EVALUATION OF *Parabacteroides distasonis* AS A PROBIOTIC AGENT IN

THE ATTENUATION OF COLORECTAL CANCER

An Undergraduate Research Scholars Thesis

By

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TABLE OF CONTENTS

ABSTRACT

Evaluation of *Parabacteroides distasonis* as a Probiotic Agent in the Attenuation of Colorectal Cancer

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The gut microbiome and its effects on its host's health has become an important factor when trying to understand that etiology of disease and therefore its diagnosis and treatment. In previous studies, the bacterial species *Parabacteroides distasonis* was found to have an inverse correlation between its gut microbiome abundance and CRC susceptibility (Barrington et al. 2018). In this study we report that oral administration of *Parabacteroides distasonis* does not provide any significant effect in the attenuation of colorectal cancer, in measures of tumor count ($p=0.5821$), average tumor size ($p=0.4496$), as well as tumor load ($p=0.9304$) when comparing a control gavage to a treatment gavage of *P. distasonis* in an AOM-induced colorectal cancer model using FVB/NJ mice fed a ketogenic diet. These results suggest that the ketogenic diet may be inhibiting the bacteria from properly colonizing the gut of the FVB mice and therefore preventing *P. distasonis* from attenuating CRC risk. Our next step would then be to use a droplet digital PCR assay to quantify absolute abundance values for the bacterial populations and confirm the population dynamics of *P. distasonis* in the gut of the mice. Ultimately, we hope to find the genetic mechanisms responsible for the regulation of the bacteria to further understand how inter-individual genetic differences effect the differential responses to dietary patterns, therefore advancing precision dietetics as a component of precision medicine.

ACKNOWLEDGEMENTS

NOMENCLATURE

CRC   Colorectal Cancer

PCR   Polymerase Chain Reaction

ddPCR   Droplet Digital Polymerase Chain Reaction

qPCR   (Real Time) Quantitative Polymerase Chain Reaction

DNA   Deoxyribose Nucleic Acid

AOM   Azoxymethane

P. distasonis   *Parabacteroides distasonis*

CHAPTER I

INTRODUCTION

Colorectal Cancer

Colorectal cancer (CRC) is the second-leading cause of cancer-related deaths in the United States, with each year about 130,000 Americans being diagnosed with the disease and nearly 50,000 mortalities (American Cancer Facts and Figures, 2017). The precise etiology of CRC is complex and largely unknown, however, an overarching hypothesis is that the disease is triggered and exacerbated by a host of environmental and genetic factors and interactions. CRC is becoming of increasing concern worldwide as several less developed countries are adopting Western lifestyles, marked by calorically dense diets, leading to a marked increase in CRC rates (Jemal et al., 2010).

Importance of Dietary Patterns on Variable Genetic Backgrounds

While genetics plays an integral role in CRC susceptibility other compounding variables can have a significant impact on CRC. Previous research has shown that 65-90% of the risk developing CRC is derived from environmental factors (Lichtenstein et al. 2000; Doll and Peto, 1981); with diet presumably having the greatest impact. Therefore understanding the interaction between diet and disease in specific genetic backgrounds is becoming increasingly important as the "one size fit all" approach to medicine proves to be less effective in certain individuals and more precise approaches to treatment is needed. While epidemiological evidence indicates that diet plays a key role in CRC risk, determining which foods specifically modify risk has been largely unsuccessful due to the complexities of inter-individual response to diet. The relationship

between dietary fat composition and serum cholesterol is a hallmark of inter-individual response to diet. Several population studies have reported that saturated fat and/or dietary cholesterol intake were positively associated with serum cholesterol (West et al., 1968). However, studies of individuals repeatedly show weak or no correlation between dietary fat composition and serum cholesterol; individuals failing to produce the same response to identical dietary interventions, suggests that diet alone could not account for serum cholesterol changes (Keys et al., 1956).

Significance of the Gut Microbiome on Health

The gut-microbiome has emerged as a focus for the physiological health of organisms through advances in DNA sequencing and bioinformatics, especially as it relates to colorectal cancer research. Bacteria and host organisms have co-evolved for at least 500 million years, with the by-products of their cooperative interaction having implications in immune response, metabolism, and reproduction to name a few (Ley et al., 2008; Benson et al., 2010). Shifts in the gut microbiota composition have been correlated with a number of conditions including CRC, inflammatory bowel disease, obesity, rheumatoid arthritis, and non-alcoholic fatty liver disease (Cho et al., 2012). Although it is likely that shifts in particular bacterial populations in the gut microbiome effect the physiology of an organism it is difficult to demonstrate a causative relationship between a bacterial species and an observed phenotype. To investigate the interaction among diet and genetics and its relationship to colorectal cancer susceptibility, a CRC model was designed using azoxymethane (AOM), a colon specific carcinogen, to induce CRC in two strains of mice, FVB/NJ (FVB) and C57BL/6J (B6), fed three different diets, an American, Ketogenic, or Standard mouse chow (See Fig.3 for diet composition data). This study reported a four-fold increase in tumor multiplicity in the FVB mice fed a ketogenic diet when compared

against the standard mouse chow; a phenotype which was not observed in any other experimental group (Fig. 1), suggesting a relationship between diet and genetic background in terms of CRC susceptibility.

