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Bacterial Community Ecology of the Colon in *Mus musculus*

Rachel Marie Nettles
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Bacterial Community Ecology of the Colon in *Mus musculus*

Rachel Marie Nettles

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

Bacterial Community Ecology of the Colon in *Mus musculus*

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The gut microbiome is a community of closely interacting microbes living in the gastrointestinal tract. Its structure has direct relevance to health. Disturbances to the microbiome, such as due to antibiotic use, have been implicated in various diseases. The goal of this study was to determine how the gut microbiome reacts to and recovers from disturbance caused by antibiotics. Because diet also influences the microbiome, this study included the interaction between diet and antibiotics. Half of the mice in each diet treatment were given antibiotics to disturb their microbiomes. After cessation of antibiotics, mice were paired in combinations within diets to determine whether the microbiomes of control mice influenced the disturbed microbiomes of formerly antibiotic mice. Chapter 1. Diet significantly altered the structure of the gut microbiome but its effect was significantly smaller than the effect of antibiotics. There was a significant interaction between diet and antibiotics; the antibiotic effect was larger in the cornstarch diet than in the glucose diet. Dysbiotic microbiomes resulting from antibiotics were characterized by an increase in *Bacteroidetes* and *Proteobacteria*, and a decrease in *Firmicutes*. Antibiotic administration also resulted in an initial increase OTU diversity, mainly because it reduced the abundance of dominant OTUs, resulting in greater evenness. Chapter 2. Seven weeks after the cessation of antibiotics (experiment termination), the effect of the antibiotics on the microbiome was still evident. The structure of the dysbiotic microbiome had not returned to that of control mice. Antibiotics significantly increased the relative abundance of some taxa and significantly decreased the relative abundance of others. It was unexpected that the taxonomic hierarchy within the microbiome did not recover after 7 weeks following cessation of antibiotics. It would appear, therefore, that antibiotics established a new, semi-stable hierarchy. Chapter 3. When paired together, the assumption was that dysbiotic microbiomes of antibiotic mice would be positively influenced by microbiomes of control mice, based on the assumption that the control mouse would act as a probiotic for the antibiotic mouse, either via coprophagy or consumption of food contaminated by feces. Contrary to that hypothesis, the microbiomes of control mice became more similar to that of antibiotic mice. One can offer at least two hypotheses to explain this result, but neither was tested. First, compared to the control microbiome, the dysbiotic microbiome may have been more stable and thus more resistant to change due to invasion by OTUs from the control microbiome. Other research has shown that dysbiotic microbiomes have a high degree of stability. If this were true, the use of probiotics is questionable. Second, one or more of the antibiotics could still have been active at the initial phase of pairing, and coprophagy caused the microbiome of the control mice to rapidly become dysbiotic. If this is true, the experiment should have been conducted with a waiting period between the cessation of antibiotic administration and pairing.

Keywords: antibiotics, community structure, competition, diet, diversity, gut, microbiome, *Mus*

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Bacterial Community Ecology of the Colon in *Mus musculus*

Rachel Nettles

Introduction

Humans are hosts to prokaryotes that live on or in virtually every part of the body, including the gut. In total, it is estimated that the human body is occupied by at least 5000 genera of bacteria, and an estimated 15,000-36,000 species (Frank et al. 2007, Rojo et al. 2016). Four major phyla account for approximately 98% of the human gastrointestinal tract microbiome: Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Frank et al. 2007, Sweeney and Morton 2013).

Bacteria differentially colonize the gastrointestinal tract, with numbers increasing with distance from the stomach. It is estimated that while there are 10^{3-4} cells/ml in the duodenum (the small intestines closest to the stomach) there are at least 10^{11} cells/ml in the colon (Walter and Ley 2011). Gastric acid inhibits the growth of bacteria in the small intestines; a low gastric acid concentration in the colon allows for larger populations (Kanno et al. 2009, Walter and Ley 2011). The colon has a high pH, a low concentration of bile salts (which can also inhibit microbial growth), and a slower rate of peristalsis, which provides an ideal environment for bacterial colonization and proliferation (Kanno et al. 2009).

The microbes of the gut are highly interactive with the host. Microbes digest the foods we cannot digest on our own. Byproducts include short chain fatty acids (SCFA), which provide an essential source of energy to the intestinal epithelial cells (Stevens and Hume 1998). Microbes engage in communication with host cells via certain metabolites (Jones et al. 2013). The host cells involved in this communication include those of the immune system. Colonization of the gut microbiome in an infant, for example, is essential for proper immune system development

(Belkaid and Hand n.d., Thaïss et al. 2016). Thus, the microbiome is key to developing a healthy, well-functioning host.

While its impact is still not fully understood, it is clear that gut prokaryotes play important roles in human health. Healthy prokaryote communities in the gut may prevent disease (Liou et al. 2013), and dysbiotic communities may precipitate a range of diseases including cancer (Dzutsev et al. 2015, Paul et al. 2015), autoimmune disorders such as rheumatoid arthritis (Rogers 2015, Zhang et al. 2015), type one diabetes (Alkanani et al. 2015, Burrows et al. 2015), gastrointestinal disorders (Becker et al. 2015, Schaubeck and Haller 2015), and many more. At least 105 diseases and disorders have been associated with changes in the human gut microbiome (Rojo et al. 2016).

The gut microbiome has frequently been considered to be a collection of taxa with individual functionalities (Zhu et al. 2010). However, the gut microbiome is, in fact, a community of closely interacting taxa that must compete for resources. Therefore, the principles of community ecology apply. In most microbial communities, distributions are uneven. A limited number of taxa are highly abundant and most are rare. This abundance hierarchy may result from a hierarchy of competitive ability, as competition does occur within the colon (Little et al. 2008, Lozupone et al. 2012). Thus, interactions among taxa help to maintain a stable community structure.

Any community may be perturbed by some external disturbance, resulting in a significantly altered community structure and, therefore, function (Connell 1978, Christian et al. 2015). There are a number of ways that a community can respond to such a disturbance. Resilient communities experience a change in structure as a response to disturbance, but are able to return to their previous state. The structure of resistant communities does not change in

response to disturbance. If a community is neither resistant nor resilient, it may remain in the altered state for a long period of time (Christian et al. 2015). This is sometimes referred to as an alternative stable state (Beisner et al. 2003).

In the case of the gut microbiome, the treatment of antibiotics represents a disruptive event. Antibiotics are known to cause community-wide changes in the gut microbiome. Dollive et al. 2013 showed that antibiotics greatly reduce the abundance of gut bacteria and result in an increase in fungal abundance. After cessation of the antibiotics bacterial abundance returned to normal levels, but did not return to a similar composition. Other studies have shown clear effects of antibiotics on gut microbiome composition and abundance (Hill et al. 2010, Hu et al. 2015). Antibiotics are also shown to have significant effects on the gut microbiome when administered at a sub-therapeutic level (Cho et al. 2012). In this study, antibiotics were used as a tool to disturb the gut microbiomes of mice. I then determined how the bacterial communities responded to, and recovered from, the disturbance.

The use of probiotics or supplementation of the gut microbiome via ingestion of beneficial bacteria, is essentially a problem of invasion. It is generally accepted that a diverse community is more resistant to invasion than a simple community (Levine and D'Antonio 1999). One might expect antibiotic treatment to simplify the gut microbiome by reducing the number of taxa and thus the antibiotic microbiome should be more invulnerable than a control microbiome. The probiotic effect in mice may occur as a consequence of deliberate or accidental coprophagy of cohabiting individuals. Accidental coprophagy would be the result of food particles coming into contact with feces by falling to the cage floor. I hypothesized that the intact microbiomes of a control mouse (not administered antibiotics) would colonize the dysbiotic microbiomes of the antibiotic mouse but that the reverse would be less likely.

Diet is also a known modulator of the gut microbiome (Turnbaugh et al. 2009b, David et al. 2014, Rojo et al. 2016, Singh et al. 2017). This is not surprising because diet is the main source of nutrition for the microbiome. Consuming a diet rich in microbiota-accessible carbohydrates increases the microbial diversity in the colon, as well as the amount of SCFAs produced by the microbes (Sonnenburg and Sonnenburg 2014). I used diet to create separate microbiomes and then determined for each the impact of, and recovery from, antibiotic disturbance.

Methods

Mice and Treatments

One hundred twenty female mice of the C57BL/6J strain at 4 weeks old (Jackson Laboratories; Sacramento, CA) were placed in small shoebox cages (7x10 inches) at five mice per cage. Cages were placed on shelving units in a dedicated mouse room. The average temperature of the room was 72.5°F, and the photoperiod was 12 hours of light and 12 hours of dark each day. Sixty mice (12 cages) were fed a diet in which the carbohydrate was supplied as cornstarch (Harlan Teklad: TD.150372; 555.6 g/kg cornstarch). Hereafter this diet will be referred to as the cornstarch diet. The cornstarch diet was composed of (by kcal): 18.4% protein, 64.9% carbohydrate, and 16.7% fat. The remaining sixty mice were fed a diet in which the carbohydrate was supplied as glucose (Harlan Teklad: TD.150373; 548.5 g/kg dextrose monohydrate). Hereafter this diet will be referred to as the glucose diet. The glucose diet was composed of (by kcal): 18.3% protein, 65.2% carbohydrate, and 16.5% fat.

Upon receipt, the mice were assigned to and fed their respective diets. Diets remained constant throughout the study. The first 14 days of this study are referred to as the Stabilization Period (Days 0-14) (Fig. 1). This allowed time for the microbiomes to adjust and stabilize to their respective diets.

Following the Stabilization Period, six cages (30 mice) within each diet group were randomly assigned to receive antibiotics. A stock solution of vancomycin (0.125g/250ml water), neomycin (0.25g/250ml water), and ampicillin (0.25g/250ml water) was supplied via drinking bottles in each cage. Bottles with antibiotics were covered with foil to reduce photo-degradation and were shaken daily. These antibiotic solutions were replaced with freshly made solution every three days. The antibiotic treatment was continued for 14 days (Days 15-28) and is referred to as

the Antibiotic Period (Fig. 1). Mice were ear-tagged to permit identification of individuals. Cages were cleaned once every week.

At the end of the Antibiotic Period, mice were placed into new cages in pairs. Treatment combinations included: control/self (C-c), control/mixed (C-a), antibiotic/mixed (c-A), and antibiotic/self (A-a) for each diet. The upper-case letters indicate the focal individual of the pair. The lower-case letters indicate the individual paired with the focal individual. Thus, the C-a and c-A pairing are the same but with different focal individuals. Cages were randomly assigned to one of ten blocks to establish a randomized complete block design. Each block consisted of each treatment combination (diet and pairing) yielding six cages per block (three from each diet). Pairing continued through the termination of the study and is referred to as the Recovery Period (Days 29-78) (Fig. 1, Fig. 2). Mice were overdosed on isoflurane at the termination of the study (Day 78).

Sampling

Samples of fecal material were taken on Days 28, 42, and 76 (Fig. 1). Day 28 is the last day of the Antibiotic Period. Day 42 is 14 days after cessation of the antibiotic treatment, and 14 days into the Recovery Period. Day 76 is the last day of the study, and is 48 days after cessation of the antibiotic treatment, and 48 days into the Recovery Period. To obtain fresh fecal samples from an individual, mice were isolated in empty cages until feces were deposited. When necessary, peristalsis was induced by hand stimulating rectal muscles. A single fecal pellet from one individual was immediately placed in a PowerSoil DNA extraction tube (MoBio, Carlsbad, USA), and stored at -20°C until extraction.

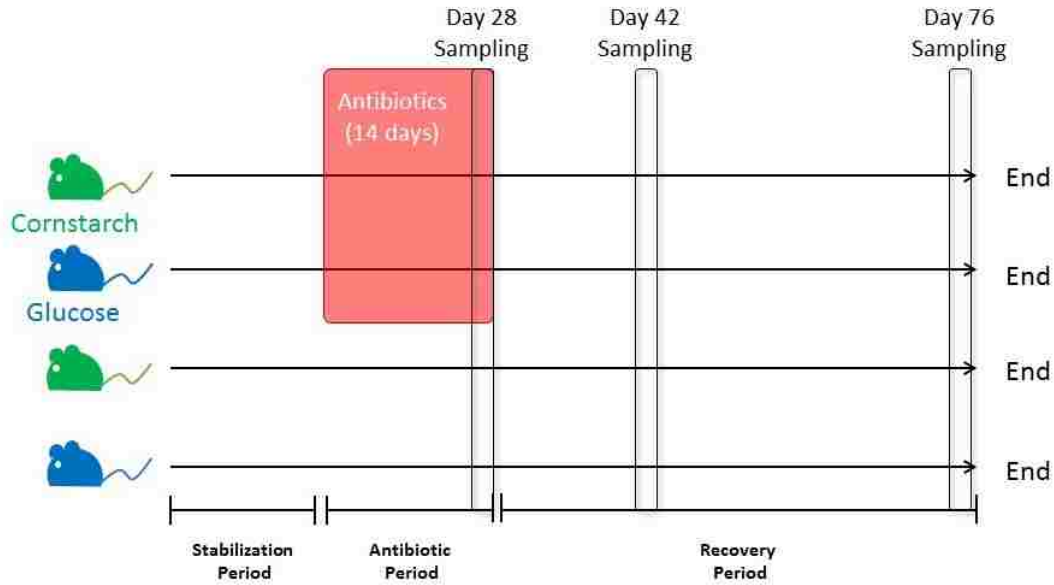


Figure 1. Timeline of chapters one and two. The Stabilization Period is 14 days. Mice from each diet were given antibiotics for 14 days which constitutes the Antibiotic Period. The Recovery Period lasted for 48 days.

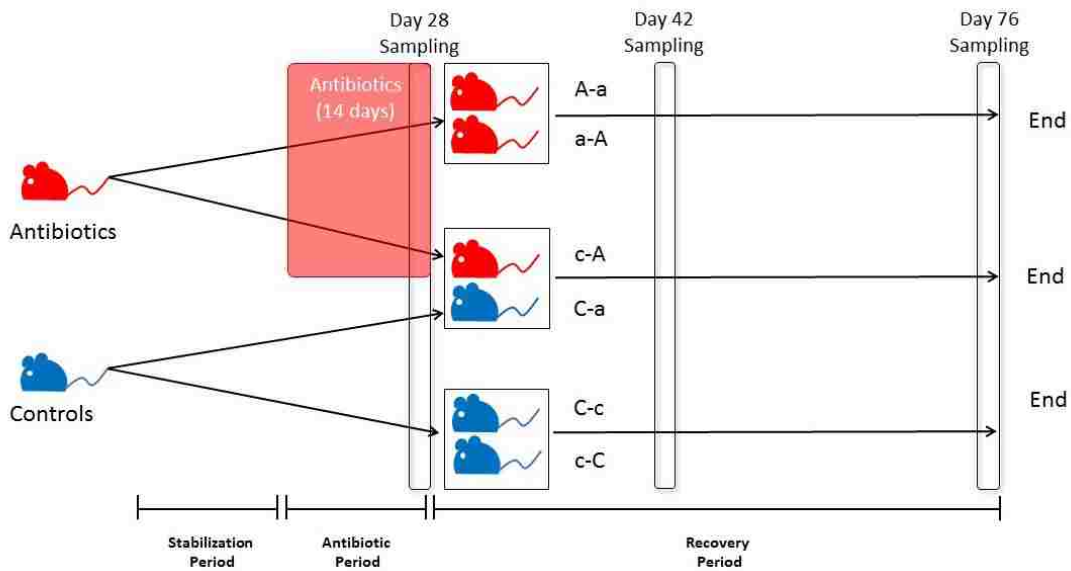


Figure 2. Timeline of chapter three. Blue mice represent control mice, while red mice represent antibiotic mice. Following the Antibiotic Period, mice were paired. The Recovery Period lasted for 48 days. Self-paired mice are designated as A-a or C-c. Mixed pair mice are designated as c-A and C-a. The capitalized letter represents the specific mouse being referenced. Pairing was assigned within diets.

Sequencing

DNA extractions were performed using the PowerSoil DNA extraction kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA). Extraction tubes were vortexed for 1.5 minutes. All other steps were completed as outlined in the PowerSoil protocol. Genomic DNA was stored at -20°C until amplification.

DNA was prepared for Miseq sequencing using a one-step PCR. AccuPrime Pfx SuperMix (Invitrogen, Carlsbad, USA) was used, in conjunction with the 515F-806R primer pair developed by Caporaso et al. 2011, targeting the 16S region. Forward primer construction is as follows from 5' to 3': Illumina adapter, 8-mer bar code, primer pad, linker, and the core primer (AATGATACGGCGACCACCGAGATCTACAC-NNNNNNNN-TATGGTAATT-GT-GTGCCAGCMGCCGCGGTAA). Reverse primer construction is as follows from 5' to 3': Illumina adapter, 8-mer bar code, primer pad, linker, and the core primer (CAAGCAGAAGACGGCATAACGAGAT-NNNNNNNN-AGTCAGTCAG-CC-GGACTACHVGGGTWTCTAAT).

The thermal cycling program used to amplify bacterial DNA was: initial activation at 95°C for 2 minutes, followed by 30 cycles of 95°C for 20s, 55°C for 15s, and 72°C for 5 minutes, with final elongation of 72°C for 10 minutes. PCR products were normalized using a SequalPrep Plate Normalization Kit (Invitrogen, Carlsbad, USA). Following normalization, PCR products were pooled and sequenced at the Brigham Young University DNA Sequencing Center (Provo, USA) on a MiSeq 2X250 (Illumina, San Diego, USA).

Sequences were processed using QIIME (Caporaso et al. 2010) and filtered using VSEARCH (Rognes et al. 2016). Sequences were excluded from the analysis if shorter than 252bp or longer than 254bp (based on target region length). Chimeras were filtered using a

denovo approach. Taxonomy was assigned using SILVA128 (Quast et al. 2013, Yilmaz et al. 2014). Singletons, doubletons, and tripletons were filtered from the OTU tables. All samples were rarefied to 3000.

