**Antibiotic induced alterations of the murine gut microbiota and subsequent effects on colonization resistance against *Clostridium difficile***

Alterations of the Murine Gut Microbiota that Lead to *Clostridium difficile* Colonization

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**5000 words with abstract**

**Science?/PNAS?/Plos Biology**

**BMC journals**

**mBio**

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**ABSTRACT**

**250 words**

**Key words (pick 6):** colonization resistance / *Clostridium difficile* /Lachnospiraceae / microbiota / Ruminococcaceae / antibiotics / CDI mouse models

**Subject Categories (pick one):** Microbial population and community ecology / Microbial ecology and functional diversity of natural habitats / Microbial ecosystem impacts / Microbe-microbe and microbe-host interactions

**INTRODUCTION funnel: microbiota and broad health/CR/cdiff/mouse studies or human studies for structur/functn so far.. then me!**

**Advantage over GF studies because in a mouse with in tact immune system that had be introduced to bacteria before…**

The microbiome, or the diverse collection of microorganisms living in and on the body, has an intimate role in determining host health. In a healthy state the bacteria-dense gastrointestinal microbiota is capable of aiding digestion, vitamin production, priming the immune system, and colonization resistance, or the ability of the microbiome to protect against pathogen colonization and outgrowth. Conversely in a dysbiotic state, which describes the \_\_ and destabilization of the microbiota, the microbiota can lead to obesity and metabolic disorders, inflammatory bowel disease, colon cancer, and susceptibility to several GI infections. The loss of colonization resistance.

CR by microbiome not fully understood, several proposed mechanisms, loss recovered by fecal transplants with cdiff, black box, need for personalized treatments (there have been studies showing that functions of certain bugs can be context dependent on other microbial members), need comprehensive study of good bacteria

There is a clear link between the microbiota and host health. The GI microbiota has been implicated in a number of roles in both health and disease. Eg. Obesity/metabolic disorders, IBD, colon cancer, susceptibility to GI infections (salmonella, VRE, cdiff); alternatively, aids digestion, vitamin production, priming immune system, maturation of GI tract, and colonization resistance. (or define CR here?)

Understanding how changes to the structure and function of the microbiota affect host health is necessary in order to understand and develop therapeutics for the disease.

For *C. difficile* in particular, where restoration of the microbiota via fecal bacterial transplants yields a 92% success rates in serious patients, understanding the ecology of the microbiota is critical to preventing disease/curing disease.. developing effective treatments. *C. difficile* infections (CDI) affect \_\_\_ individuals in the U.S. annually, resulting in \_\_ deaths and \_\_dollars. It is the number one nosocomial infection in the US, spores stick to surfaces of hospital where sick people coming in/out. Consequently, hospitalization, as well as elderly/immuno, and those on antibiotics are risk factors for CDI. Antibiotics alter the normal structure of the indigenous gut microbiota and decrease colonization resistance.

If effective multispecies probiotics are going to be designed, we need to see the whole big picture before we begin to break them down into smaller subsets…. See the overall big picture to come up with effective designs

Want to talk about mechanism or leave for discussion? Talk about studies on known mechanisms/functional studies?

We want to see who is actually participating and in which direction.

There are many studies conducted looking at CDI microbiotas in humans, where the big limitation is that you can’t see what the community looked like before *C. difficile* was introduced [my paper, manges, …]. Using mouse models of CDI we can study at the whole picture of the microbiota’s role in colonization resistance against *C. difficile*.

Cdiff Papers in mice? With the microbiota—sequencing preferably, before and after pictures?

* Sekirov—antibiotic induced perturbations of intestinal microbiota alter host susceptibility

Gap, unknown

What are the defining bacterial species/features of resistant communities vs susceptible communities?

Titrations: What changes are *necessary* in order for colonization to occur? Expect to see only changes in abundances of membership… look to see if this is true

Somewhere around here say the definition for microbiota structure

What is the role of the microbiota in CR? Need a more detailed look at the important features of a resistant versus susceptible community to colonization by *C. difficile.*

The purpose of this investigation was to determine which bacteria play significant roles in protecting against *C. difficile*, or alternatively susceptibility

See if we can identify bacteria to use in models to predict *C. difficile* colonization

These goals will be carried out by altering the mouse gut microbiota through different antibiotic treatments. Then we will test the resistance of these new communities to *C. difficile* colonization. Using a murine model system of CDI, we can observe the bacterial community in which *C. difficile* encounters/faces as it attempts to colonize the GI tract. The resultant absolute level of *C. difficile* colonization is measured on the subsequent day. Through correlation analysis and statistical modeling, we determined that several correlated/linked/related bacterial species, including Porphyromonadaceae, Lachnospiraceae, Ruminococcaceae, Clostridia.. etc\_\_, have potentially strong protective roles against *C. difficile* in mice.