To further understand this strain by diet interaction, a subsequent study was designed to understand the physiological response to diet in multiple genetic backgrounds. The physiology study incorporated four human comparable diets designed to represent dietary patterns across the globe (Fig.3). 16s phylogenetic analysis of fecal DNA extracted from the mice showed a significant 20 fold decrease in the bacterial species, *Parabacteroides distasonis* in the gut microbiome of FVB mice fed a ketogenic diet (Fig. 2). This phenotype was not observed in the other experimental groups (Barrington et al., 2018). This provided evidence that an inverse correlation exists between *P. distasonis* abundance and CRC susceptibility.
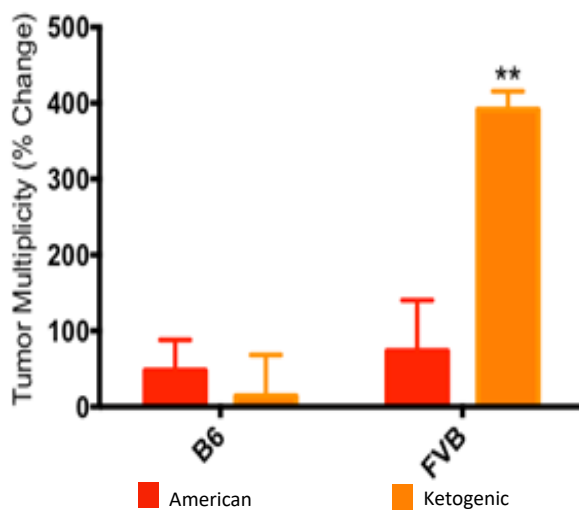

Fig 1. Change in tumor multiplicity in B6 and FVB mice fed American or ketogenic diets relative to standard diet (baseline).(Barrington et al. 2018)
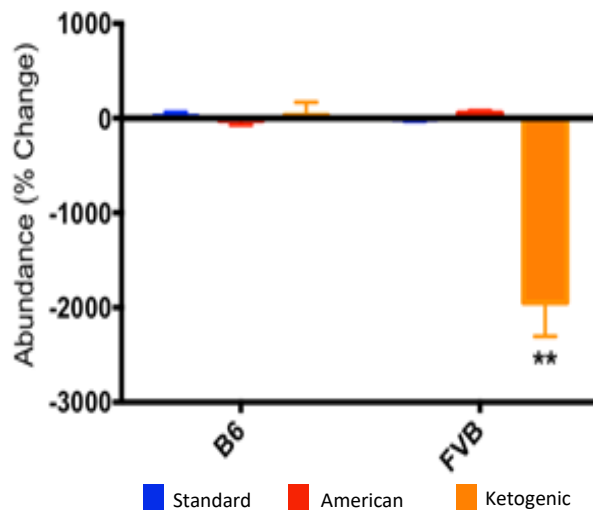
Fig. 2. Change in *Parabacteroides Distasonis* abundance before and after administration of experimental diets. (Barrington et al. 2018)
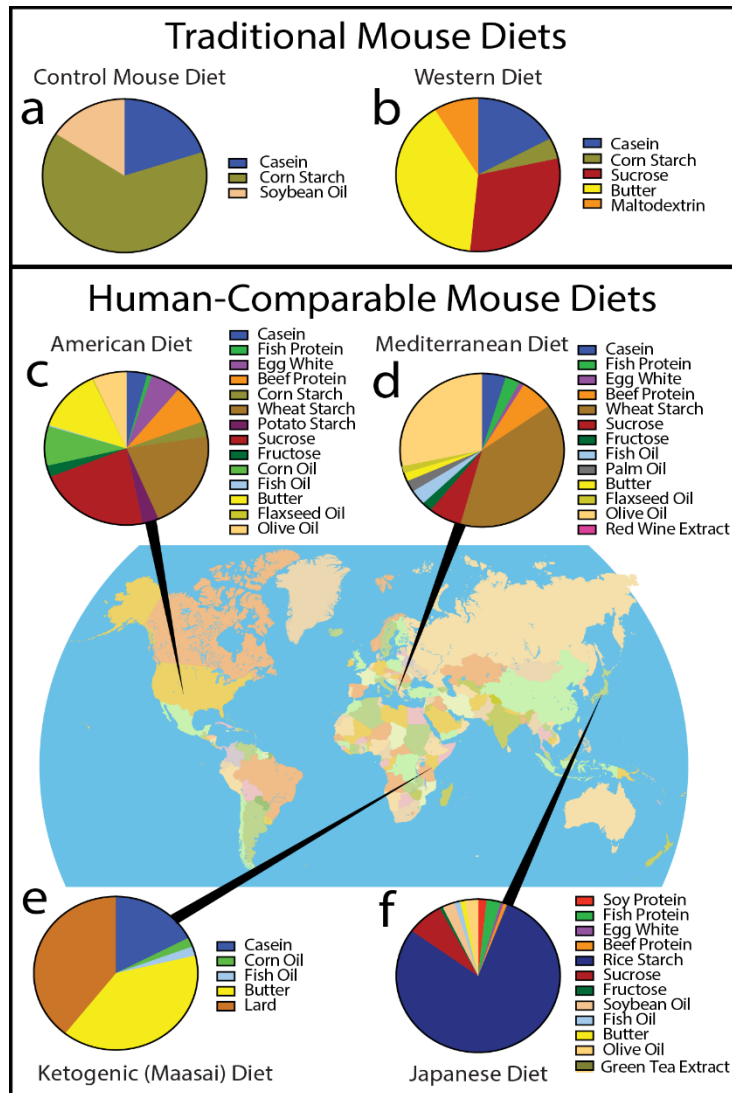
Fig. 3. Composition of designed Human-Comparable Mouse Diets and relative associated regions of consumption compared against Traditional Mouse Diets (Barrington et al. 2018)

