Reproducible Community Dynamics of the Gastrointestinal Microbiota Following Antibiotic Perturbation

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Abbreviations

V6, sixth hypervariable region of the 16S ribosomal RNA-encoding gene; AMB, amoxicillin, metronidazole and bismuth; SSU, small subunit; rRNA, ribosomal RNA; GAST, global alignment for sequence taxonomy; RDP, ribosomal database project; OTU, operational taxonomic unit; GI, gastrointestinal; SPF, specific pathogen free

Abstract

Shifts in microbial communities are implicated in the pathogenesis of a number of gastrointestinal diseases, but we have limited understanding of the mechanisms that lead to altered community structures. One difficulty with studying these mechanisms in human subjects is the inherent baseline variability of the microbiota in different individuals that arise due to varying life histories. To try and overcome this baseline variability we employed a mouse model to control host genotype, diet and other possible influences on the microbiota. This allowed us to determine if the indigenous microbiota in such mice had a stable baseline community structure and whether this community exhibited a consistent response following antibiotic administration. We employed a tag sequencing strategy targeting the V6 hypervariable region of the bacterial small-subunit (16S) ribosomal RNA combined with massively parallel sequencing to determine the community structure of the gut microbiota. Inbred mice in a controlled environment harbored a reproducible baseline community that was significantly impacted by antibiotic administration. The ability of the gut microbial community to recover to baseline following cessation of antibiotic administration varied according to the antibiotic regimen administered. Severe antibiotic pressure resulted in reproducible long-lasting alterations in the gut microbial community including a decrease in overall diversity. The finding of stereotypic responses of the indigenous microbiota to ecologic stress implies that a better understanding of the factors that govern community structure could lead to strategies for the

intentional manipulation of this ecosystem to preserve or restore a healthy microbiota.

Introduction

A highly diverse, complex community of microorganisms inhabits the gastrointestinal tract of mammals. This community, referred to as the indigenous microbiota, exists in a delicate symbiosis with the host (3, 15). A significant body of research has demonstrated that disturbances in this balance can disrupt intestinal homeostasis. Multiple disease states may arise, at least in part, in response to altered indigenous microbial communities in the gut (10, 47, 53, 56). Conversely, research on probiotics indicates the normal balance between the indigenous microbiota and the host can be protected or restored through administration of beneficial microbes (6, 45, 60).

The relationship between the indigenous microbiota and a host involves multiple interactions. The indigenous microbiota play a central role in digestion and nutrition of the host (30, 56). These microbes also affect the regulation and homeostasis of the host immune system (27, 46). As part of the innate defenses of the gastrointestinal tract, the community of indigenous microbes forms and ecologic barrier that prevents the ingress of pathogenic microorganisms. For example, the development of *Clostridium difficile*-associated colitis following antibiotic administration arises from a loss of intrinsic “colonization resistance” against pathogenic organisms (61). Antibiotic disturbance of the normal community structure of the microbiota may allow germination of environmentally acquired spores with subsequent overgrowth of the pathogen and toxin production. Alternatively, *C. difficile* colitis may develop subsequent to the expansion of low-abundance *C. difficile* populations that normally produce

82 insignificant levels of toxin. In either case, the disruption of the indigenous
83 microbiota by antibiotic administration is a key component of pathogenesis (7).
84 Murine models have provided important insights about the interactions
85 between the microbiota and the host. One consistent feature of microbiota
86 studies in human subjects is that there is significant interindividual variation in the
87 indigenous microbiota (12, 13). This variation likely arises from the accumulated
88 effects of genetic and environmental influences on the gut microbial community
89 (11). Significant baseline variation makes it difficult to conduct studies that follow
90 the dynamics of the gut microbiota in humans, especially if the goal is to discern
91 stereotypic responses to a given manipulation. Therefore, as with other areas of
92 biomedical research, murine models offer unique advantages for microbiota
93 experimentation.
94 Several recent studies describe murine models of disease in which altered
95 indigenous gut microbial communities are generated through the administration
96 of antibiotics. These altered communities can either be permissive or required for
97 the development of the model disease state, although in other cases they appear
98 to be protective (5, 9, 24, 26, 52). Although these studies have provided insight
99 into many of the host responses to the indigenous microbiota, we have
100 remarkably little information as to the exact nature of the effect of antibiotic
101 administration on the microbial communities themselves. For example, these
102 studies assume that genetically identical mice would harbor a consistent baseline
103 microbiota. Furthermore, it is also assumed that the microbiota respond in a
104 reproducible manner to the antibiotic administration resulting in consistent

