

CHARACTERIZING THE ROLE OF THE GUT MICROBIOME IN COLORECTAL CANCER

by

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ABSTRACT

The trillions of bacteria that inhabit the gastrointestinal tract, known collectively as the gut microbiome, are essential for both health and the normal functioning of the intestine. A growing literature now suggests that disruptive changes to this community are strongly associated with the development of colorectal cancer. However, it is unclear whether these disruptive changes directly contribute to disease or if they are just a consequence of colorectal cancer (CRC). Furthermore, the gut microbiome has not been explored as a potential non-invasive screen for CRC. Our hypothesis is that abnormalities in the gut microbiome can be utilized as a biomarker for detection of CRC at its earliest stages. Additionally, we postulate that these changes potentiate tumor development in the colon. To test these hypotheses, we first characterized the gut microbiome associated with human patients from three clinical groups representing three essential stages in CRC development: healthy, adenoma, and carcinoma. We demonstrated that a specific set of bacterial populations are associated with adenomas and carcinomas. The abundance of these bacterial populations was used to improve our ability to differentiate between healthy and diseased subjects and presents a viable screening tool for the earliest stages of CRC development. Next, we demonstrated using a mouse model of inflammation-driven colon cancer that there are dramatic, continual alterations in the gut microbiome during the development of tumors. By

colonizing germ-free mice with the gut microbiome from tumor-bearing mice, we determined that these changes are directly responsible for increased tumor development. Using an antibiotic cocktail, we were able to demonstrate that manipulation of this microbial community can dramatically reduce tumor burden in mice. By varying the composition of this antibiotic cocktail we generated a broad spectrum of microbial communities with varying carcinogenic capacities. This method of manipulating the gut microbiome allowed us to identify potentially protective and carcinogenic bacterial populations for further mechanistic studies. Our results demonstrate that changes to the gut microbiome can serve as an effective non-invasive screen for the early detection of colorectal cancer and that interventions that target these changes may be an effective strategy for preventing the development of colorectal cancer.

Chapter I

Introduction

Prevalence of Colorectal Cancer

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy worldwide, accounting for over a half million deaths annually [Siegel et al., 2007; Parkin et al., 2005]. The lifetime risk of developing CRC is about 1 in 20 for both men and women, however incidence and mortality rates are about 40% higher in men [American Cancer Society, 2011; Fearon et al., 2011]. Risk can become substantially higher depending on various genetic and environmental factors, which I will discuss in the following sections. It is estimated that the annual economic burden for CRC care in the United States (US) is approximately \$14 billion. This number is expected to continue to rise as average life expectancy increases and the pool of at risk population grows [Mariatto et al., 2011]. In this chapter, I will summarize what is known about the development and progression of CRC, and I will discuss current recommended screening procedures and their limitations. I will also introduce the potential role for a largely understudied variable in CRC, the gut microbiome, and discuss its potential as a novel CRC screen.

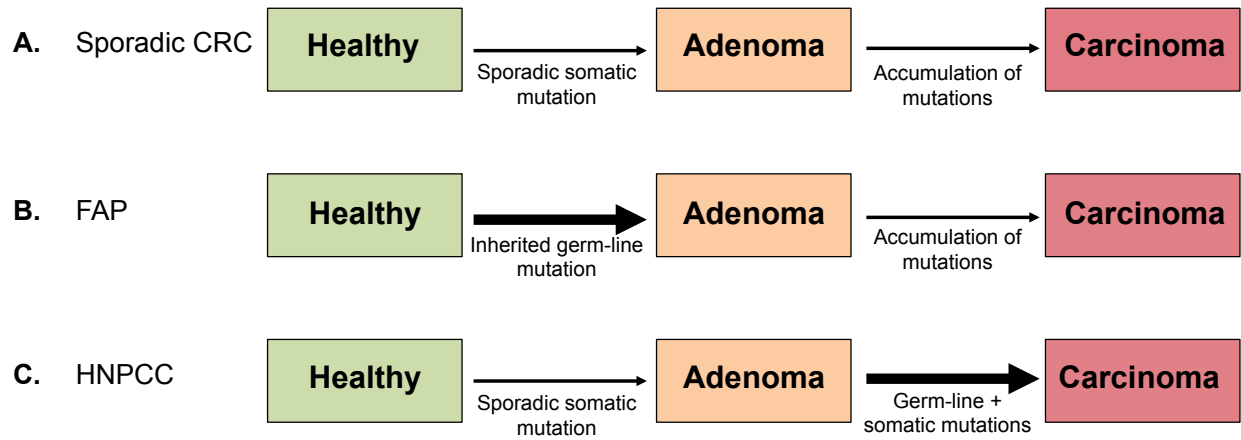


Figure 1.1. The adenoma-carcinoma progression. Modified from Fearon et al., 2011. The weight of the arrows corresponds to the rate by which a step occurs.

The Adenoma-Carcinoma Sequence

CRC development is a multistep process by which healthy epithelium develops into pre-cancerous polyps, which in turn progress into malignant carcinomas overtime (Figure 1.1). Over 95% of CRCs are adenocarcinomas that developed from glandular tissue in the colon [Stewart et al., 2006]. The large majority of these cancers arise from adenomatous polyps, which are thought to be the main precursor lesion in CRC. Adenomas are highly prevalent lesions, with over one-third of people eventually developing an adenoma in their lifetime; however, only 10% of adenomas will progress to CRC in a 10-year period [Levine et al., 2006]. An early and potentially rate limiting step to the development of an adenomas is thought to be an inactivating mutation to the adenomatous polyposis coli (APC) gene [Fearon et al., 2011]. This APC gene is an important tumor suppressor gene that regulates epithelial cell proliferation through the Wnt pathway [Goss et al., 2000]. It is estimated that 80% of sporadic adenomas have a somatic mutation in the APC gene and this mutation can be seen at the earliest stages of adenoma growth. Although it is thought to be a gatekeeper mutation in tumor development, this mutation is not sufficient for the progression to CRC; accumulation of additional mutations is necessary. Currently, our understanding of the genetic and environmental factors that promote mutations in tumor suppressor genes at each step in this progression is limited. Further work is needed to clarify the role of genetic, immunological, and environmental factors in this process.

Colorectal Cancer Screening and Surveillance

The survival rate for CRC is over 90% if diagnosis occurs while the cancer is still localized; however, survival drops dramatically when a cancer grows through the intestinal wall and becomes metastatic [Hayat et al., 2007; Ries et al., 2007]. This highlights the importance of detecting and diagnosing CRC at its earliest stages. With advancements in screening and surveillance of CRC, incidence rates have been in steady decline since the 1980s [Epey et al., 2007]. Direct inspection of the mucosa by colonoscopy is currently one of the most accurate and preferred methods for prevention of CRC in the US. Colonoscopies have a number of advantages, including the ability to examine the entire colon, remove polyps, and diagnose CRC with high sensitivity [American Cancer Society, 2011]. It is recommended that people with a history of CRC or those over the age of 50 receive colonoscopies every 10 years [Sonnenberg et al., 2000; Levin et al., 2008]. These examinations have led to a dramatic decrease in CRC mortality and it has been estimated that colonoscopy screening has the potential to completely prevent 65% of CRC cases [Brenner et al., 2007]. However, colonoscopy has a substantial risk of complications, requires significant preparation, and is invasive for the patient. Surveys have estimated that more than 30% of adults do not receive age and risk appropriate screenings, and over 50% of adults prefer other screening methods [Benson et al., 2007; Leard et al., 1997; Ling et al., 2001]. Furthermore, the healthcare costs of screening by colonoscopy are estimated in some studies to be over \$2.7 billion in the US [Fisher et al., 2006]. Thus, there is a clear need for the development of non-invasive screening methods to prioritize individuals for further evaluation by colonoscopy.

Non-invasive Screening of Colorectal Cancer

One of the most commonly utilized non-invasive screens for CRC is the guaiac fecal occult blood test (gFOBT), which detects blood in an individual's feces [Allison et al., 1996]. Occult blood in the stool can indicate the presence of advanced adenomas and carcinomas in the colon, although some non-related disorders or dietary factors may result in a false positive test. Because of the potential for false positive tests, the sensitivity for gFOBT can be as low as 9-12%, rendering it a non-feasible option for screening. However, with repeated testing, typically up to 3 fecal samples and 6 repeated tests, the sensitivity can be significantly improved [Lieberman et al., 2001; Levin et al., 2008]. Despite these limitations, gFOBT has shown to reduce CRC mortality up to 30%. CRC mortality rates could drop even further upon development of a more sensitive non-invasive screening method. Clearly, there is a need for novel biomarkers of CRC that could be used for screening.

Heredity and Family History in Colorectal Cancer

About 20-30% of CRC cases are estimated to have a significant hereditary component [Taylor et al., 2010]. Individuals with a first degree relative who has had CRC are at least two times as likely to develop CRC; moreover that risk increases if that family member developed cancer at an early age [Butterworth et al., 2006]. Two well-defined inherited syndromes that greatly increase the risk of CRC are familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). Patients with FAP have a germline inactivating mutation in the APC gene, which results in the development of hundreds of colonic polyps at an early age and inevitably leads to

CRC [Galiatsatos, et al., 2006]. Individuals with HNPCC, also known as Lynch syndrome, do not develop polyps at an early age; however, polyps that do appear tend to become malignant at a higher rate and in a short period of time [Dove-Edwin et al., 2006]. The majority of patients with FAP, and some with HNPCC, require prophylactic removal of the colon relatively early in life to prevent the inevitable onset of CRC. Together, FAP and HNPCC only make up about 5% of all CRC cases [Fearon et al., 2011], but these syndromes have allowed researchers to uncover many of the mechanisms of tumor development and progress in the colon.

Risk Factors for Colorectal Cancer

Approximately 70% of CRC cases develop sporadically and have no known causative agents. Epidemiological studies have identified several potential risk factors for CRC, including age and chronic inflammation of the gastrointestinal tract. CRC risk rises substantially with age, and there is a sharp increase in incidence at the age of 50. In fact, over 90% of CRC cases occur in people over 50 [Benson et al., 2007]. This is a clear problem because as the average life expectancy continues to rise, the pool of individuals at an increased risk of CRC also grows. Additionally, patients with chronic inflammatory diseases, such as inflammatory bowel disease, are at an increased risk. In the case of ulcerative colitis, the risk for CRC increases 1% per year after the first decade [Chambers et al., 2005; Ullman et al., 2011; Eaden et al., 2000]. The potential mechanisms by which this chronic inflammation may drive tumorigenesis will be discussed later in this chapter.

Risk Factors for Colorectal Cancer

Age

- 50 years and older

Previous cases of colorectal cancer

Family History colorectal cancer

Inheritable Syndromes

- Familial adenomatous polyposis
- Hereditary nonpolyposis colorectal cancer

Environmental factors

- Diet high in red meat
- Diet high in fat
- Diet low in fiber
- Obesity (BMI)
- Physical inactivity
- Smoking
- Alcohol

Chronic inflammation of Gastrointestinal tract

- Inflammatory bowel disease
 - Crohn's disease
 - Ulcerative colitis
-

Table 1.1 Risk factors for colorectal cancer. Summary of the known risk factors for CRC. Detail into each factor is presented in chapter I.

In addition to age and inflammation, several behavioral and environmental risk factors have been identified for CRC including obesity, physical inactivity, alcohol consumption, and diet (Table 1.1). One recent study suggested that individuals with a lifetime average of 2-4 alcoholic drinks per day have a 20% higher risk of CRC. Diet, which is closely linked to obesity, has been associated with CRC incidence in a number of studies. It has been shown that diets high in red and processed meat, or high in fat are linked to an increased risk of CRC [Butler et al., 2003]. It has been known for sometime now that high fat diets play a role in CRC. Rodents fed a high fat diet develop significantly more tumors in experimental mouse models of CRC [Wasan et al., 1997]. High fiber diets, on the other hand, have been suggested to decrease risk [Aune et al., 2011], although this effect is still debated. Support for a significant role of dietary factors in the etiology of CRC can also be seen in epidemiology studies. US-born Japanese men experience a rate of CRC that is double than the observed rate in native Japanese men [Flood et al., 2000]. This phenomenon is also seen in African Americans, who have a dramatic increase in CRC incidence compared to native Africans [O’Keefe et al., 2007]. The factor that is contributing to the disparity in CRC incidence is likely diet, which varies greatly in each of these cases. The mechanisms by which these environmental factors, such as diet, affect the development of CRC has largely remained unknown. However, a recent appreciation for the complex community of microorganisms that inhabit the gastrointestinal tract has begun to shed light on this phenomenon.

The Gut Microbiome

The human gastrointestinal tract is home to trillions of microorganisms, which outnumber human cells by 10-fold [Backhed et al., 2005]. Taken together, these microbes harbor over 100 times as many genes as their human host [Gill et al., 2006]. This has led researchers to suggest that the gut microbiome is essentially a microbial organ within the host and moreover has led some to consider the host and its microbes as a super-organism. The gut microbiome is an incredibly diverse community of microorganisms with a complex ecology that we are just beginning to understand. This community consists of bacteria, viruses, phage, and eukaryotes, which all interact with each other and the host [Guarner et al., 2003; Backhed et al., 2005]. In my thesis I will only be focusing on the bacterial component of the gut microbiome, which makes up the majority of the microbes present; however, consideration for the other members of the community is essential for a complete understanding of the underlying mechanisms of tumorigenesis. Symbiotic members of gut microbiome are often termed commensal, meaning they benefit from the relationship without affecting the host, but in reality this relationship is more mutualistic. This is supported by a wealth of literature that has determined that an intact microbial community is essential for development and health of the host [Alonso et al., 2013; Backhed et al., 2005]. The vast diversity in genetic potential harbored by the gut microbiome provides for a large arsenal of metabolic enzymes that aid the host in digestion, energy harvest, and vitamin synthesis. These microorganisms supply most of the vitamin K and water-soluble B vitamins needed by the host [Guzman et al., 2013]. Beyond digestion, the gut microbiome has a much more direct affect on the host. Extensive research has shown that the gut microbiome is needed for the proper development and maturation of the immune system. Germ-free

mice, which are completely devoid of any microbes, have a dramatically underdeveloped immune system and mucosal barrier [Mazmanian et al., 2005; Tlaskalova-Hogenova et al., 2011]. Another important feature of the gut microbiome is its ability to confer colonization resistance, which is a natural barrier that is capable of preventing the establishment of pathogenic bacteria [Vollaard et al., 1994]. Perturbations to the community can leave the host susceptible to colonization by enteric pathogens like *Clostridium difficile*, highlighting the importance of a healthy microbial community [Reeves et al., 2011; Theriot et al., 2014].

Dysbiosis and Colorectal Cancer

Although the gut microbiome is important for maintaining health in the host, it has also been associated with various diseases, including inflammatory bowel disease and obesity. In each of these diseases a phenomenon, termed dysbiosis, is observed in the gut microbiome. Dysbiosis is defined as an abnormal community structure or composition of the gut microbiome. Several recent studies have shown that this phenomenon can be observed in the feces of individuals with CRC relative to healthy controls [Chen et al., 2013; Chen et al., 2012; Kostic et al., 2012; Geng et al., 2013; Shen et al., 2013; Sobhani et al., 2011; Wang et al., 2012; Ahn et al., 2013]. Interestingly, each of these groups obtained conflicting results regarding the composition and structure of the CRC-associated community. CRC-associated dysbiosis is not limited to feces, as demonstrated by two groups who recently reported an enrichment of *Fusobacterium nucleatum* on the surface of colonic tumors relative to adjacent healthy tissue. Furthermore, it was shown that sequences from

Fusobacterium spp. could be detected in the feces of patients with CRC [Ahn et al., 2013; Kostic et al., 2013]. These data clearly show a link between the structure and composition of the gut microbiome, and CRC; however, it remains unclear whether abnormalities in the gut microbiome directly affect colon tumorigenesis or if they are simply an unrelated consequence. Regardless, it is tempting to speculate on the potential of using these abnormal changes in the gut microbiome as a way to determine if a person has CRC or is at an increased risk.

Diet, The Gut Microbiome, and CRC

Beyond dysbiosis, there are several clear links between the gut microbiome and CRC. The gut microbiome is directly associated with several important risk factors for CRC, including diet. Diet has been shown to be an important mediator of microbial community structure and composition, and certain dietary components have an important role in the function of the gut microbiome [David et al., 2013]. A significant portion of the human diet consists of carbohydrates, such as starches, cellulose, hemicellulose, and pectins, which are non-digestible by the host. When these non-digestible carbohydrates reach the colon, resident microbes ferment them and produce short chain fatty acids (SCFAs). SCFAs serve an important role in colonic health and provide important nutrients for colonocytes and other epithelial cells [Koropatkin et al., 2012]. Evidence suggests that SCFAs, specifically butyrate, also affect various physiological functions that are directly associated with cancer, including cell proliferation, angiogenesis, and apoptosis [Ruemmele et al., 2003; Segain et al., 2000; Hague et al., 1995]. In cell culture, butyrate has been shown to reduce proliferation and

induce apoptosis of tumor cells. Several studies have shown that diets high in fiber and carbohydrates can increase beneficial bacterial taxa that produce butyrate [Hague et al., 1995; Ruemmele et al., 2003]. Furthermore, butyrate has been shown to be significantly decreased in the colon of subjects with CRC [Wang et al., 2012; Chen et al., 2013]. Conversely, it has been shown that diets high in fat and low in fiber shift the structure of the gut microbiome in a potentially detrimental way [Peters et al., 1992]. These diets likely lead to a dramatic decrease in SCFA production and subsequently decrease epithelial health and mucin production [Willemsen et al., 2003; Hatayama et al., 2007]. It is also known that diets can contain various levels of toxic, carcinogenic, or mutagenic compounds. Members of the gut microbiome have the potential to both activate and detoxify these compounds. The composition and functional potential of the community could largely affect the metabolism of these chemicals and the subsequent affect on the host [Rowland, 1988]. Taken together this evidence leads us to the hypothesis that manipulation of the gut microbiome with diet could help in prevention and treatment of cancer.

Inflammation, the Gut Microbiome, and CRC

Another important link between the gut microbiome and CRC is inflammation. We have known for sometime now that chronic inflammation is directly linked to many cancers. In the gastrointestinal tract, the main driver of this inflammation is the resident microbes, their gene products, and the metabolites they produce. Gut microbes are recognized by a repertoire of pattern recognition receptors (PRRs) that are specific for conserved microbial patterns, such as components of the bacterial cell wall or nucleic

acids. Two important PRRs are Nod-like receptors (NLRs) and Toll-like receptors (TLRs), which initiate signaling cascades upon activation by microbial signals [Abreu et al., 2005]. These signal cascades activate various transcriptional regulators, including nuclear factor of kappa B (NFκB), which modulates inflammation, cell proliferation, and apoptosis [Rakoff-Nahoiium et al., 2008]. The importance of PRRs and NFκB in CRC has been extensively studied in various mouse models of tumorigenesis. Mice deficient in TLR4, which recognizes lipopolysaccharide (LPS), show a marked decrease in colon tumorigenesis [Fukata et al., 2007]. Furthermore, myeloid differentiation factor 88 (MyD88) deficient mice, which is the main adaptor protein for signaling through the majority of TLRs, show a diminished development of colorectal cancer [Uronis et al., 2009]. When NFκB is deactivated in IκB kinase (IKK) deficient mice, there is also a dramatic decrease in CRC [Greten et al., 2004]. Together these experiments clearly demonstrate that signaling through PRRs directly modulates tumorigenesis in the colon. The mechanisms by which NFκB-mediated inflammation can promote tumorigenesis include the production of pro-tumorigenic cytokines and chemokines like tumor necrosis factor alpha (TNF- α), interleukin 1 beta (IL-1β), IL-8, and IL-6 [Karin et al., 2005]. These inflammatory cytokines are produced by lamina propria myeloid cells and stimulate growth and survival of malignant cells in the gastrointestinal tract [Grivennikov et al., 2009]. Chemokine production leads to the recruitment of immune cells, such as neutrophils, to the tumor microenvironment [Fridlender et al., 2012]. It is thought that infiltration by neutrophils can increase carcinogenesis and initiate accumulation of mutations through the production of reactive oxygen species (ROS). In addition to the production of cytokines and the recruitment of immune cells, NFκB can also promote

tumorigenesis through its ability to suppress the apoptosis of pre-cancerous progenitor cells [Karin et al., 2005]. In summary, recognition of the gut microbiome by the host and subsequent inflammatory responses can directly promote tumorigenesis. However it remains largely unknown how the composition, structure, and function of the gut microbiome affect these mechanisms.

Bacteria Associated with CRC

The microbial influence on CRC is highlighted by several studies that have shown that mice raised in germ-free conditions develop significantly less tumors in various models of CRC [Dove et al., 1997; Uronis et al., 2009]. Recently, several specific members of the gut microbiome have become of particular interest in CRC (Table 1.2). One of the most intriguing of these microbes is *Fusobacterium nucleatum*, which was described previously in this chapter as being enriched on the surface of tumors. *F. nucleatum* is not a typical member of the gut microbiome; it is only seen in about 6% of healthy individuals (Unpublished data from Schloss laboratory). However, it is a normal commensal of the mouth [Signat et al., 2011]. A recent study determined that when fed to mice, this pathogen promotes tumorigenesis by recruiting myeloid cells to the tumor and generating a proinflammatory microenvironment [Kostic et al., 2013]. It still remains to be determined whether the *F. nucleatum* in the colon is the same as that in the mouth.

Several more common commensal bacteria have also been linked to CRC. A recent study demonstrated that colonic inflammation in the IL-10-deficient mouse impacts the composition of the gut microbiome, leading to an enrichment of tumor

promoting *Escherichia coli* strains [Arthur et al., 2012]. These strains have a polyketide synthase (pks) genotoxic island that increases the rate by which tumors progress. Similarly, human commensals belonging to the genus *Bacteroides*, specifically enterotoxigenic *B. fragilis* (ETBF), have been associated with inflammation and CRC [Wu et al., 2009; Sears et al., 2008]. ETBF has been shown to strongly induce colonic tumors in mice through secretion of a metalloprotease toxin and certain strains are thought to contribute to CRC risk in humans. Finally, it has been known that *Streptococcus bovis* has been linked to CRC since as early as the 1950's [Galdy et al., 2012]. Survey studies have reported a strong correlation between this bacterial species and CRC. It is clear that several microbes have a direct affect in the development and progression of CRC; however, survey studies rarely show the same bacterial profile between subjects with CRC. Furthermore, only a small percent of subjects have detectable levels of populations like *E. coli* and ETBF. This strongly suggests that there may be multiple underlying mechanisms by which the microbiome is involved in CRC and that CRC is likely a polymicrobial disease.

Bacteria Associated with Colorectal Cancer

| Bacteria | Link to colorectal cancer |
|--------------------------------|--|
| <i>Fusobacterium nucleatum</i> | Enriched on tumors and increases tumorigenesis in APC min mouse model. |
| <i>Bacteroides fragilis</i> | Enterotoxigenic <i>B. fragilis</i> has metalloprotease toxin linked to cancer. Increases tumorigenesis in APC min mouse. |
| <i>Escherichia coli</i> | Strains with polyketide synthase genotoxic island increase rate of tumors progression in Il-10 deficient mice. |
| <i>Streptococcus bovis</i> | Linked to colorectal cancer in survey-based studies of stool. |
| <i>Helicobacter hepaticus</i> | Increases tumorigenesis in APC min mice |
| <i>Helicobacter pylori</i> | Causative agent of ulcers, which increase risk of colorectal cancer |
| <i>Citrobacter rodentium</i> | Increases tumorigenesis in mouse models of colorectal cancer |

Table 1.2. Bacteria associated with colorectal cancer. Bacteria that have been associated with CRC are highlighted along with a description of their suggested involvement.

Microbial Ecology in the Gut Microbiome

The limitation of studies that have looked at the role of individual microbes in CRC is that they ignore the fact that the gut microbiome is a diverse and complex community of microorganisms. This community has a complex ecology with a wide array of microbe-microbe interactions [Konopka et al., 2009]. Interactions that occur include complex signaling, competition for resources and space, metabolic interactions, and structural interactions (i.e. biofilms) [Konopka et al., 2009]. The importance of this ecology in relation to disease can be highlighted by the syntrophic interaction between *Archaea* in periodontal disease [Lepp et al., 2004]. In this system methanogenic *Archaea* acted as a hydrogen sink to allow for the proliferation of pathogenic bacteria to a level that would not be possible in the absence of this syntrophy. In CRC, bacteria like *Fusobacterium spp.* or *E. coli* are likely heavily influenced by the bacterial community surrounding them. Their pathogenicity may rely solely on interactions with other microbes, and thus studying them out of this context may be misleading. In the subsequent chapters of my thesis, I will emphasize the importance of the entire community and the complex ecology when characterizing the role of the gut microbiome in CRC. This is not to downplay the importance of understanding the mechanisms by which individual microbes affect CRC, but we need to understand how the community as a whole affects CRC before we begin to ask more mechanistic questions.

Summary and Chapter Outline

Colorectal cancer (CRC) is one of the deadliest malignancies worldwide, affecting millions of people each year. Significant risk factors for CRC, including diet

and chronic inflammation, are intimately linked with the gut microbiome. Furthermore, the gut microbiome promotes various physiological functions that are closely associated with cancer, including cell proliferation, angiogenesis, and apoptosis. Several recent survey-based studies have reported the people with CRC have a dysbiotic gut microbiome. However, it is unclear how these changes develop over time and whether they directly affect tumorigenesis or if they are an unrelated consequence of CRC development. Therefore the primary goal of this thesis research is to understand the role of the gut microbiome in CRC. We hypothesize that the composition, structure, and functional capacity of the gut microbiome all directly affect tumor development in the colon. Additionally, we believe that understanding how dysbiosis develops over time will allow us to develop novel methods for using the gut microbiome as a biomarker for CRC.

In chapter II, we collected samples from human subjects that represented three clinical groups: healthy, adenoma, and carcinoma. We investigated the potential of using the gut microbiome as a biomarker for CRC by identifying significantly enriched bacterial populations in each group. We demonstrated that incorporation of microbial biomarkers into logit models, generated using common risk factors for CRC, and significantly improved our ability to predict the presence of both pre-cancerous adenomas and carcinomas.

