Role Of Altered Gut Microbiota In Tumor Development, Mucus Production And Inflammation In APC MIN/+ Mouse Model

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ROLE OF ALTERED GUT MICROBIOTA IN TUMOR DEVELOPMENT, MUCUS PRODUCTION AND INFLAMMATION IN $Apc^{Min/+}$ MOUSE MODEL

by

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DEDICATION

In the loving memory of my mentor, Dr. Raja Fayad, whose kind words and advice will always remain a guiding light in my future endeavors.
ACKNOWLEDGEMENTS

I am thankful to my God for providing me with the necessary strength for this great an educational pursuit and bestowing me with the necessary outlook that has led me to come in contact with some of the most important people of my professional and personal life, along with experiences that have shaped me into a better, stronger and much evolved personality. I am extremely thankful to my mentor Dr. Fayad who has always motivated me to do better than I think I can do, whose kind words and caring advices will forever resonate in my heart. I would like to thank my parents Mr. Harmel Singh and Mrs. Balbir Kaur for always understanding me and providing me with the values to follow the right paths and to be strong, sincere and loving. Their blessings and motivation has made this journey possible and have helped me to successfully finish this dissertation. I also thank them for always being a guiding light for me, for providing firm advice and at the same time, believing in me and providing me with the freedom to pursue my decisions and ideas. I would extend a special thanks to my colleague, senior and husband, Arpit Saxena, who has helped me at every step of my doctoral studies, mentored me through my early phases in my lab, helped me with experiments and teaching responsibilities and motivated me with his perseverance. I thank him for being an understanding and loving partner and for providing me with the mental support that was needed to overcome difficulty during this eventful journey. I would like to extend my loving thanks to my sister Rupinder Kaur for providing me with emotional strength and loving support, and for always being a best friend and motivating me with her cheerfulness, enthusiasm and persistence. I cannot thank
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ABSTRACT

The microbiome forms an integral part of the gut microenvironment. Once ignored, the topic has gained momentum in research during the past decade, where studies have strongly suggested the association of microbiota with health and a misbalance thereof, to many disease conditions ranging from inflammation and colitis to diabetes, obesity and colon cancer. The Human Microbiome Project (HMP, NIH common fund - 2008) has used a variety of high throughput analyses in order to study gut microbiota in health. The consortium has so far been able to isolate and characterize more than 1,300 reference bacterial strains from the human body. The large amount of data generated has led to a baseline need to address the implications of different microbial members, or groups thereof, in health and disease. The microorganisms residing in the gut comprise of bacteria, archaea, fungi and viruses that are distributed throughout the length of the gastrointestinal tract. While there will be limitations to studying all types of microorganisms owing to their overwhelming numbers and types, our study is focused only on bacterial populations of the gut, and for the purpose of convenience, terms of gut microbiota/microbiome will be used for describing gut bacteria pertaining to the mice used in our study. The overall purpose of this study is to determine the effects of alterations in the gut microbiome on tumor development and inflammation, and if it leads to recolonization of the gut by altered bacterial communities. The working hypothesis was that an alteration of bacterial microbiome occurs during tumorigenesis and manipulation of the gut microbiome externally exacerbates the clinical symptoms associated with intestinal cancer, leading
to higher gut and systemic inflammation. Specific aim 1 studied the composition of gut microbiota during the stages of tumor initiation and progression. It aimed at studying the gut microbial profiles pertaining to bacteria that reside inside the gut of the $Apc^{Min/+}$ mice during normal conditions and comparing it to control mice. Among the different bacterial phyla residing in the murine gut, the Bacteroidetes and Firmicutes comprised of dominant bacterial populations. Specific aim 2 studied the effects of external manipulation of the gut microbiome on gut health and tumorigenesis. The manipulation of the gut microbial community was used to help elucidate how alterations in the gut microbiome effect the intestinal tumor and mucus production outcomes along with their effects on the gut health in measurable terms. Specific aim 3 studied the altered inflammatory response of the mouse gut with respect to relative abundances of the Bacteroidetes and Firmicutes bacterial phyla populations.
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LIST OF ABBREVIATIONS

ANOVA ................................................................. Analysis Of Variance
APC ................................................................. Adenomatous polyposis coli
APN .............................................................. Adiponectin
CD ............................................................... Crohn’s Disease
CICC ......................................................... Chronic Inflammation induced Colon Cancer
COX-2 ............................................................ Cyclooxygenase-2
DMH ............................................................ 1,2 Dimethylhydrazine
DSS ............................................................... Dextran Sodium Sulfate
FAP ............................................................... Familial Adenomatous Polyposis
Gpx ................................................................. Glutathione peroxidase
Hes ............................................................... Hairy and Enhancer of Split
IBD ............................................................. Inflammatory Bowel Disease
IL ................................................................. Interleukin
Math ........................................................... Mouse ATonal Homolog
Min .............................................................. Multiple Intestinal Neoplasia
MUC ........................................................... Mucin
Th ............................................................... T Helper
TNF-α ........................................................ Tumor Necrosis Factor alpha
WT .............................................................. Wild Type
CHAPTER 1

INTRODUCTION
The topic of gut microbiome has recently gained attention due to research suggesting its significant role in not only maintenance of gut health but also in disease conditions such as obesity, diabetes, arthritis, colitis and cancers of the digestive system (Cho, Carter, Harari, & Pei, 2014; Dziarski, Park, Kashyap, Dowd, & Gupta, 2016; Kabeerdoss, Sandhya, & Danda, 2015; Knip and Siljander (2016)). The gut microbiome comprises of around 500-1000 species and a genetic diversity which has 100-fold more genes than humans (Dugas, Fuller, Gilbert, & Layden, 2016).

Another field that aims at answering the questions on gut microbiome and its links to health and disease is the usage of probiotics including one or more important bacteria. The bacteria *Lactobacillus acidophilus* has been implicated in a significant suppression of colon tumor incidence and size and a reduction in pro-inflammatory cytokines such as TNF-α and IFN-γ (Perdigon, Valdez, & Rachid, 1998; Urbanska, Bhathena, Martoni, & Prakash, 2009). Some studies also state that the link between the microbiome and the actual development of polyps in the gut is solely linked through the erosion of the gut lumen microenvironment and the resulting inflammation (Dianda et al., 1997).

An altered microbiome has been previously linked to inflammatory bowel disease and colorectal cancer. However, we still lack the knowledge of a characterizing the gut microbiome that relates to health or disease condition. An early event of inflammatory insult to the gut can very well result in tumorigenesis when combined with processes of bacterial DNA damage and chromatin alterations (Zhu, Michelle Luo, Jobin, & Young, 2011). Studies on mouse models such as the IL-10−/− have demonstrated a protective role of the presence of gut microbiome on inflammation, where the WT mice were rendered protected against inflammation in the gut while the germ-free (no microbiome) animals
developed colitis (S. Wu et al., 2009). It should be noted here that germ-free animals may not represent the human gut as an individual can never be completely without microbiome in the gut. From the perspective of any given disease state that can be linked to microbiome, the basis of its linkage can only be an altered microbiome. It should also be noted that the process of inflammation is the one that alters the microbiome (Uronis & Jobin, 2009), although inflammation alone is not sufficient to promote colorectal cancer and altered microbiota works hand in hand with inflammatory insults that eventually lead to cancer (Arthur & Jobin, 2013). The gut microbiome has been shown to complement AOM and DSS mouse models in causing tumorigenesis, but is yet to be elucidated if it is the increase or loss of certain bacterial populations that lead to development of polyps (Zackular et al., 2013). It is, however, not known what effect the microbial dysbiosis has at different stages of progression of tumorigenesis in the mouse, or if it leads to different consequences in normal versus tumorigenic conditions.

What is unknown?

Studies are needed to characterize the interplay between the gut microbiome, inflammation and cancer. Another aspect which may be helpful in correctly defining the microbiome and inflammation-to-cancer axis is the progression of alterations in the microbiome with worsening disease pathology. Of all the diseases that are affected by a change or loss of gut microbiome, cancer of the small and large intestine is clearly the one which can be physically and physiologically related to the presence of alterations in gut microbiome community.
AIM 1: Examine the gut microbiome composition during tumor initiation and development in \( Apc^{Min/+} \) mice.

**Rationale:** An altered gut microbiome can cause inflammation ranging from acute bouts to chronically inflamed gut. An altered microbiome, which shifts from symbiotic to a more inflammation-inducing phenotype, leads to a vicious cycle of erosion of the gut epithelial lining that, in turn, leads to more and more inflammatory processes taking place inside the gut microenvironment (Saxena et al., 2012; Wang & Zhang, 2015). Our previous study on chronic inflammation-induced colon cancer in the APNKO (Adiponectin-knockout) mice demonstrated microbiota profiles that were clearly altered during tumorigenesis (data not published). The alterations mainly pertained to the altered percentages of the *Firmicutes* and *Bacteroidetes* bacterial phyla during inflammation-induced tumorigenesis in the mice (Figure 1). As opposed to externally administered chemically-induced tumorigenesis in mice, the microbiota has not been studied with respect to a spontaneously induced tumorigenesis such as that in the \( Apc^{Min/+} \) mouse. It was recently concluded that an alteration of gut microbiome precedes polyposis in the \( Apc^{Min/+} \) mouse (Son et al., 2015). Also, it has been seen that specific pathogen-free (SPF) \( Apc^{Min/+} \) exhibit a higher tumor load and anemia with a higher infiltration of inflammatory cells specifically at advanced stages as compared to germ-free animals, indicating that a mere modulation of gut microbiome profiles can abrogate the disease condition in the gut (Y. Li et al., 2012). However, there are no studies that study the gut microbiome with respect to gut inflammation and tumorigenesis in an age/time-dependent manner. Therefore, the first aim
of the present study was directed towards characterization of alterations of microbiome profiles from early to later stages of tumorigenesis in an $Apc^{\text{Min}/+}$ mouse model.

**Hypothesis:** The gut microbiota in $Apc^{\text{Min}/+}$ mice exhibits a higher percentage of *Firmicutes* bacterial phylum and a lower percentage of *Bacteroidetes*, along with the reduction in overall bacterial diversity during onset of tumorigenesis when compared to the WT mice. Furthermore, the percentage of *Firmicutes* increases, while overall diversity decreases during the progression of tumorigenesis.

AIM 1.1: Study of the gut microbiome profiles during intestinal tumor initiation.

*Hypothesis 1.1:* The microbiome profile of $Apc^{\text{Min}/+}$ mice exhibits a higher percentage of *Firmicutes* bacteria and a lower percentage of *Bacteroidetes* as compared to WT mice at initiation of tumor development. The overall microbial diversity decreases lower in $Apc^{\text{Min}/+}$ mice as compared to their WT counterparts.

AIM 1.2: To study the profile of the gut microbiome during the progression of intestinal tumor development.

*Hypothesis 1.2:* The microbiome profile of $Apc^{\text{Min}/+}$ mice exhibit a higher percentage of *Firmicutes* bacteria and a lower percentage of *Bacteroidetes* as compared to their WT counterparts during tumor progression. *Firmicutes* population is higher during later stages of tumorigenesis as compared to initial stages of tumorigenesis. Also, the microbial diversity values are lower in $Apc^{\text{Min}/+}$ mice during tumor progression as compared to WT
mice, and within $Apc^{Min/+}$ mice at later stages of tumor development as compared to their initial stages of tumorigenesis.

**AIM 2: Determine if gut microbiome manipulation can regulate the $Apc^{Min/+}$ mouse health and tumorigenesis.**

**Rationale:** The mucus layer tends to thickness with increased diversity of the microbiota (Jakobsson et al., 2015). Our preliminary data from a study on chronic inflammation-induced colon cancer using APNKO mice suggested that the gut microbiota changes in a way that favors an increased percentage of bacteria of certain phyla (here, *Firmicutes*) as compared to others (such as *Bacteroidetes*). It has not been studied yet as to whether there is a relation between the altered percentages of especially the *Firmicutes* and *Bacteroidetes* phyla populations, and tumor number, tumor size and goblet cell numbers. It is known that the mucus layer provides a source of nutritional carbon and therefore energy to some of the intestinal flora that are able to lyse the glycans present in the mucus, making the interrelationship between the gut bacteria and the mucus layer really important (Kaur et al., 2015). Also, the metabolites produced by these microbes also influence the differentiation and function of the epithelial and immune cells in the intestinal mucosa (Kato, Kawamoto, Maruya, & Fagarasan, 2014; Shan et al., 2013). The regulation of the mucin gene expression as well as mucus production have been shown to be two levels at which the gut microbial community may modulate mucins (Comelli et al., 2008). Our recent study (Kaur, et al., 2015) also suggested that a reduction in the secretory mucin profile is associated with an inflammatory phenotype of the gut (Figure 4). Therefore, the present aim was directed
at knowing the mucus-producing phenotypes of the cells lining the gut of the experimental animals in relation to the changing profiles of the gut microbiota.

**Hypothesis:** Manipulation of the gut microbiota leads to a decrease in gut health and an increase in tumor number and size in the $Apc^{Min/+}$ mouse. Furthermore, manipulation of the gut microbiota decreases goblet cell number in the intestine and colon.

**AIM 2.1:** To determine if manipulation of gut microbiome through antibiotic administration affects disease prognosis in the $Apc^{Min/+}$ mouse.

**Hypothesis 2.1:** The manipulation of gut microbiome by externally administered antibiotics leads to a worsening of disease prognosis in the experimental mice.

**AIM 2.2:** To determine if manipulation of gut microbiome through antibiotic administration affects tumor number and size in the $Apc^{Min/+}$ mouse.

**Hypothesis 2.2:** The manipulation of the gut microbiome by externally administered antibiotics leads to an increase in tumor number and size in the $Apc^{Min/+}$ mice.

**AIM 2.3:** To determine if gut microbiome manipulation effects goblet cell numbers in small intestine and colon of $Apc^{Min/+}$ mice as compared to WT mice.

**Hypothesis 2.3:** The manipulation of the gut microbiome by externally administered antibiotics leads to a reduction in goblet cell numbers in the intestine and colon of $Apc^{Min/+}$ mice.
AIM 3: Determine if the relative abundance of *Bacteroidetes* and *Firmicutes* bacterial populations is associated with intestinal and systemic inflammation in the *Apc*<sup>Min/+</sup> mouse.

**Rationale:** The induction of cancer in the gut by chronic inflammation has always indicated towards the importance of underlying altered inflammatory processes (Wang & Zhang, 2015). Different agents that have been documented to be effective against tumors and cancers are the agents that function by downregulating important inflammatory pathways and pro-inflammatory cytokines such as IL1-β, IL-6 and TNF-α, and reduction of macrophage infiltration (Y. Li, et al., 2012; Murphy, Davis, McClellan, & Carmichael, 2011; Murphy, Davis, McClellan, Gordon, & Carmichael, 2011; Ritland et al., 1999). It has also been shown that the intestinal commensal bacteria plays a role in the development of gut microenvironmental immune system comprising both the humoral and cellular components and maintains the protective steady state immune function throughout life (Cebra, 1999; Talham, Jiang, Bos, & Cebra, 1999). It may be more appropriate to say that any alteration in comparative numbers of certain bacteria often lead to upregulation or downregulation of certain inflammatory pathways. The systemic release of microbial products as a result of the manipulation of the gut epithelium invokes an IL-23 response and a further IL-17 response in order to neutralize further invasion by the microbes, and the process is brought about by the *Firmicutes* bacteria *Clostridia sp.* (Shih et al., 2014). The bacteria especially promotes IL-17 cytokine in the small intestine which is the primary site of its attachment and survival (Omenetti & Pizarro, 2015). These Th17 cells may become autoreactive in case of intestinal epithelium insult. The bacteria *Bacteroides*
*fragilis* from the *Bacteroidetes* phylum leads to an increase in the production of IL-10 cytokine (Omenetti & Pizarro, 2015), whereas its strains have been implicated in the production of the pro-inflammatory cytokine IL-17-dependent inflammation-related colon cancer (W. Wu et al., 2015). Inflammation, which is an integral part of tumorigenesis should therefore, may strongly be correlated with a reduced bacterial diversity or numbers.

**Hypothesis:** An increase in the population of *Firmicutes* bacteria and/or a reduction in the population of *Bacteroidetes* leads to a higher inflammatory response in both the intestinal tissue and systemic circulation.

AIM 3.1: To examine if the relative abundance of *Bacteroidetes* and *Firmicutes* populations regulate intestinal tissue inflammation in *ApcMin/+* mice.

*Hypothesis 3.1:* An increase in *Firmicutes* and/or a reduction in *Bacteroidetes* bacterial populations increases the inflammatory cellular infiltration into the mucosa of the intestinal tissue of the experimental mice.

AIM 3.2: To examine if the relative abundance of *Bacteroidetes* and *Firmicutes* populations regulate intestine-secreted IL-1β, IL-17 and TNF-α in *ApcMin/+* mice.

*Hypothesis 3.2:* An increase in *Firmicutes* and/or a reduction in *Bacteroidetes* bacterial populations increases the intestine-secreted IL-1β, IL-17 and TNF-α levels in the experimental mice.
AIM 3.3: To examine if the relative abundance of *Bacteroidetes* and *Firmicutes* populations regulate systemic levels of cytokines IL-1β, IL-17 and TNF-α in *Apc"Min/"* mice. 

Hypothesis 3.3: An increase in *Firmicutes* and/or a reduction in *Bacteroidetes* bacterial populations increases systemic levels of IL-1β, IL-17 and TNF-α cytokines in the experimental mice.

**Working Model**

![Working Model](image)

**Figure 1.1. Working Model:** Figure illustrating the working model for the study where the three different aims have been depicted with their respective factors measured during the study.
The central idea of the present study is to study the role of gut microbiota (bacterial) in tumorigenesis, mucus producing phenotype and inflammation. In order to study this, we used the \( \text{Apc}^{\text{Min/+}} \) model of intestinal cancer. The \( \text{Apc}^{\text{Min/+}} \) mouse model is raised from the C57BL/6 background where a point mutation on codon 850 of the \( \text{Apc} \) (Adenomatous Polyposis Coli) gene leads to spontaneous development of polyps (adenomas) in the intestinal mucosa (Zhang et al., 2015). The mutation is responsible for the production of a truncated APC protein (2843 amino acids) that lacks its C-terminal domains. The protein functions to downregulate the Wnt signaling pathway by binding to and promoting the destruction of the \( \beta \)-catenin protein. It has been shown that altered interactions between the gut microbiota and colonic mucosa precede polyposis in the \( \text{Apc}^{\text{Min/+}} \) mouse (Son, et al., 2015). Using the basis of an altered gut microbiota preceding polyposis, we are trying to delve further into the processes that are hampered by alterations in the gut microbiota, which can be used to elucidate the ways in which the gut microbiota affects tumorigenesis.

The first aim of the study was directed towards examining the composition of gut microbiome during tumor initiation and development in the \( \text{Apc}^{\text{Min/+}} \) mouse. This aim acted as preliminary to aims 2 and 3 by characterizing and thereby defining the alterations occurring in the microbiome of \( \text{Apc}^{\text{Min/+}} \) mouse during initiation and progression of tumorigenesis. Previous studies have demonstrated an alteration in gut microbiota to be linked to a variety of conditions including diabetes, arthritis, colitis and cancer and even immune system. Conditions such as Crohn’s disease and obesity have implicated gut bacterial dysbiosis in pathogenesis (Chan, Kumar, & Mendall, 2015; Mai, Colbert, Perkins, Schatzkin, & Hursting, 2007). The characterization of the gut microbiome in the experimental \( \text{Apc}^{\text{Min/+}} \) mice helped define the abundance of different bacterial species in
the gut and their relative diversity values during the initiation and progression of tumorigenesis.

The second aim of the study was directed towards elucidating the role of an altered microbiome on pathology of the experimental animals. Antibiotics were administered to experimental mice in order to perturb the gut microbiota during the initial and progressive stages of tumorigenesis. The use of antibiotics not only help get rid of pathogenic bacteria but also deplete related micro-organisms, which fail to return to normal levels even long after the antibiotic usage subsides (Cresci & Bawden, 2015). This aim was directed towards helping in elucidating the effect of alterations in microbiome in terms of their abundance and diversity on the disease condition of the animals. This aim was also being directed towards knowing if the tumor numbers and size are related to the microbiome and its alterations. The third part of this aim helped elucidate the effects of altered gut microbiome on expression of goblet cells in the intestine and colon of mice. Our previous study (Saxena et al., 2013) has demonstrated that a reduction in goblet cell numbers leads to worsened disease pathology and reduced protection against chronic inflammation-induced colon cancer. A reduction in the number of mucus-producing goblet cells may also be altered in the $Apc^{Min/+}$ model and further worsen the disease state once they are administered antibiotics.

The third aim of the present study was aimed at demonstrating the effects of an altered microbiome on the inflammatory status of the experimental animals. Inflammation is an important route through which alterations in gut microbiota affect the overall milieu of inflammatory processes closely associated with the gut. The inflammatory response, along with being systemic, has also been demonstrated to occur at a more localized level
such as the alterations in gut microbiota leading to increased levels of pro-inflammatory cytokines such as IL-1β and TNF-α (Singh, Yeoh, Carvalho, Gewirtz, & Vijay-Kumar, 2015). In the first part of aim 3 was to study the effects of a changed microbiome on tissue-localized inflammation and infiltration occurring at the level of intestinal mucosa. The second part of the aim helped study the effects of microbiome manipulation on intestine-secreted pro-inflammatory cytokines. Whether the inflammatory effect is systemic or not, was elucidated through the third part of this aim where systemic levels of pro-inflammatory cytokines were measured.
CHAPTER 2

LITERATURE REVIEW
2.1 The $Apc^{Min/+}$ mouse model

The $Apc^{Min/+}$ mouse model is widely used by researchers to study gut inflammation and cancer. The $Apc^{Min/+}$ mouse model is raised from the C57BL/6 background where a point mutation on codon 850 of the $Apc$ (Adenomatous Polyposis Coli) gene leads to spontaneous development of polyps (adenomas) in the intestinal mucosa (Zhang, et al., 2015). The mutation is responsible for the production of a truncated APC protein (2843 amino acids) that lacks its C-terminal domains. The protein functions to downregulate the Wnt signaling pathway by binding to and promoting the destruction of the $\beta$-catenin protein. An altered APC protein thus leads to, apart from the development of intestinal polyps, dysregulation of processes like cell adhesion, cell migration, chromosome segregation and stability (McCart, Vickaryous, & Silver, 2008).

The $Apc^{Min/+}$ mouse model represents the human cancer syndrome called the Familial Adenomatous Polyposis (FAP) where the human intestine may contain several thousand adenomas by the age of 20-30 years, which corresponds to ~4-6 weeks in mouse. The $Apc^{Min/+}$ mouse exhibits more than 50 tumors along the entire length of the intestine and rarely live past the age of 21-22 weeks (Shoemaker, Gould, Luongo, Moser, & Dove, 1997), which corresponds to ~60 years of age in humans. Since all intestinal tumors in B6 Min/+ mice are benign adenomas, the premature death of these animals is associated with secondary effects of tumor growth, including severe, chronic anemia and intestinal blockage (Shoemaker, Moser, & Dove, 1995).
2.2 The Gut Microbiome

The gut microbiome is an integral and an important symbiotic system present in the gut, which comprises of bacteria, archaea, fungi and viruses residing in the gastrointestinal tract throughout its length. The gut bacteria itself comprises a major percentage of the total gut microbiome, where its numbers are in trillions. While the stomach and duodenum contain about $10^1$ to $10^2$ Colony Forming Units (CFU) per mL of bacterial forms, the jejunum and ileum comprise $10^4$ to $10^8$, and the colon has $10^{10}$ to $10^{12}$ CFU/mL of bacteria (Cresci & Bawden, 2015). The microbiome acts advantageous for the gut such that it regulates gut epithelial and endocrine cellular structure (Uribe, Alam, Johansson, Midtvedt, & Theodorsson, 1994). The commensal bacterium *B. thetaiotaomicron* VPI-5482, for example, which is a member of the gut flora, has been linked to the functional processes of the gut such as nutrition absorption, mucosal barrier function, metabolism, angiogenesis and postnatal intestinal maturation (Hooper & Gordon, 2001; Hooper et al., 2001).

Previously, many studies have demonstrated an alteration in gut microbiota to be linked to a variety of conditions including diabetes, arthritis, colitis and cancer and even immune system. Conditions such as Crohn’s disease and obesity have implicated gut bacterial dysbiosis in pathogenesis (Chan, et al., 2015; Mai, et al., 2007). The highest bacterial load is found in the distal small intestinal tract and colon - areas commonly found to be associated with disease conditions (Hooper, Midtvedt, & Gordon, 2002; Kanauchi, Mitsuyama, Araki, & Andoh, 2003). It was not clear if the bacterial dysbiosis is a cause or consequence of the disease until recently when it was shown that alterations in gut microbiome precede polyposis in the *ApcMin/+* mouse (Son, et al., 2015). An altered gut microbiome can cause inflammation ranging from acute bouts to chronically inflamed gut.
An altered microbiome, which shifts from symbiotic to a more inflammation-inducing phenotype, leads to a vicious cycle of erosion of the gut epithelial lining that, in turn, leads to more and more inflammatory processes taking place inside the gut microenvironment (Saxena, et al., 2012; Zhang, et al., 2015). A need to address and characterize host-microbe relations has been emphasized in recent years in order to elucidate the processes and factors involved in the relationship between the gut microbiome and inflammation and related tumorigenesis.

The intestinal epithelial lining has a strong relationship to the gut bacteria. The bacterial flora of the intestine helps in digesting the food (especially carbohydrates) that cannot be digested by the mammalian gut, and in turn, the intestinal epithelial cells metabolize the short-chain fatty acids that result from the bacterial fermentation of the undigested carbohydrates and use them as an energy source (Abreu, 2010).

