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The Effect of Low Dose Penicillin on Tumor Development in $Apc^{Min/+}$ Mice

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By

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DEDICATION

I dedicate this thesis to my father Larry, my mother Patricia, my grandmother Mary Ann, my grandmother Jaquelyn, and Denise. I also want to dedicate my thesis to my siblings Mariah, Aaliyah, and Kody. I want to thank all the friends and extended family that have encouraged me when it was challenging and motivated me until the end. I could not have made it this far without any of you and I am grateful for your support and love. I am honored to have accomplished something this major in my life and thankful for all of you to see it. Thank you for making my dreams possible and I will continue to make you proud as I achieve the pinnacles I intend to reach.

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ABSTRACT

Antibiotics have been our most effective weapon against bacterial infections since their discovery in the early 1900s. Their use has been critical in reducing mortality rate from infectious diseases. However, in the last few decades, the overuse of antibiotics, beginning at an early age and into adulthood, has become a growing concern globally. Penicillin is one of many extensively used antibiotics in early childhood that has been used to treat childhood infections. Recent studies showed that exposure to low dose penicillin can have adverse effects leading to chronic illness such as diabetes, allergies, inflammation, and susceptibility to obesity, with the latter two having links to colorectal cancer. In this study, our goal was to determine if exposure to low dose penicillin has an impact on the intestinal microbiota composition and inflammation and determine the impact on development in tumors of the intestine of the mice, a genetic model of intestinal tumor development. Mice were administered low dose penicillin in their drinking water, fecal samples were collected at four-week intervals to analyze changes in microbiome composition, and tumor burden was analyzed when mice were 18 weeks of age. Although other studies showed a correlation between antibiotic exposure and microbiota disruption and inflammation in the intestine, we found no significant differences in microbiota composition between exposed and control $Apc^{Min/+}$ mice. However, we found a significant increase in tumor burden specifically in female mice but not in male mice.

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INTRODUCTION

1.1 United States Statistics on Colorectal Cancer

Colorectal Cancer (CRC) is the second most common cancer and the third leading cause of cancer mortality in both women and men in the United States in 2020 [1]. The largest disparity between incidence in men and women resides in rectal tumors with a male-to-female incidence rate ratio (IRR) of 1.62; 95% CI, 1.60-1.63 and the smaller disparity in tumors in the proximal colon with IRR of 1.07; 95% CI, 1.07-1.08. Statistical data indicate that women have a higher incidence of proximal tumors (45%) when compared to men (36%) [1]. The reasons for the difference in location of tumor burden is unknown; however, it could be related to different biological and molecular characteristics that may be associated with gender. Age is associated with CRC incidence as individuals (both women and men) younger than 45 years have comparable results, while in those between the ages of 55 to 74 years, the incidence is 40-50% higher in men than in women [1].

More disparities in CRC incidence and mortality are observed based on race and ethnicity. Statistical data on the five major categories including Non-Hispanic Blacks (NHB), American Indian/Alaska Native (AI/AN), Non-Hispanic

Whites (NHW), Hispanic, and Asian/Pacific Islander (API), were collected from Purchased/Referred Care Delivery Area counties [1]. From 2012 through 2016, the incidence of CRC in NHB was approximately 20% higher than NHW, 45.7 per 100,000 population as compared to 38.6 per 100,000 population respectively. NHB incidence was nearly 50% higher than those of API classification at 30.0 per 100,000 population. From 2013 through 2017, CRC mortality rates in NHB were 40% higher than NHW (19.0 per 100,000 population to 13.8 per 100,000 population, respectively) and double those of API classification (9.5 per 100,000 population) [1]. Socioeconomic factors were the driving factor in racial disparity and were associated with access to medical care and education. Statistics from self-reported education and census-tract socioeconomic status in 2018 showed that the median income for NHB was $41,361 compared to the NHW median income of $70,642 with a poverty rate of 21% among NHB and 8% among NHW [1, 2]. Lower income populations were associated with unhealthy diet due to lack of resources, smoking, consumption of alcohol, education, and lower quality health care accounting for about 44% of the disparity [3]. Interventions that are in place to minimize CRC mortality such as early screening and detection are not as likely to be practiced by individuals with low-income and low-educational backgrounds as compared to those who come from higher income and education backgrounds [2]. The development of adenomas or CRC in NHB and NHW is not any less likely outside of associated risk factors; however, access to quality care after a positive screen is less likely for NHB and therefore has the potential to contribute to higher mortality rates [1, 2, 4].

CRC incidence is more problematic among ANs, with an incidence of 89 per 100,000 population and mortality of 40 per 100,000 population; which is double those of NHBs in the United States [1]. Another concern is that the main preventive care for those of American Indians descent is stool testing, which is very limited in detecting cancer markers; therefore, access to follow-up health care for American Indians is a major challenge that contributes to a high rate of CRC mortality [1, 5, 6].

Genetic or heritable factors play a critical role in CRC development in addition to environmental factors. Sporadic cases of CRC account for 60-65% of all CRC cases as compared to a small percentage of patients susceptible to inheritance [7, 8]. Sporadic CRC can be minimized by changes in behavior due to modifiable risk factors [9]. These modifiable factors include, poor diet, low physical activity, alcohol consumption, smoking, and obesity [7]. A sedentary lifestyle increases the risk of developing CRC by 25-50% as compared to those who maintain regular physical activity [10]. Physical activity contributes to decreased risk of CRC by minimizing fat accumulation, lower inflammation rates, and by maintaining the integrity of gut quality and hormone levels [11]. Diet also contributes to CRC development as a high fat diet is more inclined to disrupt the composition of the gut microbiota that protects the intestinal lining of the colon [12].

1.2 Global Statistics on CRC

Globally, the highest incidence of colorectal cancer (CRC) is found in different regions of Europe, Australia/New Zealand, Northern America, and Eastern Asia based on 2018 statistics [13]. In comparison, regions of Africa and Southern Asia have a low prevalence of CRC. In 2020, CRC was the third most common type of diagnosed cancer and was the second leading cause of cancer related deaths worldwide [7]. In 2020, CRC contributed to approximately 515,637 deaths among males and 419,536 deaths among females [7]. An increase in CRC in less developed countries is attributed to recent trends towards a westernized diet and lifestyle, and an association with environmental risk factors. While there is a higher incidence in more developed countries, preventive measures, and family diagnosis, and screening treatment have contributed to decreasing rates of CRC.

Notable efforts in screening and treatment have been made to offset the progressive stages of CRC and reduce risk of mortality [14]. CRC incidence has decreased within recent decades due to the emphasis on preventive screening [14, 15]. CRC develops through an adenoma-carcinoma sequence that is initiated from precursor sporadic lesions that can be removed at the early stages if found through preventive screening [16]. Based on practices by the American College of Gastroenterology (ACG), the most common forms of detection include colonoscopy, sigmoidoscopy, CT colonography and stool-based testing [16]. Endoscopic removal of early polyps during a colonoscopy has reduced CRC prevalence and mortality. Since 2018, The American Cancer Society has

recommended lowering the age for CRC screening to 45 years due to the increasing incidence of CRC in young adults [15, 17-20].

Multiple factors increase the risk of developing CRC. Sex, age, and race contribute to a large part of the development of CRC with associated risk factors including environment, quality of care, diet, and genetics [7]. In 2020, the global incidence of CRC for men was 23.4 cases per 100,000 persons which is 44% higher than the incidence of CRC for women with 16.2 cases per 100,000 persons [1, 7]. Similarly in the United States, the incidence rate for men that is 31% higher than that found in women, which correlates with the lower life expectancy in men [7].

The incidence of CRC remains higher for those over 50 years of age; however, beginning in 2000, the incidence of CRC in patients younger than 50 years has steadily increased [21, 22]. The hallmark difference is that patients younger than 50 years tend to have tumors in the left side of the colon including the descending and sigmoid colon and rectum, while those over 50 have tumors on the right side including the cecum, and ascending, proximal, and transverse colon [21]. In most European countries, CRC is more common in the proximal colon in women as compared to men [7, 23]. This is of interest because the incidence of CRC in the proximal colon was also found to increase with increasing age [23]. expectancy in men [7]. The correlation between colon tumor development and location is important to determine appropriate treatment plans for the patient [7].

1.3 Antibiotics and Low Dose Penicillin (LDP)

The increasing use of antibiotics is a concerning global phenomenon and has the potential to impact CRC prevalence in all populations [24, 25]. Antibiotics were introduced in the early 1900s and used primarily for clinical practice as shown in **Figure 1.1**.

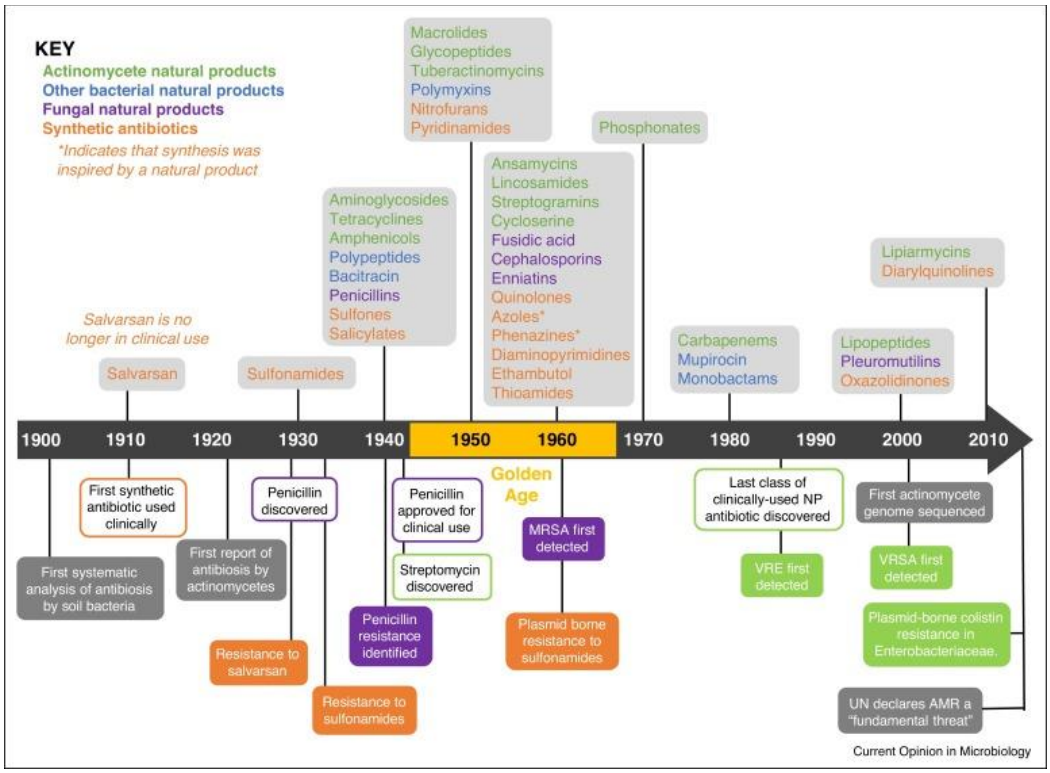


Figure 1.1 Antibiotic Introduction in Clinical Practice. Antibiotics have served in the clinical industry since the early 1900s and has advanced overtime.