Community Analysis

Community structure was investigated at both the phylum and OTU levels using Hellinger-transformed relative abundances based on the rarefied number of sequences. The effects of treatments on community structure were determined using permutational multivariate analysis of variance (PerMANOVA) in the R statistical environment (R Core Team 2013) with the vegan package (Oksanen et al. 2015). Bray-Curtis distances were used. Variation in community structure was visualized using ordination (nonmetric multidimensional scaling, NMDS) in R. The distribution among treatments of the most abundant (>1% of total Hellinger-transformed rarefied sequence reads) phyla and OTUs was determined using individual analyses of variance (ANOVA) using a false discovery rate (FDR) p-value correction (Benjamini and Hochberg 1995). ANOVAs were also calculated in R.

C-scores were calculated for OTUs that accounted for >1% of the total Hellinger-transformed rarefied sequence reads. They were calculated using fixed row sums and equiprobable columns in the R environment (R Core Team 2013) using EcoSimR (Gotelli et al. 2015).

Means were calculated for each group of interest at either the OTU or phylum level. Mean separations were accomplished using a Tukey Honest Significant Difference.

Chapter 1

Introduction

I asked in this chapter whether antibiotics could be used to disturb the gut microbiome in mice given either of the diets (cornstarch vs. glucose). To answer the question, I used data from mice on Day 28, the last day of antibiotic treatment (Fig. 1). The analyses included the variables diet and antibiotics and the interaction between the two.

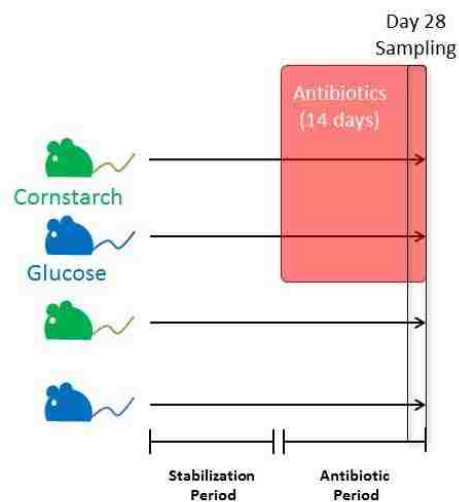


Figure 1. Timeline of chapter one. Green mice represent mice in the cornstarch diet, while blue mice represent mice in the glucose diet. The data for this chapter includes only these control and antibiotic mice at Day 28 (the last day of antibiotic treatment).

Results

The high-throughput sequencing effort revealed the presence of 2 Archaea phyla and 19 bacteria phyla (Table 1). Hereafter archaea were included in the datasets that were analyzed.

Table 1.
Phyla represented in the Day 28 dataset.

| Domain | Phylum |
|-----------------|---------------------|
| Archaea | Euryarchaeota |
| | Thaumarchaeota |
| Bacteria | Acidobacteria |
| | Actinobacteria |
| | Bacteroidetes |
| | Chloroflexi |
| | Cyanobacteria |
| | Deinococcus-Thermus |
| | Elusimicrobia |
| | Fibrobacteres |
| | Firmicutes |
| | Fusobacteria |
| | Lentisphaerae |
| | Nitrospirae |
| | Planctomycetes |
| | Proteobacteria |
| | Absconditabacteria |
| | Spirochaetae |
| | Synergistetes |
| Tenericutes | |
| Verrucomicrobia | |

Both diet and antibiotics had significant effects on the structure of the bacterial communities, and the effect of antibiotics was much larger than the effect of diet. This is seen at both the level of the OTU (Table 2, Figure 2) and the phylum (Table 3, Figure 3).

Table 2.

PerMANOVA results for Day 28 at the OTU level for both diets, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Model | R ² | P-values |
|-------------|--------------------|-----------------|-----------------|----------|----------------|----------|
| Diet | 1 | 0.40 | 0.40 | 3.83 | 0.03 | <0.01 |
| Antibiotics | 1 | 4.95 | 4.95 | 46.81 | 0.34 | <0.01 |
| Diet:AB | 1 | 0.34 | 0.34 | 3.25 | 0.02 | 0.02 |
| Residuals | 83 | 8.77 | 0.11 | | 0.61 | |
| Total | 86 | 14.47 | | | 1.00 | |

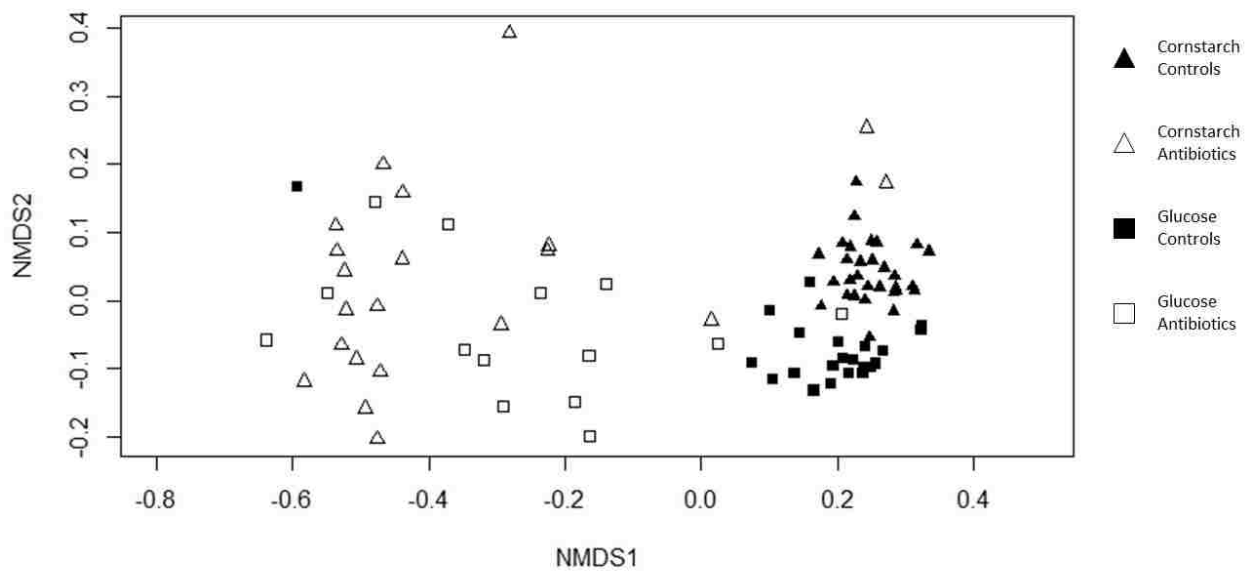


Figure 2.

Ordination of samples for Day 28 at the OTU level showing the effects of diet and antibiotics on the structure of bacterial communities.

Table 3.

PerMANOVA results for Day 28 at the phylum level for both diets, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Model | R ² | P-values |
|-------------|--------------------|-----------------|-----------------|----------|----------------|----------|
| Diet | 1 | 0.17 | 0.17 | 11.70 | 0.05 | <0.01 |
| Antibiotics | 1 | 1.97 | 1.97 | 137.10 | 0.58 | <0.01 |
| Diet:AB | 1 | 0.06 | 0.06 | 4.35 | 0.02 | 0.03 |
| Residuals | 83 | 1.20 | 0.01 | | 0.35 | |
| Total | 86 | 3.40 | | | 1.00 | |

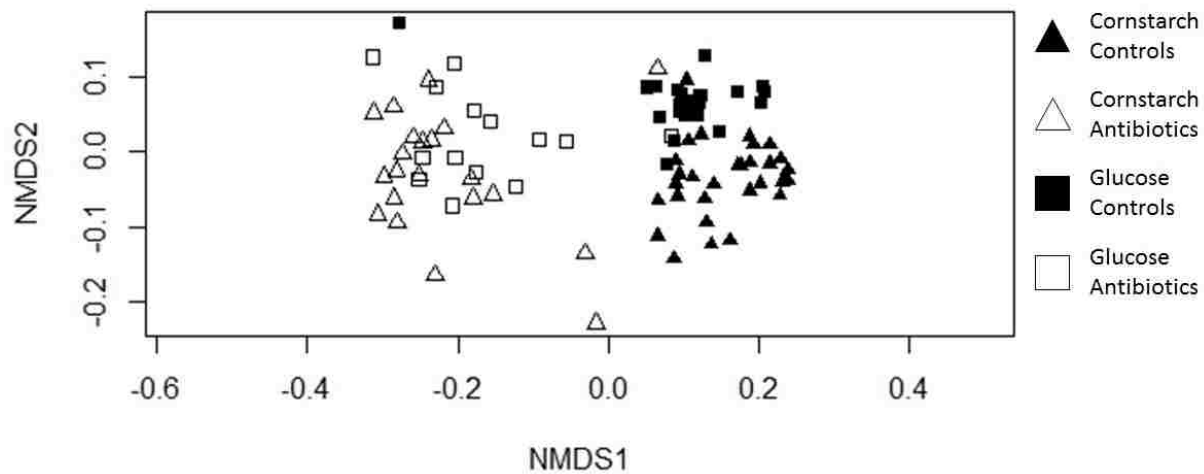


Figure 3. Ordination of samples for Day 28 at the phylum level showing the effects of diet and antibiotics on the structure of bacterial communities.

Moreover, the effect of antibiotics was larger for mice receiving the cornstarch diet compared to those receiving the glucose diet as seen from the differences in the R^2 . Again, this was true at both the OTU level (Tables 4, 5, Figure 2) and phylum level (Tables 6, 7, Figure 3).

Table 4. PerMANOVA results for Day 28 at the OTU level, cornstarch diet, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Model | R^2 | P-values |
|-------------|--------------------|-----------------|-----------------|----------|-------|----------|
| Antibiotics | 1 | 3.84 | 3.84 | 37.61 | 0.43 | <0.01 |
| Residuals | 49 | 5.00 | 0.10 | | 0.57 | |
| Total | 50 | 8.84 | | | 1.00 | |

Table 5. PerMANOVA results for Day 28 at the OTU level, glucose diet, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Model | R^2 | P-values |
|-------------|--------------------|-----------------|-----------------|----------|-------|----------|
| Antibiotics | 1 | 1.45 | 1.45 | 13.11 | 0.28 | <0.01 |
| Residuals | 34 | 3.77 | 0.11 | | 0.72 | |
| Total | 35 | 5.22 | | | 1.00 | |

Table 6.

PerMANOVA results for Day 28 at the phylum level, cornstarch diet, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Model | R ² | P-values |
|-------------|--------------------|-----------------|-----------------|----------|----------------|----------|
| Antibiotics | 1 | 1.47 | 1.47 | 107.45 | 0.68 | <0.01 |
| Residuals | 49 | 0.70 | 0.01 | | 0.32 | |
| Total | 50 | 2.18 | | | 1.00 | |

Table 7.

PerMANOVA results for Day 28 at the phylum level, glucose diet, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Model | R ² | P-values |
|-------------|--------------------|-----------------|-----------------|----------|----------------|----------|
| Antibiotics | 1 | 0.57 | 0.57 | 39.24 | 0.54 | <0.01 |
| Residuals | 34 | 0.49 | 0.01 | | 0.46 | |
| Total | 35 | 1.06 | | | 1.00 | |

The effect of antibiotics on overall community structure can be appreciated in more detail as we consider individual OTUs. According to the FDR-protected ANOVAs, the relative abundances (Hellinger-transformed rarefied sequence reads) were significantly reduced by antibiotics in both diets for BacteroidalesS24.7, *Blautia*, Lactobacillales, *Erysipelatoclostridium*, *Eubacterium nodatum* and *Ruminoclostridium*, and significantly increased by antibiotics in both diets for *Buttiauxella*, *Escherichia/Shigella*, Bacteroides-1, Bacteroides-2 and *Clostridium sensu stricto-1*. In addition, mice receiving the cornstarch diet had more OTUs that were negatively affected by antibiotics including Coriobacteriaceae, *Lactobacillus*, *Peptoclostridium* and *Clostridium sensu stricto-2*, and fewer OTUs that were positively affected by antibiotics including *Clostridium sensu stricto-2* (Tables 8, 9) compared to mice receiving the glucose diet.

Table 8.

Means of Hellinger-transformed abundances for OTUs comprising >1% in the cornstarch diet. P-values are based on FDR-protected ANOVAs.

| Taxon | Controls | Antibiotics | P-value |
|------------------------------------|----------|-------------|---------|
| <i>Bacteroidales</i> S24.7 | 0.39 | 0.19 | <0.01 |
| <i>Blautia</i> | 0.28 | 0.13 | <0.01 |
| <i>Buttiauxella</i> | 0.01 | 0.05 | <0.01 |
| Lactobacillales | 0.18 | 0.08 | <0.01 |
| <i>Erysipelatoclostridium</i> | 0.10 | 0.05 | <0.01 |
| <i>Escherichia/Shigella</i> | 0.01 | 0.36 | <0.01 |
| Bacteroides-1 | 0.00 | 0.03 | <0.01 |
| Coriobacteriaceae | 0.29 | 0.11 | <0.01 |
| <i>Lactobacillus</i> | 0.26 | 0.11 | <0.01 |
| Bacteroides-2 | 0.00 | 0.02 | <0.01 |
| <i>Eubacterium nodatum</i> | 0.20 | 0.07 | <0.01 |
| <i>Clostridium sensu stricto-1</i> | 0.01 | 0.02 | <0.01 |
| <i>Peptoclostridium</i> | 0.13 | 0.04 | <0.01 |
| <i>Clostridium sensu stricto-2</i> | 0.15 | 0.07 | <0.01 |
| <i>Ruminoclostridium</i> | 0.14 | 0.05 | <0.01 |

Table 9.

Means of Hellinger-transformed abundances for OTUs comprising >1% in the glucose diet. P-values are based on FDR-protected ANOVAs.

| Taxon | Controls | Antibiotics | P-value |
|------------------------------------|----------|-------------|---------|
| <i>Bacteroidales</i> S24.7 | 0.38 | 0.29 | 0.04 |
| <i>Blautia</i> | 0.28 | 0.18 | <0.01 |
| <i>Buttiauxella</i> | 0.01 | 0.05 | <0.01 |
| Lactobacillales | 0.07 | 0.04 | 0.07 |
| <i>Erysipelatoclostridium</i> | 0.11 | 0.05 | <0.01 |
| <i>Escherichia/Shigella</i> | 0.01 | 0.24 | <0.01 |
| Bacteroides-1 | 0.00 | 0.01 | 0.01 |
| Coriobacteriaceae | 0.07 | 0.07 | 0.75 |
| <i>Lactobacillus</i> | 0.09 | 0.07 | 0.36 |
| Bacteroides-2 | 0.00 | 0.01 | 0.04 |
| <i>Eubacterium nodatum</i> | 0.19 | 0.09 | <0.01 |
| <i>Clostridium sensu stricto-1</i> | 0.01 | 0.03 | <0.01 |
| <i>Peptoclostridium</i> | 0.08 | 0.05 | 0.11 |
| <i>Clostridium sensu stricto-2</i> | 0.01 | 0.04 | <0.01 |
| <i>Ruminoclostridium</i> | 0.21 | 0.11 | <0.01 |

The effect of antibiotics on overall community structure can also be appreciated from the distribution of the phyla. According to the FDR-protected ANOVAs, the relative abundances (Hellinger-transformed rarefied sequence reads) were significantly reduced by antibiotics in both diets for Firmicutes, and significantly increased by antibiotics in both diets for Bacteroidetes, Cyanobacteria, Fusobacteria, Planctomycetes, Proteobacteria, Absconditabacteria, Spirochaetae and Verrucomicrobia. In addition, mice receiving the cornstarch diet had additional OTUs that were positively affected by antibiotics including Chloroflexi and Euryarchaeota (Archaea), and additional OTUs that were negatively affected by antibiotics including Actinobacteria (Tables 10, 11).

Table 10.
Means of Hellinger-transformed abundances for phyla comprising >1% in the cornstarch diet. P-values are based on FDR-protected ANOVAs.

| Phylum | Controls | Antibiotics | P-value |
|--------------------------|-----------------------|-----------------------|---------|
| Euryarchaeota (Archaea) | 1.44*10 ⁻⁴ | 2.98*10 ⁻³ | <0.01 |
| Thaumarchaeota (Archaea) | 0 | 3.83*10 ⁻⁴ | 0.24 |
| Acidobacteria | 0 | 1.26*10 ⁻³ | 0.18 |
| Actinobacteria | 0.09 | 0.03 | <0.01 |
| Bacteroidetes | 0.23 | 0.39 | <0.01 |
| Chloroflexi | 2.22*10 ⁻⁵ | 1.10*10 ⁻³ | <0.01 |
| Cyanobacteria | 2.11*10 ⁻⁴ | 7.96*10 ⁻³ | <0.01 |
| Deinococcus Thermus | 0 | 1.27*10 ⁻⁴ | 0.24 |
| Elusimicrobia | 1.11*10 ⁻⁵ | 2.23*10 ⁻⁴ | 0.08 |
| Fibrobacteres | 2.00*10 ⁻⁴ | 2.69*10 ⁻³ | <0.01 |
| Firmicutes | 0.67 | 0.28 | <0.01 |
| Fusobacteria | 0 | 1.59*10 ⁻⁴ | 0.02 |
| Lentisphaerae | 0 | 3.19*10 ⁻⁵ | 0.11 |
| Nitrospirae | 0 | 3.82*10 ⁻⁴ | 0.11 |
| Planctomycetes | 1.11*10 ⁻⁴ | 1.83*10 ⁻³ | <0.01 |
| Proteobacteria | 5.37*10 ⁻³ | 0.26 | <0.01 |
| Absconditabacteria | 7.78*10 ⁻⁵ | 2.42*10 ⁻³ | <0.01 |
| Spirochaetae | 1.11*10 ⁻³ | 0.02 | <0.01 |
| Synergistetes | 4.45*10 ⁻⁵ | 9.24*10 ⁻⁴ | <0.01 |
| Tenericutes | 6.21*10 ⁻³ | 2.44*10 ⁻³ | 0.22 |
| Verrucomicrobia | 1.33*10 ⁻⁴ | 2.34*10 ⁻³ | <0.01 |

Table 11.