2.3 Antibiotic usage, gut microbiome and inflammation

Antibiotic treatments that are usually used against digestive tract infections not only target the pathogenic microbes but also the host-interactive useful microbes. It is already known that the use of antibiotics, especially broad spectrum, not only help get rid of pathogenic bacteria but also deplete related micro-organisms, which fail to return to normal levels even long after the antibiotic usage subsides (Cresci & Bawden, 2015). Microbial diversity also is seen to be reduced in the gut following antibiotic treatment which exerts detrimental effects (Lofmark, Jernberg, Jansson, & Edlund, 2006).
Antibiotic usage can severely and permanently affect 30% of the microbial population (Dethlefsen & Relman, 2011). Once the antibiotic treatment is stopped, and some resilient bacteria repopulate the gut, the final state is never a complete replacement of the initial state (Francino, 2015). Any early antibiotic treatment in humans reduces the overall diversity of the microbiota populations inside the gut which leads a dysbalance of the inflammatory molecules in the body. The dysbalance between the Th1 and Th2 paradigm was at first thought to be the main culprit in bringing out the inflammatory effects of the microbiota dysbalance. An increased Th1 activation was linked to harmful inflammatory changes (Akdis & Akdis, 2009; Oboki, Ohno, Saito, & Nakae, 2008). However recently, alterations in the gut microbiota have been linked to a dysregulation of the regulatory T cell paradigm and their associated cytokines (Shen, Hu, Kang, Tang, & Hong, 2014), where the regulation of this immunological pathway depends on the relationship between the gut microbiota and the immune system. Different cocktails of antibiotics administered to mice have led to an upregulation of either the Th2 or a Th17 response (Atarashi & Honda, 2011; Dimmitt et al., 2010), indicating that an alteration in the gut microbiota may favor one pathway and downregulate another inflammatory pathway. How the microbiota changes in the first place and then a subsequent change occurs in the inflammatory pathways during spontaneous tumorigenesis is a question that the present study aimed at answering via studying different stages of tumorigenesis.

Inflammation is an important route through which alterations in gut microbiota affect the overall milieu of inflammatory processes closely associated with the gut. Gut microbiota manipulation through antibiotic treatment has been shown to cause a decrease in the expression of the Muc2 protein, a major component of the intestinal mucus layer
(Dimmitt, et al., 2010). A thinning of the protective mucus layer leads to a direct contact between the gut lining and the gut bacteria residing in the lumen, thereby triggering innate immune responses and inflammation (Francino, 2015). The inflammatory response, along with being systemic, also occurs at a more localized level such as the alterations in gut microbiota leading to increased levels of pro-inflammatory cytokines such as IL-1β and TNF-α (Vijay-Kumar, Carvalho, Aitken, Fifadara, & Gewirtz, 2010).

For the completion of aims 2 and 3, antibiotics were administered to the treatment group of mice in three sets: 1). One cycle of antibiotic administration starting at 6 weeks of age for 10 days and sacrifice at 8 weeks; 2). Two cycles of antibiotic administration starting at 6 weeks and 10 weeks and sacrifice at 12 weeks; and 3). Three cycles of antibiotic administration starting at 6, 10 and 14 weeks of age and sacrifice at 16 weeks. Antibiotic administration at 6 weeks of age signified the manipulation of gut microbiome at the onset of tumorigenesis. This stage of tumorigenesis is marked by a gradual increase in the number of polyps in the small intestine (Puppa et al., 2011). Antibiotic administration at 6 and 10 weeks of age helped define effects of microbiome altered at the beginning of tumorigenesis (6 weeks) and then at the middle of the life span of experimental mice which is marked by progression of tumor development resulting in more pronounced symptoms of anemia and intestinal blockage. While the polyp number reaches a plateau, they only increase in size during this stage and the animals continue to lose body weight (Puppa, et al., 2011). Antibiotic treatment at 6, 10 and 14 weeks represented the alterations in microbiome throughout the processes initiation of tumorigenesis. This stage is usually marked by severe inflammation, continuation of tumor size and maximum weight loss (Puppa, et al., 2011).
2.4 Impact of antibiotics

Although antibiotics are used against pathogenic microbes, their usage affects other microbial members of the community as well. The effects that are established as a result of an altered microbiome can be long-lasting even after the antibiotic treatment has subsided (Jakobsson, et al., 2015; Jernberg, Lofmark, Edlund, & Jansson, 2007). While the more resilient bacteria are successful at returning to their pre-treatment levels, others are lost indefinitely (Willing, Russell, & Finlay, 2011). Moreover, the occurrence of co-dependence between members of the community based on differential metabolite production and utilization pathways leads to perturbation of members that are not necessarily directly targeted by the antibiotic.

The direct and indirect effects of the antibiotics are brought about at different levels and ways. The indirect effects are brought about by the changed gut microbiota. A change in the bacterial population is known to cause a change in the downstream signaling by the Pattern Recognition Receptors (PPRs) which help maintain epithelial integrity and repair process, while change in some bacterial populations that bind to Toll-like receptors, such as TLR4, are linked to an altered innate immune defenses (Willing, et al., 2011). A reduced production of short-chain fatty acids (SCFAs) is also a common feature of human metabolic profiles after antibiotic treatment (Romick-Rosendale et al., 2009; Yap et al., 2008). Changes in bacterial populations also affects differentiation of certain immune system molecules such as IL-17 and often lead to a dysregulation of the immune system (Ivanov et al., 2009). More direct effects include undesirable inflammation of the gut such as gastroenteritis (Barthel et al., 2003) and even dermatitis (J. Watanabe, Fujiwara, Sasajima, Ito, & Sonoyama, 2010).
The antibiotics used for the present study are neomycin, vancomycin and ampicillin. The antibiotic neomycin is used against both Gram-positive and Gram-negative bacteria. Being nephrotoxic, it is almost always prescribed to be consumed through the oral route. Its primary method of action is binding the duplex of RNA of bacteria (Kaul & Pilch, 2002). The antibiotic is water-soluble and has a low toxicity for animals (Waksman & Lechevalier, 1949). Vancomycin is an antibiotic which is used against Gram-positive bacteria (Gonzalez, Rubio, Romero-Vivas, Gonzalez, & Picazo, 1999; Small & Chambers, 1990). It is not absorbed by the intestinal mucosa (Van Bambek, 2006) and its acts by inhibiting the cell wall formation of the Gram-positive bacteria by binding to the terminal D-alanyl-D-alanine moieties of the N-acetyl glucosamine/N-acetyl muramic acid peptide (Chakraborty et al., 2010). The antibiotic ampicillin is active against both Gram-positive and Gram-negative bacteria and is usually no-toxic. It mechanism of action includes its penetration into the bacterial cell wall and acting as an irreversible inhibitor of the enzyme transpeptidase involved in the construction of the bacterial cell walls ultimately leading to bacterial cell lysis.

2.5 Intestinal mucus layer

The gut lining is folded into tubular invaginations called the villi in the intestine and crypts (of Leiberkuhn) in the colon. The gut lining is protected from the lumen microenvironment by a single-cell layer comprising of absorptive enterocytes (which make most of the epithelial cells), mucus-producing goblet cells, hormone-producing enteroendocrine cells and Paneth cells that produce anti-microbial products in the gut (Abreu, 2010). The four
types of the cells line the single-celled layer of the gut that faces the lumen. All the four types of cell arise from the pluripotent stem cells that lie at the base of the crypts. The differentiated cells that arise from the stem cells of the crypts slowly and continuously rise towards the crypt apex from where the single-celled layer is regularly shed and renewed to maintain a healthy gut, also providing the crypt with a feature of polarity.

The mucus in the small and large intestine is secreted by the goblet cells. It forms the first line of defense between the gut lumen microenvironment and the underlying epithelial layer and the submucosa. The mucin proteins in the gut mainly comprise of the MUC2 and MUC5AC proteins. They have properties of forming gel like coating onto the gut lumen once getting hydrated and form highly glycosylated proteins that are resistant to the digestive environment of the gut (Koboziev, Reinoso Webb, Furr, & Grisham, 2014). Changes in goblet cell function, secretion of mucins into the lumen and composition of this mucus layer are shown to be altered by an altered microbiome (Deplancke & Gaskins, 2001). The mucus layer provides a balanced ecosystem for the resident and as well as pathogenic bacteria in the intestinal lumen by acting as a source of nutrition, thus indicating a homeostatic balance between the host gut microenvironment and the microbiome associated with it (Aristoteli & Willcox, 2003). An alteration in one of them may render the other dysfunctional. It is also said that the resident bacteria may inhibit the adherence of pathogenic bacteria to the intestinal epithelial cells by increasing the production of intestinal mucus (Mack, Ahrne, Hyde, Wei, & Hollingsworth, 2003; Mack, Michail, Wei, McDougall, & Hollingsworth, 1999; Smirnova, Guo, Birchall, & Pearson, 2003).
2.6 Mucus and gut microbiome

It has been shown that the mucus layer tends to thickness with increased diversity of the microbiota (Jakobsson, et al., 2015). The mucus layer provides a source of nutritional carbon and therefore energy to some of the intestinal flora that are able to lyse the glycans present in the mucus, making the inter-relationship between the gut bacteria and the mucus layer really important (Kaur, et al., 2015). Also, the metabolites produced by these microbes also influence the differentiation and function of the epithelial and immune cells in the intestinal mucosa (Kato, et al., 2014; Shan, et al., 2013). Our recent study (Kaur, et al., 2015) also suggested that a reduction in the secretory mucin profile is associated with an inflammatory phenotype of the gut. Inflammation, which is an integral part of tumorigenesis should therefore, may strongly be correlated with a reduced bacterial diversity or numbers. Moreover, both Gram-positive and Gram-negative bacteria are known to enhance the intestinal mucin expression (Dohrman et al., 1998). The regulation of the mucin gene expression as well as mucus production have been shown to be two levels at which the gut microbial community may modulate mucins (Comelli, et al., 2008). Gut microbiota manipulation through antibiotic treatment has been shown to cause a decrease in the expression of the Muc2 protein, a major component of the intestinal mucus layer (Wlodarska et al., 2011). A thinning of the protective mucus layer leads to a direct contact between the gut lining and the gut bacteria residing in the lumen, thereby triggering innate immune responses and inflammation (Francino, 2015).
2.7 Gut microbiome and $Apc^{Min/+}$

Studies that work at elucidating the role of gut microbes in disease models, are limited, especially due to the fact that most of the important gut bacteria are difficult to grow in a laboratory setting making them unavailable for studying unless mammalian models are used. However, there is evidence that the manipulation of the gut microbiome through diet modulations, can alter the disease conditions such as those in Crohn’s disease and Inflammatory Bowel Disease (Mai, et al., 2007). Studies that use the $Apc^{Min/+}$ mouse model for studying gut diseases and their association with microbiota often report significant changes in microbiome profiles of the large intestine, but also report that differences in the polyps is only significant in the small intestine (Mai, et al., 2007) The detrimental effects are also brought about via loss of certain colonizing bacterial species that are dependent on other colonizers for processes such as nutritional interactions and removal of secondary metabolites and toxic waste products (Belenguer et al., 2006), or through loss of useful co-evolved processes brought about by the host-microbial co-dependence (Atarashi & Honda, 2011; Ferreira, Willing, & Finlay, 2011). There are no studies that study the gut microbiome with respect to gut inflammation and tumorigenesis in an age/time-dependent manner. It has been seen that specific pathogen-free (SPF) $Apc^{Min/+}$ exhibit a higher tumor load and anemia with a higher infiltration of inflammatory cells specifically at advanced stages as compared to germ-free animals, indicating that a mere modulation of gut microbiome profiles can abrogate the disease condition in the gut (Y. Li, et al., 2012).
2.8 Gut microbiome, inflammatory cytokines and tumorigenesis

It is a fact that till date, one of the most effective class of chemopreventive agents is the non-steroidal anti-inflammatory drugs (NSAIDs). The induction of cancer in the gut by chronic inflammation has always indicated towards the importance of underlying altered inflammatory processes (Zhang, et al., 2015). Different agents that have been documented to be effective against tumors and cancers are the agents that function by downregulating important inflammatory pathways and pro-inflammatory cytokines. Apart from the usual NSAIDs that function by downregulating the metabolism of the arachidonic acid through the Cyclooxygenase (COX) pathways agents such as curcumin and quercetin exert their chemopreventive effects through the downregulation of mRNA expression levels of pro-inflammatory cytokines such as IL1-β, IL-6 and TNF-α, and reduction of macrophage infiltration (Y. Li, et al., 2012; Murphy, Davis, McClellan, & Carmichael, 2011; Murphy, Davis, McClellan, Gordon, et al., 2011; Ritland, et al., 1999).

It has also been shown that the intestinal commensal bacteria play a role in the development of gut microenvironmental immune system comprising both the humoral and cellular components and maintains the protective steady state immune function throughout life (Cebra, 1999; Talham, et al., 1999). It may be more appropriate to say that any alteration in the composition of the gut microbiome leads to a dysfunctional immune system.

Certain bacterial species such as B. thetaiotaomicron bacterium have been associated with the attenuation of pro-inflammatory responses by reducing the levels of the pro-inflammatory cytokine IL-8 (Zhao et al., 2001).
Comparative numbers of certain bacteria often lead to upregulation or downregulation of certain inflammatory pathways. The systemic release of microbial products as a result of the manipulation of the gut epithelium invokes an IL-23 response and a further IL-17 response in order to neutralize further invasion by the microbes, and the process is brought about by the *Firmicutes* bacteria *Clostridia sp.* (Shih, et al., 2014). The bacteria especially promotes IL-17 cytokine in the small intestine which is the primary site of its attachment and survival (Omenetti & Pizarro, 2015). These Th17 cells may become autoreactive in case of intestinal epithelium insult. The bacteria *Bacteroides fragilis* from the *Bacteroidetes* phylum leads to an increase in the production of IL-10 cytokine (Omenetti & Pizarro, 2015), whereas its strains have been implicated in the production of the pro-inflammatory cytokine IL-17-dependent inflammation-related colon cancer (W. Wu, et al., 2015).

It is widely accepted that inflammation contributes to the development of cancer and inflammatory cells have been noted in and around tumors (Hanahan & Weinberg, 2011). A relationship between inflammation and intestinal neoplasia is supported by the facts that inflammatory bowel disease predisposes patients to intestinal carcinomas and that the anti-inflammatory drugs aspirin (Baron et al., 2003), celecoxib (Bertagnolli et al., 2006), and rofecoxib (Bertagnolli, et al., 2006), all have proven efficacy in preventing human colorectal adenoma development.

Adenoma formation has been shown to increase following adoptive transfer of pro-inflammatory lymphocytes (Rao et al., 2006) and decreased after the adoptive transfer of anti-inflammatory regulatory T-cells (Treg cells) (Erdman et al., 2005). Apart from direct effects of inflammatory cells, adenomas are also known to be impaired by the absence of
the molecule MyD88 which mediates the downstream inflammatory signaling as a response to bacterial and viral products (Rakoff-Nahoum & Medzhitov, 2007). Pro-inflammatory pathways such as those involved with IL-17 secretion are associated with many chronic inflammatory conditions such as asthma (Schnyder-Candrian et al., 2006) and inflammatory bowel disease (Yen et al., 2006), along with colon carcinogenesis (Langowski et al., 2006). IL-17, especially, has been denoted as a marker and mediator of tumor angiogenesis (Langowski, et al., 2006). Over-expression of pro-inflammatory factors such as IL-6, MCP-1, NF-κB and IL-8 can aggravate tumors (Wang & Zhang, 2015).

Mechanisms of how the microbiome dysbiosis can lead to inflammation and tumorigenesis is not clear, however, recent studies have shown that microbiota species such as the *Bacteroides fragilis* and *Escherichia coli* can cause colorectal cancer by producing virulence factors such as toxins and gene products (Arthur et al., 2012; S. Wu, et al., 2009). It has been suggested that the interplay of inflammation and tumorigenesis is modulated strongly by the gut microbiota but the mechanisms leading to the process have not elucidated. Recently, some light has been shed on the phenomena by a study which could demonstrate the process of tumorigenesis to occur in germ-free mice when transplanted with isolated gut microbiota from intestinal tumor-bearing mice (Zackular, et al., 2013).

In the context of the *ApcMin/+* mouse model, the mutual interaction of macrophages with cancer cells enhances production of inflammatory cytokines such as IL-1, IL-6 and TNF-α that transform the tumor microenvironment so that it favors the survival, growth and motility of cancer cells. Reduction in pro-inflammatory molecules such as MCP-1 leads to a decrease in the total polyp number and size in the *ApcMin/+* mouse along with
downregulating inflammatory processes in tumor tissues and surrounding tumor microenvironment (McClellan, Davis, Steiner, Day, et al., 2012). An elevation in the intestinal inflammatory cytokine (MCP-1, IL-1β, IL-6 and TNF-α) response occurs at 12 weeks of age as a result of the rapid increase in polyp number, which are further elevated with increase in polyp size. A similar study by McClellan et al (2012) (McClellan, Davis, Steiner, Enos, et al., 2012) shows an increased mRNA expression of MCP-1, IL-1β, IL-6 and TNF-α that is evident at 12 weeks of age and is consistent with the increase in polyp number that occurs at this time. After the age of 12 weeks which is associated with the largest number of small polyps, the inflammatory response continues to increase while the number of polyps remain constant which indicates that the inflammatory response after 12 weeks could largely be driven by polyp size. However, it is still unclear whether the changes in polyp number and size that occurs in this model is a result of the elevated inflammatory response or vice versa (McClellan, Davis, Steiner, Enos, et al., 2012).

2.9 The Bacteroidetes and Firmicutes

The gut microflora plays a very important role in the immunology, nutrition and pathology of an organism. A limitation in the methodical techniques and inherent biases involved in the carrying out culture-based techniques to study gut microbiome had previously limited our knowledge in better understanding the gut microbiome from the point of view of not only characterizing it as whole but also elucidating the mechanisms it can affect that may lead to various disease outcomes in living systems. New methodologies have now been developed to study the diversity of the gut microbial community, which was formerly
thought to contain merely 400-500 species. The total of 1250 Operational Taxonomic Units representing the gut bacterial species have been identified by the use of the recently developed mapping techniques (Mariat et al., 2009). In the context of humans, 80% of the mapped fecal bacteria contains three major phyla: Bacteroidetes, Firmicutes and Actinobacteria (Lay et al., 2005). The gut microbiome is a complex ecosystem containing a dominant population (>10⁹ CFU/g) of anaerobic bacterial species represented by genera of Bacteroides, Eubacterium, Bifidobacterium, Peptostreptococcus, Ruminococcus, Clostridium and Propionibacterium, and sub-dominant (< 10⁹ CFU/g) population of aerobic bacteria pertaining to the Enterobacteriaceae family, especially E. coli, and the genera Streptococcus, Enterococcus, Lactobacillus, Fusobacterium, Desulfovibrio and Methanobrevibacter (Harmsen, Raangs, He, Degener, & Welling, 2002).

The establishment and diversification of the gut microbiome is a gradual process that is required for proper development and overall health. Once the gut microbiome manifests itself completely, its stays constant during the life of a healthy individual (Franks et al., 1998) which explains the fact that an alteration in one or more of the resident microbial populations lead to pathological conditions.

The Bacteroidetes phylum is comprised of Gram-negative anaerobic and aerobic bacterial species. Some studies link the absence or reduction of, or delayed colonization of the gut by the phylum Bacteroidetes to be associated with a weakened immune system in humans. For example, a delayed postnatal colonization of Bacteroidetes population has
been associated with development of allergies in children born by Caesarian section (S. Watanabe et al., 2003).

The *Firmicutes* represent the phylum mostly containing Gram-positive bacterial species. The phylum contains both aerobic and anaerobic bacterial species.

Many *Firmicutes* are capable of producing endospores which help them survive desiccation and extreme conditions. Their capability to survive extreme conditions may be one reason that these bacteria are found in high numbers in the gut under pathological and inflammatory conditions. Comparisons of gut microbiota of lean and obese mice and lean and obese human individuals revealed that the *Bacteroidetes* significantly decrease while the *Firmicutes* significantly increase with obesity (Guo et al., 2008).

2.10 16S rRNA gene profiling and sequencing

The gut microflora plays a very important role in the immunology, nutrition and pathology of an organism. A limitation in the methodical techniques and inherent biases involved in the carrying out culture-based techniques to study gut microbiome had previously limited our knowledge in better understanding the gut microbiome from the point of view of not only characterizing it as whole but also elucidating the mechanisms it can affect that may lead to various disease outcomes in living systems. New methodologies have now been developed to study the diversity of the gut microbial community, which was formerly thought to contain merely 400-500 species. The total of 1250 Operational Taxonomic Units representing the gut bacterial species have been identified by the use of the recently developed mapping techniques (Mariat, et al., 2009). In the context of humans, 80% of the
mapped fecal bacteria contains three major phyla: Bacteroidetes, Firmicutes and Actinobacteria (Lay, et al., 2005). One of the most widely used of these methods is the 16S rRNA method. The 16S rRNA gene method is one of the earliest and most widely used methods used for phylogenetic, taxonomic and bioinformatics analyses. It targets the 16S rRNA bacterial genes to know diversity or similarities in biological samples. The 16S rRNA gene provides many advantages in microbiome analyses including its universal distribution in bacterial species, relative stability in evolution and ideal size (1500 bp). Consisting of both constant and variable regions, the 16S gene is easy to amplify used broad-range primers used against regions flanking its variable regions (Sankar, Lagier, Pontarotti, Raoult, & Fournier, 2015).

2.11 Statistical Power of the study

The microbiome analysis in our study was done using the fecal samples from experimental mice. A 16S rRNA gene sequencing technique was used to study the gut microbial populations in the animals. The sample size for the study is 4. Some of the previous studies that have been directed towards similar microbial analyses have used similar samples sizes. Russell et al (2012) (Russell et al., 2012) used n = 3-5 to study changes in the gut microbial communities in C57BL/6 mice following antibiotic treatment at neonatal and adult stages of life. The results showed significant reductions in the overall bacterial diversity and phyla abundance in antibiotic-treated mice as compared to control mice (p value<0.05). Oh et al (2016) (Oh et al., 2016) used a sample size of 5 mice per group in order to show significant differences in bacterial load and diminished rDNA in antibiotic-treated mouse fecal
samples as compared to their control counterparts. Similar studies have used n = 4-6 mice per group in order to show Significant (p value<0.01) changes in gut microbial constituents as a result of administration of the antibiotic enrofloxacin with an increase in type-2 cytokines has been shown in a study that used 4 to 6 (n) mice per group (Strzepa et al., 2016).
CHAPTER 3

GUT MICROBIOME PROFILE DURING INITIATION AND PROGRESSION OF TUMORIGENESIS IN $APC^{Min/+}$ MOUSE$^{1}$

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3.1 Abstract

An altered gut microbiome can cause inflammation ranging from acute bouts to chronically inflamed gut. An altered microbiome, which shifts from symbiotic to a more inflammation-inducing phenotype, leads to a vicious cycle of erosion of the gut epithelial lining that, in turn, leads to more and more inflammatory processes taking place inside the gut microenvironment. However, there are no studies that study the gut microbiome with respect to gut inflammation and tumorigenesis in an age/time-dependent manner. Four weeks old male $Apc^{Min/+}$ and C57BL/6 (WT) mice were obtained from the Jackson Laboratories and bred and housed at the Animal Resource Facility at University of South Carolina. Food (Purina chow) and drinking water was available to the mice ad libidum under a 12:12 hour light-dark cycle and a low-stress environment (22°C, 50% humidity and low noise). All animal care followed institutional guidelines under a protocol approved by the Institutional Animal Care and Use Committee at the University of South Carolina. Mice were monitored throughout the course of study for weight loss, diarrhea and blood in stools as a basis for calculating clinical scores (Saxena, et al., 2012). Mice were sacrificed at 8, 12 and 16 weeks. Bacterial 16S rRNA gene amplification and sequencing were performed using Polymerase Chain Reaction (PCR) amplification of the V4 region of the bacterial 16S rRNA gene. The clinical scores increased with tumor progression in the $Apc^{Min/+}$ mice and were significant as compared to the WT mice for which the score remained 0 for the course of the study. The Bacteroidetes and Firmicutes were found to be the two dominant phyla in the WT and $Apc^{Min/+}$ gut which remained so at all three experimental time points of the study. However, their overall percentages change with antibiotic treatment where Firmicutes phyla dominated with the treatment. It could be
concluded from the study that the *Firmicutes* phyla of bacteria may play a role during the initiation of tumorigenesis.

Keywords: Microbiome, Inflammation, *Bacteroidetes, Firmicutes*

### 3.2 Introduction

The gut microbiome is an integral and an important symbiotic system present in the gut, which comprises of bacteria, archaea, fungi and viruses residing in the gastrointestinal tract throughout its length. The gut bacteria itself comprises a major percentage of the total gut microbiome, where its numbers are in trillions. While the stomach and duodenum contain about $10^1$ to $10^2$ Colony Forming Units (CFU) per mL of bacterial forms, the jejunum and ileum comprise $10^4$ to $10^8$, and the colon has $10^{10}$ to $10^{12}$ CFU/mL of bacteria (Cresci & Bawden, 2015). The microbiome acts advantageous for the gut such that it regulates gut epithelial and endocrine cellular structure (Uribe, et al., 1994). Previously, many studies have demonstrated an alteration in gut microbiota to be linked to a variety of conditions including diabetes, arthritis, colitis and cancer and even immune system. Conditions such as Crohn’s disease and obesity have implicated gut bacterial dysbiosis in pathogenesis (Chan, et al., 2015; Mai, et al., 2007). It was not clear if the bacterial dysbiosis is a cause or consequence of the disease until recently when it was shown that alterations in gut microbiome precede polyposis in the *Ape* Min/+ mouse (Son, et al., 2015). An altered gut microbiome can cause inflammation ranging from acute bouts to chronically inflamed gut. An altered microbiome, which shifts from symbiotic to a more inflammation-inducing phenotype, leads to a vicious cycle of erosion of the gut epithelial lining that, in turn, leads
to more and more inflammatory processes taking place inside the gut microenvironment (Saxena, et al., 2012; Zhang, et al., 2015). A need to address and characterize host-microbe relations has been emphasized in recent years in order to elucidate the processes and factors involved in the relationship between the gut microbiome and inflammation and related tumorigenesis. The intestinal epithelial lining has a strong relationship to the gut bacteria. The bacterial flora of the intestine helps in digesting the food (especially carbohydrates) that cannot be digested by the mammalian gut, and in turn, the intestinal epithelial cells metabolize the short-chain fatty acids that result from the bacterial fermentation of the undigested carbohydrates and use them as an energy source (Abreu, 2010). There are no studies that study the gut microbiome with respect to gut inflammation and tumorigenesis in an age/time-dependent manner. How the microbiota changes in the first place and then a subsequent change occurs in the inflammatory pathways during spontaneous tumorigenesis is a question that the present study aimed at answering via studying different stages of tumorigenesis.

