EFFECT OF DIET AND GENETIC BACKGROUND ON THE GUT MICROBIOME AND COLORECTAL CANCER IN MICE

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Effect of Diet and Genetic diversity on the Gut Microbiome and Colon Cancer Severity in Mice

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Although interest in the gut microbiome is at the forefront of research in recent years, there is still much to discover about its ever-changing nature and its importance to our health. This study examined the effects of diet and genetic background on the microbial composition in the gut and tumor load in mice. Four inbred mouse strains (A/J, C57BL/6J, FVB/NJ, and NOD/ShiltJ) were selected because of their known susceptibilities to colorectal cancer. The four diets used in this experiment were Western, Japanese, ketogenic, and standard mouse chow. The mice were given four weekly azoxymethane injections to induce tumors. To analyze the gut microbial composition, DNA was extracted from feces, sequenced, and analyzed for variation in bacterial species and functional genes associated with tumor proliferation. Colons dissected from the mice postpartum were used to determine average tumor size, tumor count, and overall tumor load as it varied between strains. In the FVB strain, ketogenic diet significantly increased tumor load by over 300%. This was correlated with a decrease in relative abundance of *Clostridiales* and *Lactobacillales*. Both of these genera have been shown to synthesize compounds that inhibit tumor proliferation. A significant increase in relative abundance of the genera *Bacteroidales* and *Sphaerochaetales* was also seen in mice on the ketogenic diet. The genera *Bacteroidales* produce a certain oncogenic toxin that encourages growth of tumors in the colon. Several other genera

have been overrepresented in the microbiota of mice on ketogenic diet including *Spaerochaetales* and *Verrucmicrobiales*. These genera were absent in most mice but were present in all mice that ate a diet that increased their incidence of colorectal cancer. By identifying and understanding how the gut microbiome composition is related to cancer severity, researchers can better understand the tremendous impact the gut microbiome has on health and disease.

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NOMENCLATURE

CRC	Colorectal Cancer
DNA	Deoxyribonucleic Acid
AOM	Azoxymethane
SCFA	Short chain fatty acids

CHAPTER I

INTRODUCTION

Interest in the gut microbiome has been at the forefront of research for many years because of its ever changing nature and its importance for our health. Research indicates there are functional interactions between the host and bacteria present in the gut. The bacteria in the gut provide the host with a variety of benefits including energy extraction, mucosal immunity, and metabolism (1). The gut microbiome has been directly associated with pathological diseases, drug metabolism, drug toxicity, immunity, and even post-surgical recovery (2). Abnormalities in the microbial composition, including an increase in opportunistic pathogens and a decrease in beneficial bacteria, has been implicated in various diseases including obesity, inflammation, cancer, and even mental illnesses (3). Evidently, the gut microbial composition plays a crucial not only in colonic health but the overall health of the host organism. Understanding the gut microbiome may be key in developing pharmaceutical drugs or personalized therapeutic strategies for those with microbial composition abnormalities.

This study not only looks at how the gut microbiome is affected by diet and genetic background, but also how it is associated with colon cancer incidence. Colorectal cancer (CRC), a cancer that affects both men and women equally, is the third leading cause of death due to cancer in the United States (4). Although there have been improvements in prevention and early detection of colon cancer, there is still much to discover about CRC etiology and susceptibility factors. A recent 2-week study related colon cancer and the shifts in the microbiome of African Americans that ate low-fat, high-fiber diet or a high-fat, low-fiber diet. The results from the study indicated

more inflammation, higher mucosal proliferation rates, and less butyrate producing species in people given a high-fat, low-fiber diet (5). However, this study was done over a short period of time with no actual cancer endpoint evaluated.

Previous studies in Dr. Threadgill's lab have examined the relationship between diet, physiology, and colorectal cancer. There are several reasons that diets in this study were selected. The Japanese diet was studied because it is linked with much lower rates of colorectal cancer than the typical diet in the United States and a Western diet was studied because it is associated with high rates of colorectal cancer (6). Ketogenic diets lack carbohydrates and could reduce tumor growth by limiting access to glucose, a tumor's preferred energy source. However, research in Dr. Threadgill's lab indicated that FVB mice develop tumors at an increased rate when given the low-carb, ketogenic diet. The standard diet is a typical lab mouse chow and was used for comparison. Four mouse strains (A/J, C57BL/6J (B6), FVB/NJ, and NOD/ShiltJ) were chosen due to varying susceptibility to the carcinogen azoxymethane (AOM), which is a genetic background-dependent tumor initiator and promoter. A/J mice are very susceptible and B6 mice are very resistant to AOM. Both FVB and NOD mice are intermediate in susceptibility to AOM.