Antibiotics have been used to treat infectious bacterial diseases in humans but the use has also become widespread within the food industry [24]. Antibiotics have been incorporated in agricultural practices to treat livestock for infectious disease and as food additives to promote growth of the livestock [24, 26]. The extensive use of antibiotics has created long-term concerns regarding the integrity of our agriculture systems as 75-90% of the antibiotics present in

livestock manure could remain in the soil and water. This may result in further emergence and spread of antibiotic resistance [27] and leading to early exposure of children to these antibiotics [28, 29]. Antibiotics used in livestock have a particular viability that allows them to remain in the food products, yet there are no regulations in place to prevent extensive exposure to the antibiotics [28]. In 2011, of 5,006 selected meat samples tested by the FDA forty-seven detectable had levels of antibiotic residues, eight of which were above the permitted limits [28, 30].

Antibiotics were introduced in the 1900's and have been our most effective weapons against life threatening, infectious diseases. Although antibiotics are indispensable against bacterial infections, exposure at an early age is of concern [31, 32]. In the United States, approximately 25% of all prescriptions for children are antibiotics with the most common being amoxicillin and azithromycin [33, 34]. A study on U.S. Prescriptions [35] in 2010 indicated that by two years of age, a child in the U.S. has received approximately three antibiotic courses and up to ten antibiotic courses by ten years of age [28, 35]. Individuals in the U.S. on average, have an alarming rate of exposure to 17 antibiotic courses by the age of twenty [35]. The southern region of the U.S. experiences a higher rate of antibiotic prescriptions than the western region [35, 36]. By comparison, exposure to antibiotics in early life in Sweden is approximately 40% less than that of the U.S., suggesting that antibiotics are over prescribed in the United States [28, 36].

Among the most prescribed antibiotics, penicillin, a class of antibiotics in the beta-lactam family, is one of the most extensively used in children [31]. Studies in mice that are exposed to low dose penicillin (LDP) demonstrated negative effects on metabolic development and inflammation [28, 31, 37, 38]. Low dose penicillin caused changes in the intestinal microbiota of young mice [28, 31, 39, 40] and decreased expression of genes in the ileum of the intestinal tract [28] that were associated with the development of innate and adaptive immunity to generate cells for immune defense [28].

1.4 Antibiotics and Microbiota Associated with CRC

Infant microbiome greatly differs from that of a mature adult microbiome environment [33]. Infants have little diversity in their microbiota environment, but diversity increases overtime as new foods and environmental factors are introduced that cause the microbiota composition to shift [33, 41]. The introduction of antibiotics to a population of microbiota with low diversity poses the threat of altering compositions that have long-term effects in later life and it is important to evaluate the influence of such drugs in early life [33]. The human intestinal microbiota houses a multitude of diverse microorganisms with around 800-1000 different bacterial species and approximately 7000 different strains [24, 42]. About 95% of the microorganisms that inhabit the intestine are beneficial for pathogenic defense, with the remaining microbes having harmful properties [24]. Extensive research has been conducted to demonstrate that antibiotics have adverse effects on the composition of bacteria in the microenvironment including

the taxa of *Bacteroidetes* and *Firmicutes* [24, 43]. *Bacteroidetes* and *Firmicutes* phyla comprise the majority of the gut microbiota and are associated with lipid and carbohydrate break-down [44]. Exposure of the gut microbiota to antibiotics has the risk of reducing these prominent bacteria resulting in alterations in composition. This may lead to loss in taxa diversity and the risk of pathogenic exposure that result in antibiotic-resistant bacteria and diseases such as colitis and CRC [42, 45, 46]. Early exposure to the antibiotics tylosin and amoxicillin, in the drinking water of young mice, showed that mice were more susceptible to increased colitis, weight loss, and mortality over their lifetime [25]. The mice presented symptoms of increased blood in the fecal pellets and severe colonic tissue injury as compared to control groups without antibiotic exposure as early as 23 days after exposure, with symptoms becoming more severe after 80 days [25]. Early exposure to antibiotics may have the potential to impose long term effects on the human natural microbiota [25].

The link between microbiota and CRC is highly correlated to the *Fusobacterium* class of microbes [47], as well as the major classes of microbes *Bacteroidetes* and *Firmicutes* [48]. *Fusobacterium* is a gram-negative anaerobic bacterium that is most associated with periodontitis and intestinal diseases [47, 49]. There has been no direct correlation showing that *Fusobacterium* is the primary cause of CRC development; however, studies established that *Fusobacterium* contributes to the development and progression of CRC [47, 50]. A novel study conducted by Kostic et al. [50] established that *Fusobacterium* was

abundant in the early stages of CRC development in the colorectal adenoma. The bacterium was localized in tumor tissue but not in surrounding healthy tissues, suggesting that *Fusobacterium* is vital in the development of early CRC [50]. Recent studies further showed large numbers of *Fusobacterium* in the feces of patients with adenomas and in CRC patients as compared to healthy individuals [51].

1.5 Antibiotics and Inflammation Associated with CRC

The immune system is comprised of two major systems, adaptive immunity (acquired immunity) and innate immunity (natural immunity) [52]. Both intricate components of immunity have the potential to be affected by antibiotic exposure, thus creating long term concerns on immunity [33]. Inflammation in the microenvironment of CRC patients is one of many hallmarks of a progressive disease. Inflammation initiated by hyperactive immune cells may lead to pro-inflammatory cytokine release that can cause development of dysbiosis, colitis, inflammatory bowel disease, and Chron's disease, that are all associated with pre-cancerous properties [53]. Presence of lymphocytes in the tumor indicate that the body is attempting to fight off the pathogen; however, the abundance of myeloid-derived suppressor cells (MDSCs) and Regulatory T cells (Tregs) indicate that the prognosis of the disease is progressive [47, 54]. MDSCs flourish in environments where pathological diseases are present such as chronic infections and cancer [47]. The presence of MDSCs indicates that there is a form of resistance and strong prognosis of infection, as MDSCs have

immunosuppressive activity and contribute to the inhibition of anti-tumor immunity [47, 55, 56].

The most common inflammatory diseases associated with CRC development include ulcerative colitis (UC), Crohn's Disease (CD), and inflammatory bowel disease (IBD) [57]. Evidence shows that patients with mucosal UC after 10, 20, or 30 years, had an estimated risk of developing cancer of 2%, 8%, and 18% respectively [57, 58]. In another study, the incidence of CRC was 2.5%, 7.6%, and 10.8% at 20, 30, and 40 years of UC, respectively [59]. The risk of developing CRC from CD was reported to be 0.3%, 1.6%, and 2.4% after 5, 15, and 25 years [60]. Thus, the risk of CRC development is related to the duration of the diagnosis of disease [57]. IBD presents the most significate risk of developing CRC with a mortality rate up to 15% [61, 62]. In this study, we will test the hypothesis that exposure to low dose penicillin in early life disrupts the microbiota composition, and increases inflammation in the colon, resulting in increased tumor burden. We will test this hypothesis in the *Apc*$^{Min/+}$ mouse, a genetic model of colon carcinogenesis.

CHAPTER 2

METHODS

2.1 The $Apc^{Min/+}$ Mouse

The $Apc^{Min/+}$ mouse was used to determine the role of LDP exposure in tumor burden development. The adenomatous polyposis coli (APC) gene, found in organisms that are predisposed to colorectal cancer development [63]. It is mutated in familial adenomatous polyposis (FAP), an inherited form of CRC and in ~80% of sporadic colorectal cancers [63]. The APC gene acts as a tumor suppressor and therefore, mutations in this gene result in genetic instability within the colon [63]. The multiple intestinal neoplasia (Min) allele was identified after mutagenesis with ethylnitrosourea has a point mutation in codon 850 of the Min allele that results in an early truncation of the gene that results in the development of a phenotype that is similar to human FAP [64-66]. The $Apc^{Min/+}$ is heterozygous for this mutation and loss of the functional allele results in polyp formation within the small intestine [64]. The $Apc^{Min/+}$ mouse serves as a model for intestinal polyposis that is comparable to the human intestinal tract, with the main difference being that polyps in humans reside primarily in the colon [64]. The use of the $Apc^{Min/+}$ mouse model in this study demonstrates the effect of inflammation on tumor burden and microbiota composition in addition to the outcomes of APC mutations.

The hypothesis will test if LDP exposure will cause enhanced tumor development and contribute to inflammation and changes within gut microbiota in the *Apc*$^{Min/+}$ mice, a model predisposed for colorectal cancer development.

2.2 Experimental Design

Male Apc$^{Min/+}$ mice that were heterozygous for the Min allele were bred with female C57BI/6 mice. Offspring were genotyped to determine which ones had the Min allele, these were used for this study. LDP (2mg/mL) was administered in the drinking water. The amount of LDP administered to the mice is like that found in surface water that is commonly found in the drinking water systems of humans due to antibiotic exposure in agriculture and therefore used to model this consumption [67]. Control mice were provided with antibiotic-free water. Feces and blood serum were collected before treatment and then every two weeks during exposure to LDP. Ten male and ten female mice were used as controls, while ten male and twelve female mice were given water with LDP. Treatments are listed in **Table 2.1**. Experimental design and schedule of feces and blood collection at 4-, 8-, 12-,16-, and 18-week intervals are shown in **Figure 2.1**. Genomic DNA was extracted from feces and sent to Heflin Genomic Centre at the University of Alabama Birmingham, for microbiota 16S rDNA sequencing for analysis of composition.