105 changes in the structure and function of the microbiota responsible for the
106 observed changes in the host response. These crucial assumptions have not
107 been rigorously tested to date.
108 Early studies on the gut microbiota relied upon culture-based techniques
109 that characterizes only a small fraction of the microbial diversity present (19). The
110 introduction of molecular techniques e.g. DNA sequencing of PCR amplicons
111 from ribosomal RNA genes, allowed the detection and enumeration of
112 microorganisms that are refractory to cultivation (41, 62). Each sequence serves
113 as a proxy for the occurrence of a microbial genome in a microbial community.
114 Most of the amplicon sequences from the human gut microbiota correspond to
115 Firmicutes or Bacteroidetes (13) and their total complexity exceeds 15,000
116 different operational taxonomic units (OTUs) (42).
117 For most complex microbial communities including the gut microbiota, a
118 small number of phylotypes dominate population structures and mask the
119 appearance of many distinct but low-abundance taxa in most molecular surveys
120 (55). A meaningful comparison of microbial population structures for different
121 complex communities requires analysis of many thousands of PCR amplicon
122 sequences in order to estimate the relative abundance of different phylotypes
123 and to detect the presence of rare taxa. Recent advances in DNA sequencing
124 technology have permitted the development of methods for deep culture-
125 independent surveys of microbial diversity at relatively low cost. In this
126 communication we conducted controlled experiments to characterize the
127 changes in the community structure of the murine gastrointestinal microbiota

during antibiotic administration and to monitor the response of this community after withdrawal of drug. Using a high-throughput 16S tag sequencing approach targeting the V6 hypervariable region (21, 55), we gained an unprecedented view of the diversity present in the gut microbiota and were able to detail the dynamics of the gut microbial community during periods of ecologic stress brought on by antibiotic administration. We find that antibiotic administration results in reproducible, significant, and in some cases, long lasting, changes in the community structure of the gut microbiota. These changes most likely disturb the balanced interactions between the indigenous microbiota and the host and account for observed changes in gut homeostasis that have been shown to result from antibiotic administration in both clinical and experimental settings.

Materials and Methods

Mouse models and housing conditions. C57BL/6 IL-10$^{-/-}$ mice were from a breeding colony maintained in specific pathogen free conditions at Michigan State University (MSU), derived from mice originally purchased from Jackson Laboratories (Bar Harbor, ME). C57BL/6J wild-type mice were purchased directly from Jackson Laboratories and housed with autoclaved food, bedding, and water. For the antibiotic therapy experiments selected four- to six-week old mice were either treated with antibiotics supplemented in their food (amoxicillin [3.0 mg], metronidazole [0.69 mg] and bismuth [0.185 mg] formulated per 5 gram tablet/day/average [20 g] mouse [BioServ, Frenchtown, NJ]) or in their drinking water (cefoperazone [0.5 mg/mL] [Sigma-Aldrich]). Experiments with amoxicillin, metronidazole and bismuth were carried out at the University Research

151 Containment Facility at MSU and the experiments with cefoperazone were
152 carried out in the Unit for Laboratory Animal Medicine at the University of
153 Michigan. All experimental protocols were approved the animal use and care
154 committees at the respective institutions.
155 **Sample collection and DNA extraction.** At the conclusion of the experiments,
156 mice were euthanized by CO_2 asphyxiation. The cecum of each mouse was
157 removed and washed in phosphate-buffered saline to remove luminal contents.
158 The cecal tip was then excised, bisected, and snap-frozen in liquid nitrogen prior
159 to storage at -80º C. Genomic DNA was then extracted from cecal tip samples
160 (25-100 mg) with the QIAGEN DNeasy® Blood & Tissue Kit using a modified
161 protocol. These modifications included: (1) adding a bead beating step using
162 UltraClean™ Fecal DNA Bead Tubes (MO BIO Laboratories, Inc.) that were
163 shaken using a MiniBeadbeater-8™ (BioSpec Products, Inc.) at the
164 "homogenize" setting for one minute; (2) increasing the amount of Buffer ATL
165 used in the initial steps of the protocol (from 180 μL to 360 μL); (3) increasing the
166 volume of proteinase K used (from 20 μL to 40 μL); and (4) decreasing the
167 amount of Buffer AE used to elute the DNA at the end of the protocol (decreased
168 from 200 μL to 100 μL).
169 **Sequencing and data analysis.** The data presented here are based on 39 PCR
170 amplicon libraries sequenced in five 454 runs using the GS-FLX platform (454
171 Life Sciences, Roche Diagnostics Corp). V6 tag sequence amplicon libraries
172 were constructed as previously described (54). Primer sets corresponding to
173 967F and 1046R used in the library preparation and the permuted primer