3.3 Methods

Experimental animals and groups

Four weeks old male $Apc^{Min/+}$ and C57BL/6 (WT) mice were obtained from the Jackson Laboratories and bred and housed at the Animal Resource Facility at University of South Carolina. Food (Purina chow) and assigned to each of the following six groups with four mice each (n=4): WT- 8, 12 and 16 weeks; and $Apc^{Min/+}$- 8, 12 and 16 weeks. Food (Purina chow) and drinking water was available to the mice ad libidum under a 12:12 hour light-
dark cycle and a low-stress environment (22°C, 50% humidity and low noise). All animal care followed institutional guidelines under a protocol approved by the Institutional Animal Care and Use Committee at the University of South Carolina.

**Clinical score**

Mice were monitored throughout the course of study for weight loss, diarrhea and blood in stools. Clinical score was measured for each mouse twice during each week of the study based on our earlier published criteria (Saxena et al., 2012). Mice were sacrificed after the last clinical score measurement. Cumulative clinical scores for each mouse equaled a maximum score of 12, which was based on weight loss measurement, diarrhea and fecal hemoccult (kit, BECKMAN COULTER) with a maximum score of 4 within each of the three quantitative parameters. Score for the weight loss was based on the following published scale where 0 = 0–5% weight loss; 1 = 6–10% weight loss; 2 = 11–15% weight loss; 3 = 16–20% weight loss; and 4 = >20% weight loss. Scoring of diarrhea was as follows: 0 = well-formed pellets, 2 = pasty and semi-formed stools that do not adhere to the anus, 4 = liquid stools that adhere to the anus. Detection of blood in the stools was determined by using hemoccult kit (BECKMAN COULTER), which is a hydrogen peroxide-based kit that forms a visible blue colored complex with blood. The followings were the score rates for the fecal hemoccult: 0 = no blood, 2 = positive hemoccult, 4 = gross bleeding. The total clinical score was the summation of the individual score of weight loss, diarrhea and fecal hemoccult. The maximum score a mouse could get was 12.
Collection of tissues

Mice (n=4 for each age group and genotype) were sacrificed at 8, 12 and 16 weeks by cervical dislocation. Blood was collected before sacrifice through retro-orbital puncture, centrifuged at 10,000 rpm for 15 minutes and serum was isolated and stored at -20°C. Small intestine and colon were excised and flushed clean with PBS. 2 mm² colon tissue sections were fixed in 10% formalin and were replaced with 70% ethanol after 24 hours, followed by paraffin embedding and sectioning to obtain 5 µm thin sections on glass slides. 2 mm² intestine and colon tissue sections were incubated in RPMI medium at 37°C for 24 hours followed by centrifugation at 2500 rpm for 15 minutes. Supernatant was obtained and stored at -20°C for tissue-secreted cytokine expression analyses. The rest of the tissues were stored at -80°C for further usage. Fecal samples were snap frozen at the time of sacrifice for microbiome analysis.

Microbiome analysis

At sacrifice, fecal samples were obtained from the experimental mice to perform bacterial (luminal) microbiome analysis (n=3). Bacterial n16S rRNA gene amplification and sequencing were performed at the Alkek Center for Metagenomics and Microbiome Research, Department of Molecular Virology and Microbiology, Baylor College of Medicine (Houston, TX). Polymerase Chain Reaction (PCR) amplification of the V4 region of the bacterial 16 S rRNA gene was performed using the Illumnia Miseq sequencer. The consensus sequences obtained from the analysis were mapped into Operational Taxonomic Units (OTUs). Alpha- and beta-diversity values were obtained based on the
OTUs’ relative abundance table. Chao1 (estimator of richness) and Shannon Diversity Index (richness and evenness) were used to obtain the alpha diversity values. Weighted Unifrac (dissimilarity based on phylogenetic differences and taxonomic abundance) and Unweighted Unifrac (dissimilarity based on phylogenetic differences but not abundance) were performed to obtain the beta-diversity values for the experimental samples (He et al., 2016). Kruskal Wallis and Mann Whitney statistical analyses were performed to calculate significance in diversity and relative abundance respectively, by comparing different treatments and disease stages. A p value of less than 0.05 was considered significant.

**Statistical analysis**

Two-way analysis of variance (ANOVA), Two-way repeated measure ANOVA and One-way ANOVA were used to analyze the data with Tukey post hoc-analyses to estimate the significance of differences obtained between different experimental treatment conditions and between the experimental mouse strains used in the study. A p<0.05 were considered statistically significant. All the statistical analyses were done using SigmaStat 3.5 (SPSS, Chicago, IL). The sample size (n) was kept as 4 for the study.

### 3.4 Results

**Clinical score increases with tumor progression in Apc\textsuperscript{Min/+} mice**

Clinical score which is a culmination of diarrhea, weight loss and fecal hemoccult was plotted against the age of mice (n=4) at 8, 12 and 16 weeks (figure 3.1). Clinical score was found to increase with age of Apc\textsuperscript{Min/+} mice. Apc\textsuperscript{Min/+} mice at 6, 8, 10, 12, 14, and 16 showed
a significantly higher clinical score when compared to $Apc^{Min/+}$ mice at 5 weeks (*p<0.03, dotted line) (figure 3.1 A, B and C). $Apc^{Min/+}$ mice of 8, 10, 12, 14 and 16 weeks of age showed a significantly higher clinical score when compared to $Apc^{Min/+}$ mice at 6 weeks of age (*p<0.05). $Apc^{Min/+}$ mice of 10, 12, 14 and 16 weeks of age showed a significantly higher clinical score when compared to $Apc^{Min/+}$ mice at 8 weeks of age (**p<0.01). No significant difference was found between the clinical score of $Apc^{Min/+}$ mice at age 10, 12, 14 and 16 weeks (figure 3.1 A, B and C).

**Microbiome profile during tumor initiation**

The bacterial microbiome profiles of the experimental animals were studied without antibiotic treatment to establish the control profiles of the gut microbial community. The *Bacteroidetes* and *Firmicutes* were the two main phyla among others that were profiled through the 16S rRNA sequencing. The bacterial population including proteobacteria, firmicutes and bacteroidetes was found to fluctuate with age and genotype. The percentage of Proteobacteria was found to be significantly higher in $Apc^{Min/+}$ mice at 8 weeks (16.9%) when compared to WT mice (3.7%) and $Apc^{Min/+}$ mice at 12 (3.8%) and 16 weeks (5.1%). No significant difference was observed in the proteobacterial population between the $Apc^{Min/+}$ mice and WT mice at 12 and 16 weeks (figure 3.2 A). Percentage of firmicutes bacteria in the feces of the $Apc^{Min/+}$ mice at 8 weeks (48.8%) was significantly higher than at 12 weeks (33.2%) and 16 weeks (21.2%). Percentage of firmicutes bacteria was found to be significantly higher at 8, 12 and 16 weeks when compared to their WT counterparts (12.0%). Percentage of firmicutes population in $Apc^{Min/+}$ mice at 16 weeks (21.2%) was found to be significantly lower than the $Apc^{Min/+}$ mice at 12 weeks (33.2%) (figure 3.2 B).
Bacteroidetes showed an opposite trend in bacterial population when compared with firmicutes and proteobacteria. Percentage of bacteroidetes in Apc\textsuperscript{Min/+} mice at 8 weeks (30.6%) was found be significantly lower than both WT (83.6%) and Apc\textsuperscript{Min/+} mice at 12 (61.8%) and 16 weeks (71.8%) of age. Apc\textsuperscript{Min/+} mice at 12 weeks of age showed a significantly lower bacteroidetes population when compared to WT mice. No significant difference was observed in the bacteroidetes population of Apc\textsuperscript{Min/+} mice at 16 weeks and WT mice (figure 3.2 C).

3.5 Discussion

An alteration in gut microbiome has been previously demonstrated to be linked to a variety of conditions including diabetes, arthritis, colitis and cancer and even immune system. Conditions such as Crohn’s disease and obesity have implicated gut bacterial dysbiosis in pathogenesis (Chan, et al., 2015; Mai, et al., 2007). It was not clear if the bacterial dysbiosis is a cause or consequence of the disease until recently when it was shown that alterations in gut microbiome precede polyposis in the Apc\textsuperscript{Min/+} mouse (Son, et al., 2015). Our present study aimed at characterizing the bacterial populations that can be regarded as significant in the process of tumorigenesis. The \textit{Proteobacteria} and the \textit{Firmicutes} phylum were significantly higher in Apc\textsuperscript{Min/+} mouse during initial stages of tumorigenesis as compared to WT mice indicating that the phyla can be a precursor to and important during the initial stages of tumorigenesis. The study by Son et al (Son, et al., 2015) demonstrated a higher \textit{Bacteroidetes} population in this regard. A reason that can be cited for this discrepancy in observations is that the mice used were at initial stages of tumorigenesis and it can well be
possible that some of the $Apc^{Min/+}$ may not have even developed polyps at the age of euthanization (8 weeks). The observations however, support the higher percentages of the Bacteroidetes phylum that was observed in the WT mice at the age of 8 weeks in our study. Moreover, the observation that the $Apc^{Min/+}$ mice showing a clinical score at 5 weeks which continued to increase till 8 weeks, while the WT mice exhibited no clinical signs of disease, makes Firmicutes the likely bacterial phylum that can set the stage for tumorigenesis rather than the Bacteroidetes that are abundant in the WT mice at this age but do not lead to any clinical score.

The percentage of Proteobacteria and the Firmicutes phylum decreased significantly at 12 and 16 weeks. Percentage of Proteobacteria phylum in $Apc^{Min/+}$ mouse becomes almost similar to the WT mice. However, the percentage of Firmicutes showed a similar pattern but remains significantly higher than WT mice. Both of these phylum showed a decrease with increase in tumorigenesis. The restoration of these phylum levels to almost those of the WT mice may be seen as a defense mechanism of the digestive tract under the absence of any external manipulation of the gut microbiome. Concomitantly, we also observed an increase in the percentage of Bacteroidetes with age further strengthening the idea that higher percentage of Proteobacteria and Firmicutes and lower percentage of Bacteroidetes could set a stage for tumorigenesis and then body defense mechanism tries to restore the gut microbiome (figure 3.2). However, clinical scores for the tumor-bearing mice continued to increase until 16 weeks (figure 3.1), an indication that levels of Proteobacteria, Firmicutes and Bacteroidetes may not correlate with disease prognosis once the process of tumorigenesis starts. This finding is also corroborated by the observation of the species richness values that were not significantly different between
experimental time points within the two animal groups. However, the overall diversity between the Apc\textsuperscript{Min/+} and WT mice was significant whether species abundance is taken into account or not.

3.6 Conclusion

In conclusion, it can be stated that reduced abundance of the phylum Firmicutes can be cited as one of the factors that set the conditions for initiation of tumorigenesis in the mouse gut although the two phyla Firmicutes and Bacteroidetes do not bear any significant correlation to the clinical scores for the tumor-bearing mice during the progression of tumorigenesis. With an absence of any external manipulation of the gut microbiome, the species present in the normal mice may not be similar to that present in tumor-bearing mice at 8, 12 and 16 weeks of age. However, phylogenetically, the species members residing in a tumor-bearing gut may significantly be different and diverse from those residing in a normal gut. A bigger samples size may help elucidate interesting links between the major gut microbial members and tumorigenesis with respect to phylogenetic and diversity analyses.

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technical support and experimental animals for the study. We would also like to acknowledge Center of Biomedical Research Excellence (COBRE), NIH Center for Colon Cancer Research, USC for funding the study. The study sponsor has no personal and financial interest and involvement in any section of the manuscript.

3.7 Figure Legends

**Figure 3.1. Clinical score (A–C)** Clinical scores for \( Apc^{Min/+} \) plotted for different time points during the study (n=4). The scores were calculated out of twelve points; four points for each parameter: weight loss, diarrhea and hemoccult. Two-way repeated measure of analysis of variance (ANOVA) was used to calculate the significant difference between the clinical score for \( Apc^{Min/+} \) mice between different time points throughout the length of the experiment. \#p<0.03 \( Apc^{Min/+} \) vs \( Apc^{Min/+} \) 5 weeks, *p<0.05 vs \( Apc^{Min/+} \) at 6 weeks and **p<0.01 vs \( Apc^{Min/+} \) at 8 weeks.

**Figure 3.2. Intestinal microbiome phylum profiles.** Relative percentages of the major phyla obtained from fecal 16S rRNA (V4 region) sequencing analysis from experimental mice (n=4): (A) Proteobacteria, (B) Firmicutes and (C) Bacteroidetes (C). (Percentage values represent fractions of depicted bacteria out of the total microbial pool). Two-way analysis of variance (ANOVA) was used to calculate the significant difference between the percentage of bacteria in \( Apc^{Min/+} \) control and WT control mice at age 8, 12 and 16 weeks. *p<0.05 vs Apc-min 8 weeks, **p<0.05 Apc-min (12 weeks vs 16 weeks) and #p<0.01 Apc-min vs WT.
Figure 3.1. Clinical score (A–C) Clinical scores for $Apc^{Min/+}$ plotted for different time points during the study (n=4). The scores were calculated out of twelve points; four points for each parameter: weight loss, diarrhea and hemoccult. Two-way repeated measure of analysis of variance (ANOVA) was used to calculate the significant difference between the clinical score for $Apc^{Min/+}$ mice between different time points throughout the length of the experiment. $^\#p<0.03$ $Apc^{Min/+}$ vs $Apc^{Min/+}$ 5 weeks, $^*p<0.05$ vs $Apc^{Min/+}$ at 6 weeks and $^{**}p<0.01$ vs $Apc^{Min/+}$ at 8 weeks.
Figure 3.2. Intestinal microbiome phylum profiles. Relative percentages of the major phyla obtained from fecal 16S rRNA (V4 region) sequencing analysis from experimental mice (n=4): (A) Proteobacteria, (B) Firmicutes and (C) Bacteroidetes. (Percentage values represent fractions of depicted bacteria out of the total microbial pool). Two-way analysis of variance (ANOVA) was used to calculate the significant difference between the percentage of bacteria in Apc\textsuperscript{Min/+} control and WT control mice at age 8, 12 and 16 weeks. *p<0.05 vs Apc-min 8 weeks, **p<0.05 Apc-min (12 weeks vs 16 weeks) and #p<0.01 Apc-min vs WT.
CHAPTER 4

EFFECT OF GUT MICROBIOME MANIPULATION ON GUT HEALTH AND TUMORIGENESIS IN $APC^{Min/+}$ MOUSE$^2$

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4.1 Abstract

The mucus layer acts as a site of residence for the gut microbiota. The metabolites produced by the microbes influence the differentiation and function of the epithelial and immune cells in the intestinal mucosa. The gut microbiota can regulate mucin gene expression and mucus production. Our recent study suggested a reduction in the secretory mucin profile to be associated with an inflammatory phenotype of the gut. 4 weeks old \( Apc^{Min/+} \) and C57BL/6 (WT) mice were housed at the Animal Resource Facility, University of South Carolina with access to food and water \textit{ad libidum}. The \( Apc^{Min/+} \) and C56BL/6 mice were assigned to the following groups: WT-Control (no antibiotic)-8 weeks, WT-Antibiotic (ABT)-8 weeks, WT-Control-12 weeks, WT-ABT-12 weeks, WT-Control-16 weeks, WT-ABT-16 weeks, \( Apc^{Min/+}\)-Control-8 weeks, \( Apc^{Min/+}\)-ABT-8 weeks, \( Apc^{Min/+}\)-Control-12 weeks, \( Apc^{Min/+}\)-ABT-12 weeks, \( Apc^{Min/+}\)-Control-16 weeks and \( Apc^{Min/+}\)-ABT-16 weeks. An antibiotic mixture containing Vancomycin, Neomycin and Ampicillin (each 1 mg/mL) was administered to experimental mice through drinking water starting at the 5\textsuperscript{th}, 10\textsuperscript{th} and 14\textsuperscript{th} weeks of age for 10 days followed by normal drinking water. Mice were monitored twice during each week of the study for weight loss, diarrhea and blood in stools to serve as basis for calculating clinical scores. Small intestine and colon from the animals was excised, flushed with PBS and tumor number and area were counted using 1\% Methyl Blue stain. Small intestinal samples were stained with Alcian Blue dye solution for quantifying mucus-containing goblet cells. The clinical scores for the animals increased with antibiotic treatment except for WT-C mice for which the scores were 0 throughout the study. The highest clinical scores were exhibited with tumorigenesis as well as microbiome manipulation. Polyp counts for the \( Apc^{Min/+} \) mice increased with antibiotic treatment. A
significantly higher percentage of the *Firmicutes* phylum was observed with microbiome manipulation. The goblet to epithelial cell ratio was significantly reduced in both $Apc^{Min/+}$ and WT mice with antibiotic treatment during all three experimental time points. Our results suggest that *Firmicutes* within the gut microbiome plays a regulatory role in colonic epithelial to goblet cell differentiation and tumorigenesis.

Keywords: Microbiome, polyps, tumor, *Firmicutes, Apc^{Min/+}*

4.2 Introduction

An altered gut microbiome can cause inflammation ranging from acute bouts to chronically inflamed gut. An altered microbiome, which shifts from symbiotic to a more inflammation-inducing phenotype, leads to a vicious cycle of erosion of the gut epithelial lining that, in turn, leads to more and more inflammatory processes taking place inside the gut microenvironment (Saxena, et al., 2012; Zhang, et al., 2015). A need to address and characterize host-microbe relations has been emphasized in recent years in order to elucidate the processes and factors involved in the relationship between the gut microbiome and inflammation and related tumorigenesis. Inflammation is an important route through which alterations in gut microbiota affect the overall milieu of inflammatory processes closely associated with the gut. Gut microbiota manipulation through antibiotic treatment has been shown to cause a decrease in the expression of the Muc2 protein, a major component of the intestinal mucus layer (Dimmitt, et al., 2010). A thinning of the
A thinning of the protective mucus layer leads to a direct contact between the gut lining and the gut bacteria residing in the lumen, thereby triggering innate immune responses and inflammation (Francino, 2015). The inflammatory response, along with being systemic, also occurs at a more localized level such as the alterations in gut microbiota leading to increased levels of pro-inflammatory cytokines such as IL-1β and TNF-α (Vijay-Kumar, et al., 2010). It has been shown that the mucus layer tends to thickness with increased diversity of the microbiota (Jakobsson, et al., 2015). The mucus layer provides a source of nutritional carbon and therefore energy to some of the intestinal flora that are able to lyse the glycans present in the mucus, making the inter-relationship between the gut bacteria and the mucus layer really important (Kaur, et al., 2015). Also, the metabolites produced by these microbes also influence the differentiation and function of the epithelial and immune cells in the intestinal mucosa (Kato, et al., 2014; Shan, et al., 2013). Our recent study (Kaur, et al., 2015) also suggested that a reduction in the secretory mucin profile is associated with an inflammatory phenotype of the gut. Both Gram-positive and Gram-negative bacteria are known to enhance the intestinal mucin expression (Dohrman, et al., 1998). The regulation of the mucin gene expression as well as mucus production have been shown to be two levels at which the gut microbial community may modulate mucins (Comelli, et al., 2008). Gut microbiota manipulation through antibiotic treatment has been shown to cause a decrease in the expression of the Muc2 protein, a major component of the intestinal mucus layer (Wlodarska, et al., 2011). A thinning of the protective mucus layer leads to a direct contact between the gut lining and the gut bacteria residing in the lumen, thereby triggering innate immune responses and inflammation (Francino, 2015).
Studies that work at elucidating the role of gut microbes in disease models, have been limited, especially due to the fact that most of the important gut bacteria are difficult to grow in a laboratory setting making them unavailable for studying unless mammalian models are used. However, there are evidences that the manipulation of the gut microbiome through diet modulations, can alter the disease conditions such as those in Crohn’s disease and Inflammatory Bowel Disease (Mai, et al., 2007). Studies that use the \( Apc^{Min/+} \) mouse model for studying gut diseases and their association with microbiota often report significant changes in microbiome profiles of the large intestine, but also report that differences in the polyps is only significant in the small intestine (Mai, et al., 2007) The detrimental effects are also brought about via loss of certain colonizing bacterial species that are dependent on other colonizers for processes such as nutritional interactions and removal of secondary metabolites and toxic waste products (Belenguer, et al., 2006), or through loss of useful co-evolved processes brought about by the host-microbial co-dependence (Atarashi & Honda, 2011; Ferreira, et al., 2011). There are no studies that study the gut microbiome with respect to gut inflammation and tumorigenesis in an age/time-dependent manner.

4.3 Materials and methods

Experimental animals and groups, Collection of tissues

4 weeks old \( Apc^{Min/+} \) and C57BL/6 (WT) mice were obtained from Jackson Laboratories and bred in-house at the Animal Resource Facility, University of South Carolina. Food
(Purina chow) and drinking water was available to the mice *ad libidum* under a 12:12 hour light-dark cycle and a low-stress environment (22°C, 50% humidity and low noise). The $Apc^{Min/+}$ and C56BL/6 mice were assigned to the following twelve groups with 4 mice in each group (n=4): WT-Control (no antibiotic)-8 weeks, WT-Antibiotic (ABT)-8 weeks, WT-Control-12 weeks, WT-ABT-12 weeks, WT-Control-16 weeks, WT-ABT-16 weeks, $Apc^{Min/+}$-Control-8 weeks, $Apc^{Min/+}$-ABT-8 weeks, $Apc^{Min/+}$-Control-12 weeks, $Apc^{Min/+}$-ABT-12 weeks, $Apc^{Min/+}$-Control-16 weeks and $Apc^{Min/+}$-ABT-16 weeks. Experimental animals from aim 1 were used for aim 2 as control animals. All animal care followed institutional guidelines under a protocol approved by the Institutional Animal Care and Use Committee at the University of South Carolina.

**Collection of tissues**

Mice (n=4 separate for each age group, genotype and treatment group) were sacrificed at 8, 12 and 16 weeks by cervical dislocation. The control mice for the present experiment were the same as used under aim1. Blood was collected before sacrifice through retro-orbital puncture, centrifuged at 10,000 rpm for 15 minutes and serum was isolated and stored at -20°C. Small intestine and colon were excised and flushed clean with PBS. 2 mm² colon tissue sections were fixed in 10% formalin and were replaced with 70% ethanol after 24 hours, followed by paraffin embedding and sectioning to obtain 5 µm thin sections on glass slides. 2 mm² intestine and colon tissue sections were incubated in RPMI medium at 37°C for 24 hours followed by centrifugation at 2500 rpm for 15 minutes. Supernatant was obtained and stored at -20°C for tissue-secreted cytokine expression analyses. The rest of
the tissues were stored at -80°C for further usage. Fecal samples were snap frozen at the time of sacrifice for microbiome analysis.

**Microbiome analysis**

At sacrifice, fecal samples were obtained from the experimental mice to perform bacterial (luminal) microbiome analysis (n=3). Bacterial 16S rRNA gene amplification and sequencing were performed at the Alkek Center for Metagenomics and Microbiome Research, Department of Molecular Virology and Microbiology, Baylor College of Medicine (Houston, TX). Polymerase Chain Reaction (PCR) amplification of the V4 region of the bacterial 16S rRNA gene was performed using the Illumnia Miseq sequencer. The consensus sequences obtained from the analysis were mapped into Operational Taxonomic Units (OTUs). Alpha- and beta-diversity values were obtained based on the OTUs’ relative abundance table. Chao1 (estimator of richness) and Shannon Diversity Index (richness and evenness) were used to obtain the alpha diversity values. Weighted Unifrac (dissimilarity based on phylogenetic differences and taxonomic abundance) and Unweighted Unifrac (dissimilarity based on phylogenetic differences but not abundance) were performed to obtain the beta-diversity values for the experimental samples (He, et al., 2016). Kruskal Wallis and Mann Whitney statistical analyses were performed to calculate significance in diversity and relative abundance respectively, by comparing different treatments and disease stages. A p value of less than 0.05 was considered significant.
Antibiotic treatment

An antibiotic mixture containing Vancomycin, Neomycin and Streptomycin (each 1 mg/mL) was administered to experimental mice belonging to the antibiotic treatment groups. While neomycin and streptomycin act against Gram-negative bacteria, vancomycin is used against Gram-positive bacteria. The antibiotics mixture was administered to the mice through drinking water starting at the 5th, 10th and 14th weeks of age for 10 days followed by normal drinking water.