**MATERIALS AND METHODS**

**Animal Care**

We used 5-8 week old C57Bl/6 mice for all our experiments. These mice were housed in our SPF room in the animal facility at the University of Michigan. All animal-related protocols and experiments were approved by the University Committee on Use and Care of Animals at the University of Michigan.

**Antibiotic administration**

Mice were administered one of seven different antibiotics, including cefoperazone, vancomycin, metronidazole, streptomycin, ciprofloxacin, ampicillin, and clindamycin. The route of administration depended on the specific antibiotic. Cefoperazone (either 0.5mg/ml, 0.3mg/ml, or 0.1mg/ml), vancomycin (0.625mg/ml, 0.3mg/ml, or 0.1mg/ml), streptomycin (5mg/ml, 0.5mg/ml, or 0.1mg/ml), metronidazole (0.5mg/ml), and ampicillin (0.5mg/ml) were all administered in the mouse drinking water for 5 days. Ciprofloxacin (10 mg/kg) was administered via oral gavage, and clindamycin (10 mg/kg) was administered via IP injection. All antibiotic treated mice were given 24 hours of recovery on regular drinking water prior to *C. difficile* challenge.

***C. difficile* Preparation andChallenge**

In all our experiments we used *C. difficile* strain 630Δerm [cite dena lyras]. The spores used were prepared from a single large batch made following previously established protocols [cite paul?]. Spores were quantified within the week prior to *C. difficile* challenge for each experiment [established protocol I can cite?]. Spores were quantified by boiling a small volume at 65°C for 20 minutes to kill off residual vegetative cells. The volume was then diluted and plated on TCCFA plates. These were incubated at 37°C anaerobically overnight. The next day *C. difficile* colony forming units (CFU) were counted and the concentration of spores in the working stock was back calculated. On the day of challenge, *C. difficile* spores, at a final concentration of 103, were administered to mice via oral gavage. Immediately following this challenge, the remaining *C. difficile* inoculum was diluted in a series and plated to confirm the correct dosage.

**Sample Collection and Plating**

Fecal samples were freshly collected for each mouse immediately prior to the administration of any antibiotic, during any antibiotic recovery days, and days 0 and 1 post *C. difficile* challenge. Each fecal sample on day 1 was weighed and diluted under anaerobic conditions with anaerobic PBS 1:10 based on pellet weight. A dilution series was then plated onto TCCFA plates [cite plates] and incubated at 37°C anaerobically overnight. The colony forming units (CFU) were counted and calculated the subsequent day.

**DNA Extraction and Sequencing**

Total bacterial DNA was extracted from each untreated and day 0 stool sample using the MOBIO PowerSoil®-htp 96 Well Soil DNA Isolation Kit. Sequencing preparation and sequencing was carried out as previously described (Kozich JJ et al. 2013). Briefly, the V4 region of the 16S rRNA gene was amplified, purified, and pooled in equimolar concentrations. These libraries were then mixed with Illumina-prepared PhiX control libraries. Sequencing was performed using Illumina’s MiSeq platform to produce paired 250-nt reads. Mock/error/error of run?

**Sequence Curation**

These sequences were curated using mothur as previously described (Kozich JJ et al and Schloss PD et al). The full pipeline is also demonstrated online at the mother website (<http://www.mothur.org/wiki/MiSeq_SOP>). Sequences were binned into operational taxonomic units (OTUs) using a 3% dissimilarity cutoff. Taxonomic assignments were determined by using a naïve Bayesian classifier with the Ribosomal Database Project (RDP) training set (version 9) requiring an 80% bootstrap confidence score. In order to avoid biases due to uneven sampling, samples were normalized to 1,625 sequences per samples [Schloss, reducing effects]. For those samples with more than 1,625 sequences per samples, the range was \_\_, the mean was \_\_, the median was \_\_, the median absolute deviation was \_\_. All 16S rRNA gene sequence data and the MIMARKS table are available at \_\_.

**Statistical Analysis & Modeling**

Correlation analysis was performed using Spearman’s rank based correlation coefficient, ρ, for non-normally distributed data. This metric was only calculated for OTUs whose average abundance across all samples was greater than 0.05%.