Here, we evaluate the effect of diet on gut microbiome in genetically diverse individuals to find associations between bacterial species, functional genes, and tumor load.

CHAPTER II

METHODS

The samples used in this experiment were collected from a previous experiment that analyzed the incidence of colorectal cancer in two inbred mouse strains. Briefly, four-week old mice were ordered from Jackson Laboratory. Mice were fed diets for ten weeks, followed by four weekly injections of AOM (10 mg/kg). Mice remained on diets for 10 weeks following the final injection and then were necropsied. Upon necropsy, colons were fixed and stored in ethanol for analysis.

To determine tumor count, tumor average size, and overall tumor load, the colons were stained with 0.1% methylene blue and visualized at 10x under a compound microscope. The tumors were counted and the diameter was determined using digital calipers.

To analyze gut microbial composition, DNA was extracted from the feces using a FastDNA Spin Kit for Soil. This soil extraction kit was used because it produces the largest and most diverse DNA yields in comparison to typical DNA extraction kits (4). The purified DNA was then quantified using a Qubit dsDNA HS Assay Kit and Qubit fluoremeter. The Qubit HS Assay kit accurately determines how much double-stranded DNA was extracted from the feces samples. A resuspension buffer was used to normalize the DNA to a concentration of 20 ng/μl to reduce bias that could be introduced from using different DNA concentrations. The DNA was sheared to be 350 base pairs in length. Once the DNA library preparation was performed, sequencing of the microbiome DNA was executed using an Illumina NextSeq 500 sequencer. After the DNA was

sequenced, a bioinformatic analysis was performed using Illumina 16S Metagenomics Analysis software. This software performs phylogenetic mapping based on 16S ribosomal DNA. Bacterial genera were compared to diet and tumor load to explore the relationships between microbiota composition and colorectal cancer occurrences.

CHAPTER III

RESULTS

Colon Tumor Load and Size Data

Colon tumor load and size were determined for FVB and B6 mice on a Western, Mediterranean, Japanese and ketogenic diet. When compared to the standard diet (the control), the data indicates that there was a statistically significant increase in tumor load and count in FVB mice fed ketogenic diet ($p<0.05$). Interestingly, the size of tumors did not increase with an increase in tumor load. While there was an increase in tumor load on all diets compared to standard diet, the results were not statistically significant. The mice on the ketogenic had a 293% increase in tumor count (Fig.1) and a 362% ($p=0.01$) increase in tumor load (Fig.2).

Fig 1. Tumor Count in FVB mice on various diets

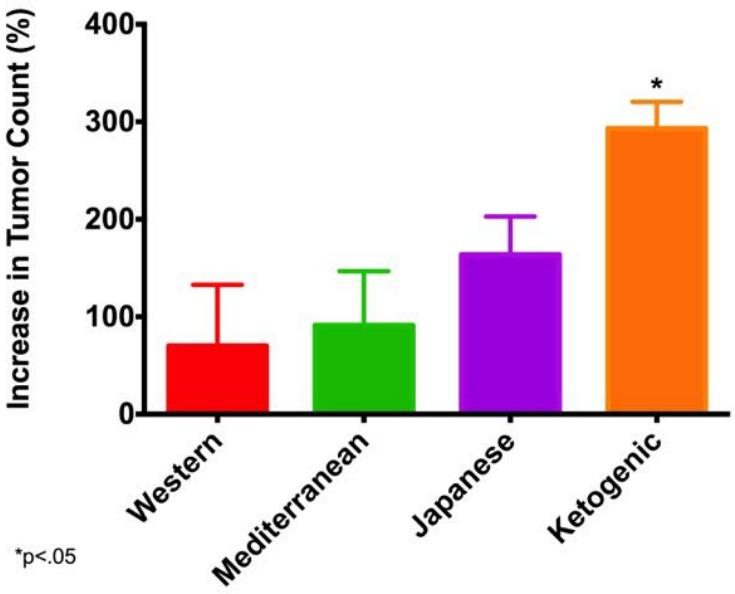
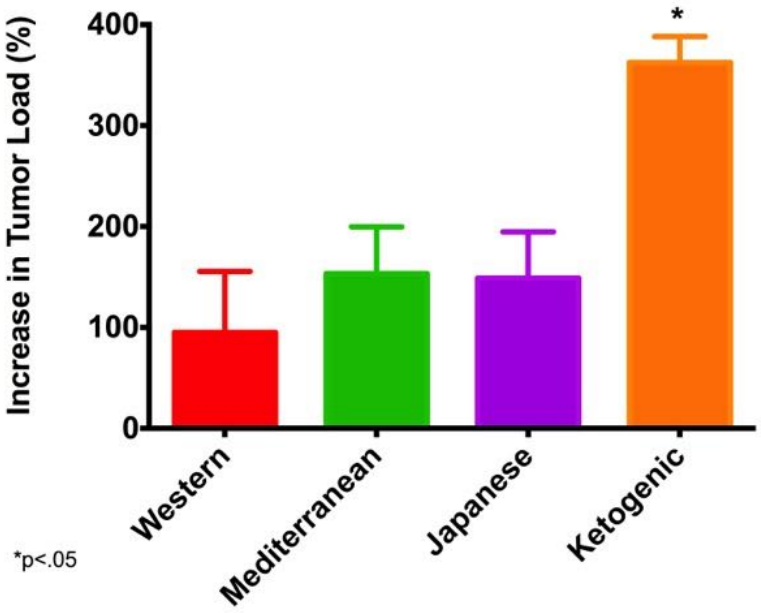