Clinical score

Mice were monitored throughout the course of study for weight loss, diarrhea and blood in stools. Clinical score was measured for each mouse twice during each week of the study. Mice were sacrificed after the last clinical score measurement. Cumulative clinical scores for each mouse equaled a maximum score of 12, which was based on weight loss measurement, diarrhea and fecal hemoccult (kit, BECKMAN COULTER) with a maximum score of 4 within each of the three quantitative parameters. Score for the weight loss was based on the following published scale where 0 = 0–5% weight loss; 1 = 6–10% weight loss; 2 = 11–15% weight loss; 3 = 16–20% weight loss; and 4 = >20% weight loss. Scoring of diarrhea was as follows: 0 = well-formed pellets, 2 = pasty and semi-formed stools that do not adhere to the anus, 4 = liquid stools that adhere to the anus. Detection of blood in the stools was determined by using hemoccult kit (BECKMAN COULTER), which is a hydrogen peroxide-based kit that forms a visible blue colored complex with blood. The followings were the score rates for the fecal hemoccult: 0 = no blood, 2 =
positive hemoccult, 4 = gross bleeding. The total clinical score was the summation of the individual score of weight loss, diarrhea and fecal hemoccult. The maximum score a mouse could get was 12.

**Polyp count**

Small intestine and colon from the experimental animals were excised and flushed with PBS. Tumor number and area was counted using 1% Methyl Blue stain under the light microscope for all mice in different groups and significant difference was calculated. Tumor number and/or area were also correlated with the manipulation of the microbiome.

**Goblet to epithelial cell ratio**

Small intestinal samples (0.5 cm) were embedded in paraffin wax and sectioned using a microtome into 10 µm sections obtained on glass slides. Standard deparaffinization procedure was followed using xylene and gradations of ethanol for the small intestinal sections. Alcian Blue dye solution for staining mucus-containing goblet cells was prepared by dissolving the dye powder (8GX) at 1% concentration in 3% acetic acid solution and setting the pH at 2.5. Nuclear Fast Red solution for staining the epithelial cells of the mucosa were prepared at a final concentration of 0.1% dissolved in 5% aluminum sulfate solution. Tissues were firstly stained with Alcian Blue and then counterstained with Nuclear Fast Red solution. Goblet to epithelial cell ratio was calculated per crypt with ten crypts per section and five sections per group.

**Statistical analysis**

Two-way analysis of variance (ANOVA), Two-way repeated measure ANOVA and One-way ANOVA were used to analyze the data with Tukey post hoc-analyses to estimate the
significance of differences obtained between different experimental treatment conditions and between the experimental mouse strains used in the study. A p<0.05 were considered statistically significant. All the statistical analyses were done using SigmaStat 3.5 (SPSS, Chicago, IL). The sample size was kept as 4 for the study.

4.4 Results

Clinical scores

Clinical scores were recorded twice per week during the course of study for all experimental animals to know the disease prognosis and health. Mice sacrificed at 8 weeks of age (figure 4.1 A): \( Apc^{Min/+} \) mice ABT group showed significantly higher clinical score than both \( Apc^{Min/+} \) control and WT-ABT group at 6 weeks. \( Apc^{Min/+} \) mice at 8 weeks showed significantly higher clinical score than 6 weeks and WT-ABT group. Lastly, \( Apc^{Min/+} \) mice ABT group showed significantly higher clinical score than WT-ABT mice at 8 weeks.

Mice sacrificed at 12 weeks of age (figure 4.1 B): \( Apc^{Min/+} \) mice at 6 and 8 weeks of age showed a significantly lower clinical score than WT-ABT group. \( Apc^{Min/+} \) mice at 8, 10 and 12 weeks of age showed a significantly higher clinical when compared to \( Apc^{Min/+} \) mice at 6 weeks of age. \( Apc^{Min/+} \) mice at 10 and 12 weeks of age showed a significantly higher clinical score than \( Apc^{Min/+} \) mice at 8 weeks of age. \( Apc^{Min/+} \) mice ABT group at 6 and 8 weeks of age showed a significantly higher clinical score than \( Apc^{Min/+} \) mice at the same age respectively. \( Apc^{Min/+} \) mice ABT group at 12 weeks of age showed a significantly
higher clinical score than WT-ABT group. No significant difference was observed between other treatment groups.

Mice sacrificed at 16 weeks of age (figure 4.1 C): \(Apc^{Min/+}\) mice at 6 weeks of age showed a significantly lower clinical score than WT-ABT group. \(Apc^{Min/+}\) mice at 8, 10, 12, 14 and 16 weeks of age showed a significantly higher clinical score than \(Apc^{Min/+}\) mice at 6 weeks of age. \(Apc^{Min/+}\) mice at 16 weeks of age showed a significantly higher clinical score than \(Apc^{Min/+}\) ABT mice at 8 weeks of age. \(Apc^{Min/+}\) ABT group at 6, 8, 12 and 16 weeks of age showed a significantly higher clinical score than \(Apc^{Min/+}\) mice at the same age respectively. \(Apc^{Min/+}\) mice ABT group at 16 weeks of age showed a significantly higher clinical score than \(Apc^{Min/+}\) ABT group at 6, 8, 12 and 14 weeks of age.

**Polyp count**

The total polyp count in \(Apc^{Min/+}\) ABT and \(Apc^{Min/+}\) control mice group at 12 and 16 weeks of age was significantly higher when compared to the mice within the same group at 8 weeks. \(Apc^{Min/+}\) ABT and \(Apc^{Min/+}\) control at 16 weeks of age showed a significant increase in the polyp count when compared to the mice belonging to the same group at 12 weeks of age. Only \(Apc^{Min/+}\) ABT mice at 16 weeks of age showing a significantly higher polyp count when compared to the \(Apc^{Min/+}\) control at the same age (figure 4.2A). No significant difference was observed in the polyp count of \(Apc^{Min/+}\) ABT and \(Apc^{Min/+}\) control at 8 and 12 weeks of age.
Polyp count (<1 mm²)

No significant difference was observed between the ABT-treated and control $Apc^{Min/+}$ mice for the number of intestinal polyps less than 1 mm² area, at 8, 12 and 16 weeks. Also no significance was observed within the $Apc^{Min/+}$ ABT and $Apc^{Min/+}$ control group at 8, 12 and 16 weeks of age (figure 4.2B).

Polyp count (>1 mm²)

Intestinal polyps measuring more than 1 mm² in area were significantly increased in $Apc^{Min/+}$ ABT and $Apc^{Min/+}$ control mice group at 12 and 16 weeks of age was significantly higher when compared to the mice within the same group at 8 weeks. $Apc^{Min/+}$ ABT and $Apc^{Min/+}$ control at 16 weeks of age showed a significant increase in the polyp count (>1 mm²) when compared to the mice belonging to the same group at 12 weeks of age. Only $Apc^{Min/+}$ ABT mice at 16 weeks of age showed a significantly higher polyp count (>1 mm²) when compared to the $Apc^{Min/+}$ control at the same age (figure 4.2B). No significant difference was observed in the polyp count of $Apc^{Min/+}$ ABT and $Apc^{Min/+}$ control at 8 and 12 weeks of age.

Goblet cells

Goblet to epithelial cell ratio provide an indirect measure in order to quantify mucus production in the small intestine which is closely related to the gut resident bacterial community although the mechanisms for the same are not known. The goblet to epithelial
cell ratio decreased significantly at 12 and 16 weeks of age and increased significantly at 12 and 16 weeks of age in WT control group and WT ABT group respectively when compared within the respective group at 8 weeks of age. Goblet to epithelial cell ratio was found to be significantly higher in WT control group at 8, 12 and 16 weeks when compared to WT ABT group at the same age (figure 4.3 A and B). No significant difference was observed in the goblet to epithelial cell ratio with other group or with age.

The goblet to epithelial cell ratio decreased significantly at 12 and 16 weeks of age in \( A\text{pc}^{\text{Min/+/}} \)-Control and \( A\text{pc}^{\text{Min/+/}} \)-ABT group when compared within the respective group at 8 weeks of age. The ratio was also found to be significantly lower in \( A\text{pc}^{\text{Min/+/}} \) control group at 16 weeks of age when compared with 12 weeks of age within the same group. Goblet to epithelial cell ratio was found to be significantly higher in \( A\text{pc}^{\text{Min/+/}} \) control group at 8, 12 and 16 weeks when compared to \( A\text{pc}^{\text{Min/+/}} \) ABT group at the same age (figure 4.3 A and C). No significant difference was observed in the goblet to epithelial cell ratio with other group or within group with age.

The bacterial population including proteobacteria, firmicutes and bacteroidetes was found to fluctuate with age, genotype and antibiotic treatment. The percentage of Proteobacteria was found be significantly higher in \( A\text{pc}^{\text{Min/+/}} \) control mice at 8 weeks when compared to WT control mice and \( A\text{pc}^{\text{Min/+/}} \) control mice at 12 and 16 weeks. No significant difference was observed in the proteobacterial population between the \( A\text{pc}^{\text{Min/+/}} \) and WT control mice at 12 and 16 weeks. Percentage of proteobacteria in \( A\text{pc}^{\text{Min/+/}} \) control mice at 8 weeks was found to be significantly higher when compared to \( A\text{pc}^{\text{Min/+/}} \) ABT treated mice at the same age. However, at 16 weeks the percentage of proteobacteria in \( A\text{pc}^{\text{Min/+/}} \) control mice were
found to be significantly lower than Apc$^{\text{Min}+/+}$ ABT treated mice at the same age. No significance in the proteobacterial population was observed between other groups with change in either treatment, age or genotype. The percentage of firmicutes bacteria in WT ABT treated mice, Apc$^{\text{Min}+/+}$ control mice and Apc$^{\text{Min}+/+}$ ABT treated mice at age 8, 12 and 16 weeks was found to be significantly higher than the WT control mice. The percentage of firmicutes bacteria in WT ABT treated and Apc$^{\text{Min}+/+}$ ABT treated mice at age 8, 12 and 16 weeks was significantly higher than the Apc$^{\text{Min}+/+}$ control mice at the respective age. Percentage of firmicutes bacteria in the feces of the Apc$^{\text{Min}+/+}$ mice at 8 weeks was significantly higher than at 12 weeks and 16 weeks. Percentage of firmicutes bacteria was found to be significantly higher at 8, 12 and 16 weeks when compared to their WT counterparts. Percentage of firmicutes population in Apc$^{\text{Min}+/+}$ mice at 16 weeks was found be significantly lower than the Apc$^{\text{Min}+/+}$ mice at 12 weeks. No significant difference was observed in the firmicutes population of WT ABT treated and Apc$^{\text{Min}+/+}$ ABT treated mice with age. The percentage of bacteroidetes bacteria in WT ABT treated mice, Apc$^{\text{Min}+/+}$ control mice and Apc$^{\text{Min}+/+}$ ABT treated mice at age 8, 12 and 16 weeks was found to be significantly lower than the WT control mice. Percentage of bacteroidetes in Apc$^{\text{Min}+/+}$ mice at 8 weeks was found be significantly lower than both WT and Apc$^{\text{Min}+/+}$ mice at 12 and 16 weeks of age. No significant difference was observed in the bacteroidetes population at 16 weeks between Apc$^{\text{Min}+/+}$ mice and WT mice. The percentage of bacteroidetes bacteria in WT ABT treated and Apc$^{\text{Min}+/+}$ ABT treated mice at age 8, 12 and 16 weeks was found to be significantly lower than the Apc$^{\text{Min}+/+}$ control mice. No significant difference was observed in the bacteroidetes population of WT ABT treated and Apc$^{\text{Min}+/+}$ ABT treated mice with age.
4.5 Discussion

Our study aimed at following the processes of tumorigenesis and its resulting disease prognosis and intestinal mucus secretion phenotype upon microbial manipulation at three different time points representing different stages of tumorigenesis. The antibiotic treatment lead to a significant increase in the percentage of the *Firmicutes* population. In our previous study, the phylum was observed to be present abundantly during the initial stages of tumorigenesis while decreasing in abundance gradually from 12 weeks to 16 weeks. The phylum showed a similar trend in the control groups while continuing to remain high even at 12 (tumor development) and 16 weeks (later stages of tumorigenesis) of age with antibiotic treatment. Our data may corroborate other studies that demonstrated that even when the antibiotic treatment is stopped, and some resilient bacteria repopulate the gut, the final state is never a complete replacement of the initial state (Francino, 2015). Our clinical score continued to remain high at 12 and 16 weeks for the $Apc^{Min/+}$ with and without antibiotic treatment and for WT mice with antibiotic treatment. The observation that after 8 weeks, the clinical scores of antibiotic treated $Apc^{Min/+}$ were not significantly different from those of the control $Apc^{Min/+}$ mice indicates towards the insult incurred by the process of tumorigenesis in the intestinal mucosa. Thus, the physical erosion of the mucosa and the inflammatory insult by the process of tumorigenesis are not additive during tumorigenic conditions. However, the antibiotic treatment can lead to an increase in the numbers of polyps. Moreover, the clinical scores did not correlate to the three time points of antibiotic administration to the mice, indicating that neither the timing of antibiotic administration nor the levels of the *Firmicutes* may have any effect on the disease prognosis. This is not however the case with the WT phenotype, where antibiotic treatment did produce a clinical
score even without tumorigenesis during 8, 12 and 16 weeks as compared to the WT mice without treatment. This indicates towards a more physical effect of microbiome manipulation on the gut that may lead to the same clinical symptoms that are present with processes of inflammation and tumorigenesis.

While the external manipulation of the gut microbiome may have physical impacts, it surely affects the physiology of the intestine as well, as can be seen from the data on the polyp numbers of the \( Apc^{Min/+} \) mice. While the manipulation of gut microbiome significantly increased the small intestinal total polyp counts at 16 weeks, the numbers were not significantly increased between antibiotic and control groups of 8 and 12 weeks. The antibiotic treatment also significantly increased the polyp numbers at 16 weeks as compared to those at 12 weeks with antibiotic treatment. The effects of the gut microbiome manipulation followed a similar trend in case of polyps that measured more than 1 mm in diameter, while had no significant effect on polyps less than 1 mm diameter. The effects of gut microbiome manipulation were more pronounced at 16 weeks of age as, the polyp numbers at 12 weeks with or without antibiotics were significantly increased as compared to those at 8 weeks with or without antibiotics, there was no significant difference between the polyp numbers at 12 weeks with and without antibiotics. Interestingly, the manipulation of the gut microbiome could significantly increase the polyp numbers from 12 to 16 weeks in the \( Apc^{Min/+} \) mice, there was no significant difference between the polyp numbers at 12 and 16 weeks without antibiotics - a usual characteristic of tumorigenesis in the \( Apc^{Min/+} \) without any treatment, where the polyps only increase in their size and not numbers from 12 weeks to later stages of tumorigenesis (McClellan, Davis, Steiner, Enos, et al., 2012).
The manipulation of the gut microbiome through antibiotics also significantly decreased the mucus-producing goblet cell numbers (measured by goblet to epithelial cell ratio) at all three time points of the present study. While the effect was significant only between antibiotic treatment and control within the $Apc^{Min/+}$ and WT mice at 8 weeks, the microbial manipulation could lower the goblet to epithelial cell ratio in the $Apc^{Min/+}$ mice as compared to the WT mice. This may again reflect the additive effects of inflammation, genetic susceptibility to polyp generation and the physical intestinal insult by the antibiotic treatment – an effect that was not observed in case of the WT mice.

4.6 Conclusion

It can be concluded that alterations in the gut microbiome community through antibiotic treatment can render the gut susceptible to an altered re-establishment of microbial community. The effects of the microbiome manipulation protocol are more obvious on the total number of polyp numbers in the gut and the on the goblet to epithelial cell ratios in the intestinal. While under no external treatment, an $Apc^{Min/+}$ may exhibit only an increase in polyp size during progression of tumorigenesis towards its later stages, the effect of an externally manipulated $Apc^{Min/+}$ microbiome may result in an increase is both polyp numbers and size as the process of tumorigenesis progress. While the ratio of the intestinal goblet to epithelia cells is significantly reduced as a result of gut microbial manipulation in both $Apc^{Min/+}$ and WT mice, the effect is more obvious in the tumor-bearing mice as compared to the WT mice.
Acknowledgements

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4.7 Figure Legends

**Figure 4.1. Clinical score with antibiotic treatment. (A–C)** Clinical scores for $Apc^{Min/+}$ mice at different time points (8, 12 and 16 weeks) ($n=4$ for each group) during the study. The scores were calculated out of twelve points; four points for each parameter: weight loss, diarrhea and hemoccult. Two-way repeated measure analysis of variance (ANOVA) was applied to calculate the significant difference between the clinical scores between different groups. #p<0.03 vs WT-ABT, *p<0.04 vs $Apc^{Min/+}$ control group at 6 weeks, $p<0.05$ vs $Apc^{Min/+}$ within the same age group and $p<0.05$ $Apc^{Min/+}$ ABT at 16 weeks vs $Apc^{Min/+}$ ABT at 14, 12, 10, 8 and 6 weeks of age.

**Figure 4.2. Tumor Quantification:** (A) Graph representing the small intestine polyp count in $Apc^{Min/+}$-Control and $Apc^{Min/+}$-ABT at 8 weeks, 12 weeks and 16 weeks after 1, 2 and 3 cycles of antibiotics respectively. (B) Graph showing polyp number (size < 1mm$^2$) at 8 weeks, 12 weeks and 16 weeks. (C) Graph showing polyp number (size < 1mm$^2$) at 8 weeks, 12 weeks and 16 weeks. Two-way repeated measure analysis of variance (ANOVA) was applied to calculate the significant difference between the total polyp, small polyp and large polyp count between different groups at different ages. *p<0.01 vs 8 weeks within the same group. **p<0.02 16 weeks vs 12 weeks within the same treatment group. #p<0.01 $Apc^{Min/+}$-Control vs $Apc^{Min/+}$-ABT at 16 weeks of age.
**Figure 4.3. Goblet and epithelial cell production:** Figure illustrates (A) Alcian blue staining for small intestine tissue sections of WT and $Apc^{Min/+}$ mice with and without antibiotic treatment. Blue and pink staining indicates goblet and epithelial cells respectively. (B) and (C): Graphs showing goblet to epithelial cell ratio per view per group for WT (B) and $Apc^{Min/+}$ (C) mice. Two-way and two-way ANOVA was used to determine the significant difference in goblet to epithelial cell ratio between and within groups. $^*$p value<0.05 vs 8 weeks within treatment group, $^{**}$p<0.01 $Apc^{Min/+}$ control mice 16 vs 12 weeks. $^{#}$p<0.01 control vs ABT group.

**Figure 4.4. Intestinal microbiome phylum profiles.** Relative percentages of the major phyla obtained from fecal 16S rRNA (V4 region) sequencing analysis from experimental mice at (A) Proteobacteria, (B) Firmicutes and (C) Bacteroidetes (C). (Percentage values represent fractions of depicted bacteria out of the total microbial pool). Two-way analysis of variance (ANOVA) was used to calculate the significant difference between the percentage of bacteria in $Apc^{Min/+}$ control, $Apc^{Min/+}$ ABT treated, WT ABT treated and WT control mice at age 8, 12 and 16 weeks. $^*$p<0.05 vs $Apc^{Min/+}$ 8 weeks, $^{**}$p<0.05 $Apc^{Min/+}$ (12 weeks vs 16 weeks) and $^{#}$p<0.01 $Apc^{Min/+}$ or $Apc^{Min/+}$ ABT treated or WT ABT treated vs WT.$^{5}$p<0.001 WT ABT treated and $Apc^{Min/+}$ ABT treated vs $Apc^{Min/+}$ control.
Figure 4.1. Clinical score with antibiotic treatment. (A–C) Clinical scores for $Apc^{Min/+}$ mice at different time points (8, 12 and 16 weeks) (n=4 for each group) during the study. The scores were calculated out of twelve points; four points for each parameter: weight loss, diarrhea and hemoccult. Two-way repeated measure analysis of variance (ANOVA) was applied to calculate the significant difference between the clinical scores between different groups. #p<0.03 vs WT-ABT, *p<0.04 vs $Apc^{Min/+}$-Control group at 6 weeks, **p<0.03 $Apc^{Min/+}$-ABT 16 weeks vs 12 and 8 weeks, $^3p<0.05$ vs $Apc^{Min/+}$ within the same age group and $^4p<0.05$ $Apc^{Min/+}$-ABT at 16 weeks vs $Apc^{Min/+}$-ABT at 14, 12, 10, 8 and 6 weeks of age.
**Figure 4.2. Tumor Quantification:** (A) Graph representing the small intestine polyp count in $Apc^{Min/+}$-Control and $Apc^{Min/+}$-ABT at 8 weeks, 12 weeks and 16 weeks after 1, 2 and 3 cycles of antibiotics respectively. (B) Graph showing polyp number (size < 1mm$^2$) at 8 weeks, 12 weeks and 16 weeks. (C) Graph showing polyp number (size < 1mm$^2$) at 8 weeks, 12 weeks and 16 weeks. Two-way repeated measure analysis of variance (ANOVA) was applied to calculate the significant difference between the total polyp, small polyp and large polyp count between different groups at different ages. *$p<0.01$ vs 8 weeks within the same group. **$p<0.02$ 16 weeks vs 12 weeks within the same treatment group. #$p<0.01$ $Apc^{Min/+}$-Control vs $Apc^{Min/+}$-ABT at 16 weeks of age.
A

WT-C       WT-ABT

8 weeks

12 weeks

16 weeks

Ap<sup>Min/+</sup>-C       Ap<sup>Min/+</sup>-ABT

8 weeks

12 weeks

16 weeks
Figure 4.3. Goblet and epithelial cell production: Figure illustrates (A) Alcian blue staining for small intestine tissue sections of WT and $Apc^{Min/+}$ mice with and without antibiotic treatment. Blue and pink staining indicates goblet and epithelial cells respectively. (B) and (C): Graphs showing goblet to epithelial cell ratio per view per group for WT (B) and $Apc^{Min/+}$ (C) mice. Two-way and two-way ANOVA was used to determine the significant difference in goblet to epithelial cell ratio between and within groups. *p value<0.05 vs 8 weeks within treatment group, **p<0.01 $Apc^{Min/+}$ control mice 16 vs 12 weeks. #p<0.01 control vs ABT group.
Figure 4.4. **Intestinal microbiome phylum profiles.** Relative percentages of the major phyla obtained from fecal 16S rRNA (V4 region) sequencing analysis from experimental mice at (A) Proteobacteria, (B) Firmicutes and (C) Bacteroidetes. (Percentage values represent fractions of depicted bacteria out of the total microbial pool). Dotted line represents WT-C group as a comparative baseline. Two-way analysis of variance (ANOVA) was used to calculate the significant difference between the percentage of bacteria in ApcMin/+ control, ApcMin/+ ABT treated, WT ABT treated and WT control mice at age 8, 12 and 16 weeks. *p<0.05 vs Apc-min 8 weeks, **p<0.05 Apc-min (12 weeks vs 16 weeks) and #p<0.01 Apc-min or ApcMin/+ ABT treated or WT ABT treated vs WT. $p<0.001$ WT ABT treated and ApcMin/+ ABT treated vs ApcMin/+ control.
CHAPTER 5

ASSOCIATION OF BACTEROIDETES AND FIRMICUTES WITH INFLAMMATION IN $APC^{MIN/+}$ MOUSE$^3$

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5.1 Abstract

The induction of cancer in the gut by chronic inflammation has always indicated towards the importance of underlying altered inflammatory processes. Apart from chemopreventive agents that act by modulating and alleviating harmful inflammation, the intestinal commensal bacteria also plays a role in the development of gut microenvironmental immune system and maintains a protective steady state immune function throughout life. The systemic release of microbial products as a result of insults to gut epithelium has been associated with inflammation-related colon cancer and the present study aimed at characterizing this association. 4 weeks old $Apc^{Min/+}$ and C57BL/6 (WT) mice were housed at the Animal Resource Facility, University of South Carolina with access to food and water ad libidum. The $Apc^{Min/+}$ and C56BL/6 mice were assigned to the following groups: WT-Control (no antibiotic)-8 weeks, WT-Antibiotic (ABT)-8 weeks, WT-Control-12 weeks, WT-ABT-12 weeks, WT-Control-16 weeks, WT-ABT-16 weeks, $Apc^{Min/+}$-Control-8 weeks, $Apc^{Min/+}$-ABT-8 weeks, $Apc^{Min/+}$-Control-12 weeks, $Apc^{Min/+}$-ABT-12 weeks, $Apc^{Min/+}$-Control-16 weeks and $Apc^{Min/+}$-ABT-16 weeks. An antibiotic mixture containing Vancomycin, Neomycin and Ampicillin (each 1 mg/mL) was administered to experimental mice through drinking water starting at the 5th, 10th and 14th weeks of age for 10 days followed by normal drinking water. Small intestinal paraffin-embedded sections were used for performing hematoxylin and eosin staining to determine the histopathology of small intestine and colon epithelial layer and for calculating crypt depth to villus ratio (CVR). Spleen weight was also measured to quantify general inflammation. The antibiotic treatment caused a physical insult to the intestinal mucosa.
and caused a reduction in spleen weight. The CVR increased significantly with antibiotic treatment in the WT mice as compared to controls at all three time points, while its increase was significant only at 12 weeks in case of $Apc^{Min/+}$ mice. The overall of percentage of *Firmicutes* increased with antibiotic treatment. It could be concluded that the effects of an altered microbiome may have both physical and physiological effects during initiation and progression of tumorigenesis. These effects may manifest as an insult to the intestinal mucosa, reduced spleen size and increased CVR, and may also be enhanced by an increased percentage of the *Firmicutes* phylum.

Keywords: *Firmicutes*, inflammation, polyp, gut microbiome, crypts

5.2 Introduction

A need to address and characterize host-microbe relations has been emphasized in recent years in order to elucidate the processes and factors involved in the relationship between the gut microbiome and inflammation and related tumorigenesis. Alterations in the gut microbiota have been linked to a dysregulation of the regulatory T cell paradigm and their associated cytokines (Shen, et al., 2014), where the regulation of this immunological pathway depends on the relationship between the gut microbiota and the immune system. There have been no studies that study the gut microbiome with respect to gut inflammation and tumorigenesis in an age/time-dependent manner. It has been seen that specific pathogen-free (SPF) $Apc^{Min/+}$ exhibit a higher tumor load and anemia with a higher infiltration of inflammatory cells specifically at advanced stages as compared to
germ-free animals, indicating that a mere modulation of gut microbiome profiles can abrogate the disease condition in the gut (Y. Li, et al., 2012).