[Statistical significance with a FDR correction? Need to say?]. Feature selection, random forest, negative binomial model. We are NOT using the zero inflated one because we think all our zeros represent true zeros--high number of untreated samples resulting in high reports of *C. difficile* level of colonization of 0 CFU/g feces in the data. This was chosen over Poisson regression model due to the over-distribution of the data (variance > mean).

All statistical analyses were conducted using R version 3.0.1.

Using data from the original antibiotic treatments groups, OTUs were selected for addition into the model following several criteria. First all OTUs were present in abundance at a minimum of 1% in at least one or more samples. Second, these bacteria were among the top \_\_ % of OTUs with the strongest positive correlation or among the top \_\_% of OTUs with the strongest negative correlation withsubsequent *C. difficile* colonization. Third, we used the random forest feature selection algorithm to determine influential bacteria in predicting *C. difficile* levels. Finally, candidates in our model were screened for strong correlations with each other using sparCC [reference]. This was performed to eliminate redundancy within the model. This screening process allowed us to narrow our model down to X number of OTUs.

**RESULTS**

**Levels of colonization resistance are associated with the initial structure of the gut microbiota.** Using untreated mice as well as those treated with seven representatives from six classes of antibiotics (**Table 1**) we generated distinct and reproducible community structures that were assessed for their resistance to *C. difficile* colonization (**Figure 2a**). 16S rRNA gene sequencing of the communities sampled from the feces of the mice sampled at the time of *C. difficile* inoculation revealed six distinct community structures (AMOVA, p<0.001). The community structures of the beta-lactam-treated mice (i.e. cefoperazone and ampicillin) were not significantly different from each other (AMOVA, p=0.37). In addition, the untreated and ciprofloxacin-treated microbiota were not significantly different from each other (AMOVA, p=0.12). Although both pairs of treatment groups were not significantly different across the entire community, there did appear to be differences among the low-abundance taxa (**Figure 2a**). All other comparisons between the community structures were significantly different from each other (AMOVA, p<0.001). Subsequently, 24 hours after inoculation *C. difficile* was undetectable in the untreated mice or the ciprofloxacin-treated mice; however it had successfully colonized the mice in all of the other treatment groups to similarly high levels (107-108 CFU/g) **(Figure 2a)**. We calculated the diversity of all of the communities using the Shannon and the Inverse Simpson indices (**Supplemental Figure 1**) and observed a significant negative correlation with subsequent *C. difficile* colonization (both p<0.001). These results suggest that multiple microbiota structures are capable of being colonized by *C. difficile* and that diversity may be an important factor in resistance to invasion.

**Correlation analysis reveals potentially protective bacteria.** To identify bacterial taxa that could be associated with resistance or susceptibility to *C. difficile*, we measured the correlation between the relative abundance of each OTU on the day of inoculation with the level of *C. difficile* colonization 24 hours later across all treatment groups (**Figure 3**). OTUs with negative a correlation were associated with providing resistance against *C. difficile* (N=XX) and those with a positive correlation were associated with susceptibility (N=XX). Among various bacterial families, three were consistently negatively correlated with *C. difficile* colonization: Porphyromonadaceae (ρaverage= -0.57), Lachnospiraceae (ρaverage= -0.39), Ruminococcaceae (ρaverage= -0.47). OTUs from one bacterial phylum, the Proteobacteria, had a consistent strong positive relationship with *C. difficile* colonization. These included OTUs associated with the *Pseudomonas* (ρ= 0.31)*,* *Rhodobacter* (ρ= 0.31)*,* and *Escherichia* (ρ= 0.56) genera. Other notable OTUs that were found at the extremes of the distribution of correlation scores included those affiliated with the genus *Lactobacillus* (order Lactobacillales; OTU23, ρ= -0.73), *Alistipes* (order Bacteroidales; OTU20, ρ= -0.78), *Enterorhabdus* (phylum Actinobacterium; OTU29, ρ= -0.70), and *Streptococcus* (order Lactobacillales; OTU78, ρ= 0.36). Overall, these results suggest that several larger groups of related OTUs, particularly within the Firmicutes phylum, may participate in colonization resistance against *C. difficile*, while broadly many members of the Proteobacteria phylum may be involved in susceptibility.

**Smaller perturbations of the microbiota result in maintenance of CR.** Because most of our original treatments resulted in similarly high levels of *C. difficile,* we hypothesized that applying smaller antibiotic perturbations to the microbiota would result in reduced sensitivity to *C. difficile*. To test this, we titrated the doses of cefoperazone, streptomycin, and vancomycin given to the mice (**Supplemental Table 1)**. Colonization levels decreased in the mice receiving lower doses of cefoperazone and streptomycin in support of our hypothesis; while these levels were similar across mice receiving vancomycin, regardless of the dose (**Figure** **4**). Next, we hypothesized that the populations we previously identified as being associated with protection against colonization would be elevated in the mice with decreased colonization and those associated with susceptibility would be less abundant.

