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

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**Abstract**

There is growing evidence that individuals with colonic adenomas and carcinomas harbor a distinct microbiota. These alterations may allow the outgrowth of populations that induce mutations or exacerbate inflammation. In addition, it is likely that the loss of key populations may result in the loss of protective functions that are provided for by a healthy microbiota. Using an inflammation-based murine model of colorectal cancer we explored the host-microbiota relationship to better understand the role of various populations through the process of tumorigenesis. By perturbing the microbiota with mixtures of antibiotics that targeted distinct groups of bacteria we observed that it was possible to predict the number of tumors that the animals would harbor by the end of the model. It was apparent that distinct microbiota could lead to similar numbers of tumors and that variation in the composition of the microbiota could also lead to wide variation in the number of colonic tumors that formed. Furthermore, without altering the number of bacteria in the colon, we were able to fully suppress tumor formation using a combination of metronidazole and streptomycin. Finally, by altering when the antibiotics were given to the model we showed that the role of the microbiota in tumorigenesis is most pronounced during the period of inflammation rather than in the processing of the mutagen. These results suggest that altering the structure and function of the gut microbiota can arrest the course of colorectal cancer.

**Keywords:** azoxymethane, dextran sodium sulfate, 16S rRNA gene sequencing,

microbial ecology, microbiome

**Introduction**

The mammalian gastrointestinal tract is home to a complex and dynamic community of microorganisms, termed the gut microbiome, which is essential for maintaining host health ([1](#_ENREF_1)). Among the bacterial populations that make up the gut microbiome there are complex interactions that have an important effect on host health ([2-4](#_ENREF_2)). The number of diseases that are associated with abnormalities in the gut microbiome highlights the importance of these ecological interactions ([5-7](#_ENREF_5)). Over the last several years it has been well documented that perturbations to this community are associated with colorectal cancer (CRC) in humans and mice ([8-15](#_ENREF_8)). We have previously shown that CRC-associated changes in the gut microbiome directly potentiate colon tumorigenesis in a mouse model of CRC ([16](#_ENREF_16)). In that study we observed clear shifts in the microbiome that could be associated with a stepwise progression in the number of tumors that were found in the colon. In addition, we showed that transfer of the tumor-associated microbiota to germ-free mice resulted in increased tumor formation relative to germ-free mice receiving the microbiota of healthy mice. These results were supported by a subsequent study where we colonized germ-free mice with the microbiota of human donors and observed significant variation in the number of tumors that formed ([17](#_ENREF_17)). Combined, these results demonstrate that the microbiota interact with the host to potentiate tumorigenesis. A critical question that remains unanswered is what factors and ecological principles mediate the gut microbiome's influence on this process. 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.

Several bacterial populations including *E. coli*, *Bacteroides fragilis*, and *Fusobacteirum nucleatum* have been shown to directly influence the process of tumorigenesis in the colon. The mechanisms by which bacteria potentiate these processes range from the production of carcinogenic toxins ([18](#_ENREF_18), [19](#_ENREF_19)) to direct manipulation of the inflammatory status in a tumor's microenvironment ([20](#_ENREF_20), [21](#_ENREF_21)). Although individual bacterial populations undoubtedly modulate colorectal cancer, there are likely a myriad of commensal bacteria that work together to influence tumorigenesis in the colon. This is supported by several studies that have explored the gut microbiome associated with individuals with CRC ([8-15](#_ENREF_8), [22](#_ENREF_22)). With each study, the number of CRC-associated bacterial populations that likely play a role in tumorigenesis continues to grow. This is likely due to the fact that there is significant functional redundancy within the gut microbiome and various bacterial populations may fill similar roles in tumorigenesis ([23-25](#_ENREF_23)). Furthermore, some bacterial populations have been hypothesized be protective against CRC ([26](#_ENREF_26), [27](#_ENREF_27)). This protective phenotype may be mediated through metabolite production, induction of immunotolerance, or an ability to outcompete pathogenic bacteria ([28](#_ENREF_28)). 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 to be polymicrobial.