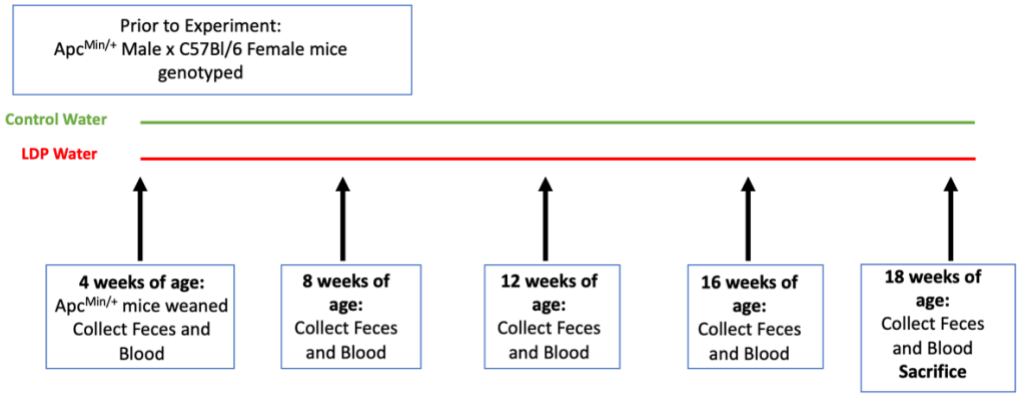


Figure 2.1 Experimental Design of $Apc^{Min/+}$ Mice Treatment.

Table 2.1 Mouse Model and Treatment Groups

Mouse	Water Treatment Group	Gender	Age	Number of Mice
ApcMin/+	Control	Male	18 weeks	10
ApcMin/+	LDP	Male	18 weeks	10
ApcMin/+	Control	Female	18 weeks	10
ApcMin/+	LDP	Female	18 weeks	12

2.3 Microbiota Alpha Diversity

Alpha Diversity associated with microbiota data is a measure of species richness, or how many different types of species are within a population. Richness is measured by the amount of diversity present within a population [68]. Less diversity within the population is indicated by a lower evenness Pielou score as compared to a higher evenness Pielou score symbolizing the abundance in diversity. Studies have shown that a correlation between age and gut microbiota

alpha diversity. Studies have shown that a correlation between age and gut microbiota alpha diversity from the birth of the mouse pups into adulthood exists [69-71]. The integrity of alpha diversity within a population is maintained in progressive ages of mouse models; however, the association of disease overtime will contribute to the changes in the composition [71]. Other studies have reported links between gender and gut microbiota that are similar between humans and mice and established that women typically show a higher microbial diversity than men. This may contribute to gender differences in the emergence of different diseases [70-72].

Graphpad Prism was used to calculate alpha diversity at different time points (TPs) throughout the experiment. Alpha diversity was analyzed using an unpaired t-test with Welch's correction without assuming equal standard deviations. Significance level was calculated based on a p-value < 0.05%.

2.4 Calculation of Beta Diversity

Beta diversity within the intestinal microbiome compares the bacterial population in a group of mice at a specific time point to a different time point of the same mice [68]. This test establishes how different two populations are from one another. Both unweighted (qualitative) and weighted (quantitative) UniFrac measurements are reflected in data collection [73]. The unweighted UniFrac measure is the distance between two different populations by the fraction of the genetic diversity between the two, while a weighted UniFrac measure is more specific to the overlap within the two populations [73]. Measuring the beta

diversity between two populations is calculated by the fixation index (F_{ST}) which compares the distance between two communities based on the genetic diversity within each community to the total combined genetic diversity of the communities [74].

In this study the beta diversity of the microbiota at the start of the study of all mice was determined in timepoint one (purple dots). Comparison of these two groups showed the greatest differences prior to the start of the experiment and shortly after the mice received treatment. Mice in timepoint one, before the start of the treatment had similar microbiota compositions; however, in timepoint two after one round of treatment, each group shared different microbiota changes. Beta diversity was measured using QIIME 2.0 analysis software. Significance level alpha of diversity was set at p-value < 0.05%.

2.5 Taxonomic Analysis of Microbiota Composition

Dysbiosis within the microbiota of the intestine plays a large part in the pathogenesis of immunological diseases that are influenced by gender differences [75]. Dysbiosis is caused due to a reduction of microbiota diversity and the loss of beneficial bacteria such as the Bacteroidetes and Firmicutes [75]. To determine the differences between male and female mice, the composition of the microbiota can be analyzed and classified at phylum level. This would allow us to assess the overall effects these differences may exert at the hormonal level. Studies have shown that differences in sex-specific hormonal profiles, can affect the functions of the gut microbiota [76, 77]. There is also evidence

suggesting microorganisms in the gut microbiome may be associated with the recirculation of sex steroid hormones including estrogens and androgens [76, 78]. Sex-specific differences within the phylum level of the intestinal microbiota are particularly centered around the *Bacteroidetes* and *Firmicutes* phylums as they play important roles in the homeostasis of normal intestinal health [79].

Bacteroidetes and *Firmicutes* are the most abundant phylums in the gut microbiome [44]. The *Bacteroidetes* phylum have an impact on dietary effects as many of the species in the phylum produce carbohydrate-active enzymes [44, 80]. The enzymes generated by the *Bacteroidetes* phylum act as signal peptides to degrade glycans that are unable to breech the bacterial cell wall [44]. This function is important for many diets, and studies have shown that *Bacteroidetes* phylum are more active in gut microbiomes of obese individuals as compared to lean individuals [81]. The *Firmicutes* phylum consists mostly of gram positive organisms as compared to the *Bacteroidetes* phylum that is mostly composed of gram negative organisms [82]. Similar to *Bacteroidetes*, *Firmicutes* are also involved in the breakdown of carbohydrates and fiber in the diet [82].

To determine the changes in microbiota composition, the relative abundance of the bacterial phylums were compared between the different treatment groups and timepoints of experimentation. When analyzing the data, bacterial groups with less than 0.005% amongst all treatment groups were removed and determined as non-detectable. Data were analyzed and collected using Graphpad Prism software. At the phylum level there were 11 different

bacterial groups while at the genus level a total of 52 different species were detected.

2.6 Effect of LDP on Tumor Burden and Tumor Microenvironment

CRC is associated with multiple factors that promote tumor development and disrupt the microenvironment of the intestinal tissue. Mouse models have demonstrated the impact of cells in the tumor microenvironment and immunological responses on tumor burden [83]. Increases in Regulatory T Cells (Treg) and myeloid derived suppressor cells (MDSC) immune cells indicate progression of a growing tumor [83-85]. Increased macrophage infiltration into the tumor microenvironment correlates to tumor growth [84]. Increases in immunosuppressive cells in the tumor microenvironment suggest the need to target the suppressive immune cell populations as a means to decrease the development of tumor burden [83]. Increased immune cells, indicates elevated production of cytokines associated with tumor progression which can further influence recruitment of new immune cells to increase tumor burden [86]. Transforming growth factor-beta (TGF-β), is a major cytokine that is associated with tumor suppression in early and late stages of cancer development and triggered by the presence of immunosuppressive cells [86].

2.7 Preparation of Tissue Samples and Staining Procedure

Tissue was collected from mice at the end of the 18-week experiment and fixed using 4% Paraformaldehyde. Tissues were then embedded in 13%

acrylamide and ammonium and sectioned using vibratome technology. Sections were transferred to a 96 well plate with PBS until ready for staining.

Each immune cell group was stained with specific antibody markers to detect presence using Confocal Microscopy. **Table 2.2** shows the different markers associated with each immune cell. All immune cell stains followed the same protocol, however cell types with two primary antibodies had a few additional steps. For cell types with one primary antibody marker, each tissue was washed in PBS/0.01M Glycine/0.1% Triton-X three times for 30 minutes each. For an hour the tissues were placed in 5% BSA/PBS and then transferred to 5% Normal Donkey Serum (1%BSA/PBS) for another hour. The tissues were stained and incubated with the primary antibody (1%BSA/PBS)(1:100) at 37ºC an hour and then left to sit overnight. The tissues were washed twice for 15 minutes each with 1%BSA/PBS and then placed in 5% Normal Donkey Serum (1%BSA/PBS) for one hour. The tissues were then stained and incubated with the secondary antibody (1%BSA/PBS)(1:100) at 37ºC for an hour. All samples were wrapped in foil from this step on. The tissue samples were washed twice for 15 minutes each with 1%BSA/PBS. If the immune cell stain has two primary antibodies, after this step they are then placed back in 5% Normal Donkey Serum for an hour before the second primary antibody is added and the steps are followed again. If the immune cell stain has only one primary antibody, they are then rinsed with PBS for 15 minutes and then placed on the shaker for two hours with Phalloidin (PBS 1:50). The tissues were then rinsed with PBS three

times for 15 minutes each and placed in DAPI (PBS 1:5000) on the shaker for 15 minutes. Finally, the sections were rinsed with PBS and mounted with DABCO.

2.8 Immune Cell Imaging Procedure

Immune cells were observed using the Zeiss LSM 510 META microscope. The images were captured with the Carl Zeiss Laser Scanning Microscope LSM510 Version 4.2 software. The microscope was set up by first turning on all Laser Diode 405, Argon/2, HeNe1, HeNe2 and the imaging software. The slide was placed inside of the microscope and the image was focused. Once the image was focused, the image was viewed on the software. A series of calibrations were conducted, first setting the parameters with the red channel. The pinhole was set to one Airy Unit and the lookup table was selected to establish the dynamic range of the image. The detector gain, amplifier offset, and laser intensity were adjusted for the best image. Once this was set, the next two channels were set by using the same optical slice from the red channel and the same measurements were adjusted. Once the channels were set, the lookup table was removed, and all three lasers were imaged together for a final image. Measurements were adjusted to remove excess noise if present. All sections were observed in the 20x water objective and tumor burden was counted as well as the immune cells. All images were exported as Tiff files once all the images were collected.