The titrated antibiotic doses clearly resulted in distinct community structures that were variations of the original dose (**Figure 5**). The cefoperazone titrations resulted in a shift in dominance from the Bacteroidetes to the Firmicutes phylum with increasing antibiotic dose. Most notable was the increase in OTUs associated with resistance including the Porphyromonadaceae and *Alistipes* and a reduction in OTUs associated with susceptibility including members of the Proteobacteria. The relative abundance of other OTUs associated with protection including the Lactobacillus, Lachnospiraceae, and Ruminococcaceae did not change as expected based on the initial correlation analysis; however, these OTUs were present at levels similar to the untreated mice. The streptomycin titrations did not result in profound shifts in the overall community structure, but did yield increased resistance. Rather, there were individual populations that were gained or lost across the gradient. Notably, mice receiving the lowest dose retained higher levels of *Alistipes* and Lachnospiraceae, which we previously found to be associated with colonization resistance. The relative abundances of the Porphyromonodaceae and Ruminococcaceae were unchanged, but were present at similarly high levels to those found in the untreated mice. Although the vancomycin titration yielded a number of large changes in the abundance of individual populations, there was no recovery of colonization resistance. The relative abundance of many of the populations associated with resistance increased with a decreased vancomycin dosage; however, with the exception of the *Lactobacillus* populations, they did not reach the levels observed in the untreated mice. In addition, all of the vancomycin-treated mice had high levels of OTUs affiliated with the *Akkermansia* and Enterobacteriaceae. Together, these results suggest that individual populations are not sufficient to provide colonization resistance. Rather, resistance is likely a product of the overall composition of the community.

We next wanted to determine whether associations we observed with the seven antibiotics at a single dose were supported by the results we observed in the titration experiments. Using only the new titration groups, we again performed correlation analysis of species level OTUs at inoculation with the *C. difficile* colonization levels the next day (**Figure 6**). Among many of the taxa previously identified as having significant relationships with *C. difficile*, we observed similar trends across the new titration data set, most notably with the titration treatment groups of cefoperazone and vancomycin.

Overall, cefoperazone titration treatments supported the correlation results from the original data set. The most positively correlated OTUs were *Pseudomonas* (OTU65), *E. coli* (OTU11), and *Clostridium* (OTU61), while the top 8 most negatively correlated were all different Porphyromonadaceae OTUs (OTU3, 4, 6, 8, 13, 2, 7, 12). Vancomycin titration treatments also supported the significant correlations found from the original data set. Among those with correlations smaller than -0.80, 7 Porphyromonadaceae OTUs (OTUs 19, 12, 3, 4, 2, 1, 13), Lachnospiraceae OTU39, and *Alistipes* OTU20 supported significant results found in the original data. Similarly, *Escherichia* (OTU11, ρ= 0.84) had the strongest positive correlation with subsequent *C. difficile* levels. In addition, the new vancomycin titration treatment results showed that *Akkermansia* had a strong positive relationship (ρ=0.77). The relative abundance of *Akkermansia*, a member of the Verrucomicrobia phylum, bloomed significantly following treatment with any of the 3 doses from <5% to as much as 50% of the community (p=?, **Figure 5**). Streptomycin titration treatment experiment results had the most sign discrepancies for direction of relationship (positive or negative) compared with the original antibiotic results [quantify?].

However, those discrepancies that were significant were found among the strongest positively correlated OTUs.

This included Bacteroidetes OTU16 (ρ=0.61) and *Bacteroides* OTU5 (ρ=0.72). *Akkermansia* OTU10 was also strongly positively correlated (ρ=0.64). *E. coli* OTU11 was near absent from the streptomycin treated communities (**Figure 5**), and thus we did not observe a strong association with *C. difficile* colonization as in most previous antibiotic treatments. Some of the strongest negative correlations were among several Porphyromonadaceae (OTUs 6, 18, 13), *Turicibacter* OTU17 (ρ=-0.70), and *Alistipes* OTU20 (ρ=-0.69).

All of the statistically significant negative correlations from the titration results support findings from the original antibiotic treatments.