Parabacteroides distasonis

The commensal bacterium of interest, Parabacteroides distasonis, is an understudied organism and it is therefore difficult to predict the phenotypic effects this bacterium might have regarding a host of CRC symptoms. *P. distasonis* has been shown as a potential attenuating agent in dextran sulphate sodium (DSS) induced colitis for BALB/c mice upon gavage of the bacteria (Kverka et al., 2010), however, contradictory results have also been reported when *P. distasonis*

lysates were administered to *Pglyrp*-deficient mice with DSS induced colitis with a depleted intestinal microflora utilizing a 3-week long treatment of oral ciprofloxacin and metronidazole (Dziarski et al., 2015). While there are substantial experimental differences between the two studies; the shown attenuating effects of *P. distasonis* on colitis and the correlations found between an increase in tumor multiplicity and decrease in abundance of this bacteria holds promise for *Parabacteroides distasonis* as a potential probiotic agent to facilitate gut health. We therefore, hypothesize that *Parabacteroides distasonis* plays a protective role against colorectal cancer thus, a reduction in the abundance of *P. distasonis* in FVB mice fed the ketogenic diet increases their inherent risk of developing CRC.

CHAPTER II

METHODS

Animal Husbandry and Treatment Administration

All animal handling protocols were done in accordance with the Animal Use Protocol IACUC 2016-0339 - "Clinical phenotyping in the Collaborative Cross" (Reference Number: 055227). Forty four-week old FVB/NJ mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were randomized into two experimental groups, a control and a treatment group and studied over a course of 6 months. After acclimating for two-weeks, the 40 mice were exposed to a Maasai/Ketogenic diet (D12052706), designed in collaboration with Research Diets, Inc. (New Brunswick, NJ). The diet was formulated with dairy sources consumed by the Masaai people (Mann *et al.* 1964) as well as menhaden and corn oil in order to maintain a state of ketosis as well as maintain essential lipids in the mice respectively. Treatment of mice with either a control gavage of sterile working solution or the treatment gavage containing *Parabacteroides distasonis* began in parallel with the initiation of the ketogenic diet. Mice were administered control or treatment gavage of 100µl on a biweekly basis until necropsy. Upon the 7th week of diet and treatment administration, mice were given four weekly injections of AOM (10mg/kg). Mice remained on diets for 20 weeks following the final injection and were then necropsied for final sample collection.

Bacterial Preparation

As an obligate anaerobe, growth of *Parabacteroides distasonis* required an anaerobic gas mixture of *80% N_2 - 10% CO_2 - 10% H_2*, using an aerobic chamber in a BSL 2 laboratory setup.

P. distasonis was ordered from ATCC (ATCC® 8503™) in the freeze-dried format and stored at -20°C. Under anaerobic conditions, aliquots of the bacteria were re-hydrated using 0.5 mL of the Anaerobe Systems Reinforced Clostridial Broth (AS-606) media. Under our anaerobic conditions approximately one week was required for the bacteria to recover, after which 1 mL of the solution was used to inoculate new cultures. 20% glycerol stocks of the bacteria were then prepared from the RCB broth media and 80% glycerol solutions. 20% glycerol stocks were then stored at -80°C.

P. distasonis developed best on Brucella Blood plates from Anaerobic Systems worked best in growing *P. distasonis* as these plates are supplemented with hemin and metathione (Vitamin K1). To initiate pure *P. distasonis* growth, an aliquot of the glycerol stock solutions of the bacteria was thawed and 40 µL of the solution was pipetted onto a Brucella blood plate, and was allowed to incubate for 2-3 days. The subsequent growth of pure *P. distasonis* was used to streak bacterial lawns for the treatment gavage; approximately 3-4 days was required for appropriate growth. A plate was reserved to streak new plates for the proceeding treatment. To maintain bacterial specificity and limit contamination; frozen pure *P. distasonis* aliquots were used to restart growth approximately once a month.