Means of Hellinger-transformed abundances for OTUs comprising >1% in the glucose diet. P-values are based on FDR-protected ANOVAs.

| Phylum | Controls | Antibiotics | P-value |
|--------------------------|-----------------------|-----------------------|---------|
| Euryarchaeota (Archaea) | 8.37×10^{-4} | 1.65×10^{-3} | 0.27 |
| Thaumarchaeota (Archaea) | 0 | 0 | N/A |
| Acidobacteria | 0 | 1.67×10^{-4} | 0.26 |
| Actinobacteria | 0.01 | 0.02 | 0.12 |
| Bacteroidetes | 0.27 | 0.40 | <0.01 |
| Chloroflexi | 2.44×10^{-4} | 7.16×10^{-4} | 0.15 |
| Cyanobacteria | 3.80×10^{-4} | 7.45×10^{-3} | <0.01 |
| Deinococcus Thermus | 0 | 0 | N/A |
| Elusimicrobia | 3.04×10^{-5} | 9.60×10^{-5} | 0.45 |
| Fibrobacteres | 1.05×10^{-3} | 2.73×10^{-3} | 0.16 |
| Firmicutes | 0.70 | 0.37 | <0.01 |
| Fusobacteria | 0 | 1.00×10^{-3} | <0.01 |
| Lentisphaerae | 0 | 2.40×10^{-5} | 0.26 |
| Nitrospirae | 0 | 0 | N/A |
| Planctomycetes | 4.57×10^{-4} | 2.06×10^{-3} | 0.05 |
| Proteobacteria | 0.01 | 0.17 | <0.01 |
| Absconditabacteria | 5.01×10^{-4} | 2.42×10^{-3} | <0.01 |
| Spirochaetae | 2.81×10^{-3} | 0.02 | <0.01 |
| Synergistetes | 1.52×10^{-4} | 1.08×10^{-3} | 0.02 |
| Tenericutes | 2.23×10^{-3} | 2.06×10^{-3} | 0.80 |
| Verrucomicrobia | 5.48×10^{-4} | 2.01×10^{-3} | 0.02 |

Diet and antibiotics also influenced various measures of bacterial diversity. The cornstarch diet produced a lower Shannon-Wiener index and antibiotics increased the Shannon-Wiener index (Table 12).

Table 12.

Means of diversity measures of OTUs for Day 28.

| Diet | AB | Shannon-Wiener | Effective Number of OTUs | Pielou's Evenness |
|------------|-------------|----------------|--------------------------|-------------------|
| Cornstarch | Control | 4.95 | 143.91 | 0.9818 |
| | Antibiotics | 5.80 | 350.39 | 0.9906 |
| Glucose | Control | 5.20 | 192.60 | 0.9841 |
| | Antibiotics | 5.81 | 345.61 | 0.9904 |

For the Shannon-Wiener index, both diet and antibiotics were significant but, as seen from the sums of squares, the effect of antibiotics was much larger than the effect of diet (Table 13).

Table 13.
ANOVA results of Shannon-Wiener diversity index of OTUs on Day 28, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean Square | F | P |
|-------------|--------------------|-----------------|-------------|--------|-------|
| Diet | 1 | 0.50 | 0.50 | 5.45 | 0.02 |
| Antibiotics | 1 | 11.77 | 11.77 | 128.81 | <0.01 |
| Diet:AB | 1 | 0.28 | 0.28 | 3.10 | 0.08 |
| Residuals | 83 | 7.59 | 0.09 | | |

Essentially the same result is seen for the effective number of OTUs (Table 12), except in this case the effect of diet was not significant (Table 14).

Table 14.
ANOVA results of effective number of OTUs on Day 28, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean Square | F | P-values |
|-------------|--------------------|-----------------|-------------|--------|----------|
| Diet | 1 | 15827 | 15827 | 2.99 | 0.09 |
| Antibiotics | 1 | 707963 | 707963 | 133.71 | <0.01 |
| Diet:AB | 1 | 14454 | 14454 | 2.73 | 0.10 |
| Residuals | 83 | 439464 | 5295 | | |

There was a significant interaction between diet and antibiotics for Pielou's evenness index (Table 15) such that the positive effect of antibiotics on evenness was greater in the cornstarch diet than in the glucose diet as can be appreciated from the means (Table 12) as well as from the sums of squares (Tables 16, 17).

Table 15.

ANOVA results of Pielou's evenness index of OTUs on Day 28, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean Square | F | P |
|-------------|--------------------|-----------------------|-----------------------|--------|-------|
| Diet | 1 | 3.66×10^{-5} | 3.66×10^{-5} | 6.22 | 0.01 |
| Antibiotics | 1 | 1.27×10^{-3} | 1.27×10^{-3} | 215.30 | <0.01 |
| Diet:AB | 1 | 3.27×10^{-5} | 3.27×10^{-5} | 5.56 | 0.02 |
| Residuals | 83 | 4.88×10^{-4} | 5.88×10^{-6} | | |

Table 16.

ANOVA results of Pielou's evenness index of OTUs on Day 28, excluding mixed pairs. Analysis includes only mice on the cornstarch diet.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Value | P-values |
|-------------|--------------------|-----------------------|-----------------------|----------|----------|
| Antibiotics | 1 | 9.68×10^{-4} | 9.68×10^{-4} | 204.7 | <0.01 |
| Residuals | 49 | 2.32×10^{-4} | 4.73×10^{-6} | | |

Table 17.

ANOVA results of Pielou's evenness index of OTUs on Day 28, excluding mixed pairs. Analysis includes only mice on the glucose diet.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Value | P-values |
|-------------|--------------------|-----------------------|-----------------------|----------|----------|
| Antibiotics | 1 | 3.40×10^{-4} | 3.40×10^{-4} | 45.12 | <0.01 |
| Residuals | 34 | 2.57×10^{-4} | 7.54×10^{-6} | | |

As seen from the means and 95% confidence intervals, the Chao2 richness was significantly affected by both diet and antibiotics (Table 18). Antibiotics increased Chao2, but the effect was larger in the cornstarch diet than in the glucose diet (Table 18). Antibiotics produced higher species richness values at both the individual sample level as well as at the treatment group level (Table 19).

Table 18.

Mean (95% confidence interval) Chao2 richness.

| Diet | AB | Mean | 95% CI |
|------------|-------------|------|-----------------|
| Cornstarch | Control | 666 | 662.5 – 669.3 |
| | Antibiotics | 1233 | 1229.8 – 1236.4 |
| Glucose | Control | 884 | 881.1 – 886.5 |
| | Antibiotics | 1037 | 1025.4 – 1049.2 |

Table 19.

Means of species richness for Day 28 data by treatment.

| Diet | Treatment | Total Richness | | Sample Richness | |
|------------|-------------|----------------|---|-----------------|---|
| Cornstarch | Control | 667 | A | 139 | A |
| | Antibiotics | 1247 | B | 396 | B |
| Glucose | Control | 885 | A | 292 | A |
| | Antibiotics | 1100 | B | 329 | B |

Non-random interactions among OTUs were documented by calculating C-scores. Co-occurrence of OTUs was greater than expected by chance in the mice given antibiotics and fed a glucose diet (Table 20).

Table 20.

C-scores of bacterial communities comprising OTUs >1% of Hellinger-transformed abundances on Day 28.

| Diet | AB | Observed | Simulated | Standardized Effect Size | P-value |
|------------|-------------|----------|-----------|--------------------------|---------|
| Cornstarch | Control | 2.96 | 3.48 | -1.13 | 0.12 |
| | Antibiotics | 1.39 | 1.35 | 0.34 | 0.42 |
| Glucose | Control | 3.38 | 3.43 | -0.12 | 0.43 |
| | Antibiotics | 0.75 | 1.39 | -3.42 | <0.01 |

Discussion

As I hypothesized, both diet and antibiotics influenced the bacterial communities in the mouse colon as determined on Day 28, the end of the 14-day period of antibiotic treatment. In general, the effect of antibiotics was much larger than the effect of diet, and there were frequent interactions between diet and antibiotics such that the effect of antibiotics was larger in mice receiving the cornstarch diet compared to those receiving the glucose diet. The one exception to this was the non-random associations as indicated by C-scores, for which significant non-randomness was found only in the antibiotic-treated mice given the glucose diet.

Antibiotics significantly affected the relative abundances of many phyla. Here I focus on three of the most abundant phyla: Firmicutes, Bacteroidetes, and Proteobacteria. In general, antibiotics created a dysbiotic, potentially disease-prone state in the antibiotic mice. The Firmicutes to Bacteroidetes ratio is often cited as an indicator for obesity: the higher the ratio, the more likely it will be associated with obesity (Abdallah Ismail et al. 2011). In this study, antibiotics resulted in a decrease in the abundance of Firmicutes and increase in abundance of Bacteroidetes, suggesting that antibiotics did not result in this form of dysbiosis. The effect of diet was relatively small compared to the effect of antibiotics. The Firmicutes to Bacteroidetes ratio was 2.9 in the mice given the cornstarch diet, and 2.6 in the mice given the glucose diet. However, there are many conflicting reports that suggest that dysbiosis cannot be characterized simply as an altered Firmicutes to Bacteroidetes ratio (Ley et al. 2006, Duncan et al. 2008, Turnbaugh et al. 2009). For example, reductions in Firmicutes has been associated with Crohn's disease (Manichanh et al. 2006). Moreover, Proteobacteria increased significantly with antibiotics, and an increase in the abundance of this phylum is considered a key indicator of a dysbiotic microbiome that could indicate increased susceptibility to disease (Shin et al. 2015),

including inflammatory bowel disease and Crohn's disease (Lepage et al. 2011, Mondot et al. 2011). In some cases, a proliferation of Proteobacteria can induce colitis which may persist even after elimination of the bacteria (Carvalho et al. 2012).

Changes due to antibiotics in the Firmicutes in mice given both diets can be attributed to the decrease in relative abundances of *Blautia*, *Erysipelatoclostridium*, *Eubacterium nodatum*, and *Ruminoclostridium*. However, the microbiomes of mice given the cornstarch diet had three additional significant decreases in the Firmicutes OTUs than the glucose, including Lactobacillales, *Lactobacillus*, and *Peptoclostridium*. This supports the finding that the effect of antibiotics was larger in the cornstarch mice than in the glucose mice.

Changes in the Bacteroidetes in both diets can be attributed to increases in relative abundances of Bacteroides-1 and Bacteroides-2. However, there was a large decrease in the BacteroidalesS24.7. This suggests that while the most abundant OTU in the Bacteroides decreased, the OTUs in lower abundance increased to create an overall increase at the phylum level. This could be the result of an uneven community becoming more even through the effect of antibiotics.

Changes in the Proteobacteria in both diets can be attributed to an increase in *Buttiauxella*, and *Escherichia/Shigella*.

Antibiotics resulted in greater diversity in the microbiomes of mice given either diet, stemming from both an increase in evenness and richness. The increase in evenness could have resulted from a release from competition among the less abundant OTUs when the most abundant OTUs were impacted by the antibiotics. Thus, antibiotics may have leveled the playing field, allowing for a slight reorganization of the hierarchical structure (Connell 1978, Lozupone et al. 2012). The increase in species richness may be explained in two ways. Firstly, singletons,

doubleton, and tripletons were excluded from all analyses. The reduction in relative abundance of the top OTUs may have allowed the rarer OTUs to be detected during sequencing, where before they were vastly outnumbered. Secondly, it is possible that the abundances of these rare OTUs increased as a result of the competitive release caused by antibiotics. Because of the increase in the abundance (read numbers) of these rare OTUs they were no longer excluded from the analysis, resulting in an inflation of richness.

These results indicate that antibiotics did strongly perturb the colonic microbiome and thus could be used as a tool to achieve the overall objective of the study, which was to determine how the microbiome responds to, and recovers from, disturbance. In addition, these results indicate that diet also sufficiently affected the colonic microbiomes of the mice. Because these tools have been shown to be effective, I will now explore the changes in these communities over time, and how that is affected by pairing.

Chapter 2

Introduction

In this chapter I ask whether dysbiotic microbiomes recover over time to the pre-dysbiotic state. I hypothesize that, over time, the dysbiotic microbiome of the antibiotic mice will converge with the intact microbiome of the control mice. I compare control and antibiotic mice in self-pairs (C-c and A-a) across all time points.

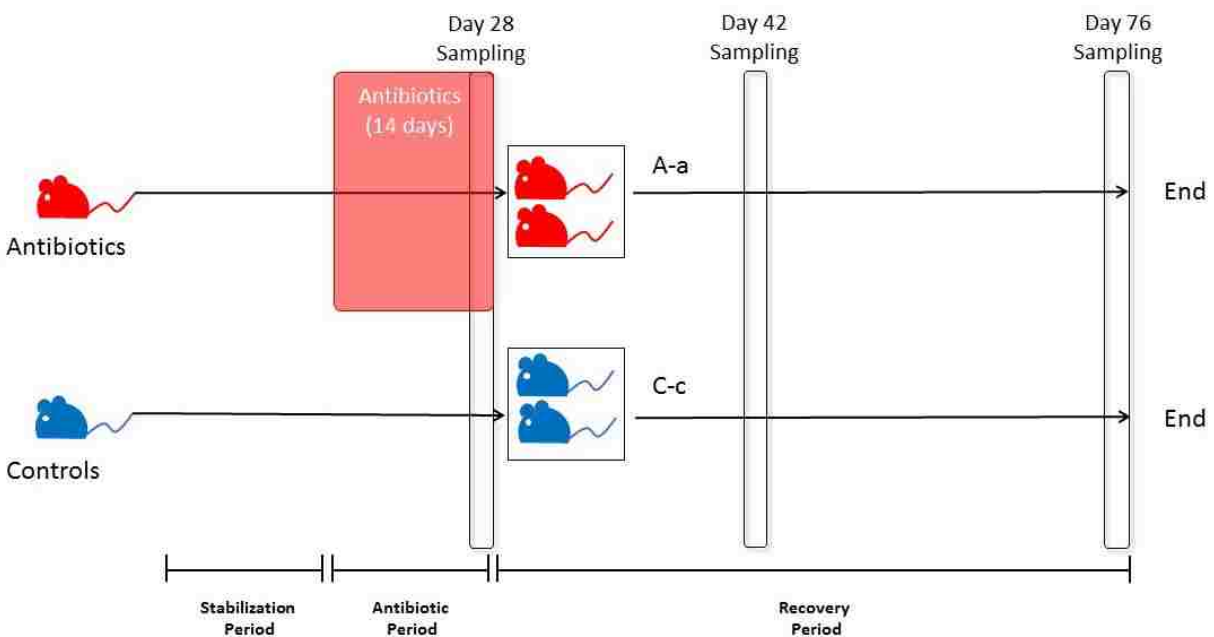


Figure 1.

Timeline of chapter 1. The red mouse represents an antibiotic mouse, while the blue mouse represents a control mouse. At the end of the antibiotic period, mice are paired within their respective diets. This chapter uses data for self-paired control and antibiotic mice at all sampling points.

Results

From Day 28, when the antibiotic treatment was terminated, the structure of the bacterial communities based on OTUs continued to change from Day 28 to Day 42 and again from Day 42 to Day 76. This was true for mice on both the cornstarch diet (Table 1) and the glucose diet (Table 2).

Table 1.
PerMANOVA results across time at the OTU level, cornstarch diet, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Model | R ² | P-values |
|-------------|--------------------|-----------------|-----------------|----------|----------------|----------|
| Antibiotics | 1 | 5.71 | 5.71 | 53.94 | 0.28 | <0.01 |
| Time | 2 | 3.88 | 1.94 | 18.39 | 0.19 | <0.01 |
| AB:Time | 2 | 2.09 | 1.05 | 9.92 | 0.10 | <0.01 |
| Residuals | 85 | 8.98 | 0.11 | | 0.43 | |
| Total | 90 | 20.65 | | | 1.00 | |

Table 2.
PerMANOVA results across time at the OTU level, glucose diet, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Model | R ² | P-values |
|-------------|--------------------|-----------------|-----------------|----------|----------------|----------|
| Antibiotics | 1 | 3.85 | 3.85 | 37.05 | 0.22 | <0.01 |
| Time | 2 | 4.54 | 2.27 | 21.80 | 0.26 | <0.01 |
| AB:Time | 2 | 1.77 | 0.89 | 8.53 | 0.10 | <0.01 |
| Residuals | 70 | 7.28 | 0.10 | | 0.42 | |
| Total | 75 | 17.45 | | | 1.00 | |

This effect of time and the interaction between time and antibiotics can be appreciated from the ordinations, which show that while the samples from the control mice (no antibiotics) remained relatively tightly clustered despite the passage of time, the samples from the mice previously given antibiotics changed with time but remained distinct from the controls (Figures 2, 3). For both diets, the distances between communities of control mice and of mice that had

received antibiotics were relatively small at Day 28, large at Day 42, and relatively small again at Day 76.