The significance of inflammation as a process underlying and crucial to cancer is demonstrated by the fact that till date, one of the most effective class of chemopreventive agents is the non-steroidal anti-inflammatory drugs (NSAIDs). The induction of cancer in the gut by chronic inflammation has always indicated towards the importance of underlying altered inflammatory processes (Zhang, et al., 2015). Different agents that have been documented to be effective against tumors and cancers are the agents that function by downregulating important inflammatory pathways and pro-inflammatory cytokines. It has also been shown that the intestinal commensal bacteria play a role in the development of the immune system of the gut microenvironment comprising both the humoral and cellular components and maintains the protective steady state immune function throughout life (Cebra, 1999; Talham, et al., 1999). It may be more appropriate to say that any alteration in the composition of the gut microbiome leads to a dysfunctional immune system. Certain bacterial species such as *B. thetaiotaomicron* bacterium have been associated with the attenuation of pro-inflammatory responses by reducing the levels of the pro-inflammatory cytokine IL-8 (Zhao, et al., 2001). Comparative numbers of certain bacteria often lead to upregulation or downregulation of certain inflammatory pathways. The systemic release of microbial products as a result of the manipulation of the gut epithelium invokes an IL-23 response and a further IL-17 response in order to neutralize further invasion by the microbes, and the process is brought about by the bacteria *Clostridia sp.* (Shih, et al., 2014). Other bacterial species may also be involved with anti-inflammatory effects such as the bacteria *Bacteroides fragilis* from the *Bacteroidetes* phylum leads to an increase in the
production of IL-10 cytokine (Omenetti & Pizarro, 2015). It has been suggested that the interplay of inflammation and tumorigenesis is modulated strongly by the gut microbiota but the mechanisms leading to the process have not elucidated. Recently, some light has been shed on the phenomena by a study which could demonstrate the process of tumorigenesis to occur in germ-free mice when transplanted with isolated gut microbiota from intestinal tumor-bearing mice (Zackular, et al., 2013).

The establishment and diversification of the gut microbiome is a gradual process that is required for proper development and overall health. Once the gut microbiome manifests itself completely, its stays constant during the life of a healthy individual (Franks, et al., 1998) which explains the fact that an alteration in one or more of the resident microbial populations lead to pathological conditions.

The Bacteroidetes phylum is comprised of Gram-negative anaerobic and aerobic bacterial species. Some studies link the absence or reduction of, or delayed colonization of the gut by the phylum Bacteroidetes to be associated with a weakened immune system in humans. For example, a delayed postnatal colonization of Bacteroidetes population has been associated with development of allergies in children born by Caesarian section (S. Watanabe, et al., 2003).

The Firmicutes represent the phylum mostly containing Gram-positive bacterial species. The phylum contains both aerobic and anaerobic bacterial species.

Many Firmicutes are capable of producing endospores which help them survive desiccation and extreme conditions. Their capability to survive extreme conditions may be one reason that these bacteria are found in high numbers in the gut under pathological and
inflammatory conditions. Comparisons of gut microbiota of lean and obese mice and lean
and obese human individuals revealed that the Bacteroidetes significantly decrease while
the Firmicutes significantly increase with obesity (Guo, et al., 2008).

An altered ratio between the two main gut bacterial phyla Bacteroidetes and
Firmicutes may help elucidate at least way through which the gut changes during initiation
of progression of tumorigenesis.

5.3 Material and Methods

Experimental animals and groups

4 weeks old Apc\(^{Min/+}\) and C57BL/6 (WT) mice were obtained from Jackson Laboratories
and bred in-house at the Animal Resource Facility, University of South Carolina. Food
(Purina chow) and drinking water was available to the mice ad libitum under a 12:12 hour
light-dark cycle and a low-stress environment (22°C, 50% humidity and low noise). The
Apc\(^{Min/+}\) and C56BL/6 mice were assigned to the following twelve groups with four mice
in each group (n=4): WT-Control (no antibiotic)-8 weeks, WT-Antibiotic (ABT)-8 weeks,
WT-Control-12 weeks, WT-ABT-12 weeks, WT-Control-16 weeks, WT-ABT-16 weeks,
Apc\(^{Min/+}\)-Control-8 weeks, Apc\(^{Min/+}\)-ABT-8 weeks, Apc\(^{Min/+}\)-Control-12 weeks, Apc\(^{Min/+}\)-
ABT-12 weeks, Apc\(^{Min/+}\)-Control-16 weeks and Apc\(^{Min/+}\)-ABT-16 weeks. Experimental
animals from aim 1 were used for aim 2 as control animals. All animal care followed
institutional guidelines under a protocol approved by the Institutional Animal Care and Use Committee at the University of South Carolina.

**Histopathology**

Small intestinal paraffin-embedded sections from aim 2 were used for performing hematoxylin and eosin staining in order to determine the morphology of small intestine and colon epithelial layer. Histopathology were quantified visually in a double-blind condition using light microscopy by a pathologist. Localized inflammatory status were indicated by inflammatory cells present in the mucosal layers of intestine and colon. Aberrant villi and crypts were also used as indicators of intestinal and colonic insults due to inflammatory response.

Hematoxylin and eosin staining was used to observe the morphology of the intestinal mucosa and the disease severity was quantified on the basis of inflammation, immune cell infiltration into the mucosa and degree of tumor. Histopathology was quantified based on the scoring system indicating the severity of disease and constituting inflammation and immune cell infiltration. This was on the scale of 8 where highest score of 4 was given for each parameter, where 0 = no infiltration or no inflammation; 2 = moderate infiltration or inflammation; and 4 = severe inflammation with distorted crypts or infiltration and formation of lymphatic follicles (Saxena et al., 2009). All the images were taken in 20X magnification with Nikon e600 microscope. Two investigators in blinded fashion measured the scores independently and the average was plotted in a graph.”
Crypt depth to villus height ratio

Paraffin-embedded 10 µm small intestinal sections were deparaffinized and stained with hematoxylin and eosin. Digitized images of the tissues were obtained using a digital camera connected to a light microscope. The images were analyzed in a double blinded manner using the digital image software ImageJ, and crypt depth and villus height were measured (mm/mm) to calculate the crypt depth to villus height ratio.

Spleen weight

Spleens were excised from the animals at the time of sacrifice and their weights were recorded to estimate levels of systemic inflammation. The spleen samples were then rinsed with PBS and snap frozen.

Enzyme-linked Immunosorbent Assay

Enzyme-linked Immunosorbent Assay (ELISA) was performed to calculate the levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-17 using kits (BD Pharmingen). The ELISA were performed on serum to know the levels of systemic cytokines. At the time of sacrifice, 1 cm sections of ileum and distal colon were excised from the experimental mice and incubated in 1 mL of RPMI medium at 37°C for 24 hours. The medium incubated with the excised tissues was then collected into separate centrifuge tubes and centrifuged at 4000 rpm at 25°C for 15 minutes. The supernatant collected from the centrifuged contents served as the sample containing small intestine or colon tissue-secreted cytokines.
Statistical analysis

Two-way analysis of variance (ANOVA), Two-way repeated measure ANOVA and One-way ANOVA were used to analyze the data with Tukey post hoc-analyses to estimate the significance of differences obtained between different experimental treatment conditions and between the experimental mouse strains used in the study. A p<0.05 were considered statistically significant. All the statistical analyses were done using SigmaStat 3.5 (SPSS, Chicago, IL).

5.4. Results

Histopathology

Histopathology of the small intestine was revealed by hematoxylin and eosin staining. Signs of inflammation were observed during all stages of tumorigenesis in the intestinal tissue of \(Apc^{Min/+}\) mice. Hyperplasia was observed in all \(Apc^{Min/+}\) mice at 8 weeks with and without antibiotic treatment. Mucosal dysplasia, blunted villi and increased monocytic inflammation was observed at 12 weeks. High grade chronic-type inflammation as shown by the presence of plasma cells and macrophages but no neutrophils was observed at 16 weeks in the \(Apc^{Min/+}\) mice without antibiotic treatment. High grade dysplasia and dysplastic lesions were observed with antibiotic treatment in the \(Apc^{Min/+}\) mice at 16 weeks as compared to low-grade dysplasia that was observed at 16 weeks without antibiotic treatment (figure 5.1 A).
Histopathology scoring was used to determine quantitative difference in the histopathology of the colon of the mice belonging to different groups and with age. Degree of inflammation and immune cell infiltration was found to be significantly higher in $Apc^{Min/+}$ ABT group when compared to $Apc^{Min/+}$ control and WT ABT group. All the groups showed significantly higher clinical score than WT control group. Colon of the mice belonging to the $Apc^{Min/+}$ control, $Apc^{Min/+}$ ABT and WT ABT group showed a significantly higher inflammation and immune cell infiltration when compared to mice within the same group at 8 weeks. No significant difference was found between other groups and within group with age (figure 5.1 B).

**Spleen Weight**

Spleen weight was assessed as a measure of systemic inflammation along with serum cytokines. Spleen weight of $Apc^{Min/+}$ ABT group at 16 weeks and $Apc^{Min/+}$ control group at 12 and 16 weeks was found to be significantly higher than the mice within the same groups at 8 weeks of age. Spleen weight of $Apc^{Min/+}$ ABT and $Apc^{Min/+}$ control group mice at 16 weeks of age was found to be significantly higher than both WT control mice and within the same group at 12 weeks of age. $Apc^{Min/+}$ control group mice was found to have a significantly higher spleen weight as compared to the WT control mice (figure 5.2A).
Crypt depth to villus height ratio (CVR)

The crypt depth to villus ratio (CVR) was calculated as a measure of intestinal inflammation for all animals. The CVR ratio in both $Apc^{Min/+}$ and WT group with ABT at 12 and 16 weeks was found to be significantly higher than the mice within the same group at 8 weeks of age. The CVR ratio of WT and $Apc^{Min/+}$ with ABT treatment was 12 weeks of age was found to be significantly higher than the control mice with the same genotype. Spleen weight of the WT mice with ABT treatment was significantly higher at 16 weeks of age when compared to WT control at the same age. $Apc^{Min/+}$ control mice at 16 weeks of age was found to have significantly higher CVR ratio when compared to the control mice at 12 weeks of age with the same genotype. NO significance was found between other treatment groups and with age in the same treatment group with same or different genotype (figure 5.3 A and B).

Cytokines

The pro-inflammatory cytokines TNF-α, IL-1β and IL-17 did not show any significant difference between the experimental groups when measured for their levels in the serum (systemic) (figure 5.2 B,C and D) and as secreted by the small intestine and colon (tissue-level) (figure 5.3 C, D, E and F)suggesting that an alteration in gut microbiome may not significantly affect the systemic and tissue-level inflammation.
5.5. Discussion

The present study aimed at defining the inflammatory effects of an altered gut microbiome with relation to stages of tumorigenesis. We have found previously that the manipulation of the gut microbiome through usage of antibiotics significantly decreases the goblet to epithelial cell ratio in the small intestine of \( Apc^{Min/+} \) and WT experimental mice. A reduction in the number of goblet cells reduces the production of the protective intestinal mucus, which thins over time until eventually lost thus exposing the underlying mucosa to possible physical erosion by the presence of antibiotics in the intestinal lumen. In the present study, the physical harmful effects of gut microbiome manipulation were observed to lead to the recruitment of certain inflammatory cells to the eroded mucosal sights. While damaged crypts result in the WT mice, the \( Apc^{Min/+} \) mice exhibit effects ranging from low-grade dysplasia to high-grade dysplasia, neutrophil and monocyte infiltration and villus edema, corroborating our previous findings that the use of antibiotics has an additive effect on gut health along with factors such as inflammation, polyp numbers and mucus production. The spleen weights were significantly reduced with antibiotic treatment only in the \( Apc^{Min/+} \) mice, which indicates that the antibiotic treatment does not have any effect under normal conditions (Prior, Gander, Irache, & Gamazo, 2005; Reikvam et al., 2011). The significant difference in the spleen size between antibiotic treatment and control within the \( Apc^{Min/+} \) mice may reflect an effect of reduced immune system function as a result of inflammation caused due to tumorigenesis. Interestingly, the spleen weights were significantly higher in the \( Apc^{Min/+} \) as compared to those of the WT mice at 16 weeks, which may support the idea that a longer period of microbiome manipulation may lead an
even lower spleen weight in the WT but is not able to decrease it to the same level under tumorigenic conditions. However, our results from cytokine analyses failed to reiterate our spleen results as the serum cytokine levels were not significantly altered following antibiotic administration in both WT and $Apc^{Min/+}$ mice. A higher samples size may help better standardize the effects of the antibiotics and microbiome manipulation on spleen size during tumorigenesis.

The CVR was also not significantly different between antibiotic-treated and control groups at 8 weeks, indicating a relatively balanced inflammatory status of the experimental animals at the initiation of tumorigenesis. The CVR increases significantly in the treatment group as compared to the control groups of animals at 12 weeks indicating an inflammatory-inducing effect of antibiotics on both the strains of mice. An absence of any difference between the antibiotic-treatment and control in the $Apc^{Min/+}$ mice at 16 weeks is probably due to similarities between the measurements for antibiotic treatment and control. This observation, when compared to the WT mice indicates a healthier intestinal CVR during the absence of antibiotics which is abrogated once the WT intestine is exposed to the antibiotics. However, an absence of secretion of pro-inflammatory cytokines by the small intestine and colon indicates towards a more physical effect of the antibiotic treatment than that on the physiology of the intestinal and colon tissues. A larger sample size may help to elucidate the physiological effects more coherently.
5.6. Conclusion

In conclusion, the effects of gut microbiome manipulation through antibiotic treatment could not be established on the $Apc^{Min/+}$ mouse clinical scores, spleen weights and CVR, and only some of the physical effects of erosion and insult to the intestinal mucosa could be established. A larger samples size will be required to support or negate the findings of the present study.

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5.7. Figure Legends

Figure 5.1. Histopathology: (A) Hemeatoxylin and eosin stained small intestinal tissues belonging to the WT and $Apc^{Min/+}$ mice with and without antibiotic treatment at different ages of mice. (B) Histopathology scoring determining the degree of inflammation and immune cell infiltration was plotted for different treatment group at different age. Two-way analysis of variance (ANOVA) was used to calculate the significant difference between different treatment groups at different ages. *p<0.05- Apc-min-ABT vs Apc-min-
C, *p<0.05- Apc-min-C vs WT-ABT, #p<0.01- Apc-min-ABT vs WT-ABT, ***p<0.05 16 weeks vs 8 weeks.

**Figure 5.2. Systemic inflammation:** Figure illustrating systemic inflammation in the form of (A) spleen weights (grams) for all four experimental groups and serum pro-inflammatory cytokine (B) IL-17, (C) IL-1β and (D) TNF-α levels for the experimental groups. Two-way analysis of variance (ANOVA) was used to calculate the significant difference between different treatment groups at different ages. *p value<0.05 vs 8 weeks within the same treatment group. #p<0.05 vs WT control and **p<0.05 16 weeks vs 12 weeks.

**Figure 5.3. Tissue-level inflammation:** Figure illustrating tissue-level inflammation in the form of small intestinal crypt depth to villus height ratio (CVR) between different experimental groups for (A) WT and (B) ApcMin/+ mice; and small intestine-secreted levels of pro-inflammatory cytokines (C) IL-17, (D) Il-1β and (E) TNF-α; and colon-secreted levels of pro-inflammatory cytokines (F) IL-17, (G) IL-1β and (H) TNF-α. Two-way analysis of variance (ANOVA) was used to calculate the significant difference between different treatment groups at different ages. *p value<0.04 vs 8 weeks, #p<0.01 ABT vs control group and **p<0.05 16 vs 12 weeks within the same group.
A  

Control  

WT-C  WT-ABT  

8 weeks  

12 weeks  

16 weeks  

Antibiotics  

Apc\textsuperscript{Min/+}-C  Apc\textsuperscript{Min/+}-ABT  

8 weeks  

12 weeks  

16 weeks
Figure 5.1. Histopathology: (A) Hemeatoxylin and eosin stained small intestinal tissues belonging to the WT and Apc\textsuperscript{Min/+} mice with and without antibiotic treatment at different ages of mice. (B) Histopathology scoring determining the degree of inflammation and immune cell infiltration was plotted for different treatment group at different age. Two-way analysis of variance (ANOVA) was used to calculate the significant difference between different treatment groups at different ages. *p<0.05- Apc-min-ABT vs Apc-min-C, **p<0.05- Apc-min-C vs WT-ABT, #p<0.01- Apc-min-ABT vs WT-ABT, ***p<0.05 16 weeks vs 8 weeks.
Figure 5.2. Systemic inflammation: Figure illustrating systemic inflammation in the form of (A) spleen weights (grams) for all four experimental groups and serum pro-inflammatory cytokine (B) IL-17, (C) IL-1β and (D) TNF-α levels for the experimental groups. Two-way analysis of variance (ANOVA) was used to calculate the significant difference between different treatment groups at different ages. *p value<0.05 vs 8 weeks within the same treatment group, #p<0.05 vs WT control and between Apc\(^{Min/+}\)-C vs Apc\(^{Min/+}\)-ABT, and **p<0.05 16 weeks vs 12 weeks.
Figure 5.3. Tissue-level inflammation: Figure illustrating tissue-level inflammation in the form of small intestinal crypt depth to villus height ratio (CVR) between different experimental groups for (A) WT and (B) ApcMin/+ mice; and small intestine-secreted levels of pro-inflammatory cytokines (C) IL-17, (D) IL-1β and (E) TNF-α; and colon-secreted levels of pro-inflammatory cytokines (F) IL-17, (G) IL-1β and (H) TNF-α. Two-way analysis of variance (ANOVA) was used to calculate the significant difference between different treatment groups at different ages. *p value<0.04 vs 8 weeks, #p<0.01 ABT vs control group and **p<0.05 16 vs 12 weeks within the same group.
CHAPTER 6

OVERALL DISCUSSION
Our first experiment was aimed at studying alterations in the gut microbiome at the stages of tumor initiation and progression in an age-dependent manner using a spontaneously-developing tumor mouse model. We hypothesized that an increase in the levels of *Firmicutes* and a reduction in the *Bacteroidetes* levels would be associated with tumorigenesis. We also hypothesized that the process of tumorigenesis will result in lower diversity values of gut microbiome. We found that the *Firmicutes* were significantly higher than the *Bacteroidetes* during tumorigenesis at all three ages – 8, 12 and 16 weeks, indicating that the *Firmicutes* may be precursors to tumorigenesis. While this was true, the diversity values (supplemental data) were not significantly different between tumor-bearing and control mice, indicating that the overall diversity may not be indicative of tumorigenesis as can be the altered levels of certain phyla. The *Firmicutes* phylum was the only factor that was seen to be increased in the tumor-bearing mice as compared to the WT mice. The fact that the WT mice did not show any clinical score makes *Firmicutes* the likely bacterial phylum that can set the stage for tumorigenesis. The observation that the clinical scores for tumor-bearing mice continued to increase until 16 weeks may indicate that levels of *Firmicutes* may not correlate with disease prognosis once the process of tumorigenesis begins.

The relationship between altered percentages of the *Firmicutes* and *Bacteroidetes* phyla populations, and tumor number, tumor size and mucus-producing goblet cell numbers has not been previously examined. The regulation of the mucin gene expression as well as mucus production have been shown to be two levels at which the gut microbial community may modulate mucins (Comelli, et al., 2008). Our second study was aimed at following the processes of tumorigenesis, resulting disease prognosis, and intestinal mucus
secretion phenotype after microbial manipulation at three different time points – 8, 12 and 16 weeks. We hypothesized that the manipulation of the gut microbiota would lead to a loss in gut health and an increase in tumor number and size in the ApcMin/+ mouse. Furthermore, manipulation of the gut microbiota would decrease goblet cell number in the intestine and colon. While our first study showed a progressive decrease in the percentage of Firmicutes population in tumor-bearing mice under normal conditions, the phylum showed a similar trend in the control groups while continuing to remain high even at 12 (tumor development) and 16 weeks (later stages of tumorigenesis) of age with antibiotic treatment. Our data may support other studies that demonstrated that even when the antibiotic treatment is stopped, and some resilient bacteria repopulate the gut, the final state is never a complete replacement of the initial state (Francino, 2015). Our clinical score continued to remain high at 12 and 16 weeks for the ApcMin/+ with and without antibiotic treatment and for WT mice with antibiotic treatment and correlated to the time points of antibiotic administration to the mice. The external manipulation of the gut microbiome induced effects on the physiology of the intestine as well, as can be seen from the data on the polyp numbers of the ApcMin/+ mice. The manipulation of gut microbiome significantly increased the polyp numbers at 16 weeks as compared to those at 12 weeks with antibiotic treatment. This implicates a role for the antibiotic treatment in the later stages of tumorigenesis, which is not seen under tumorigenic conditions without antibiotic treatment where the polyps increase in their size and not numbers from 12 weeks to later stages of tumorigenesis (McClellan, Davis, Steiner, Enos, et al., 2012). The effects of antibiotic treatment or gut microbiome manipulation followed a similar trend in case of polyps that
measured more than 1 mm in diameter, while had no significant effect on polyps less than 1 mm diameter.

Our study also demonstrated a significant reduction in the ratio of intestinal goblet to epithelial cells at all three time points, indicating the potential for the gut microbiome to influence mucus-producing phenotype of the cells comprising the intestinal mucosa. Histopathological observations of intestinal mucosa in the third part of the present study revealed a significant increment in the levels of inflammation and cellular infiltration in antibiotic-treated $Apcc^{Min/+}$ mice as compared to the control $Apcc^{Min/+}$ and antibiotic-treated WT mice at 8, 12 and 16 weeks indicating an inflammatory effect of antibiotic treatment, which is more pronounced during tumorigenic conditions. The levels of inflammation and cellular infiltration were significantly higher at 16 weeks as compared to those at 8 weeks for $Apcc^{Min/+}$-ABT, $Apcc^{Min/+}$-C and WT-ABT mice, but not at 12 weeks, indicating that not only the inflammatory responses following antibiotic treatment may exacerbate during tumor-bearing conditions and but antibiotic treatment may bring about significant inflammatory responses even in control conditions. A loss in the protective mucus in the gut lumen may be a reason for such an effect, however, we did not investigate such an interrelationship directly. We hypothesized for aim 3, an increase in systemic and tissue-level inflammation with gut microbial manipulation with antibiotic administration. We found that spleen weight which is an indicator of systemic inflammation, increased significantly at 12 and 16 weeks as compared to 8 weeks in the $Apcc^{Min/+}$ mice under control conditions and antibiotic-treatment conditions which indicates an increased inflammatory response of tumorigenesis and microbiome manipulation. However, within 12 and 16 weeks, the spleen weights were significantly reduced with antibiotic administration, an
effect that negated our hypothesis of an increased systemic inflammation with antibiotic administration. Our data on serum cytokines was not conclusive on systemic inflammation. We also hypothesized an increase in tissue-level inflammation with antibiotic-mediated gut microbial manipulation. We measured CVR for an estimate of the same. The gut microbial manipulation significantly increased the CVR for both \(Apc^{Min/+}\) and WT mice at 12 and 16 weeks as compared to 8 weeks indicating a shift towards a more pronounced tissue-inflammatory response at 12 and 16 weeks. The antibiotic treatment increased the CVR significantly at 12 and 16 weeks for the WT mice but only at 12 weeks for the tumor-bearing \(Apc^{Min/+}\) mice, suggesting that gut microbial manipulation may be important for tissue level inflammation during progression of tumorigenesis. However, our data on tissue-secreted cytokines could not corroborate our findings on CVR indicating towards an effects of antibiotics exposure on intestinal mucosal morphology rather than on its secreted cytokines.

**Summary**

By means of this dissertation, we demonstrated that while certain members of the gut microbiome and any alterations thereof may seem to affect the process of tumorigenesis, there numbers and relative numbers may not affect the disease prognosis. The way by which the gut microbiome is altered may also play an important role in the way we see processes that are linked to tumorigenesis in the gut most importantly the physical aspects of the gut lumen. An externally manipulated gut microbiome specifically through antibiotics can disrupt the mucosal lining producing, under normal conditions, symptoms same as those manifested under tumor-bearing conditions. The effects of the method of gut microbiome manipulation through antibiotics seems to be more due to the physical effects
of the antibiotic rather than an altered gut microbiome. The polyp number increased with antibiotic administration as compared to untreated tumor-bearing conditions. Other physiological alterations and possible routes through which intestinal insult can result is the reduction in the size of the villi lining the absorptive surface of the intestinal lumen and hampering the development of goblet cells that produce protective mucus on the luminal surface. While antibiotics used to manipulate the gut microbiome may be broad-ranged and less specific, certain phyla of bacteria may be more susceptible to the same combination of the antibiotics as compared to others. In our study, while the *Bacteroidetes* phylum of bacteria is severely susceptible to the antibiotic treatment leading to negligible levels in the experimental groups with microbiome manipulation, *Firmicutes* phylum seem to thrive more than in normal conditions with the use of antibiotics. However, larger samples sizes with specific pathogen-free experimental conditions could be helpful for better understanding the role of certain classes of gut microbial community in processes such as tumorigenesis and inflammation.

**Future directions**

Studies on topics of the present dissertation need to be highly controlled and specific. The usage of specific pathogen-free animals or antibiotics against specific microbial community/communities will be necessary to better understand the role of specific classes of gut microbiome in disease processes such and inflammation and tumorigenesis. Another highly controlled and specific method to elucidate the role(s) of gut microbiota on inflammation and tumorigenesis and other disease conditions is the use of germ-free animals.


