Cardiometabolic Cohorts

### NCSU (Cohort 1)

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### TAMU (Cohort 2)

**Appendix Figure 26. Timelines of cardiometabolic studies.** Months are listed below timelines. Each cohort began when mice were five weeks old. MRI indicates body composition measurement. GTT indicates glucose tolerance test. ITT indicates insulin tolerance test.
APPENDIX B. DIET PREPARATION AND COMPOSITION

Diet Preparation

Diets were based on national food intake data and previous studies. Diets were manufactured by Research Diets, Inc. (New Brunswick, NJ) in collaboration with Senior Scientist, Michael Pellizzon. Diets were designed to mimic human diets as closely as possible, matching both the macronutrient content and ingredients. A wide range of ingredients were used to formulate the mouse diets including egg, casein, beef, soy, fish protein, rice starch, wheat starch, corn starch, potato starch, and various types of fats.

Mediterranean and Japanese Diets (1961)

Mediterranean and Japanese diets were formulated with data from the “Food Balance Sheets” produced by the Food and Agriculture Organization (FAO) of the United Nations [99]. Red wine extract and green tea extract were added to the Mediterranean and Japanese diets, respectively, to match the average amount of wine/tea consumed per person in those areas. Exact fatty acid compositions were not available from the FAO so this data was obtained from previous studies [172, 173].

Western Diet (2005)

The Western Diet was formulated similarly to those above, but using the USDA’s “Dietary Assessment of Major Food Trends” [100]. This resource was used because it was more complete than the FAO data.

Ketogenic Diet
The ketogenic diet was based on a previous study [101]. Fish oil was added to the diet to supplement omega-3 fatty acids.

Appendix Table 5. Diet ingredients.
Appendix Table 6. Macronutrient ratios of diets and relative contribution of ingredients.

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<td>4.9</td>
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<tr>
<td>Cellulose, BW200 (Insoluble) (g/kg)</td>
<td>70.6</td>
<td>47.6</td>
<td>9.7</td>
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<td>61.5</td>
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<td>Insulin (Starch) (g/kg)</td>
<td>23.3</td>
<td>15.8</td>
<td>36.7</td>
<td>25.1</td>
<td>6.9</td>
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<td>Total fiber(g/kg) (includes starch sources)</td>
<td>97.5</td>
<td>59.4</td>
<td>48.3</td>
<td>94.3</td>
<td>26.3</td>
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<td>Cholesterol (mg/kg)</td>
<td>71.3</td>
<td>239.8</td>
<td>225.3</td>
<td>617.6</td>
<td>2096.2</td>
<td>3422.1</td>
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<td>Red Wine Extract (mg/kg)</td>
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<td>Green Tea Extract (mg/kg)</td>
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<td>0</td>
<td>207.9</td>
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<tr>
<td>BHQ (mg/3641 kcal)</td>
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<td>4.9</td>
<td>4.9</td>
<td>4.9</td>
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<tr>
<td>Betacarotene (mg/3641 kcal)</td>
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<td>1.00</td>
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Appendix Figure 27. Macronutrient compositions of diets.
APPENDIX C. FOOD CONSUMPTION PROTOCOL

MATERIALS:
- coffee filter to act as spill paper
- scale
- weigh boats
- notebook, pens
- diet,
- jars and lids with holes to allow feeding access
- wire-bottom cage (urine analysis chambers)

To weigh food in:

1. Lay clean, labeled spill paper under each cage. Make sure the edges lay flat and it is centered under the appropriate cage. Flatness of bedding and spill paper is important because rodents can reach down through the bottom of the cage and grab the paper, inevitably shredding it to bits.
2. Tare scale.
3. Place appropriate amount of food in jar and weigh jar with food in it.
4. Record value in the “Food in” column.

To weigh food out and spill:

1. Return all animals to their original housing taking care not to disturb food still in the cage/food jar or the spill papers.
2. Open cage and remove food jar.
3. Make sure there are no chunks of food on bottom of cage. If there are, retrieve it and place it back in jar.
4. Place jar and leftover food on scale and weigh (remove glass food cup weight for mice beforehand).

5. Record value in “Food out” column.

6. Also record any abnormalities such as wet or urine-soaked food in comments section.

**Weigh spill:**

1. Place weigh boat on scale and tare.

2. Carefully lift spill paper off bedding, curling edges so food crumbs don’t fall.

3. Sift spill so that it accumulates in the center of the paper.

4. Using a gloved hand and/or the folded index card, separate feces from spill. Make sure there are no food bits stuck to feces.

5. Once all feces is removed from the paper, collect food in center of paper.

6. If spill is wet with urine, you must allow it to thoroughly dry before weighing. Set the spill paper aside overnight and weigh it the next day.