**Allowing recovery of identified groups restores colonization resistance.** For each antibiotic treatment, a control group of mice receiving antibiotics without inoculation of *C. difficile* was maintained. The microbiota’s of animals treated with metronidazole underwent a drastic shift in the microbiota structure with an additional 5 days of recovery. We observed changes in relative abundance of several OTUs that we previously observed had strong relationships with subsequent *C. difficile* colonization (**Figure 7**). Notably there were increases in overall abundance of the levels of Porphyromonadaceae, Ruminococcaceae, and Lachnospiraceae, and decreases in levels of Enterobacteriaceae. Based on these changes, we hypothesized that delaying *C. difficile* challenge to allow these potentially important changes in microbiota structure to occur would lead to a decrease in colonization. On Day 1 post challenge of these recovered mice, we observed a significant decrease in *C. difficile* level of CFU/g feces (p=?). These results strengthen the importance of Porphyromonadaceae, Lachnospiraceae, Ruminococcaceae, and Enterobacteriaceae in setting up the right environment of negatively and positively correlated bacteria to affect the outcome of *C. difficile* colonization.

**Modeling to predict colonization outcomes.** Given our ability to make general predictions of *C. difficile* colonization based on the microbiota, we next designed a model to calculate expected colonization levels given the relative abundance of a specific OTU subset. We identified this subset using only the original data set to build the initial model [**Materials & Methods**]. This model included a total of \_\_ OTUs listed in **Table/Figure X**.

OTU27—Ruminoccocus lactaris (94%) and Blautia glucerasea (94%, enriched with inulin, a prebiotic/soluble fiber, which also enhances bifidobacteria), blautia schinkii (94%), bacteroides xylanolyticus (93%), clostridium hathewayi (93%)

OTU39—Eubacterium ventriosum (94%), eubacterium rectale (93%), roseburia intestinalis/hominis/faecis (93%) (positively correlated to differences observed in discordant MZ twins for BMI—more abundant in higher BMI siblings… these are all butyrate producers, also capable of degrading fiber)

FOS—fructooligosaccharides produced by degredation of inulin or polyfructose

**Inulin and fructo-oligosaccharides have divergent effects on colitis and commensal microbiota in HLA-B27 transgenic rats.**

### Abstract

Modulation of intestinal microbiota by non-digestible carbohydrates may reduce inflammation in inflammatory bowel disease (IBD). The aim of the present study was to assess the effects of inulin and fructo-oligosaccharides (FOS) on intestinal microbiota and colitis in HLA-B27 transgenic rats, a well-validated rodent model for IBD. In this study, 4-week-old rats were fed 8 g/kg body weight inulin or FOS for 12 weeks, or not. Faeces were collected at 4 and 16 weeks of age; and caecal samples were collected at necropsy. The effects of inulin and FOS on chronic intestinal inflammation were assessed using a gross gut score, histology score and levels of mucosal IL-1β. Intestinal microbiota were characterised by quantitative PCR and denaturing gradient gel electrophoresis. Colitis was significantly reduced in all FOS-fed rats compared to the control diet, whereas inulin decreased chronic intestinal inflammation in only half the number of animals. Quantitative analysis of caecal microbiota demonstrated that inulin increased the numbers of total bacteria and the Bacteroides-Prevotella-Porphyromonas group, FOS increased bifidobacteria, and both fructans decreased Clostridium cluster XI. In the faecal samples, both inulin and FOS decreased total bacteria, Bacteroides-Prevotella-Porphyromonas group, and Clostridium clusters XI and XIVa. FOS increased Bifidobacterium spp., and mediated a decrease of gene copies of Enterobacteriaceae and Clostridium difficile toxin B in faeces. SCFA concentrations in the faecal and caecal samples were unaffected by the diets. In conclusion, FOS increased the abundance of Bifidobacterium spp., whereas both fructans reduced Clostridium cluster XI and C. difficile toxin gene expression, correlating with a reduction of chronic intestinal inflammation.

Testing of our model with titration data, and then the ampicillin delay experiments?

**DISCUSSION—maybe make a point somewhere about how some whole groups like clostridia/Clostridiales/rumino&lachno, porphyros can appear to be protective, while for positive correlations/bad bugs its more species specific.. coincidence?**

Strong correlations, whether positive or negative, could indicate significant relationships between specific bacterial members and *C. difficile.*