Preparation of Working Solution and Treatment Gavage

The gavage vehicle was prepared with a sterile working solution comprised of 50 µl phosphate buffer saline (PBS), 1 mg of Soybean Trypsin Inhibitor (Sigma Aldrich, Product Number: : T9128-500MG), and 50 µl of 0.15M sodium bicarbonate buffer per mouse. A 1.5mg pellet of *Parabacteroides distasonis* was added to the vehicle for the treatment group. Vehicle aliquots

appropriate for 30 mice were prepared of the sterile working solution and stored in -20°C until ready for gavage. Bacteria was harvested from matured plates within a BSL 2 Biosafety cabinet using cell scrapers and suspending the bacteria within a PBS solution. The bacteria was pelleted by centrifuge at 14k x g for 10 minutes and 45mg was collected for a treatment gavage aliquot sufficient for 30 mice (1.5mg per mouse).

Sample and Data Collection

To analyze phenotypic differences between experimental groups, feces, weight, and MRI data were collected at pre-diet (initial), middle, and post-necropsy (final) time points. Fresh feces was flash frozen in liquid nitrogen after collection. Upon necropsy feces was collected from the colon and flash frozen in liquid nitrogen. Colons were palpated for tumors, fixed in formalin for 24 hours, and stored in 70% ethanol for later processing.

Evaluation of Colorectal Tumorigenesis

To determine tumor count, tumor average size, and overall tumor load, the colons were stained with 0.1% methylene blue and visualized under a compound microscope. Tumors were counted and size determined using digital calipers.

Microbiome Analysis and Droplet Digital PCR

To analyze gut microbial composition, DNA was extracted from feces via a FastDNA Spin Kit for Soil. This kit was determined to be most effective in producing the largest and most diverse DNA yields in comparison to other available kits, using a Qubit dsDNA HS Assay Kit and Qubit Fluoremeter to assess yield and purity. The DNA was digested with the restriction enzyme ApoI,

which recognizes the 5' RAATTY 3' site outside of the DNA template designed from our *P. distasonis* specific primers (F: GCGGTGGAACTTGGACTTAATA, R: GCCACCAAGTCCTCCAATAG).

A droplet digital PCR assay was designed using the BIO-RAD QX200™ system as a system to have an absolute quantification of *P. distasonis* as an absolute measure of abundance of the bacteria. The system relies on two elements: positive and negative fluorescence data from the sample droplets and data fitting to a Poisson distribution. A temperature gradient and serial dilution using known positive and negative controls were utilized to optimize the assay's experimental conditions. The Reaction Mix and cycling conditions for the assay are as outlined in *table 1*. We utilized the dye based EvaGreen® Supermix ddPCR followed by data acquisition and analysis using the QuantaSoft™ software.

Table 1. Preparation of the ddPCR Reaction Mix

Component	Volume per Reaction, µl
2x QX200 ddPCR EvaGreen Supermix	10
Forward Primer	2
Reverse Primer	2
Diluted Restriction Enzyme	1
DNA Template	2
Water	5
Total Volume	22

Statistical Analysis

Statistical analysis of data points were done using the JMP statistical software from SAS. Pearson's Chi-square test was used to calculate the *p*-values for the treatment's effect on tumor penetrance. Two-way ANOVA was performed to calculate *p*-values for tumor load (total sum of

tumor size), average tumor size, tumor count, mean body fat %, and body fat % for the cohort

with treatment and sex as factors for all tests $p < 0.05$ was used for significance threshold.

CHAPTER III

RESULTS

Animal Husbandry and Survival

Five mice were excluded from final analyses due to technical issues unrelated to the experimental design.