In chapter III, we characterized the gut microbiome in a mouse model of CRC and determined that mice develop dysbiosis that mirrors that seen in humans. Using machine-learning algorithms, we were able to accurately predict the presence of tumors in the colon based solely on the structure of the gut microbiome. Using germ-free mice

we demonstrated for the first time that tumor-associated changes in the gut microbiome increase tumorigenesis in the colon. Additionally, manipulation of this community with antibiotics dramatically reduced tumor incidence in mice.

To further explore the effect of community structure on tumorigenesis, we treated mice with a wide array of different antibiotic treatments. In chapter IV, we identify several potentially carcinogenic and protective bacterial populations in the gut microbiome. We also show that changes in community structure and diversity have a dramatic affect on tumor incidence in the colon. Using antibiotics as an intervention, we demonstrate that modulation of the gut microbiome at the first signs of dysbiosis can reduce tumorigenesis. This strongly suggests that targeting of the gut microbiome for therapeutic purposes may be an effective strategy for CRC prevention. Chapter V includes a summary of the results, future areas of study, and conclusions including a proposed model overview.

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CHAPTER II

The Human Gut Microbiome as a Screening Tool for Colorectal Cancer

Abstract

Recent studies have suggested that the gut microbiome may be an important factor in the development of colorectal cancer (CRC). Abnormalities in the gut microbiome have been reported in patients with CRC; however, this microbial community has not been explored as a potential screen for early stage disease. We characterized the gut microbiome in patients from three clinical groups representing the stages of CRC development: health, adenoma, and carcinoma. Analysis of the gut microbiome from stool samples revealed both an enrichment and depletion of several bacterial populations associated with adenomas and carcinomas. Combined with known clinical risk factors of CRC (e.g. BMI, age, race), data from the gut microbiome significantly improved the ability to differentiate between healthy, adenoma, and carcinoma clinical groups relative to risk factors alone. Using Bayesian methods, we determined that using gut microbiome data as a screening tool improved the pre-test to post-test probability of adenoma over 50-fold. For example, the pre-test probability in a 65 year-old was 0.17% and, after using the microbiome data, this increased to 10.67% (1 in 9 chance of having an adenoma). Taken together the results of our study demonstrate the feasibility of using the composition of the gut microbiome to detect the

presence of precancerous and cancerous lesions. Furthermore, these results support the need for more cross sectional studies with diverse populations and linkage to other stool markers, dietary data, and personal health information.

Introduction

Worldwide, colorectal cancer (CRC) is the third most commonly diagnosed malignancy and accounts for over a half million deaths annually [Parkin et al., 2005]. Development of CRC is a stepwise process by which localized precancerous adenomatous polyps (adenomas) develop in the colon and progress into invasive and metastatic cancerous tumors (carcinomas) overtime [Fearon, 2011; Jass, 2007]. Development of carcinomas is largely preventable if adenomas are detected and removed [Levin et al., 2008], with a CRC survival rate exceeding 90% if the diagnosis occurs while the disease is still localized. However, there is a dramatic decline in survival following invasion and metastasis [SEER program, 2013]. Thus, early detection at the adenoma stage of this disease has been critical for successful treatment and survival.

From 1975 to 2010, death rates from colorectal cancer have steadily decreased in the United States, with a 2.8% average annual decline [SEER program, 2013]. Screening with high sensitivity fecal occult blood testing (FOBT), sigmoidoscopy, and colonoscopy has improved survival rates and is recommended for adults 50 to 75 years of age [Whitlock et al., 2008]. In particular, colonoscopies allow for full examination of the bowel with the opportunity for same-session colonic biopsies and removal of polyps.

However, over 30% of adults in the US do not receive age and risk-appropriate screenings and surveys indicate that 50-60% of adults prefer non-invasive screening methods [Benson, 2007; Learn et al., 1997; Ling et al., 2001]. Lack of compliance with these recommendations may be due in part to the intrusiveness and uncomfortable nature of the colonoscopy procedure. Furthermore, the healthcare costs of screening for CRC by colonoscopy are considerable, ranging from \$800 to \$3160 per procedure in 2012 which was undergone by more than 48 million 50-75 year-old Americans [Joseph et al., 2012; Howden and Meyer, 2012]. Therefore, there is a need to develop cost-effective non-invasive screening methods to prioritize individuals for further evaluation by colonoscopy. One of the most commonly used non-invasive screening procedures is the guaiac fecal occult blood test (gFOBT), which detects blood in an individual's feces [Allison et al., 1996]. Occult blood in stool can indicate the presence of advanced adenomas and carcinomas in the colon, but can also indicate a wide variety of other disorders and factors that may lead to false positive tests [Young et al., 2002]. Although the specificity of the method ranges from 87-98% [Allison et al., 1996], the sensitivity can be as low as 9-12% [Collings et al., 2005; Imperiale et al., 2004]. With repeated testing using multiple stool samples and regular screening intervals, sensitivity can be dramatically improved [Lieberman et al., 2001; Levin et al., 2008]. Despite these limitations, gFOBT has been shown to reduce mortality from CRC by 15 to 33%, highlighting the effectiveness of non-invasive screening measures [Hardcastle et al., 1996; Kronborg et al., 1996; Mandel et al., 1999; Mandel et al., 2000].

Approximately 70% of CRC cases develop spontaneously and are of unknown etiology [Fearon, 2011]. Factors associated with increased risk of CRC include diet,

alcohol, and chronic inflammation of the gastrointestinal tract [Chambers et al., 2005; Huxley et al., 2009; Larsson et al., 2005; Slattery, 2000]. Recently, there has been increasing appreciation for a largely understudied variable in CRC, the gut microbiome. This collection of symbiotic microorganisms inhabits the gastrointestinal tract and is associated with diseases such as obesity and inflammatory bowel disease [Turnbaugh et al., 2006; Manichanh et al., 2006]. In animal studies, evidence suggests that through interaction with the immune system, production of cancer-associated metabolites, and the release of genotoxic virulence factors, bacteria can directly contribute to the development of CRC [Zackular et al., 2013; Arthur et al., 2012; Couturier-Maillard et al., 2013; Kostic et al., 2013]. Furthermore, in human studies, patients with CRC have an abnormal gut microbiome structure when compared to healthy patients [Chen et al., 2013; Kostic et al., 2012; Geng et al., 2013; Shen et al., 2010; Sobhani et al., 2011; Wang et al., 2012; Ahn et al., 2013]. Taken together, this suggests that the gut microbiome might be a candidate biomarker for early detection of CRC.

We hypothesized that using novel microbiome biomarkers of CRC in concert with known clinical risk factors could improve the ability to identify candidates for colonoscopy. We compared the microbiome of healthy individuals, persons with adenomas, and patients with colorectal carcinomas. We sequenced the V4 region of the 16S rRNA gene from the feces of each individual using the Illumina MiSeq sequencing platform. The resulting data were used to test our hypothesis that the incorporation of microbiome data would significantly improve the ability to distinguish among the three types of individuals, beyond clinical (demographic) data and FOBT

results. This analysis demonstrates that the microbiome provides a powerful source of biomarkers for identifying individuals harboring adenomas and carcinomas.

| | Healthy | Adenoma | Cancer | P-value |
|------------------------------------|----------------|----------------|---------------|----------------|
| Age, years (mean, SD) | 55.3 (9.2) | 61.3 (11.1) | 59.4 (11.0) | 0.080 |
| Gender (n, %): Men | 11 (37%) | 18 (60%) | 21 (70%) | 0.029 |
| Women | 19 (63%) | 12 (40%) | 9 (30%) | |
| Race/Ethnicity: Non-Hispanic white | 21 (70%) | 27 (90%) | 28 (93%) | 0.026 |
| Other | 9 (30%) | 3 (10%) | 2 (7%) | |
| Body mass index (mean, SD) | 26.6 (5.2) | 27.4 (4.4) | 30.7 (7.2) | 0.022 |
| Current medication use (n, %) | 23 (77%) | 21 (70%) | 26 (87%) | 0.295 |
| Positive FOBT (n, %) | 0 (0%) | 4 (13%) | 22 (73%) | 0.001 |

Table 2.1. Characteristics of subjects in each clinical group. Breakdown of clinical data from each of the clinical groups. Body mass index (BMI) was calculated based on height and weight (kg/m²).

Results

Selection of Subjects

Colonoscopies were performed and fecal samples were collected from subjects in 4 locations: Toronto (Ontario, Canada), Boston (Massachusetts, USA), Houston (Texas, USA), and Ann Arbor (Michigan, USA). All participants collected a whole evacuated stool after following the usual dietary and medication restrictions for 24 hours. Following endoscopic examination, patients without colonic abnormalities were designated as healthy (n=30). Examinations that revealed the presence of lesions resulted in a biopsy and subsequent diagnosis of adenoma (n=30) or carcinoma (n=30). For each patient, clinical data were collected including demographic information and the results of the gFOBT (Table 2.1). There were no significant differences in age or current medication use among the three patient groups. However, among our samples, men, whites, and those with greater BMI were more likely to have colorectal cancer (Table 1).

Comparison of healthy and adenoma clinical groups

We utilized logit regression models to differentiate between patients in the healthy and adenoma clinical groups. Preliminary models were generated using age, gender, race/ethnicity, BMI, and medication use as independent variables. For these subjects, both age and race were significantly associated with the presence of adenomas (AUC=0.713; 95% CI: 0.580-0.845; p=0.009). There were also differences in the gut microbiome between individuals with and without adenomas. Relative to healthy subjects, subjects with adenomas had higher relative abundances of operational taxonomic units (OTUs) affiliated with the Ruminococcaceae (OTUs 21 and 60) and

Porphyromonadaceae (OTUs 1901 and 1903); they had lower relative abundances of OTUs affiliated with the *Bacteroides* (OTUs 1889 and 1913), Lachnospiraceae (OTU 36), Clostridiales (OTU 38), and *Clostridium* (OTUs 20, 97, 99) (Figure 2.1). The model that yielded the greatest differentiation between adenoma and healthy groups included age, race, and 5 OTUs (OTUs 38, 99, 136, 1889, 1913) (Figure 2.2A). The addition of these 5 OTUs significantly improved the predictive ability of the model beyond that of age and race only (AUC=0.896; 95% CI: 0.816-0.976; p=0.002) (Figure 2.2B). These results demonstrate that differences in the gut microbiome could be detected at pre-cancerous stages of CRC development and this signal may improve our ability to predict the presence of colonic adenomas.

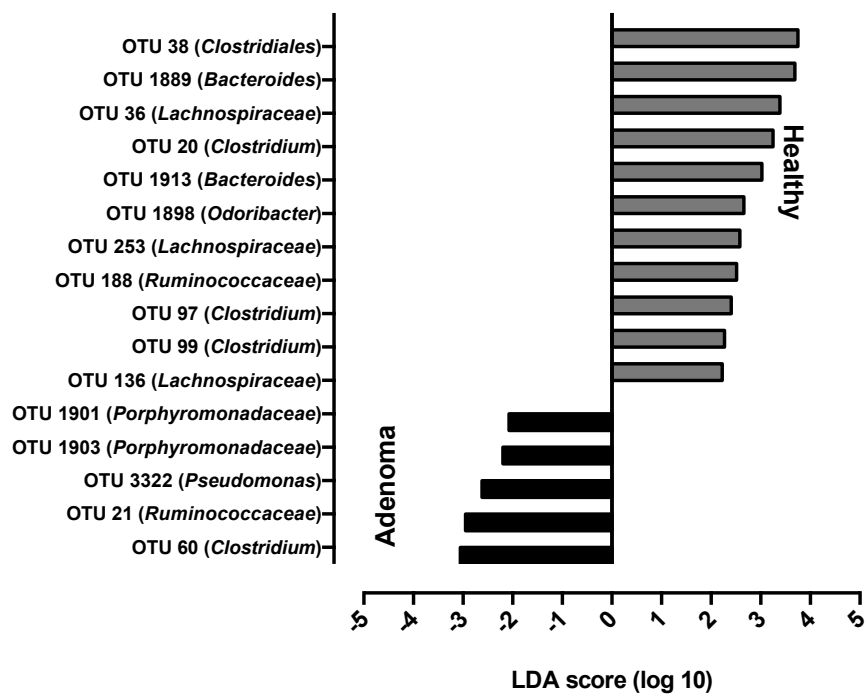


Figure 2.1. Microbial biomarkers for healthy and adenoma clinical groups. OTUs that were differentially abundant in healthy and adenoma clinical groups. LDA scores for significant OTUs are shown. OTU number and Taxonomic group based on RDP classification are represented.

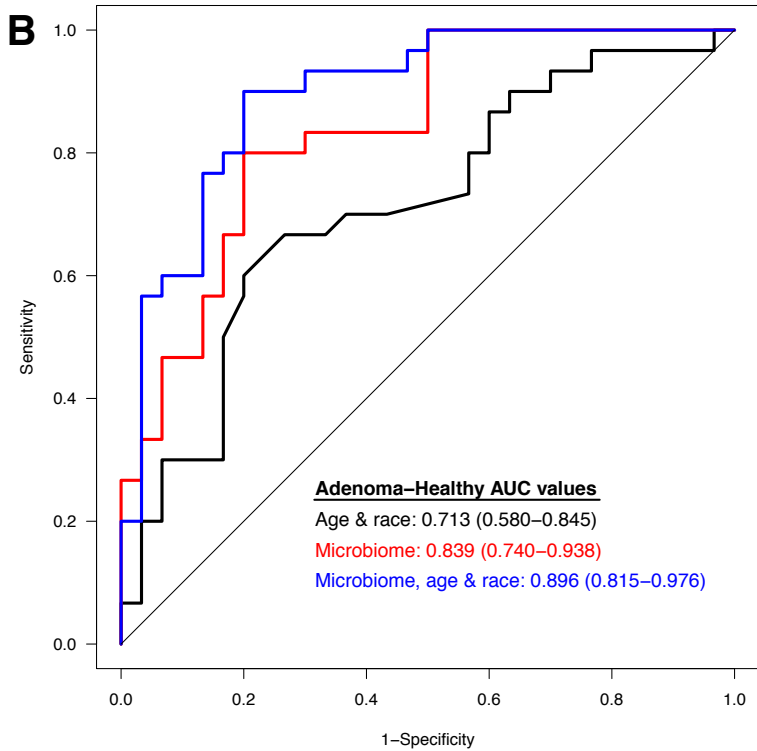
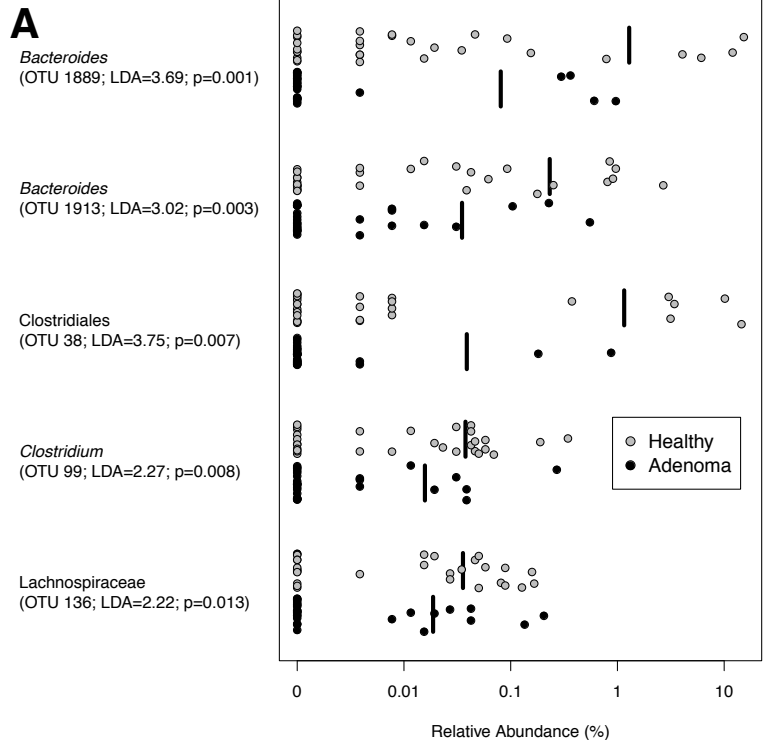


Figure 2.2. Microbiome improves accuracy of predictive models for healthy and adenoma. A. Relative abundance of differentially abundant OTUs for all healthy (n=30; grey) and adenoma (n=30; black) subjects. Vertical black line represents mean. **B.** ROC curves for microbial biomarkers alone, clinical data alone, and microbial biomarkers with clinical data. The straight line represents the null model.

Comparison of healthy and carcinoma clinical groups

Next, we generated logit models using clinical and microbiome data to differentiate between patients in the healthy and carcinoma groups. Age, race, and BMI were predictive of carcinomas (AUC=0.798; 95% CI: 0.686-0.910; $p < 0.001$). We observed that relative to healthy subjects, subjects with carcinomas had higher abundances of OTUs associated with *Fusobacterium* (OTU 2458), *Porphyromonas* (OTU 1905), and Enterobacteriaceae (OTU 2479); they had lower relative abundances of OTUs affiliated with the *Bacteroides* (OTU 1889), Lachnospiraceae (OTUs 23, 30, 253, 136), and Clostridiales (OTU 42) (Figure 2.3). To test the hypothesis that the gut microbiome could improve our ability to predict the presence of carcinomas, we added these OTUs to the logit model we generated based on the subjects' age, race, and BMI (Figure 2.4B). The model with the greatest discriminatory ability included age, race, BMI and 6 OTUs (OTUs 136, 1901, 1905, 1913, 2479, 2458; Figure 2.4A). This model significantly improved the ability to distinguish between healthy and carcinoma compared to the model containing age, race and BMI only (AUC=0.922; 95% CI: 0.858-0.986; $p = 0.012$; (Figure 2.4B). These results suggest that the relative abundance of six bacterial populations differentiate healthy gut tissue from the presence of cancerous lesions in the colon.

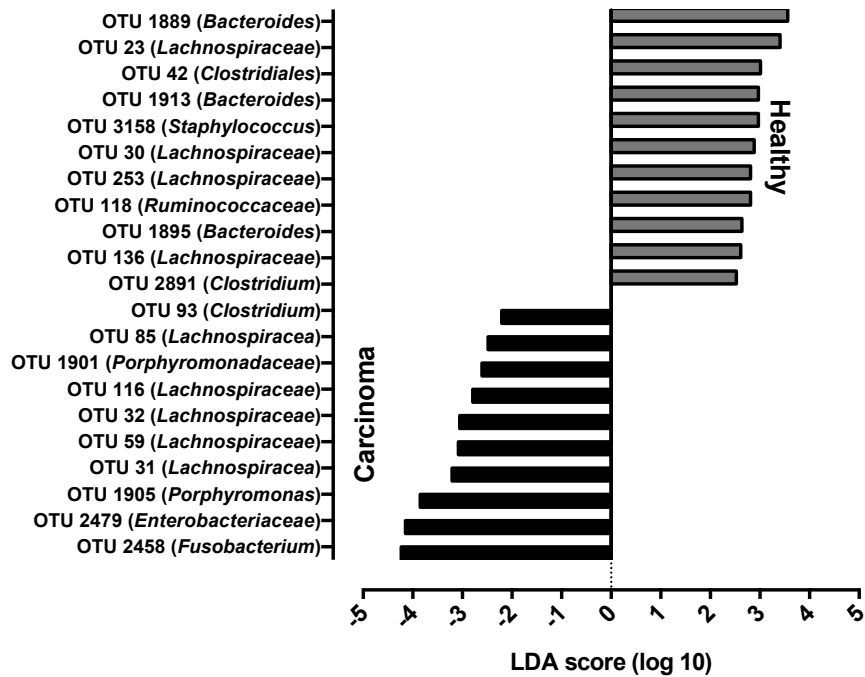


Figure 2.3. Microbial biomarkers for healthy and carcinoma clinical groups. OTUs that were differentially abundant in healthy and carcinoma clinical groups. LDA scores for significant OTUs are shown. OTU number and Taxonomic group based on RDP classification are represented.

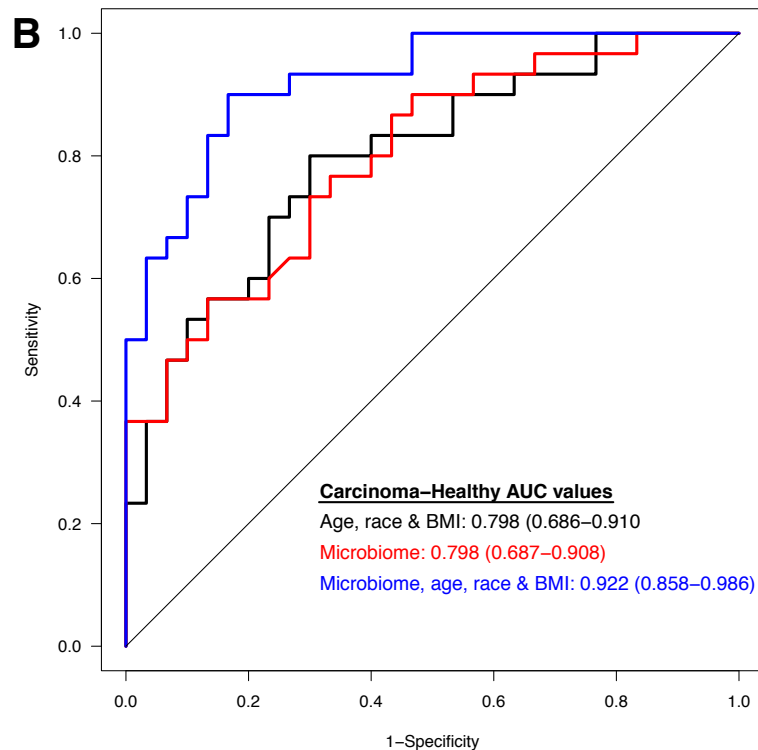
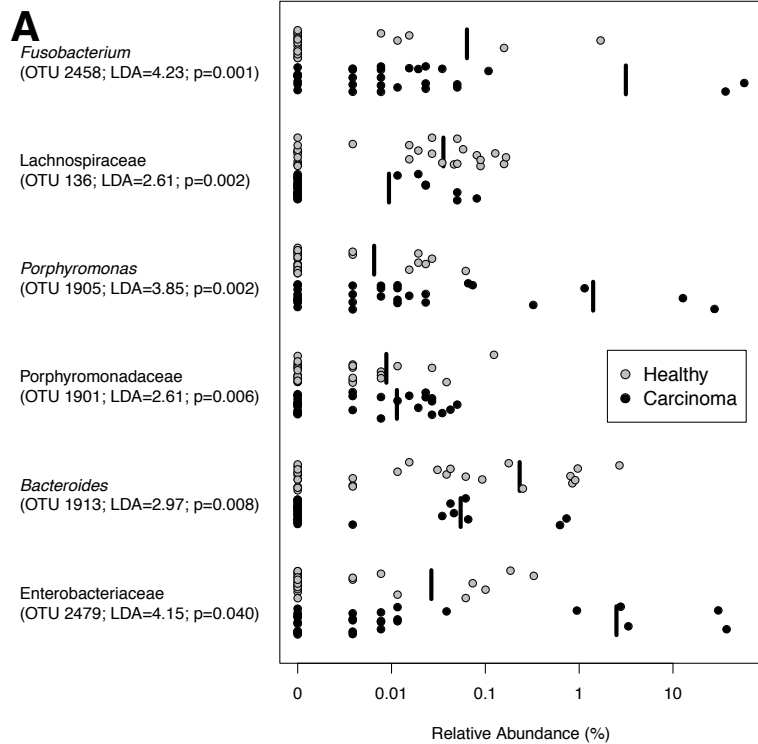


Figure 2.4 Microbiome improves accuracy of predictive models for healthy and carcinoma. **A.** Relative abundance of differentially abundant OTUs for all healthy (n=30; grey) and carcinoma (n=30; black) subjects. Vertical black line represents mean. **B.** ROC curves for microbial biomarkers alone, clinical data alone, and microbial biomarkers with clinical data. The straight line represents the null model.

Comparison of healthy individuals to those with colonic lesions

Next, we explored the ability of the gut microbiome to differentiate between healthy subjects and those with either adenoma or carcinomas. Thus, we combined the clinical and microbiome data from adenoma and carcinoma subjects to create a combined colonic lesion group. We then generated a logit model to differentiate between healthy subjects and the colonic lesion group. Clinical variables that were predictive of colonic lesion were age, gender, and race (AUC=0.754; 95% CI: 0.648-0.859) (Figure 2.6). To test the hypothesis that the gut microbiome could improve our ability to predict the presence of colonic lesions regardless of stage, we added 6 OTUs (OTU 136, 253, 1889, 1897, 1913, 2891) (Figure 2.5) to this logit model. Age, gender, race, and these 6 OTUs significantly improved the ability to distinguish between the healthy and colonic lesion combined groups (AUC=0.936; 95% CI: 0.887-0.985; $p < 0.0001$) (Figure 2.6). These results demonstrate that the gut microbiome can improve the ability to discriminate between healthy subjects and colonic lesions independent of stage.

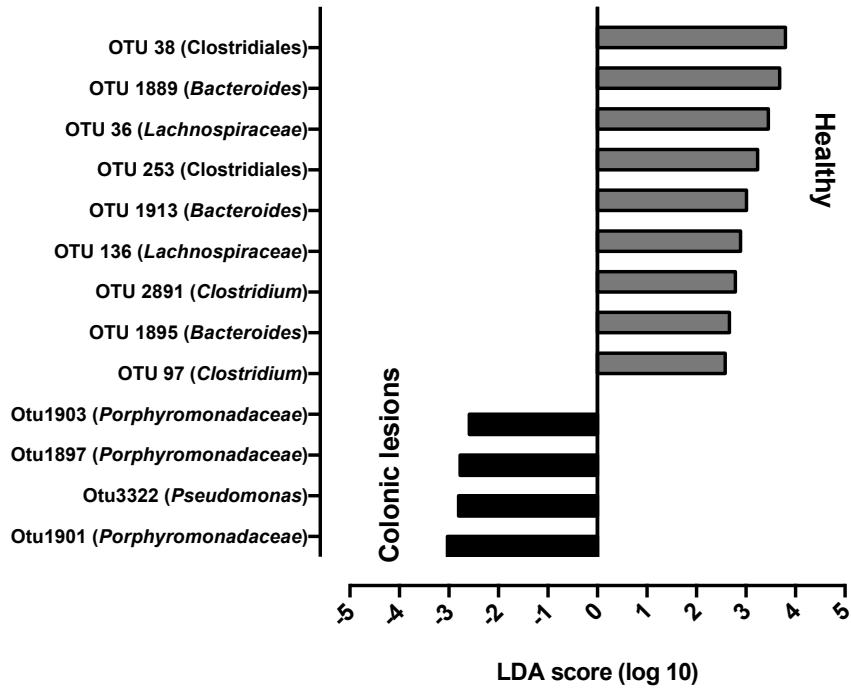


Figure 2.5. Microbial biomarkers for healthy and colonic lesion clinical groups. OTUs that were differentially abundant in adenoma and carcinoma clinical groups. LDA scores for significant OTUs are shown. OTU number and Taxonomic group based on RDP classification are represented.

