**Manipulation of the Gut Microbiota Reveals Role of Gut Microbiota in Colon Tumorigenesis**

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**Abstract**

There is growing evidence that individuals with colonic adenomas and carcinomas harbor a distinct microbiota. Alterations to the gut microbiota may allow the outgrowth of bacterial populations that induce genomic mutations or exacerbate tumor-promoting inflammation. In addition, it is likely that the loss of key bacterial populations may result in the loss of protective functions that are normally provided by the microbiota. We explored the role of the gut microbiota in colon tumorigenesis using an inflammation-based murine model. We observed that perturbing the microbiota with different combinations of antibiotics did not change the bacterial load but reduced the number of tumors at the end of the model. Using the random forest machine learning algorithm we successfully modeled the number of tumors that developed over the course of the model based on the composition of the microbiota at the beginning. The timing of antibiotic treatment was an important determinant of tumor outcome as colon tumorigenesis was arrested with the use of antibiotics during the inflammation period of the murine model. Together, these results indicate that it is possible to predict colon tumorigenesis based on the composition of the microbiota and that altering the gut microbiota can alter the course of tumorigenesis.

**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 microbiota, which is essential for maintaining host health 1. There are complex interactions among bacterial populations in the gut that have an important effect on host health 2-4. The number of diseases that are associated with abnormalities in the gut microbiota highlights the importance of these ecological interactions 5-7. 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. We have previously shown that CRC-associated changes in the gut microbiota directly potentiate colon tumorigenesis in a mouse model of CRC 16. In that study we observed clear shifts in the microbiota that were associated with a stepwise progression in the number of tumors that developed 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 that received 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 that different starting communities yielded significant variation in the number of tumors that formed 17. Combined, these results demonstrate that the microbiota interact with the host to affect tumor susceptibility. A critical question that remains unanswered is what factors and ecological principles mediate the gut microbiota's influence on tumor development. Deciphering how changes in microbial community composition and structure alters gut homeostasis, and subsequently modulate tumorigenesis, is an essential step in understanding the etiology of CRC.

Several bacterial populations including *E. coli*, *Bacteroides fragilis*, and *Fusobacterium nucleatum* have been shown to directly influence tumor development in the colon. The mechanisms by which bacteria potentiate these processes range from the production of carcinogenic toxins 18,19 to direct manipulation of the inflammatory status of the tumor microenvironment 20,21. Although individual bacterial populations undoubtedly modulate colorectal carcinogenesis, 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 microbiota associated with individuals with CRC 8-15,22. 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 microbiota and various bacterial populations may fill similar roles in tumorigenesis 23-25. Furthermore, some bacterial populations have been hypothesized to be protective against CRC 26,27. This protective phenotype may be mediated through metabolite production, induction of immunotolerance, or an ability to outcompete pathogenic bacteria 28. We hypothesize that multiple bacteria in the gut microbiota have the potential to play pro-tumorigenic or tumor-suppressive roles; thus, the gut microbiota's influence on CRC is likely to be driven by complex interactions within the microbiota and the colonic epithelium.

We have shown that conventionally-raised mice treated with a cocktail of metronidazole, streptomycin, and vancomycin in their drinking water had a significant decrease in tumor numbers using an inflammation-based model of CRC 22. In the current study, we explored how differential alterations in the microbiota by different antibiotic treatments affected the composition of the microbiota and how changes in bacterial community structure affected tumor susceptibility. Our results confirmed our hypothesis that the microbiota is capable of driving tumorigenesis and that an antibiotic-based intervention during tumor induction can arrest tumorigenesis. Our analysis further supports the model that individual bacterial populations play an important role in CRC, but the ecological interactions and community structure of the gut microbiota mediate the capacity to modulate tumorigenesis.

**Results**

***Antibiotic perturbation of the gut microbiota modulates tumorigenicity.*** We subjected specific pathogen-free (SPF) 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 16,17,29 (Figure 1A). To determine how differential changes in the gut microbiota affected tumorigenesis, we manipulated the microbiota by administering seven different antibiotic combinations for the length of the model (Intervention 1; Figure 1A) and then quantified the effects of the treatments on the number of tumors observed at the end of the model (Figure 1BC). 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. The three antibiotics were selected based on their reported ability to target general groups of bacteria including anaerobes (metronidazole), Gram-negatives (streptomycin), and Gram-positives (vancomycin). Upon necropsy we observed that perturbation of the microbiota through the use of antibiotics yielded a differential capacity for colon tumorigenesis (Figures 1BC). Sequencing the 16S rRNA genes that were present in the feces of conventional and antibiotic-treated mice demonstrated that the different antibiotic treatments generated different bacterial communities prior to AOM injection (Figure 1D); however, the composition of these communities could not have been predicted by the spectrum of the antibiotic that was used to treat the mice. The eight community structures generated by using the untreated mice and those that received one of the seven antibiotic combinations were all significantly different from each other (all P<0.05 by AMOVA with Benjimani-Hochberg correction). These results indicated that the communities are distinct from each other (Figure 1E) and varied in their ability to drive tumorigenesis.