Table 2.2 Immune Cell Primary and Secondary Antibody Markers

Cell Type	Primary Antibody Marker	Primary Antibody source	Secondary Antibody Marker
MDSC	Gr1	rat	cy2 donkey anti-rat
	Cd11b	goat	cy3 donkey anti-goat
Macrophage	F4/80	rat	cy2 donkey anti-rat
Mast cell	FceR1a	rabbit	cy3 donkey anti-rabbit
	CD117	rat	cy2 donkey anti-rat
T-reg	CD4	rat	cy2 donkey anti-rat
	FOXP3	rabbit	cy3 donkey anti-rabbit
All Leukocytes	CD 45	rat	cy2 donkey anti-rat

The number of cells with specific cell surface markers were counted in the GraphPad Prism software to analyze the data obtained from immune cell counts, using unpaired t-test with Welch's correction without assuming equal standard deviations. Statistical significance was set at $p<0.05$.

CHAPTER 3

RESULTS

3.1 Alpha Diversity

Analysis of microbiota composition four weeks after LDP, **Figure 3.1**, showed that there was no significant difference in alpha diversity between control and LDP treatment male mice, or female mice. While there were no significant differences observed, it is important to note that control male groups had a wider range of diversity, while the diversity between the microbial population in the LDP male microbiota had a consistently higher diversity. The microbial populations between the control and LDP female groups both showed high levels of diversity after initial treatment. Notable differences between the diversity range are evident between males and females in which the female populations have a higher level of diversity than that of male groups.

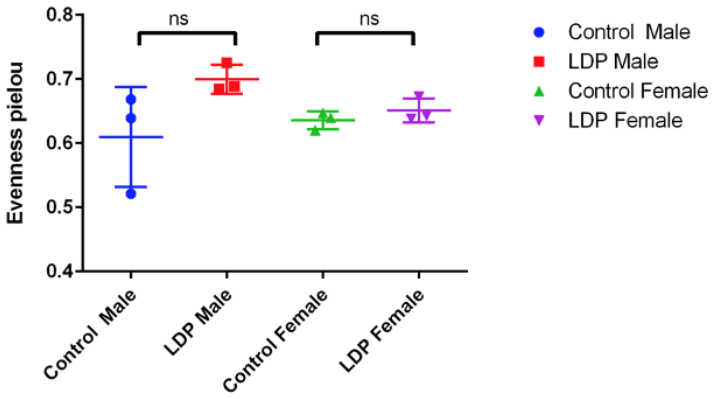


Figure 3.1 Analysis of Alpha Diversity Start of Experiment (TP1). The figure shows data collected at the start of the experiment before LDP treatment. There was no significant difference found in the control and LDP males, and not significant difference found in control and LDP female groups.

After 4 weeks of treatment at timepoint 2, there was no significant difference between the male control and LDP-treated mice; however, there is a significant difference between the female control and LDP-treated mice with a p-value of 0.0147, **Figure 3.2**. The difference in microbiota diversity of male and female mice poses an interesting question as to why female mice were more susceptible to LDP treatment than the male mice. It is possible that differences in hormones and genetic makeup that differ between genders may be associated with this observation [1, 75].

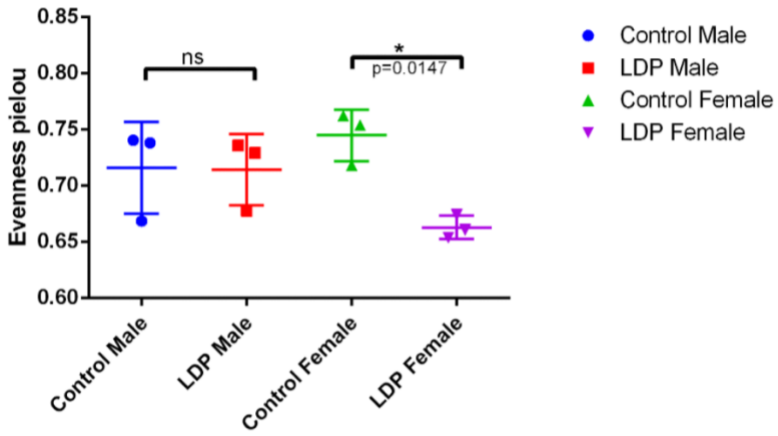


Figure 3.2 Analysis of Alpha Diversity after first exposure (TP2). The figure shows data after the first exposure to LDP treatment. There was no significant difference found in the control and LDP males, but a significant difference in diversity was found between control and LDP female groups.

At timepoint 5 after 18 weeks of treatment, **Figure 3.3**, showed that no significant differences between control and LDP-treated male and female mice. It is important to mention that differences in alpha diversity were observed within each population as the mice aged overtime. The female groups were more consistent in their microbial populations as compared to alpha diversity observed in male mice. LDP treated groups have less diversity within their microbial populations and a more expansion within their alpha diversity.

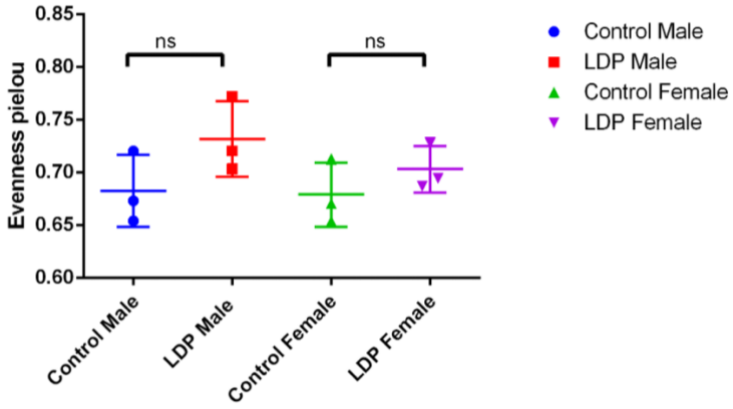


Figure 3.3 Analysis of Alpha Diversity end of experiment (TP5). Figure shows data at the end of the study. There was no significant difference found in the control and LDP males, and not significant difference found in control and LDP female groups.

3.2 Beta Diversity

Beta diversity of each treatment group at timepoint one prior to treatment and timepoint two after one round of treatment was analyzed using QIIME 2.0. The results in **Figure 3.4**, does not indicate a significant difference in the composition of the gut microbiota among treatment groups. The unweighted beta diversity demonstrates more clustering of groups in comparison to the weighted beta diversity. As expected in timepoint one, beta diversity measurements for all

mice are clustered close together, indicating that their microbiotas are similar in composition. At timepoint two, mice were treated with LDP, the beta diversity values of each group trend towards difference however the differences, did not reach a statistical significance. The most significant change among treatment groups is the weighted beta diversity between control male and LDP-treated males. The distance within both groups indicates changes in the phylum composition; however, the composition is not statistically significant to allow determination of the difference. A similar trend is apparent in the unweighted beta diversity calculated among the control female and LDP-treated female groups. It is important to establish both types of data to allow the ability to identify relative phylogenetic lineage and the diversity between environments.

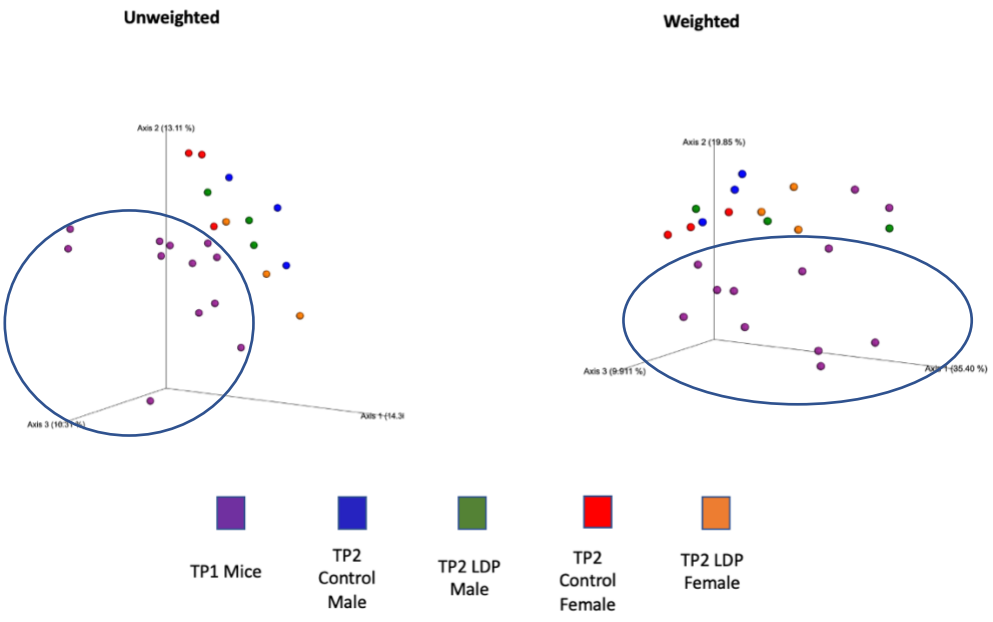


Figure 3.4 Analysis of Beta Diversity. Beta diversity graphs show the correlation between TP1 and TP2 differences in microbiota compositions in all treatment groups. Both unweighted and weighted beta diversity data does not show significant differences between TP1 and TP2 for all treatment groups. Circled areas indicate TP1 bacteria.

3.3 Taxonomic Analysis

At timepoint one, before the start of LDP exposure, the relative abundance or frequency of a bacteria is expressed as the percentage composition of an organism relative to the total number of organisms that were analyzed [87]. *Bacteroidetes* and *Firmicutes* were the most abundant phylum's among each treatment group with minimal differences among samples taken at this timepoint, **Figure 3.5**. Phylum name and species number can be observed in **Table 3.1**.