Tumor Penetrance

To determine the treatment gavage's effect on tumor penetrance, the percentage of mice with at least one tumor and used Pearson's chi-square-test to calculate p-value. No significant difference in effect of the treatment gavage on penetrance ($p=0.1570$) was observed, with penetrance being 88.88% and 100% for the control and *P. distasonis* gavage respectively (Fig. 4). The overall penetrance of the cohort being 94.29%.
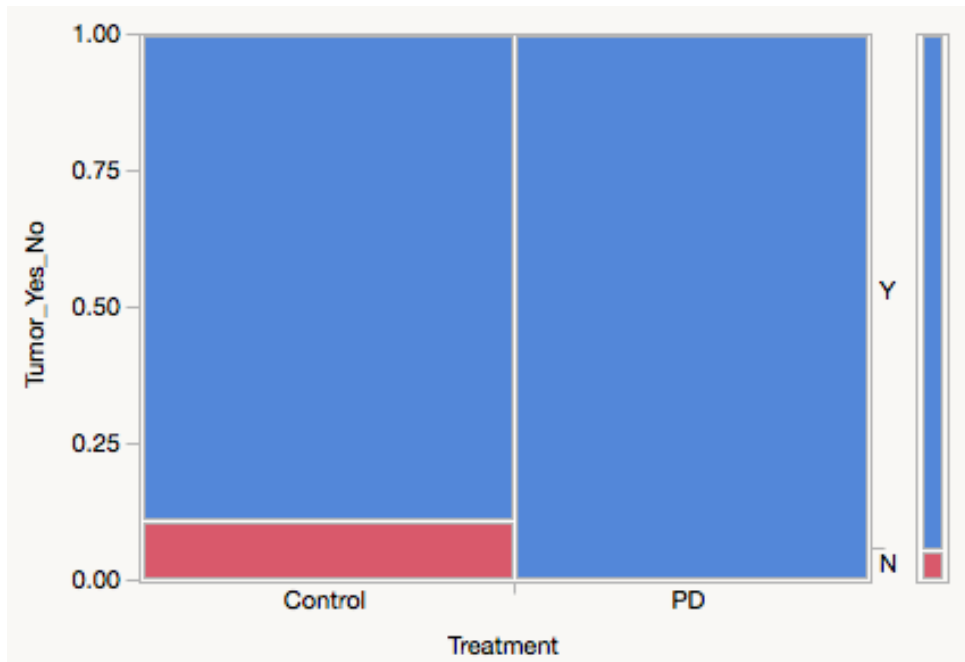

Fig. 4. Effects of treatment gavage on penetrance. "Y" and "N" defined as a mouse with at least 1 tumor and no tumors respectively.

Tumor Count

No significant difference was found in tumor count per mouse between control and *P. distasonis* treatment groups (*p*=0.5821). The control group and the treatment group showed an average tumor count per mouse of 2.666 and 2.352 tumors respectively (Fig. 5). There was a significant difference, however, between sexes (*p*=0.0156). With females and males showing an average of 3.125 and 2.000 tumors per mouse respectively. No significance was observed when looking at a sex by diet interaction (*p*=0.5821), with females on the control and treatment gavage measuring an average 3.333 and 2.857 tumors per mouse respectively. Males on the control and treatment gavage both measuring an average of 2.000 tumors per mouse.
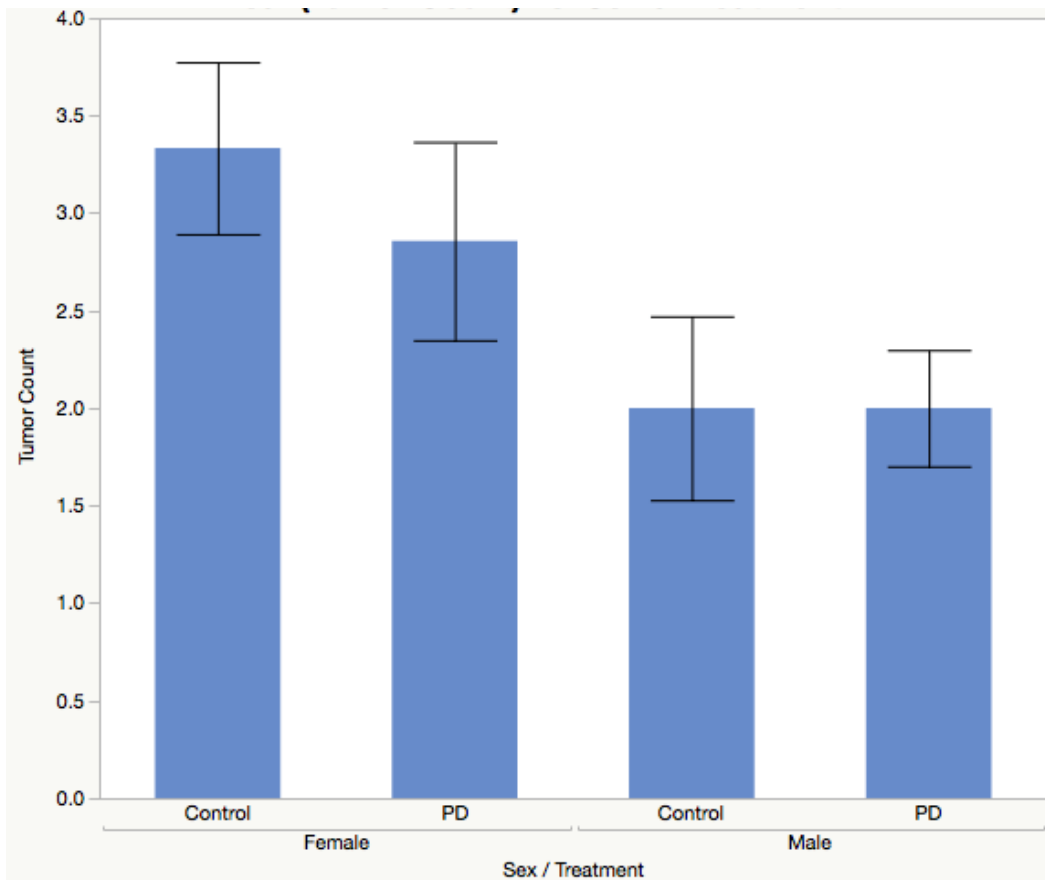