***Tumor burden can be predicted from the initial microbiota.*** Tumor burden can be predicted from the initial microbiota. Serial collection of fecal samples allowed us to ascertain the composition of the microbiota for each mouse and associate it with the number of tumors that developed 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 developed at the end of the model. The model that included all 685 OTUs explained 53.9% of the variation in the tumor counts. We then sorted the OTUs by their importance in the random forest model as determined by the percent reduction in the mean squared error when that OTU was removed from the model and. There was a peak in the amount of the variation explained in the observed tumor counts when we used the twelve most important OTUs (Supplemental Figure 1). The simplified model explained 67.7% of the variation in the observed tumor counts (Figure 2). These twelve OTUs included members of the Firmicutes (OTUs 1, 66, 99, and 185), Bacteroidetes (OTUs 14, 67, 79, and 107), Proteobacteria (OTUs 7, 36, and 72), and Tenericutes (OTU 9). With the exception of OTUs affiliated with members of the *Lactobacillus* (OTU 1) and Betaproteobacteria (OTU 7), each of the OTUs was associated with increased tumor burden (Figure 3). The relative abundance of the *Lactobacillus*-affiliated OTU at the start of the model was inversely proportional to the tumor burden at the end of the model. There was not a clear relationship between the initial relative abundance of the *Betaproteobacteria*-affiliated OTU and the final tumor burden. Interestingly, tumorigenesis was not exclusively dependent on the presence of any of the OTUs. In other words, tumors could form even when an OTU was below the limit of detection. This suggests that the role of the microbiota in driving tumor formation was context dependent. More broadly, the random forest model 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.

***Tumor burden can be predicted from the microbiota at the end of the model.*** 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 (Supplemental Figure 2). The simplified model included eight OTUs and explained 65.6% of the variation in the tumor counts (Supplementary Figure 3). This was comparable to what we observed when we modeled tumor counts based on the initial community composition. This model utilized the relative abundance data from OTUs affiliated with members of the Firmicutes (OTU 85), Bacteroidetes (OTUs 7, 19, 28, 29, and 51), Proteobacteria (OTU 7), and candidate phylum Saccharibacteria (OTU 192; Supplementary Figure 4). Interestingly, of the OTUs that were predictive of the number of tumor counts using the initial and final community composition data, only one of the OTUs overlapped, which was affiliated with the Betaproteobacteria (OTU 7). The distinction between OTUs that were predictive of tumor burden using the community composition at the beginning and end of the model suggests that the communities that gave rise to tumors were different from those that were enriched in a tumor-laden environment.

***The microbial community is dynamic during inflammation-associated tumorigenesis.*** 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. In the current study, however, there was a non-significant association between the change in the community structure as measured by the YC metric of community structure similarity and tumor burden (=0.26, P=0.08; Figure 4A). We did observe that mice that did not receive antibiotics and those that received the Δvancomycin and Δmetronidazole treatments changed the most over the course of the model. When we identified those OTUs whose relative abundances changed the most across each treatment group, we found that OTUs affiliated with the *Lactobacillus* (OTU 1) and Enterobacteriaceae (OTU 2) were consistently among the most dynamic OTUs across the treatment groups (Figure 4). Interestingly the initial relative abundance of the *Lactobacillus*-affiliated OTU was predictive of the final tumor burden, but the final relative abundance of neither OTU was predictive of final tumor burden. These data suggest that the magnitude of change that occurs across a microbial community during tumorigenesis is not strongly associated with tumor burden. Instead, the relative abundance of a subset of populations within the community dictate tumor burden.

***Antibiotic intervention during inflammation reduces tumorigenesis.*** The results of our current study and our previous investigations of the role of the gut microbiota in colonic tumorigenesis have suggested that by manipulating the gut microbiota, it would be possible to manipulate tumorigenesis 16,17. To further validate these results, we performed two additional antibiotic intervention experiments. We first treated mice with the vancomycin, metronidazole and streptomycin two weeks prior to the administration of AOM and up until the first round of DSS and then removed the antibiotic cocktail for the remainder of the model (Intervention 2; Figure 1A). We found that these mice had a similar tumor burden to untreated mice (Figure 5). Next, we treated mice after the first round of DSS administration with the antibiotic cocktail until the end of the model. Our previous work found that the period following the first round of DSS coincided with a period when inflammatory responses were the greatest and there were aberrant changes in the gut microbiota 16 (Intervention 3; Figure 1A). With these mice, we found that the intervention resulted in a significant decrease in the number of tumors (Figure 5). These results suggest that the gut microbiota-mediated effect on CRC is independent of AOM-mediated carcinogenesis. Furthermore, it shows that targeting the gut microbiota at later stages of tumor growth is a viable option for minimizing tumorigenesis and highlights microbiota manipulation as a potential therapeutic in CRC.