Altering the composition of the microbiome in an inflammation-based mouse model of CRC resulted in varying numbers of colonic tumors. We also treated conventionally-raised mice with a cocktail of metronidazole, streptomycin, and vancomycin in their drinking water while they received the tumor-inducing treatment and observed a significant decrease in tumorigenesis ([22](#_ENREF_22)). In the current study, 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. To better understand the composition of the microbiome and its dynamics, we extended this result by permuting the composition of the antibiotic cocktail and associated changes in the microbiome with the number of tumors that formed. These results confirmed our hypothesis that the microbiome is capable of driving tumorigenesis. To further test this hypothesis, we designed an antiboitic-based intervention to determine whether it was possible to arrest tumorigenesis. 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.*** We subjected conventionally-raised C57BL/6 mice to an inflammation-based model of colorectal cancer that utilizes azoxymethane (AOM) as a mutagen and dextran sodium sulfate (DSS) to induce inflammation ([16](#_ENREF_16), [17](#_ENREF_17)) (Figure 1A). As a test of the hypothesis that the microbiota are involved in tumorigenesis, we manipulated the microbiota by administering eight antibiotic treatment groups and then quantified the effects of the treatments on the number of tumors observed at the end of the model. Specifically, we treated mice with (i) no antibiotics, (ii) metronidazole, streptomycin, and vancomycin (all antibiotics), (iii) streptomycin and vancomycin ( metronidazole), (iv) metronidazole and vancomycin ( streptomycin), (v) metronidazole and streptomycin ( vancomycin), (vi) metronidazole, (vii) streptomycin, and (viii) vancomycin. Quantitative PCR targeting the 16S rRNA gene indicated that the number of copies of the 16S rRNA gene did not differ between the treatment groups. The three antibiotics were selected because they were thought to target different groups of bacteria including anaerobes (metronidazole), Gram-negatives (streptomycin), and Gram-positives (vancomycin). By sequencing the 16S rRNA genes that were present in the feces of the mice that were treated with the antibiotics it was clear that these treatments generated different starting communities (Figure 1B); however, the composition of these communities was not a predictable result of the antibiotic that was used to treat the mice. In support of our hypothesis we observed that antibiotic treatment resulted in a decrease in tumor multiplicity relative to the mice that did not receive antibiotics (Figure 1CD). These results demonstrated that perturbation of the microbiota through the use of antibiotics yields a differential capacity for colon tumorigenesis.

***The final tumor burden can be predicted from the initial microbiota.*** Because we obtained serial fecal samples it was possible to ascertain the composition of the microbiota for each mouse as well as the number of tumors in its colon at the end of the model. Using the 16S rRNA gene sequence data generated from feces collected on the day of AOM injection, we assigned the sequences to operational taxonomic units (OTUs) that were defined as a group of sequences that, on average, were not more than 3% different from each other. We then used the regression-based random forest machine learning algorithm to identify OTUs that would enable us to predict the number of tumors that were found in the same mice at the end of the model. The model that included OTUs that had an average relative abundance greater than 1.5% resulted in the greatest percentage of the variance explained (Supplementary Figure 1). This model included 15 OTUs and explained 62.6% of the variation in the tumor counts (Figure 2). As suggested in Figure 2A, when the OTUs were sorted in decreasing order by their Gini coefficient, there was a jump between the sixth and seventh OTUs. In fact, when we reconstructed the model using only the six OTUs that provided the largest Gini coefficients, the model explained 62.8% of the variation in the tumor counts. These six OTUs included members of the Firmicutes (OTU 6), Bacteroidetes (OTUs 4 and 19), Proteobacteria (OTU 3), and Tenericutes (OTUs 34 and 35). Increased numbers of tumors were associated with decreases in the relative abundance of relatives of the Enterobacteriaceae (OTU 3), *Ureaplasma* (OTU 34), and *Lactobacillus* (OTU 6) and increases in the relative abundance of the *Anaeroplasma* (OTU 35), Porphyromonadaceae (OTU 4), and *Prevotella* (OTU 19). Our random forest modeling demonstrated that it was possible to predict the number of tumors at the end of the model based on the composition of the microbiota at the beginning of the model.