Table 3.1 Bacteria Phylum Bergey's Manual of Systemic Bacteriology 1st Edition

Name of Phylum	Number of Species	Number of Genera
Aquificae	27	12
Xenobacteria	29	11
Chrysogenetes	1	1
Thermomicrobia	13	6
Cyanobacteria	78	62
Chlorobia	17	6
Proteobacteria	1644	366
Firmicutes	2474	255
Planctomycetes etc.	13	5
Spirochaetes	92	13
Fibrobacter	5	3
Bacteroids	130	20
Flavobacteria	72	15
Sphingobacteria	76	22
Fusobacteria	29	6
Verrucomicrobia	5	2

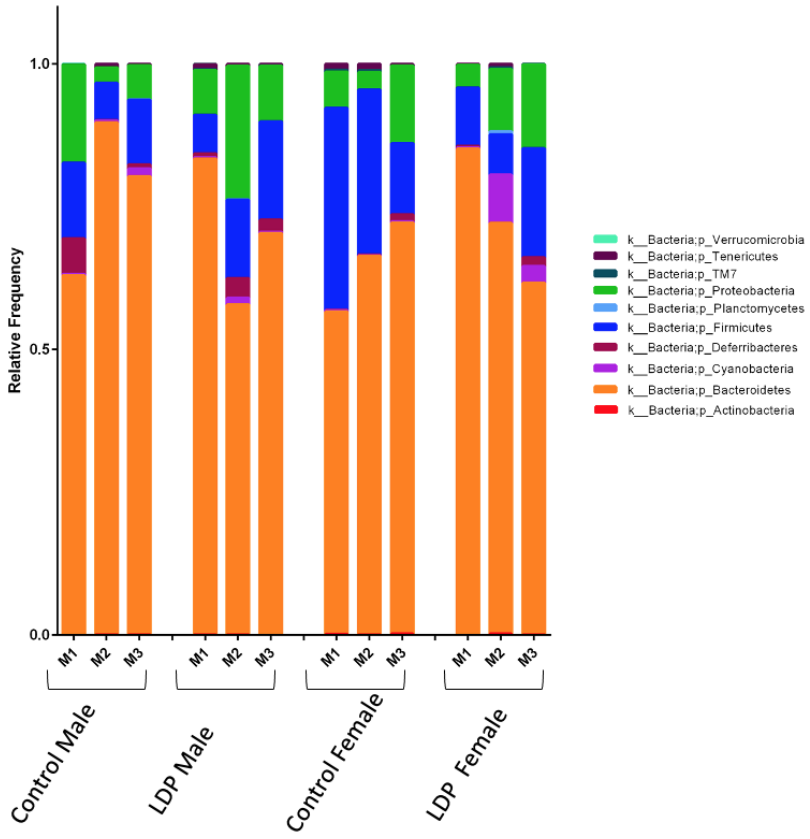


Figure 3.5 Phylum analysis of microbiota start of experiment (TP1). Microbiota graph shows the relative frequency of microbiota composition within each treatment group. TP1 shows that each treatment group contains a similar composition with minor variations prior to treatment.

At timepoint two, after four weeks on LDP, shown in **Figure 3.6**, there is a significant decrease in the *Bacteroidetes* phylum and an increase in the *Firmicutes* phylum among all groups except for the LDP-treated female mice. These findings suggest that in respect to LDP, change in microbiota composition are correlated with a decrease in *Bacteroidetes* and an increase in *Firmicutes* [25, 28]. The data suggest that there are different compositional changes between *Bacteroidetes* and *Firmicutes* from timepoint one, **Figure 3.5**, and

timepoint two, **Figure 3.6**. It is important to note that as the mice mature overtime, the composition of the microbiota naturally changes in response to environmental factors and drug exposure. Among all treatment groups in **Figure 3.6**, the LDP-treated male and control female mice showed significant decreases in the *Bacteroidetes* phylum and increases in *Firmicutes* phylum. The exact reason is not known; however, it is possible that hormonal differences between gender might be the underlying cause [76]. The loss of *Planctomycetes* and emergence of an undetectable but different bacterial phylum (yellow) in timepoint two, suggests that the composition of the microbiome has been altered to introduce new species. Although the microbiome changes overtime, the loss of a species and emergence of a new species can be detrimental to the overall health and well-being of the mouse. Interestingly enough certain species that are lost may never return as shown in **Figure 3.7**, where *Planctomycete* phylum is not observed to re-establish itself in the microbiota.

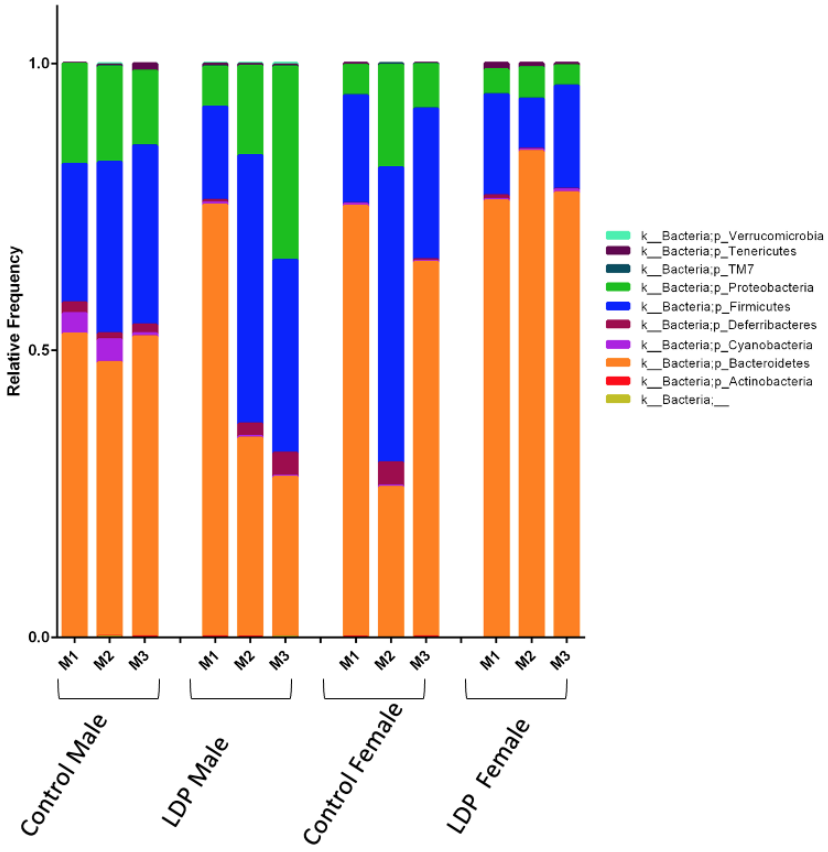


Figure 3.6 Phylum analysis of microbiota after first exposure (TP2). Microbiota graph shows the relative frequency of microbiota composition within each treatment group. TP2 shows the changes within treatment groups after the first administration of LDP. Changes are evident in both control groups and LDP male groups; however minimal changes are observed in LDP female groups.

At the end of the 18-week experiment, timepoint five, analysis of fecal microbiota showed an increase in *Bacteroidetes* phylum as shown in **Figure 3.7**. The resurgence of the *Bacteroidetes* phylum in the LDP-treated male mice suggests that the microbiome adopts to the environmental changes and re-establishes its ability to fight pathogens. The most striking observation in

microbiota composition at timepoint five is the continued loss of the *Planctomycete* phylum that was observed in timepoint one prior to treatment **Figure 3.5**, the loss of the unidentified phylum from timepoint two, after first exposure, **Figure 3.6**, and the loss of the *Verrucomicrobia* phylum in timepoint five, at the end of treatment**, Figure 3.7**. Overtime, the gut microbiota was altered as the mice matured, however the introduction of environmental stressors such as LDP, have contributed to a decrease in the diversity among the groups that were exposed to LDP.

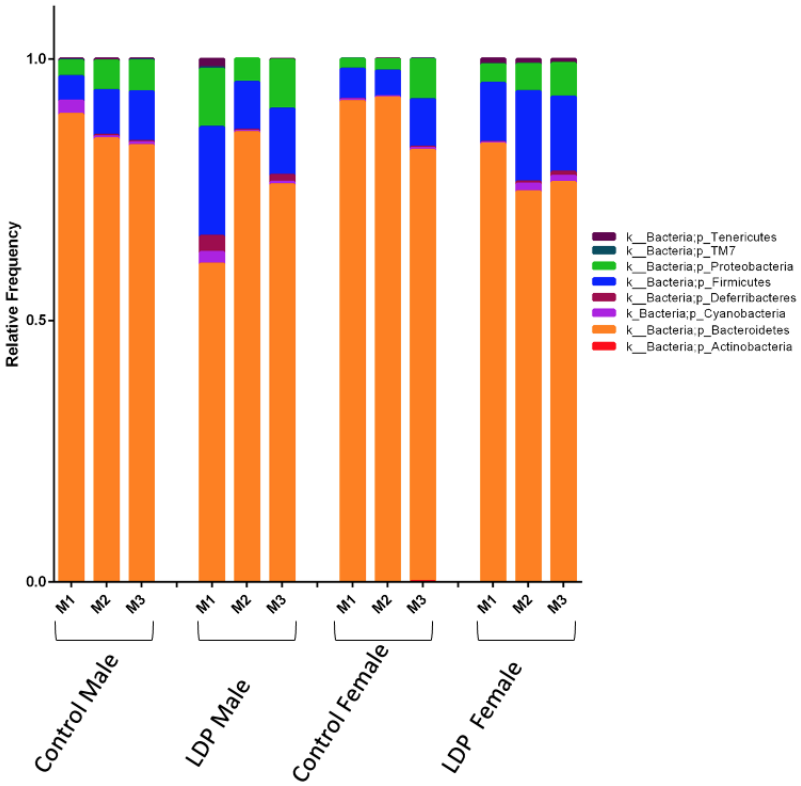


Figure 3.7 Phylum analysis of microbiota end of experiment (TP5). Microbiota graph shows the relative frequency of microbiota composition within each treatment group. TP5 shows the microbiota composition at the end of the 18-week experiment between all

treatment groups. It is evident that each treatment group has a balanced microbiota composition like that in TP1 but with less diversity.