**Discussion**

In the present study, we established the importance of the microbial community structure in determining the extent of tumorigenesis. We demonstrated that manipulation of the murine gut microbiota with different antibiotic cocktails resulted in distinct community structures that were associated with disparate levels of tumorigenesis. To determine whether the microbiota was involved in possibly converting the AOM to a carcinogenic metabolite or involved in the inflammation process we restricted the application of antibiotics to alter the microbiota during these phases of the model. We determined that the gut microbiota affects tumorigenesis via a mechanism that does not involve AOM-induced carcinogenesis. Our experiments also demonstrated that targeting the gut microbiota at the emergence of dysbiosis (i.e. after the first round of DSS in the AOM/DSS model) is a viable strategy for the amelioration of colon tumorigenesis. Such a result offers hope that by altering a person's gut microbiota it may be possible to alter their risk of developing colon cancer.

Our analysis suggests that community-wide changes affect the process of tumorigenesis in the murine gut. To investigate this process, we manipulated the gut microbiota by applying various antibiotic cocktails. One risk of this approach is that the antibiotic perturbation could reduce the overall bacterial load and confound the analysis. We previously analyzed the feces of mice receiving all three antibiotics using a culture-independent quantitative PCR approach and observed a non-significant reduction in the bacterial load 22. This result agrees with other studies that have used similar antibiotic cocktails to study the role of the microbiota in colitis 30,31. Meanwhile, others have seen a small but significant decrease in bacterial load which varied along the gastrointestinal tract 32. Considering our previous result and the fact that we observed we observed a relatively consistent relationship between bacterial populations in the gut and tumor burden, it is unlikely that differences in the bacterial load of the colon is responsible for the observed results. An alternative approach would involve colonizing germ-free mice with defined cocktails of bacteria or from murine or human donors. The challenge of this approach is that the immune system would still be altered from a normal state and it is difficult to dictate the final structure of a transplanted community 17. By pursuing various approaches to generate variation in the initial community, it is clear that gut microbiota are involved in protecting against and exacerbating colonic tumorigenesis.

There has been a focus on identifying specific 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 18,19,21. *F. nucleatum*, which has been detected on the surface of over 50% adenomas, can promote inflammation within the tumor microenvironment in multiple intestinal neoplasia mice 10,20. 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 33. 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 role of the gut microbiota in CRC is likely polymicrobial in nature. Our results support this hypothesis, as we demonstrated that different community structures were associated with similar levels of tumorigenesis in mice. When we examined the relative abundance of bacterial populations associated with increased tumor burden, we never observed consistent enrichment of any one population across all treatment groups (Figure 3). Similar to a previous study exploring the role of the gut microbiota in shaping resistance against *Clostridium difficile* colonization 34, we found that the context of the gut microbiota is important in predicting the eventual tumor burden. Such a result suggests that there may be redundancy in tumor-modulating roles amongst different bacteria populations within the gut microbiota.

As described above, there has been considerable effort to identify bacteria and their products that cause colon cancer. In contrast, our results indicate a need to focus on protective populations. We consistently observed that that the relative abundance of a *Lactobacillus*-affiliated OTU (OTU 1) was predictive of a low tumor burden (Figure 3). Various *Lactobacillus* strains are widely used as probiotics to reduce inflammation in the gastrointestinal tract. These bacteria have been shown to reduce inflammation in mouse models of colitis 35, necrotizing enterocolitis 36, and graft-versus-host disease 37. *Lactobacillus* spp. enhance epithelial barrier function by inducing the production of mucus and tight junction proteins 38,39 and can modulate the host's immune response by suppressing the expression of the proinflammatory cytokine IL-17 40. The clinical significance of this result is unclear, however, considering we observed suppression of tumorigenesis when the microbiota had levels of *Lactobacillus* that were higher than the 0.1 to 1% relative abundance commonly observed in the feces of humans 41. Regardless, a better understanding of the possible protective role of *Latobacillus* in limiting tumorigenesis may be useful in developing probiotic and prebiotic therapies.