7. Make a crease in the middle of the paper on one edge to form a spout from which you will pour the spill.

8. Carefully pour the spill into the weigh boat.

9. Record spill value on the data sheet in the column marked “Spill”.
### APPENDIX D. BETA-HYDROXYBUTYRATE CONCENTRATIONS

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<tr>
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<th>FVB</th>
<th>NOD</th>
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<tr>
<td>BHB Conc.</td>
<td>Keto</td>
<td>Stand</td>
<td>Keto</td>
<td>Stand</td>
</tr>
<tr>
<td>Mean (mM)</td>
<td>4.85</td>
<td>0.35</td>
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<tr>
<td>Std. Error</td>
<td>0.04</td>
<td>0.02</td>
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Appendix Table 7. Comparison of beta-hydroxybutyrate (BHB) concentrations by diet and strain. Samples were collected after mice had been fed ketogenic (keto) or standard (stand) diet for three months.
APPENDIX E. FEEDER COMPARISON

Appendix Figure 28. Change in body fat after two months by feeder type, diet, and strain. Effect of different feeders on adiposity was assessed in the same facility in 5 FVB and B6 mice of each sex. The results indicate feeder type contributed to adiposity differences between NCSU and TAMU cardiometabolic studies. Open feeders were used for the TAMU cardiometabolic cohort and all cancer cohorts. Closed feeders were used in the NCSU cardiometabolic cohort. Significance p<0.05 indicated by *, p<0.01 indicated by **. A non-significant trend was detected for feeder type for B6 mice on standard diet (p=0.075).
Appendix Figure 29. Feeder Comparison. Closed feeders were used for the NCSU cohort of the cardiometabolic study. Open feeders were used for the TAMU cohort of the cardiometabolic and all cancer studies. Closed feeders limit eating access to one mouse at a time and a wire screen in the metal dish prevents mice from spreading food. Open feeders allowed all mice to eat simultaneously.
# APPENDIX F. qPCR PRIMER SEQUENCES AND RESULTS

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<td>Ucp1 F</td>
<td>CGACTCAGTCCAAGAGTACTTCTCTTC</td>
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<tr>
<td>Ucp1 R</td>
<td>GCCGGCTGAGATCTTTGTTTC</td>
</tr>
<tr>
<td>Ucp2 F</td>
<td>TCCCCCTGTTGATGTGGTCAA</td>
</tr>
<tr>
<td>Ucp2 R</td>
<td>CAGTGACCTGCGCTGTGGTA</td>
</tr>
<tr>
<td>Ucp3 F</td>
<td>CCTACGACATCATCAAGGAGAAGTT</td>
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<tr>
<td>Ucp3 R</td>
<td>TCCAAAAGAGACAAAGTGAA</td>
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<tr>
<td>Ucp5 F</td>
<td>TCACAACTGCTCAGCGTG</td>
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<td>Ucp5 R</td>
<td>GGTGCTTCTTTGTAATATCATAAACG</td>
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<tr>
<td>Cox1 F</td>
<td>TGCTTACACCACATGAAACA</td>
</tr>
<tr>
<td>Cox1 R</td>
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</tr>
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<td>Pgc1a F</td>
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</tr>
<tr>
<td>Pgc1a R</td>
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<td>Hprt R</td>
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Appendix Table 8. Primers sequences used for qPCR assays.
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<th>Diet</th>
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<th>Gene</th>
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<th>Std Error</th>
<th>Difference</th>
<th>p-value</th>
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<td>Standard</td>
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Appendix Table 9. Expression comparison of thermogenesis-related genes in A strain mice on ketogenic vs. standard diet. Gene expression is normalized to Hprt. No genes matched the expected direction change or reached significance threshold for explanation of increased metabolic rate in A strain mice fed ketogenic diet.
APPENDIX G. SURVIVAL IN CANCER STUDY COHORTS

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Appendix Table 10. Survival rate in Cancer Study NCSU Cohort 1.

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Appendix Table 11. Survival rate in Cancer Study NCSU Cohort 2.

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<td>100%</td>
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Appendix Table 12. Survival rate in Cancer Study TAMU Cohort.
### APPENDIX H. STATISTICAL EFFECTS SUMMARY FOR CANCER STUDIES

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**Appendix Table 13.** Effects of variables influencing microbiota composition at the phylum level.

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<tr>
<td>Cohort<em>Diet</em>Gender</td>
<td>5.748</td>
<td>0</td>
</tr>
<tr>
<td>Strain*Gender</td>
<td>3.607</td>
<td>0.00025</td>
</tr>
</tbody>
</table>

**Appendix Table 14.** Effects of variables influencing microbiota composition at the species level.