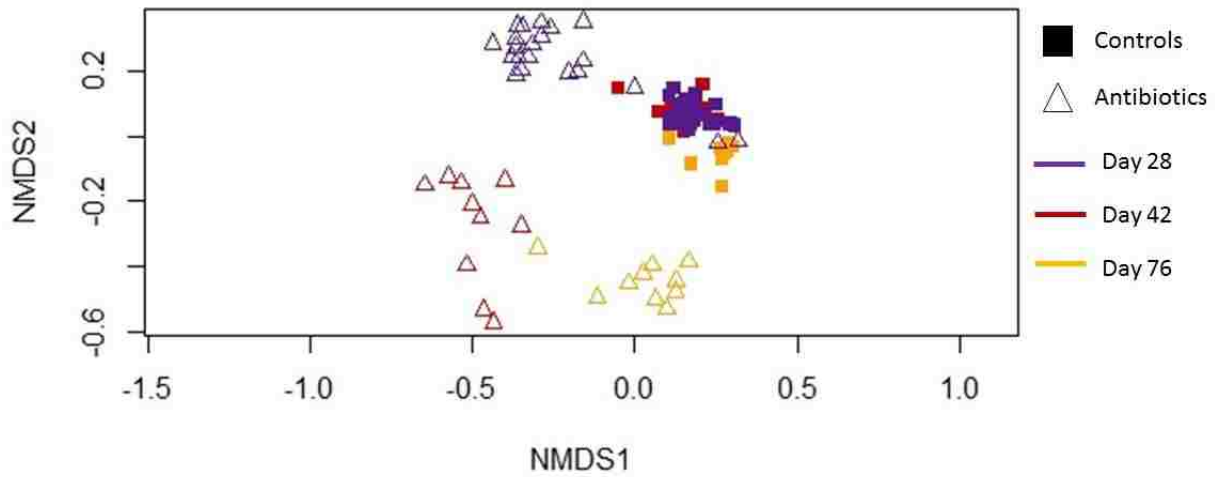


Figure 2. Ordination of colonic bacterial community structure based on the OTUs of C-c mice and A-a mice given the cornstarch diet on all sampling days.

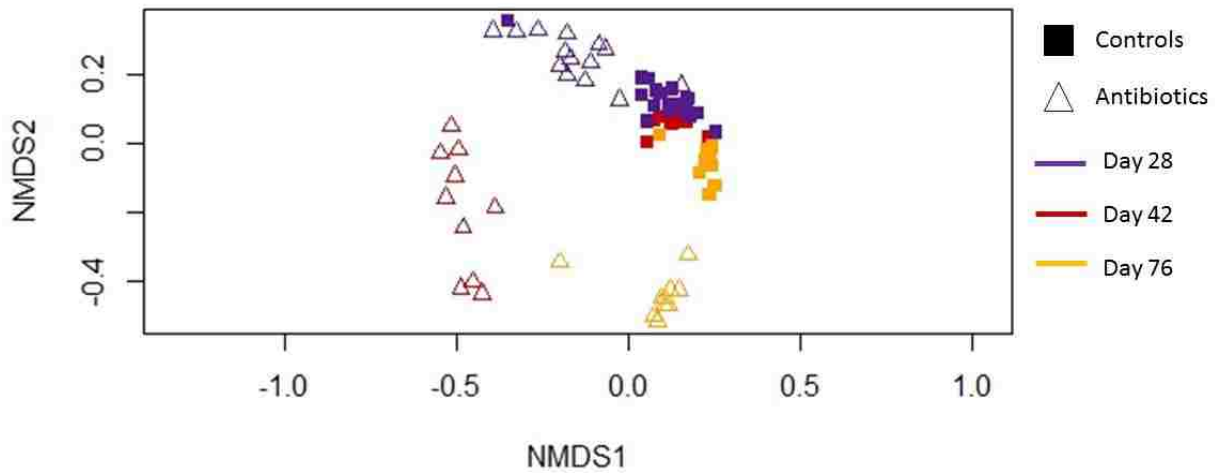


Figure 3. Ordination of colonic bacterial community structure based on the OTUs of C-c mice and A-a mice given the glucose diet on all sampling days.

Similar results were obtained when the data were analyzed at the level of phylum (ordinations not shown). Again, from Day 28 upon cessation of the antibiotic treatment, the structure of the bacterial communities based on phyla continued to change at Day 42 and Day 76. This was true for mice on both the cornstarch diet (Table 3) and the glucose diet (Table 4).

Table 3.
PerMANOVA results across time at the phylum level, cornstarch diet, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Model | R ² | P-values |
|-------------|--------------------|-----------------|-----------------|----------|----------------|----------|
| Antibiotics | 1 | 1.72 | 1.72 | 130.06 | 0.42 | <0.01 |
| Time | 2 | 0.61 | 0.30 | 23.03 | 0.15 | <0.01 |
| AB:Time | 2 | 0.67 | 0.33 | 25.34 | 0.16 | <0.01 |
| Residuals | 85 | 1.12 | 0.01 | | 0.27 | |
| Total | 90 | 4.12 | | | 1.00 | |

Table 4.
PerMANOVA results across time at the phylum level, glucose diet, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Model | R ² | P-values |
|-------------|--------------------|-----------------|-----------------|----------|----------------|----------|
| Antibiotics | 1 | 0.97 | 0.97 | 99.49 | 0.36 | <0.01 |
| Time | 2 | 0.55 | 0.27 | 28.14 | 0.20 | <0.01 |
| AB:Time | 2 | 0.51 | 0.25 | 26.17 | 0.19 | <0.01 |
| Residuals | 70 | 0.68 | 0.01 | | 0.25 | |
| Total | 75 | 2.71 | | | 1.00 | |

Irrespective of time, antibiotics had a significant effect on bacterial community structure for mice given both the cornstarch diet and the glucose diet, at both levels of bacterial OTUs (Table 5) and phyla (Table 6).

Table 5.

Summary of separate PerMANOVA models for each time x diet combination at the OTU level, excluding mixed pairs.

| Groups | Factor | Degrees Freedom | Sum of Squares | Mean of Squares | F Model | R ² | P-Value | |
|--------|--------|--------------------|-------------------|--------------------|---------|----------------|---------|-------|
| Day 28 | CS | Antibiotics | 1 | 3.84 | 3.84 | 37.6 | 0.43 | <0.01 |
| | | Residuals | 49 | 5.00 | 0.10 | | 0.67 | |
| | | Total | 50 | 8.84 | | | 1.00 | |
| | GL | Antibiotics | 1 | 1.45 | 1.45 | 13.11 | 0.28 | <0.01 |
| | | Residuals | 34 | 3.77 | 0.11 | | 0.72 | |
| | | Total | 35 | 5.22 | | | 1.00 | |
| Day 42 | CS | Antibiotics | 1 | 2.35 | 2.35 | 17.00 | 0.49 | <0.01 |
| | | Residuals | 18 | 2.48 | 0.14 | | 0.51 | |
| | | Total | 19 | 4.83 | | | 1.00 | |
| | GL | Antibiotics | 1 | 2.45 | 2.45 | 18.42 | 0.51 | <0.01 |
| | | Residuals | 18 | 2.40 | 0.13 | | 0.49 | |
| | | Total | 19 | 4.85 | | | 1.00 | |
| Day 76 | CS | Antibiotics | 1 | 1.51 | 1.51 | 18.23 | 0.50 | <0.01 |
| | | Residuals | 18 | 1.49 | 0.08 | | 0.50 | |
| | | Total | 19 | 3.00 | | | 1.00 | |
| | GL | Antibiotics | 1 | 1.55 | 1.55 | 25.04 | 0.58 | <0.01 |
| | | Residuals | 18 | 1.12 | 0.06 | | 0.42 | |
| | | Total | 19 | 2.67 | | | 1.00 | |

Table 6.

Summary of separate PerMANOVA models for each time x diet combination at the phylum level, excluding mixed pairs.

| Groups | Factor | Degrees Freedom | Sum of Squares | Mean of Squares | F Model | R ² | P-Value |
|--------|----------------|-----------------|----------------|-----------------|---------|----------------|---------|
| Day 28 | CS Antibiotics | 1 | 1.47 | 1.47 | 102.45 | 0.68 | <0.01 |
| | Residuals | 49 | 0.70 | 0.01 | | 0.32 | |
| | Total | 50 | 2.18 | | | 1.00 | |
| | GL Antibiotics | 1 | 0.57 | 0.57 | 39.24 | 0.54 | <0.01 |
| | Residuals | 34 | 0.49 | 0.01 | | 0.46 | |
| | Total | 35 | 1.06 | | | 1.00 | |
| Day 42 | CS Antibiotics | 1 | 0.83 | 0.83 | 61.81 | 0.77 | <0.01 |
| | Residuals | 18 | 0.24 | 0.01 | | 0.23 | |
| | Total | 19 | 1.08 | | | 1.00 | |
| | GL Antibiotics | 1 | 0.84 | 0.84 | 105.63 | 0.85 | <0.01 |
| | Residuals | 18 | 0.14 | 0.01 | | 0.15 | |
| | Total | 19 | 0.98 | | | 1.00 | |
| Day 76 | CS Antibiotics | 1 | 0.07 | 0.07 | 6.89 | 0.28 | <0.01 |
| | Residuals | 18 | 0.18 | 0.01 | | 0.72 | |
| | Total | 19 | 0.24 | | | 1.00 | |
| | GL Antibiotics | 1 | 0.04 | 0.04 | 15.06 | 0.46 | <0.01 |
| | Residuals | 18 | 0.05 | 0.00 | | 0.54 | |
| | Total | 19 | 0.09 | | | 1.00 | |

The interactions between antibiotics and time with respect to overall community structure can be explained by the analysis of individual OTUs and individual phyla for each diet (Tables 7-10). For example, for the mice given the glucose diet, the abundance of the OTU BacteroidalesS24.7 (and several other OTUs) was much higher in the control compared to the antibiotic mice at Day 28, but this difference declined with time (Table 7). The same was true of OTUs in mice given the cornstarch diet (Table 8) and of prokaryote phyla in mice given either diet (Tables 9, 10). One notable exception to this was the OTU *Escherichia/Shigella*, for which the difference between antibiotics remained large throughout the time course (Tables 7, 8). Because that OTU is a member of the Proteobacteria, the same pattern of non-diminishing

difference between antibiotic and control mice is evident for that phylum in mice given either diet (Tables 9, 10).

Table 7.

Means of Hellinger-transformed, read numbers, and R² and P values from the ANOVAs for the OTUs contributing more than 1% of the community. Glucose diet.

| OTU | Antibiotics | Day | | | R ² & P | | | |
|-----------------------------------|-------------|--------|--------|--------|--------------------|--------|----------|----------|
| | | 28 | 42 | 76 | Antibiotics | Day | Day x AB | Residual |
| BacteroidalesS24.7 | C | 0.3727 | 0.2199 | 0.3811 | 0.3674 | 0.2488 | 0.1122 | 0.2715 |
| | AB | 0.0079 | 0.0478 | 0.2913 | 0.0000 | 0.0000 | 0.0000 | |
| <i>Blautia</i> | C | 0.2648 | 0.2022 | 0.2762 | 0.3740 | 0.1146 | 0.1210 | 0.3903 |
| | AB | 0.0018 | 0.1369 | 0.1814 | 0.0000 | 0.0002 | 0.0001 | |
| <i>Buttiauxella</i> | C | 0.0253 | 0.0018 | 0.0120 | 0.1953 | 0.3480 | 0.3166 | 0.1400 |
| | AB | 0.5363 | 0.0032 | 0.0539 | 0.0000 | 0.0000 | 0.0000 | |
| Lactobacillales | C | 0.1425 | 0.1706 | 0.0740 | 0.0155 | 0.3645 | 0.0540 | 0.5660 |
| | AB | 0.1665 | 0.1022 | 0.0387 | 0.1825 | 0.0000 | 0.0475 | |
| <i>Erysipelatoclostridium</i> | C | 0.1312 | 0.0901 | 0.1109 | 0.0378 | 0.3285 | 0.2774 | 0.3562 |
| | AB | 0.2821 | 0.1443 | 0.0455 | 0.0104 | 0.0000 | 0.0000 | |
| <i>Escherichia/Shigella</i> | C | 0.0000 | 0.1650 | 0.0084 | 0.3444 | 0.0773 | 0.0174 | 0.5609 |
| | AB | 0.2190 | 0.2900 | 0.2435 | 0.0000 | 0.0133 | 0.3587 | |
| Bacteroides1 | C | 0.0000 | 0.4185 | 0.0020 | 0.0001 | 0.8493 | 0.0128 | 0.1378 |
| | AB | 0.0000 | 0.3340 | 0.0128 | 0.8746 | 0.0000 | 0.0506 | |
| Coriobacteriaceae | C | 0.1506 | 0.1849 | 0.0699 | 0.0756 | 0.3092 | 0.1716 | 0.4435 |
| | AB | 0.0018 | 0.1466 | 0.0749 | 0.0013 | 0.0000 | 0.0000 | |
| <i>Lactobacillus</i> | C | 0.2121 | 0.2325 | 0.0915 | 0.3072 | 0.0979 | 0.1981 | 0.3968 |
| | AB | 0.0037 | 0.0547 | 0.0710 | 0.0000 | 0.0006 | 0.0000 | |
| Bacteroides2 | C | 0.0000 | 0.3201 | 0.0044 | 0.0358 | 0.8854 | 0.0356 | 0.0433 |
| | AB | 0.0000 | 0.4845 | 0.0145 | 0.0000 | 0.0000 | 0.0000 | |
| <i>Eubacterium nodatum</i> | C | 0.2802 | 0.1965 | 0.1927 | 0.6057 | 0.0009 | 0.1643 | 0.2291 |
| | AB | 0.0055 | 0.0915 | 0.0940 | 0.0000 | 0.8746 | 0.0000 | |
| <i>Clostridium sensu stricto1</i> | C | 0.0106 | 0.0073 | 0.0064 | 0.2119 | 0.0818 | 0.0903 | 0.6159 |
| | AB | 0.1825 | 0.1250 | 0.0314 | 0.0000 | 0.0151 | 0.0104 | |
| <i>Peptoclostridium</i> | C | 0.1537 | 0.1424 | 0.0791 | 0.3175 | 0.0928 | 0.1378 | 0.4519 |
| | AB | 0.0175 | 0.0635 | 0.0505 | 0.0000 | 0.0019 | 0.0001 | |
| <i>Clostridium sensu stricto2</i> | C | 0.0055 | 0.0037 | 0.0053 | 0.1370 | 0.1601 | 0.1612 | 0.5417 |
| | AB | 0.1859 | 0.0032 | 0.0379 | 0.0001 | 0.0002 | 0.0002 | |
| <i>Ruminiclostridium</i> | C | 0.1803 | 0.1494 | 0.2122 | 0.5383 | 0.1320 | 0.0275 | 0.3022 |
| | AB | 0.0026 | 0.0291 | 0.1085 | 0.0000 | 0.0000 | 0.0519 | |

Table 8.

Means of Hellinger-transformed, read numbers, and R² and P values from the ANOVAs for the OTUs contributing more than 1% of the community. Cornstarch diet.

| OTU | Antibiotics | Day | | | R ² & P | | | |
|------------------------------------|-------------|--------|--------|--------|--------------------|--------|----------|----------|
| | | 28 | 42 | 76 | Antibiotics | Day | Day x AB | Residual |
| BacteroidalesS24.7 | C | 0.4112 | 0.2477 | 0.3854 | 0.5213 | 0.0847 | 0.0849 | 0.3091 |
| | AB | 0.0062 | 0.1034 | 0.1928 | 0.0000 | 0.0001 | 0.0001 | |
| <i>Blautia</i> | C | 0.2584 | 0.2168 | 0.2786 | 0.4418 | 0.0518 | 0.0493 | 0.4570 |
| | AB | 0.0018 | 0.1248 | 0.1254 | 0.0000 | 0.0156 | 0.0181 | |
| <i>Buttiauxella</i> | C | 0.0259 | 0.0000 | 0.0099 | 0.1903 | 0.3983 | 0.3619 | 0.0494 |
| | AB | 0.6184 | 0.0044 | 0.0546 | 0.0000 | 0.0000 | 0.0000 | |
| Lactobacillales | C | 0.1900 | 0.1649 | 0.1827 | 0.0726 | 0.1107 | 0.0951 | 0.7216 |
| | AB | 0.2380 | 0.1165 | 0.0843 | 0.0071 | 0.0040 | 0.0081 | |
| <i>Erysipelatoclostridium</i> | C | 0.0846 | 0.0828 | 0.0963 | 0.0796 | 0.2933 | 0.4126 | 0.2145 |
| | AB | 0.3100 | 0.1697 | 0.0460 | 0.0000 | 0.0000 | 0.0000 | |
| <i>Escherichia/Shigella</i> | C | 0.0055 | 0.1304 | 0.0051 | 0.5203 | 0.0980 | 0.1445 | 0.2372 |
| | AB | 0.1007 | 0.2420 | 0.3627 | 0.0000 | 0.0000 | 0.0000 | |
| Bacteroides1 | C | 0.0018 | 0.3662 | 0.0033 | 0.0038 | 0.7501 | 0.0072 | 0.2389 |
| | AB | 0.0000 | 0.3261 | 0.0311 | 0.2717 | 0.0000 | 0.2889 | |
| Coriobacteriaceae | C | 0.2711 | 0.2074 | 0.2856 | 0.4243 | 0.0368 | 0.0723 | 0.4666 |
| | AB | 0.0037 | 0.1511 | 0.1144 | 0.0000 | 0.0511 | 0.0040 | |
| <i>Lactobacillus</i> | C | 0.2007 | 0.1983 | 0.2583 | 0.4814 | 0.0899 | 0.0335 | 0.3953 |
| | AB | 0.0000 | 0.1167 | 0.1063 | 0.0000 | 0.0003 | 0.0418 | |
| Bacteroides2 | C | 0.0018 | 0.2227 | 0.0030 | 0.0465 | 0.5300 | 0.0134 | 0.4100 |
| | AB | 0.0692 | 0.3253 | 0.0248 | 0.0043 | 0.0000 | 0.2727 | |
| <i>Eubacterium nodatum</i> | C | 0.2310 | 0.1705 | 0.2012 | 0.5869 | 0.0012 | 0.0728 | 0.3392 |
| | AB | 0.0307 | 0.1089 | 0.0698 | 0.0000 | 0.8602 | 0.0005 | |
| <i>Clostridium sensu stricto</i> 1 | C | 0.0076 | 0.0018 | 0.0054 | 0.1457 | 0.0541 | 0.0699 | 0.7303 |
| | AB | 0.0730 | 0.1100 | 0.0249 | 0.0002 | 0.0584 | 0.0280 | |
| <i>Peptoclostridium</i> | C | 0.1085 | 0.1046 | 0.1293 | 0.5241 | 0.0320 | 0.0215 | 0.4224 |
| | AB | 0.0172 | 0.0562 | 0.0446 | 0.0000 | 0.0563 | 0.1366 | |
| <i>Clostridium sensu stricto</i> 2 | C | 0.1500 | 0.1078 | 0.1542 | 0.1600 | 0.0800 | 0.0234 | 0.7366 |
| | AB | 0.1297 | 0.0167 | 0.0660 | 0.0001 | 0.0181 | 0.2768 | |
| <i>Ruminiclostridium</i> | C | 0.1486 | 0.1239 | 0.1442 | 0.6604 | 0.0190 | 0.0215 | 0.2991 |
| | AB | 0.0055 | 0.0286 | 0.0478 | 0.0000 | 0.0845 | 0.0623 | |

Table 9.