To further demonstrate this point, analysis of the gut microbiota composition at the genus level provided more insight on the various changes associated with the gain and loss of bacterial species. Analysis of each phylum showed that there are more strains of bacteria that belong to the *Proteobacteria* phylum (16) than those that belong to *Bacteroidetes* (8) or *Firmicutes* (10) phylum in timepoint one, **Figure 3.8**. Although analysis based on phylum comparisons alone showed that the *Bacteroidetes* and *Firmicutes* were more abundant, analysis at the genus level showed that there are more identifiable classifications within the microbiota. For timepoint one, **Figure 3.8**, all treatment groups show similar composition with high diversity.

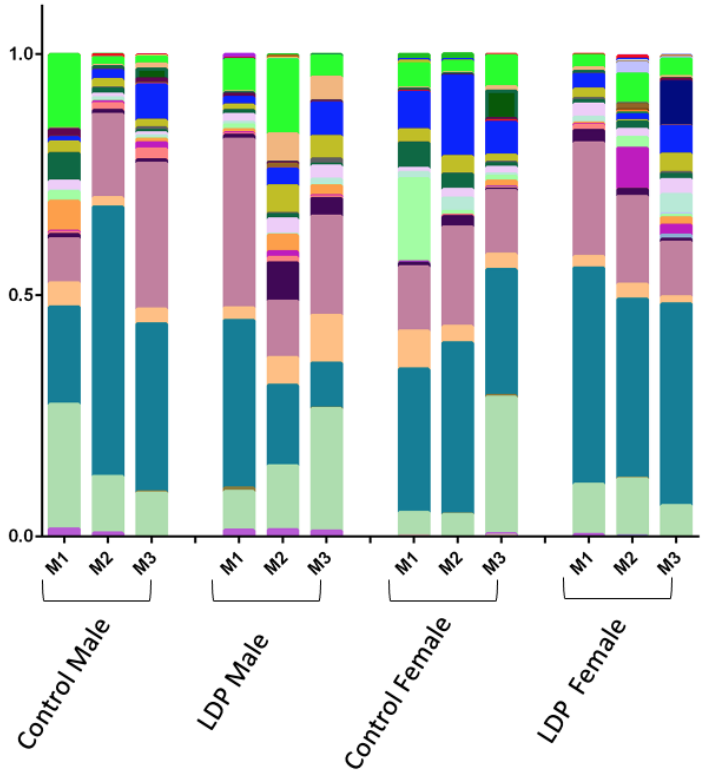
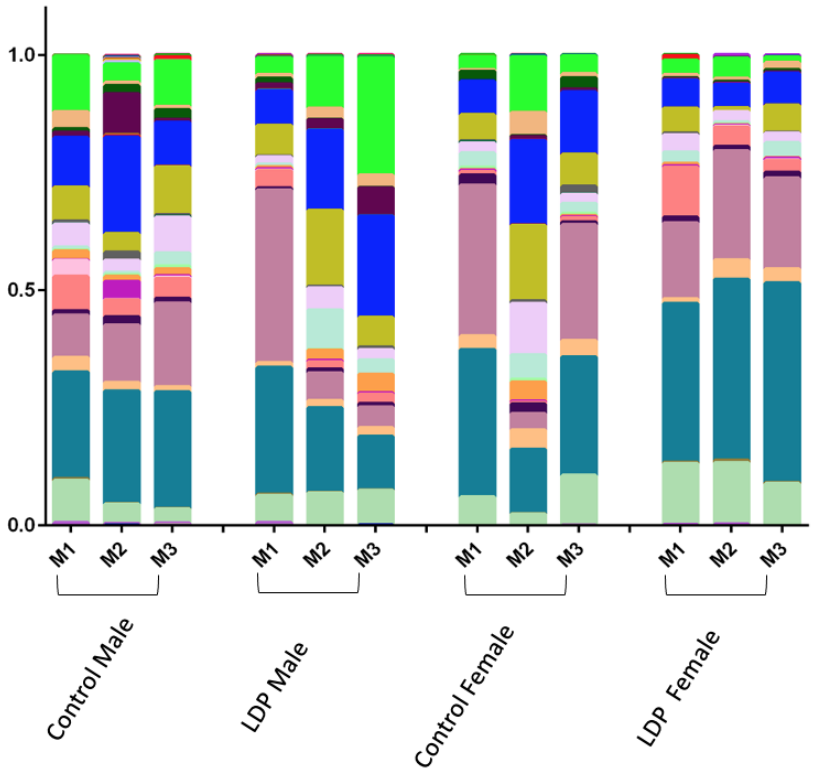


Figure 3.8 Analysis of genus level start of experiment (TP1). Microbiota graph shows the relative frequency of microbiota composition at the genus level within each treatment group. TP1 shows that each treatment group contains a similar composition with minor variations prior to treatment.

At timepoint two, after exposure to LDP, the data in **Figure 3.9**, shows that the microbiota strains among all treatment groups began to lose diversity as the *Proteobacteria* phylum reduced to twelve species, *Bacteroidetes* remained at eight species and *Firmicutes* remained at ten species. The *Proteobacteria* phylum is known to increase in inflammation within the gut microenvironment as shown in mouse models of colitis [88-90]. The decrease in species within *Proteobacteria* phylum suggests that exposure to LDP has adverse effects on the capability of the *Proteobacteria* to clear infection [91]. Among the treatment

groups in **Figure 3.9**, both male and female LDP-treated mice have less diversity among species as compared to that in timepoint one in **Figure 3.8**. In both control male and female groups the relative diversity is maintained, indicating that the initial administration of LDP greatly influenced the composition of the microbiota.



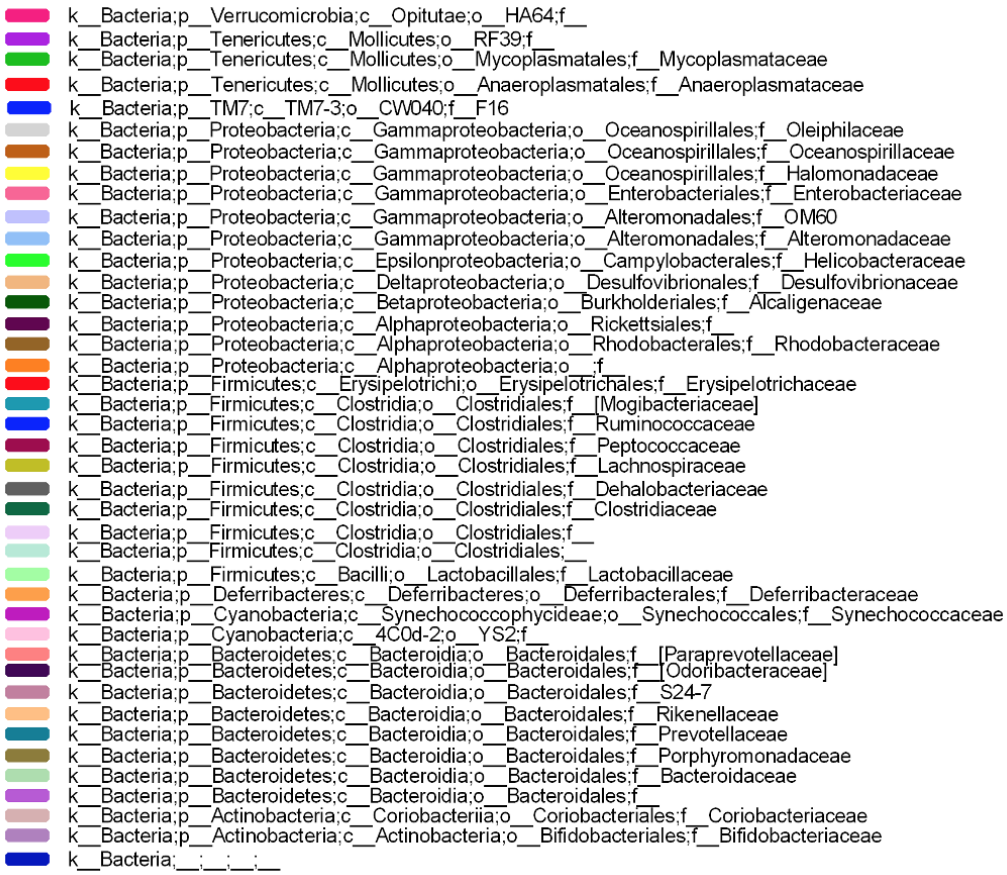


Figure 3.9 Analysis of genus level after first exposure (TP2).Microbiota graph shows the relative frequency of microbiota composition at the genus level within each treatment group. TP2 shows the changes within treatment groups after the first administration of LDP. Minimal changes are evident in all treatment groups; however, changes within the diversity of composition are evident.

At the end of the study in timepoint five, **Figure 3.10**, the composition of *Proteobacteria* phylum has decreased to six species, *Bacteroidetes* remained with eight species, while *Firmicutes* also remained at ten species which can be shown in **Figure 3.11**. Each treatment group had similar bacterial composition, however both LDP-treated male and female mice continued to have less diversity than the control male and female groups. The persistence of *Bacteroidetes* and

Firmicutes phylum species is consistent with the abundance of these species within normal gut microbiota [44].