***The final tumor burden can be predicted from the final microbiota.*** Similar to our analysis using the initial composition of the microbiota, we developed a random forest regression model to predict the number of tumors in the mice based on the composition of the microbiota at the end of the model. The model included 11 OTUs after we again applied a filter requiring each OTU to have an average relative abundance of at least 1.5%. The model explained 52.9% of the variation in the tumor counts (Supplementary Figure 2), which is less than we observed when we modeled tumor counts based on the initial community composition. The seven most important OTUs in the model explained 55.4% of the variation and included *Odoribacter* (OTU 70), *Bacteroides* (OTU 5), *Lactobacillus* (OTU 6), Enterobacteriaceae (OTU 3), *Alloprevotella* (OTU 14), *Prevotella* (OTU 19), and Betaproteobacteria (OTU 17) (Supplementary Figure 3). Interestingly, of the OTUs that were predictive of the number of tumor counts using the baseline and final community composition data, only three of the OTUs overlapped. These included *Lactobacillus* (OTU 6), Enterobacteriaceae (OTU 3), and *Prevotella* (OTU 19). These results confirm those of our earlier study where we used mice that did not receive antibiotics and the sequencing was performed using the 454 platform ([16](#_ENREF_16)).

***The microbial community is dynamic.*** Using mice that were colonized with human feces, we previously reported that tumor burden was associated with the amount of change in the community structure over the course of the AOM-DSS model ([17](#_ENREF_17)). In the current study, however, there was a non-significant association between the change in the community structure as measured by the θYC metric of community structure similarity and tumor burden (ρ=0.26, P=0.08; Figure 4A). We did observe that mice that did not receive antibiotics and those that received the vancomycin and metronidazole treatments changed the most over the course of the model. Interestingly, when we investigated the temporal progression of the three OTUs that were most important for predicting the number of tumors based on the starting and final community structure (i.e. OTUs 3, 6, and 19; Figure 2B) we observed a dynamic progression across the model. These data suggest that the magnitude of change that occurs in a microbial community during tumorigenesis may not influence tumor burden. Instead, specific changes in community structure and the abundance of tumor-associated bacterial populations mediate tumor burden.

***Antibiotic intervention narrows possible mechanisms of microbiome involvement in tumorigenesis.*** The AOM-DSS model has been shown to reproduce the patterns seen in human CRC, but the microbial contribution has not been elucidated. To determine whether the gut microbiome facilitates tumorigenesis by modulating AOM-induced mutations or DSS-induced 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 1A). We found that these mice had a similar tumor burden to untreated mice (Figure 5). Next, we treated mice before the second round of DSS administration, following the initiation of AOM/DSS mediated tumorigenesis and inflammation (Figure 1A). In this treatment, there was a significant decrease in the number of tumors (Figure 5). These results suggest that the gut microbiome mediated affect on CRC is independent of AOM-mediated 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.

**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 outgrowth 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 *E. coli*, *Fusobacterium nucleatum* and enterotoxigenic *Bacteroides fragilis* (ETBF) have been linked to CRC in humans ([18](#_ENREF_18), [19](#_ENREF_19), [21](#_ENREF_21)). *F. nuceleatum* 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 ([10](#_ENREF_10), [20](#_ENREF_20)). 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 ([29](#_ENREF_29)). 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 it is unlikely that there is a single 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 ∆metronidazole) showing a consistent enrichment. The same was observed with potentially protective populations across all treatment groups that developed significantly less tumors (All antibiotics, ∆vancomycin, ∆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 (∆metranidazole and ∆vancomycin). Interestingly, one treatment group (∆vancomycin) developed fewer tumors despite a similar increase in this potentially tumor-modulating bacterial clade. A recent study by Arthur and colleagues ([18](#_ENREF_18)) showed that in an IL-10-deficient colitis-associated mouse model of CRC; there is an enrichment of *Enterobacteriaceae* associated with inflammation. This leads to an expansion of *E. coli* populations 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 ([30-32](#_ENREF_30)). 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 *what is this???*]. 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 ([26](#_ENREF_26)). SCFA, specifically butyrate, are important nutrients for colonocytes and they also possess anti-inflammatory and anti-tumor properties. Furthermore, *Enterococcus* and *Streptococcus* species have been linked to down-regulating the inflammatory response in the colon ([33](#_ENREF_33), [34](#_ENREF_34)). 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 ([16](#_ENREF_16), [17](#_ENREF_17)). 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 ([16](#_ENREF_16)). 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), metronidazole (n=5) (streptomycin and vancomycin), streptomycin (n=5) (metronidazole and vancomycin), 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 1A. Tumors were enumerated at the end of the model.