Fig 2. Tumor Load in FVB mice on various diets



In B6 mice, statistically significant results were not found for any diet effect on tumor load, tumor count or average tumor size. AOM treatment in B6 mice is less penetrant than in FVB. The lack of statistically significant results could indicate lack of effect of diet or insufficient power to detect differences.

Differences in Fecal Microbial Communities in FVB Mice on Ketogenic, Western, and Standard Diets Based on Taxonomic Comparisons

Gut microbiome composition was analyzed using Illumina's 16S Metagenomics Analysis to determine bacterial genera differences between samples. Results have been finalized for FVB mice. Computational analysis for the B6 mice is in progress.

Fig. 3 Percent Relative Abundance of Genera in FVB mice on Ketogenic and Western Diets

Diet	*Clostridiales*	*Bacillales*	*Bacteroidales*	*Camplobacteraceae*
Western	-60% *p=0.002	+119% *p=0.001	+41% *p=0.04	+2250% *p=0.002
Ketogenic	-33% p=0.204	+43% *p=0.001	+29% p=0.001	-25% p=0.992

Diet	*Lactobacillales*	*Flavobacteriales*	*Sphaerochaetales*	*Verrumicrobiales*
Western	-77% *p=0.03	+103% p=0.001	-100% p=0.83	-66% *p=0.003
Ketogenic	-84% p=0.06	-63% *p=0.02	+20580% *p=0.001	+21% p=0.60

At the order level in FVB mice, all of the samples had a microbial population that predominated in *Bacteroidales, Firmicutes,* and *Clostridiales*, as expected. The relative abundance of *Bacteroidales* in FVB mice on Western and ketogenic diets showed a marked increase when compared to the control group, FVB mice on standard mouse chow. As seen in Fig. 3, the mice on the Western diet had a 41% increase in the relative abundance of *Bacteroidales* (p=0.04), and mice on the ketogenic diet had a 29.2% in the relative abundance of *Bacteroidales* (p=0.0006). The relative abundance of *Clostridiales* (p=0.0024) in mice with the Western diet was a 60.3% decrease and a 33.4% decrease in abundance of *Clostridiales* (p=0.2006) in mice on a ketogenic diet. The *Clostridiales* were underrepresented in the mice with a ketogenic or Western diet, both of which had an increase in CRC tumor load. Compared to the control group, both mice on a ketogenic and Western diet had an increase in the order *Bacillales*. As seen in Fig. 3, mice on a Western diet had a 119.3% increase and mice on a ketogenic diet had a 43% increase compared to the mice on the standard diet. Two other groups, *Fusobacterium* and *Lactobacillales* were significantly less prevalent in mice on a ketogenic and Western diets. Remarkably, *Flavobacteriales* (p=0.0001) increased in relative abundance in the mice on a Western diet and

decreased in relative abundance in the mice on a ketogenic diet. *Verrucmicrobiales* also showed differences based on diet. Mice on a Western diet had a 66.3% decrease in relative abundance and mice on a ketogenic diet had a 21% increase in relative abundance.

The diversity of the bacterial species is also an important indicator of CRC proliferation. Compared to the standard diet, the mice on the ketogenic diet had a significant decrease in number of species detected even after normalizing for differences in number of hits between samples. This reduction of bacterial species in mice on the ketogenic diet could have an association with the increased CRC susceptibility.