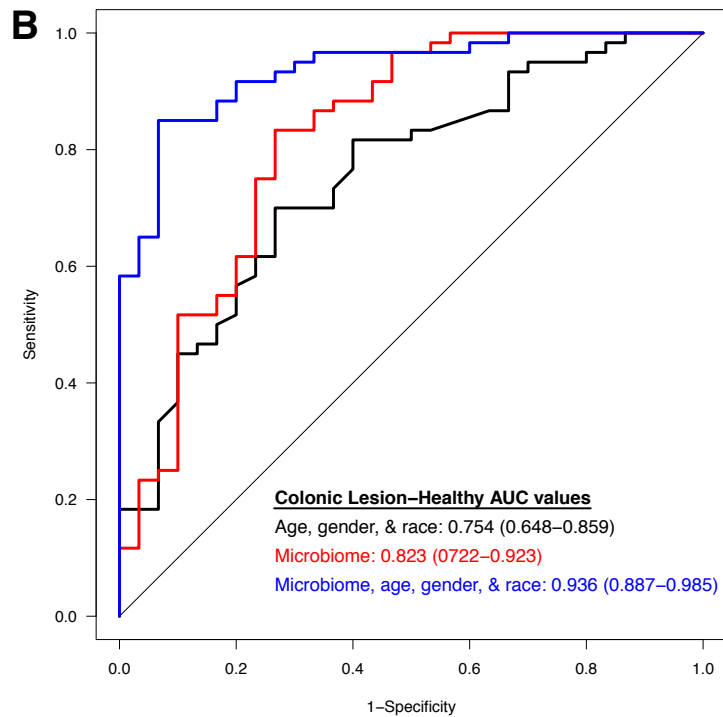
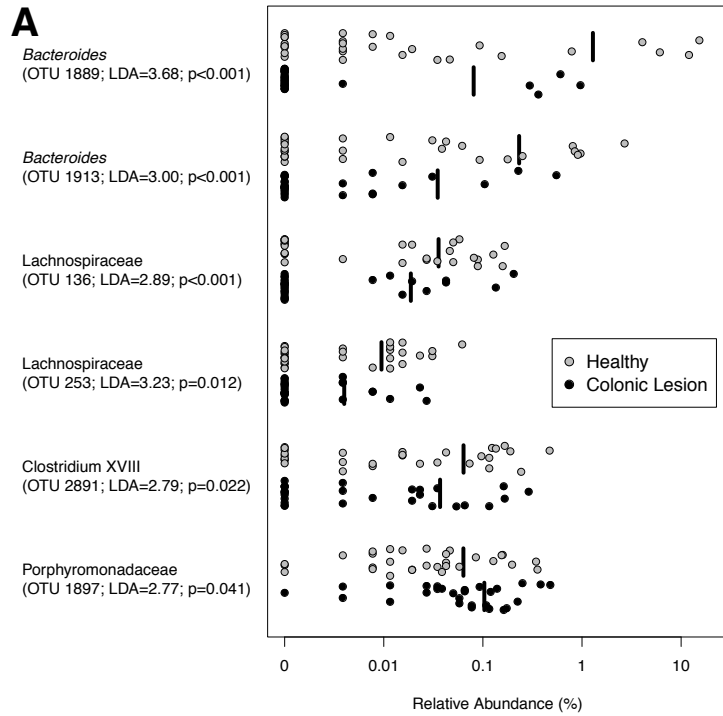


Figure 2.6. Microbiome improves accuracy of predictive models for healthy and colonic lesions. **A.** Relative abundance of differentially abundant OTUs for healthy ($n=30$; grey) subjects and those with lesions ($n=60$; black). Vertical black line represents mean. **B.** ROC curves for microbial biomarkers alone, clinical data alone, and microbial biomarkers with clinical data. The straight line represents the null model.

Comparison of adenoma and carcinoma clinical groups

Finally, we generated logit models using clinical and microbiome data to differentiate between patients in the adenoma and carcinoma groups. A patient's BMI was the only clinical variable that discriminated between the adenoma and carcinoma clinical groups (AUC=0.658; 95% CI: 0.518-0.799; $p=0.023$). When examining populations within the gut microbiome, relative to subjects with adenomas, those with carcinomas harbored higher relative abundances of OTUs that affiliated with the *Fusobacterium* (OTU 2458), *Bacteroides* (OTU 1882), *Phascolarctobacterium* (OTU 2395), and *Porphyromonas* (OTU 1905). In contrast, OTUs affiliated with *Blautia* (OTU 9), and Lachnospiraceae (OTU 12 and 23) were more abundant in subjects with adenomas (Figure 2.7). Next, we constructed a logit model to differentiate between the adenoma and carcinoma clinical groups using BMI with microbiome data. The model that provided the greatest differentiation between carcinoma and adenoma included BMI and 4 OTUs (OTUs 1905, 2395, 2458, 3235; Figure 2.8A). This model provided significantly greater discrimination than BMI alone (AUC=0.963; 95% CI: 0.921-1.00; ($p<0.001$; Figure 2.8B). Examination of the relative abundance of OTUs associated with the *Fusobacterium* genera revealed no significant associations between *Fusobacterium* and the stage or location of carcinomas. Together, these results demonstrated that the gut microbiome could be used to differentiate between the presence of precancerous and cancerous lesions in the colon.

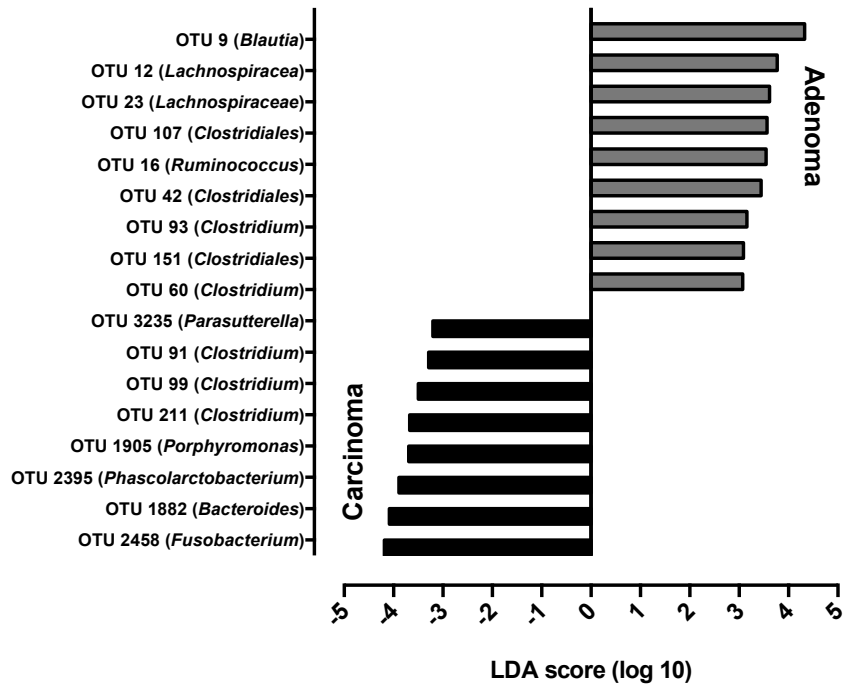


Figure 2.7. Microbial biomarkers for adenoma and carcinoma clinical groups. OTUs that were differentially abundant in adenoma and carcinoma clinical groups. LDA scores for significant OTUs are shown. OTU number and Taxonomic group based on RDP classification are represented.

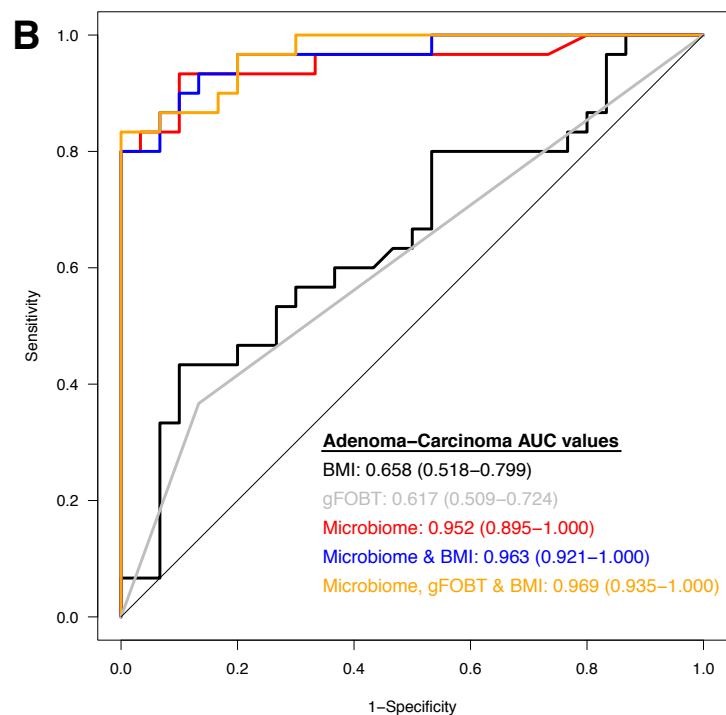
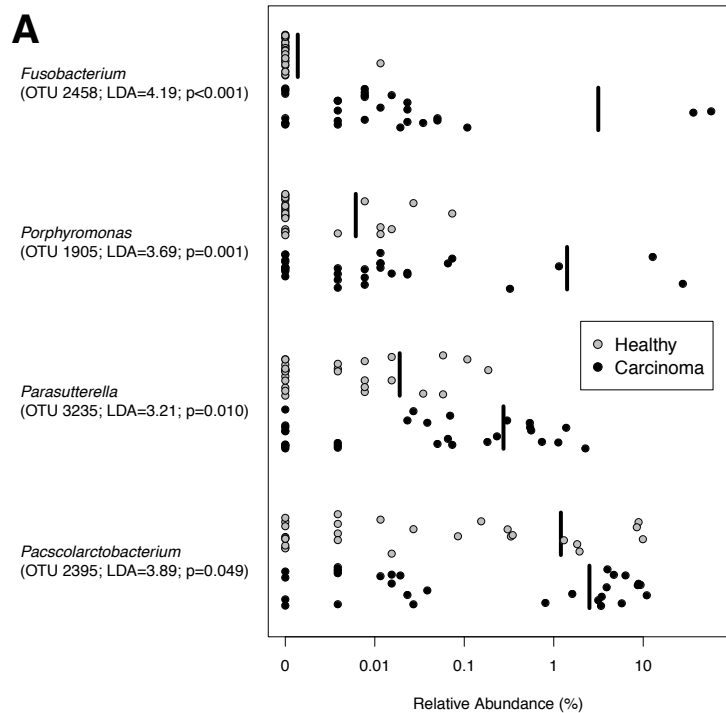


Figure 2.8. Microbiome improves accuracy of predictive models for adenoma and carcinoma. **A.** Relative abundance of differentially abundant OTUs for adenoma ($n=30$; grey) and carcinoma ($n=30$; black) subjects. Vertical black line represents mean. **B.** ROC curves for microbial biomarkers alone, clinical data alone, FOBT alone, microbial biomarkers with clinical data, and microbial biomarkers with FOBT and clinical data. For each comparison, the straight line represents the null model.

Complementing gFOBT test with microbiome-based models

Because gFOBT is the most common, non-invasive screening tool for CRC, we evaluated whether the microbiome-based models could be improved by including gFOBT results. The gFOBT test had 100% specificity in our study when comparing healthy individuals to those with colonic lesions. That is, patients without colonic lesions tested negative on the gFOBT. In an analysis comparing adenoma and carcinoma groups, the odds ratio for gFOBT was 3.76 (95% CI 1.04-13.65) when entered as a single explanatory variable, with AUC=0.617. In contrast, the microbiome data alone yielded an AUC of 0.952. The model combining BMI, gFOBT, and the microbiome data (OTUs 1905, 2395, 2458, 3235) provided excellent discriminatory ability (AUC=0.969; 95% CI: 0.935-1.000; Figure 2.8B). These results demonstrate that the gut microbiome dramatically outperforms gFOBT in discriminating between adenoma and carcinoma clinical groups in our sample of subjects.

Application of Microbiome Results to Population Data

To further test the capacity of the gut microbiome as a CRC screening candidate, we extracted data from Surveillance, Epidemiology and End Results (SEER) for age-specific incidence rates of CRC in the United States. Since likely candidates for CRC screening would target identification of early stage disease (adenoma), we designed a preliminary screening test based on the 5 OTUs (OTUs 38, 99, 136, 1889, 1913), which were enriched in healthy subjects compared to patients with adenomas. Persons who had any detectable levels (Relative abundance > 0) of these 5 OTUs were more likely to have healthy colons and constituted a negative test. Using a Bayesian model, we calculated the positive likelihood ratio for this preliminary screening test and applied it to

population probabilities of CRC for each age group (Table 2.2). The likelihood ratio of this test was 71 (95% CI: 64.78, 77.22) (sensitivity=23.3% [7/30], specificity=100% [30/30]). As can be seen in Table 2.2, individuals who are 65 years of age had a pre-test probability of CRC of 0.17% based on nationwide SEER data. When we applied the OTU test to this age group, the probability of adenoma was 10.67% after knowing the microbiome data (1 in 9 chance of having an adenoma). For people 50 years of age, the results suggest a one in 26 chance of having an adenoma with a positive OTU test, and for adults 80 years of age; a positive OTU test yielded a 1 in 5 chance of having an adenoma. Together, these results demonstrate that our preliminary screen dramatically improves the ability to predict the presence of an adenoma.

For comparison purposes, we assessed the pre-to-post-test probabilities of detecting adenoma based on the gFOBT results in this sample. The likelihood ratio of a positive gFOBT was 41 (95% CI: 34.75 - 47.25), which was lower than the likelihood ratio of a positive microbiome test (i.e., LR+=71). For a person who is 65 years of age with a positive gFOBT, the post-test probability of adenoma was 6.46%, indicating a 1 in 15 chance of having an adenoma. This contrasts with the 10.67% probability of adenoma (1 in 9 chance) using a positive microbiome test in the same 65-year old. While both tests had good specificity in this sample, the sensitivity of the microbiome test was greater than the sensitivity of the gFOBT.

| Age At Diagnosis (years) | Incidence Rate (per 100,000 people)* | Pre-Test Probability | Pre-Test Odds | Post-Test Odds** | Post-Test Probability | 95% Confidence Interval for Post-Test Probability |
|--------------------------|--------------------------------------|----------------------|---------------|------------------|-----------------------|---|
| 35-39 | 8.20 | 0.0001 | 0.0001 | 0.0058 | 0.0058 | 0.005-0.006 |
| 40-44 | 15.78 | 0.0002 | 0.0002 | 0.0112 | 0.0111 | 0.010-0.012 |
| 45-49 | 29.11 | 0.0003 | 0.0003 | 0.0207 | 0.0203 | 0.019-0.023 |
| 50-54 | 55.81 | 0.0006 | 0.0006 | 0.0396 | 0.0381 | 0.036-0.043 |
| 55-59 | 76.99 | 0.0008 | 0.0008 | 0.0547 | 0.0519 | 0.045-0.060 |
| 60-64 | 112.05 | 0.0011 | 0.0011 | 0.0796 | 0.0738 | 0.073-0.087 |
| 65-69 | 168.04 | 0.0017 | 0.0017 | 0.1195 | 0.1067 | 0.109-0.130 |
| 70-74 | 223.40 | 0.0022 | 0.0022 | 0.1590 | 0.1372 | 0.145-0.173 |
| 75-79 | 283.25 | 0.0028 | 0.0028 | 0.2017 | 0.1678 | 0.184-0.219 |
| 80-84 | 337.10 | 0.0034 | 0.0034 | 0.2401 | 0.1936 | 0.219-0.261 |
| 85+ | 376.39 | 0.0038 | 0.0038 | 0.2682 | 0.2115 | 0.245-0.292 |

* Based on Surveillance, Epidemiology and End Results data, Years 2000-2010.

** Using Likelihood Ratio of a positive test = 71.

Table 2.2. Post-test probability of microbiome-based adenoma screen. The probability of having an adenoma is listed for each age group prior to and after the incorporation of the results of a preliminary screen test based on microbiome relative abundance data. Adenoma test based on 5 OTUs (OTUs 38, 99, 136, 1889, 1913).

Discussion

Our results suggest that relative abundance data from the human gut microbiome differentiates individuals with healthy colons from those with adenomas and carcinomas. Most importantly, there was a significant difference in the gut microbiome of people with colonic adenomas compared to those with healthy colons. This has considerable importance in secondary prevention because screening for early stage colorectal cancer hinges on the ability to detect early pathologic changes. In this regard, we found that failure to detect at least 1 of the 5 OTUs served as a signal of the presence of adenoma. The probability of having an adenoma rose over 50-fold with this added information regarding microbiome. Taken with the existing literature regarding the importance of the gut microbiome in health and disease, our study further suggests that the microbiome may play a crucial role in the etiology of colorectal cancer.

A strength of our study design was that we collected samples from three clinical groups that represented the multistage progression in CRC (healthy, adenoma, and carcinoma). This allowed us to identify a panel of bacterial populations that could indicate both the progression from healthy tissue to adenoma and the progression from adenoma to carcinoma. Interestingly, when we looked at each patient, we rarely observed significant enrichment of every bacterial population among the OTUs incorporated in the logit models. For example, 11 of the 30 carcinoma patients had no detectable levels of *Fusobacterium*. However using the relative abundance data for the remaining panel of microbial biomarkers, such as *Porphyromonas*, *Bacteroides*, and Enterobacteriaceae, we were able to accurately classify these subjects. This strongly

suggests that there may be multiple underlying mechanisms by which the microbiome is involved in CRC and that CRC is likely a polymicrobial disease.

Our findings are supported by previous evidence. Three research groups reported that *Fusobacterium* spp. were enriched on the surface of tumors compared to adjacent healthy tissue [Kostic et al., 2012; Castellarin et al., 2012; Rubinstein et al., 2013]. Building upon these clinical studies, animal and tissue culture-based studies have provided evidence that *Fusobacterium* may contribute to tumor multiplicity through the recruitment of immune cells to tumors [Kostic et al., 2013; Rubinstein et al., 2013]. These mechanistic studies agree with our findings that *Fusobacterium* may be a marker for the presence of tumors. In addition, enterotoxigenic *Bacteroides fragilis* (ETBF), a pathogenic variant of a common commensal, has been shown to directly influence the development of CRC in murine genetic models through the production of a metalloprotease toxin [Sears et al., 2008]. In our samples, subjects with carcinomas showed an increase in the relative abundance of one *Bacteroides* population (OTU 1882) compared to subjects with adenomas. However, PCR-based screens for the toxin producing genes did not reveal the presence of ETBF. Additionally, we observed a significant decrease in the relative abundance of *Bacteroides* populations (OTUs 1889, 1913) associated with the advancement of tumorigenesis. Finally, a polyketide synthetase operon from *E. coli*, was shown to influence the progression of tumors using a murine model of inflammation-derived tumorigenesis [Arthur et al., 2012, Swidsinski et al., 1998]. Although we did see an enrichment for non-*E. coli* Enterobacteriaceae in the carcinoma subjects relative to the healthy subjects, we were unable to detect significant differences in the relative abundance of *E. coli* across the three clinical groups.

It is tempting to speculate on the enrichment of *Fusobacterium* and *Porphyromonas* spp. in subjects with CRC. Both of these bacterial taxa are common commensals of the mouth and a wealth of literature has linked them to chronic inflammation and periodontal disease [Signat et al., 2011; Deshpande et al., 1999; Darveau and Tanner, 1997; Han et al., 2000]. The mouth is a reservoir for these pathogens, allowing for colonization of the gastrointestinal tract under abnormal environmental conditions. During colorectal carcinogenesis, dramatic physiological changes occur in the microenvironment of colonic lesions [Peddareddigari et al., 2010]. Tumor-associated fluxes in nutrients and shifts in inflammatory mediators may favor colonization by opportunistic pathogens such as *Fusobacterium* and *Porphyromonas*. As demonstrated by Kostic and colleagues, colonization by such pathogens can support the development and progression of CRC [Kostic et al., 2013, Rubinstein et al., 2013]. We were unable to detect a significant association between either population and carcinoma severity or location. Additional studies are needed to examine how and at what stage these bacterial populations are affecting the development of CRC and how they may be linked to the oral microbiome and related to oral disease.

As highlighted above, there is a clear association with the enrichment of pathogenic bacterial populations and colon tumorigenesis; however, in the present study we emphasize that the depletion of potentially protective bacteria likely plays a similar role CRC pathology. We identified several bacterial populations that were significantly depleted in CRC. Individuals with both adenomas and carcinomas showed a dramatic loss in OTUs associated with the genera *Clostridium* and *Bacteroides*, and the family *Lachnospiraceae* [Atarashi et al., 2011; Smith et al., 2013; Round et al.,

2010]. Each of these bacterial taxa are well known producers of short chain fatty acids (SCFAs) in the colon. SCFAs are important microbial metabolites that supply nutrients to colonocytes and help maintain epithelial health and homeostasis. Specifically the SCFA, butyrate, has been shown to have substantial anti-tumorigenic properties including the ability to inhibit tumor cell proliferation, initiate apoptosis in tumor cells [Hague et al., 1995; Ruemmele et al., 2003], and mediate T-regulatory cell homeostasis [Smith et al., 2013]. Loss of these important bacterial populations in concert with an enrichment of pathogenic populations likely plays a synergistic role in potentiating tumorigenesis.

Although our results are important, there are limitations to the investigation. A larger, more diverse sample of individuals is needed to augment and validate our findings. Furthermore, although our study clearly demonstrates the viability of using the gut microbiome as a biomarker for CRC, we cannot assess the bacterial populations' role in causation or the mechanisms by which these populations affect the development and progression of CRC. Regardless, the feasibility, lack of invasive procedures, ability to be complement existing screening methods (e.g. gFOBT), and the strength of signal seen in this study support the further investigation and application of microbial biomarkers from stool as a method for CRC screening.

Material and Methods

Study design and sample collection

As part of the National Cancer Institute-funded Early Detection Research Network (EDRN), the Great Lakes-New England Clinical Epidemiological Center (GLNE CEC) created a biorepository that included whole evacuated stool for studies on potential molecular markers for the detection of colonic precancerous and cancerous conditions and cancer risk assessment. Eligible patients were 18 years of age or older, able to tolerate 58 ml of blood removal at two time points, willing to complete an gFOBT kit, able to provide informed consent, and had colonoscopy and histologically confirmed colonic disease status. Patients were excluded if known HIV or chronic viral hepatitis, known HNPCC or FAP, inflammatory bowel disease, any surgery, radiation or chemotherapy for their current colorectal cancer or colonic adenoma. Colonic disease status was defined as normal (no personal or family history of colon cancer, adenomas), colonic adenoma (colonoscopic and histologically confirmed adenoma), and colorectal adenocarcinoma (colonoscopic and histologically confirmed colorectal adenocarcinoma).

All participants collected a whole evacuated stool in a hat with no preservatives. Immediately after collection, the patient prepared a gFOBT six-panel kit (Sensa Hemocult II, Beckman-Coulter, Palo Alto, CA) from different areas of the stool. The whole stool was then packaged and shipped to the processing center along with the gFOBT. The gFOBT was processed and interpreted as soon as it arrived at the processing center. If any of the six wells were positive, the kit was recorded as positive for the participant. The whole stool was homogenized and aliquoted into equal volumes

and stored at -80°C . All participants had intact colonic lesions at time of stool collection. Study participants provided their stool sample between one and four weeks after their colonoscopy preparation. We were provided with 90 stool samples and linked data randomly chosen from disease groups of healthy (n=30), colonic adenoma (n=30), and colonic adenocarcinoma (n=30).

DNA extraction and 16S rRNA gene sequencing

Microbial genomic DNA was extracted using the PowerSoil-htp 96 Well Soil DNA isolation kit (Mo Bio Laboratories) using an EPMotion 5075 pipetting system. The V4 region of the 16S rRNA gene from each sample was amplified and sequenced using the Illumina MiSeq Personal Sequencing platform as described elsewhere [Kozich et al., 2013]. Sequences were curated as described previously using the mothur software package [Schloss et al., 2009]. Briefly, we reduced sequencing and PCR errors, aligned the resulting sequences to the SILVA 16S rRNA sequence database [Pruesse et al., 2007], and removed any chimeric sequences flagged by UCHIME [Edgar et al., 2011]. After curation, we obtained between 25,953 and 404,696 sequences per sample (median=95464), with a median length of 253 bp. To limit effects of uneven sampling, we rarefied the dataset to 25,958 sequences per sample. Parallel sequencing of a mock community revealed an error rate of 0.03%. All fastq files and the MIMARKS spreadsheet are available at <http://www.mothur.org/MicrobiomeBiomarkerCRC>.

Gut Microbiome Biomarker Discovery Analysis

Sequences were clustered into OTUs at a 97% similarity cutoff and the relative abundance was calculated for OTUs in each sample. All sequences were classified using a naïve Bayesian classifier trained against the RDP training set version 9 (<http://sourceforge.net/projects/rdp-classifier/>) and OTUs were assigned a classification based on which taxonomy had the majority consensus of sequences within a given OTU [Wang et al., 2007]. Differentially abundant OTUs were selected using the biomarker discovery algorithm, LEfSe (Linear discriminant analysis Effect Size) for each pairwise comparison of clinical groups [Segata et al., 2011] (Healthy vs. Adenoma, Healthy vs. Carcinoma, Adenoma vs. Carcinoma, Healthy vs. Colonic lesion) using a maximum P-value of 0.05 and a minimum linear discriminant analysis (LDA) score of 2.0.