**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) ([35](#_ENREF_35)). 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 rRNA gene copy number, not actual bacterial numbers.

**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 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, sequenced using the Illumina MiSeq Personal Sequencing platform, and curated as described previously using the mothur software package ([36](#_ENREF_36), [37](#_ENREF_37)). Briefly, we reduced sequencing and PCR errors by requiring reads to fully overlap and in cases where base calls conflicted, we broke the conflict by requiring one base call to have a PHRED quality score 6 units higher than the other otherwise the base call was replaced with an ambiguous base call in the contig. Any reads containing ambiguous base calls were culled. Sequences were aligned to a customized version of the SILVA 16S rRNA sequence database ([38](#_ENREF_38)) and were screened to insure that they correctly overlapped within the V4 region. Chimeric sequences were identified using the de novo implementation of UCHIME and they were culled ([39](#_ENREF_39)). The resulting sequences had a median length of 253 nt and we rarefied to 2,500 sequences per sample to limit effects of uneven sampling. A mock community was sequenced and processed in parallel to the fecal samples. Based on the mock community data we observed a sequencing error rate of 0.05%. The complete analysis methods and this document as an R-executable document are available at https://github.com/SchlossLab/Zackular\_AbAOMDSS\_GutMicrobes\_2015. All FASTQ sequence data can be obtained from the Sequence Read Archive at NCBI (Need to deposit data).

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**Figure legends**

**Figure 1. Antibiotic perturbation drives changes in microbial community structure and final tumor burden.** The AOM-DSS model was administered to C57BL/6 mice reared under standard pathogen free (SPF) conditions with different antibiotic perturbations; Black arrows indicate fecal samples that used for our analysis (A). The mice were treated with all possible combinations of metronidazole, streptomycin, and vancomycin to create eight treatment groups, which resulted in considerable differences in the taxonomic composition of the gut communities at the start of the model (Day 0) (B). These communities resulted in a continuum of tumor burden in the mice (C and D).

**Figure 2. A random forest model successfully predicted the number of tumors in the mice at the end of the model (A) based on their microbiota composition at the start of the model (B).** The OTUs in B are ranked in decreasing order of their mean decrease in the mean squared error. The relationships between the first 6 OTUs and the number of tumors found in those mice are shown in Figure 3.

**Figure 3. Relationship between the initial relative abundance of the most informative OTUs from the random forest model with the number of tumors found in the mice at the end of the model.** The vertical gray line indicates the limit of detection.

**Figure 4. The murine microbiota is dynamic but the amount of change is not associated with the final number of tumors.** The structure of the gut microbiota associated with untreated and the metronidazole and vancoymcin-treated mice changed the most throughout the model as measured using the YC distance metric (A). OTUs 3, 6, and 19 were among the most salient features for predicting tumor burden at the beginning and end of the model (B). The plotting symbols and characters are the same as those used in Figure 1. In panel B, the median relative abundance is indicated by the plotting symbol and the range of observed relative abundances is plotted by the vertical bar. The vertical blue regions indicate when the DSS treatments were applied.

**Figure 5. 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 with enumeration of tumors performed at the end point of the model (A). Representative images of tumors in the distal colon of mice from each treatment group (B).

**Supplementary Figure 1. Quality of random forest regression fit as a function of the minimum average relative abundance an OTU must have to be included in the model.** The integers displayed across the plot indicate the number of OTUs that were included in the model. Because a minimum average relative abundance of 1.5% yielded the best R2, it was used for the remainder of the analysis.

**Supplemental Figure 2. A random forest model successfully predicted the number of tumors in the mice at the end of the model (A) based on their microbiota composition at the start end of the model (B).** The OTUs in B are ranked in decreasing order of their Gini coefficient. The relationships between the first 6 OTUs and the number of tumors found in those mice are shown in Supplemental Figure 3.

**Supplemental Figure 3. Relationship between the initial relative abundance of the most informative OTUs from the random forest model with the number of tumors found in the mice at the end of the model.** The vertical gray line indicates the limit of detection.