**--also somewhere, ecoli are typically on avg (\_\_% of the community, typically wayyyy outnumbered… whats the avg relabund of all those CR OTUs in an untreated mouse, how does that compare to the abundance of those bacteria I’ve implicated in –CR) …point is that Proteobacteria are usually outnumbered… don’t have a chance, gut is suited to bact/firm (which not surprisingly make up the major players in CR).. I bet those other outlier bad bugs are also in low abund in untreated mice (?). but when the gut environment changes for w/e reason, can give those rarer bad ones/proteo/esp e. coli a chance to take hold. They could just be individually opportunistic or work synergistically with cdiff. If individually opportunistic, it might be that they are facing pretty much a common enemy which is the lach/rum/clos/porphs and end up having similar techniques/niches as a result of that common enemy. Working synergistically—eg. b. theta sialidase where it cleaves off resources for cdiff.**

**-therapeutic: continuously supply probiotics while lowering inflammation of the host (get host side back to normal state in tandem, via drugs? diet)… so attack the issue of CDI from both the microbiota end and host end… microbiota-host-pathogen triangle… …squeeze it out (eg. clindamycin clearance? If compared pathology of host at diff days along 10d for clindamycin vs cef/met/amp (these other ones that are high in cdiff coloniz), is host or toxin diff?... why does clinda clear after being CDhigh but no other ABX?)**

* + Recap major findings, answer to the question

Together these data allowed us to make better predictions about the colonization resistance ability of any given microbiota

Porphyromonadaceae, Lachnospiraceae, Ruminococcaceae, Clostridia, Alistipes, etc were found to have strong potential resistance abilities against *C. difficile*. Conversely, several members of the Proteobacteria phylum, including Pseudomonas, Streptococcus, and especially *E. coli* have strong potential positive impacts for *C. difficile* colonization. These results were supported through experiments in which the original doses of cefoperazone, streptomycin, and (vancomycin) were titrated to alter the overall abundances but not membership of the community from the starting dose. These subtler structural changes allowed for greater range of colonization levels of *C. difficile* as well as further/fine-tuned support for our original results. Furthermore, a known change to occur in important bacterial populations, we were able to reduce the level of colonization by *C. difficile*.

Correlation analyses and modeling techniques statistically confirm these results.

* + How answer supported by results
    - See this by correlation analysis, modeling, supported across several antibiotic regimens

Go into how these findings are similar to other papers/work? Buffie, reeves, theriot, me, etc, see list.

* + How relates to other studies
    - Can I apply my model to my human data?
    - Antonopoulos et al-reproducible community dynamics
    - Antunes et al (finlay)—abx treatment on intestinal metabalome
    - Sekirov—antibiotic induced perturbations of intestinal microbiota
    - Buffie et al (pamer)-single dose clinda
    - Reeves/theriot/robinson—clindamycin/cef/cocktail/antbiotic administration alters the community structure
    - Croswell—prolonge impact of abx on intestinal microbial ecology salmonella
    - Lawley-targeted restoration
    - Rea- effect of broad and narrow spectrum antimicrobials
    - Ferreira—intestinal microbiota plays a role in salmonella induced colitis
    - Reeves with clone libraries and cef
    - Theriot?—at least the metabolome paper—before and after with metabolites and cef, also does she look at anything taxonomically?
    - Buffie et al—single dose of clindamycin (diff strain of cdiff, other differences in systems/mice), how do their results compare

Any interesting points about the results? Mechanisms? Competition—ask matt if they are good? Proteobacteria could definitely play a role in susceptibility—creating an environment fit for cdiff… what ways could all those other bugs be making a bad environment for cdiff? Using up bile acids/germinants.., with those bugs gone freeing up sugars, … maybe connecting a lot more to some of caseys metabolome paper, and how mine would fit that

* + Limitations
    - No smaller intervals? Of cdiff levels, or changes in microbiota
    - Porphyromonadaceae is more prevalent in mice… so might not be as relevant for humans
    - Different strains of cdiff have different microbiota requirements to protect?
    - Doesn’t get at function… ?
  + Contribution to the field, positive spins
    - PROBIOTICS
    - Possible global effects? CR against other GI pathogens?

**Many of the relationships we observed in our study between specific bacteria and *C. difficile* are supported in the literature. –see my comments in this section**

Within the Porphyromonadaceae, only OTU505 had a significant positive relationship with subsequent *C. difficile* colonization (ρ= +0.21), which by blastn shares 99% identity with *Coprobacter fastidiosus.* Similarly within the Lachnospiraceae family, only one OTU had a positive relationship with *C. difficile* colonization (OTU174, ρ= +0.22). The Lachnospiraceae group also contained an OTU that had an exceptionally strong negative correlation (OTU39, ρ= +0.77). The Clostridia class was largely composed of the order Clostridiales (n=6), of which only one had a positive relationship with *C. difficile* colonization (OTU154, ρ= +0.26).