approach for sequencing multiple libraries within a single GS-FLX 454 run without use of a physical partition are described in Huber et al., 2007 (21). Primers were trimmed off and all sequences without an exact match to the forward primer, shorter than 50 nt, or containing ambiguous base calls were removed as low quality reads. Sequences were organized in a relational database, and operational taxonomic units were created by aligning with MUSCLE and clustering with DOTUR as described in Huber et al., 2007 (14, 21, 50). Taxonomic assignments were made through direct comparison of tags to a reference database of close to 200,000 distinct V6 sequences and using a consensus of the nearest tags in a global alignment of tags and reference sequences (22).

Quantitative PCR. Quantitative PCR reactions were used to separately assay the quantity of rRNA operons in the DNA samples relative to a singe-copy host gene (mouse TNF-α). A portion of the 16S rRNA encoding gene from *Helicobacter hepaticus* 3B1 was cloned and used as a positive control (between positions 331 and 797 based on *Escherichia coli* numbering of the 16S rRNA gene). A 264-bp portion of the gene encoding TNF- α from *Mus musculus* was also cloned and used as a positive control for the host gene target (between positions 6455 and 6718 of the mouse TNF- α encoding gene; GenBank accession number Y00467). Plasmids were purified from each clone and a 10-fold dilution series was used to determine detection limits of the assay as well as provide standard curves for absolute quantification in the qPCR reactions (range of 10^1-10^7 copies per reaction). Assay volumes were comprised of the

LightCycler® 480 Probes Master reaction mix (Roche) at 1x concentration, and appropriate primer-probe sets to increase specificity of the detected signals from the sample DNA (100 ng). For detection of the bacterial signal 100 nanomoles of each of the forward and reverse primers and the fluorogenic probe were included in the reactions. Sequences for the forward primer (5'-TCCTACGGGAGGCAGCAGT-3'), the reverse primer (5'-GGACTACCAGGGTATCTAATCCTGTT-3'), and the probe (5'-[6-FAM]-CGTATTACCGCGGCTGCTGGCAC-[TAMRA]-3') were based on Nadkarni et al., 2002 (39). Final assay volumes of 20 μL were dispensed in triplicate in 96-well plates. Signals were detected with a LightCycler 480 instrument (Roche). The reaction conditions for amplification of DNA were 95 °C for 10 minutes and 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Detection of the host signal used 200 nanomoles of the forward (TNFa_mu_se; 5'-GGCTTTCCGAATTCACTGGAG-3') and reverse primers (TNFa_mu_as; 5'-CCCCGGCCTTCCAAATAAA-3'), and 100 nanomoles of the probe (TNFa_mu_probe; 5'-[Cy5]-ATGTCCATTCCTGAGTTCTGCAAAGGGA-[Iowa Black RQ™]-3') adapted from Nitsch et al., 2001 (40). Amplification of the host signal began with an incubation at 95°C for 10 minutes, followed by 45 cycles of 95°C for 20 seconds and 64°C for 30 seconds. Comparison in relative bacterial load was performed via the $\Delta\Delta C_T$ method normalizing the 16S signal to the host signal (51).