Fig. 5. Effects of sex/treatment on average tumor count per mouse

Average Tumor Size

No significant difference was found in average tumor size between control and *P. distasonis* treatment groups ($p=0.4496$). The control group and the treatment group showed mean tumor sizes of 0.897mm and 0.956mm respectively (Fig. 6). No significant difference was found in average tumor size when looking between sexes as well as treatment by sex ($p=0.807$ and $p=0.199$ respectively). Average size between the sexes were measured to be 0.935mm and 0.921mm respectively. Females and Males on the control gavage measured average tumor sizes of 0.857mm and 0.948mm respectively. Females and males on the *P. distasonis* treatment gavage measured 1.034mm and 0.902mm respectively.
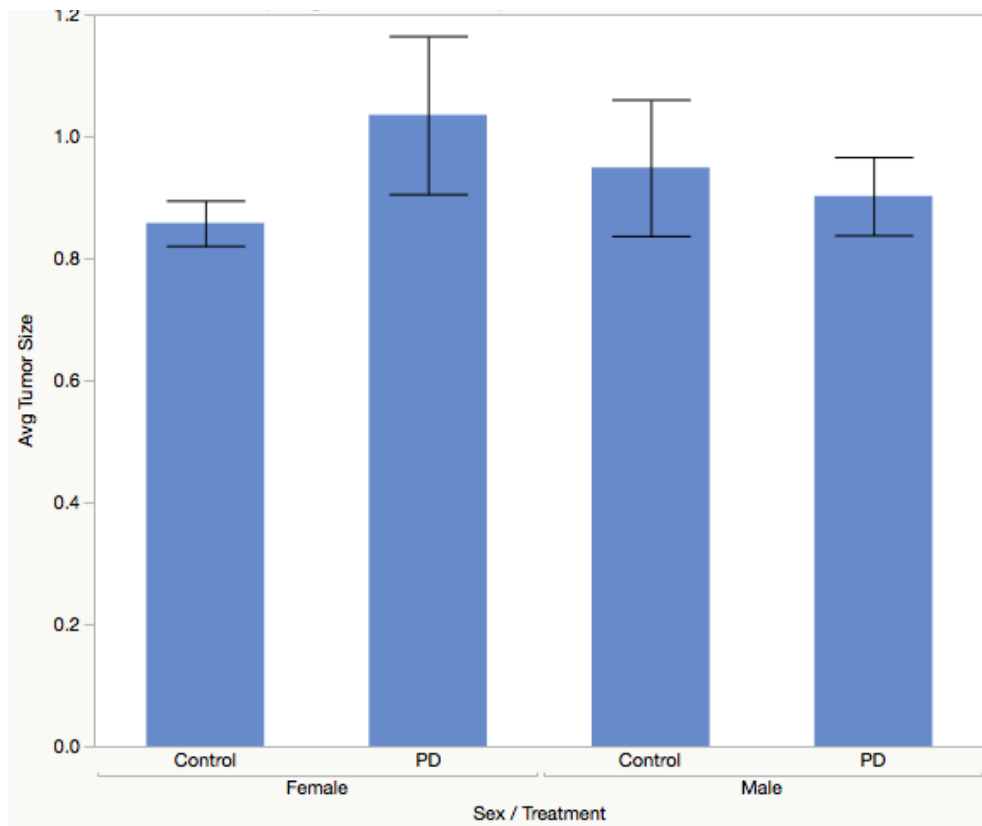

Fig. 6. Effects of Sex/Treatment on average tumor size. Error bar demotes 1 standard error from the mean.