It is striking that we were able to quantitatively predict the tumor burden that resulted at the end of our 73-day model based on the community composition at the start of the model. The random forest regression modeling approach is non-parametric and accounts for the nonlinearities and interactions within the dataset to identify a subset of OTUs that are predictive of tumor burden. An added advantage of this approach is that cross-validation is built into the model generation procedure limiting the risks of over fitting the model to the data 42. The regression-based approach has been used with microbiome data to predict *Clostridium difficile* colonization 34 and to assign a microbiome-based age to malnourished children 43. Given the significant heterogeneity that we observe in gut microbiota, regression-based random forest models are a powerful tool to identify subsets of communities that are associated with disease.

Dysbiosis of the gut microbiota generates a pro-inflammatory environment which results in a self-reinforcing pathogenic cascade between the gut microbiota and the host 16,17. In this study, we demonstrated that antibiotic manipulation of the gut microbiota during the onset of inflammation can significantly decrease tumorigenesis in mice. This highlights the efficacy of targeting the gut microbiota in CRC. Additional studies are needed to explore the viability of manipulating the gut microbiota 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 and carried out in accordance with the approved guidelines.

**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. 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 (N=12), all antibiotics (n=9) (metronidazole, streptomycin, and vancomycin), metronidazole (n=5) (streptomycin and vancomycin), streptomycin (n=5) (metronidazole and vancomycin), vancomycin (n=5) (metronidazole and streptomycin), metronidazole only (N=5), streptomycin only (N=5), 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.

**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 44,45. 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 46 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 47. 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\_SciReports\_2015. All FASTQ sequence data can be obtained from the Sequence Read Archive at NCBI (Accession SRP056144).

**Statistical analysis.** The microbiota data were analyzed using the R project for statistical computing. All R source code is available on our GitHub repository at https://github.com/SchlossLab/Zackular\_AbAOMDSS\_SciReports\_2015. All random forest models were made using the randomForest package with 10,000 trees 42. Diagnostic plots indicated that the percent of the variance explained had stabilized with this number of trees. Comparison of tumor counts were made by carrying out non-parametric pairwise Wilcoxon tests. The resulting p-values were corrected for multiple comparisons using the Benjamini-Hochberg procedure using an experiment-wide Type I error rate of 0.05.

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**Contributions** All authors contributed to the design of the experiments. JPZ and NTB carried out the experiments and generated the data. JPZ and PDS analyzed the data. All authors participated in interpreting the results. JPZ and PDS wrote the manuscript and NTB and GYC helped with the final editing of the text.

**Competing financial interests** The authors declare no competing financial interests.

**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 which were applied during the period covered by each of the rectangles; 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 a continuum of tumor burden in the mice (C and D). The stars indicate which treatments yielded a significantly (P<0.05) different number of tumors when compared to the treatment with the vertical line. The antibiotic treatments resulted in variation in the taxonomic structure of the communities at the start of the model (Day 0) (D). The two dimensional NMDS ordination had a stress of 0.20 and explained 84.0% of the variation in the distances (E).

**Figure 2. A random forest model successfully predicted the number of tumors in the mice at the end of the model based on their microbiota composition at the start of the model.** The model explains 67.7% of the variation in the data.

**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. Panels are ordered in decreasing order of the percent increase in the mean squared error of the model when that OTU was removed. The color and shape of the plotting symbols corresponds to those used in Figure 2.

**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 1 and 2 were among the most dynamic OTUs across all treatment groups; here we depict the change in their relative abundance across the model for those treatment groups that experienced the greatest overall change in community structure (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).

**Supplemental Figure 1. Effect of pruning the number of OTUs included in the random forest model for predicting the number of tumors at the end of the model based on the microbiota found at the start of the model.** The order of OTUs was set by the percent increase in mean square error when that OTU was removed from the model. The percent of the variance explained here indicates the quality of the fit when the top features were used to generate a model. The star indicates the number of OTUs that resulted in the model explaining the maximum percent of the variance.

**Supplemental Figure 2. Effect of pruning the number of OTUs included in the random forest model for predicting the number of tumors at the end of the model based on the microbiota found at the end of the model.** The order of OTUs was set by the percent increase in mean square error when that OTU was removed from the model. The percent of the variance explained here indicates the quality of the fit when the top features were used to generate a model. The star indicates the number of OTUs that resulted in the model explaining the maximum percent of the variance.

**Supplemental Figure 3. A random forest model successfully predicted the number of tumors in the mice at the end of the model based on their microbiota composition at the end of the model.** The model explains 65.6% of the variation in the data.

**Supplemental Figure 4. Relationship between the final 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. Panels are ordered in decreasing order of the percent increase in the mean squared error of the model when that OTU was removed. The color and shape of the plotting symbols corresponds to those used in Figure S3.