Figure S1: Alpha diversity values $Apc^{Min/+}$-C and WT-C: Figure illustrating box plots of alpha diversity values obtained between $Apc^{Min/+}$ and WT mice under control conditions and significance values obtained by Kruskal-Wallis statistical analysis where p value $>0.05$ was considered significant. (OTUs: Observed Taxonomic Units between the two groups; Chao1 index: Estimator of richness between the two groups; and Shannon index: Estimator of richness and evenness between the two groups). The three horizontal lines contained within each box plot represent the third quartile, median and first quartile (from top to bottom). The vertical lines on top and bottom of the box plot represent the maximum and minimum values plotted respectively.
Figure S2: Alpha diversity values. Figure illustrating box plots of alpha diversity values obtained between (A) $Apc^{Min/+}$-ABT and $Apc^{Min/+}$-C; (B) WT-ABT and WT-C; and (C) WT-C/ABT and $Apc^{Min/+}$-C/ABT. Significance values were obtained by Kruskal-Wallis statistical analysis where p value > 0.05 was considered significant. (OTUs: Observed Taxonomic Units between the two groups; Chao1 index: Estimator of richness between the two groups; and Shannon index: Estimator of richness and evenness between the two groups). The three horizontal lines contained within each box plot represent the third quartile, median and first quartile (from top to bottom) respectively. The vertical lines on top and bottom of the box plot represent the maximum and minimum values plotted respectively.
APPENDIX B

DETAILED PROTOCOLS
Alcian Blue Staining Protocol

Solutions and Reagents:

3% Acetic Acid Solution:
   Glacial acetic acid -------------- 3 mL
   Distilled water ---------------- 97 mL

Alcian Blue Solution (pH 2.5):
   Alcian blue, 8GX ---------------- 1 g
   Acetic acid, 3% solution -------- 100 mL
   Mix well and adjust pH to 2.5 using acetic acid.

0.1% Nuclear Fast Red Solution:
   Nuclear fast red ----------------- 0.1 g
   Aluminum sulfate--------------- 5 g
   Distilled water --------------- 100 mL
   Dissolve aluminum sulfate in water. Add nuclear fast red and slowly heat to boil and cool. Filter and add a grain of thymol as a preservative.

Procedure:

1. Deparaffinize slides and hydrate to distilled water.
2. Stain in Alcian Blue solution for 30 minutes.
3. Wash in running tap water for 2 minutes.
4. Rinse in distilled water.
5. Counterstain in nuclear fast red solution for 5 minutes.
6. Wash in running tap water for 1 minute.
7. Dehydrate and through 95% alcohol, 2 changes of absolute alcohol, 3 minutes each.
8. Clear in xylene or xylene substitute.
9. Mount with resinous mounting medium.
Hematoxylin and Eosin Staining Protocol

1. Deparaffinize in Xylene I and II and III (3 minutes)
2. Rehydrate
   a. EtOH 100% (3 minutes)
   b. EtOH 100% (3 minutes)
   c. EtOH 95% (3 minutes)
   d. EtOH 95% (3 minutes)
   e. EtOH 70% (3 minutes)
3. Rinse in distilled water (5 minutes)
4. Stain in hematoxylin (6 minutes) *Filter before each use to remove oxidized particles*
5. Rinse in running tap water (15 minutes)
6. Decolorize in acid alcohol (1 second)
7. Rinse well in tap water (5 minutes)
8. Immerse in ammonia water (3 Seconds)
9. Rinse in tap water (5 minutes)
10. Counterstain in Eosin (15 seconds)
11. Dehydrate
    a. EtOH 95 % (3 minutes) *Discard after each use* 
    b. EtOH 95% (3 minutes)
    c. EtOH 100 % (3 minutes)
    d. EtOH 100 % (3 minutes)
12. Clear in Xylene I and II (5 minutes)
Role of altered gut microbiome on tumor development, mucus production and inflammation in $Apc^{Min/+}$ mouse

Submitted by:
KAMALJEET KAUR
Summary

Background and significance: The microbiome forms an integral part of the gut microenvironment. Once ignored, the topic has gained momentum in research during the past decade, where studies have strongly suggested the association of microbiota with health and a misbalance thereof, to many disease conditions ranging from inflammation and colitis to diabetes, obesity and colon cancer. The Human Microbiome Project (HMP, NIH common fund - 2008) has used a variety of high throughput analyses in order to study gut microbiota in health. The consortium has so far been able to isolate and characterize more than 1,300 reference bacterial strains from the human body. The large amount of data generated has led to a baseline need to address the implications of different microbial members, or groups thereof, in health and disease. The microorganisms residing in the gut comprise of bacteria, archaea, fungi and viruses that are distributed throughout the length of the gastrointestinal tract. While there will be limitations to studying all types of microorganisms owing to their overwhelming numbers and types, our study is focused only on bacterial populations of the gut, and for the purpose of convenience, terms of gut microbiota/microbiome will be used for describing gut bacteria pertaining to the mice used in our study. The overall purpose of this study is to determine the effects of alterations in the gut microbiome on tumor development and inflammation, and if it leads to recolonization of the gut by altered bacterial communities. The working hypothesis is that an alteration of bacterial microbiome will occur during tumorigenesis and manipulation of the gut microbiome externally will exacerbate the clinical symptoms associated with intestinal cancer, leading to higher gut and systemic inflammation.
To test this hypothesis, the following specific aims will be used:

**Specific Aim 1:** To examine the composition of gut microbiome during tumor initiation and development in the $Apc^{Min/+}$ mouse.

**Specific Aim 2:** To determine if gut microbiome manipulation can regulate the $Apc^{Min/+}$ mouse gut health and tumorigenesis.

**Specific Aim 3:** To determine if the relative abundance of Bacteroidetes and Firmicutes bacterial populations is associated with intestinal and systemic inflammation in the $Apc^{Min/+}$ mouse.

The *primary objectives* of this study are to: 1) determine whether the gut microbiome profiles change during early and later stages of tumorigenesis, 2) determine the effect of external manipulation of gut microbiome on tumor load and size, and on mucus-producing goblet cell population in the gut, 3) study any alterations in the bacterial populations pertaining to the *Bacteroidetes* and *Firmicutes* phyla that recolonize after external manipulations of the gut microbiome, 4) determine if altered *Bacteroidetes* and *Firmicutes* populations increases tissue inflammation in the intestine, 5) determine if altered *Bacteroidetes* and *Firmicutes* populations lead to an increase in circulatory cytokines. The *rationale* behind our study are the research studies that have been published and our preliminary data. It has been shown that the gut microbiota accelerates tumor growth in the $Apc^{Min/+}$ mice through the c-jun/JNK and STAT3 phosphorylation pathways and causes a vicious cycle of inflammation in the intestine (Y. Li, et al., 2012). Recently, it has also been demonstrated that alterations in the gut microbiota precede tumor development in the
\(Apc^{Min/+}\) mice (Son, et al., 2015) which is due to the resulting alterations in interactions of gut microbiota and gut mucosa. The novelty of the present study is in answering the question as to how the gut microbiome shifts from early to later stages of tumorigenesis. Studies do indicate that the gut microbiota can be a potential target in the amelioration of cancer progression but it is not known how the microbiome paradigm of the gut shifts with respect to progression in tumorigenesis. This study will also be directed towards understanding inflammatory changes and changes in mucus producing function of the gut and their relation to a change in quantifiable disease severity and microbiota alterations.
CHAPTER 1

Introduction and Aims

The topic of gut microbiome has recently gained attention due to research suggesting its significant role in not only maintenance of gut health but also in disease conditions such as obesity, diabetes, arthritis, colitis and cancers of the digestive system (Cho, et al., 2014; Dziarski, et al., 2016; Kabeerdoss, et al., 2015; Knip & Siljander, 2016). The gut microbiome comprises of around 500-1000 species and a genetic diversity which has 100-fold more genes than humans (Dugas, et al., 2016).

Another field that aims at answering the questions on gut microbiome and its links to health and disease is the usage of probiotics including one or more important bacteria. The bacteria *Lactobacillus acidophilus* has been implicated in a significant suppression of colon tumor incidence and size and a reduction in pro-inflammatory cytokines such as TNF-α and IFN-γ (Perdigon, et al., 1998; Urbanska, et al., 2009). Some studies also state that the link between the microbiome and the actual development of polyps in the gut is solely linked through the erosion of the gut lumen microenvironment and the resulting inflammation (Dianda, et al., 1997).

An altered microbiome has been previously linked to inflammatory bowel disease and colorectal cancer. However, we still lack the knowledge of a characterizing the gut microbiome that relates to health or disease condition. An early event of inflammatory insult to the gut can very well result in tumorigenesis when combined with processes of bacterial DNA damage and chromatin alterations (Zhu, et al., 2011). Studies on mouse models such as the IL-10−/− have demonstrated a protective role of the presence of gut
microbiome on inflammation, where the WT mice were rendered protected against inflammation in the gut while the germ-free (no microbiome) animals developed colitis (S. Wu, et al., 2009). It should be noted here that a germ-free animals may not represent the human gut as an individual can never be completely without microbiome in the gut. From the perspective of any given disease state that can be linked to microbiome, the basis of its linkage can only be an altered microbiome. It should also be noted that the process of inflammation is the one that alters the microbiome (Uronis & Jobin, 2009), although inflammation alone is not sufficient to promote colorectal cancer and altered microbiota works hand in hand with inflammatory insults that will eventually lead to cancer (Arthur & Jobin, 2013). The gut microbiome has been shown to complement AOM and DSS mouse models in causing tumorigenesis, but is yet to be elucidated if it is the increase or loss of certain bacterial populations that lead to development of polyps (Zackular, et al., 2013). It is, however, not known what effect the microbial dysbiosis has at different stages of progression of tumorigenesis in the mouse, or if it leads to different consequences in normal versus tumorigenic conditions.

**What is unknown?**

Studies are needed to characterize the interplay between the gut microbiome, inflammation and cancer. Another aspect which will be helpful in correctly defining the microbiome and inflammation-to-cancer axis will be the progression of alterations in the microbiome with worsening disease pathology. Of all the diseases that are affected by a change or loss of gut microbiome, cancer of the small and large intestine is clearly the one which can be
physically and physiologically related to the presence of alterations in gut microbiome community.

**AIM 1: To examine the composition of gut microbiome during tumor initiation and development in the Apc\(^{\text{Min/+}}\) mouse.**

**Rationale:** An altered gut microbiome can cause inflammation ranging from acute bouts to chronically inflamed gut. An altered microbiome, which shifts from symbiotic to a more inflammation-inducing phenotype, leads to a vicious cycle of erosion of the gut epithelial lining that, in turn, leads to more and more inflammatory processes taking place inside the gut microenvironment (Saxena, et al., 2012; Wang & Zhang, 2015). Our previous study on chronic inflammation-induced colon cancer in the APNKO (Adiponectin-knockout) mice demonstrated microbiota profiles that were clearly altered during tumorigenesis (data not published). The alterations mainly pertained to the altered percentages of the *Firmicutes* and *Bacteroidetes* bacterial phyla during inflammation-induced tumorigenesis in the mice (Figure 1). As opposed to externally administered chemically-induced tumorigenesis in mice, the microbiota has not been studied with respect to a spontaneously induced tumorigenesis such as that in the *Apc\(^{\text{Min/+}}\) mouse. It was recently concluded that an alteration of gut microbiome precedes polyposis in the *Apc\(^{\text{Min/+}}\) mouse (Son, et al., 2015). Also, it has been seen that specific pathogen-free (SPF) *Apc\(^{\text{Min/+}}\) exhibit a higher tumor load and anemia with a higher infiltration of inflammatory cells specifically at advanced stages as compared to germ-free animals, indicating that a mere modulation of gut microbiome profiles can abrogate the disease condition in the gut (Y. Li, et al., 2012).
However, there are no studies that study the gut microbiome with respect to gut inflammation and tumorigenesis in an age/time-dependent manner. Therefore, the first aim of the present study will be directed towards characterization of alterations of microbiome profiles from early to later stages of tumorigenesis in an \( Apc^{\text{Min/+}} \) mouse model.

**Hypothesis:** The gut microbiota in \( Apc^{\text{Min/+}} \) mice will exhibit a higher percentage of *Firmicutes* bacterial phyla and a reduction in the percentage of *Bacteroidetes*, along with reduction in overall bacterial diversity during onset of tumorigenesis when compared to the WT mice. Furthermore, the percentage of *Firmicutes* will increase, while overall diversity will decrease during the progression of tumorigenesis.

AIM 1.1: To study the profile of the gut microbiome during intestinal tumor initiation.

**Hypothesis 1.1:** The microbiome profile of \( Apc^{\text{Min/+}} \) mice will exhibit a higher percentage of *Firmicutes* bacteria and a lower percentage of *Bacteroidetes* as compared to WT mice at initiation of tumor development. The overall microbial diversity will be lower in \( Apc^{\text{Min/+}} \) mice as compared to their WT counterparts.

AIM 1.2: To study the profile of the gut microbiome during the progression of intestinal tumor development.

**Hypothesis 1.2:** The microbiome profile of \( Apc^{\text{Min/+}} \) mice will exhibit a higher percentage of *Firmicutes* bacteria and a lower percentage of *Bacteroidetes* as compared to their WT counterparts during tumor progression. *Firmicutes* population will be higher during later stages of tumorigenesis as compared to initial stages of tumorigenesis. Also, the microbial diversity values will be lower in \( Apc^{\text{Min/+}} \) mice during tumor progression as compared to
WT mice, and within \( Apc^{Min/+} \) mice at later stages of tumor development as compared to their initial stages of tumorigenesis.

**AIM 2: To determine if gut microbiome manipulation can regulate the \( Apc^{Min/+} \) mouse health and tumorigenesis.**

**Rationale:** The mucus layer tends to thickness with increased diversity of the microbiota (Jakobsson, et al., 2015). Our preliminary data from a study on chronic inflammation-induced colon cancer using APNKO mice suggested that the gut microbiota changes in a way that favors an increased percentage of bacteria of certain phyla (here, \( \text{Firmicutes} \)) as compared to others (such as \( \text{Bacteroidetes} \)). It has not been studied yet as to whether there is a relation between the altered percentages of especially the \( \text{Firmicutes} \) and \( \text{Bacteroidetes} \) phyla populations, and tumor number, tumor size and goblet cell numbers. It is known that the mucus layer provides a source of nutritional carbon and therefore energy to some of the intestinal flora that are able to lyse the glycans present in the mucus, making the inter-relationship between the gut bacteria and the mucus layer really important (H. Li et al., 2015). Also, the metabolites produced by these microbes also influence the differentiation and function of the epithelial and immune cells in the intestinal mucosa (Kato, et al., 2014; Shan, et al., 2013). The regulation of the mucin gene expression as well as mucus production have been shown to be two levels at which the gut microbial community may modulate mucins (Comelli, et al., 2008). Our recent study (Kaur, et al., 2015) also suggested that a reduction in the secretory mucin profile is associated with an inflammatory phenotype of the gut (Figure 4). Therefore, the present aim will be directed at knowing the
mucus-producing phenotypes of the cells lining the gut of the experimental animals in relation to the changing profiles of the gut microbiota.

**Hypothesis:** Manipulation of the gut microbiota will lead to a decrease in gut health and an increase in tumor number and size in the $Apc^{Min/+}$ mouse. Furthermore, manipulation of the gut microbiota will decrease goblet cell number in the intestine and colon.

**AIM 2.1:** To determine if manipulation of gut microbiome through antibiotic administration affects disease prognosis in the $Apc^{Min/+}$ mouse.

**Hypothesis 2.1:** The manipulation of gut microbiome by externally administered antibiotics will lead to a worsening of disease prognosis in the experimental mice.

**AIM 2.2:** To determine if manipulation of gut microbiome through antibiotic administration affects tumor number and size in the $Apc^{Min/+}$ mouse.

**Hypothesis 2.2:** The manipulation of the gut microbiome by externally administered antibiotics will lead to an increase in tumor number and size in $Apc^{Min/+}$ mice.

**AIM 2.3:** To determine if gut microbiome manipulation effects goblet cell numbers in small intestine and colon of $Apc^{Min/+}$ mice as compared to WT mice.

**Hypothesis 2.3:** The manipulation of the gut microbiome by externally administered antibiotics will lead to a reduction in goblet cell numbers in the intestine and colon of $Apc^{Min/+}$ mice.
AIM 3: To determine if the relative abundance of *Bacteroidetes* and *Firmicutes* bacterial populations is associated with intestinal and systemic inflammation in the *ApcMin/+* mouse.

**Rationale:** The induction of cancer in the gut by chronic inflammation has always indicated towards the importance of underlying altered inflammatory processes (Wang & Zhang, 2015). Different agents that have been documented to be effective against tumors and cancers are the agents that function by downregulating important inflammatory pathways and pro-inflammatory cytokines such as IL1-β, IL-6 and TNF-α, and reduction of macrophage infiltration (Y. Li, et al., 2012; Murphy, Davis, McCellan, & Carmichael, 2011; Murphy, Davis, McCellan, Gordon, et al., 2011; Ritland, et al., 1999). It has also been shown that the intestinal commensal bacteria plays a role in the development of gut microenvironmental immune system comprising both the humoral and cellular components and maintains the protective steady state immune function throughout life (Cebra, 1999; Talham, et al., 1999). It may be more appropriate to say that any alteration in comparative numbers of certain bacteria often lead to upregulation or downregulation of certain inflammatory pathways. The systemic release of microbial products as a result of the manipulation of the gut epithelium invokes an IL-23 response and a further IL-17 response in order to neutralize further invasion by the microbes, and the process is brought about by the *Firmicutes* bacteria *Clostridia sp.* (Shih, et al., 2014). The bacteria especially promotes IL-17 cytokine in the small intestine which is the primary site of its attachment and survival (Omenetti & Pizarro, 2015). These Th17 cells may become autoreactive in case of intestinal epithelium insult. The bacteria *Bacteroides fragilis* from the *Bacteroidetes* phylum leads to an increase in the production of IL-10 cytokine (Omenetti & Pizarro,
2015), whereas its strains have been implicated in the production of the pro-inflammatory cytokine IL-17-dependent inflammation-related colon cancer (W. Wu, et al., 2015). Inflammation, which is an integral part of tumorigenesis should therefore, may strongly be correlated with a reduced bacterial diversity or numbers.

**Hypothesis:** An increase in the population of *Firmicutes* bacteria and/or a reduction in the population of *Bacteroidetes* will lead to a higher inflammatory response in both the intestinal tissue and systemic circulation.

AIM 3.1: To examine if the relative abundance of *Bacteroidetes* and *Firmicutes* populations regulate intestinal tissue inflammation in the ApcMin/+ mouse.

*Hypothesis 3.1:* An increase in *Firmicutes* and/or a reduction in *Bacteroidetes* bacterial populations will increase the inflammatory cellular infiltration into the mucosa of the intestinal tissue of the experimental mice.

AIM 3.2: To examine if the relative abundance of *Bacteroidetes* and *Firmicutes* populations regulate intestine-secreted IL-1β, IL-17 and TNF-α in ApcMin/+ mice.

*Hypothesis 3.2:* An increase in *Firmicutes* and/or a reduction in *Bacteroidetes* bacterial populations will increase the intestine-secreted IL-1β, IL-17 and TNF-α levels in the experimental mice.

AIM 3.3: To examine if the relative abundance of *Bacteroidetes* and *Firmicutes* populations regulate systemic levels of cytokines IL-1β, IL-17 and TNF-α in ApcMin/+ mice.
Hypothesis 3.3: An increase in Firmicutes and/or a reduction in Bacteroidetes bacterial populations will increase systemic levels of IL-1β, IL-17 and TNF-α cytokines in the experimental mice.

Limitations and Pitfalls:

1. The ApcMin/+ mouse model is different from the DSS-DMH chronic inflammation-induced cancer model (preliminary data). Therefore, microbiome profile of the ApcMin/+ model may be different from that of the DSS-DMH model of chronic inflammation-induced cancer model.

2. The microbiome profiles to be studied will be obtained from the fecal samples and will be assumed to represent the bacterial repertoire of both small intestine and colon of the experimental animals. Microbiome of the small intestine may be different from that of the colon.

3. The effects of antibiotic administration on goblet cell production and inflammatory index in the ApcMin/+ mouse model may be very different from those seen as a result of a single bout of inflammation in the DSS-induced colitis model (preliminary data).

4. The ApcMin/+ mouse model is majorly an intestinal mouse model and most of the polyps are seen in the small intestine. Thus, changes in goblet cell expression and inflammatory index may be significant only in the small intestine of the experimental mice.

5. Administration of antibiotics to the experimental animals may lead to an increase in intestine-secreted pro-inflammatory cytokines due erosion of the epithelial layer but may not necessarily change them systemically.
6. The study has a small sample size which may lead to lesser significant values of the results that will be obtained from the study.

**Working model**

The central idea of the present study is to study the role of gut microbiota (bacterial) in tumorigenesis, mucus producing phenotype and inflammation. In order to study this, we will be using the $Apc^{Min/+}$ model of intestinal cancer. The $Apc^{Min/+}$ mouse model is raised
from the C57BL/6 background where a point mutation on codon 850 of the \textit{Apc} (Adenomatous Polyposis Coli) gene leads to spontaneous development of polyps (adenomas) in the intestinal mucosa (Zhang, et al., 2015). The mutation is responsible for the production of a truncated APC protein (2843 amino acids) that lacks its C-terminal domains. The protein functions to downregulate the Wnt signaling pathway by binding to and promoting the destruction of the β-catenin protein. It has been shown that altered interactions between the gut microbiota and colonic mucosa precede polyposis in the \textit{Apc}^{Min/+} mouse (Son, et al., 2015). Using the basis of an altered gut microbiota preceding polyposis, we are trying to delve further into the processes that are hampered by alterations in the gut microbiota, which can be used to elucidate the ways in which the gut microbiota affects tumorigenesis.

The first aim of the study will be directed towards examining the composition of gut microbiome during tumor initiation and development in \textit{Apc}^{Min/+} mouse. This aim will act as preliminary to aims 2 and 3 by characterizing and thereby defining the alterations occurring in the microbiome of \textit{Apc}^{Min/+} mouse during initiation and progression of tumorigenesis. Previous studies have demonstrated an alteration in gut microbiota to be linked to a variety of conditions including diabetes, arthritis, colitis and cancer and even immune system. Conditions such as Crohn’s disease and obesity have implicated gut bacterial dysbiosis in pathogenesis (Chan, et al., 2015; Mai, et al., 2007). The characterization of the gut microbiome in the experimental \textit{Apc}^{Min/+} mice will help define the abundance of different bacterial species in the gut and their relative diversity values during the initiation and progression of tumorigenesis.
The second aim of the study will be directed towards elucidating the role of an altered microbiome on pathology of the experimental animals. Antibiotics will be administered to experimental mice in order to perturb the gut microbiota during the initial and progressive stages of tumorigenesis. The use of antibiotics not only help get rid of pathogenic bacteria but also deplete related micro-organisms, which fail to return to normal levels even long after the antibiotic usage subsides (Cresci & Bawden, 2015). This aim will thus help in elucidating the effect of alterations in microbiome in terms of their abundance and diversity on the disease condition of the animals. This aim will also be directed towards knowing if the tumor numbers and size are related to the microbiome and its alterations. The third part of this aim will elucidate the effects of altered gut microbiome on expression of goblet cells in the intestine and colon of mice. Our previous study (Saxena, et al., 2013) has demonstrated that a reduction in goblet cell numbers leads to worsened disease pathology and reduced protection against chronic inflammation-induced colon cancer. A reduction in the number of mucus-producing goblet cells may also be altered in the \( Apc^{Min/+} \) model and further worsen the disease state once they are administered antibiotics.

The third aim of the present study will demonstrate the effects of an altered microbiome on the inflammatory status of the experimental animals. Inflammation is an important route through which alterations in gut microbiota affect the overall milieu of inflammatory processes closely associated with the gut. The inflammatory response, along with being systemic, has also been demonstrated to occur at a more localized level such as the alterations in gut microbiota leading to increased levels of pro-inflammatory cytokines such as IL-1\( \beta \) and TNF-\( \alpha \) (Singh, et al., 2015). In the first part of aim 3 we will study the effects of a changed microbiome on tissue-localized inflammation and infiltration
occurring at the level of intestinal mucosa. The second part of the aim will help study the
effects of microbiome manipulation on intestine-secreted pro-inflammatory cytokines.
Whether the inflammatory effect is systemic or not, will be elucidated through the third
part of this aim where systemic levels of pro-inflammatory cytokines will be measured.

CHAPTER 2

Review of Literature

The $Apc^{Min/+}$ mouse model

The $Apc^{Min/+}$ mouse model is widely used by researchers to study gut inflammation and
cancer. The $Apc^{Min/+}$ mouse model is raised from the C57BL/6 background where a point
mutation on codon 850 of the $Apc$ (Adenomatous Polyposis Coli) gene leads to
spontaneous development of polyps (adenomas) in the intestinal mucosa (Zhang, et al.,
2015). The mutation is responsible for the production of a truncated APC protein (2843
amino acids) that lacks its C-terminal domains. The protein functions to downregulate the
Wnt signaling pathway by binding to and promoting the destruction of the $\beta$-catenin
protein. An altered APC protein thus leads to, apart from the development of intestinal
polyps, dysregulation of processes like cell adhesion, cell migration, chromosome
segregation and stability (McCart, et al., 2008).

The $Apc^{Min/+}$ mouse model represents the human cancer syndrome called the
Familial Adenomatous Polyposis (FAP) where the human intestine may contain several
thousand adenomas by the age of 20-30 years, which corresponds to $\sim$4-6 weeks in mouse.
The Apc\textsuperscript{Min/+} mouse exhibits more than 50 tumors along the entire length of the intestine and rarely live past the age of 21-22 weeks (Shoemaker, et al., 1997), which corresponds to ~60 years of age in humans. Since all intestinal tumors in B6 Min/+ mice are benign adenomas, the premature death of these animals is associated with secondary effects of tumor growth, including severe, chronic anemia and intestinal blockage (Shoemaker, et al., 1995).