Several broader groups of bacteria can work together (in some combination) to provide CR …possible that proteobacteria in general are bad …These results indicate that many related species within the larger/broader bacterial groups may be able to serve in providing colonization resistance against *C. difficile.*

Other taxons in Figure 3 had more species specific correlations. For instance the Actinobacteria phylum included *Enterorhabdus* (OTU29, ρ= -0.70), Coriobacteriaceae (OTU50, ρ= -0.50), *Bifodbacterium* (OTU22, ρ= -0.34), *Actinomyces* (OTU58, ρ= +0.23). The Lactobacillales group is divided into two negatively correlated *Lactobacillus* OTUs (OTUs 23, 21, ρ= -0.73, -0.40 respectively) and two positively correlated *Streptococcus* OTUs (OTUs 78, 79). Similarly, the Bacteroidales group included species at both extremes, for example, an *Alistipes* (OTU20, ρ= -0.78) and a *Bacteroides* species (OTU44, ρ= +0.34). [END sent, all the other bad bugs look more like random smaller level taxonomic classification divisions, as in it’s a genus/species level effect type deal]

**--functional redundancy? Across related large groups like porphyros?**

**--mostly Firmicutes—better competitors with cdiff? Because similar nutrients resources?**

**Also functional redundancy with a highly diverse community… not just about who possibly but also ab being diverse—emphasizes community as a whole**

**Discussion** Do not recapitulate the results, but discuss their significance against the background of existing knowledge, and identify clearly those aspects that are novel. The final paragraph should highlight the main conclusion(s), and provide some indication of the direction future research should take. This section may be divided into subheadings to assist the reader. Results and Discussion may be combined.

**ACKNOWLEDGEMENTS**

We would like to thank Dena Lyras for providing the 630Δerm strain used in this study***.*** We would also like to thank Mary A. M. Rogers for her assistance with statistical questions. This work was supported by several grants from the National Institutes for Health 1R01GM099514 (PDS), R01HG005975 (PDS), U19AI090871 (VBY, PDS), and P30DK034933 (PDS, VBY). The funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**CONFLICT OF INTEREST STATEMENT**

All authors have no conflict of interest to report.

**REFERENCES**

**References**

Authors are responsible for the accuracy of the references. Published articles as well as those in press (please state the name of the journal and enclose a copy of the manuscript) may be included. In the text of the manuscript, a reference should be cited by author and year of publication eg (Bailey & Kowalchuk, 2006) and (Heidelberg et al, 1994) and listed at the end of the paper in alphabetical order of first author. References should be listed and journal titles abbreviated according to the style used by Index Medicus, examples are given below. All authors should be quoted for papers with up to six authors; for papers with more than seven authors, the first six only should be quoted, followed by *et al*.

**Journal article:**

Cho JC, Kim MW, Lee DH, Kim SJ. (1997). Response of bacterial communities to changes in composition of extracellular organic carbon from phytoplankton in Daechung reservoir (Korea). Arch Hydrobiol 138:559–576.

**FIGURE LEGENDS**

**Table 1. Description of Antibiotics used in this study.** The number of animals per group (n) are shown for each antibiotic and dose.

**Figure 2.** Bar graph**.** Significantly distinguishing OTUs for each abx treatment to show community types---RF using antibiotic categories for topdose data

--OR show community by just showing those with the most significant correlations

**Figure 3. Correlation analysis of bacterial species present on Day 0 with *C. difficile* levels on Day 1.** Species level OTUs (3% cutoff) found on Day 0 were correlated with the level of *C. difficile* CFU/g feces on Day 1 using Spearman’s rank based correlation coefficient. Only OTUs with an average abundance of at least 0.05% across all original treatments were considered in the correlation analysis. All correlations shown in this graph are significant (p<0.05). The lowest common classification among bacterial species was used to group bacteria as shown on the x-axis along with the number OTUs (n) belonging to that classification.

**Supplemental Figure 1 (?).** Also correlation results of Shannon/inv simpson (what’s the difference why choose one), showing actual levels in bar graph and then the correlation plot

**Supplemental Table 1. Titration amounts for each antibiotic.** The number of animals per group (n) are shown for each antibiotic and dose.

**Figure 4. Levels of *C. difficile* present on Day 1 for each titration treatment.** The amount of *C. difficile* was measured by CFU/gram of fecal sample collected from mice treated with **A)** cefoperazone titrations, **B)** streptomycin titrations, or **C)** vancomycin titrations.