Means of Hellinger-transformed, read numbers, and R² and P values from the ANOVAs for phyla. Glucose diet.

| Phylum | Antibiotics | Day | | | R ² & P | | | |
|-----------------------------------|-------------|--------|--------|--------|--------------------|--------|----------|----------|
| | | 28 | 42 | 76 | Antibiotics | Day | Day x AB | Residual |
| Archaea Euryarchaeota | C | 0.0003 | 0.0000 | 0.0008 | 0.0070 | 0.1290 | 0.0193 | 0.8446 |
| | AB | 0.0002 | 0.0000 | 0.0016 | 0.4990 | 0.0141 | 0.4990 | |
| Archaea Thaumarchaeota | C | 0.0000 | 0.0000 | 0.0000 | | | | |
| | AB | 0.0000 | 0.0000 | 0.0000 | | | | |
| Bacteria Acidobacteria | C | 0.0000 | 0.0000 | 0.0000 | 0.0165 | 0.0187 | 0.0238 | 0.9410 |
| | AB | 0.0000 | 0.0000 | 0.0002 | 0.3340 | 0.5310 | 0.4845 | |
| Bacteria Actinobacteria | C | 0.0323 | 0.0407 | 0.0089 | 0.0269 | 0.1885 | 0.1422 | 0.6424 |
| | AB | 0.0000 | 0.0271 | 0.0166 | 0.1299 | 0.0004 | 0.0027 | |
| Bacteria Bacteroidetes | C | 0.2531 | 0.3842 | 0.2682 | 0.0002 | 0.4282 | 0.2521 | 0.3196 |
| | AB | 0.0510 | 0.3919 | 0.4025 | 0.8399 | 0.0000 | 0.0000 | |
| Bacteria Chloroflexi | C | 0.0000 | 0.0000 | 0.0002 | 0.0189 | 0.1247 | 0.0361 | 0.8203 |
| | AB | 0.0000 | 0.0000 | 0.0007 | 0.2892 | 0.0141 | 0.2985 | |
| Bacteria Cyanobacteria | C | 0.0001 | 0.0000 | 0.0004 | 0.1273 | 0.1858 | 0.1934 | 0.4935 |
| | AB | 0.0000 | 0.0000 | 0.0075 | 0.0003 | 0.0001 | 0.0001 | |
| Bacteria Deinococcus-Thermus | C | 0.0000 | 0.0000 | 0.0000 | | | | |
| | AB | 0.0000 | 0.0000 | 0.0000 | | | | |
| Bacteria Elusimicrobia | C | 0.0000 | 0.0000 | 0.0000 | 0.0022 | 0.0239 | 0.0164 | 0.9574 |
| | AB | 0.0000 | 0.0000 | 0.0001 | 0.7009 | 0.4845 | 0.5729 | |
| Bacteria Fibrobacteres | C | 0.0004 | 0.0000 | 0.0011 | 0.0174 | 0.1275 | 0.0340 | 0.8210 |
| | AB | 0.0004 | 0.0000 | 0.0027 | 0.2985 | 0.0141 | 0.3103 | |
| Bacteria Firmicutes | C | 0.7052 | 0.5333 | 0.7019 | 0.5145 | 0.0441 | 0.0994 | 0.3420 |
| | AB | 0.4735 | 0.4545 | 0.3678 | 0.0000 | 0.0267 | 0.0004 | |
| Bacteria Fusobacteria | C | 0.0000 | 0.0000 | 0.0000 | 0.0791 | 0.0900 | 0.1145 | 0.7164 |
| | AB | 0.0000 | 0.0000 | 0.0010 | 0.0141 | 0.0277 | 0.0141 | |
| Bacteria Lentisphaerae | C | 0.0000 | 0.0000 | 0.0000 | 0.0165 | 0.0187 | 0.0238 | 0.9410 |
| | AB | 0.0000 | 0.0000 | 0.0000 | 0.3340 | 0.5310 | 0.4845 | |
| Bacteria Nitrospirae | C | 0.0000 | 0.0000 | 0.0000 | | | | |
| | AB | 0.0000 | 0.0000 | 0.0000 | | | | |
| Bacteria Planctomycetes | C | 0.0001 | 0.0000 | 0.0005 | 0.0394 | 0.1194 | 0.0621 | 0.7791 |
| | AB | 0.0001 | 0.0000 | 0.0021 | 0.0960 | 0.0141 | 0.0996 | |
| Bacteria Proteobacteria | C | 0.0049 | 0.0402 | 0.0117 | 0.4967 | 0.1575 | 0.2137 | 0.1321 |
| | AB | 0.4734 | 0.1262 | 0.1720 | 0.0000 | 0.0000 | 0.0000 | |
| Bacteria SR1 (Absconditabacteria) | C | 0.0002 | 0.0000 | 0.0005 | 0.0860 | 0.2246 | 0.1236 | 0.5658 |
| | AB | 0.0003 | 0.0000 | 0.0024 | 0.0046 | 0.0000 | 0.0028 | |
| Bacteria Spirochaetae | C | 0.0004 | 0.0001 | 0.0028 | 0.1225 | 0.2900 | 0.1898 | 0.3976 |
| | AB | 0.0006 | 0.0001 | 0.0175 | 0.0001 | 0.0000 | 0.0000 | |
| Bacteria Synergistetes | C | 0.0001 | 0.0000 | 0.0002 | 0.0609 | 0.1162 | 0.0867 | 0.7362 |
| | AB | 0.0001 | 0.0000 | 0.0011 | 0.0316 | 0.0141 | 0.0332 | |
| Bacteria Tenericutes | C | 0.0027 | 0.0014 | 0.0022 | 0.1241 | 0.0964 | 0.0776 | 0.7019 |
| | AB | 0.0002 | 0.0000 | 0.0021 | 0.0024 | 0.0213 | 0.0392 | |
| Bacteria Verrucomicrobia | C | 0.0001 | 0.0000 | 0.0005 | 0.0506 | 0.2030 | 0.0833 | 0.6631 |
| | AB | 0.0001 | 0.0000 | 0.0020 | 0.0378 | 0.0003 | 0.0277 | |

Table 10.

Means of Hellinger-transformed, read numbers, and R² and P values from the ANOVAs for phyla. Cornstarch diet.

| Phylum | Antibiotics | Day | | | R ² & P | | | |
|--------------------------------------|-------------|--------|--------|--------|--------------------|--------|----------|----------|
| | | 28 | 42 | 76 | Antibiotics | Day | Day x AB | Residual |
| Archaea Euryarchaeota | C | 0.0001 | 0.0000 | 0.0001 | 0.1218 | 0.0947 | 0.1002 | 0.6833 |
| | AB | 0.0003 | 0.0000 | 0.0030 | 0.0005 | 0.0077 | 0.0063 | |
| Archaea Thaumarchaeota | C | 0.0000 | 0.0000 | 0.0000 | 0.0136 | 0.0108 | 0.0127 | 0.9630 |
| | AB | 0.0000 | 0.0000 | 0.0004 | 0.3424 | 0.6321 | 0.6120 | |
| Bacteria Acidobacteria | C | 0.0000 | 0.0000 | 0.0000 | 0.0196 | 0.0157 | 0.0183 | 0.9464 |
| | AB | 0.0000 | 0.0000 | 0.0013 | 0.2521 | 0.5705 | 0.5162 | |
| Bacteria Actinobacteria | C | 0.0977 | 0.0857 | 0.0919 | 0.3331 | 0.0093 | 0.0260 | 0.6317 |
| | AB | 0.0001 | 0.0400 | 0.0324 | 0.0000 | 0.6062 | 0.2474 | |
| Bacteria Bacteroidetes | C | 0.2720 | 0.4530 | 0.2278 | 0.0123 | 0.3280 | 0.2289 | 0.4308 |
| | AB | 0.0624 | 0.4226 | 0.3872 | 0.1723 | 0.0000 | 0.0000 | |
| Bacteria Chloroflexi | C | 0.0001 | 0.0000 | 0.0000 | 0.1063 | 0.0915 | 0.1119 | 0.6903 |
| | AB | 0.0000 | 0.0000 | 0.0011 | 0.0013 | 0.0094 | 0.0037 | |
| Bacteria Cyanobacteria | C | 0.0002 | 0.0000 | 0.0002 | 0.1615 | 0.1412 | 0.1556 | 0.5417 |
| | AB | 0.0001 | 0.0000 | 0.0080 | 0.0000 | 0.0002 | 0.0001 | |
| Bacteria Deinococcus-Thermus | C | 0.0000 | 0.0000 | 0.0000 | 0.0136 | 0.0108 | 0.0127 | 0.9630 |
| | AB | 0.0000 | 0.0000 | 0.0001 | 0.3424 | 0.6321 | 0.6120 | |
| Bacteria Elusimicrobia | C | 0.0000 | 0.0000 | 0.0000 | 0.0330 | 0.0333 | 0.0315 | 0.9022 |
| | AB | 0.0000 | 0.0000 | 0.0002 | 0.1249 | 0.2811 | 0.2997 | |
| Bacteria Fibrobacteres | C | 0.0003 | 0.0000 | 0.0002 | 0.1740 | 0.1668 | 0.1775 | 0.4818 |
| | AB | 0.0002 | 0.0000 | 0.0027 | 0.0000 | 0.0000 | 0.0000 | |
| Bacteria Firmicutes | C | 0.6192 | 0.4354 | 0.6667 | 0.3861 | 0.0535 | 0.1842 | 0.3761 |
| | AB | 0.5083 | 0.4268 | 0.2805 | 0.0000 | 0.0069 | 0.0000 | |
| Bacteria Fusobacteria | C | 0.0000 | 0.0000 | 0.0000 | 0.0585 | 0.0467 | 0.0547 | 0.8400 |
| | AB | 0.0000 | 0.0000 | 0.0002 | 0.0290 | 0.1468 | 0.1104 | |
| Bacteria Lentisphaerae | C | 0.0000 | 0.0000 | 0.0000 | 0.0416 | 0.0119 | 0.0125 | 0.9340 |
| | AB | 0.0000 | 0.0000 | 0.0000 | 0.0913 | 0.6120 | 0.6120 | |
| Bacteria Nitrospirae | C | 0.0000 | 0.0000 | 0.0000 | 0.0266 | 0.0213 | 0.0249 | 0.9272 |
| | AB | 0.0000 | 0.0000 | 0.0004 | 0.1723 | 0.4537 | 0.3929 | |
| Bacteria Planctomycetes | C | 0.0002 | 0.0000 | 0.0001 | 0.1085 | 0.0970 | 0.1139 | 0.6806 |
| | AB | 0.0001 | 0.0000 | 0.0018 | 0.0011 | 0.0069 | 0.0032 | |
| Bacteria Proteobacteria | C | 0.0074 | 0.0243 | 0.0054 | 0.5930 | 0.0947 | 0.1156 | 0.1967 |
| | AB | 0.4277 | 0.1103 | 0.2550 | 0.0000 | 0.0000 | 0.0000 | |
| Bacteria SR1 (Absconditabacteria) | C | 0.0001 | 0.0001 | 0.0001 | 0.1724 | 0.1441 | 0.1703 | 0.5131 |
| | AB | 0.0001 | 0.0000 | 0.0024 | 0.0000 | 0.0001 | 0.0000 | |
| Bacteria Spirochaetae | C | 0.0011 | 0.0001 | 0.0011 | 0.2134 | 0.2096 | 0.2152 | 0.3618 |
| | AB | 0.0005 | 0.0002 | 0.0174 | 0.0000 | 0.0000 | 0.0000 | |
| Bacteria Synergistetes | C | 0.0001 | 0.0000 | 0.0000 | 0.1202 | 0.1171 | 0.1407 | 0.6220 |
| | AB | 0.0000 | 0.0000 | 0.0009 | 0.0003 | 0.0016 | 0.0005 | |
| Bacteria Tenericutes | C | 0.0014 | 0.0013 | 0.0062 | 0.0350 | 0.0535 | 0.0061 | 0.9054 |
| | AB | 0.0001 | 0.0001 | 0.0024 | 0.1156 | 0.1308 | 0.7515 | |
| Bacteria Verrucomicrobia | C | 0.0001 | 0.0000 | 0.0001 | 0.0903 | 0.0877 | 0.0905 | 0.7315 |
| | AB | 0.0001 | 0.0000 | 0.0023 | 0.0037 | 0.0142 | 0.0126 | |

Means of the various diversity indices are given in Table 11. All three indices produced the same pattern. At Day 28, diversity increased as a consequence of antibiotics, but by Day 42 the mice administered antibiotics had lower diversity. The significance of these time x antibiotics interactions for the various diversity measures are given in Tables 12-17.

Table 11.
Means of the diversity indices.

| Groups | Factor | Shannon- Wiener | Effective Numbers | Pielou's Evenness |
|--------|-------------|--------------------|----------------------|----------------------|
| Day 28 | CS Control | 4.95 | 143.91 | 0.9817981 |
| | Antibiotics | 5.80 | 350.39 | 0.9906495 |
| | GL Control | 5.20 | 192.60 | 0.9841280 |
| | Antibiotics | 5.81 | 345.61 | 0.9904360 |
| Day 42 | CS Control | 5.04 | 158.98 | 0.9824603 |
| | Antibiotics | 3.73 | 47.95 | 0.9496097 |
| | GL Control | 5.10 | 165.70 | 0.9826814 |
| | Antibiotics | 3.83 | 55.33 | 0.9527866 |
| Day 76 | CS Control | 4.51 | 93.51 | 0.9775098 |
| | Antibiotics | 3.90 | 51.22 | 0.9693588 |
| | GL Control | 4.61 | 103.24 | 0.9791350 |
| | Antibiotics | 3.71 | 42.37 | 0.9728883 |

Table 12.
ANOVA results of Shannon-Wiener diversity index based on OTUs for mice given the cornstarch diet across all time points, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Value | P-values |
|-------------|-----------------------|--------------------|--------------------|----------|----------|
| Time | 2 | 22.96 | 11.48 | 105.57 | <0.01 |
| Antibiotics | 1 | 0.05 | 0.05 | 0.49 | 0.49 |
| Time:AB | 2 | 19.38 | 9.69 | 89.12 | <0.01 |
| Residuals | 85 | 9.24 | 0.11 | | |

Table 13.

ANOVA results of Shannon-Wiener diversity index based on OTUs for mice given the glucose diet across all time points, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Value | P-values |
|-------------|--------------------|-----------------|-----------------|----------|----------|
| Time | 2 | 23.34 | 11.67 | 88.74 | <0.01 |
| Antibiotics | 1 | 3.35 | 3.35 | 25.47 | <0.01 |
| Time:AB | 2 | 13.58 | 6.79 | 51.63 | <0.01 |
| Residuals | 70 | 9.20 | 0.13 | | |

Table 14.

ANOVA results of effective numbers of OTUs for mice given the cornstarch diet across all time points, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Value | P-values |
|-------------|--------------------|-----------------|-----------------|----------|----------|
| Time | 2 | 497302 | 248651 | 89.45 | <0.01 |
| Antibiotics | 1 | 100550 | 100550 | 36.17 | <0.01 |
| Time:AB | 2 | 454837 | 227419 | 81.814 | <0.01 |
| Residuals | 85 | 236275 | 2780 | | |

Table 15.

ANOVA results of effective numbers of OTUs for mice given the glucose diet across all time points, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Value | P-values |
|-------------|--------------------|-----------------|-----------------|----------|----------|
| Time | 2 | 512302 | 256151 | 70.66 | <0.01 |
| Antibiotics | 1 | 704 | 704 | 0.19 | 0.66 |
| Time:AB | 2 | 268664 | 134332 | 37.06 | <0.01 |
| Residuals | 70 | 253748 | 3625 | | |

Table 16.

ANOVA results of Pielou's evenness index based on OTUs for mice given the cornstarch diet across all time points, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Value | P-values |
|-------------|--------------------|-----------------------|-----------------------|----------|----------|
| Time | 2 | 5.78×10^{-3} | 2.89×10^{-3} | 58.32 | <0.01 |
| Antibiotics | 1 | 7.13×10^{-4} | 7.13×10^{-4} | 14.40 | <0.01 |
| Time:AB | 2 | 6.29×10^{-3} | 3.14×10^{-3} | 63.48 | <0.01 |
| Residuals | 85 | 4.21×10^{-3} | 4.95×10^{-5} | | |

Table 17.