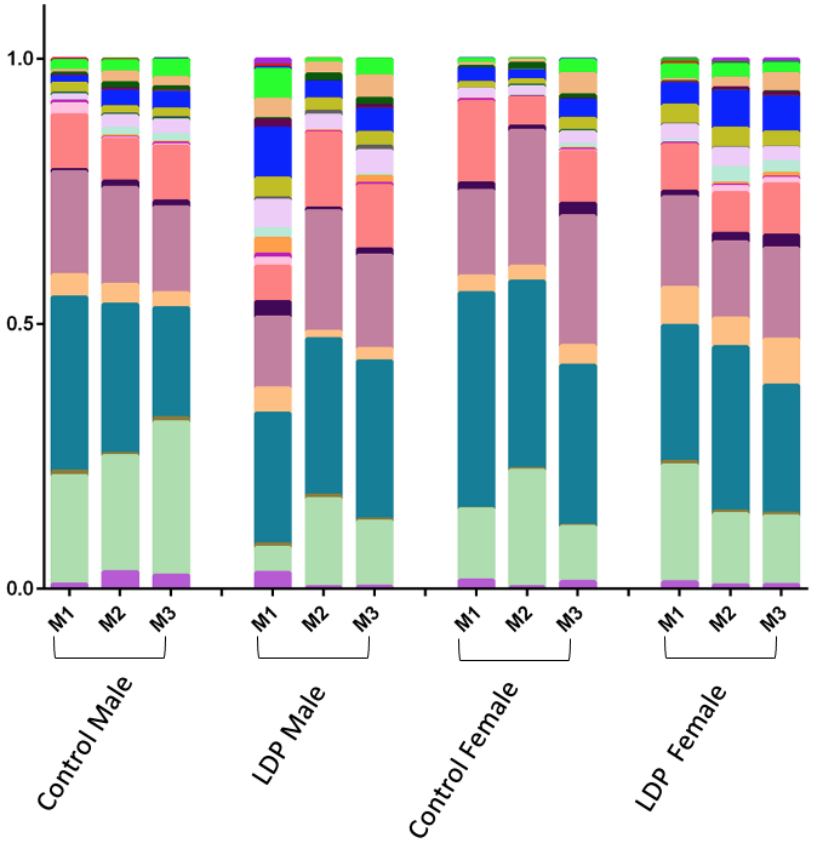


Figure 3.10 Analysis of genus level end of experiment (TP5). Microbiota graph shows the relative frequency of microbiota composition at the genus level within each treatment group. TP5 shows the microbiota composition at the end of the 18-week experiment between all treatment groups. It is evident that each treatment group has a balanced microbiota composition like that in TP1 but with less diversity. Each group has a similar composition as well indicating no significant difference in the administration of LDP.

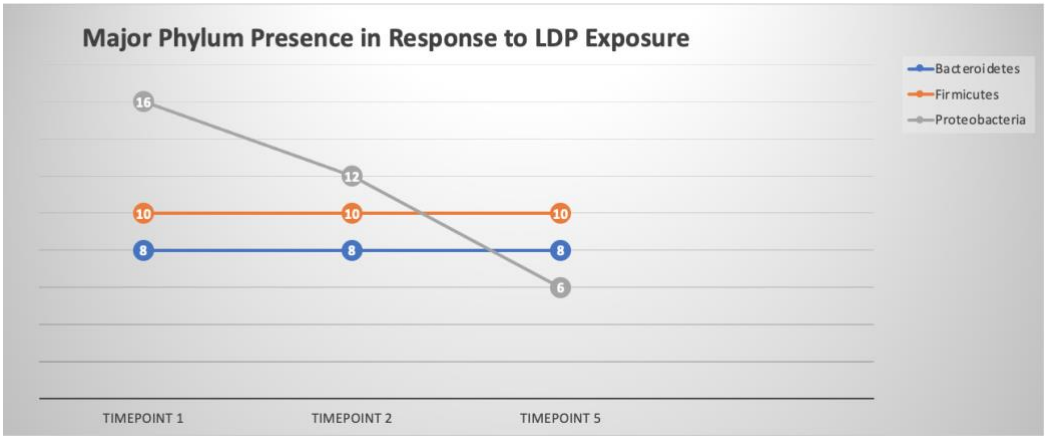


Figure 3.11 Major Phylum in Response to LDP Exposure. This figure shows how over the three timepoints from timepoint 1 (prior to LDP exposure), timepoint 2 (beginning of LDP exposure), and timepoint 5 (end of 18-week exposure). *Bacteroidetes* and *Firmicutes* maintain the same number

of species throughout the exposure, while *Proteobacteria* significantly decreases in bacterial species composition overtime. In all groups, bacterial species are introduced and removed overtime.

3.4 Results of Immune Cell Analysis in the Tumor Microenvironment

Figure 3.12 shows the tumor burden between all tumor development within mice groups as well as a comparison between male and female tumor development. While there was not a significant difference on overall tumor burden for all mice groups, it is evident that the tumor burden significantly increased among the *Apc*$^{Min/+}$ LDP-treated and control female mice. The increase in development of tumor burden may be associated to the Apc mutation found in these mice as it is a predisposition to the development of colorectal cancer as discussed earlier. The LDP-treated and control male mice have similar trends toward increase however, gender differences may be associated with the development between male and female. This data suggests that *Apc*$^{Min/+}$ female mice predisposed to colorectal cancer development when exposed to LDP, are more susceptible to tumor development.

Figure 3.13 shows the overall immune cell count among the different treatment groups. As expected, we found trends towards increased immune cell infiltration among the LDP-treated (female and male) mice except in leukocyte cell number for female mice as compared to control female mice. Significant difference was detected in the mast cells populations between LDP and control male mice, indicating a major increase in the mast cell population. Differences in mast cell significance between male and female mice, may be contributed to gender differences and dosage of penicillin. Increase in immune cells overall, in

the LDP-treated mice suggests an increased trend of inflammation in the intestinal environment in response to LDP treatment. As previously discussed, an increase in MDSCs, Tregs, and macrophages, indicate the presence of disease, inflammation within the colon tissue [47, 54]. The data suggest that treatment with LDP at a young age can lead to increased intestinal inflammation [28, 40, 92] that can subsequently be linked to increased tumor development in a *Apc*$^{Min/+}$ mice. The leukocyte immune cells collected within this study can be observed in **Figure 3.14** to represent the trend in difference between the LDP-treated and control female mice.

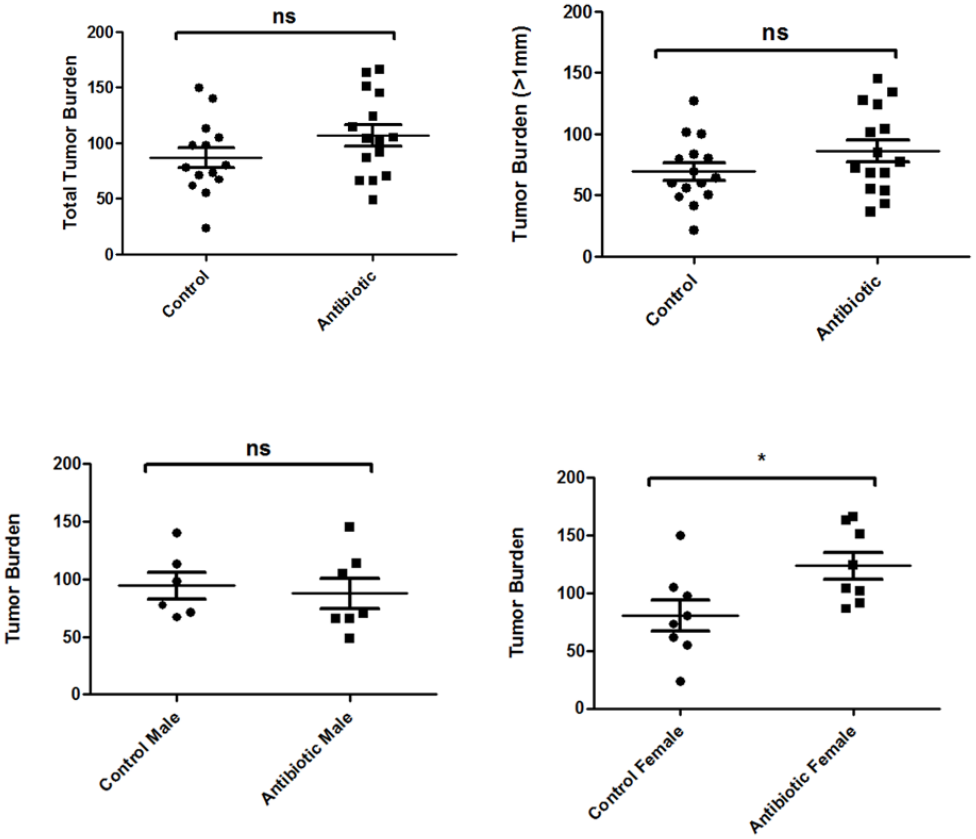


Figure 3.12 Tumor Burden between LDP-treated and Control Mice. The figure shows that there was no significant difference between the size of tumor development in all mice groups; however, there was a significant increase in tumor burden among LDP-treated and control female mice groups.

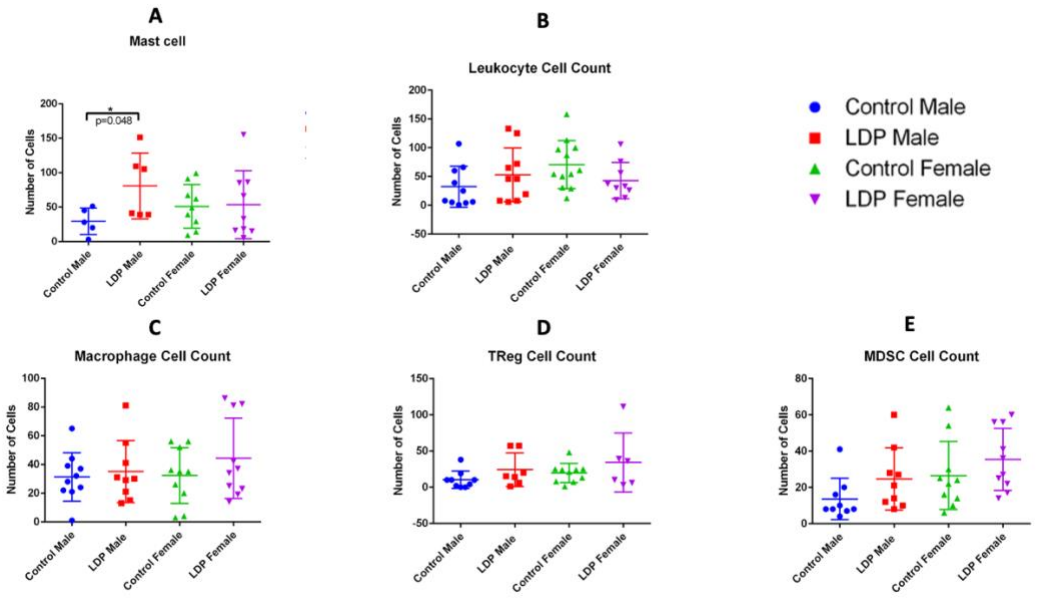


Figure 3.13 Immune Cell Analysis. Major immune cells investigated for the experimental design of this project include, mast, leukocyte, macrophage, Treg, and MDSC. Within each cell count group excluding mast cells for male groups, no significant difference was detected; however, there are visual comparisons to be made between different cell count group