CHAPTER IV

CONCLUSION

In this study, we compared the composition of gut microbiota in genetically diverse mouse strains on four different human-relevant diets. Analysis of tumor load, count and size revealed significant impacts of FVB mice eating a ketogenic diet versus the control. No statistically significant effects were observed in FVB mice eating other diets. No significant diet effects were observed in B6 mice. This could be because B6 mice are genetically more resistant to tumor proliferation from the AOM treatment. In the future, the B6 mice could be further tested to determine whether significant diet effects exist. Microbiome analysis revealed correlations between microbiota abundance on the level of order and tumor load and size in FVB mice fed a ketogenic diet. Once completed, microbiota analysis in B6 mice can be compared to the results obtained from FVB.

Our findings show that FVB mice on a ketogenic diet had a significant increase in tumor load and tumor count. There was not a statistically significant increase in size in mice on any of the diets. An increase in size would indicate an increase in tumor growth rate, whereas an increase in count indicates more tumor initiation. This data conveys that FVB mice on a ketogenic diet experienced a higher tumor initiation rate but not an increase in tumor growth rate. More diet effects may be present, however, we may not have the power to detect this effect. Testing a larger cohort of mice on each of the diets could help determine any additional or subtle diet effects.

Susceptibility to CRC has also been associated with a decrease in fecal bacterial diversity (7). This indicates a lack of balance in the bacterial composition residing in the gut. The results in this study have supported this association. FVB mice on a ketogenic diet are more susceptible to CRC and also have a decrease in diversity. This decrease in microbial diversity was among the strongest associations with tumor load in FVB mice. The gut requires a large diversity of bacterial species to effectively maintain all of the processes it has a role in. A reduced bacterial diversity can cause abnormalities in microbial composition such as an imbalance between "healthy" and "bad" bacteria (7).

Here we report that members of the order *Clostridiales* have lower colonization rates in mice fed a ketogenic or Western diet when compared to the mice on the standard diet. This group of anaerobic bacteria utilizes a fermentative metabolism of both carbohydrates and amino acids. The dietary carbohydrates they ferment produce short-chain fatty acids (SCFA) as a byproduct, including acetate, propionate and butyrate. Butyrate is a potent histone deacetylase used as the main energy source in the colonic epithelium (1). It has been shown to lead to colonic cancer prevention as well as offering important epithelial regulatory activities. (1). As expected, the mice on Western and ketogenic diets both showed a decrease in relative abundance of *Clostridiales*. However, this was only associated with a significant increase in tumor load in ketogenic diet fed mice.

Previous studies have identified associations between 4-cresol, a phenolic compound produced by *Lactobacillales*, and CRC proliferation. *Lactobacillales*, specifically *Lactobacillus*

acidophilus, can also change the pH of the gut environment, preventing harmful bacteria from colonizing. Mice on the ketogenic and Western diet had a decrease in the number of *Lactobacillales* that thrived in their gut. Studies have shown that 4-cresol produced by *Lactobacillales* decreases the proliferation of CRC. This compound is synthesized by decarboxylation of 4-hydroxyphenylacetate (1). There is not much data that indicates why 4-cresol decreases CRC proliferation. Future experiments should be conducted to determine why 4-cresol has such an effect on CRC proliferation.

In our study, increased abundance of *Bacteroidales* was also associated with CRC. The members if this order produce fragilysin, an oncogenic bacterial toxin (8). Fragilysin has been shown to induce colonic epithelial cell proliferation and expression of the oncogene c-Myc (8). The group *Bacteroidales* was found to be more abundant in the gut microbiome of mice on a ketogenic and Western diet when compared to a standard diet.

Two genera, *Sphaerochaetales* and *Verrucmicrobiales*, had statistically significant increases in relative abundance in mice on a ketogenic diet and decrease in relative abundance in mice on a western diet. There was a 200-fold increase in *Sphaerochaetales* in mice on a ketogenic diet. These two genera that had unique shifts in both mice on western and ketogenic diets has no current research on their connection with CRC. Their relationships with CRC susceptibility should be further researched to determine if this relationship is causal.

This study determined that tumor load is significantly increased in FVB mice fed a ketogenic diet. This change was associated with shifts in microbial type and a decrease in overall species

diversity. Novel associations were found between CRC and bacterial abundance on the level of order. These included a 200-fold increase in *Sphaerochaetales* in mice fed ketogenic diet. Ultimately, the results demonstrate novel associations between microbiome composition and CRC incidence. Further studies can validate these associations to determine causality.

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