ANOVA results of Pielou's evenness index based on OTUs for mice given the glucose diet across all time points, excluding mixed pairs.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Value | P-values |
|-------------|--------------------|-----------------------|-----------------------|----------|----------|
| Time | 2 | 4.33×10^{-3} | 2.16×10^{-3} | 37.32 | <0.01 |
| Antibiotics | 1 | 1.32×10^{-3} | 1.32×10^{-3} | 22.84 | <0.01 |
| Time:AB | 2 | 4.14×10^{-3} | 2.07×10^{-3} | 35.70 | <0.01 |
| Residuals | 70 | 4.06×10^{-3} | 5.80×10^{-5} | | |

Chao2 richness exhibited virtually the same pattern as the other diversity indices in that at Day 28 diversity increased as a consequence of antibiotics, but by Day 42 the mice administered antibiotics had lower diversity (Table 18).

Table 18.

Mean (95% confidence interval) of Chao2 richness.

| Groups | Factor | Chao2 | 95% CI | |
|--------|--------|-------------|--------|-----------------|
| Day 28 | CS | Control | 666 | 662.5 – 669.3 |
| | | Antibiotics | 1233 | 1299.8 – 1236.4 |
| | GL | Control | 884 | 881.1 – 886.5 |
| | | Antibiotics | 1037 | 1025.4 – 1049.2 |
| Day 42 | CS | Control | 658.1 | 653.0 - 663.2 |
| | | Antibiotics | 432.5 | 426.8 – 438.2 |
| | GL | Control | 621 | 618.8 – 623.2 |
| | | Antibiotics | 515.7 | 510.4 – 521.0 |
| Day 76 | CS | Control | 361.6 | 358.5 – 364.7 |
| | | Antibiotics | 318.0 | 310.2 – 325.8 |
| | GL | Control | 309.6 | 287.8 – 331.4 |
| | | Antibiotics | 262.5 | 250.2 – 274.8 |

Non-random interactions among OTUs were documented by calculating C-scores. Co-occurrence of OTUs was greater than expected by chance in both control and antibiotic mice given the cornstarch diet at Day 42 (Tables 19, 20), and antibiotic mice given the glucose diet at

Day 28 (Table 22). There were no significant C-scores for control mice given the glucose diet (Table 21).

Table 19.

C-scores of bacterial communities comprising OTUs >1% of Hellinger-transformed abundances for control mice on the cornstarch diet over time.

| Time | Observed Index | Simulated Index | Standardized Effect Size | P-Value |
|--------|----------------|-----------------|--------------------------|---------|
| Day 28 | 2.96 | 3.48 | -1.13 | 0.12 |
| Day 42 | 0.12 | 0.34 | -2.01 | 0.03 |
| Day 76 | 0 | 0.02 | -0.49 | 0.81 |

Table 20.

C-scores of bacterial communities comprising OTUs >1% of Hellinger-transformed abundances for mice that had received antibiotics and on the cornstarch diet over time.

| Time | Observed Index | Simulated Index | Standardized Effect Size | P-Value |
|--------|----------------|-----------------|--------------------------|---------|
| Day 28 | 1.39 | 1.35 | 0.34 | 0.42 |
| Day 42 | 1.41 | 1.92 | -3.15 | <0.01 |
| Day 76 | 0.91 | 0.92 | -0.07 | 0.46 |

Table 21.

C-scores of bacterial communities comprising OTUs >1% of Hellinger-transformed abundances for control mice on the glucose diet over time.

| Time | Observed Index | Simulated Index | Standardized Effect Size | P-Value |
|--------|----------------|-----------------|--------------------------|---------|
| Day 28 | 3.38 | 3.43 | -0.12 | 0.43 |
| Day 42 | 0.24 | 0.24 | 0.02 | 0.54 |
| Day 76 | 0.12 | 0.13 | -0.04 | 0.50 |

Table 22.

C-scores of bacterial communities comprising OTUs >1% of Hellinger-transformed abundances for mice that had received antibiotics and on the glucose diet over time.

| Time | Observed Index | Simulated Index | Standardized Effect Size | P-Value |
|--------|----------------|-----------------|--------------------------|---------|
| Day 28 | 0.75 | 1.39 | -3.42 | <0.01 |
| Day 42 | 1.58 | 1.86 | -1.10 | 0.15 |
| Day 76 | 0.99 | 0.99 | -0.13 | 0.40 |

Discussion

For both diets, the ordinations of bacterial communities indicated that the distances between communities of control mice and of mice that had received antibiotics were relatively small at Day 28, large at Day 42, and relatively small again at Day 76. This indicates that the antibiotic effect was larger 14 days following the cessation of antibiotic treatment (Day 42) than it was on the day of cessation (Day 28). Moreover, it indicates that even nearly 7 weeks after the cessation of antibiotic treatment (Day 76) the antibiotic effect was still evident. Thus, in the time course of this experiment, the colonic microbiome of the mice given antibiotics, irrespective of diet, did not return to “normal” even after 7 weeks without antibiotics. These findings are consistent with other studies (Hill et al. 2010, Dethlefsen and Relman 2011). Antibiotics was a significant factor in the PerMANOVA model at both time points for both diets, further suggesting a lack of complete recovery. In the next chapter, I address whether the microbiome of a mouse given antibiotics recovers more rapidly if housed with a control mouse (not given antibiotics).

The disturbance of the colonic microbiome by antibiotic administration involved significant increases in the relative abundance of Proteobacteria. This was consistent in both diets across time. The increase in Proteobacteria can be ascribed to an increase in the *Escherichia/Shigella* OTU. Significant decreases due to antibiotics in the relative abundance of Firmicutes in mice given antibiotics also occurred in both diets and all time points. This can be attributed to decreases of *Blauttia*, Lactobacillales, *Lactobacillus*, *Eubacterium nodatum*, and *Ruminoclostridium* in both diets. In mice given either diet and administered antibiotics, Bacteroidetes significantly increased over time. This can be attributed to BacteroidalesS24.7 and Bacteroides1. In control mice consuming the cornstarch diet, Bacteroidetes significantly decreased over time, whereas in the glucose diet Bacteroidetes slightly increased over time.

These differences can be attributed to Bacteroidales^{S24.7}, Bacteroides¹, and Bacteroides², which each behaved differently according to diet and time.

The net result of these shifts was that at Day 28 the diversity was significantly greater in mice given antibiotics, but subsequently (Days 42, 76) the diversity was significantly lower. Change in diversity was apparently not due simply to gains or losses of OTUs, but involved change in the evenness of the community. It was rather unexpected that the microbiome did not recover after 7 weeks following cessation of antibiotic treatment. Antibiotics suppressed some OTUs, allowing competitive release of others, but the cessation of antibiotic treatment did not result in a rapid reestablishment of the former hierarchy despite the fact that no major OTUs were entirely lost from the microbiome. The large shift in hierarchical relationships due to antibiotics established among the OTUs apparently resulted in the establishment of a new, semi-stable microbiome, possibly by influencing the nature of interactions among OTUs (Modi et al. 2014).

Thus, disturbances to the gut microbiome may have negative consequences for human health because their results may be long-lasting (Nobel et al. 2015). This is perhaps why there is some desire to develop antibiotics with more restricted activity, allowing most of the microbiome to remain intact (Yao et al. 2016). Even in that case, however, it is difficult to predict just how the complex community that is the microbiome will react to the loss of just a few OTUs. A complete reordering of the hierarchies within the microbiome may still result.

The use of antibiotics may have consequences beyond the reorganization of the prokaryote microbiome. Guts also contain other organisms including protozoa (Khelaifia and Drancourt 2012) and fungi (Suhr and Hallen-Adams 2015). Interactions obviously exist among prokaryotes, protozoa and fungi, and the perturbation of one of these communities is likely to

have effects on the other two (Hoffmann et al. 2013, Kumar et al. 2015). While interactions have begun to be explored, there is still much we do not know concerning the nature of these interactions.

Chapter 3

Introduction

In this chapter I ask whether pairing with a control mouse will ameliorate the perturbation by antibiotics. I hypothesize that, over time, the dysbiotic microbiome of a c-A mouse will converge with the intact microbiome of a C-a mouse, while the A-a mice will not. In order to test this hypothesis, I made comparisons across all pairing types (C-c, C-a, c-A, and A-a) across two time points (Day 42 and Day 76).

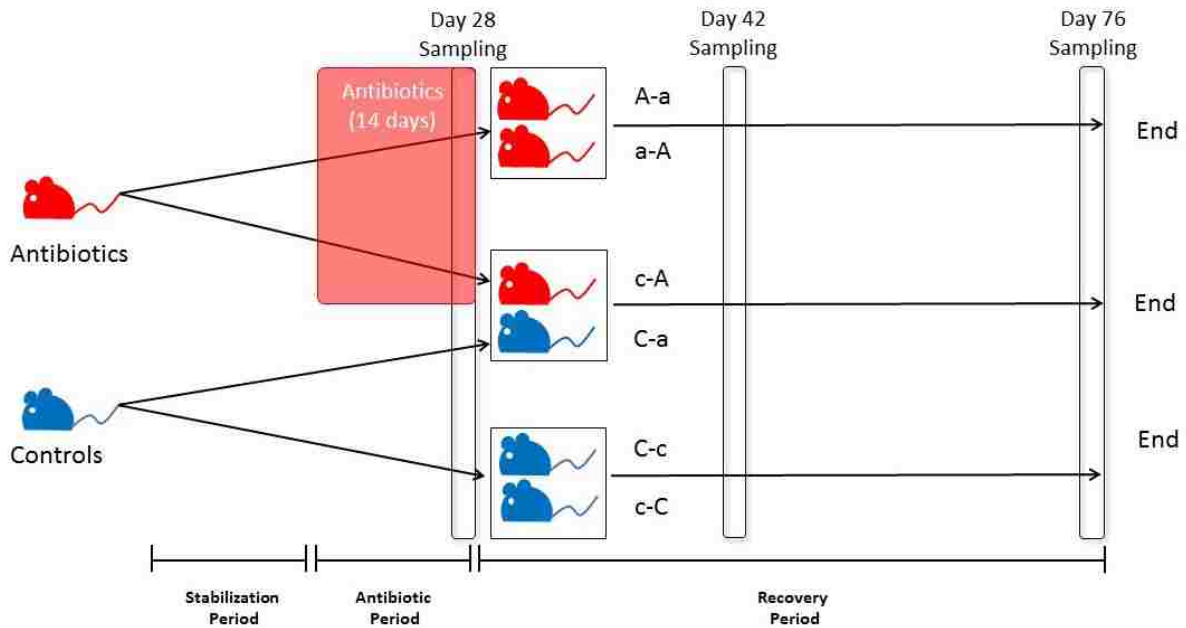


Figure 1.

Timeline of chapter 3. The red mouse represents an antibiotic mouse, while the blue mouse represents a control mouse. At the end of the antibiotic period, mice are paired within their respective diets. Pairing refers to either “Self” pairing (C-c or A-a) or “Mixed” pairing (C-a or c-A) in which C,c = control, A,a = antibiotic, upper case = focal animal, lower case = the animal paired with the focal animal. This chapter uses data for self-paired control and antibiotic mice at Day 42 and Day 76.

Results

The analyses of the communities based on OTUs indicated that all main effects of antibiotics, time, and pairing, and all interactions were significant (Tables 1, 2). The analyses based on phyla essentially returned the same results (data not shown).

Table 1.

PerMANOVA of bacterial communities at the OTU level in mice give the cornstarch diet. Pairing refers to either “Self” pairing (C-c or A-a) or “Mixed” pairing (C-a or c-A) in which C,c = control, A,a = antibiotic, upper case = focal animal, lower case = the animal paired with the focal animal. Time was either Day 42 or 76. The end of the antibiotic period was Day 28.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Model | R ² | P-values |
|--------------|--------------------|-----------------|-----------------|----------|----------------|----------|
| Antibiotics | 1 | 1.64 | 1.64 | 13.68 | 0.08 | <0.01 |
| Time | 1 | 5.92 | 5.92 | 49.35 | 0.29 | <0.01 |
| Pair | 1 | 1.39 | 1.39 | 11.56 | 0.07 | <0.01 |
| AB:Time | 1 | 0.32 | 0.32 | 2.69 | 0.02 | 0.02 |
| AB:Pair | 1 | 1.73 | 1.73 | 14.39 | 0.08 | <0.01 |
| Time:Pair | 1 | 0.39 | 0.39 | 3.22 | 0.02 | <0.01 |
| AB:Time:Pair | 1 | 0.33 | 0.33 | 2.78 | 0.02 | 0.03 |
| Residuals | 72 | 8.64 | 0.12 | | 0.42 | |
| Total | 79 | 20.36 | | | 1.00 | |

Table 2.

PerMANOVA of bacterial communities at the OTU level in mice give the glucose diet. Pairing refers to either “Self” pairing (C-c or A-a) or “Mixed” pairing (C-a or c-A) in which C,c = control, A,a = antibiotic, upper case = focal animal, lower case = the animal paired with the focal animal. Time was either Day 42 or 76. The end of the antibiotic period was Day 28.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Model | R ² | P-values |
|--------------|--------------------|-----------------|-----------------|----------|----------------|----------|
| Antibiotics | 1 | 1.73 | 1.73 | 14.41 | 0.08 | <0.01 |
| Time | 1 | 6.28 | 6.28 | 52.20 | 0.30 | <0.01 |
| Pair | 1 | 1.34 | 1.34 | 11.11 | 0.06 | <0.01 |
| AB:Time | 1 | 0.33 | 0.33 | 2.74 | 0.02 | 0.03 |
| AB:Pair | 1 | 1.75 | 1.75 | 14.51 | 0.08 | <0.01 |
| Time:Pair | 1 | 0.27 | 0.27 | 2.23 | 0.01 | 0.05 |
| AB:Time:Pair | 1 | 0.37 | 0.37 | 3.06 | 0.02 | 0.02 |
| Residuals | 72 | 8.66 | 0.12 | 0.42 | | |
| Total | 79 | 20.73 | | 1.00 | | |

Because of the significant three-factor interaction, I examined the effect of antibiotics and pairing for the two times (Day 42, Day 76) separately. In this case, there were significant interactions between antibiotics and pairing for both diets at Day 42 (Tables 3, 4) as well as both diets at Day 76 (Tables 5, 6).

Table 3.

PerMANOVA of bacterial communities at the OTU level at Day 42 in mice give the cornstarch diet. Pairing refers to either “Self” pairing (C-c or A-a) or “Mixed” pairing (C-a or c-A) in which C,c = control, A,a = antibiotic, upper case = focal animal, lower case = the animal paired with the focal animal.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Model | R ² | P-values |
|-------------|--------------------|-----------------|-----------------|----------|----------------|----------|
| Pair | 1 | 1.09 | 1.09 | 6.93 | 0.12 | <0.01 |
| Antibiotics | 1 | 1.32 | 1.32 | 8.40 | 0.14 | <0.01 |
| Pair:AB | 1 | 1.15 | 1.15 | 7.3 | 0.12 | <0.01 |
| Residuals | 36 | 5.68 | 0.16 | | 0.61 | |
| Total | 39 | 9.24 | | | 1.00 | |

Table 4.

PerMANOVA of bacterial communities at the OTU level at Day 42 in mice give the glucose diet. Pairing refers to either “Self” pairing (C-c or A-a) or “Mixed” pairing (C-a or c-A) in which C,c = control, A,a = antibiotic, upper case = focal animal, lower case = the animal paired with the focal animal.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Model | R ² | P-values |
|-------------|--------------------|-----------------|-----------------|----------|----------------|----------|
| Pair | 1 | 0.10 | 0.10 | 5.92 | 0.10 | <0.01 |
| Antibiotics | 1 | 1.32 | 1.32 | 7.85 | 0.14 | <0.01 |
| Pair:AB | 1 | 1.28 | 1.28 | 7.61 | 0.13 | <0.01 |
| Residuals | 36 | 6.07 | 0.17 | | 0.63 | |
| Total | 39 | 9.67 | | | 1.00 | |

Table 5.

PerMANOVA of bacterial communities at the OTU level at Day 76 in mice give the cornstarch diet. Pairing refers to either “Self” pairing (C-c or A-a) or “Mixed” pairing (C-a or c-A) in which C,c = control, A,a = antibiotic, upper case = focal animal, lower case = the animal paired with the focal animal.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Model | R ² | P-values |
|-------------|--------------------|-----------------|-----------------|----------|----------------|----------|
| Pair | 1 | 0.68 | 0.68 | 8.25 | 0.13 | <0.01 |
| Antibiotics | 1 | 0.64 | 0.64 | 7.78 | 0.12 | <0.01 |
| Pair:AB | 1 | 0.91 | 0.91 | 11.10 | 0.18 | <0.01 |
| Residuals | 36 | 2.97 | 0.08 | | 0.57 | |
| Total | 39 | 5.20 | | | 1.00 | |

Table 6.

PerMANOVA of bacterial communities at the OTU level at Day 76 in mice give the glucose diet. Pairing refers to either “Self” pairing (C-c or A-a) or “Mixed” pairing (C-a or c-A) in which C,c = control, A,a = antibiotic, upper case = focal animal, lower case = the animal paired with the focal animal.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Model | R ² | P-values |
|-------------|--------------------|-----------------|-----------------|----------|----------------|----------|
| Pair | 1 | 0.61 | 0.61 | 8.42 | 0.13 | <0.01 |
| Antibiotics | 1 | 0.74 | 0.74 | 10.27 | 0.16 | <0.01 |
| Pair:AB | 1 | 0.83 | 0.83 | 11.54 | 0.17 | <0.01 |
| Residuals | 36 | 2.60 | 0.07 | | 0.54 | |
| Total | 39 | 4.78 | | | 1.00 | |

The nature of these interactions is revealed in the ordinations. For the cornstarch diet at Day 42, the C-c mice were clearly distinct from all other mice, and the other three treatments (C-a, c-A, A-a) were clustered together. Thus, the control mice paired with antibiotic mice were more like antibiotic mice than like control mice, and the antibiotic mice paired with control mice were more like antibiotic mice than control mice (Figure 2). In other words, antibiotic mice had a greater effect on control mice than control mice had on antibiotic mice.