Tumor Load

Defined as the total sum of tumor size per mouse, the tumor load between control and *P. distasonis* gavage treatments showed no significant difference ($p=0.9304$). With average tumor load being 2.446mm *and* 2.402mm for the P. distasonis and Control groups respectively. Similarly, no significance was found when looking at the effects of both the treatment and sex ($p=0.7926$). Females and Males on the control gavage measured 2.886mm and 1.918mm for average tumor load respectively. Females and Males on the treatment gavage measured 3.060mm and 1.832mm respectively for average tumor load. A significant difference was found, however, when looking at the effect of only sex on tumor load ($p=0.0326$). With average tumor load being 2.96mm and 1.873mm for female and male tumor load respectively (Fig. 7).
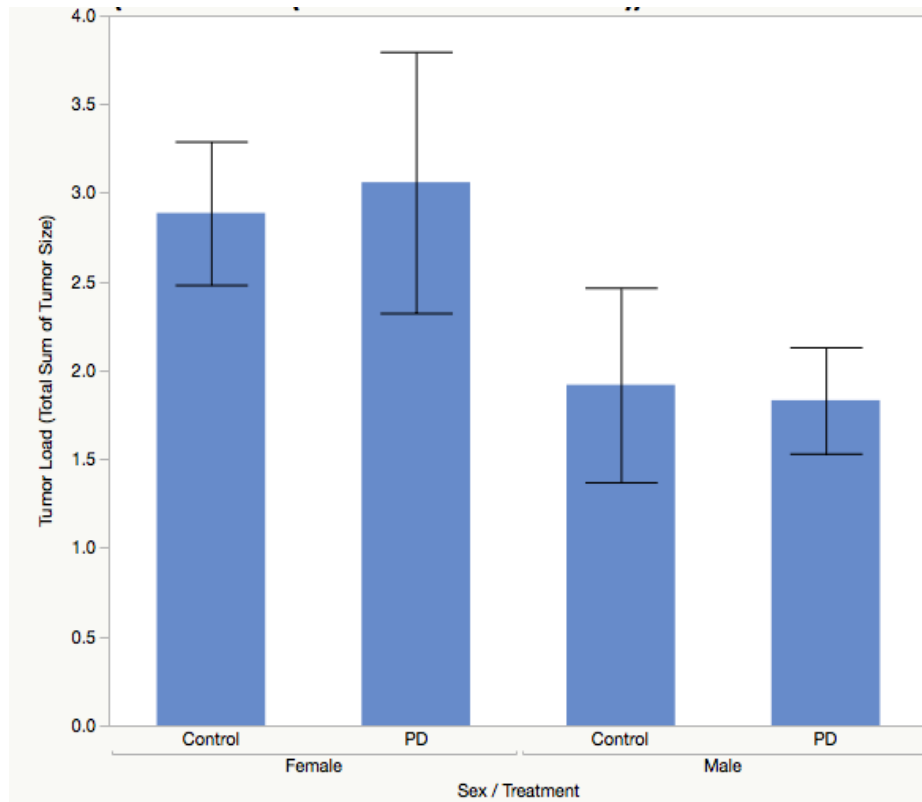

Fig. 7. Effects of Sex/Treatment on Mean Tumor Load. Error bar demotes 1 standard error from the mean.

Body Fat

No significant difference in body fat percentage gain was measure between control and treatment

groups (Fig. 8). However, a significant difference in body fat percentage gain was shown

between sexes, with males gaining an average 0.187% body fat and females an average 0.104%
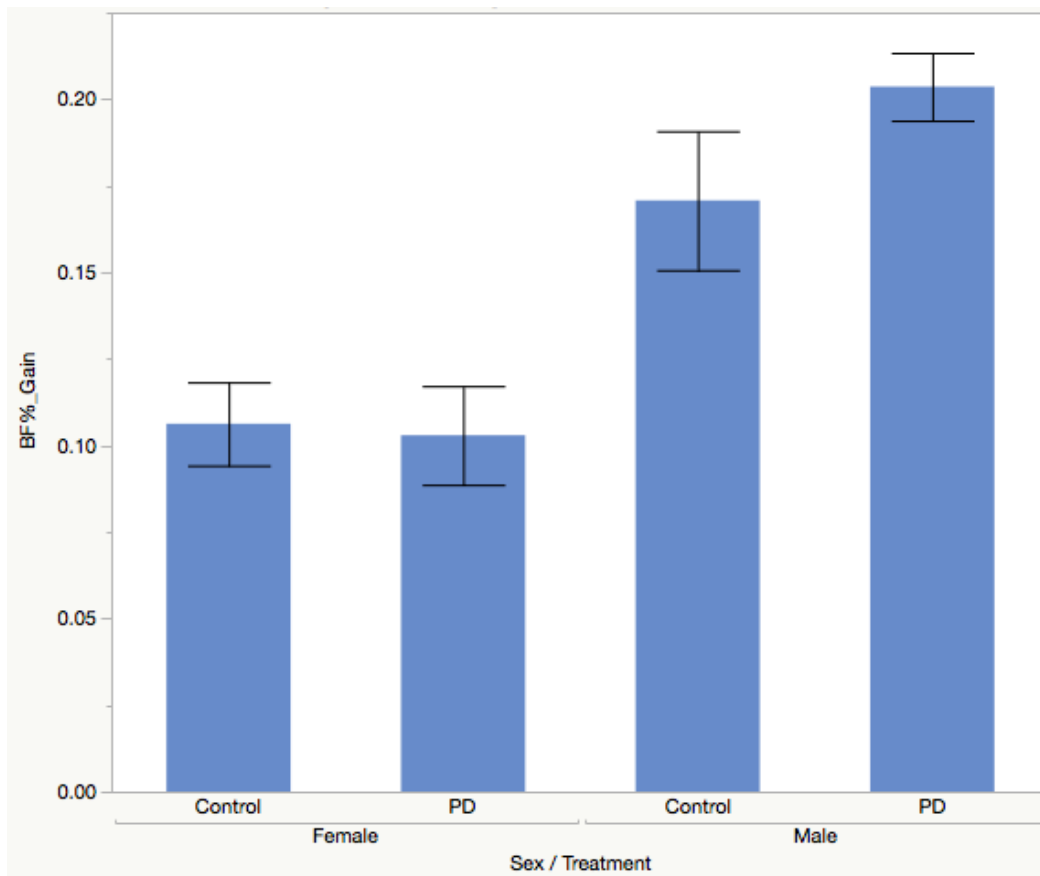
(p<0.0001).