Data Analyses

Analyses of patient-level characteristics across the three clinical groups utilized Pearson chi-square test for categorical data and one-way ANOVA for continuous variables. Clinical variables evaluated were age, gender, race/ethnicity, body mass index (BMI, kg/m²), and current medications. One missing value for BMI was imputed. Logit models were generated using both clinical and microbiome data as independent variables to contrast differences across disease groups (i.e., healthy versus adenomas; healthy versus cancer; adenomas versus cancer). OTUs demonstrating the highest LDAs and smallest p-values were entered into a logit model and their ability to discriminate group classification was evaluated using area under the receiver operator characteristic (ROC) curve. We used a maximum of 6 OTUs for each model to avoid potentially over-fitting the model. Differences between nested models were compared

using the test for the equality of ROC areas [DeLong et al., 1988]. Data were available on gFOBt status and therefore, this was entered as an independent variable when comparing adenoma versus carcinoma. We tested using an experiment wide error rate (i.e. α) of 0.05 and performed 2-tailed tests. Analyses were conducted in Stata/MP 13.1.

We utilized Bayesian methods to estimate the probability of adenoma based on relative abundance data taken from the gut microbiome [Linnet, 1988]. We utilized data from the 5 OTUs found to differentiate adenoma from healthy colons as the basis of a preliminary screening test. Sensitivity, specificity and positive likelihood ratios were calculated based on our study results, with failure to detect any appreciable level of any of these 5 OTUs (0 relative abundance) indicating possible pathology (i.e., positive test). Since the false positive rate of this test was 0%, we applied a continuity correction of 0.1 to each cell and calculated the likelihood ratio of a positive test and the 95% confidence intervals using standard methods [Simel et al., 1991]. The likelihood ratio was then applied to the pre-test probability of CRC based on national Surveillance, Epidemiology and End Results (SEER) data, years 2000-2010.

Acknowledgements

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CHAPTER III

The Gut Microbiome Modulates Colon Tumorigenesis

Abstract

Recent studies have shown that individuals with colorectal cancer have an altered gut microbiome when compared to healthy controls. It remains unclear whether these differences are a response to tumorigenesis or actively drive tumorigenesis. To determine the role of the gut microbiome in the development of colorectal cancer, we characterized the gut microbiome in a murine model of inflammation-associated colorectal cancer that mirrors what is seen in humans. We followed the development of an abnormal microbial community structure associated with inflammation and tumorigenesis in the colon. Tumor-bearing mice showed enrichment in operational taxonomic units (OTUs) affiliated with members of the *Bacteroides*, *Odoribacter*, and *Turicibacter* genera, and decreases in OTUs affiliated with members of the Prevotellaceae and Porphyromonadaceae families. Colonization of germ-free mice with microbiota from tumor-bearing mice significantly increased tumorigenesis in the colon compared to animals colonized with a healthy gut microbiome from untreated mice. Furthermore, at the end of the model, germ-free mice colonized with microbiota from tumor-bearing mice harbored a higher relative abundance of populations associated

with tumor formation in conventional animals. Manipulation of the gut microbiome with antibiotics resulted in a dramatic decrease in both the number and size of tumors. Our results demonstrate that changes in the gut microbiome associated with inflammation and tumorigenesis directly contribute to tumorigenesis and suggest that interventions affecting the composition of the microbiome may contribute to a strategy to prevent the development or progression of colon cancer.

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies worldwide, resulting in over a half million deaths annually [Parking et al., 2005]. Significant risk factors for CRC include diets rich in red and processed meat, alcohol consumption, and chronic inflammation of the gastrointestinal tract [Chambers et al., 2005; Huxley et al., 2009; Larsson et al., 2005; Slattery, 2000]. Each of these factors is closely associated with changes in composition and function of the complex community of microorganisms that inhabits our gastrointestinal tract. This community, known as the gut microbiome, promotes various physiological functions that are associated with cancer, including cell proliferation, angiogenesis, and apoptosis [Cheesman et al., 2011; Dolara et al., 2002; Stappenbeck et al., 2002; Rakoff-Nahoum et al., 2007]. Therefore, we hypothesized that the composition, structure, and functional capacity of the gut microbiome all directly affect tumor development in the colon.

Several recent studies have addressed this hypothesis by characterizing the composition of the gut microbiome associated with patients with CRC [Chen et al., 2013; Chen et al., 2012; Kostic et al., 2012; Geng et al., 2013; Shen et al., 2010;

Sobhani et al., 2011; Wang et al., 2012]. Using culture-independent approaches, each of these studies observed a significant shift in the composition of the gut microbiome in patients with CRC when compared to healthy controls. This phenomenon referred to as dysbiosis, can be observed in both the luminal microbiome from feces and the mucosal-associated microbiome from tumor biopsies. Interestingly, each of these studies obtained conflicting results regarding the composition and structure of the CRC-associated microbial community. Furthermore, there are no bacterial populations that have been consistently identified across each study that can be attributed to the development or presence of CRC. These data clearly show an association between abnormalities in the gut microbiome and CRC; however, the conflicting results point out the need for a mechanistic understanding of the role of the gut microbiome in this process.

The combination of factors that could lead to dysbiosis is complex and not well understood. In addition, the effect of the development of this abnormal community on colon tumorigenesis remains unclear. Recent evidence suggests that certain strains of *Bacteroides fragilis* and *E. coli* can directly affect tumor development in the colon through the production of virulence factors (e.g. toxins, gene products) [Arthur et al., 2012; Wu et al., 2009]. Furthermore, bacterial populations that produce the short chain fatty acid, butyrate, have anti-tumor effects in the colon by promoting apoptosis of colonic cancer cells [Hague et al., 1995; Ruemmele et al., 2003]. We reason that dysbiosis of the gut microbiome leads to both enrichment of cancer-promoting bacterial populations and loss of protective populations. Thus, understanding the changes in the

gut microbiome on a community-wide scale will be essential for understanding colon tumor development.

The gut microbiome is also likely to contribute to CRC through the initiation of inflammation. The link between inflammation and cancer is well established, and patients with inflammatory bowel diseases, such as ulcerative colitis, are at a greater risk of developing CRC in their lifetime. In the case of ulcerative colitis, the risk for cancer is related to both duration and severity of inflammation with an increasing rate of 0.5-1% per year after the first decade [Chambers et al., 2005; Ullman et al., 2011; Eaden et al., 2000]. Chronic inflammation of the colon leads to the production of various inflammatory cytokines and reactive oxygen species that work in concert to generate a tumor microenvironment that promotes carcinogenesis [Ullman et al., 2011; Arthur et al., 2013; Lin et al., 2007]. It has been suggested that this process is microbially driven, but it is unclear how the normally beneficial gut microbiome becomes inflammatory.

To determine the role of the gut microbiome in inflammation and colon tumorigenesis, we used a well-established model of colitis-associated CRC that recapitulates the progression from chronic inflammation to dysplasia and adenocarcinoma in humans [De Robertis et al., 2011]. We characterized the dynamics of the gut microbiome in this model and demonstrated that community-wide changes promote tumorigenesis in the colon. Our data support a model in which epithelial cell mutation and inflammatory perturbations to the gut microbiome lead to the development of an abnormal microbial community with enhanced tumor-promoting activity.

Results

Inflammation-associated colon tumorigenesis.

We were able to replicate an inflammation-based murine model of tumorigenesis in specific pathogen free (SPF) C57BL/6 mice (n=22) using an intraperitoneal injection of the chemical carcinogen azoxymethane (AOM) followed by three subsequent rounds of water-administered 2% dextran sodium sulfate (DSS) treatment [Tanaka et al., 2003; Chen et al., 2008] (Figure 3.1). The mice showed a consistent pattern of weight loss following each round of DSS treatment, with the most pronounced change occurring after the first round of DSS (Figure 3.2). We did not observe macroscopic tumors following the first round of DSS administration; however, we did observe increased infiltration by immune cells, significant epithelial damage, and submucosal edema (Figure 3.2). In addition, we observed a significant increase in the pro-inflammatory mediators macrophage inflammatory protein 2 (MIP-2), interferon gamma (IFN- γ), tumor necrosis factor alpha (TNF- α), interleukin 6 (IL-6), and IL-1 β (Figure 3.2). Macroscopic tumors and epithelial hyperplasia were apparent following the second round of DSS (Figure 3.1B, Figure 3.2). At the end of the model, the cohort had a median of 14.5 tumors per mouse (n=12), the majority of which were greater than 1 mm in diameter and located in the distal colon and rectum (Figure 3.2). These results demonstrate that our cohort of AOM/DSS treated mice developed a substantial number of colonic tumors with complete penetrance that could be detected as early as 7 weeks after AOM injection.

To determine whether tumor incidence and penetrance was dependent on the gut microbiome, we treated mice (n=9) with an antibiotic cocktail of metronidazole, vancomycin, and streptomycin *ad libitum* for 2 weeks prior to AOM and then throughout

the model, including the days of AOM injection and throughout the DSS treatment and recovery periods. Antibiotic-treated mice had significantly fewer tumors in the colon relative to the untreated mice (Fisher exact test: $p < 0.001$; Fig. 3.1B). Tumors that were present in antibiotic-treated mice were also significantly smaller than those observed in untreated mice (Student's t-test: $p = 0.002$; Fig. 3.1C, Figure 3.8). These results suggest that specific populations within the microbiome were essential for tumorigenesis. To determine whether the relative change in bacterial density following antibiotic treatment was due to a change in bacterial load, we performed qPCR on the 16S rRNA gene from stool samples of antibiotic-treated mice. The number of 16S rRNA gene copies per mg of feces was not significantly different from untreated stool samples ($p = 0.21$, Figure 3.6). Combined, these results indicate that changes to the structure of the community rather than total bacterial numbers affected tumorigenesis.

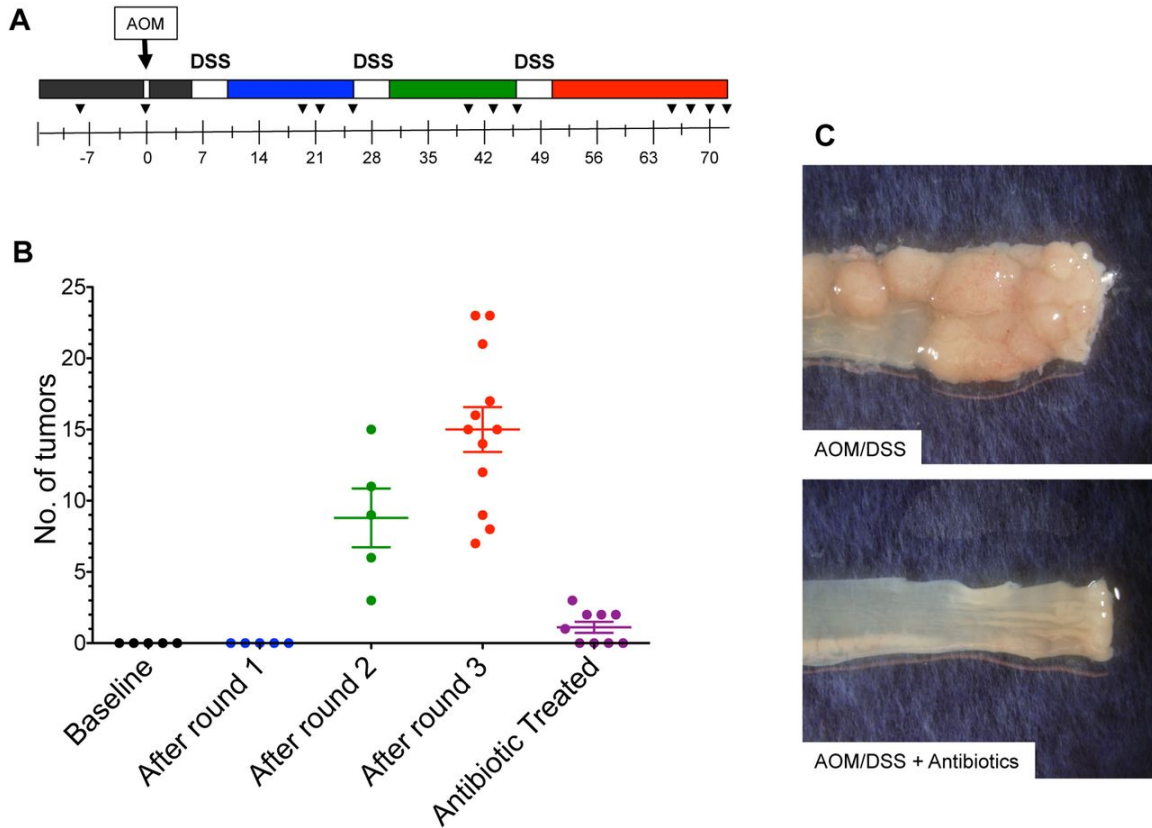


Figure 3.1. Development of a dysbiotic gut microbiome during colon tumorigenesis. Microbiome analysis was performed on fecal samples from 10 representative mice and color coded as indicated in Figure 3.1A. **A.** Inverse Simpson's diversity index. **B.** Observed community richness estimate. Statistical analysis was performed using repeated measured paired group analysis of variance. **C.** NMDS ordination based on θ_{YC} distances for all 10 mice during the AOM/DSS model. (AMOVA; $P < 0.01$) **D.** Average θ_{YC} distance within (black) and between (grey) phase of the model. Statistical analysis was performed using Wilcoxon test. *, $P < 0.01$. Error bars represent \pm SEM.

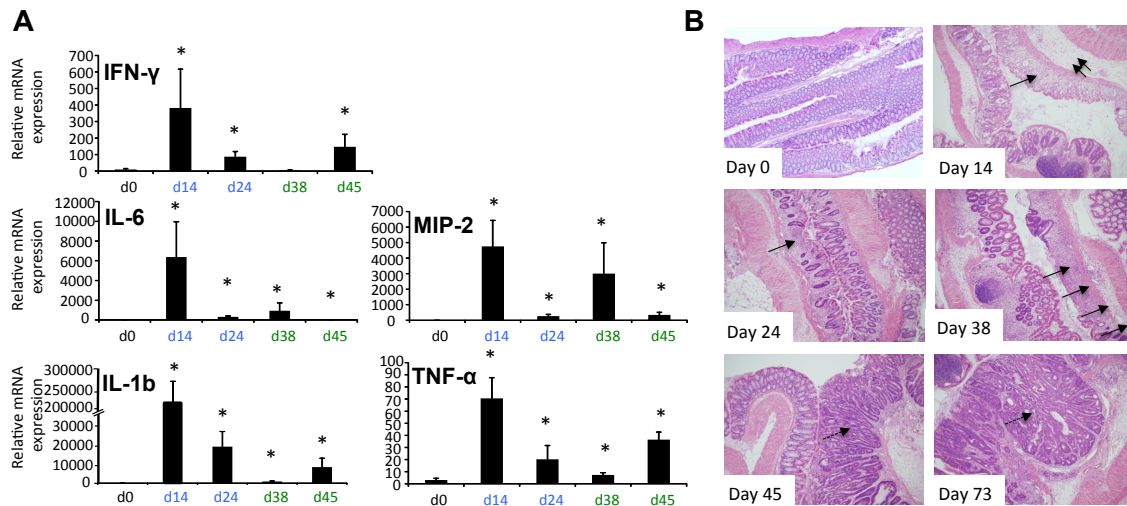


Figure 3.2. Colonic inflammation during tumorigenesis. **A.** Relative cytokine expression levels in colon. Statistical analysis was performed using a two-tailed Student's t-test. *, $P < 0.01$. **B.** Representative H&E stained histological sections from colon of mice during AOM/DSS tumor induction protocol. Single arrows indicate mucosal ulceration, double arrows indicate submucosa edema, and dashed arrows indicate adenoma. Error bars represent SD.

Significant shifts in the microbiome are associated with colon tumorigenesis. To further test the hypothesis that specific changes in the microbial community structure were associated with inflammation and tumorigenesis, we examined the dynamics of the gut microbiome throughout the model using stool samples from a subset of the original cohort of conventional mice treated with AOM/DSS in Figure 3.1 (n=10). We used the fecal samples taken prior to AOM injection as a baseline control for each mouse and then took samples following each subsequent round of DSS administration (Figure 3.1A). Mice showed a significant decrease in microbial diversity in the gut microbiome following the first round of DSS administration through tumor development ($P < 0.001$; Figure 3.3A and 3.3B). Ordination of the distances between fecal samples showed that at the time of euthanization, tumor-bearing mice developed a significantly altered microbiome that clustered separately from that in baseline samples taken prior to the first round of DSS (Figure 3.3C). Further examination of fecal samples collected at various time points during the AOM/DSS tumor induction protocol revealed that significant alterations in the microbiome could be observed as early as the first round of DSS administration in 7 of the 10 mice. Each round of DSS treatment resulted in a significant change in the structure of the microbiome (Figure 3.3D). Fecal samples taken from tumor-bearing mice after the third round of DSS until the time of euthanization also clustered separately from earlier samples. The distances between clusters were significantly higher than the distance within clusters (Figure 3.3D). These clusters were observed using OTU and phylogenetic-based metrics of β -diversity (i.e. Θ_{YC} and unweighted or weighted UniFrac) and could be distinguished from one another using the Random Forest machine learning algorithm (Accuracy for each group:

Baseline: 100%, DSS round 1: 72.4%, DSS round 2: 71.9%, DSS round 3: 80.6%). These results highlighted the association between a dramatically altered microbiome structure and the presence of tumors.

To determine the effect of inflammation on the microbial community independent of tumorigenesis, we treated mice with three rounds of DSS without the AOM injection (n=5). Mice treated with DSS in the absence of AOM did not develop colonic tumors. There was an initial community shift following the first round of DSS, but the subsequent stepwise shifts that occurred in AOM/DSS-treated mice were not observed in mice treated with DSS only (Figure 3.7). Furthermore, we did not observe the sustained drop in microbial diversity that was observed in AOM/DSS-treated animals (Figure 3.7). These results suggest that inflammation alone is insufficient to cause microbial community changes. Rather, the synergistic effects of the AOM/DSS model are necessary for the development of the altered microbiome structure and tumorigenesis. We next identified which operational taxonomic units (OTUs) were responsible for the dramatic shifts to the microbial community structure during inflammation and tumorigenesis (Figure 3.4). Consistent with our communitywide β -diversity analyses, we observed changes in 37 bacterial populations (after excluding OTUs representing < 0.5% of the community) during the time course of the model relative to baseline samples prior to treatment. Fecal samples taken after the first round of DSS were enriched in the relative abundance of OTUs affiliated with members of the genus *Bacteroides* (OTUs 1 and 13). We also observed significant decrease in the relative abundance of OTUs associated with members of the genus *Prevotella* and unclassified genera within the family Porphyromonadaceae. Following the second round of DSS, we

observed a further loss of the same *Prevotella* (OTUs 4 and 5) and Porphyromonadaceae (OTUs 7, 12, 15, 22, 31, and 48) and the continued enrichment of *Bacteroides* (OTUs 1 and 13). Samples taken from mice following the third round of DSS showed significant differences compared to those taken following the first round of DSS and healthy baseline mice (Figure 3.4; all $P < 0.001$ by AMOVA). Tumor-bearing mice showed enrichment in OTUs affiliated with *Bacteroides* (OTUs 1), *Odoribacter* (OTU 3), and *Turicibacter* (OTU 20). Additionally, in tumor-bearing mice we detected a marked bloom of a member of the Erysipelotrichaceae family (OTU 26), which was undetectable in all of the mice prior to the second round of DSS when tumors are not evident. Simultaneous with the blooming of several bacterial populations, there was a significant decrease in the relative abundance of OTUs associated with members of the genus *Prevotella* (OTUs 4 and 5) and the family Porphyromonadaceae (OTUs 7, 12, 15, 22, 31, and 48). An OTU associated with the *Bacteroides* genus (OTU 13), which bloomed during the onset of inflammation, significantly decreased following the third round of DSS. Mice that were treated with DSS in the absence of AOM, showed no significant changes in specific bacterial populations when compared to untreated mice. These results strongly suggest that both inflammation and tumorigenesis promote gut microbiome dysbiosis, as highlighted by major shifts in bacterial populations from a wide range of taxonomic groups.

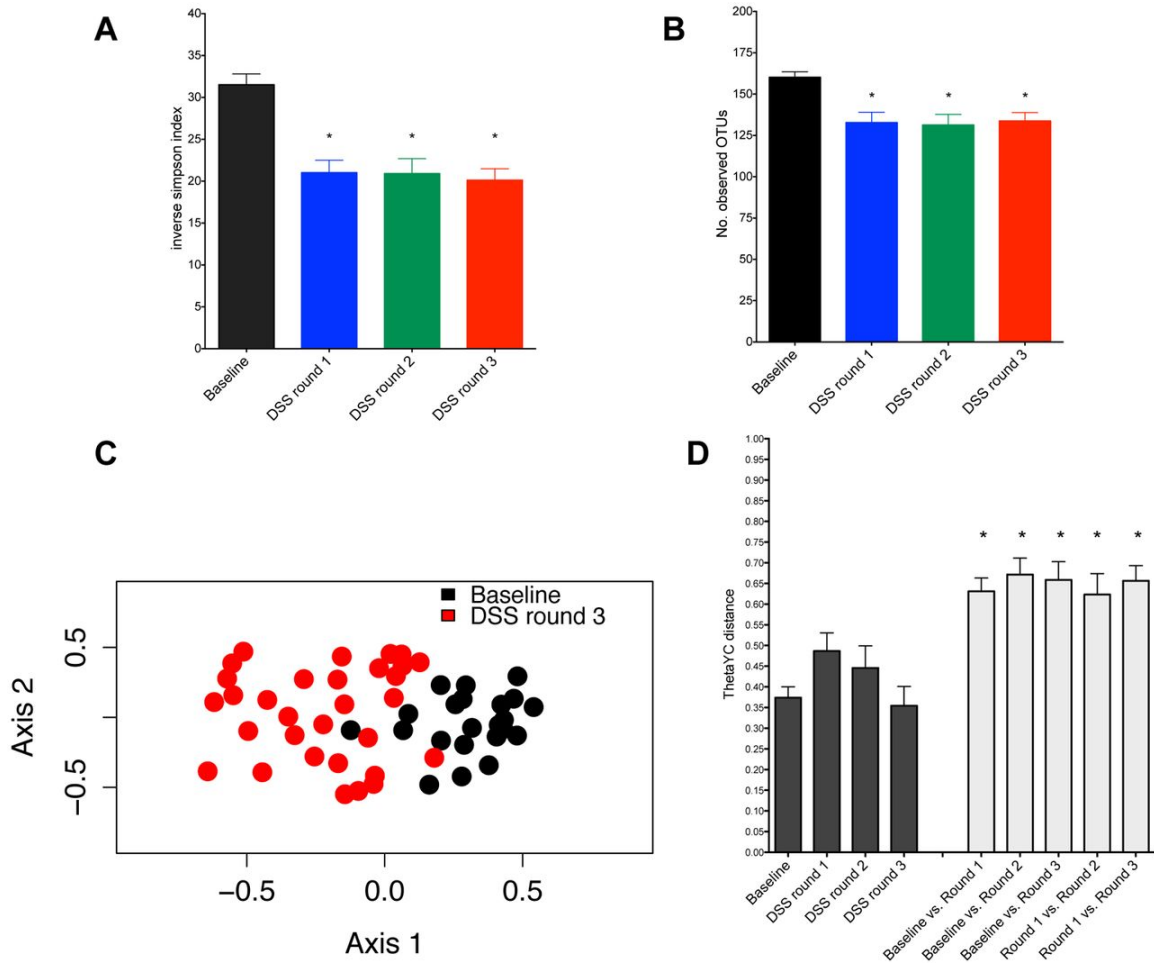


Figure 3.3. Development of a dysbiotic gut microbiome during colon tumorigenesis. Microbiome analysis was performed on fecal samples from 10 representative mice and color coded as indicated in Figure 1A. **A.** Inverse Simpson's diversity index. **B.** Observed community richness estimate. Statistical analysis was performed using repeated measured paired group analysis of variance. **C.** NMDS ordination based on θ_{YC} distances for all 10 mice during the AOM/DSS model. (AMOVA; $P < 0.01$) **D.** Average θ_{YC} distance within (black) and between (grey) phase of the model. Statistical analysis was performed using Wilcoxon test. *, $P < 0.01$. Error bars represent +/- SEM.