**The Gut Microbiome**

The gut microbiome is an integral and an important symbiotic system present in the gut, which comprises of bacteria, archaea, fungi and viruses residing in the gastrointestinal tract throughout its length. The gut bacteria itself comprises a major percentage of the total gut microbiome, where its numbers are in trillions. While the stomach and duodenum contain about $10^1$ to $10^2$ Colony Forming Units (CFU) per mL of bacterial forms, the jejunum and ileum comprise $10^4$ to $10^8$, and the colon has $10^{10}$ to $10^{12}$ CFU/mL of bacteria (Cresci & Bawden, 2015). The microbiome acts advantageous for the gut such that it regulates gut epithelial and endocrine cellular structure (Uribe, et al., 1994). The commensal bacterium *B. thetaiotaomicron* VPI-5482, for example, which is a member of the gut flora, has been linked to the functional processes of the gut such as nutrition absorption, mucosal barrier function, metabolism, angiogenesis and postnatal intestinal maturation (Hooper & Gordon, 2001; Hooper, et al., 2001).

Previously, many studies have demonstrated an alteration in gut microbiota to be linked to a variety of conditions including diabetes, arthritis, colitis and cancer and even
immune system. Conditions such as Crohn’s disease and obesity have implicated gut bacterial dysbiosis in pathogenesis (Chan, et al., 2015; Mai, et al., 2007). The highest bacterial load is found in the distal small intestinal tract and colon - areas commonly found to be associated with disease conditions (Hooper, et al., 2002; Kanauchi, et al., 2003). It was not clear if the bacterial dysbiosis is a cause or consequence of the disease until recently when it was shown that alterations in gut microbiome precede polyposis in the Apc\(^{Min/+}\) mouse (Son, et al., 2015). An altered gut microbiome can cause inflammation ranging from acute bouts to chronically inflamed gut. An altered microbiome, which shifts from symbiotic to a more inflammation-inducing phenotype, leads to a vicious cycle of erosion of the gut epithelial lining that, in turn, leads to more and more inflammatory processes taking place inside the gut microenvironment (Saxena, et al., 2012; Zhang, et al., 2015). A need to address and characterize host-microbe relations has been emphasized in recent years in order to elucidate the processes and factors involved in the relationship between the gut microbiome and inflammation and related tumorigenesis.

The intestinal epithelial lining has a strong relationship to the gut bacteria. The bacterial flora of the intestine helps in digesting the food (especially carbohydrates) that cannot be digested by the mammalian gut, and in turn, the intestinal epithelial cells metabolize the short-chain fatty acids that result from the bacterial fermentation of the undigested carbohydrates and use them as an energy source (Abreu, 2010).
Antibiotic usage, gut microbiome and inflammation

Antibiotic treatments that are usually used against digestive tract infections not only target the pathogenic microbes but also the host-interactive useful microbes. It is already known that the use of antibiotics, especially broad spectrum, not only help get rid of pathogenic bacteria but also deplete related micro-organisms, which fail to return to normal levels even long after the antibiotic usage subsides (Cresci & Bawden, 2015). Microbial diversity also is seen to be reduced in the gut following antibiotic treatment which exerts detrimental effects (Lofmark, et al., 2006).

Antibiotic usage can severely and permanently affect 30% of the microbial population (Dethlefsen & Relman, 2011). Once the antibiotic treatment is stopped, and some resilient bacteria repopulate the gut, the final state is never a complete replacement of the initial state (Francino, 2015). Any early antibiotic treatment in humans reduces the overall diversity of the microbiota populations inside the gut which leads a disbalance of the inflammatory molecules in the body. The disbalance between the Th1 and Th2 paradigm was at first thought to be the main culprit in bringing out the inflammatory effects of the microbiota dysbalance. An increased Th1 activation was linked to harmful inflammatory changes (Akdis & Akdis, 2009; Oboki, et al., 2008). However recently, alterations in the gut microbiota have been linked to a dysregulation of the regulatory T cell paradigm and their associated cytokines (Shen, et al., 2014), where the regulation of this immunological pathway depends on the relationship between the gut microbiota and the immune system. Different cocktails of antibiotics administered to mice have led to an upregulation of either the Th2 or a Th17 response (Atarashi & Honda, 2011; Dimmitt, et al., 2010), indicating that an alteration in the gut microbiota may favor one pathway and
downregulate another inflammatory pathway. How the microbiota changes in the first place and then a subsequent change occurs in the inflammatory pathways during spontaneous tumorigenesis is a question that the present study will be aiming to answer via studying different stages of tumorigenesis.

Inflammation is an important route through which alterations in gut microbiota affect the overall milieu of inflammatory processes closely associated with the gut. Gut microbiota manipulation through antibiotic treatment has been shown to cause a decrease in the expression of the Muc2 protein, a major component of the intestinal mucus layer (Dimmitt, et al., 2010). A thinning of the protective mucus layer leads to a direct contact between the gut lining and the gut bacteria residing in the lumen, thereby triggering innate immune responses and inflammation (Francino, 2015). The inflammatory response, along with being systemic, also occurs at a more localized level such as the alterations in gut microbiota leading to increased levels of pro-inflammatory cytokines such as IL-1β and TNF-α (Vijay-Kumar, et al., 2010).

For the completion of aims 2 and 3, antibiotics will administered to the treatment group of mice in three sets: 1). One cycle of antibiotic administration starting at 6 weeks of age for 10 days and sacrifice at 8 weeks; 2). Two cycles of antibiotic administration starting at 6 weeks and 10 weeks and sacrifice at 12 weeks; and 3). Three cycles of antibiotic administration starting at 6, 10 and 14 weeks of age and sacrifice at 16 weeks. Antibiotic administration at 6 weeks of age will signify the manipulation of gut microbiome at the onset of tumorigenesis. This stage of tumorigenesis is marked by a gradual increase in the number of polyps in the small intestine (Puppa, et al., 2011). Antibiotic administration at 6 and 10 weeks of age will help define effects of microbiome altered at
the beginning of tumorigenesis (6 weeks) and then at the middle of the life span of experimental mice which is marked by progression of tumor development resulting in more pronounced symptoms of anemia and intestinal blockage. While the polyp number reaches a plateau, they only increase in size during this stage and the animals continue to lose body weight (Puppa, et al., 2011). Antibiotic treatment at 6, 10 and 14 weeks will represent the alterations in microbiome throughout the processes initiation of tumorigenesis. This stage is marked by severe inflammation, continuation of tumor size and maximum weight loss (Puppa, et al., 2011).

**Impact of antibiotics**

Although antibiotics are used against pathogenic microbes, their usage affects other microbial members of the community as well. The effects that are established as a result of an altered microbiome can be long-lasting even after the antibiotic treatment has subsided (Jakobsson, et al., 2015; Jernberg, et al., 2007). While the more resilient bacteria are successful at returning to their pre-treatment levels, others are lost indefinitely (Willing, et al., 2011). Moreover, the occurrence of co-dependence between members of the community based on differential metabolite production and utilization pathways leads to perturbation of members that are not necessarily directly targeted by the antibiotic.

The direct and indirect effects of the antibiotics are brought about at different levels and ways. The indirect effects are brought about by the changed gut microbiota. A change in the bacterial population is known to cause a change in the downstream signaling by the Pattern Recognition Receptors (PPRs) which help maintain epithelial integrity and repair
process, while change in some bacterial populations that bind to Toll-like receptors, such as TLR4, are linked to an altered innate immune defenses (Willing, et al., 2011). A reduced production of short-chain fatty acids (SCFAs) is also a common feature of human metabolic profiles after antibiotic treatment (Romick-Rosendale, et al., 2009; Yap, et al., 2008). Changes in bacterial populations also affects differentiation of certain immune system molecules such as IL-17 and often lead to a dysregulation of the immune system (Ivanov, et al., 2009).

More direct effects include undesirable inflammation of the gut such as gastroenteritis (Barthel, et al., 2003) and even dermatitis (J. Watanabe, et al., 2010).

**Intestinal mucus layer**

The gut lining is folded into tubular invaginations called the villi in the intestine and crypts (of Leiberkuhn) in the colon. The gut lining is protected from the lumen microenvironment by a single-cell layer comprising of absorptive enterocytes (which make most of the epithelial cells), mucus-producing goblet cells, hormone-producing enteroendocrine cells and Paneth cells that produce anti-microbial products in the gut (Abreu, 2010). The four types of the cells line the single-celled layer of the gut that faces the lumen. All the four types of cell arise from the pluripotent stem cells that lie at the base of the crypts. The differentiated cells that arise from the stem cells of the crypts slowly and continuously rise towards the crypt apex from where the single-celled layer is regularly shed and renewed to maintain a healthy gut, also providing the crypt with a feature of polarity.

The mucus in the small and large intestine is secreted by the goblet cells. It forms the first line of defense between the gut lumen microenvironment and the underlying
epithelial layer and the submucosa. The mucin proteins in the gut mainly comprise of the MUC2 and MUC5AC proteins. They have properties of forming gel like coating onto the gut lumen once getting hydrated and form highly glycosylated proteins that are resistant to the digestive environment of the gut (Koboziev, et al., 2014). Changes in goblet cell function, secretion of mucins into the lumen and composition of this mucus layer are shown to be altered by an altered microbiome (Deplancke & Gaskins, 2001). The mucus layer provides a balanced ecosystem for the resident and as well as pathogenic bacteria in the intestinal lumen by acting as a source of nutrition, thus indicating a homeostatic balance between the host gut microenvironment and the microbiome associated with it (Aristoteli & Willcox, 2003). An alteration in one of them may render the other dysfunctional. It is also said that the resident bacteria may inhibit the adherence of pathogenic bacteria to the intestinal epithelial cells by increasing the production of intestinal mucus (Mack, et al., 2003; Mack, et al., 1999; Smirnova, et al., 2003).

**Mucus and gut microbiome**

It has been shown that the mucus layer tends to thickness with increased diversity of the microbiota (Jakobsson, et al., 2015). The mucus layer provides a source of nutritional carbon and therefore energy to some of the intestinal flora that are able to lyse the glycans present in the mucus, making the inter-relationship between the gut bacteria and the mucus layer really important (H. Li, et al., 2015). Also, the metabolites produced by these microbes also influence the differentiation and function of the epithelial and immune cells in the intestinal mucosa (Kato, et al., 2014; Shan, et al., 2013). Our recent study (Kaur, et
also suggested that a reduction in the secretory mucin profile is associated with an inflammatory phenotype of the gut. Inflammation, which is an integral part of tumorigenesis should therefore, may strongly be correlated with a reduced bacterial diversity or numbers. Moreover, both Gram-positive and Gram-negative bacteria are known to enhance the intestinal mucin expression (Dohrman, et al., 1998). The regulation of the mucin gene expression as well as mucus production have been shown to be two levels at which the gut microbial community may modulate mucins (Comelli, et al., 2008).

Gut microbiota manipulation through antibiotic treatment has been shown to cause a decrease in the expression of the Muc2 protein, a major component of the intestinal mucus layer (Wlodarska, et al., 2011). A thinning of the protective mucus layer leads to a direct contact between the gut lining and the gut bacteria residing in the lumen, thereby triggering innate immune responses and inflammation (Francino, 2015).

**Gut microbiome and Apc-min**

Studies that work at elucidating the role of gut microbes in disease models, have been limited, especially due to the fact that most of the important gut bacteria are difficult to grow in a laboratory setting making them unavailable for studying unless mammalian models are used. However, there are evidences that the manipulation of the gut microbiome through diet modulations, can alter the disease conditions such as those in Crohn’s disease and Inflammatory Bowel Disease (Mai, et al., 2007). Studies that use the \( Apc^{Min/+} \) mouse model for studying gut diseases and their association with microbiota often report significant changes in microbiome profiles of the large intestine, but also report that
differences in the polyps is only significant in the small intestine (Mai, et al., 2007) The detrimental effects are also brought about via loss of certain colonizing bacterial species that are dependent on other colonizers for processes such as nutritional interactions and removal of secondary metabolites and toxic waste products (Belenguer, et al., 2006), or through loss of useful co-evolved processes brought about by the host-microbial co-dependence (Atarashi & Honda, 2011; Ferreira, et al., 2011). There are no studies that study the gut microbiome with respect to gut inflammation and tumorigenesis in an age/time-dependent manner. It has been seen that specific pathogen-free (SPF) Apc\textsuperscript{Min/+} exhibit a higher tumor load and anemia with a higher infiltration of inflammatory cells specifically at advanced stages as compared to germ-free animals, indicating that a mere modulation of gut microbiome profiles can abrogate the disease condition in the gut (Y. Li, et al., 2012).

**Gut microbiome, inflammatory cytokines and tumorigenesis**

It is a fact that till date, one of the most effective class of chemopreventive agents is the non-steroidal anti-inflammatory drugs (NSAIDs). The induction of cancer in the gut by chronic inflammation has always indicated towards the importance of underlying altered inflammatory processes (Zhang, et al., 2015). Different agents that have been documented to be effective against tumors and cancers are the agents that function by downregulating important inflammatory pathways and pro-inflammatory cytokines. Apart from the usual NSAIDs that function by downregulating the metabolism of the arachidonic acid through the Cyclooxygenase (COX) pathways agents such as curcumin and quercetin exert their
chemopreventive effects through the downregulation of mRNA expression levels of pro-inflammatory cytokines such as IL1-β, IL-6 and TNF-α, and reduction of macrophage infiltration (Y. Li, et al., 2012; Murphy, Davis, McClellan, & Carmichael, 2011; Murphy, Davis, McClellan, Gordon, et al., 2011; Ritland, et al., 1999).

It has also been shown that the intestinal commensal bacteria plays a role in the development of gut microenvironmental immune system comprising both the humoral and cellular components and maintains the protective steady state immune function throughout life (Cebra, 1999; Talham, et al., 1999). It may be more appropriate to say that any alteration in the composition of the gut microbiome will lead to a dysfunctional immune system.

Certain bacterial species such as *B. thetaiotaomicron* bacterium have been associated with the attenuation of pro-inflammatory responses by reducing the levels of the pro-inflammatory cytokine IL-8 (Zhao, et al., 2001).

Comparative numbers of certain bacteria often lead to upregulation or downregulation of certain inflammatory pathways. The systemic release of microbial products as a result of the manipulation of the gut epithelium invokes an IL-23 response and a further IL-17 response in order to neutralize further invasion by the microbes, and the process is brought about by the *Firmicutes* bacteria *Clostridia sp.* (Shih, et al., 2014). The bacteria especially promotes IL-17 cytokine in the small intestine which is the primary site of its attachment and survival (Omenetti & Pizarro, 2015). These Th17 cells may become autoreactive in case of intestinal epithelium insult. The bacteria *Bacteroides fragilis* from the *Bacteroidetes* phylum leads to an increase in the production of IL-10.
cytokine (Omenetti & Pizarro, 2015), whereas its strains have been implicated in the production of the pro-inflammatory cytokine IL-17-dependent inflammation-related colon cancer (W. Wu, et al., 2015).

It is widely accepted that inflammation contributes to the development of cancer and inflammatory cells have been noted in and around tumors (Hanahan & Weinberg, 2011). A relationship between inflammation and intestinal neoplasia is supported by the facts that inflammatory bowel disease predisposes patients to intestinal carcinomas and that the anti-inflammatory drugs aspirin (Baron, et al., 2003), celecoxib (Bertagnolli, et al., 2006), and rofecoxib (Bertagnolli, et al., 2006), all have proven efficacy in preventing human colorectal adenoma development.

Adenoma formation has been shown to increase following adoptive transfer of pro-inflammatory lymphocytes (Rao, et al., 2006) and decreased after the adoptive transfer of anti-inflammatory regulatory T-cells (Treg cells) (Erdman, et al., 2005). Apart from direct effects of inflammatory cells, adenomas are also known to be impaired by the absence of the molecule MyD88 which mediates the downstream inflammatory signaling as a response to bacterial and viral products (Rakoff-Nahoum & Medzhitov, 2007). Pro-inflammatory pathways such as those involved with IL-17 secretion are associated with many chronic inflammatory conditions such as asthma (Schnyder-Candrian, et al., 2006) and inflammatory bowel disease (Yen, et al., 2006), along with colon carcinogenesis (Langowski, et al., 2006). IL-17, especially, has been denoted as a marker and mediator of tumor angiogenesis (Langowski, et al., 2006). Over-expression of pro-inflammatory factors such as IL-6, MCP-1, NF-κB and IL-8 can aggravate tumors (Wang & Zhang, 2015).
In the context of the \( Apc^{Min/+} \) mouse model, the mutual interaction of macrophages with cancer cells enhances production of inflammatory cytokines such as IL-1, IL-6 and TNF-\( \alpha \) that transform the tumor microenvironment so that it favors the survival, growth and motility of cancer cells. Reduction in pro-inflammatory molecules such as MCP-1 leads to a decrease in the total polyp number and size in the \( Apc^{Min/+} \) mouse along with downregulating inflammatory processes in tumor tissues and surrounding tumor microenvironment (McClellan, Davis, Steiner, Day, et al., 2012). An elevation in the intestinal inflammatory cytokine (MCP-1, IL-1\( \beta \), IL-6 and TNF-\( \alpha \)) response occurs at 12 weeks of age as a result of the rapid increase in polyp number, which are further elevated with increase in polyp size. A similar study by McClellan et al (2012) (McClellan, Davis, Steiner, Enos, et al., 2012) shows an increased mRNA expression of MCP-1, IL-1b, IL-6 and TNF-\( \alpha \) that is evident at 12 weeks of age and is consistent with the increase in polyp number that occurs at this time. After the age of 12 weeks which is associated with the largest number of small polyps, the inflammatory response continues to increase while the number of polyps remain constant which indicates that the inflammatory response after 12 weeks could largely be driven by polyp size. However, it is still unclear whether the changes in polyp number and size that occurs in this model is a result of the elevated inflammatory response or vice versa (McClellan, Davis, Steiner, Enos, et al., 2012).

16S rRNA gene profiling and sequencing

The 16S rRNA gene method is one of the earliest and most widely used methods used for phylogenetic, taxonomic and bioinformatics analyses. It targets the 16S rRNA bacterial
genes to know diversity or similarities in biological samples. The 16S rRNA gene provides many advantages in microbiome analyses including its universal distribution in bacterial species, relative stability in evolution and ideal size (1500 bp). Consisting of both constant and variable regions, the 16S gene is easy to amplify used broad-range primers used against regions flanking its variable regions (Sankar, et al., 2015).

**Statistical Power of the study**

The microbiome analysis in our study will be done using the fecal samples from experimental mice. A 16S rRNA gene sequencing technique will be used to study the gut microbial populations in the animals. The sample size for the study is 4. Some of the previous studies that have been directed towards similar microbial analyses have used similar samples sizes. Russell et al (2012) (Russell, et al., 2012) used n = 3-5 to study changes in the gut microbial communities in C57BL/6 mice following antibiotic treatment at neonatal and adult stages of life. The results showed significant reductions in the overall bacterial diversity and phyla abundance in antibiotic-treated mice as compared to control mice (p value<0.05). Oh et al (2016) (Oh, et al., 2016) used a sample size of 5 mice per group in order to show significant differences in bacterial load and diminished rDNA in antibiotic-treated mouse fecal samples as compared to their control counterparts. Similar studies have used n = 4-6 mice per group in order to show Significant (p value<0.01) changes in gut microbial constituents as a result of administration of the antibiotic enrofloxacin with an increase in type-2 cytokines has been shown in a study that used 4 to 6 (n) mice per group (Strzepa, et al., 2016).
PRELIMINARY DATA

Preliminary data for Aim 1:

Chronic inflammation and colon cancer was induced in Dextran Sodium Sulphate (DSS) + DMH treatment group in both APNKO (n=5) and WT (n=5) mice by administering DSS dissolved in their drinking water for five days followed by five days of regular drinking water along with administering a single injection of DMH intraperitoneally once a week for 12 weeks at a concentration of 20mg/kg body weight of mice at the beginning of DSS treatment. Fecal samples were collected at the time of sacrifice and were analyzed by sequencing the V3 variable region of bacterial 16S rRNA gene to profile the composition of the microbiome (Figure 1).
Figure 1. Bacterial phylum profiles are altered during cancer development. The figure illustrates distribution of different bacterial phyla (percentages) in colons tissues of WT (C57BL/6) and APNKO mice belonging to control and DSS+DMH treatment groups.

**Preliminary data for Aim 2:**

Clinical score which is a summation of weight loss, diarrhea and fecal hemoccult was determined during the length of the study thrice a week till the date of sacrifice (day 153). We found a significant increase in the clinical score of the APNKO mice on day 30 and then at day 67 which remained significantly high till the end of the study as compared to WT mice given the same treatment (Figure 2).
Figure 2. Clinical score of increases during tumor development. Clinical score for DSS+DMH treatment was plotted against different time points of the study. The score was calculated out of a total of twelve points; four points each for the observations of weight loss, diarrhea and hemoccult. Two-way repeated measure analysis of variance (ANOVA) was applied to calculate the significant difference between the clinical score for APNKO and WT mice throughout the length of the study. **p<0.05, #p<0.001, *p<0.03.

The data shown in figures 1 and 2 demonstrate that there may be a correlation between the abundance of Firmicutes bacterial phylum and clinical score such that an increased abundance of Firmicutes may be associated with disease severity in colon cancer.

Mice were sacrificed on day 153 and the colon was excised and flushed with PBS. Colon was cut open longitudinally and stained with 2% methylene blue to count the tumor number and tumor area. Significant increase in the tumor number and area was found in the APNKO mice as compared to the WT mice (Figure 3).
Figure 3. Tumor development in APNKO and WT mice treated with DSS+DMH. (A) Average number of tumors counted per mice per group. (B) Average tumor area in mm² counted for each mouse per group plotted in the order of their severity. One-way ANOVA was used to calculate significant difference between APNKO and WT mice. *p<0.05.

The data shown in figures 1 and 3 demonstrate that there may also be a correlation between the abundance of *Firmicutes* bacterial phylum and tumor number and size such that an increased abundance of *Firmicutes* may be associated with a higher tumor load in colon cancer.

Mice colon were harvested in 2 mm² sections and were embedded in embedded in paraffin. 5 µm sections were cut using a microtome. Standard deparaffinization procedure was followed using xylene and gradation of ethanol. Alcian blue solution (1 %) of pH 2.5 in 3 % acetic acid and nuclear fast red in aluminum sulfate was prepared. Tissues were stained with Alcian blue and counterstained with nuclear fast red solution. Goblet to epithelial cell ratio was counted per crypt with ten crypts per section and five sections per group (Figure 4). The staining revealed a reduction in the number of mucus-producing goblet cells in the DSS+DMH group both in APNKO and WT groups.
Figure 4. Goblet to epithelial cell ratio is decreased in colon cancer. Descending colon, 2 mm² sections of the mice stained with Alcian blue dye representing goblet cells (blue) in APNKO and WT groups belonging to Control and DSS+DMH groups.

The data shown in figures 1 and 4 may indicate a correlation between the abundance of *Firmicutes* bacterial phylum and a decreased expression of mucus-producing phenotype of the cells lining the gut epithelium which renders a reduced protection from epithelial insult and inflammation during cancer.
**Preliminary data for Aim 3:**

Mice colon paraffinized sections were cut using a microtome into 5 µm thickness. Standard deparaffinization procedure was followed using xylene and gradation of ethanol. Hematoxylin and Eosin staining was performed to highlight the general tissue structure (Figure 5). The DSS+DMH group revealed increased cellular infiltration into the colonic mucosal layers indicative of an increased inflammatory index.

Figure 5. Inflammation and infiltration of colon epithelium. H&E stained sections of the descending colon of APNKO and WT mice in DSS+DMH group with 20X magnification (scale bar=120 µm).
RESEARCH DESIGN AND METHODS

Overall Research Design: The overall purpose of the present study is to elucidate the role of alteration in the gut microbiome on disease severity in an intestinal cancer model of mouse. The study will firstly comprise of a preliminary data on characterizing the alterations in the gut microbiome of the $Apc^{Min/+}$ mouse. An external manipulation of the microbiome with the help of an antibiotic cocktail will then be used in order to control the microbiome manipulation cycles and accurately understanding the role of the microbial alteration on health, tumorigenesis, mucus production and inflammation. So far, no study has demonstrated these effects brought about by an altered gut microbiome throughout the development of tumor.

The first aim of the study will be directed towards examining the composition of gut microbiome during tumor initiation and development in $Apc^{Min/+}$ mouse. This aim will act as preliminary to aims 2 and 3 by characterizing and thereby defining the alterations occurring in the microbiome of $Apc^{Min/+}$ mouse during initiation and progression of tumorigenesis. The characterization of the gut microbiome in the experimental $Apc^{Min/+}$ mice will help define the abundance of different bacterial species in the gut and their relative diversity values during the initiation and progression of tumorigenesis.

The second aim of the study will be directed towards elucidating the role of a manipulated microbiome on pathology of the experimental animals. Antibiotics will be administered to experimental mice in order to perturb the gut microbiota during the initial and progressive stages of tumorigenesis. This aim will thus help in elucidating the effect
of alterations in microbiome in terms of their abundance and diversity on the disease condition, tumorigenesis and goblet cell expression levels in the animals.

The third aim of the present study will demonstrate the effects of microbial manipulation on the inflammatory status of the experimental animals. In the first part of aim 3 we will study the effects of an altered microbiome on tissue-localized inflammation and infiltration occurring at the level of intestinal mucosa. The second part of the aim will help study the effects of microbiome alteration on intestine-secreted pro-inflammatory cytokines. Whether the inflammatory effect is systemic or not, will be elucidated through the third part of this aim where systemic levels of pro-inflammatory cytokines will be measured.

A timeline for the present study is provided below:

![Study timeline and time points](image)

Figure 6. Study timeline and time points.