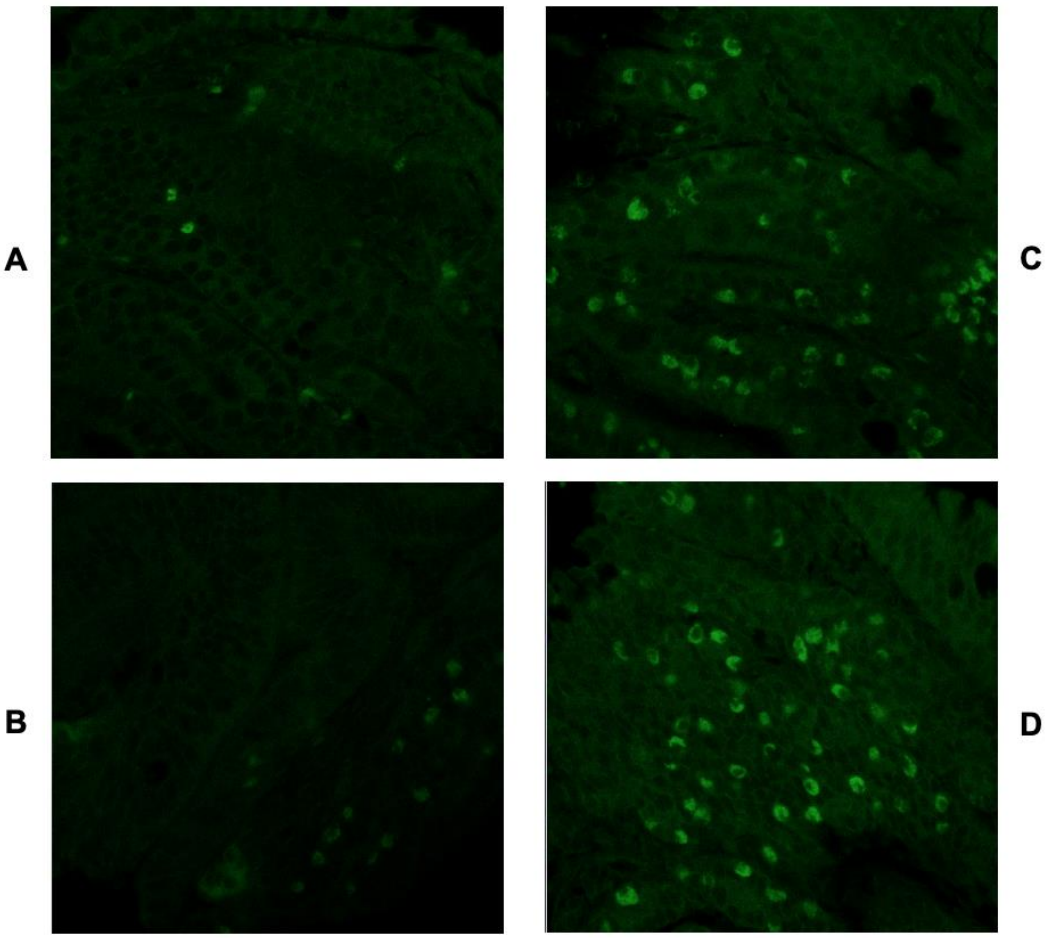


Figure 3.14 Leukocyte Immune Cells. Male mice control groups (A), male mice LDP-treated group (B), female control group (C), female LDP-treated group (D). This image shows a trend towards significant difference between the LDP-treated and control female mice.

CHAPTER 4

CONCLUSIONS

6.1 Conclusion

The use of antibiotics has resulted in revolutionary advancements in our ability to treat infectious diseases; however, their overuse poses a major threat to the human population in terms of acquired resistance and their long-term effects on the microbiome [24]. There is considerable evidence that shows a correlation between short-term exposure to antibiotics and resistance of organisms within microbial populations in the intestine [32]. Extended exposure and overuse of antibiotics may have the potential to inflict mutations and genetic changes within the microbiome causing bacteria to become resistant to the drugs resulting in an increase in the rate of infection [24, 28, 93]. Compelling evidence demonstrates that the administration of LDP to mice one week before birth until weaning results in increased weight and fat mass in their adult life; however, the intestinal microenvironment returned to a normal state four weeks after antibiotic withdrawal [28, 40, 92]. LDP administered at the early stages of weaning in these mouse models exhibited long term affects in the function and viability of the pups in their adult life [28]. Increasing the dosage period of LDP beyond a four-week time frame may demonstrate a persistent change in gut microbiome composition, which is the primary focus of this study.

Our data suggest that there are some significant differences between early and late stages of LDP exposure among the microenvironment; however, tumor burden was significantly increased among female LDP-treated mice. It was hypothesized that exposure to low dose penicillin in early life disrupts the microbiota composition, and increases inflammation in the colon, resulting in increased tumor burden. Although not all changes were significant, there were other changes that may explain these observations.

A significant difference was detected in the alpha diversity before and after LDP-treatment for timepoint one or timepoint two between female control and LDP-treated mice indicating that LDP alters the gut microbiota within the initial exposure period. This is consistent with findings found in a study conducted by Boursi et. al where an increased risk of CRC occurred with an increased dose of penicillin in human epidemiological studies [94]. The study suggests that initial use of penicillin >10 years prior to diagnosis correlated with CRC patients as compared to those who were not exposed. While there was a significant correlation to penicillin exposure and increased changes to intestinal bacteria diversity in the study by Boursi et al, this study showed that LDP did not greatly impact tumor burden except in female mice. These results suggest that the lower concentrations of penicillin may not have as much effect as higher concentrations of penicillin, as compared to chronic exposure, resulting in low effects on microbiota composition.

Beta diversity within the intestinal microbiota showed trends toward significant difference between the start of the study prior to LDP exposure and

the first exposure to LDP. Evident changes among the beta diversity were observed **Figure 3.4**, after the start of LDP exposure as the timepoint two microbiota analysis for each mice group begin to spread from one another indicating difference. The gradual increase in difference among the absence and initiation of LDP exposure could be correlated to the low dose of penicillin and may have more significant changes if a higher dose was administered.

Similar findings within the intestinal microbiota compositional changes from timepoint 1, 2, and 5, indicate that the first dosing of LDP has minimal effects that disrupt the microbiota, but the change in composition returns to a balanced state similar to timepoint. The return of bacteria within the microbiota environment is a complex process in which the bacteria use genes known as resistomes to maintain their species and return after disruption occurs [95]. This is extremely important for the health and integrity of the gut microbiota and ensures that these bacteria are not lost; however, if long term exposure occurs, antibiotic resistance may cause changes in the bacterial resistance [96, 97]. This phenomenon has been proven in other studies showing the difference in microbiota compositions of antibiotic treated mice versus untreated with increasing numbers of *Firmicutes* and *Bacteroidetes,* which in turn lowers the diversity of the microbiome further [33, 39]. Treatment with the LDP in the mice significantly decreased the diversity of the microbiota in the early stages of treatment and changed composition of the bacteria after antibiotics were administered and returned to normal states following short term exposure [39]. Investigation of these changes is important for the long-term effects on the

overall health of an individual, whether certain types of bacteria are more beneficial in protection than others.

Among the many immune cells analyzed in this study, mast cells show a significant correlation between male LDP exposed and control groups. The correlation of mast cell increase in male LDP groups may be associated with hormonal differences between male and female; however, the direct cause is unknown.

Among female LDP-treated mice, tumor burden was significantly increased as compared to those that were not exposed to LDP. While there were increases in all mice, the most significant increases were among the female LDP-treated mice. Increases in tumor burden among female mice suggest that LDP exposure has altering effects within the intestinal environment and may be associated with gender involving hormone differences. The data suggest that administering antibiotics, even at low doses, increases tumor burden within female *Apc*$^{Min/+}$ mice predisposed to colorectal cancer development. LDP water treatment resulted in decreased diversity as shown in **Figure 3.11** this is important and emphasizes that there are problems inherent with even low doses of penicillin.

Disruption to gut microbiota and other adverse effects due to LDP and antibiotic exposure has been shown in many studies [25, 28, 31, 45], but the mice did not have mutations such as the Apc gene that predisposed them to colorectal cancer development; therefore, our results suggest that in addition to

administration of LDP, mutations for CRC enhance the overall effect of the intestinal microbiota.

6.2 Limitations

It is difficult to control the amount of water each mouse consumes and therefore it is difficult to determine the exact dose of LDP each mouse consumed daily. This limitation may be the result in low significance in some groups of mice. Increasing the dose of penicillin in the water or using direct injection of penicillin to further control the dosing, may well result in increased significance. Injecting the antibiotic directly is a more controlled and measured direction of study and could present more accurate results.

It is evident that the microbiota composition in the intestine does change after the first administration of antibiotic and returns to a balanced composition at the end of the study; however, determining at what point the microbiota composition returns to a state of balance has not been determined. To investigate this question, all time point fecal samples would need to be analyzed, but this becomes costly and not related to the hypothesis in question.

6.3 Future Directions

Future directions for experiments include, increasing the dosage of penicillin from a low 2 mg/ml to a higher dose, extending the periods of antibiotic exposure with higher concentration, and the investigation of different types of antibiotics.

Increasing the dosage of penicillin, administered in the water or by injection, may have altering results compared to the lower dose of penicillin in this study. The changes within the microbiota composition and immune cell populations found in this study suggest that a higher dosage of penicillin may have more of an effect on tumor burden and further changes in the microbiota composition.

It is evident that at 18-weeks at the close of the study, the microbiota composition returned to a state similar to TP1. Therefore, increasing the length of time for LDP exposure is not sufficient to see different results, but with a higher dosage, results may be more significant. Conducting this study with the use of different antibiotics at higher concentrations would not only show differing outcomes but would provide insight into the effect of different types of antibiotics and association to tumor burden in the colon.

Overall, this study shows the correlation of inflammation, tumor burden, and disruption of intestinal microbiota composition in response to LDP exposure indicating an increase in tumor burden among mice predisposed to colorectal cancer development.

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