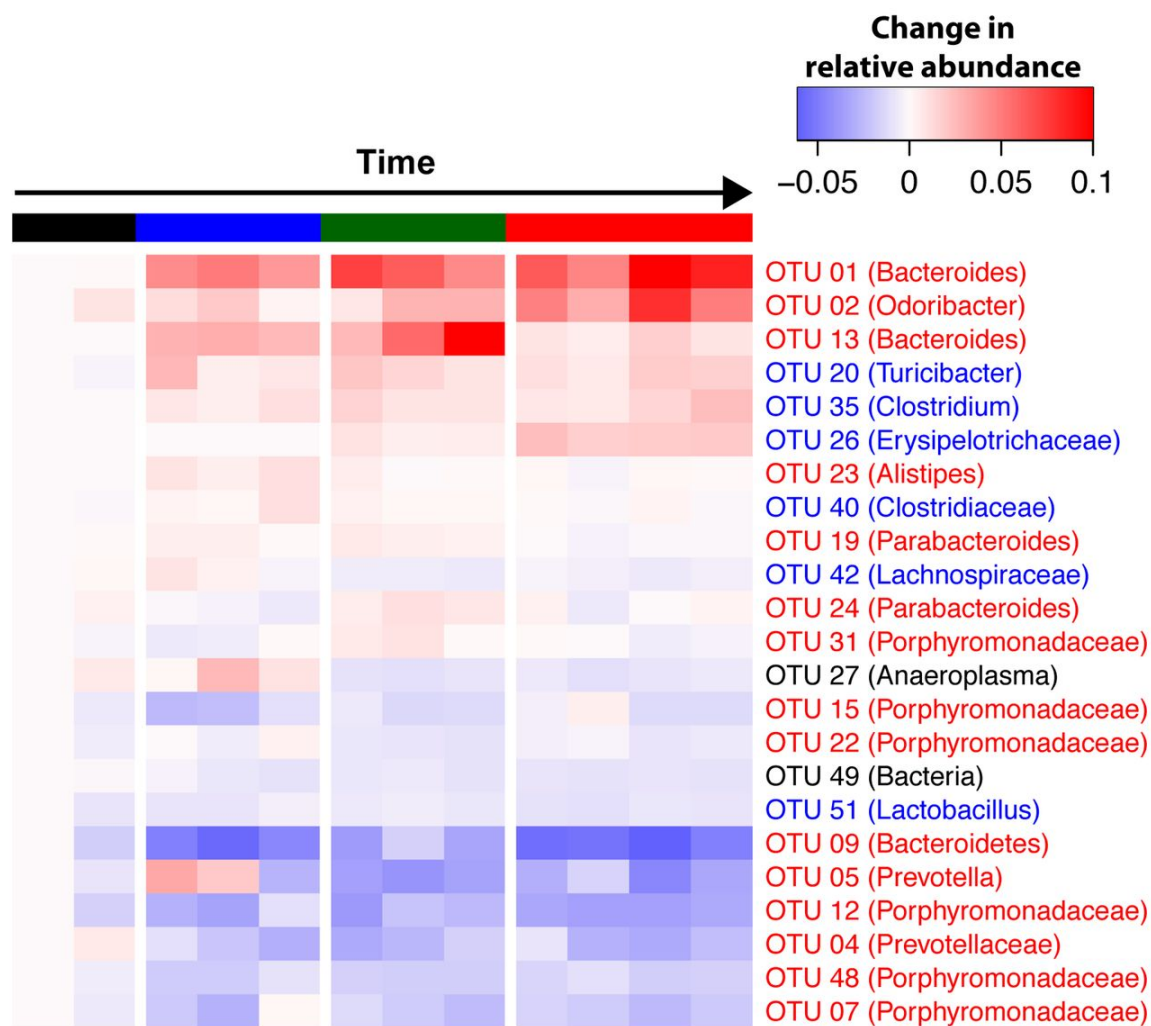


Figure 3.4. Change in relative abundance for OTUs that are significantly different from the time of AOM administration. Heatmap shows change in OTU abundance relative to samples taken at baseline time points. Average OTU abundance between mice for each OTU was calculated for each time point. Timeline is colored into the following groups: Baseline samples (prior to AOM): black, following the first round of DSS: blue, following the second round of DSS: green, Following the third round of DSS: red. OTU number and Taxonomic group based on RDP classification are represented for each row and each OTU is colored based on phylum (Firmicutes: blue, Bacteroidetes: red, Tenericutes: black). Repeated measures paired group analysis of variance was used to identify significantly altered OTUs.

We hypothesized that the variability in tumor burden among AOM/DSS-treated mice was associated with variability in the gut microbiome between mice (coefficient of variation for tumor burden=37.9; Figure 3.1C). We identified an OTU related to an unclassified genus within the family Porphyromonadaceae (OTU 12) that was negatively correlated with tumor burden (Spearman correlation=-0.73, p-value<0.05). The relative abundance of this bacterial population decreased with each round of DSS and this drop in abundance was more pronounced in mice with higher tumor burden. These results suggest that alterations in the relative abundance of specific bacterial populations were associated not only with the incidence of tumors, but also their prevalence.

Tumor-associated alterations in the microbiome increase tumorigenesis in germ-free mice.

To determine whether the community-wide microbiome changes directly contributed to tumor incidence in the colon, we colonized germ-free mice with either the healthy microbiome of untreated mice or the microbiome of tumor-bearing mice from Figure 3.1 following administration of the AOM/DSS model. To ensure that mice were repeatedly inoculated and stably colonized, we transferred fresh feces and bedding to two groups of germ-free mice (n=10/group). One group was housed with the bedding from healthy, untreated SPF mice, and a second group was housed with bedding from tumor-bearing AOM/DSS treated mice. To minimize litter effects, each group was comprised of two cages of 5 mice collected from separate litters that were randomly assigned to each of the cages. Following a three-week colonization period, mice were treated with AOM/DSS under germ-free conditions, as described above (Figure 3.1). All

bacterial phyla and 90% (62 of 69) of genus-level taxa detected in donor samples were detected within the recipient germ-free mice (Table 3.2), which is higher than has been previously reported [Turnbaugh et al., 2009; Smith et al., 2013]. Furthermore, 81% of the sequences we obtained from the donor mice belonged to OTUs that were found in the recipient germ-free mice. Following AOM/DSS treatment, mice colonized with the microbiome of tumor-bearing mice had a 2-fold increase in tumor burden ($p=0.002$) relative to mice colonized with a healthy microbiome (Figure 3.5). Additionally, tumors from these mice were significantly larger than those observed in recipients of a healthy microbiome ($p=0.002$; 3.8). Similar to our results with SPF mice, germ-free mice colonized with the community of tumor-bearing mice had a significantly less diverse gut microbiome ($P<0.001$). Using community wide β -diversity analyses we determined that following a three-week colonization period with these two treatments of bedding resulted in two distinct microbial community structures (AMOVA; $p<0.001$; Figure 3.5C). Germ-free mice colonized with the microbiome of tumor-bearing mice showed significant enrichment in the relative abundance of OTUs affiliated with the genera *Bacteroides* (OTU 1) and the family Erysipelotrichaceae (OTU 26). Additionally, these germ-free mice had significantly fewer Porphyromonadaceae (OTU 12) when compared to germ-free mice colonized with bedding from healthy mice. Finally, germ-free mice colonized with a healthy microbiome successfully recapitulated the community dynamics seen in conventional mice during tumorigenesis. We observed significant changes in 34 OTUs following the AOM/DSS model. Similar to tumor-bearing conventional mice, germfree tumor-bearing mice showed enrichment in OTUs affiliated with members of the *Bacteroides* (OTUs 1), *Odoribacter* (OTU 3), *Turicibacter* (OTU 20), and a bloom in

Erysipelotrichaceae (OTU 26), following AOM/DSS administration. There was also significant decrease in the relative abundance of OTUs associated with members of the genus *Prevotella* (OTUs 4 and 5) and the family Porphyromonadaceae (OTUs 6, 7, and 12). These results demonstrate that alterations to the gut microbiome that were associated with chronic inflammation and tumorigenesis in SPF mice were transmitted to germfree mice and can exacerbate colon tumorigenesis.

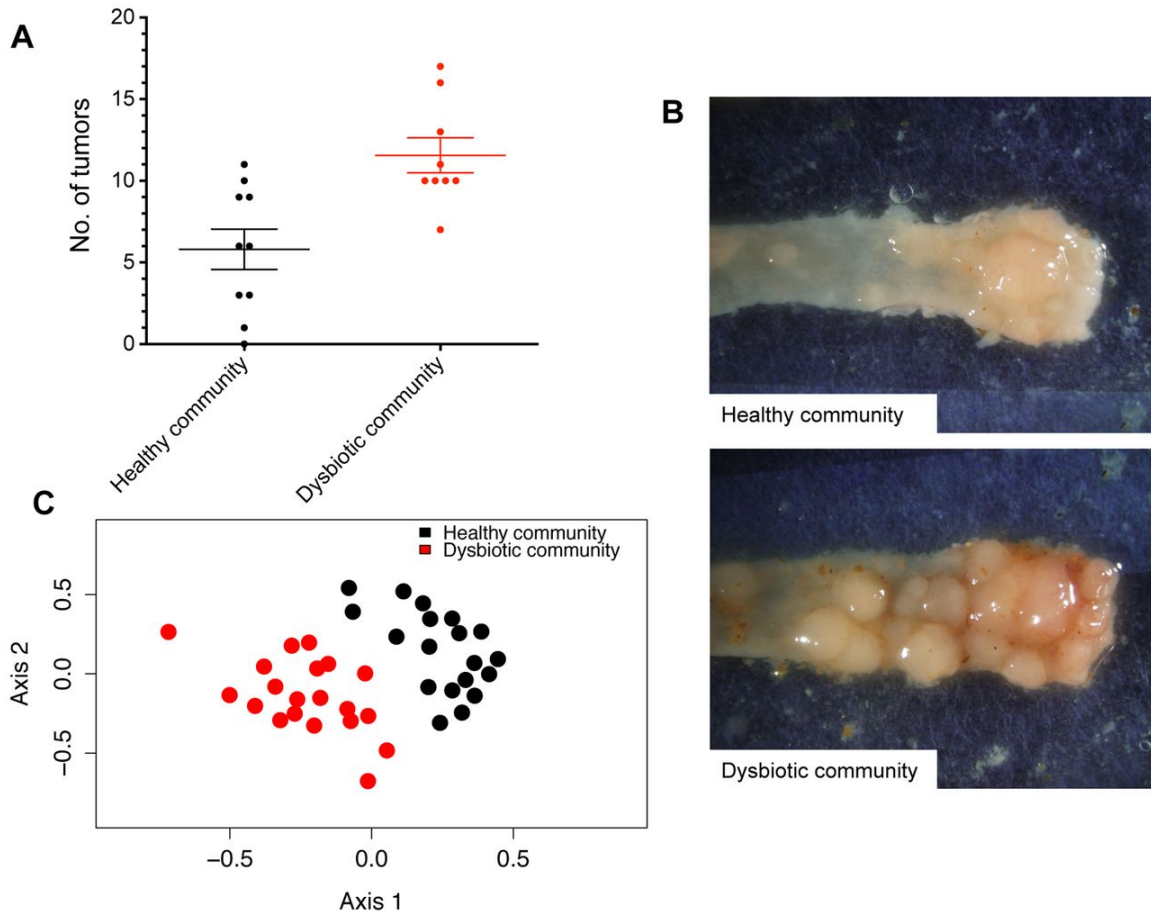


Figure 3.5. Tumor-associated gut microbiome alterations exacerbate tumorigenesis in germ-free mice. **A.** Number of tumors observed at the end of the model when germ-free mice were colonized using bedding from healthy mice (Healthy community) and mice with tumors (Dysbiotic community). Tumors counts were taken following AOM/DSS treatment. **B.** Representative images of tumors in the distal colon of mice colonized with a healthy microbiome (n=10) or the microbiome of tumor-bearing mice (n = 9). **C.** NMDS ordination based on θ_{yc} distances for all 19 mice following a three-week colonization period with a healthy microbiome (Healthy community) or the microbiome of tumor-bearing mice (Dysbiotic community). Error bars represent +/- SEM.

Discussion

In the present study we established a causal role for the gut microbiome in exacerbating tumor formation in an inflammation-based model of tumorigenesis. Manipulation of the microbiome using antibiotics reduced tumor formation, which highlighted the importance of bacterial driven factors in tumorigenesis. We demonstrated dynamic changes in the microbial community structure associated with dysbiosis, which occurs prior to the first signs of macroscopic tumor formation. We established the synergistic affect of AOM- and DSS-induced inflammation and tumorigenesis in driving microbial community changes that occur in a stepwise fashion. Finally, transfer of microbiota from tumor-bearing mice into germfree mice significantly increased the number and size of tumors compared to that in germ-free mice inoculated with healthy microbiota. Our experiments also demonstrated dramatic shifts in the relative abundance of bacterial populations, including those related to the genus *Bacteroides*, which were associated with increased tumorigenesis.

Several recent studies have compared the gut microbiome of patients with CRC to healthy controls [Chen et al., 2013; Chen et al., 2012; Kostic et al., 2012; Geng et al., 2013; Shen et al., 2010; Sobhani et al., 2011; Wang et al., 2012]. These studies have consistently demonstrated significant differences in the microbial community structure of patients with CRC, but each study has disagreed in terms of the specific gut microbiome composition and profile associated with CRC. The inability to identify a consensus community profile or etiological agent is likely due to the large variation in the structure of the microbiome across individuals and the improbability of there being one community profile or bacterial population that is associated with all CRCs. We were

able to reduce the inter-individual variation and the diversity of cancer types using a murine model of inflammation-induced CRC. Unlike the human cross-sectional studies, we demonstrated dynamic changes in the microbiome during the development of inflammation and tumorigenesis and that these changes directly cause disease.

Based on this study, the gut microbiome complements the activity of AOM and DSS to cause tumorigenesis, but the underlying mechanisms driving microbial-mediated tumorigenesis observed remain to be elucidated. Although a number of bacterial populations have altered relative abundances throughout the model, it is as yet unclear whether there is an increase in bacterial populations that induce inflammation or a loss of populations that produce anti-inflammatory signals and help maintain immune homeostasis in the gut. Regardless, an increasingly inflammatory environment would generate a self-reinforcing pathogenic cascade between the gut microbiome and the host, fostering the development of cancer through the development of, for example, genotoxic reactive oxygen species and pro-tumor inflammatory mediators (e.g. TNF- α , IL-6, IL-1 β , IL-23). In addition to the role of the gut microbiome in inflammation, changes mediated by chronic inflammation and tumorigenesis could lead to the enrichment of bacterial populations [Couturier-Maillard et al., 2013] that have a direct role in tumor development through the production metabolites, antigens, virulence factors and other potential tumor-promoting gene products. A recent study by Arthur *et al.* [Arthur et al., 2012] demonstrated that colonic inflammation in the IL-10-deficient mouse impacts the composition of the gut microbiome, leading to an enrichment of tumor-promoting *E. coli* strains. Although we did not detect any significant changes in populations related to the genus *Escherichia*, it is likely that the microbial community

alterations we observed in our tumor model are enriched with populations that fill a similar role. Specifically, marked increases in *Bacteroides* sp. in our study may contribute to tumorigenesis. Human commensals belonging to the genus *Bacteroides*, specifically enterotoxigenic *B. fragilis* (ETBF), have been associated with inflammation and CRC [Wu et al., 2009; Sears et al., 2008]. ETBF has been shown to strongly induce colonic tumors in multiple intestinal neoplasia mice through secretion of a metalloprotease toxin and certain strains are thought to contribute to CRC risk in humans. We did not detect ETBF in the murine gut microbiome (data not shown), but it is possible that similar processes are occurring during tumorigenesis in mice.

Chronic inflammation and tumorigenesis are also likely to lead to the loss of members of the gut microbiome that are important for maintaining epithelial health and immune homeostasis. In this study, we observed a dramatic decrease in OTUs from unclassified genera within the family Porphyromonadaceae. We hypothesize that these bacterial populations serve a protective role and are important mediators of gut health in the murine gut microbiome. One mechanism of protection could be through the fermentation of complex carbohydrates (e.g. fiber) into short chain fatty acids (SCFA) such as butyrate. Butyrate reduces inflammation [Segain et al., 2000] and inhibits growth and induces apoptosis in cancer cells [Hague et al., 1995; Rummelle et al., 2003]. Therefore, loss of butyrate-producing populations in the gut could increase both inflammation and tumorigenesis. This is supported by extensive epidemiological data that demonstrates a link between diets high in fiber and decreased CRC risk [Aune et al., 2011]. Furthermore, recent studies have shown that both individuals who consume low fiber diets or are diagnosed with CRC have a lower level of SCFAs in their feces

[O'Keefe et al., 2009]. It is also possible that members of the family Porphyromonadaceae are important mediators of anti-inflammatory signals in the gut. A loss of such anti-inflammatory populations would lead to a dramatic intensification of inflammation in the gut during DSS induced colitis and a marked increase in tumor-promoting signals.

It is important to note that the gut microbiome is an extremely complex and diverse community and therefore, it is unlikely that a single bacterial population is responsible for driving tumorigenesis or that one CRC-associated microbiome can be found in all CRC patients. Rather, as our data suggest, a community-wide effect involving the gain and loss of bacterial populations and general metabolic functions likely plays a critical role in CRC development. As we demonstrated in this study, changes in the entire gut microbiome can dramatically alter tumor burden, and identifying the mechanisms behind this phenomenon will be critical for addressing how the microbiome can be altered therapeutically to reduce colon tumorigenesis.

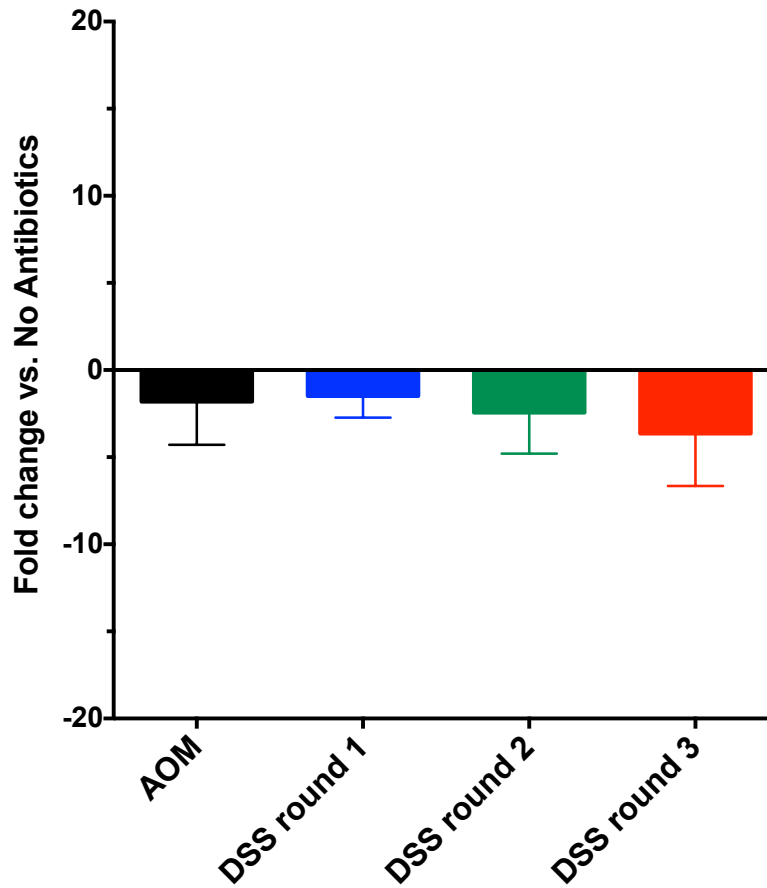


Figure 3.6. 16S rRNA quantitative qPCR. Relative fold decrease in 16S rRNA copy number during antibiotic treatment compared to baseline untreated samples. All time points showed no significant decrease compared to baseline untreated samples ($p = 0.21$). Error bars represent SEM.

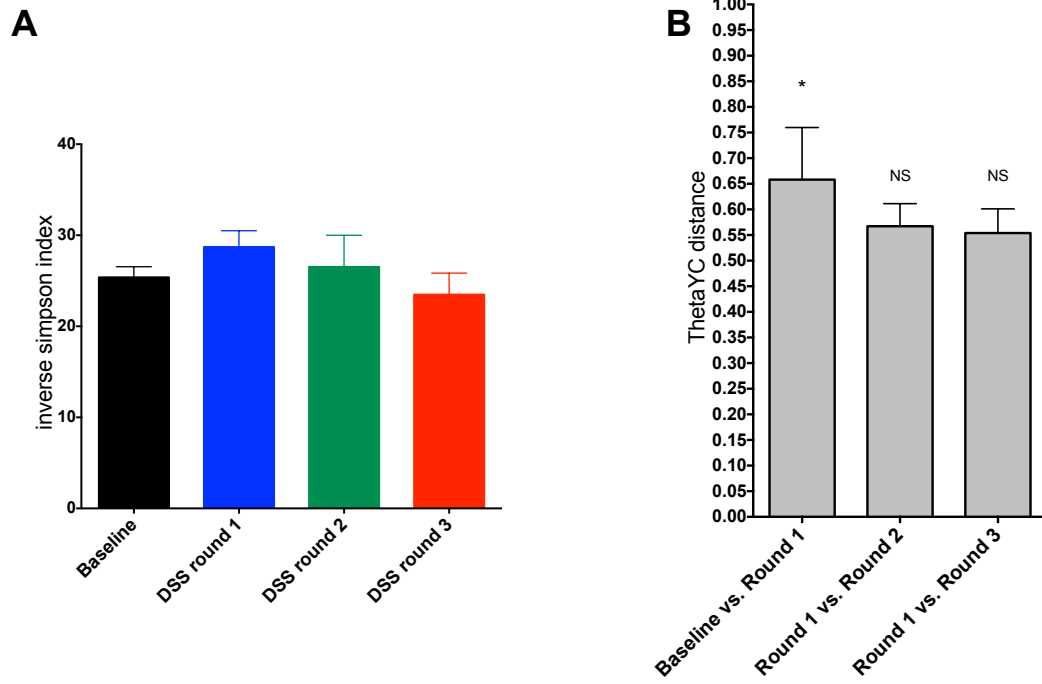


Figure 3.7. Microbiome alterations associated with DSS induced inflammation. A. Inverse Simpson's diversity index. **B.** Average θ_{YC} distance between phase of the model. Error bars represent SEM.

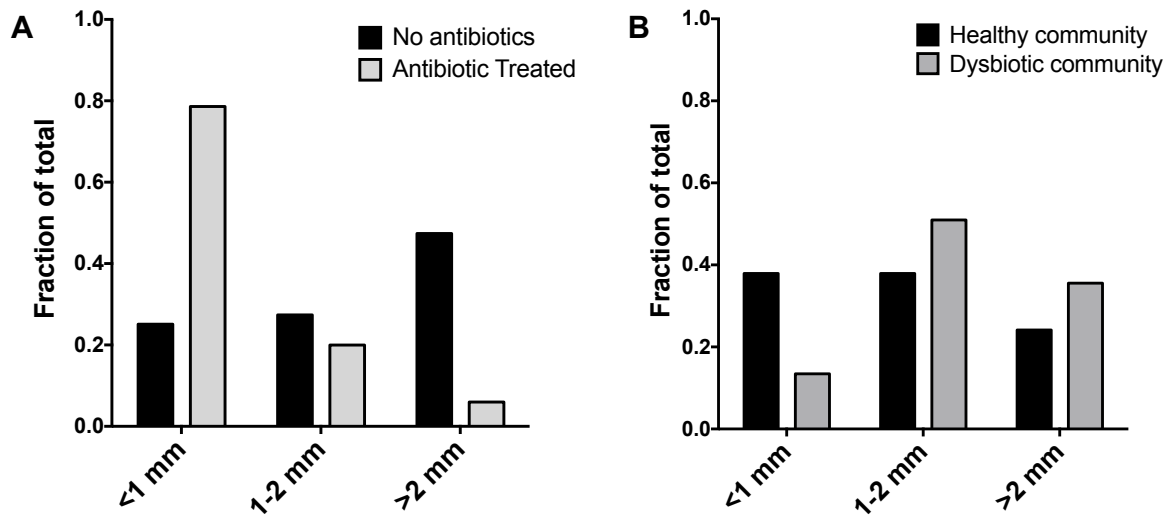


Figure 3.8. Tumor size for antibiotic treated and germ-free mice. Measurement of the largest dimension of each tumor (mm) was performed using calipers for each mouse following AOM/DSS administration. (A) Antibiotic-treated mice (n=10) versus conventional mice (n=12). P = 0.002. (B) Germfree mice colonized with a healthy community (n=10) versus a dysbiotic community (n=10). P = 0.0019. Statistical analysis of tumor count data was performed using Fisher's exact test.

| OTU# | Taxonomy (level) | Baseline | DSS Round 1 | DSS Round 2 | DSS Round 3 |
|------|------------------------------|----------|-------------|-------------|-------------|
| 1 | <i>Bacteroides</i> (genus) | 3.99* | 8.32 | 9.97 | 11.35 |
| 3 | <i>Odoribacter</i> (genus) | 1.42 | 2.02 | 3.06 | 5.98* |
| 4 | Prevotellaceae(family) | 5.48* | 3.07 | 2.67 | 3.18 |
| 5 | <i>Prevotella</i> (genus) | 5.03 | 6.01 | 1.76* | 2.44* |
| 6 | Porphyromonadaceae (family) | 4.99* | 2.19 | 2.01 | 4.52 |
| 7 | Porphyromonadaceae (family) | 4.15 | 3.06 | 2.61 | 2.6 |
| 9 | Bacteroidetes (phylum) | 6.2 | 2.05* | 4.09 | 1.71 |
| 12 | Porphyromonadaceae (family) | 3.56 | 2.08 | 1.52 | 1.05 |
| 13 | <i>Bacteroides</i> (genus) | 0.07 | 2.84 | 6.39 | 1.01 |
| 14 | <i>Lactobacillus</i> (genus) | 0.98 | 1.69 | 2.2 | 2.89 |
| 15 | Porphyromonadaceae (family) | 2.51 | 0.98 | 1.77 | 2.14 |
| 18 | Clostridiales (order) | 1.39 | 1.78 | 0.58 | 0.82 |
| 20 | <i>Turicibacter</i> (genus) | 0.16 | 1.57 | 1.69 | 1.54 |
| 22 | Porphyromonadaceae (family) | 0.99 | 1.23 | 0.42 | 0.69* |
| 23 | <i>Alistipes</i> (genus) | 0.51 | 1.31 | 0.71 | 0.55* |
| 26 | Erysipelotrichaceae (family) | 0 | 0 | 0.69* | 2.03* |
| 27 | <i>Anaeroplasma</i> (genus) | 1.51 | 2.31 | 0.21* | 0.44* |
| 35 | <i>Clostridium</i> (genus) | 0* | 0.8 | 1.06 | 1.39 |
| 36 | Bacteria (kingdom) | 0 | 0.06 | 0.54 | 1.16* |
| 42 | Lachnospiraceae (family) | 0.71 | 0.91 | 0.08* | 0.22 |
| 48 | Porphyromonadaceae (family) | 1.58 | 0.36 | 0.14 | 0.36 |
| 49 | Bacteria (kingdom) | 0.92 | 0.31 | 0.19 | 0.08 |
| 51 | <i>Lactobacillus</i> (genus) | 0.72 | 0.47 | 0.5 | 0.22 |
| 56 | Porphyromonadaceae (family) | 0.7 | 0.46 | 0.27 | 0.41 |
| 59 | Bacteroidales (order) | 0.35 | 0.09 | 0.21 | 0.14 |
| 62 | Alphaproteobacteria (order) | 0.42* | 0.12 | 0.05 | 0.09 |
| 66 | Porphyromonadaceae (family) | 0.67 | 0.49 | 0.17* | 0.32 |
| 69 | <i>Barnesiella</i> (genus) | 0.52* | 0.12 | 0.41* | 0.14 |
| 70 | Clostridiales (order) | 0.39 | 0.72 | 0.24 | 0.08 |
| 72 | Porphyromonadaceae (family) | 0.15 | 0.09 | 0.62* | 0.28* |
| 81 | Lachnospiraceae (family) | 0.43 | 0.15 | 0.16 | 0.11 |
| 85 | <i>Clostridium</i> (genus) | 0.02 | 0.16 | 0.48 | 0 |
| 86 | Bacteroidales (order) | 0.65* | 0.07 | 0.16 | 0.12 |
| 87 | Porphyromonadaceae (family) | 0.31 | 0.25 | 0.13 | 0.12 |
| 94 | Porphyromonadaceae (family) | 0.41 | 0.5 | 0.18 | 0.11* |
| 98 | Bacteria (kingdom) | 0.35* | 0.12 | 0.07 | 0.09 |