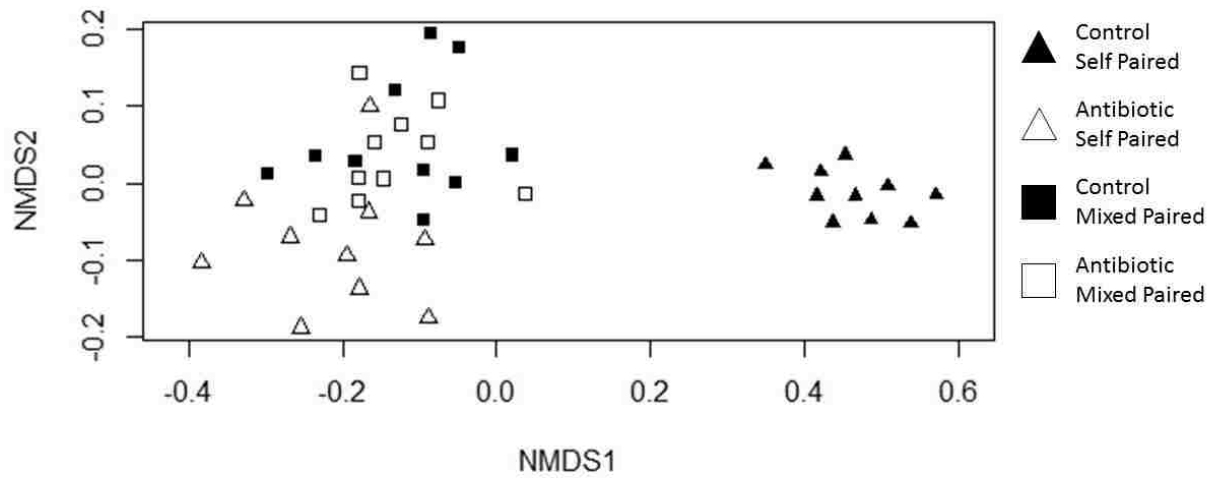


Figure 2.
Ordination of bacterial communities based on OTUs on Day 42 for mice given the cornstarch diet.

The same pattern held for mice given the cornstarch diet at Day 76 (Figure 3), the mice given the glucose diet at Day 42 (Figure 4), and the mice given the glucose diet at Day 76 (Figure 5).

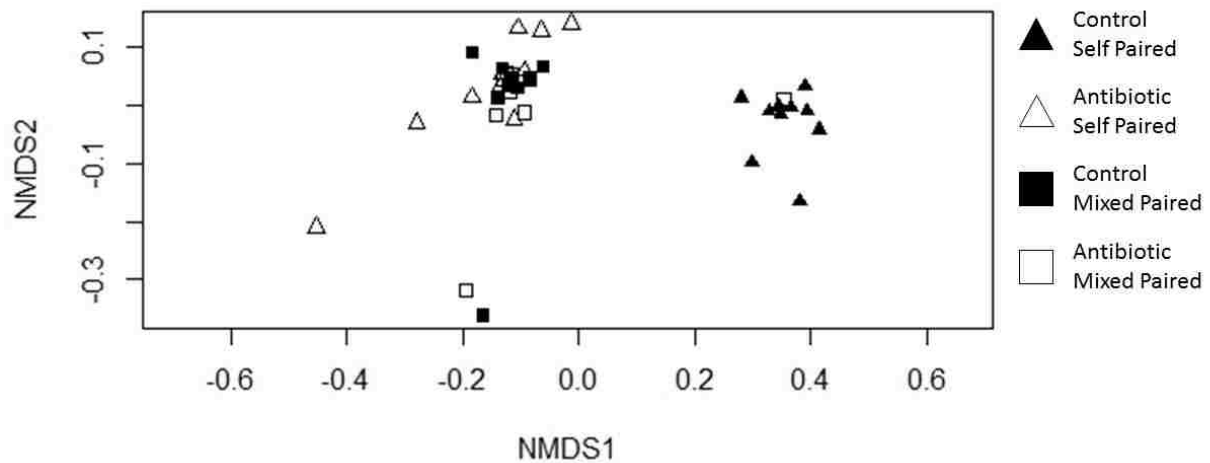


Figure 3.
Ordination of bacterial communities based on OTUs on Day 76 for mice given the cornstarch diet.

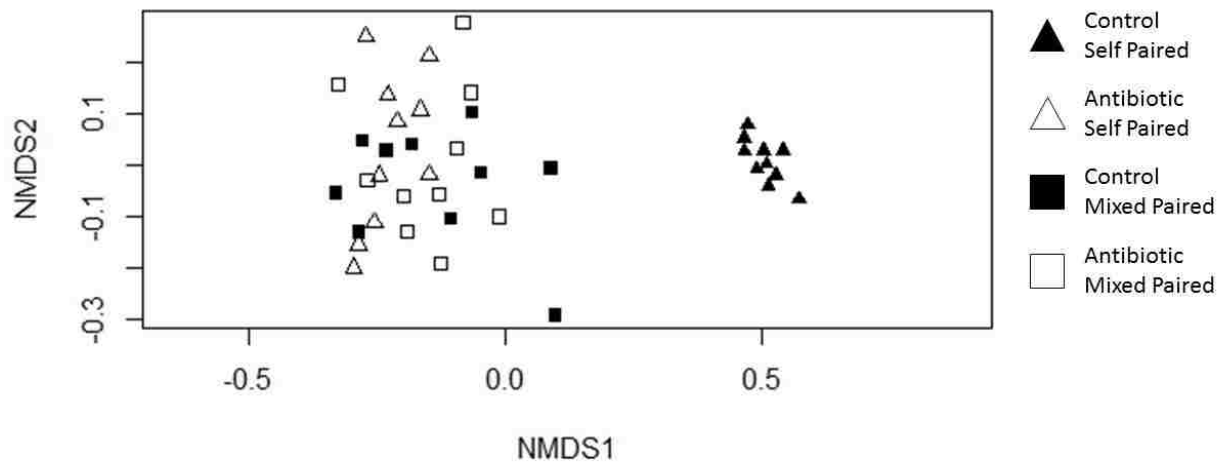


Figure 4. Ordination of bacterial communities based on OTUs on Day 42 for mice given the glucose diet.

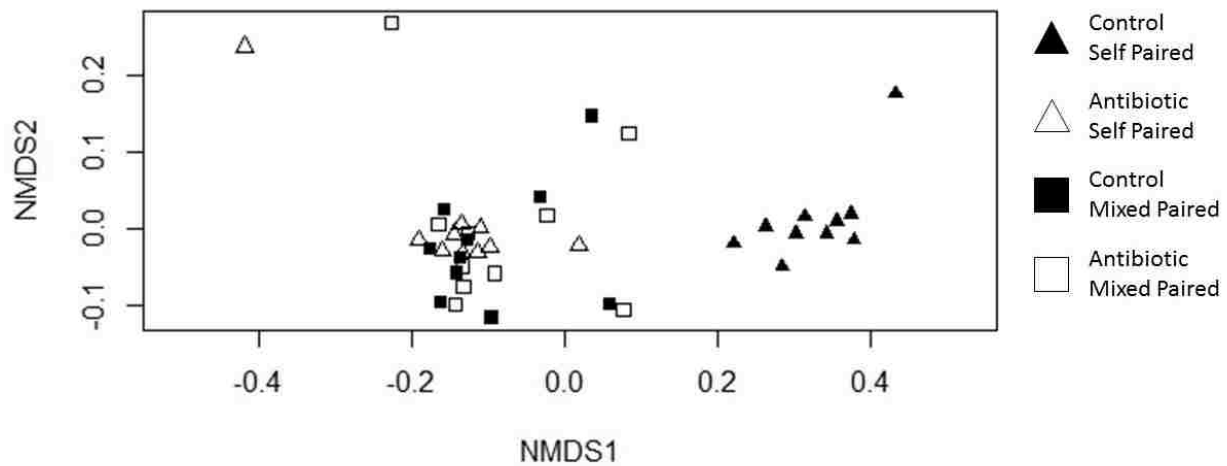


Figure 5. Ordination of bacterial communities based on OTUs on Day 76 for mice given the glucose diet.

This pattern in which the colonic microbiomes of the C-c mice are distinct from the those of the mice in the other treatments is also evident in the distribution of the OTUs among the different pairs at both the OTU level and the phylum level. For example, for mice given the cornstarch diet at Day 42 (Table 7), some OTUs were significantly more abundant in C-c mice

than in C-a, c-A, and A-a mice (BacteroidalesS24.7, *Blautia*, Coriobacteriaceae, *Lactobacillus*, *Eubacterium nodatum*, *Ruminiclostridium*), and some were significantly less abundant (*Buttiauxella*, *Erysipelatoclostridium*, *Clostridium sensu stricto1*).

Table 7.

Means of Hellinger-transformed sequence reads (relative abundance) for OTUs contributing more than 1% of the community. Different letters indicate relative abundances are significantly ($P < 0.05$) different. Day 42. Cornstarch diet.

| OTU | C-c | C-a | c-A | A-a |
|-----------------------------------|-------------|--------------|--------------|-------------|
| BacteroidalesS24.7 | 0.4112 a | 0.0308 b | 0.0199 b | 0.0062 b |
| <i>Blautia</i> | 0.2584 a | 0.0577 b | 0.0154 b | 0.0018 b |
| <i>Buttiauxella</i> | 0.0259 b | 0.5766 a | 0.5647 a | 0.6184 a |
| Lactobacillales | 0.1900 a | 0.1975 a | 0.2261 a | 0.2380 a |
| <i>Erysipelatoclostridium</i> | 0.0846 c | 0.2253 b | 0.2526 ab | 0.3100 a |
| <i>Escherichia/Shigella</i> | 0.0055 b | 0.0130 b | 0.0143 b | 0.1007 a |
| Bacteroides1 | 0.0018 a | 0.0000 a | 0.0000 a | 0.0000 a |
| Coriobacteriaceae | 0.2711 a | 0.0300 b | 0.0266 b | 0.0037 b |
| <i>Lactobacillus</i> | 0.2007 a | 0.0662 b | 0.0558 b | 0.0000 b |
| Bacteroides2 | 0.0018 a | 0.0000 a | 0.0018 a | 0.0692 a |
| <i>Eubacterium nodatum</i> | 0.2310 a | 0.0358 b | 0.0265 b | 0.0307 b |
| <i>Clostridium sensu stricto1</i> | 0.0076 b | 0.3076 a | 0.3411 a | 0.0730 b |
| <i>Peptoclostridium</i> | 0.1085 a | 0.0752 ab | 0.0925 a | 0.0172 b |
| <i>Clostridium sensu stricto2</i> | 0.1500 a | 0.2029 a | 0.1993 a | 0.1297 a |
| <i>Ruminiclostridium</i> | 0.1486 a | 0.0018 b | 0.0073 b | 0.0055 b |

A similar pattern was observed for the distribution of OTUs among the pairings for mice given the cornstarch diet at Day 76, mice given the glucose diet at Day 42, and mice given the

glucose diet at Day 76 with slight variation in the OTUs contributing to the pattern (Tables 8, 9, 10, respectively).

Table 8.

Means of Hellinger-transformed sequence reads (relative abundance) for OTUs contributing more than 1% of the community. Different letters indicate relative abundances are significantly ($P < 0.05$) different. Day 76. Cornstarch diet.

| OTU | C-c | C-a | c-A | A-a |
|------------------------------------|--------|---------|--------|--------|
| BacteroidalesS24.7 | 0.2477 | 0.01655 | 0.0424 | 0.1034 |
| | a | b | b | b |
| <i>Blautia</i> | 0.2168 | 0.1168 | 0.1256 | 0.1248 |
| | a | b | b | b |
| <i>Buttiauxella</i> | 0 | 0.0018 | 0.0086 | 0.0044 |
| | a | a | a | a |
| Lactobacillales | 0.1649 | 0.1287 | 0.1084 | 0.1165 |
| | a | a | a | a |
| <i>Erysipelatoclostridium</i> | 0.0828 | 0.1495 | 0.1384 | 0.1697 |
| | b | a | ab | a |
| <i>Escherichia/Shigella</i> | 0.1304 | 0.2276 | 0.2501 | 0.2420 |
| | b | a | a | a |
| Bacteroides1 | 0.3662 | 0.4555 | 0.4661 | 0.3261 |
| | a | a | a | a |
| Coriobacteriaceae | 0.2074 | 0.1519 | 0.1644 | 0.1511 |
| | a | a | a | a |
| <i>Lactobacillus</i> | 0.1983 | 0.1216 | 0.1072 | 0.1167 |
| | a | b | b | b |
| Bacteroides2 | 0.2227 | 0.2578 | 0.2803 | 0.3253 |
| | a | a | a | a |
| <i>Eubacterium nodatum</i> | 0.1705 | 0.0903 | 0.0870 | 0.1089 |
| | a | b | b | ab |
| <i>Clostridium sensu stricto</i> 1 | 0.0018 | 0.0678 | 0.0688 | 0.1010 |
| | b | ab | ab | a |
| <i>Peptoclostridium</i> | 0.1046 | 0.0577 | 0.0781 | 0.0562 |
| | a | b | ab | b |
| <i>Clostridium sensu stricto</i> 2 | 0.1078 | 0.0081 | 0.0221 | 0.0167 |
| | a | b | b | b |
| <i>Rumiclostridium</i> | 0.1239 | 0.0081 | 0.0195 | 0.0286 |
| | a | b | b | b |

Table 9.

Means of Hellinger-transformed sequence reads (relative abundance) for OTUs contributing more than 1% of the community. Different letters indicate relative abundances are significantly ($P < 0.05$) different. Day 42. Glucose diet.

| OTU | C-c | C-a | c-A | A-a |
|------------------------------------|-------------|--------------|--------------|--------------|
| BacteroidalesS24.7 | 0.3727 a | 0.0185 b | 0.0099 b | 0.0079 b |
| <i>Blautia</i> | 0.2648 a | 0.0259 b | 0.0073 b | 0.0018 b |
| <i>Buttiauxella</i> | 0.0253 b | 0.5993 a | 0.6030 a | 0.5363 a |
| Lactobacillales | 0.1425 a | 0.1964 a | 0.2126 a | 0.1665 a |
| <i>Erysipelatoclostridium</i> | 0.1312 b | 0.2090 ab | 0.2338 a | 0.2821 a |
| <i>Escherichia/Shigella</i> | 0.0000 b | 0.0102 b | 0.0279 ab | 0.2190 a |
| Bacteroides1 | 0.0000 a | 0.0000 a | 0.0000 a | 0.0000 a |
| Coriobacteriaceae | 0.1506 a | 0.0162 b | 0.0018 b | 0.0018 b |
| <i>Lactobacillus</i> | 0.2121 a | 0.0068 b | 0.0073 b | 0.0037 b |
| Bacteroides2 | 0.0000 a | 0.0000 a | 0.0045 a | 0.0000 a |
| <i>Eubacterium nodatum</i> | 0.2802 a | 0.0381 b | 0.0399 b | 0.0055 b |
| <i>Clostridium sensu stricto</i> 1 | 0.0106 c | 0.3783 a | 0.2972 ab | 0.1825 bc |
| <i>Peptoclostridium</i> | 0.1537 a | 0.0663 bc | 0.0852 b | 0.0175 c |
| <i>Clostridium sensu stricto</i> 2 | 0.0055 b | 0.1352 ab | 0.1116 ab | 0.1859 a |
| <i>Ruminiclostridium</i> | 0.1803 a | 0.0118 b | 0.0174 b | 0.0026 b |

Table 10.

Means of Hellinger-transformed sequence reads (relative abundance) for OTUs contributing more than 1% of the community. Different letters indicate relative abundances are significantly ($P < 0.05$) different. Day 76. Glucose diet.

| OTU | C-c | C-a | c-A | A-a |
|-----------------------------------|-------------|--------------|--------------|--------------|
| BacteroidalesS24.7 | 0.2199 a | 0.0516 b | 0.0403 b | 0.0478 b |
| <i>Blautia</i> | 0.2022 a | 0.1615 a | 0.1649 a | 0.1369 a |
| <i>Buttiauxella</i> | 0.0018 a | 0.0032 a | 0.0018 a | 0.0032 a |
| Lactobacillales | 0.1706 a | 0.1215 a | 0.1552 a | 0.1022 a |
| <i>Erysipelatoclostridium</i> | 0.0901 b | 0.1672 a | 0.1480 ab | 0.1443 ab |
| <i>Escherichia/Shigella</i> | 0.1650 b | 0.2449 ab | 0.2659 a | 0.2900 a |
| Bacteroides1 | 0.4185 a | 0.3957 a | 0.4069 a | 0.3340 a |
| Coriobacteriaceae | 0.1849 a | 0.1439 a | 0.1534 a | 0.1466 a |
| <i>Lactobacillus</i> | 0.2325 a | 0.1156 b | 0.1318 b | 0.0547 b |
| Bacteroides2 | 0.3201 b | 0.4422 a | 0.4240 a | 0.4845 a |
| <i>Eubacterium nodatum</i> | 0.1965 a | 0.0978 b | 0.0866 b | 0.0915 b |
| <i>Clostridium sensu stricto1</i> | 0.0073 b | 0.1516 a | 0.1166 a | 0.1250 a |
| <i>Peptoclostridium</i> | 0.1424 a | 0.0805 b | 0.0760 b | 0.0635 b |
| <i>Clostridium sensu stricto2</i> | 0.0037 a | 0.0000 a | 0.0018 a | 0.0032 a |
| <i>Ruminiclostridium</i> | 0.1494 a | 0.0142 b | 0.0342 b | 0.0291 b |

Similar patterns were observed among the different pairs at the phylum level for mice given cornstarch and glucose diets, and at Days 42 and 76 (data not shown).