**Figure 5. Relative abundances for titration groups.** Microbiota on Day 0.

**Figure 6. Heatmap comparing correlation analysis between the original data set and titration data sets.** Species level OTUs (3% cutoff) found on Day 0 were correlated with the level of *C. difficile* CFU/g feces on Day 1 using Spearman’s rank based correlation coefficient. Only OTUs with an average abundance of at least 0.1% across all original treatments were considered in the correlation analysis. All correlations shown in this graph are significant (p<0.05). The lowest common classification among bacterial species was used to group bacteria shown….

**Figure 7. Differences in day 0s for metronidazole. –maybe better way to show because it takes up a lot of space**

**Supplemental Figure 2**. Timeline for metro delay.

**FIGURES (temporary figs, see separate files for actual graphs)**

topdose2_tx2_sorted.pdf

**Figure 2.** Bar graph**.**

topdose2_CFU.pdf

**Figure2b.** CFU for each of the treatments in bargraph form

corr_allSig_topdose2.pdf

**Figure 3. Correlation analysis of bacterial species present on Day 0 with *C. difficile* levels on Day 1.** Species level OTUs (3% cutoff) found on Day 0 were correlated with the level of *C. difficile* CFU/g feces on Day 1 using Spearman’s rank based correlation coefficient. Only OTUs with an average abundance of at least 0.05% across all original treatments were considered in the correlation analysis. All correlations shown in this graph are significant (p<0.05). The lowest common classification among bacterial species was used to group bacteria as shown on the x-axis along with the number OTUs (n) belonging to that classification. Add line through 0, also add possibly “with cdiff CFU day 1”on the y and maybe an x label that says “3% level otus on day 0”

figures.pdf

**Figure 4. Levels of *C. difficile* present on Day 1 for each titration treatment.** The amount of *C. difficile* was measured by CFU/gram of fecal sample collected from mice treated with **A)** cefoperazone titrations, **B)** streptomycin titrations, or **C)** vancomycin titrations.

allceftitr_tx2.pdfallstreptitr_tx2.pdfallvanctitr_tx2.pdf

**Figure 5. Relative abundances for titration groups.** Microbiota on Day 0.

corr_heatmap_0.01rel_topdose2_newtitr.pdfAlso reorder this by classification and see what happens

**Figure 6. Heatmap comparing correlation analysis between the original data set and titration data sets.**

**metro_d0s_tx2.pdf**

**Figure 7. Differences in day 0s for metronidazole.**

**Supplemental Figure 2**. Timeline for metro delay.

**TABLES**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Antibiotic** | **Dose** | **Class** | **Mechanism** |  | **Primary Target** |
| Vancomycin (n=6) | 0.625 mg/ml | Glycopeptide | Inhibits peptidoglycan synthesis |  | Gram + |
| Streptomycin (n=8) | 5.0 mg/ml | Aminoglycoside | Inhibits protein synthesis |  | Gram +/- |
| Cefoperazone (n=4) | 0.5 mg/ml | Cephalosporin β-Lactam | Inhibits peptidoglycan synthesis |  | Gram + |
| Ampicillin (n=6) | 0.5 mg/ml | Penicillin  β-Lactam | Inhibits bacterial cell wall synthesis |  | Gram +/- |
| Metronidazole (n=7) | 1.0 mg/ml | Nitromidazole | Incorporates in bacterial DNA, making unstable |  | Anaerobes |
| Clindamycin (n=11) | 10 mg/kg | Lincosamide | Inhibits protein synthesis |  | Anaerobes |
| Ciprofloxacin (n=5) | 10 mg/kg | Fluoroquinolone | Inhibits DNA gyrase |  | Gram +/- |

**Table 1. Description of Antibiotics used in this study.** The number of animals per group (n) are shown for each antibiotic and dose. [change order?? Of abx]

**SUPPLEMENTARY INFORMATION**

**Supplemental Figure 1 (?).** Also correlation results of Shannon/inv simpson (what’s the difference why choose one), showing actual levels in bar graph and then the correlation plot

|  |  |  |  |
| --- | --- | --- | --- |
| **Antibiotic** | **Original Dose** | **Titration Doses** | |
| Cefoperazone | 0.5 mg/ml (n=4) | 0.3 mg/ml (n=12) | 0.1 mg/ml (n=6) |
| Streptomycin | 5.0 mg/ml (n=8) | 0.5 mg/ml (n=9) | 0.1 mg/ml (n=10) |
| Vancomycin | 0.625 mg/ml (n=6) | 0.3 mg/ml (n=8) | 0.1 mg/ml (n=9) |

**Supplemental Table 1. Titration amounts for each antibiotic.** The number of animals per group (n) are shown for each antibiotic and dose.