Table 3.1. Average relative abundance of significantly altered OTUs during AOM/DSS tumor induction. OTUs included in table are significantly altered from baseline over time. Significance was determined using repeated measure paired treatment analysis of variance and correcting for multiple comparisons using an experiment-wise error rate of 0.01. Taxonomic group based on RDP classification is represented for each OTU. * indicates significant alterations from DSS round 1.

| OTU # | Taxonomy (level) | Baseline | DSS Round 3 |
|-------|------------------------------|----------|-------------|
| 1 | <i>Bacteroides</i> (genus) | 1.2 | 7.75 |
| 3 | <i>Odoribacter</i> (genus) | 1.87 | 11.23 |
| 4 | Prevotellaceae(family) | 3.9 | 2.08 |
| 6 | Porphyromonadaceae (family) | 1.66 | 0.45 |
| 7 | Porphyromonadaceae (family) | 2.83 | 1.49 |
| 9 | Bacteroidetes (phylum) | 0.16 | 2.53 |
| 11 | Erysipelotrichaceae (family) | 3.7 | 0.2 |
| 18 | Clostridiales (order) | 3.18 | 0.93 |
| 20 | <i>Turicibacter</i> (genus) | 0 | 2.04 |
| 21 | <i>Ruminococcus</i> (genus) | 2.46 | 0.21 |
| 22 | Porphyromonadaceae (family) | 2.16 | 0.48 |
| 26 | Erysipelotrichaceae (family) | 0 | 4.18 |
| 28 | Bacteroidales (order) | 4.1 | 0.16 |
| 30 | Porphyromonadaceae (family) | 2.97 | 1.42 |
| 32 | <i>Akkermansia</i> (genus) | 1.05 | 0.2 |
| 39 | <i>Odoribacter</i> (genus) | 0.75 | 1.83 |
| 40 | Clostridiaceae (family) | 0.01 | 1.33 |
| 41 | Bacteroidetes (phylum) | 0.32 | 5.65 |
| 42 | Lachnospiraceae (family) | 3.33 | 0.11 |
| 45 | Porphyromonadaceae (family) | 1.72 | 0.6 |
| 47 | Porphyromonadaceae (family) | 0.51 | 0.1 |
| 49 | Bacteria (kingdom) | 1.69 | 0.43 |
| 53 | Clostridiales (order) | 0.46 | 0.1 |
| 62 | Alphaproteobacteria (class) | 1.26 | 0.08 |
| 67 | Porphyromonadaceae (family) | 0.07 | 0.57 |
| 69 | <i>Barnesiella</i> (genus) | 0.57 | 0.13 |
| 81 | Lachnospiraceae (family) | 0.5 | 0.07 |
| 90 | Bacteria (kingdom) | 0.87 | 0.14 |
| 96 | Clostridiales (order) | 0.86 | 0.05 |
| 99 | Lachnospiraceae (family) | 0.97 | 0.15 |

Table 3.2. Average relative abundance of OTUs for baseline and DSS round 3 in germ-free mice conventionalized with healthy microbiota. OTUs included in table are significantly altered from baseline over time in mice conventionalized with bedding from healthy mice. Significance was determined using repeated measure paired treatment analysis of variance and correcting for multiple comparisons using an experiment-wise error rate of 0.01. Taxonomic group based on RDP classification is represented for each OTU.

Materials & Methods

Animals and animal care.

Studies were conducted on adult (8 to 12 week old) age-matched male C57BL/6 mice that were bred and maintained under SPF or germ-free conditions as specified above. Mice were co-housed in groups of five. Both SPF and germfree mice were fed the same autoclaved chow diet. All animal experiments were approved by the University Committee on Use and Care of Animals at the University of Michigan.

Inflammation-induced colon tumorigenesis.

Eight to 12-week-old mice received a single intraperitoneal (i.p.) injection of azoxymethane (10 mg/kg). Water containing 2% DSS was administered to mice beginning on day 5 for 5 days followed by 16 days of water. This was repeated twice for a total of 3 rounds of DSS [Tanaka et al., 2003; Chen et al., 2008]. Mice were euthanized on days 14, 24, 38, and 45 for intermediate time point analysis. The remaining mice were euthanized 3 weeks after the third round of DSS administration for tumor counting.

Histological analysis.

At necropsy, all colons were harvested, flushed of luminal contents, and cut open longitudinally to count and measure tumors. The largest dimension of each tumor was measured with calipers. Tumors were categorized based on size (<1mm, 1-2mm, >2mm). Colons were then jellyrolled, fixed in Carnoy's solution, and embedded in

paraffin. Five-micrometer sections were used for H&E staining and slides were analyzed under 100X magnification.

RNA isolation and cytokine analysis.

Distal colon tissue was homogenized and total RNA isolated using the Nucleospin RNA kit (Macherey-Nagel) cDNA was synthesized using iScript (Bio-Rad) and the cDNA was then used for quantitative qPCR using SYBR Green Expression Assay (Applied Biosystems).

Antibiotic treatment.

Mice were treated with an antibiotic cocktail of metronidazole (0.75 g/L), vancomycin (0.5 g/L), and streptomycin (2 g/L) in their drinking water for 2 weeks prior to and throughout the duration of AOM/DSS administration.

Germ-free colonization.

Eight-week-old C57BL/6 germ-free male mice were used. Fresh feces and bedding were collected from untreated and AOM/DSS-treated tumor-bearing mice and immediately transferred to cages of germ-free mice two weeks prior to AOM injection to allow stable colonization. Germfree mice were divided into two treatment groups, one group receiving bedding from untreated, healthy, the other group receiving bedding from AOM/DSS-treated tumor-bearing mice. To ensure that there were no cage effects, each treatment group was comprised of two cages of mice. The mice were obtained from separate litters and randomly assigned to the four cages. Mice were given three

weeks following colonization with feces and then treated with AOM/DSS to induce tumors as described above. Three weeks after the last round of DSS, mice were euthanized and colons were harvested as described above.

DNA extraction.

Fecal samples were collected daily from the mice throughout the AOM/DSS protocol and immediately frozen for storage at -20°C. We selected 12 fecal samples distributed over the 73-day timeline of the AOM/DSS model for 10 representative mice taken from five replicate cages. Microbial genomic DNA was extracted using the PowerSoil-htp 96 Well Soil DNA Isolation Kit (MO BIO laboratories) using an EpMotion 5075.

16S rRNA gene sequencing and curation.

The V35 region of the 16S rRNA gene from each sample was amplified and sequenced using the 454 Titanium sequencing platform at the Baylor College of Medicine Human Genome Sequencing Center as described elsewhere (http://www.mothur.org/wiki/454_SOP). We curated our sequences as described previously using the mothur software package [Schloss et al., 2012; Schloss et al., 2009; Schloss et al., 2011]. Briefly, we denoised sequences using the PyroNoise algorithm after trimming each flowgram to 450 flows [Quince et al., 2009], aligned the resulting sequences to a reference alignment derived from the SILVA 16S rRNA sequence database [Pruesse et al., 2007], and removed sequences that were flagged as possible chimeras by UCHIME [Edgar et al., 2011] or that did not align to the V35

region. After curating the sequence data, we obtained between 6 and 10,742 sequences (median = 5681) with a median length of 253 bp. To minimize biased effects of uneven sampling, we rarefied to 1,800 sequences per sample. Seven samples either did not pass through sequence curation or had less than 1,800 sequences and were therefore not used for further analysis. Parallel sequencing of a mock community allowed us to measure a median error rate of 0.06%.

Analysis of the microbiome.

Sequences were clustered into OTUs based on a 3% distance cutoff using the average neighbor algorithm. All sequences were classified using the RDP training set version 9 (<http://sourceforge.net/projects/rdp-classifier/>) and OTUs were assigned a classification based on which taxonomy had the majority consensus of sequences within a given OTU using a naïve Bayesian classifier [Wang et al., 2007]. Microbial diversity was calculated using inverse Simpson index [Magurran, 1988] and the observed number of OTUs. To calculate β -diversity, we used the θ_{YC} distance metric with OTU frequency data [Yue et al., 2005] and we calculated UniFrac statistics [Lozupone et al., 2005] using neighbor joining phylogenetic trees generated using the non-heuristic neighbor joining algorithm implemented in Clearcut [Shenaman et al., 2006]. Analysis of molecular variance (AMOVA) was performed to determine significance between the community structures of different groups of samples based on θ_{YC} and UniFrac distance matrices [Martin, 2002]. To identify OTUs important for driving differences between groups (baseline, after DSS round 1, after DSS round 2, and after DSS round 3), we used a repeated measure paired treatment analysis of

variance for each OTU and corrected for multiple comparisons using an experiment-wise error rate of 0.01 [Benjamini et al., 1995]. Additionally, we identified features (OTUs) important for each group using the machine learning algorithm random forest as implemented in R (<http://CRAN.R-project.org>) [Liaw et al., 2002]. The abundance-based Jaccard dissimilarity index indicates the fraction of all sequences that affiliate with OTUs that are shared between two communities and was used to calculate the fraction of OTUs that were shared between donor samples and germ-free recipient samples [Chao et al., 2006]. All sff files and the MIMARKS spreadsheet are available at http://www.mothur.org/aomdss_dynamics/.

16S rRNA quantitative PCR (qPCR) analysis.

Relative bacterial loads in stool samples were quantified by qPCR analysis of bacterial genomic DNA using KAPA SYBR-fast Master Mix (KAPA biosciences) and universal 16S rRNA gene primers (F: ACTCCTACGGGAGGCAGCAGT. R: ATTACCGCGGCTGCTGGC.) [Vaishnava et al., 2011]. Samples were normalized to fecal mass and relative fold change was determined using untreated stool samples for each replicate mouse (n=5). Note that qPCR measures relative fold change of 16S gene copy number, not actual bacterial numbers.

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Chapter IV

Manipulation of the Gut Microbiome Reveals Role for Microbial Community Structure in Colon Tumorigenesis

Introduction

The mammalian gastrointestinal tract is home to a complex and dynamic community of microorganisms, termed the gut microbiome, which is essential for the health of the host [Backhed et al., 2005]. Over the last several years it has been well document that abnormalities in this community are associated with colorectal cancer in humans and mice [Chen et al., 2013; Chen, et al., 2012; Kostic et al., 2012; Geng et al., 2013; Shen et al., 2013; Sobhani et al., 2011; Wang et al., 2012; Ahn et al., 2013]. We have previously demonstrated that in a mouse model, CRC-associated changes in the gut microbiome directly potentiate colon tumorigenesis [Zackular et al., 2013]. A critical question that remains unanswered is what factors and ecological principles mediate the gut microbiome's influence on this process [Fearon, 2011].

One strategy that has been used to answer this question is through the examination of individual bacteria populations within the gut microbiome. It has been

demonstrated that several common commensal bacteria have the capability to directly influence the process of tumor development and progression in the colon. The mechanisms by which bacteria potentiate these processes range from the production of carcinogenic toxins [Arthur et al., 2012; Sears et al., 2008] to direct manipulation of the inflammatory status in a tumors microenvironment [Kostic et al., 2013; Rubinstein et al., 2014]. Furthermore, some bacterial populations have been hypothesized be protective against CRC [Louis and Flint, 2009; Arthur et al., 2011; Appleyard et al., 2011]. This may be mediated through metabolite production, induction of immunotolerance, or an ability to outcompete pathogenic bacteria [Zhu et al., 2011].

Although there have been several associations between members of the gut microbiome and colorectal cancer, there is likely to be many bacteria that can modulate tumorigenesis in the colon. This is supported by studies that have identified abnormalities in the microbial communities associated with CRC in humans [Chen et al., 2013; Chen, et al., 2012; Kostic et al., 2012; Geng et al., 2013; Shen et al., 2013; Sobhani et al., 2011; Wang et al., 2012; Ahn et al., 2013]. Each of these studies reported abnormal shifts in the gut microbiome, termed dysbiosis; however, there were no CRC-associated bacterial populations that were consistently identified across all studies. This could be due to the fact that there is significant functional redundancy within the gut microbiome and different bacterial populations may fill similar roles in tumorigenesis [Lepage et al., 2011; Turnbaugh et al., 2009; Qin et al., 2010]. We hypothesize that multiple bacteria in the gut microbiome have the potential to play antagonistic or protective roles in tumorigenesis; thus, the gut microbiome's influence on CRC is likely polymicrobial.

Within host-associated microbial communities there are complex interactions between individual bacterial populations that have an important effect on host health [Levy and Bordenstein, 2013, Marino et al., 2013, Lepp et al., 2004]. The number of diseases that are associated with abnormalities in the gut microbiome highlights the importance of these ecological interactions [Turnbaugh et al. 2006; Tamboli et al.; 2004, Saulnier et al., 2011]. Deciphering how changes in community composition and structure disturb these interactions, and subsequently modulate tumorigenesis, is an essential step in understanding the etiology of CRC. In our previous study, treatment with antibiotics dramatically decreased tumorigenesis without a significant decrease in overall bacterial burden in the colon. Our results suggest that microbial community structure may play an important role in both down modulating and enhancing tumorigenesis. However, it remains unclear if manipulation of the community structure directly determines the carcinogenicity of the gut microbiome.

Previously, we found that when mice received metronidazole, streptomycin, and vancomycin in their drinking water and were then treated to induce inflammation-associated CRC, there was a significant decrease in tumorigenesis [Zackular et al, 2013]. Here we explored this result further by altering the composition of this antibiotic cocktail to test the hypothesis that the gut microbiome structure mediates tumor multiplicity and severity. Furthermore, we sequenced the V4 region of the 16S rRNA gene from treated mice in each group and determined that each treatment generated non-overlapping microbial community structures with varying levels of carcinogenicity. Using antibiotics to intervene following the first signs of dysbiosis, we demonstrated the feasibility of targeting the gut microbiome as a therapeutic for CRC. Our analysis

supports a model by which individual bacterial populations play an important role in CRC, but the ecological interactions and community structure of the gut microbiome mediate the capacity to modulate tumorigenesis.

Results

Antibiotic perturbation of the gut microbiome modulates tumorigenicity.

To test the hypothesis that gut microbiome structure mediates tumorigenesis, we administered an assortment of antibiotic treatments to specific-pathogen-free (SPF) C57BL/6 mice and treated them with the azoxymethane (AOM) and DSS inflammation-based model of CRC [Zackular et al., 2013] (Figure 4.1A). We treated mice with all possible combinations of metronidazole, streptomycin, and vancomycin to create eight treatment groups: no antibiotics, all antibiotics (metronidazole, streptomycin, and vancomycin), Δ metronidazole (streptomycin and vancomycin), Δ streptomycin (metronidazole and vancomycin), Δ vancomycin (metronidazole and streptomycin), metronidazole only, streptomycin only, and vancomycin only. Each treatment group showed a significant decrease in tumor multiplicity compared to mice that did not receive antibiotics; however, all of the antibiotic treatments resulted in varying levels of tumorigenesis (Figure 4.1A). These results demonstrate that distinctive antibiotic treatments yield a differential capacity for colon tumorigenesis.

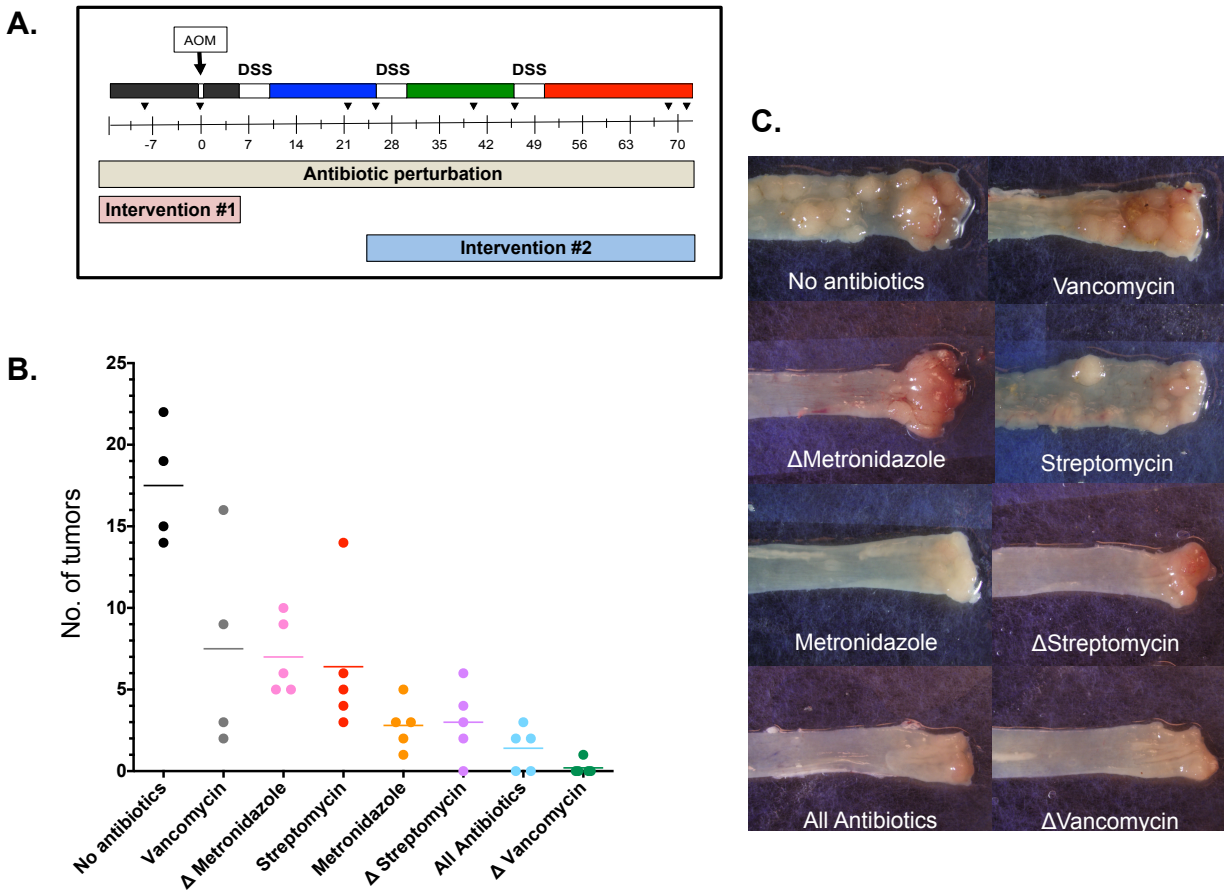


Figure 4.1. Antibiotic perturbation of the gut microbiome modulates tumorigenicity. **A.** Mice were injected with azoxymethane (AOM) on day 1 followed by 3 subsequent rounds of water administered 2% DSS. Colons were harvested 73 days after AOM and tumors grossly counted. Black wedges indicate fecal samples used for gut microbiome analysis. Antibiotic perturbation was started 2 week prior to AOM injection and continued throughout the model. **B.** Mice were treated with all possible combinations of metronidazole, streptomycin, and vancomycin to create eight treatment groups. Tumors were enumerated at the end of the model. **C.** Representative images of tumors in the distal colon of mice from each treatment group.

Marked structural changes associated with each antibiotic treatment could be observed at the phylum level (Figure 4.2C). Untreated control mice were dominated by the phyla Bacteroidetes (65±5.7%), Firmicutes (24±4.6%), and Proteobacteria (3±1.1%) (Figure 4.2A). The mice that received the full antibiotic cocktail primarily harbored Firmicutes (92±3%), particularly from the family *Lactobacillaceae*. In contrast, the mice that received only metronidazole had similar levels of Bacteroidetes (74±4%) and Firmicutes (7±1%) as control mice, but there was a significant bloom in Proteobacteria (9±0.08%). The Δ metronidazole and Δ streptomycin treatments led to the dominance of Proteobacteria (66±5% and 75±5%, respectively). The Δ vancomycin treated mice harbored Firmicutes (48±8%) and Proteobacteria (48±12%). The mice that received only vancomycin were composed of bacterial populations belonging to the phyla Firmicutes (12±3%), Proteobacteria (38±2%), Tenericutes (18±6%), and Verrucomicrobia (27±12%). These results demonstrate that antibiotic treatments perturbed the structure of the microbial community, leading to 7 distinct communities that were associated with varying levels of tumorigenesis.

To examine the role of the microbial community in this variation in tumorigenesis, we first considered the diversity of the gut microbiome using fecal samples taken at the end of the model (Figure 4.1A). Each antibiotic treatment resulted in a significant decrease in the microbial diversity compared to mice that did not receive antibiotics. Mice treated with streptomycin or metronidazole alone showed intermediate levels of diversity that were significantly higher than the remaining treatments (Figure 4.2A). However, there was no direct correlation between changes in diversity and tumorigenicity of the microbial community. To determine if there was an association

with a decrease in bacterial burden in the colon and tumorigenesis, we next looked at overall bacterial numbers in the feces. Using qPCR of the 16S rRNA gene we determined that there was not a significant decrease in overall bacterial numbers following treatment. These results indicate that the mere bacterial load or diversity of the community was insufficient to explain the differences in tumor burden.

Next, we characterized the differences in the structures of the communities by calculating the distance between samples using a metric that incorporates the OTU membership and relative abundance (i.e. θ_{YC}) [Yue and Clayton, 2005]. Each antibiotic treatment group harbored a significantly altered microbiome at the end point of the model compared to untreated animals (Figure 4.2B). Furthermore, each treatment resulted in a significantly different community structure, as each treatment group clustered separately from one another (Figure 4.2B). Similar results were observed when we used phylogenetic-based metrics of β -diversity (i.e. θ_{YC} and weighted and unweighted UniFrac). These results indicate that highly disparate community structures can come from mice that harbor similar numbers of tumors.

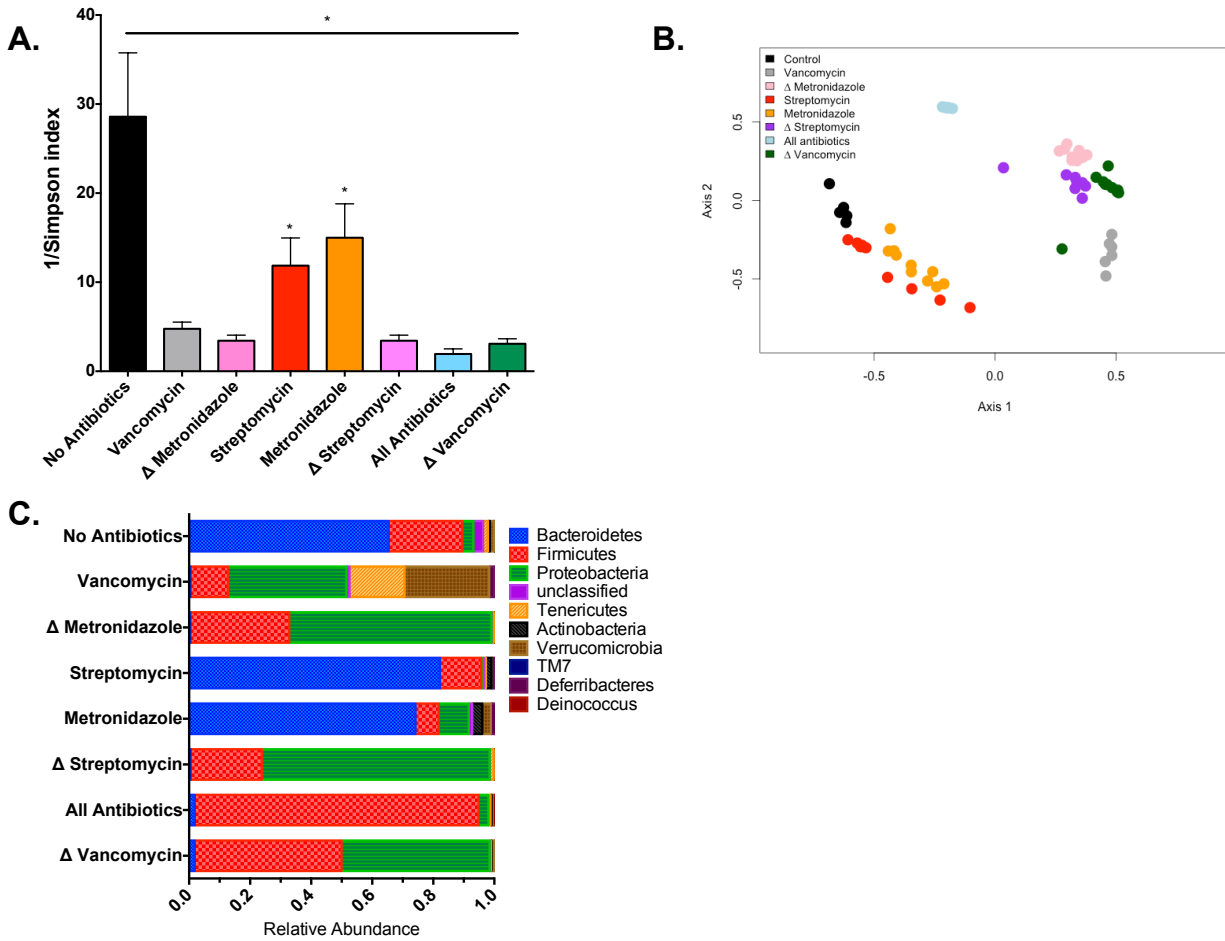


Figure 4.2. Antibiotic perturbation drives changes in microbial community structure. Microbiome analysis was performed on fecal samples from each mouse on days indicated in Figure 1A. **A.** Inverse Simpson's diversity index of fecal samples taken at the endpoint of the model. **B.** NMDS ordination based on θ_{yc} distances for all mice following the third DSS administration. (AMOVA; $P < 0.01$; Stress = 0.175). **C.** Phylum level relative abundance for each treatment group. Relative abundance represents the average structure of each treatment group at the endpoint of the model.