The various diversity measures are given in Tables 11 and 12 (cornstarch diet) and Tables 13, and 14 (glucose diet). In general, for the control (C) mice, pairing with antibiotic (A) mice resulted in a very large and significant decrease in evenness, Shannon-Wiener diversity index, effective number of OTUs and Chao2 richness in both diets. On the other hand, for the antibiotic

(A) mice, pairing with control (C) mice either had a modest positive impact (Day 42) or essentially no impact (Day 76) on these metrics. Thus, there was a significant interaction between pairing and antibiotics for both diets with respect to Shannon-Wiener index (Tables 15, 16), effective numbers of OTUs (Tables 17, 18) and Pielou's evenness (Tables 19, 20).

Table 11.
Means of the diversity indices on Days 42 and 76 for mice given the cornstarch diet.

| Treatments | | Shannon | Effective Numbers | Pielou's Evenness |
|------------|----------|---------|----------------------|----------------------|
| 42 | C Self | 5.04 | 158.98 | 0.9824603 |
| | Mixed | 4.13 | 67.53 | 0.9605767 |
| | AB Mixed | 3.98 | 58.80 | 0.9571185 |
| | Self | 3.73 | 47.95 | 0.9496097 |
| 76 | C Self | 4.51 | 93.51 | 0.9775098 |
| | Mixed | 3.75 | 46.05 | 0.9718017 |
| | AB Mixed | 3.85 | 50.05 | 0.9714507 |
| | Self | 3.90 | 51.22 | 0.9693588 |

Table 12.
Chao2 means on Days 42 and 76 for mice given the cornstarch diet.

| Treatments | | Chao2 | 95% CI |
|------------|----------|-------|-------------|
| 42 | C Self | 515.1 | 506.4-532.4 |
| | Mixed | 341.0 | 336.1-345.9 |
| | AB Mixed | 302.5 | 292.5-312.5 |
| | Self | 280.3 | 272.1-288.5 |
| 76 | C Self | 249.2 | 229.9-268.5 |
| | Mixed | 172.4 | 151.0-193.8 |
| | AB Mixed | 205.8 | 192.2-219.4 |
| | Self | 208.9 | 201.6-216.2 |

Table 13.

Means of diversity indices in Days 42 and 76 for mice given the glucose diet.

| Treatments | | Shannon | Effective Numbers | Pielou's Evenness |
|------------|----------|---------|----------------------|----------------------|
| 42 | C Self | 5.10 | 165.70 | 0.98268 |
| | Mixed | 3.98 | 58.01 | 0.96044 |
| | AB Mixed | 4.20 | 72.45 | 0.96426 |
| | Self | 3.83 | 55.33 | 0.95279 |
| 76 | C Self | 4.61 | 103.24 | 0.97914 |
| | Mixed | 3.74 | 43.27 | 0.97463 |
| | AB Mixed | 3.81 | 47.88 | 0.97288 |
| | Self | 3.71 | 42.37 | 0.97289 |

Table 14.

Chao2 means on Days 42 and 76 for mice given the glucose diet.

| Treatments | | Chao2 | 95% CI |
|------------|----------|-------|-------------|
| 42 | C Self | 491.0 | 496.9-485.1 |
| | Mixed | 324.3 | 318.3-330.3 |
| | AB Mixed | 369.3 | 360.3-378.3 |
| | Self | 300.4 | 291.0-309.8 |
| 76 | C Self | 272.0 | 255.2-288.8 |
| | Mixed | 145.7 | 149.1-142.3 |
| | AB Mixed | 204.2 | 201.3-207.1 |
| | Self | 151.8 | 135.3-168.3 |

Table 15.

ANOVA results of Shannon-Wiener diversity index based on OTUs of mice given the cornstarch diet.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Value | P-values |
|--------------|-----------------------|--------------------|--------------------|----------|----------|
| Time | 1 | 0.95 | 0.95 | 5.88 | 0.02 |
| Antibiotics | 1 | 4.84 | 4.84 | 29.92 | <0.01 |
| Pair | 1 | 2.72 | 2.72 | 16.81 | <0.01 |
| Time:AB | 1 | 1.14 | 1.14 | 7.07 | <0.01 |
| Time:Pair | 1 | 0.03 | 0.03 | 0.19 | 0.66 |
| AB:Pair | 1 | 4.40 | 4.40 | 27.22 | <0.01 |
| Time:AB:Pair | 1 | 0.26 | 0.26 | 1.61 | 0.21 |
| Residuals | 72 | 11.64 | 0.16 | | |

Table 16.

ANOVA results of Shannon-Wiener diversity index based on OTUs of mice given the glucose diet.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Value | P-values |
|--------------|--------------------|-----------------|-----------------|----------|----------|
| Time | 1 | 1.96 | 1.96 | 13.48 | <0.01 |
| Antibiotics | 1 | 4.37 | 4.37 | 30.10 | <0.01 |
| Pair | 1 | 2.81 | 2.81 | 19.36 | <0.01 |
| Time:AB | 1 | 0.06 | 0.06 | 0.39 | 0.53 |
| Time:Pair | 1 | 0.00 | 0.00 | 0.00 | 0.97 |
| AB:Pair | 1 | 7.54 | 7.54 | 51.93 | <0.01 |
| Time:AB:Pair | 1 | 0.33 | 0.33 | 2.25 | 0.14 |
| Residuals | 72 | 10.45 | 0.15 | | |

Table 17.

ANOVA results of the effective numbers of OTUs of mice given the cornstarch diet.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Value | P-values |
|--------------|--------------------|-----------------|-----------------|----------|----------|
| Time | 1 | 10675 | 10675 | 16.42 | <0.01 |
| Antibiotics | 1 | 31224 | 31224 | 48.04 | <0.01 |
| Pair | 1 | 20876 | 20876 | 32.12 | <0.01 |
| Time:AB | 1 | 8297 | 8297 | 12.77 | <0.01 |
| Time:Pair | 1 | 1278 | 1278 | 1.97 | 0.17 |
| AB:Pair | 1 | 27600 | 27600 | 42.46 | <0.01 |
| Time:AB:Pair | 1 | 3923 | 3923 | 6.04 | 0.02 |
| Residuals | 72 | 46799 | 650 | | |

Table 18.

ANOVA results of the effective numbers of OTUs of mice given the glucose diet.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Value | P-values |
|--------------|--------------------|-----------------|-----------------|----------|----------|
| Time | 1 | 16452 | 16452 | 28.61 | <0.01 |
| Antibiotics | 1 | 28949 | 28949 | 50.35 | <0.01 |
| Pair | 1 | 2696 | 2696 | 45.73 | <0.01 |
| Time:AB | 1 | 1968 | 1968 | 3.42 | 0.07 |
| Time:Pair | 1 | 1629 | 1629 | 2.83 | 0.10 |
| AB:Pair | 1 | 45266 | 45266 | 78.73 | <0.01 |
| Time:AB:Pair | 1 | 4399 | 4399 | 7.65 | <0.01 |
| Residuals | 72 | 41399 | 575 | | |

Table 19.

ANOVA results of Pielou's evenness based on OTUS of mice given the cornstarch diet.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Value | P-values |
|--------------|--------------------|-----------------------|-----------------------|----------|----------|
| Time | 1 | 2.04*10 ⁻³ | 2.04*10 ⁻³ | 19.92 | 0.02 |
| Antibiotics | 1 | 2.51*10 ⁻³ | 2.51*10 ⁻³ | 24.57 | <0.01 |
| Pair | 1 | 4.05*10 ⁻⁴ | 4.05*10 ⁻⁴ | 3.96 | <0.01 |
| Time:AB | 1 | 9.67*10 ⁻⁴ | 9.67*10 ⁻⁴ | 9.46 | <0.01 |
| Time:Pair | 1 | 1.45*10 ⁻⁴ | 1.45*10 ⁻⁴ | 1.42 | 0.24 |
| AB:Pair | 1 | 1.73*10 ⁻³ | 1.73*10 ⁻³ | 16.92 | <0.01 |
| Time:AB:Pair | 1 | 5.83*10 ⁻⁴ | 5.83*10 ⁻⁴ | 5.7 | 0.02 |
| Residuals | 72 | 7.36*10 ⁻³ | 1.02*10 ⁻⁴ | | |

Table 20.

ANOVA results of Pielou's evenness based on OTUs of mice given the glucose diet.

| Factor | Degrees of Freedom | Sums of Squares | Mean of Squares | F. Value | P-values |
|--------------|--------------------|-----------------------|-----------------------|----------|----------|
| Time | 1 | 1.94*10 ⁻³ | 1.94*10 ⁻³ | 23.01 | <0.01 |
| Antibiotics | 1 | 1.45*10 ⁻³ | 1.45*10 ⁻³ | 17.25 | <0.01 |
| Pair | 1 | 2.92*10 ⁻⁴ | 2.92*10 ⁻⁴ | 3.47 | 0.07 |
| Time:AB | 1 | 4.09*10 ⁻³ | 4.09*10 ⁻³ | 4.86 | 0.03 |
| Time:Pair | 1 | 4.87*10 ⁻⁵ | 4.87*10 ⁻⁵ | 0.58 | 0.45 |
| AB:Pair | 1 | 1.83*10 ⁻³ | 1.83*10 ⁻³ | 21.69 | <0.01 |
| Time:AB:Pair | 1 | 1.07*10 ⁻³ | 1.07*10 ⁻³ | 12.68 | <0.01 |
| Residuals | 72 | 6.06*10 ⁻³ | 8.41*10 ⁻⁵ | | |

Non-random interactions among OTUs were documented by calculating C-scores. Co-occurrence of OTUs was greater than expected by chance more frequently at Day 42 than at Day 76, particularly in mice given the cornstarch diet (Tables 21-24). However, the number of non-random interactions did not appear to be different in the microbiomes of C-c mice compared to those in C-a, c-A, or A-a mice.

Table 21.
C-scores, Day 42, cornstarch diet.

| Pairing | Observed Index | Simulated Index | Standardized Effect Size | P-Value |
|---------|----------------|-----------------|--------------------------|---------|
| C-c | 0.12 | 0.34 | -2.01 | 0.03 |
| C-a | 1.44 | 1.94 | -1.96 | 0.04 |
| A-c | 1.19 | 1.49 | -1.57 | 0.08 |
| A-a | 1.41 | 1.92 | -3.15 | <0.01 |

Table 22.
C-scores, Day 42, glucose diet.

| Pairing | Observed Index | Simulated Index | Standardized Effect Size | P-Value |
|---------|----------------|-----------------|--------------------------|---------|
| C-c | 0.24 | 0.24 | 0.02 | 0.54 |
| C-a | 1.35 | 2.16 | -3.12 | <0.01 |
| A-c | 1.79 | 2.08 | -1.11 | 0.14 |
| A-a | 1.58 | 1.86 | -1.1 | 0.15 |

Table 23.
C-scores, Day 76, cornstarch diet.

| Pairing | Observed Index | Simulated Index | Standardized Effect Size | P-Value |
|---------|----------------|-----------------|--------------------------|---------|
| C-c | 0 | 0.02 | -0.49 | 0.81 |
| C-a | 0.67 | 0.6 | 0.51 | 0.34 |
| A-c | 0.52 | 0.68 | -1.15 | 0.14 |
| A-a | 0.91 | 0.92 | -0.07 | 0.46 |

Table 24.
C-scores, Day 76, glucose diet.

| Pairing | Observed Index | Simulated Index | Standardized Effect Size | P-Value |
|---------|----------------|-----------------|--------------------------|---------|
| C-c | 0.12 | 0.13 | -0.04 | 0.5 |
| C-a | 0.58 | 0.45 | 1.06 | 0.16 |
| A-c | 0.75 | 0.52 | 2.08 | 0.01 |
| A-a | 0.99 | 0.99 | -0.13 | 0.4 |

Discussion

I tested the hypothesis that the dysbiotic microbiome of a mouse previously given antibiotics would return to “normal” more rapidly when housed with a control mouse (never given antibiotics) than when housed with another mouse previously given antibiotics. The hypothesis is based on the assumption that the control mouse would act effectively as a probiotic for the antibiotic mouse, either as the antibiotic mouse consumed the feces of the control mouse (Takahashi et al. 1985) or consumed food contaminated by feces of the control mouse. However, even after 7 weeks post antibiotic treatment, antibiotic mice had a greater effect on the microbiome structure of control mice than control mice had on microbiomes of antibiotic mice; control mice paired with antibiotic mice possessed dysbiotic microbiomes within 2 weeks and lasting at least 7 weeks (termination of the study).

OTUs that contributed to the differences between a C-c mouse and C-a, c-A, and A-a mice in the cornstarch diet at both time points include Bacteroidales, *Blautia*, *Lactobacillus*, *Eubacterium nodatum*, and *Ruminoclostridium*. OTUs that contributed to the differences between a C-c mouse and C-a, c-A, and A-a mice in the glucose diet at both time points include Bacteroidales, *Lactobacillus*, *Eubacterium nodatum*, and *Ruminoclostridium*.

C-c mice had higher diversities than C-a mice. However, C-a mice maintained a higher diversity than c-A and A-a mice at Day 42. At Day 76, however, C-a mice had a lower diversity than the c-A and A-a mice. This suggests that their microbiome was still converging to the c-A mice’s microbiome at Day 42. For c-A mice, pairing had either a slightly positive impact at Day 42, or no impact at Day 76. Day 42 appears to be transitional state of both the C-a and c-A mice’s microbiomes. The nature of the effect of pairing changes from Day 42 to Day 76.

In the cornstarch diet at Day 42, C-c, C-a, and A-a mice all had greater than expected co-occurrence. However, in the glucose diet only the C-a mice at Day 42 had a significant C-score. At Day 76, none of the groups in the cornstarch diet had significant C-scores. This further suggests that the microbiomes were in a transitional state at Day 42. In the glucose diet, only the C-a mice had greater than expected co-occurrence at Day 42. However, at Day 76 the C-a mice no longer has a significant C-score. c-A mice has a significant C-score at Day 76, indicating that there was less than expected co-occurrence. The microbiomes appeared to be in some flux throughout the study.

Human studies have suggested that individuals living in close proximity to each other have gut microbiomes that are more similar than expected (Song et al. 2013). This study confirms that pairing two mice in a single cage can have that effect as well. The surprising result, however, was that the mouse with the dysbiotic microbiome influenced the mouse with the control microbiome far more than the reverse.

One can offer at least two hypotheses to explain this result, but neither was tested. First, compared to the control microbiome, the dysbiotic microbiome may have been more stable and thus more resistant to change due to invasion by OTUs from the control microbiome. Other research has also shown that dysbiotic microbiomes have a high degree of stability (Hill et al. 2010, Dethlefsen and Relman 2011). Certainly, if this hypothesis were true, the use of probiotics is called into question. Second, it is possible that one or more of the antibiotics were still active at the initial phase of pairing, and coprophagy caused the microbiome of the control mice to rapidly become dysbiotic. If this is true, the experiment should have been conducted with a waiting period between the cessation of antibiotic administration and pairing. The half-lives of the antibiotics used are: 30 minutes (vancomycin), approximately one hour (for ampicillin), and

3.2 hours (for neomycin in rabbits) (Liu et al. 1990, Jin et al. 2005, Vesga et al. 2010). Thus, the antibiotics may have had biological activity for some small period of time following cessation of antibiotic treatment on Day 28. If control mice consumed feces or urine of antibiotic mice, and if the feces or urine contained active antibiotics (Takahashi et al. 1985), there was a way for control mice to receive a dose of antibiotics. However, one must assume that during that same period of time, if antibiotic mice were consuming feces of control mice, antibiotic mice would have been ingesting an inoculum of the “normal” microbiome. Despite this, the structure of the microbiome of c-A mice was similar to that of A-a mice, suggesting that the control mouse housed with the antibiotic mouse had no significant probiotic effect.

Future Directions

Our 16S primers detected a total of seven archaeal OTUs. For this study, these OTUs were included in the bacterial data sets, as these were not in sufficient abundance to warrant an additional dataset. In the future when studying community interactions, it would be interesting to employ archaeal-specific primer sets to sufficiently sample this community. Using archaeal-specific primers would yield enough data to warrant further community analysis.

Unfortunately, read numbers are not an accurate predictor of biomass. In the future, it would be beneficial to complete a fatty acid analysis to estimate biomass of both bacteria and fungi in the colon (Frostegard and Baath 1996). This would allow for abundance comparisons between communities, as well as to elucidate treatment effects on abundances.

Originally the focus of this thesis was on the interactions between fungi and bacteria in the colon. Unfortunately, due to difficulties in the PCR process and sequencing, I did not receive valid fungal data that could be used for this thesis. In the future, studies of the interactions between bacteria and fungi will be of great value to the scientific community. This could be accomplished by both sequencing and biomass measurements of both communities.

During this study, I attempted to collect blood samples upon animal sacrifice for the comparison of inflammation between treatments. Blood samples were sent to an independent lab to quantify white blood cell counts as a marker of inflammation. Unfortunately, only a small fraction of these samples yielded data which made the analysis invalid. In future studies concerning antibiotics blood samples should be collected to determine inflammation levels in the mice. When mice were dissected, I observed some mice had inflamed kidneys. These mice had received antibiotics.

In addition to the measurements made over the course of this study, there are some measurements that would have been beneficial to obtain. These include mouse weights over time, amount of food consumed, and amount of antibiotic water consumed. The mouse weights over time would have been particularly interesting because of the known phenomenon that dysbiotic microbiomes can contribute to weight gain (Ridaura et al. 2013).

In addition to these future directions, it would be interesting to do a lifetime study of these mice. By the completion of this study the dysbiotic microbiomes caused by the antibiotics never made a complete recovery. A lifetime study would be interesting to see how long it takes for these microbiomes to fully recover, if they ever do.

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