**AIM 1: To examine the composition of gut microbiome during tumor initiation and development in $Apc^{Min/+}$ mouse.**
Rationale: An altered gut microbiome can cause inflammation ranging from acute bouts to chronically inflamed gut. An altered microbiome, which shifts from symbiotic to a more inflammation-inducing phenotype, leads to a vicious cycle of erosion of the gut epithelial lining that, in turn, leads to more and more inflammatory processes taking place inside the gut microenvironment (Saxena, et al., 2012; Zhang, et al., 2015). Our previous study on chronic inflammation-induced colon cancer in the APNKO (Adiponectin-knockout) mice demonstrated microbiota profiles that were clearly altered during tumorigenesis. The alterations mainly pertained to the altered percentages of the *Firmicutes* and *Bacteroidetes* bacterial phyla during inflammation-induced tumorigenesis in the mice (Figure 1). As opposed to externally administered chemically-induced tumorigenesis in mice, the microbiota has not been studied with respect to a spontaneously induced tumorigenesis such as that in the *Apc*\(^{Min/+}\) mouse. It was recently concluded that an alteration of gut microbiome precedes polyposis in the *Apc*\(^{Min/+}\) mouse (Son, et al., 2015). Also, it has been seen that specific pathogen-free (SPF) *Apc*\(^{Min/+}\) exhibit a higher tumor load and anemia with a higher infiltration of inflammatory cells specifically at advanced stages as compared to germ-free animals, indicating that a mere modulation of gut microbiome profiles can abrogate the disease condition in the gut (Y. Li, et al., 2012). However, there are no studies that study the gut microbiome with respect to gut inflammation and tumorigenesis in an age/time- dependent manner. Therefore, the first aim of the present study will be directed towards characterization of alterations of microbiome profiles from early to later stages of tumorigenesis in an *Apc*\(^{Min/+}\) mouse model.

AIM 1.1: To study the gut microbiome profiles of *Apc*\(^{Min/+}\) mice during intestinal tumor initiation.
AIM 1.2: To study the gut microbiome profiles for $Apc^{Min/+}$ mice during intestinal tumor progression.

AIM 1.3: To study the gut microbiome profiles of $Apc^{Min/+}$ mice during peak of intestinal tumorigenesis.

**Experimental Design Specific Aim 1:** Aim 1 will study the composition of the gut microbiome during initiation and development of tumorigenesis in $Apc^{Min/+}$ mouse model. Four weeks old male $Apc^{Min/+}$ and C57BL/6 (WT) mice will be housed at the Animal Resource Facility, USC. Food (Purina chow) and drinking water will be available to the mice ad libidum under a 12:12 hour light-dark cycle and a low-stress environment (22°C, 50% humidity and low noise). Cumulative clinical scores will be obtained for each mouse twice a week with a maximum clinical score of 12. The clinical score for each mouse will be based on weight loss measurement, diarrhea and fecal hemoccult. The experimental animals will be sacrificed at 8, 12 and 16 weeks as per figure 6, without any antibiotic treatment, and microbiome profiles will be studied using fecal samples at each of the three time points.

**Animal Model and Handling:** Four weeks old $Apc^{Min/+}$ mice will be obtained from the Jackson Laboratories and bred at the Animal Resource Facility at University of South Carolina. Food (Purina chow) and drinking water will be available to the mice ad libidum under a 12:12 hour light-dark cycle and a low-stress environment (22°C, 50% humidity and low noise). All animal care followed institutional guidelines under a protocol approved by the Institutional Animal Care and Use Committee at the University of South Carolina. Cumulative clinical scores will be obtained for each mouse twice a week with a maximum
clinical score of 12. The clinical score for each mouse will be based on weight loss measurement, diarrhea and fecal hemoccult with a maximum score of 4 within each of the three quantitative parameters. $Apc^{Min/+}$ and WT mice will be randomLy assigned to three groups (n=4): 1) 8 weeks; 2) 12 weeks; 3) 16 weeks.

Mice will be monitored throughout the course of study for weight loss, diarrhea and fecal hemoccult. Mice will sacrificed at the three different time points by cervical dislocation. Blood will be collected before sacrifice through retro-orbital puncture, centrifuged at 10,000 rpm for 15 minutes and serum will be isolated and stored at -20°C. Small intestine and colon will be excised and flushed clean with PBS. 2 mm$^2$ colon tissue sections will be fixed in 10% formalin and after 24 hours will be replaced with 70% ethanol followed by paraffin embedding and sectioning to obtain 5 µm thin sections on glass slides. 2 mm$^2$ intestine and colon tissue sections will be incubated in RPMI medium at 37°C for 24 hours followed by centrifugation at 2500 rpm for 15 minutes. Supernatant will be obtained and stored at -20°C for tissue-secreted cytokine expression analyses. The rest of tissues will be stored at -80°C for further usage. Fecal samples will be snap frozen at the time of sacrifice for microbiome analysis.

**Material and methods:**
Clinical score

Clinical score will be measured for each mouse twice during each week of the study. Mice will be sacrificed after the last clinical score measurement. Cumulative clinical scores will be obtained for each mouse twice a week with a maximum clinical score of 12. The clinical score for each mouse will be based on weight loss measurement, diarrhea and fecal hemoccult (kit, BECKMAN COULTER) with a maximum score of 4 within each of the three quantitative parameters. The clinical score will be calculated by the following method:

Weight loss -  
0 = 0-5% weight loss;  
1 = 6-10% weight loss;  
2 = 11-15% weight loss;  
3 = 16-20% weight loss;  
4 = >20% weight loss.

Diarrhea -
0 = well-formed pellets,
2 = pasty and semi-formed stools that do not adhere to anus
4 = liquid stools that adhere to the anus

Fecal Hemoccult - 0 = no blood, 2 = positive hemoccult,
4 = gross bleeding

Table 1. Clinical score measurement criteria

Tissue and serum collection

Mice will sacrificed at the three different time points by cervical dislocation. Blood will be collected before sacrifice through retro-orbital puncture, centrifuged at 10,000 rpm for 15 minutes and serum will be isolated and stored at -20°C. Small intestine and colon will be excised and flushed clean with PBS. 2 mm² small intestine and colon tissue sections will be fixed in 10% formalin and after 24 hours will be replaced with 70% ethanol followed
by paraffin embedding and sectioning to obtain 5 µm thin sections on glass slides. 2 mm² intestine and colon tissue sections will be incubated in RPMI medium at 37°C for 24 hours followed by centrifugation at 2500 rpm for 15 minutes. Supernatant will be obtained and stored at -20°C for tissue-secreted cytokine expression analyses. The rest of tissues will be stored at -80°C for further usage. Fecal samples will be snap frozen at the time of sacrifice for microbiome analysis.

16S rRNA-based Metagenomics

At sacrifice, fecal samples will be obtained from the experimental mice to perform bacterial (luminal) microbiome analysis. Bacterial n16S rRNA gene amplification and sequencing will be performed at the Alkek Center for Metagenomics and Microbiome Research, Department of Molecular Virology and Microbiology, Baylor College of Medicine (Houston, TX). Polymerase Chain Reaction (PCR) amplification of the V4 region of the bacterial 16 S rRNA gene will be performed using the Illumnia Miseq sequencer. The consensus sequences obtained from the analysis will be mapped into Operational Taxonomic Units (OTUs). Alpha- and beta-diversity values will be obtained based on the OTUs’ relative abundance table. Chao1 (estimator of richness) and Shannon Diversity Index (richness and evenness) will be used to obtain the alpha diversity values. Weighted Unifrac (dissimilarity based on phylogenetic differences and taxonomic abundance) and Unweighted Unifrac (dissimilarity based on phylogenetic differences but not abundance) will be performed to obtain the beta-diversity values for the experimental samples (He, et al., 2016). Kruskal Wallis and Mann Whitney statistical analyses will be performed to
calculate significance in diversity and relative abundance respectively, by comparing
different treatments and disease stages. A p value of less than 0.05 will be considered
significant.

**Primary Outcomes:**

*Alpha diversity*

The metagenomics Chao1 and Shannon indices will be used to measure the alpha diversity
values for different groups of experimental animals. Alpha diversity values will be used to
measure total species richness within the individual fecal samples (Weir et al., 2013).

*Beta diversity*

The metagenomics Unifract index will be used to measure the beta diversity values among
the different experimental groups. The beta diversity values will be used to measure the
degree of diversity among the different experimental groups and thus will give a measure
of difference in diversity between different groups (Weir, et al., 2013).

*Relative abundance of different bacterial phyla*

The relative abundance of different phyla will be plotted for each of the experimental
groups in order to characterize the dominance of one or more phyla during initiation and
development of tumorigenesis and under normal conditions.
AIM 2: To determine if gut microbiome manipulation can regulate $Apc^{Min/+}$ mouse health and tumorigenesis.

**Rationale:** The mucus layer tends to thickness with increased diversity of the microbiota (Jakobsson, et al., 2015). Our preliminary data from a study on chronic inflammation-induced colon cancer using APNKO mice suggested that the gut microbiota changes in a way that favors an increased percentage of bacteria of certain phyla (here, *Firmicutes*) as compared to others (such as *Bacteroidetes*). It is not been studied yet as to whether there is a relation between the altered percentages of especially the *Firmicutes* and *Bacteroidetes* phyla populations, and tumor number, tumor size and goblet cell numbers. It is known that the mucus layer provides a source of nutritional carbon and therefore energy to some of the intestinal flora that are able to lyse the glycans present in the mucus, making the inter-relationship between the gut bacteria and the mucus layer really important (H. Li, et al., 2015). Also, the metabolites produced by these microbes also influence the differentiation and function of the epithelial and immune cells in the intestinal mucosa (Kato, et al., 2014). The regulation of the mucin gene expression as well as mucus production have been shown to be two levels at which the gut microbial community may modulate mucins (Comelli, et al., 2008). Our previous study (Saxena, et al., 2013) also suggested that a reduction in the secretory mucin profile is associated with an inflammatory phenotype of the gut (Figure 4). Therefore, the present aim will be directed at knowing the mucus-producing phenotypes of the cells lining the gut of the experimental animals in relation to the changing profiles of the gut microbiota.

AIM 2.1: To determine if gut microbiome manipulation effects intestinal tumor number and size in small intestine and colon of $Apc^{Min/+}$ mice.
AIM 2.2: To determine if gut microbiome manipulation effects goblet cell numbers in small intestine and colon of \(Apc^{Min/+}\) mice as compared to C57BL/6 mice.

AIM 2.3: To determine if gut microbiome manipulation leads to a differential recolonization of \(Bacteroidetes\) and \(Firmicutes\) populations of bacteria \(Apc^{Min/+}\) mice as compared to C57BL/6 mice.

**Experimental Design Specific Aim 2:** Aim 2 will study the effects of manipulation of the gut microbiome primarily on health in terms of disease severity and tumorigenesis in terms if tumor number and size. Secondarily, aim 2 will also be directed towards quantifying the number of goblet cells in the small intestine and colon of the experimental animals.

The first part of aim 2 will be to study the role of manipulation of microbiota on the health of the experimental animals. The gut health of the animals will be monitored by clinical score which will be calculated in the same way as that in aim 1.

The second part of aim 2 will decipher the role of an altered microbiome via administration of antibiotics to the animals, on tumor number and size throughout the process of tumorigenesis.

The third part of aim 2 will be directed towards determining the role of microbiome manipulation on the expression of mucus-producing goblet cells in the epithelium of small intestine and colon of the experimental animals.

Aim 2 will provide a mechanistic way of deciphering the role of an altered microbiome on three of the processes which define a disease state pertaining to the gut.
Animal Model and Handling: 4 weeks old $Apc^{Min/+}$ and C57BL/6 (WT) mice will be obtained from Jackson Laboratories and bred in-house at the Animal Resource Facility, University of South Carolina. Food (Purina chow) and drinking water will be available to the mice ad libidum under a 12:12 hour light-dark cycle and a low-stress environment (22°C, 50% humidity and low noise). The $Apc^{Min/+}$ and C56BL/6 mice will be assigned to the following groups: WT-Control (no antibiotic)-8 weeks, WT-Antibiotic (ABT)-8 weeks, WT-Control-12 weeks, WT-ABT-12 weeks, WT-Control-16 weeks, WT-ABT-16 weeks, $Apc^{Min/+}$-Control-8 weeks, $Apc^{Min/+}$-ABT-8 weeks, $Apc^{Min/+}$-Control-12 weeks, $Apc^{Min/+}$-ABT-12 weeks, $Apc^{Min/+}$-Control-16 weeks and $Apc^{Min/+}$-ABT-16 weeks. All animal care will follow institutional guidelines under a protocol approved by the Institutional Animal Care and Use Committee at the University of South Carolina. Cumulative clinical scores will be obtained for each mouse twice a week with a maximum clinical score of 12. The clinical score for each mouse will be based on weight loss measurement, diarrhea and fecal hemoccult with a maximum score of 4 within each of the three quantitative parameters. The $Apc^{Min/+}$-Control animals to be used in aim 2 will be the same as those used in aim 1.
Mice will be monitored throughout the course of study for weight loss, diarrhea and fecal hemoccult. Mice will sacrificed at the three different time points by cervical dislocation. Blood will be collected before sacrifice through retro-orbital puncture, centrifuged at 10,000 rpm for 15 minutes and serum will be isolated and stored at -20°C. Small intestine and colon will be excised and flushed clean with PBS. 2 mm2 colon tissue sections will be fixed in 10% formalin and after 24 hours will be replaced with 70% ethanol followed by paraffin embedding and sectioning to obtain 5 µm thin sections on glass slides. 2 mm2 intestine and colon tissue sections will be incubated in RPMI medium at 37°C for 24 hours followed by centrifugation at 2500 rpm for 15 minutes. Supernatant will be obtained and stored at -20°C for tissue-secreted cytokine expression analyses. The rest of tissues will be stored at -80°C for further usage. Fecal samples will be snap frozen at the time of sacrifice for microbiome analysis.

**Material and methods:**

**Antibiotic administration:** An antibiotic mixture containing Vancomycin, Neomycin and Streptomycin (each 1 mg/mL) will be administered to experimental mice under the antibiotic treatment groups. While neomycin and streptomycin act against Gram-negative bacteria, vancomycin is used against Gram-positive bacteria. The antibiotics mixture will
be administered to the mice through drinking water starting at the 5th, 10th and 14th weeks for 10 days followed by normal drinking water.

**Clinical score:** Clinical score will be measured for each mouse twice during each week of the study. Mice will be sacrificed after the last clinical score measurement. Cumulative clinical scores will be obtained for each mouse twice a week with a maximum clinical score of 12. The clinical score for each mouse will be based on weight loss measurement, diarrhea and fecal hemoccult (kit, BECKMAN COULTER) with a maximum score of 4 within each of the three quantitative parameters. Score for the weight loss is based on the following published scale where 0 = 0–5% weight loss; 1 = 6–10% weight loss; 2 = 11–15% weight loss; 3 = 16–20% weight loss; and 4 = >20% weight loss. Scoring of diarrhea is as follows: 0 = well-formed pellets, 2 = pasty and semi-formed stools that do not adhere to the anus, 4 = liquid stools that adhere to the anus. Detection of blood in the stools will be determined using hemoccult kit (BECKMAN COULTER). The higher intensity of blue color indicates greater bleeding. The followings are the score rates for the fecal hemoccult: 0 = no blood, 2 = positive hemoccult, 4 = gross bleeding. The total clinical score will be the summation of the individual score of weight loss, diarrhea and fecal hemoccult. The maximum score a mouse could get is 12.

**Tissue and serum collection:** Mice will sacrificed at the three different time points by cervical dislocation. Blood will be collected before sacrifice through retro-orbital puncture, centrifuged at 10,000 rpm for 15 minutes and serum will be isolated and stored at -20°C.
Small intestine and colon will be excised and flushed clean with PBS. 2 mm² small intestine and colon tissue sections will be fixed in 10% formalin and after 24 hours will be replaced with 70% ethanol followed by paraffin embedding and sectioning to obtain 5 µm thin sections on glass slides. 2 mm² intestine and colon tissue sections will be incubated in RPMI medium at 37°C for 24 hours followed by centrifugation at 2500 rpm for 15 minutes. Supernatant will be obtained and stored at -20°C for tissue-secreted cytokine expression analyses. The rest of tissues will be stored at -80°C for further usage. Fecal samples will be snap frozen at the time of sacrifice for microbiome analysis.

**Tumor Number and Area:** Small intestine and colon from the experimental animals will be excised and flushed with PBS. Tumor number and area will be counted using 1% Methyl Blue stain under the light microscope for all mice in different groups and significant difference will be calculated.

**Alcian Blue staining:** Standard deparaffinization procedure will be followed using xylene and gradations of ethanol. Alcian Blue dye solution for staining mucus-containing goblet cells will be prepared by dissolving the dye powder (8GX) at 1% concentration in 3% acetic acid solution and setting the pH at 2.5. Nuclear Fast Red solution for staining the epithelial cells of the mucosa will be prepared at a final concentration of 0.1% dissolved in 5% aluminium sulfate solution. Tissues will firstly be stained with Alcian Blue and then counterstained with Nuclear Fast Red solution. Goblet to epithelial cell ratio will be counted per crypt with ten crypts per section and five sections per group.
**Statistical analysis:** Two-way analysis of variance (ANOVA), Two-way repeated measure ANOVA and One-way ANOVA will be used to analyze the data with Tukey post hoc-analyses. A p<0.05 will be considered statistically significant. All the statistical analyses will be done using SigmaStat 3.5 (SPSS, Chicago, IL).

**PRIMARY OUTCOMES:**

**Clinical score**

Clinical score will be measured for each mouse twice during each week of the study. Mice will be sacrificed after the last clinical score measurement. Cumulative clinical scores will be obtained for each mouse twice a week with a maximum clinical score of 12. The clinical score for each mouse will be based on weight loss measurement, diarrhea and fecal hemoccult (kit, BECKMAN COULTER) with a maximum score of 4 within each of the three quantitative parameters. Score for the weight loss is based on the following published scale where 0 = 0–5% weight loss; 1 = 6–10% weight loss; 2 = 11–15% weight loss; 3 = 16–20% weight loss; and 4 = >20% weight loss. Scoring of diarrhea is as follows: 0 = well-formed pellets, 2 = pasty and semi-formed stools that do not adhere to the anus, 4 = liquid stools that adhere to the anus. Detection of blood in the stools will be determined using hemoccult kit (BECKMAN COULTER). The higher intensity of blue color indicates greater bleeding. The followings are the score rates for the fecal hemoccult: 0 = no blood, 2 = positive hemoccult, 4 = gross bleeding. The total clinical score will be the summation of the individual score of weight loss, diarrhea and fecal hemoccult. The maximum score
a mouse could get is 12. The clinical score will be studied as an effect due to the perturbation of microbiome.

**Tumor number and area**

Small intestine and colon from the experimental animals will be excised and flushed with PBS. Tumor number and area will be counted using 1% Methyl Blue stain under the light microscope for all mice in different groups and significant difference will be calculated. Tumor number and/or area will also be correlated with the manipulation of the microbiome.

**Secondary outcomes:**

**Goblet cells**

Goblet cells counted through Alcian Blue staining will form a secondary outcome of aim 2 in the present study. Since goblet cells form a mucus-producing phenotype of the gut lining, their presence is associated with better prognosis of disease. A manipulation of microbiome may work to reduce the numbers of goblet cells in small intestine and colon.

**AIM 3: To determine if a change in relative abundance Bacteroidetes and Fimicutes population regulate(s) systemic inflammation in Apc$^{Min/+}$ mice.**

**Rationale:** The induction of cancer in the gut by chronic inflammation has always indicated towards the importance of underlying altered inflammatory processes (Zhang, et al., 2015). Different agents that have been documented to be effective against tumors and
cancers are the agents that function by downregulating important inflammatory pathways and pro-inflammatory cytokines such as IL1-β, IL-6 and TNF-α, and reduction of macrophage infiltration (Y. Li, et al., 2012). It has also been shown that the intestinal commensal bacteria plays a role in the development of immune system of the gut microenvironment comprising both the humoral and cellular components and maintains the protective steady state immune function throughout life (Cebra, 1999). It may be more appropriate to say that any alteration numbers of certain bacteria often lead to upregulation or downregulation of certain inflammatory pathways. The systemic release of microbial products as a result of the perturbation of the gut epithelium invokes an IL-23 response and a further IL-17 response in order to neutralize further invasion by the microbes, and the process is brought about by the Firmicutes bacteria Clostridia sp. (Shih, et al., 2014). The bacteria especially promotes IL-17 cytokine in the small intestine which is the primary site of its attachment and survival (Omenetti & Pizarro, 2015). These Th17 cells may become autoreactive in case of intestinal epithelium insult. The bacteria Bacteroides fragilis from the Bacteroidetes phylum leads to an increase in the production of IL-10 cytokine, whereas its strains have been implicated in the production of the pro-inflammatory cytokine IL-17-dependent inflammation-related colon cancer (W. Wu, et al., 2015). Inflammation, which is an integral part of tumorigenesis should therefore, may strongly be correlated with a reduced bacterial diversity or numbers.

AIM 3.1: To examine if a change in Bacteroidetes and Firmicutes populations regulate intestinal goblet cell numbers in Apc\(^{Min/+}\) mice.

AIM 3.2: To examine if a change in Bacteroidetes and Firmicutes populations regulate systemic levels of cytokines IL-6 and TNF-α in Apc\(^{Min/+}\) mice.
Experimental Design Specific Aim 3: The experimental design for aim 3 will be the same as that for aim 2. The experimental animals and groups will be the same as those used for aim 2.

The first part of aim 3 will be directed towards elucidating the effect of an altered microbiome on the process of inflammation localized at the level of epithelial lining in the form of intestinal insult and undesirable infiltration of immune cells at the gut lining.

The second part of aim 3 will elucidate an inflammatory effect of microbiome dysbiosis which can be studied through the measuring tissue-secreted cytokines as a result of the aforesaid localized inflammation.

The third part of aim 3 is aimed at studying an inflammatory response of the body to microbiome dysbiosis at a systemic level.

Material and methods:

Tissue and serum collection: The experimental tissue and serum collected in aim 1 and 2 will also be used in aim 3.

16S rRNA-based Metagenomics

At sacrifice, fecal samples will be obtained from the experimental mice to perform bacterial (luminal) microbiome analysis. Bacterial n16S rRNA gene amplification and sequencing will be performed at the Alkek Center for Metagenomics and Microbiome Research, Department of Molecular Virology and Microbiology, Baylor College of Medicine (Houston, TX). Polymerase Chain Reaction (PCR) amplification of the V4 region of the
bacterial 16S rRNA gene will be performed using the Illumnia Miseq sequencer. The consensus sequences obtained from the analysis will be mapped into Operational Taxonomic Units (OTUs). Alpha- and beta-diversity values will be obtained based on the OTUs’ relative abundance table. Chaol (estimator of richness) and Shannon Diversity Index (richness and evenness) will be used to obtain the alpha diversity values. Weighted Unifrac (dissimilarity based on phylogenetic differences and taxonomic abundance) and Unweighted Unifrac (dissimilarity based on phylogenetic differences but not abundance) will be performed to obtain the beta-diversity values for the experimental samples (He, et al., 2016). Kruskal Wallis and Mann Whitney statistical analyses will be performed to calculate significance in diversity and relative abundance respectively, by comparing different treatments and disease stages. A p value of less than 0.05 will be considered significant.

**Hematoxylin and Eosin staining:** Paraffin embedded 5 µm sections from aim 2 will be used for performing Hematoxylin and Eosin staining in order to determine the morphology of small intestine and colon epithelial layer. Histopathology will be quantified visually in a double-blind condition using light microscopy. Localized inflammatory status will be indicated by inflammatory cells present in the mucosal layers of intestine and colon. Aberrant villi and crypts will also be used as indicators of intestinal and colonic insults due to inflammatory response.
**Enzyme-linked Immunosorbent Assay (ELISA):** Supernatents obtained from 2 mm² small intestinal tissues kept in RPMI for 24 hours at 37°C will be used to perform ELISA to quantify small-intestinal secreted pro-inflammatory cytokines IL-1β, IL-17 and TNF-α.

Serum samples obtained from the experimental animals at the time of sacrifice will be used to quantify systemic levels inflammatory response by measurement of systemic levels of pro-inflammatory cytokines IL-1β, IL-17 and TNF-α.

**Statistical Analysis:** Two-way analysis of variance (ANOVA), Two-way repeated measure ANOVA and One-way ANOVA will be used to analyze the data with Tukey post hoc-analyses. A p<0.05 will be considered statistically significant. All the statistical analyses will be done using SigmaStat 3.5 (SPSS, Chicago, IL).

**Primary Outcomes:**

*Relative abundance of Firmicutes and Bacteroidetes*

The inflammatory status is often associated with the abundance of *Firmicutes* population that tends to further increase the levels of inflammation. Thus, quantification of *Firmicutes* will be an important primary outcome of aim 3.

*Visually quantifiable levels of mucosal inflammation*
Abnormal infiltration by immune cells at the mucosal layer can be used as a measure to determine the effect of an altered microbiome on an inflammatory response at the mucosal level.

**Tissue-localized inflammatory response**

Certain measurable quantities of pro-inflammatory cytokines can be secreted locally by the intestinal tissue as a response to either the localized mucosal layer infiltration by immune cells or due to increased levels of systemic inflammation. 1 cm long sections of small intestine will be excised from the experimental mice at the time of sacrifice and incubated in 1 mL of RPMI buffer in 12-well plates at 37°C overnight. The tissue supernatant will be centrifuged at 4000 rpm for 10 minutes at 15°C and will be stored at -20°C till analysis.

**Systemic inflammatory response**

Systemic levels of pro-inflammatory cytokine proteins IL-1β, IL-17 and TNF-α will be measured to quantify inflammation as a result of perturbed microbiome at systemic levels.