Identification of tumor modulating bacterial populations in the gut microbiome

The clear differences in community structure and composition coupled with the disparate levels of tumorigenesis provided us with an opportunity to identify potentially protective or tumor-promoting bacterial populations. To identify these tumor-modulating OTUs, we partitioned the treatment groups into high tumor incidence (Δ metronidazole, streptomycin only, and vancomycin only; median number of tumors=5.5) and low tumor incidence (All antibiotics and Δ vancomycin, Δ streptomycin and metronidazole only median number of tumors=2.0). We then used LEfSe to identify differentially abundant bacterial populations that were associated with an increased or decreased tumor burden in mice (Figure 4.3). We observed that four OTUs were significantly enriched among treatment groups with a higher tumor burden (Figure 4.3). Among the mice in the treatments with a low tumor burden there were 12 OTUs that were significantly enriched (Figure 4.3). Using Spearman correlation analysis we confirmed that these bacterial populations were highly correlated with an increased or decreased tumor burden in mice. These results indicate that there are populations that are associated with promoting or protecting against tumorigenesis.

Interestingly, when we investigated the relative abundance of these OTUs for each treatment group, we observed that the distribution of these OTUs was patchy across the treatment groups (Figure 4.4). For example, among the three groups with the most tumors, β -Proteobacteria (OTU 25) was highly abundant among the Δ metronidazole group, significantly less abundant in vancomycin only group, and completely absent from the streptomycin only group. In contrast, *Mucinispirillum* (OTU 87) was absent in the Δ metronidazole group, but elevated in the other two treatment

groups. This result suggests that there was not a single bacterial population promoting tumorigenesis in mice. Instead it is likely that there are populations of tumor promoting bacteria and tumor suppressing bacteria that can interact or serve redundant roles to modulate tumor burden.

Microbiome dynamics reveal potential associations between bacterial populations that mediate tumorigenesis

Comparison of the communities in the initial and final fecal samples collected from each treatment group indicated that the groups varied in the amount of change over the course of the model. The mice that received streptomycin or vancomycin only and the Δ streptomycin mice had significantly less structural change overtime compared to control mice (Fig. 4.4A). Meanwhile, the mice that received the full cocktail maintained a constant community structure throughout the model. Conversely, the Δ metronidazole and Δ vancomycin mice had the highest level of variation across the model (Figure 4.4A). The amount that the gut microbiome changed was not directly correlated with tumor burden ($\rho = 0.15$, $p = 0.41$). This result indicated that the destabilization of the initial community structure and the resulting host response was not associated with tumor burden.

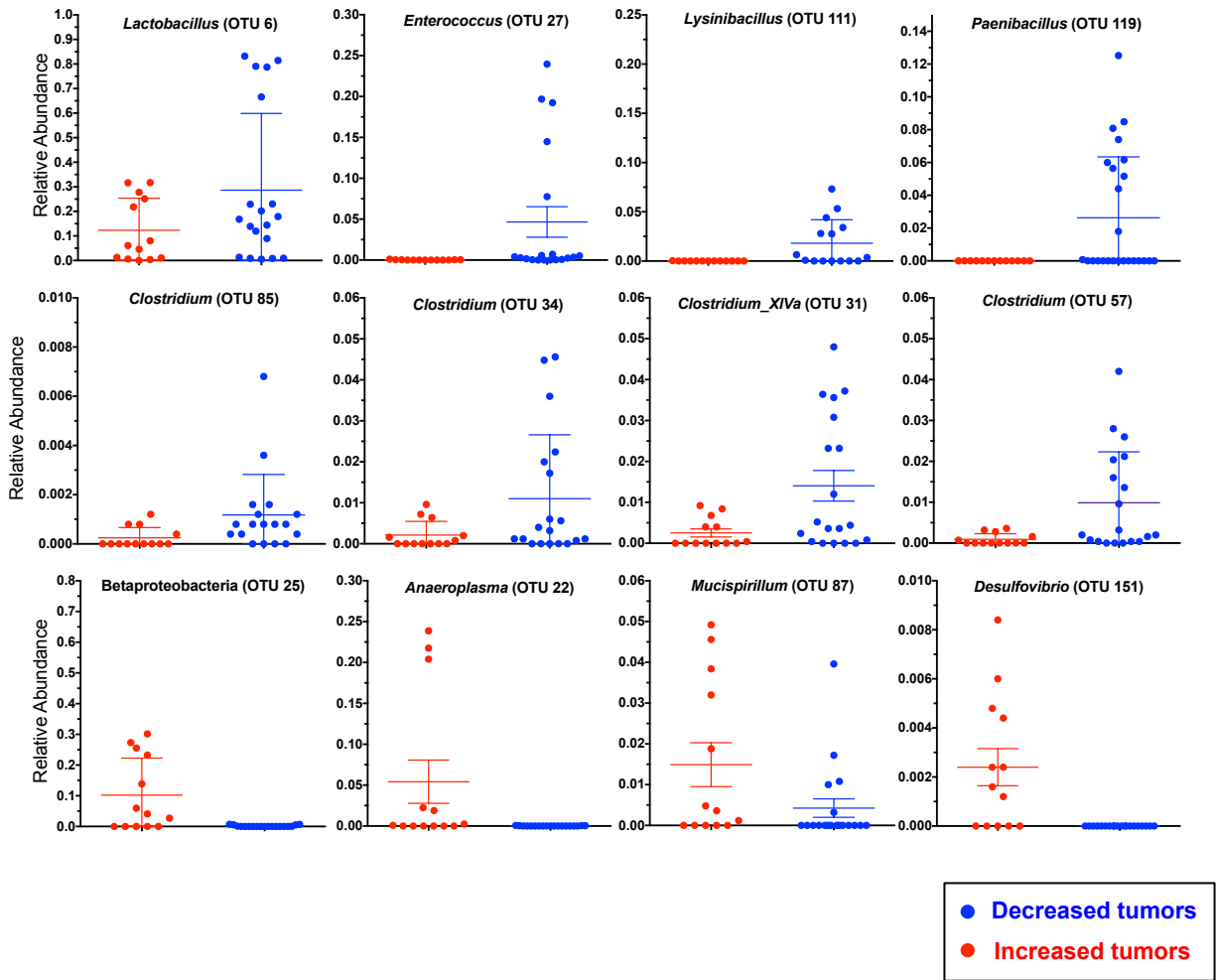


Figure 4.3. Analysis of bacterial populations associated with increased and decreased tumorigenesis. Antibiotic treatment groups were partitioned into two groups based on tumor burden: high tumor burden (Δ metronidazole, vancomycin only, and streptomycin only) and low tumor burden (All antibiotics, Δ streptomycin, Δ vancomycin, and metronidazole). LEfSe analysis was performed for stool samples from each mouse at the endpoint of the model. Strip charts show relative abundance for OTUs with LDA-value greater than 3.5. Error bars represent +/- standard deviation

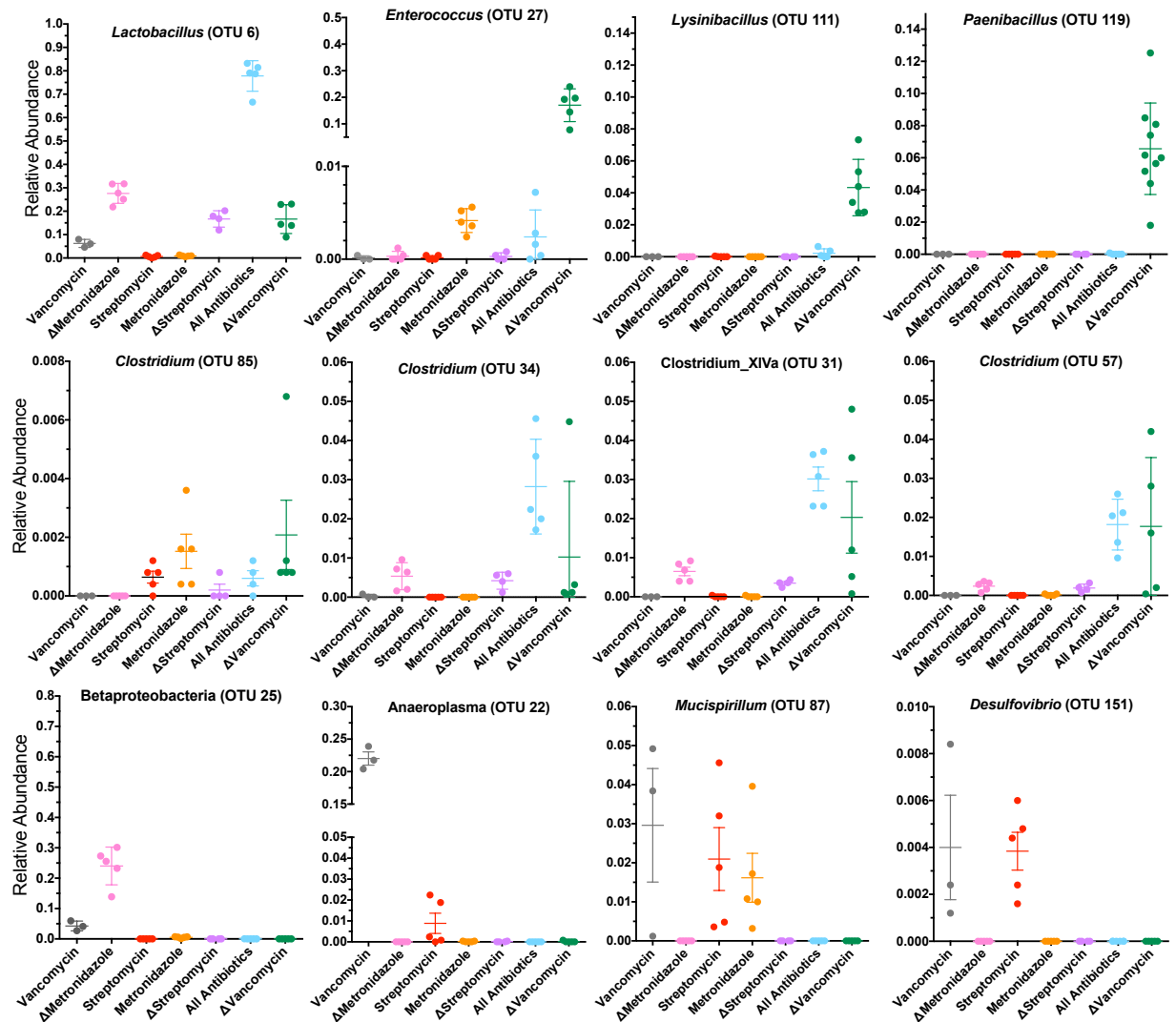


Figure 4.4. Relative abundance of significantly enriched OTUs for each treatment group. Strip charts show relative abundance of OTUs identified with LEfSe analysis for each treatment group separately. Abundance data is taken from endpoint of the model for each mouse. Error bars represent +/- standard deviation

We determined whether the OTU-level changes across the model could reveal underlying mechanisms associated with this dramatic difference in tumorigenicity between treatment groups. Thus, we performed repeated-measures paired group analysis of variance to identify features from within the microbiome that were significantly enriched or depleted overtime for each treatment group. We used fecal samples from day 0 and compared those to samples at the endpoint of the model for the Δ metronidazole and Δ vancomycin treated groups since these communities showed the greatest change over the course of the model (Figure 4.5A). In both groups there was a significant enrichment of an OTU associated with the *Enterobacteriaceae* (OTU 3) family. In the Δ metronidazole-treated mice, this enrichment was associated with the simultaneous depletion in the relative abundance of *Clostridium* (OTUs 31, 34, and 57), *Streptococcus* (OTU 92), and *Enterococcus* (OTU 27). However, Δ vancomycin-treated mice only showed a significant decrease in OTUs associated with *Lactobacillus* (OTU 6), while maintaining steady levels in each of the depleted populations observed in Δ metronidazole treated mice. We also observed enrichment in the relative abundance of *Turicibacter* (OTU 91) and Bacillales (OTU 225) in Δ vancomycin treated mice. These results support the hypothesis that the balance of tumor promoting and inhibiting populations are responsible for the final tumor burden.

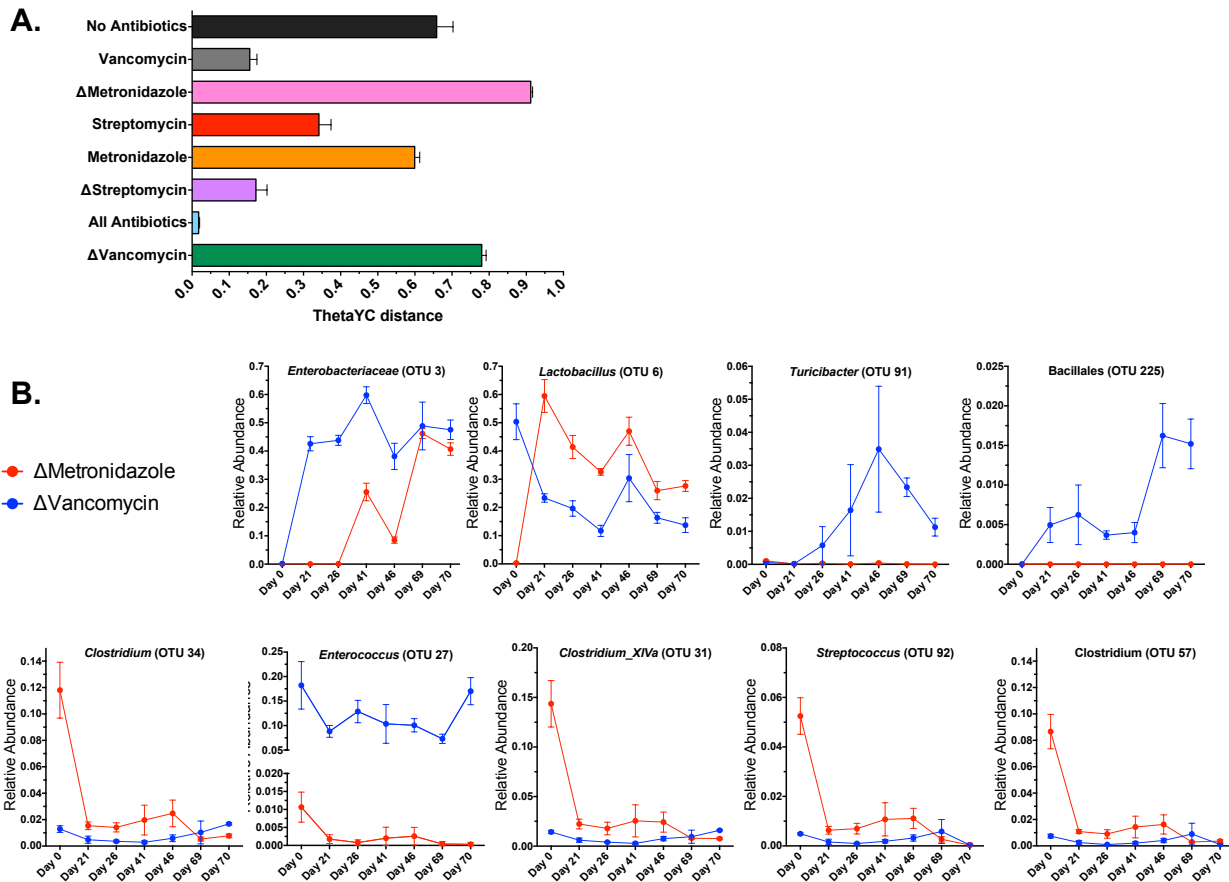


Figure 4.5. Gut microbiome dynamics during tumorigenesis for Δmetronidazole and Δvancomycin treatments groups. **A.** Average change in the gut microbiome community structure over the time course of tumorigenesis. Distances calculated using θ_{yc} distances. **B.** and **C.** Change in relative abundance over the time course of tumorigenesis for Δmetronidazole and Δvancomycin treatment groups. OTUs with relative abundances that were significantly different at day 70 compared to day 0 are shown. Repeated-measures paired group analysis of variance was used to identify significantly altered OTUs. Error bars represent +/- standard error.

Antibiotic intervention narrows possible mechanisms of microbiome involvement in tumorigenesis

The AOM-DSS model closely mirrors the patterns seen in human CRC. AOM induces DNA damage and the DSS induces inflammation. To determine whether the gut microbiome facilitates tumorigenesis by modulating AOM-induced mutations or inflammation, we performed two antibiotic intervention experiments. We first treated mice with the full antibiotic cocktail two weeks prior to the administration of AOM and up until the first round of DSS (Figure 4.1A). We found that these mice had a similar tumor burden to untreated mice (Figure 4.6A). Next, we treated mice before the second round of DSS administration with the all antibiotic cocktail (Figure 4.1A). In this treatment, there was a significant decrease in the number of tumors. Together, these results suggest that the gut microbiome's affect on CRC is independent of AOM carcinogenesis. Furthermore, it shows that targeting the gut microbiome at later stages of tumor growth is a viable option for minimizing tumorigenesis and highlights microbiome manipulation as a potential therapeutic in CRC.

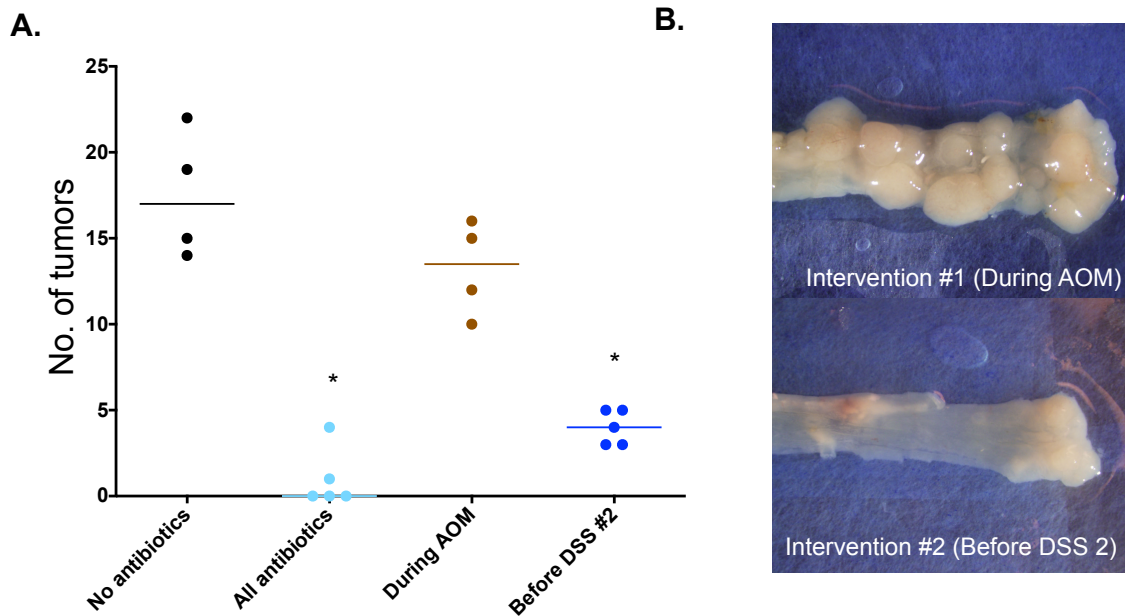


Figure 4.6. Antibiotic intervention prior to second administration of DSS alleviates tumor burden. Interventions with an antibiotic cocktail of metronidazole, vancomycin, and streptomycin were performed as depicted in Figure 1A. **A.** Tumors were enumerated at the end point of the model. Median tumor counts are shown for each treatment group. **B.** Representative images of tumors in the distal colon of mice from each treatment group. Statistical analysis was performed using Wilcoxon test. *, $P < 0.01$

Discussion

In the present study, we established the importance of the microbial community structure in mediating the gut microbiome's capacity for tumorigenesis. We demonstrated that manipulation of the murine gut microbiome with an assortment of antibiotic treatments resulted in non-overlapping community structures with a disparate level of tumorigenesis. Enrichment in the relative abundance of several bacterial populations was associated with high and low levels of colonic tumors. We determined that out growth of potentially inflammatory members of the gut microbiome only mediated increased tumorigenesis when there was a corresponding decrease in potentially protective, butyrate producing, bacteria. By perturbing the community at various time points in the AOM/DSS model, we determined that the gut microbiome is likely potentiating tumorigenesis independent of AOM-carcinogenesis. Our experiments also demonstrated that targeting the gut microbiome at the first signs of dysbiosis is a viable strategy for the amelioration of colon tumorigenesis.

In recent years, there has been a focus on identifying bacterial populations that are etiologic agents of CRC. Several commensal bacteria, including *Fusobacterium nucleatum* and enterotoxigenic *Bacteroides fragilis* (ETBF) have been linked to CRC in humans [Arthur et al., 2013; Rubinstein et al., 2013, Sears et al., 2008]. *F. nucleatum* can manipulate the inflammatory environment on in the tumor microenvironment in multiple intestinal neoplasia mice and in the studied population has been detected on the surface of over 50% of adenomas [Kostic et al., 2012; Kostic et al., 2013]. ETBF increases tumor multiplicity in the colon of multiple intestinal neoplasia mice through the action of a secreted metalloprotease toxin. It has been estimated that between 5-35%

of people carry ETBF [Housseau and Sears, 2010]. Although there is substantial evidence for a role in potentiating tumorigenesis, the fact that each of these bacteria is only associated with a fraction of CRCs suggests that there isn't likely one microbial agent that causes cancer. Rather, the gut microbiome's role in CRC is likely polymicrobial. The results in the present study support this hypothesis, as we demonstrated that non-overlapping community structures could confer similar levels of tumorigenesis in mice. When we examined the relative abundance of bacterial populations associated with increased tumor burden, we never observed all three treatment groups with high tumor levels (vancomycin only, streptomycin only, and Δ metronidazole) showing a consistent enrichment. The same was observed with potentially protective populations across all treatment groups that developed significantly less tumors (All antibiotics, Δ vancomycin, Δ streptomycin, and metronidazole only). This suggests that various bacteria within the gut microbiome may confer the same function and be playing redundant tumor-modulating roles.

During the time course of tumorigenesis we observed a marked increase in members of the *Enterobacteriaceae* associated with two antibiotic treatment groups (Δ metronidazole and Δ vancomycin). Interestingly, one treatment group (Δ vancomycin) developed significantly less tumors despite a similar increase in this potentially tumor-modulating bacterial clade. A recent study by Arthur and colleagues showed that in an IL-10-deficient colitis-associated mouse model of CRC; there is an enrichment of *Enterobacteriaceae* associated with inflammation [Arthur et al., 2012]. This leads to an expansion of *E. coli* strains with genotoxic capabilities and a consequential increase in tumor multiplicity and invasion. Furthermore, members of the *Enterobacteriaceae* have

been shown to perpetuate inflammation in several inflammatory diseases, including ulcerative colitis, which increase an individual's risk of developing CRC [Rolhion and Darfeuille-Michaud, 2007; Garrett et al., 2007; Rooks et al., 2014]. When we further examined the two antibiotic treatment groups, we observed that mice with an increased tumor burden had a corresponding decrease in several potentially anti-inflammatory and butyrate producing bacterial populations. These observations support a model by which the pathogenicity of individual members of the gut microbiome is mediated by the community structure and ecological interactions within the gut microbiome. We hypothesize that inflammatory and carcinogenic commensal bacteria, such as *Enterobacteriaceae*, can only mediate a pathogenic phenotype if the context of the community structure is conducive.

One mechanism by which community structure likely mediates tumorigenicity is through shifts in the balance of immunomodulatory metabolites and signals. During health, the gut microbiome is an important mediator of immunotolerance, but when the balance of pro- and anti-inflammatory signals is disrupted gut pathologies can arise [Kelly et al., 2005]. In our mice, *Enterobacteriaceae* is likely acting as an inflammatory member of the gut microbiome. We only observed an increase in tumorigenesis when there was a corresponding depletion of potentially protective members of the genera *Clostridium*, *Enterococcus*, and *Streptococcus*. Members of the *Clostridium* are known producers of short chain fatty acids (SCFA) in the colon [Louis et al., 2009]. SCFA, specifically butyrate, are important nutrients for colonocytes and they also possess anti-inflammatory and anti-tumor properties [Louis et al., 2009]. Furthermore, *Enterococcus* and *Streptococcus* species have been linked to down-regulating the inflammatory

response in the colon [Wang et al., 2008; Kaci et al, 2011]. It is likely that these bacterial populations have the ability to antagonize inflammatory clades (e.g. *Enterobacteriaceae*) and confer protection; however, when perturbation to the microbial community structure disrupts this homeostasis, these opportunistic pathogens can potentiate tumorigenesis

In our previous work, we demonstrated that dysbiosis of the gut microbiome generates a pro-inflammatory environment which results in a self-reinforcing pathogenic cascade between the gut microbiome and the host [Zackular et al., 2013]. In this study we demonstrated that antibiotic manipulation of the gut microbiome after the initiation of inflammation and tumorigenesis can significantly decreased tumorigenesis in mice. This highlights the efficacy of targeting the gut microbiome in CRC. Additional studies are needed to explore the viability of manipulating the gut microbiome in CRC with methods such as diet, probiotics, and prebiotics

Materials & Methods

Animals and animal care.

Studies were conducted using adult (8 to 12 week old) age-matched C57BL/6 male mice that were maintained under SPF conditions. Mice were co-housed in groups of five and fed the same autoclaved chow diet. All animal experiments were approved by the University Committee on Use and Care of Animals at the University of Michigan.

Inflammation-induced colon tumorigenesis.

Mice received a single intraperitoneal (i.p.) injection of azoxymethane (10 mg/kg). Water containing 2% DSS was administered to mice beginning on day 5 for 5 days followed by 16 days of water. This was repeated twice for a total of 3 rounds of DSS [Zackular et al., 2013]. Mice were euthanized 3 weeks after the third round of DSS administration for tumor counting. At necropsy, all colons were harvested, flushed of luminal contents, and cut open longitudinally to count and measure tumors.

Antibiotic treatment.