Fig. 8. Effects of sex/treatment on body fat percentage gain. Error bar denotes 1 standard error from the
mean.

CHAPTER IV

DISCUSSION

This study aimed to elucidate the effects of the bacteria *Parabacteroides distasonis* on AOM-induced colon cancer in FVB mice fed a ketogenic diet. The abundance of the bacteria *P. distasonis* was previously found to be negatively correlated to tumor multiplicity, and thus was hypothesized to have a potential protective probiotic effect on colorectal cancer. We found that by administering this bacteria via gavage to an AOM injected FVB mouse no significant effects were found between the control and treatment groups in tumor penetrance, average tumor count, size or load. Interestingly, a significant difference in tumor count between sexes was observed, however not in a sex by treatment manner.

Here we report that despite the administration of large amounts of the *Parabacteroides distasonis* bacteria there was no significant difference in AOM induced colorectal cancer progression. Colitis is a known facilitative factor in the progression of colorectal cancer and previous studies have shown that through the oral administration of *P. distasonis* antigens resulted in an attenuation in murine colitis (Kverka et al. 2011). Thus coupled with previous research correlating low abundance of this bacteria to a high tumor multiplicity we hoped to demonstrate a causative role in *P. distasonis* in CRC attenuation and prevention. This was not observed. Contradictory research in the effects of *P. distasonis* has also been reported in previous research as administration of the bacteria to non-germ free microflora depleted mice showed enhanced DSS-induced colonic inflammation. If *P. distasonis* did in fact have an enhancing effect on colitis we would then expect to see an increase in colon tumor multiplicity, however,

this was also not observed. Thus we suspect that the bacteria failed to colonize the gut-microflora community. Based on previous diet research, the ketogenic diet most likely creates a hostile environment that inhibits the proliferation and maintenance of *P. distasonis* populations in the gut of FVB mice (Barrington et al. 2018). Therefore we postulate that despite the administration of the bacteria, the environment in which the bacteria are placed was not conducive to establishing a functioning population and therefore inhibited the response to *P. distasonis*. Our next step is to quantify the amount of *P. distasonis* in the gut-microbiome of both control and treatment gavage groups to evaluate the success of the gavage technique.

Current efforts to quantify the abundance of *P. distasonis* have been unsuccessful in quantifying the bacteria in fecal samples. A quantitative-PCR assay was initially designed, however, was determined to lack the sensitivity necessary to accurately quantify the relative abundance of the bacteria. To increase a sensitivity, a nested-qPCR assay was then designed, however, similar problems in detection of *P. distasonis* from fecal samples were observed as before. A droplet digital PCR assay, with its high sensitivity and accuracy was determined to be the most effective assay for determining an absolute abundance of the bacteria, while qPCR assays would only give relative abundance.

Significant effects of sex on tumor proliferation were observed. Specifically in the effect of sex on tumor count and tumor load, with females being significantly higher for both variables. This is inconsistent with current literature as it males have been shown to demonstrate a higher degree of tumor multiplicity as well as load in AOM models of colorectal cancer (Lee et. al 2016). This

suggests that the oral administration of *P. distasonis* may have resulted in a divergent response between sexes in regards to colorectal cancer progression.

Administration of *P. distasonis* did not seem to play any significant role in body fat percent change of the FVB mice fed a ketogenic diet. Unsurprisingly, sex played a significant role in the percentage of body fat gain with male FVB mice showing a significantly higher increase in body fat when compared to females. Which is consistent with previous studies in which demonstrated protective factors against obesity in female mice (Hong et al. 2009)

Ultimately, if it is determined that *Parabacteroides distasonis* was unable to colonize the gut-microbiome of FVB mice fed a ketogenic diet we will redesign our AOM model of CRC to utilize germ-free FVB mice and selectively recolonize the gut-microbiome with *P. distasonis.* This will help us to better understand how the bacteria effects colorectal cancer when properly colonized in the gut. If a causal relationship is demonstrated between *P. distasonis* abundance and resistance to CRC an intercross (F2) population derived from FVB and B6 parental strains would be generated to investigate the significant differences observed in tumor multiplicity between these groups (Barrington et al. 2018) when fed ketogenic diet. This will identify the genetic loci regulating *Parabacteroides distasonis* abundance.

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