Mice were treated with all possible combinations of metronidazole (0.75 g/L), streptomycin (2 g/L), and vancomycin (0.5 g/L) to create eight treatment groups: no antibiotics, all antibiotics (n=5) (metronidazole, streptomycin, and vancomycin), Δ metronidazole (n=5) (streptomycin and vancomycin), Δ streptomycin (n=5) (metronidazole and vancomycin), Δ vancomycin (n=5) (metronidazole and streptomycin), metronidazole only, streptomycin only, and vancomycin only (n=3). Antibiotics were

administered in mouse drinking water for 2 weeks prior to and throughout the duration of AOM/DSS administration, unless otherwise specified in Figure 4.1A. Tumors were enumerated at the end of the model.

DNA extraction and 16S rRNA gene sequencing

Fecal samples were collected daily from the mice throughout the AOM/DSS protocol and immediately frozen for storage at -20°C. For each mouse, 8 fecal samples distributed over the 73-day timeline of the AOM/DSS model were selected for analysis (Figure 4.1A). Microbial genomic DNA was extracted using the PowerSoil-htp 96 Well Soil DNA Isolation Kit (MO BIO laboratories) using an EpMotion 5075. The V4 region of the 16S rRNA gene from each sample was amplified and sequenced using the Illumina MiSeq Personal Sequencing platform as described elsewhere [Kozich et al., 2013]. Sequences were curated as described previously using the mothur software package [Schloss et al., 2009]. Briefly, we reduced sequencing and PCR errors, aligned the resulting sequences to the SILVA 16S rRNA sequence database [Pruesse et al., 2007], and removed any chimeric sequences flagged by UCHIME [Edgar, 2011]. Sequences had a median length of 253 bp and we rarefied to 2,500 sequences per sample to limit effects of uneven sampling.

16S rRNA quantitative PCR (qPCR) analysis.

Relative bacterial loads were quantified by qPCR analysis of bacterial genomic DNA using KAPA SYBR-fast Master Mix (KAPA biosciences) and universal 16S rRNA gene primers (F: ACTCCTACGGGAGGCAGCAGT. R: ATTACCGCGGCTGCTGGC.)

[Vaishnava et al., 2011]. Samples were normalized to fecal mass and relative fold change was determined using untreated stool samples for each replicate mouse. Note that qPCR measures relative fold change of 16S gene copy number, not actual bacterial numbers.

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Chapter V

Discussion

Overview

CRC is one of the most commonly diagnosed malignancies worldwide, affecting millions of people each year. Significant risk factors for CRC such as fatty diets, physical inactivity, and chronic inflammation have been associated with altered composition, metabolism, and function of the resident gut microbiome. Furthermore, various physiological functions that are associated with cancer, including cell proliferation, angiogenesis, and apoptosis, are modulated by the gut microbiome. Recent survey based studies have reported an association between dysbiosis of the gut microbiome and CRC [Chen et al., 2013; Chen et al., 2012; Kostic et al., 2012; Geng et al., 2013; Shen et al., 2013; Sobhani et al., 2011; Wang et al., 2012; Ahn et al., 2013]. However, these studies have not explored the potential of using the gut microbiome as a biomarker for CRC. Furthermore, it remains unclear if dysbiosis of the gut microbiome directly modulates tumorigenesis in the colon or if they are just a consequence of physiological changes in the tumor microenvironment. The research presented in this dissertation revealed the potential of the gut microbiome as a non-

invasive biomarker for CRC development. In addition, we determined that dysbiosis has a causal role in the development of CRC and we further uncovered the factors important for gut microbiome mediated modulation of tumorigenesis. In this chapter, I will discuss the overall impact of the findings in this dissertation and elucidate the potential mechanisms by which the gut microbiome modulates CRC. I will then provide a proposed model and discuss the future directions of this research.

Dysbiosis of the Gut Microbiome is Predictive of CRC

Early detection of CRC is essential for the long-term prognosis of patients. Currently, it is estimated that over 30% of adults do not receive risk appropriate screenings and over 50% of people prefer non-invasive screening methods [Benson et al., 2007; Leard et al., 1997; Ling et al., 2001]. This highlights a clear need for the development of novel non-invasive screening methods for the detection of CRC. It is well established that dysbiosis of the gut microbiome is associated with CRC patients, but the viability of using the gut microbiome as a potential screen remains unexplored. In chapter II, to test the hypothesis that the gut microbiome can be used as a biomarker for the early detection of CRC, we acquired stool samples from the early detection research network (EDRN), an initiative created to discover biomarkers for cancer. The EDRN has a wealth of stool samples and clinical metadata from patients who have been diagnosed with various stages of CRC. In our study, we selected samples from patients that were diagnosed with adenomas, carcinomas, and healthy controls. A benefit to this design was that it allowed us to analyze changes in the gut microbiome that were associated with each step in the multistage progression of CRC (Figure 1.1).

Using microbial biomarker discovery algorithms [Segata et al., 2011], we were able to identify several bacterial populations that were differentially abundant between each clinical group. Using the relative abundances of these bacteria, we generated logit regression models using both clinical and microbiome data for each subject. We included microbial biomarkers that were both enriched and depleted for each comparison. This is important because we believe that in addition to increases in populations, depletion of members of the gut microbiome are equally as important for the biology and detection of CRC. For each comparison we made, we observed a significant improvement in our ability to predict the presence of adenomas and carcinomas upon inclusion of the microbiome data. Furthermore, we determined that using gut microbiome data as a screening tool improved the pre-test to post-test probability of adenoma over 50-fold. These findings demonstrated, for the first time, that the gut microbiome has the capability to serve as an accurate screening tool for early detection of CRC. Our results strongly suggest that consideration must be given to the gut microbiome in concert with clinical data and existing non-invasive diagnostics to yield the most accurate diagnosis of CRC.

The Dynamics of Dysbiosis

Based on the data from this dissertation and studies performed by several other groups, it was clear that shifts in the gut microbiome are associated with CRC. However, it still remained unclear whether these changes were driving tumorigenesis or if they were just a consequence of this disease. We could not easily test this in humans, so in chapter III, we investigated the role of gut microbiome dysbiosis in a

mouse model of CRC. We used a well characterized model of inflammation associated CRC and examined the dynamics of the gut microbiome during tumorigenesis. Similar to what has been observed in humans with CRC, tumor-bearing mice harbored a dramatically altered microbial community. A major caveat to our study in chapter II was that we only surveyed each community at one time point. This cross-sectional design did not allow us to assess the changes in a CRC-associated microbiome over the time course of CRC development. We hypothesized that the dynamics of the gut microbiome during tumorigenesis would reveal the underlying mechanisms driving the development and progression of dysbiosis. To test this, we sampled each mouse daily, which enabled us to see the changes in the microbiome during tumorigenesis. Results from this longitudinal study revealed that there was a stepwise progression in the gut microbiome, which ultimately led to what we classified as a tumor-associated dysbiosis. We also determined that DSS-induced inflammation alone did not lead to the development of tumors and was insufficient for the development of a tumor-associated dysbiosis. In order to develop a tumor-associated dysbiosis, AOM and DSS were both needed. This is important because Arthur and colleagues have suggested that inflammation alone is sufficient to generate a tumor-associated dysbiosis [Arthur et al., 2012]. Our results support a model by which the synergistic effects of inflammation and tumorigenesis are necessary for the development of an altered microbiome structure and tumorigenesis. We believe that the physiological changes that occur in the mucosa during inflammation and tumor development contribute collectively to dysbiosis.

The Gut Microbiome Modulates Colon Tumorigenesis

Dysbiosis of the gut microbiome has been associated with many diseases, including obesity, inflammatory bowel disease, and CRC [Turnbaugh et al., 2006; Tamboli et al., 2004]. Although correlations have been observed between dysbiosis and disease, it is unclear if a dysbiotic microbiome induces disease progression, e.g. tumorigenesis. In chapter III, I tested the hypothesis that tumor-associated changes to the gut microbiome directly contribute to tumorigenesis. Germ-free mice were inoculated with microbiota from healthy and tumor-bearing mice and were run through the AOM/DSS model. Mice that received a dysbiotic gut microbiome developed significantly more tumors than those receiving a healthy microbiota. For the first time, we revealed a direct causative role of tumor-associated changes in the gut microbiome in the development of CRC. This new information indicated that the interactions between inflammation, the tumor microenvironment, and subsequent changes in the gut microbiome create the conditions that result in colon tumors.

Studies previous to the work presented in my thesis have focused on the role of individual bacterial pathogens on tumor development. These types of studies largely ignore the complexity, dynamics, and ecology of the gut microbiome. We hypothesized that the structure of the microbial community was the essential variable driving tumorigenesis in the colon. To test this we perturbed the gut microbiome with an antibiotic cocktail of metronidazole, streptomycin, and vancomycin. This modulation of the gut microbiome resulted in a dramatic decrease in tumor burden in the colons of mice. Importantly, treatment with this cocktail did not result in a significant decrease in overall bacterial load. This strongly supported our hypothesis that the presence of a resident microbial community isn't sufficient for the development of tumors; instead the

specific structure of the community is the driving factor. In chapter IV, we further tested this hypothesis by manipulating the structure of the resident gut microbiome in mice and measuring subsequent effects on tumorigenesis. To generate different community structures we treated mice with various combinations of the antibiotics that were used in chapter III (metronidazole, streptomycin, and vancomycin). Each antibiotic treatment resulted in a significantly different community that had varying capacities to modulate tumorigenesis. Interestingly, although antibiotic treatment groups harbored significantly different structures, some groups had similar levels of tumorigenesis. When we examined individual bacterial populations within treatment groups with high or low tumor burdens, we never observed the enrichment of an individual population across all groups with similar levels of tumors. Comparison of the gut microbiome dynamics within a low and high tumor burden treatment groups, revealed similar enrichment of the potentially inflammatory *Enterobacteriaceae* clade. However, increased tumorigenicity associated with *Enterobacteriaceae* was only observed when there was a corresponding decrease in protective bacterial populations. The observations made in chapter IV, suggest that various bacteria within the gut microbiome confer the same function and be playing redundant tumor-modulating roles. Furthermore, these results support a model by which the pathogenicity of individual members of the gut microbiome is mediated by the community structure and ecological interactions with other members in the gut microbiome. We hypothesize that inflammatory and carcinogenic commensal bacteria, such as *Enterobacteriaceae*, can only mediate a pathogenic phenotype if the context of the community structure is conducive.

Potential Mechanisms of Gut Microbiome-Mediated Tumorigenesis (CRC)

The specific mechanisms by which dysbiosis of the gut microbiome promotes tumorigenesis remain largely unclear. We hypothesized that dysbiosis of the gut microbiome likely leads to enrichment in tumor-promoting bacteria and loss of tumor-repressing bacteria. One mechanism by which certain bacterial populations drive tumorigenesis is through the induction of an inflammatory immune response. An inflammatory environment fosters the development of cancer through the production of genotoxic reactive oxygen species and pro-tumor inflammatory mediators, such as TNF- α , IL-6, IL-1 β , and IL-23. A recent study by Kostic and colleagues reported such a phenomenon with the common mouth commensal, *Fusobacterium nucleatum* [Kostic et al., 2013]. They reported that *F. nucleatum* was enriched in tumor biopsies from CRC patients. Using a mouse model, they determined that *F. nucleatum* increases tumorigenesis through the recruitment of immune cells and subsequent generation of a pro-inflammatory environment. In our mouse studies we did not observe any *Fusobacterium*, but when we looked at the stool of CRC patients in chapter II, we observed significantly higher levels of *Fusobacterium* OTUs when compared to healthy and adenoma patients. It is likely that the microbial community alterations we observed in chapter III and IV are enriched with populations that fill a similar role.

Another potential mechanism for the development of a pro-inflammatory environment in the colon is through the degradation of host-secreted mucin by resident microbes. Mucin is a host glycol-protein that is secreted by goblet cells in the gastrointestinal tract and acts as a protective liner between the host epithelium and the resident gut microbiome [Hollingsworth et al., 2004]. When this layer of mucin is

compromised, microorganisms can invade the host epithelium and cause inflammation. Furthermore, it is hypothesized that mucins bind and sequester cytokines, growth factors, and other mediators of inflammation [Hollingsworth et al., 2004]. Upon degradation of mucin these inflammatory factors may be released and stimulate an inflammatory response. In chapter III and IV, we observed significant enrichment of bacterial populations belonging to the *Akkermansia* and *Mucispirillum* genera. These bacteria were both enriched during the time course of tumorigenesis and antibiotic treated mice with increased tumor burden showed marked out growth of *Mucispirillum*. Members of the *Akkermansia* and *Mucispirillum* genera are known mucin-degrading bacteria. We hypothesize that this increase in relative abundance of these bacterial populations may result in a thinning of the protective mucin layer. Disruption of this mucin layer has been shown to enhance the ability of opportunistic pathogens to invade the mucosa [Ganesh et al., 2013]. We postulate that during tumorigenesis, abnormal levels of mucin degradation could subsequently lead to a dramatic increase in invasion by gut microbiota, release of mucin-associated inflammatory factors, and a subsequent reubust inflammatory response. Additional studies are needed to identify the role of *Akkermansia* and *Mucispirillum spp.* in inflammation and tumorigenesis.

In addition to contributing to inflammation, dysbiotic microbiomes are likely enriched in bacteria with a more direct role in tumor development through the production of toxins, virulence factors, and other tumor-promoting gene products. A recent study by Arthur and colleagues demonstrated that chronic inflammation in an IL-10 deficient mouse alters the gut microbiome, leading to an enrichment of tumor-promoting *E. coli* [Arthur et al., 2012]. These strains of *E. coli* harbor a polyketide

synthase (pks) genotoxic island, which produces Colibactin, a requirement for increased tumor multiplicity and invasion in this model. In each of my mouse and human studies we did not detect any significant changes in the genus *Escherichia*, but it is likely that other bacterial populations are playing a similar role. Specifically, we observed a marked increase in *Bacteroides* species during tumorigenesis in chapter III. Following the first signs of tumors, this population became enriched to over 10% of the community. Furthermore, there was a correlation between the relative abundance of this OTU at the endpoint of the model and an increased tumor burden. Members of the *Bacteroides* genus, including enterotoxigenic *B. fragilis* (ETBF), have been associated with diarrheal disease, inflammation, and CRC [Sears et al., 2008]. Specifically it is estimated that 5-35% of adults carry ETBF, which produces a metalloprotease toxin, *B. fragilis* toxin (BFT). ETBF has been strongly associated with colonic tumors in multiple-intestinal-neoplasia mice through the secretion of this toxin, which augments the host cell cycle [Wu et al., 2009]. We did not detect ETBF our mice, but it is possible that similar processes are occurring during tumorigenesis in our mice.

We hypothesize that an equally important mechanism driving tumorigenesis in the colon is the loss of members of the gut microbiome that are important for maintaining epithelial health and immune homeostasis. Recent evidence suggests that anti-inflammatory members of the gut microbiome are an important variable in the maintenance of gut homeostasis. Homeostasis is necessary in order to tolerate the large number of potentially pro-inflammatory, TLR stimulating resident bacteria. Resident bacteria mediate immunotolerance in several ways, including direct inhibition of inflammatory pathways. A classic example is the anti-inflammatory role of the

common human commensal *B. thetaiotaomicron* in the gut. Studies have shown that *B. thetaiotaomicron* can antagonize NFκB mediated inflammation by targeting the active NFκB subunit, RelA, for transport out of the nucleus [Kelly et al., 2004]. This prevents downstream NFκB mediated cytokine and chemokine production. Presumably, a loss of *B. thetaiotaomicron*, and other NFκB-modulating bacteria would lead to an increase in the inflammatory response to resident microbes. Another immunomodulatory mechanism mediated by the gut microbiome is the production of anti-inflammatory metabolites, such as butyrate. Butyrate is a type of short-chain fatty acid (SCFA), which are by-products of bacterial fermentation of complex carbohydrates in our diet (e.g. fiber). Butyrate has been shown to reduce inflammation in the gastrointestinal tract through several mechanisms. It can inhibit NFκB signaling through the inhibition of IκBα degradation [Segain et al., 2000] and also stimulate T-regulatory cell mediated homeostasis in the lamina propria. In addition to its anti-inflammatory properties, butyrate also has more direct effects on tumorigenesis, including inhibition of growth and induction of apoptosis in cancer cells [Hague et al., 1995; Ruemmele et al., 2003]. Therefore, the loss of butyrate-producing populations in the gut microbiome could increase both inflammation and tumorigenesis. In chapter III, we observed a dramatic decrease in several OTUs from unclassified genera within the family *Porphyromonadaceae*. Furthermore, in chapter IV when performing microbial biomarker discovery analysis we identified several bacterial populations that were significantly depleted in CRC. Individuals with carcinomas showed a dramatic loss in OTUs associated with the genera *Clostridium* and *Bacteroides*, and the family *Lachnospiraceae* [Louis et al., 2009; Atarashi et al., 2011; Smith et al., 2013; Round et

al., 2010]. Each of these bacterial taxa are well known producers of SCFA in the colon. We hypothesize that loss of these important bacterial populations in concert with an enrichment of pathogenic populations likely plays a synergistic role in potentiating tumorigenesis.

Based on the findings presented in my thesis and the synthesis of evidence from the literature, I have proposed a model for the role of dysbiosis in CRC (Figure 5.1). In summary, shifts in the structure of the gut microbiome lead to an imbalance between pro- and anti-inflammatory signals in the gut and a loss of intestinal homeostasis. In the case of tumorigenesis, this leads to an increase in bacterial populations that are inflammatory and a loss of populations that produce anti-inflammatory signals. These shifts in the microbial community initiate a strong pro-inflammatory response, which in turn results in further perpetuation of dysbiosis. The synergistic effect of these two forces generates a self-reinforcing inflammatory cascade between the gut microbiome and the host. This inflammatory environment fosters the development of cancer through the production of genotoxic reactive oxygen species and inflammatory mediators, such as TNF- α , IL-6, IL-1 β , and IL-23. A chronic inflammatory environment in concert with the tumor development can lead to the enrichment of bacterial populations that have a direct role in tumor progression through the production of toxins, virulence factors, antigens, and other tumor-promoting gene products.

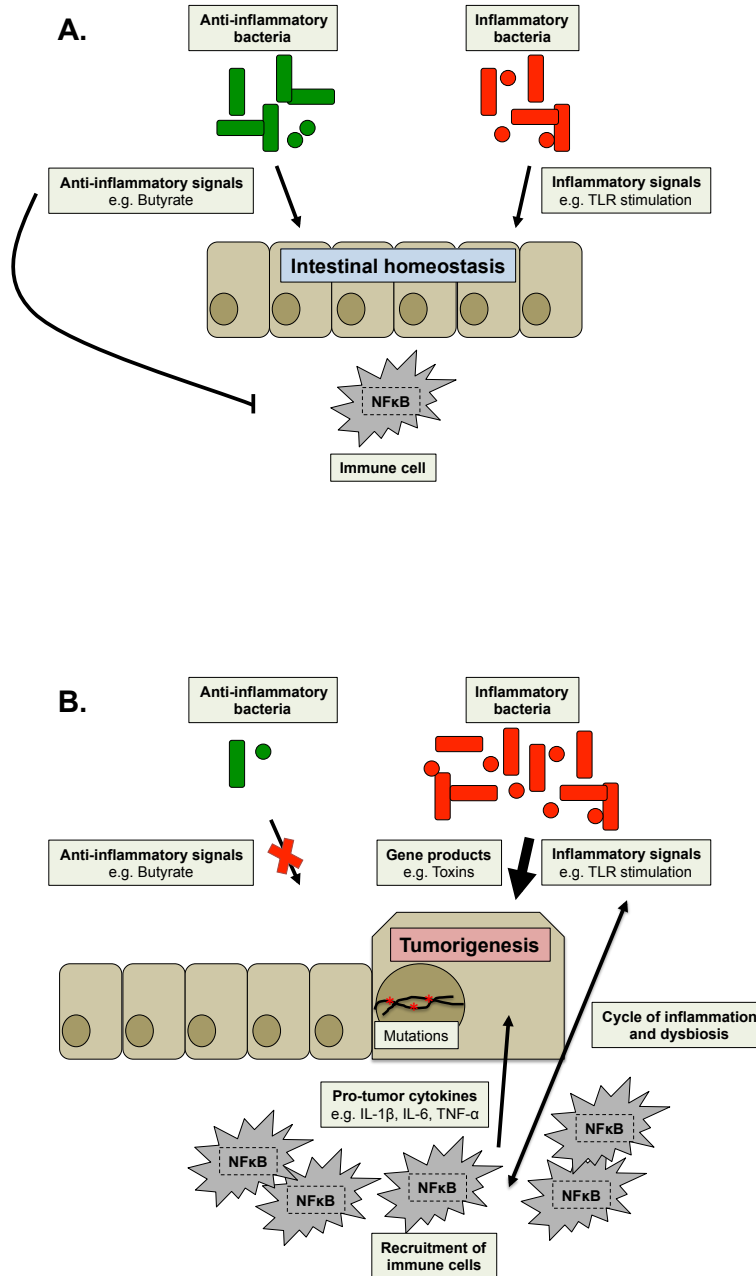


Figure 5.1. Proposed model of gut microbiome mediated modulation of tumorigenesis. **A.** Interaction between the gut microbiome and mucosa during intestinal homeostasis. There is a balance between pro and anti-inflammatory signals from the gut microbiome mediating immune tolerance and homeostasis. **B.** The gut during dysbiosis and tumor development. There is an increase in pro-inflammatory bacteria and a loss of protective bacteria. This leads to increased toxin production and inflammation. Inflammation creates a pro-tumor environment and further perpetuated dysbiosis, generating a pathogenic cycle.

The Gut Microbiome as a Therapeutic Target for CRC

Based on our proposed model, we postulated that the gut microbiome is a viable therapeutic target for CRC. In chapter III we modulated the gut microbiome with a cocktail of antibiotics during the time course of tumorigenesis. The results of this experiment provided a proof of principle that direct targeting of the gut microbiome can reduce colon tumorigenesis. To further explore the potential of the gut microbiome as a therapeutic target, we explored the effect of targeting the community in a more clinically relevant way. In chapter IV, mice were treated with an antibiotic cocktail (metronidazole, streptomycin, and vancomycin) following the first signs of dysbiosis in the gut microbiome. The results of this experiment revealed that modulation of the gut microbiome following dysbiosis significantly minimized tumor development in mice. This is an important finding because it demonstrates the concept that the gut microbiome is a viable therapeutic intervention in CRC. In a clinical setting, it is unlikely that broad-spectrum antibiotics would be recommended as a practical preventative measure for CRC. Instead we suggest that manipulation of the gut microbiome be performed by using methods such as prebiotics, probiotics, and changes in diet. It is known that diets high in fiber can lead to an increase in beneficial microbes that produce anti-cancer metabolites, such as butyrate.

In order to identify specific microbes that could be targeted for enrichment or probiotic therapies, it is necessary to perform studies that identify protective bacterial populations. Towards this goal, in chapter IV we used various antibiotic therapies to generate communities with differing protective and carcinogenic properties. By focusing on the groups that developed the fewest tumors, we identified several potential bacterial

populations with potential protective properties. These bacteria included members of the genera *Lactobacillus*, *Enterococcus*, and *Clostridium*, and members of the family *Porphyromonadaceae*. *Lactobacillus spp.* have long been used as probiotics because of their ability to enhance epithelial barrier function, produce antimicrobial products, and elicit an anti-inflammatory response in the gut [Fernandez et al., 2011]. It is possible that *Lactobacillus spp.* may also provide protection in the context of CRC. Members of the *Clostridium* genus and *Porphyromonadaceae* family are known producers of butyrate in the colon [Louis et al., 2009; Atarshi et al., 2013] and as described earlier, this likely provides a strong anti-tumor effect. It still remains unclear if these populations actively provide protection against tumor development. Furthermore, additional studies are needed to identify the mechanisms by which they confer resistance to CRC. Once these questions are answered it will be important to explore their potential as probiotics and determine diets that enrich these populations.

Future Directions

One of the inherent limitations of the work presented in this thesis is our inability to assess the gut microbiome at a functional level. Using 16S rRNA sequences we are only given a broad view of bacterial populations and we can only speculate on the functional activity of the community. In order understand the principal mechanisms behind the role of the gut microbiome in CRC; we will need to focus on the specific functions being performed by the community. My hypothesis is that during tumorigenesis there are functional changes to the gut microbiome that lead to the production of pro-tumor gene products and metabolites. Furthermore, there is a loss of

protective gene products and metabolites. Two methods that can be used to test this hypothesis are transcriptomics and metabolomics. Transcriptomics is a way to capture the mRNA from a bacterial community. This can allow for a better understanding of what genes are being expressed in the gut microbiome during tumorigenesis. Metabolomics is a method to measure the concentration of metabolites being produced by bacterial communities. I propose that by analyzing the expression profile and metabolite production of the gut microbiome during tumorigenesis, we will be able to identify important functions driving tumorigenesis.

In the work described in this thesis we identified several bacterial populations that were associated with an increased or decreased tumor burden. Outside of this correlation and synthesis of previous literature, we were unable to pinpoint specific features, gene products, or mechanisms by which these bacterial populations modulated tumor development or progression. In order to begin to answer these questions, I propose that we perform targeted culturing of several bacterial populations that we identified in this thesis. Those populations include *Bacteroides spp.*, *Mucispirillum spp.*, *Porphyromonadaceae spp.*, *Clostridium spp.*, and *Lactobacillus spp.*, among others. By using 16S rRNA sequences from chapter III and IV, we can perform plate wash PCR as described by Stevenson and colleagues [Stevenson et al., 2004]. This will allow us to identify and target these specific populations. I next propose that we perform gnotobiotic experiments with these bacterial populations. Using the germ-free mouse facility at the University of Michigan, we can mono-associate mice with these strains and determine their carcinogenicity. Since it is difficult to determine if bacterial populations are protective using a germ-free model, we can also introduce

potentially protective isolates, like *Lactobacillus*, to conventional mice by repeated gavage. I expect that we will be able to isolate the majority of our target bacterial populations and determine their tumor modulating ability. Following these experiments, we can begin to perform in vitro assays to identifying the genes involved in modulating tumorigenesis using genetic screens and genomics. Identification of these tumor modulating genes and bacteria will give us important insight into the microbial influence of tumor development. Additionally, this will allow us to determine the feasibility of using probiotics in CRC.

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