Results

Antibiotic administration alters the structure of the gut microbiota

To characterize the impact of antibiotic administration on the composition of the gut microbiota, a combination of amoxicillin, metronidazole and bismuth (AMB) was administered to C57BL/6 IL-10$^{-/-}$ mice via their chow for 10 days (Figure 1). The C57BL/6 IL-10$^{-/-}$ strain was chosen as it is utilized as model of inflammatory bowel disease that is responsive to antibiotic therapy (33). The microbial community in one group of mice was assessed immediately following the 10-day treatment, while a second group of mice was switched back to drug free chow for two weeks before microbial community analysis. The microbiota from mice in both groups were compared to a group of control mice that had been fed conventional chow for the duration of the study.

We used a massively-parallel pyrosequencing strategy to retrieve sequences of the V6 hypervariable region of the small-subunit (SSU) rRNA gene (55) to determine the composition of the microbial community associated with the cecal mucosal of these was mice. These SSU sequence tags are generated by PCR amplification and function as proxies for the presence of individual phylotypes present in a given community. The use of pyrosequencing permitted characterization of a greater number of phylotypes than previously practical via PCR amplification, cloning and capillary sequencing of SSU genes. GAST (Global Alignment for Sequence Taxonomy) provided taxonomic assignments for each of the tag sequences (22).

240 We collected a total of 1,006,137 sequence tags were generated from
241 representative samples (9 control, 2 treated and 10 treated followed by
242 recovery). The vast majority of the sequence tags recovered from the cecal
243 community of control animals affiliated with the phyla Bacteroidetes and
244 Firmicutes, with only about 1% belonging to Proteobacteria (Figure 2, Table S1).
245 In the antibiotic-treated animals however, the majority of tags (73,010 of 102,822,
246 [71%]) were Proteobacteria. Two specific tags, assigned to the family
247 Enterobacteriaceae accounted for 67,717 (93%) of these Proteobacteria tags
248 (Table S2). In the control animals, these two tags represented only 106 out of a
249 total of 5214 (2%) tags belonging to Proteobacteria.
250 In the animals whose gut microbial community was allowed to recover via
251 a two-week antibiotic-free period, Firmicutes and Bacteroidetes returned to
252 dominance (70% and 22% of the total number of tags, respectively).
253 Proteobacteria decreased to 5.77% of the total, greater than the 1.2% in the
254 animals that never received antibiotics, but much less than the 73% that they
255 comprised at the end of the AMB treatment (Figure 2). The relative increase in
256 Proteobacteria resulted from increases in tags that mapped to several taxonomic
257 groups within the phylum (Table S1). The two Enterobacteriaceae tags that were
258 dominant in the AMB-treated mice were encountered only 201 times out of a total
259 of 26,964 (0.75%) Proteobacteria tags (Table S2).
260 A global comparison of all of the gut microbial communities in each of the
261 animals was performed by calculating the Bray-Curtis measure of community
262 similarity (34). This index is based upon presence/absence and relative

abundance of each phylotype encountered the mucosa-associated communities. We calculated the average Bray-Curtis similarity for each pair-wise comparison of the control, treated and recovered animals (Table 1). ANOVA of these Bray-Curtis values confirmed that the mucosa-associated microbiota from antibiotic treated animals differed significantly from both the control animals and the recovered animals ($p < 0.05$). The average Bray-Curtis similarity comparing communities from antibiotic-treated animals to communities from the other two experimental groups was significantly lower.

Variability in the murine gut microbiota

Despite the significant differences in the gut microbiota between control animals and animals that received the triple antibiotic cocktail, there was still inter-animal variation noted within each experimental group. The animals in this experiment were selected from a breeding colony maintained at Michigan State University over a time period of approximately 5 months. The animals therefore came from several different litters born to separate mothers.

To determine the degree of similarity amongst animals that shared as many variables as possible, we sequenced and compared 48,594 V6 sequence tags from the mucosa-associated microbiota located in the cecae of three age-matched, wild-type C57BL/6 mice purchased from a commercial vendor. Figure 3 depicts the results of taxonomic assignments and Bray-Curtis measures of community similarity based upon presence/absence and relative phylotype abundance from the mucosa-associated community of each animal. As observed previously, Firmicutes and Bacteroidetes dominated the microbial communities in

286 the cecae of each of the three animals. All three communities displayed similar
287 phylotype distributions at all taxonomic levels with Bray-Curtis similarities > 0.9
288 for all pairwise community comparisons. We recovered approximately 16,000
289 tags from each community (Figure 3). Using an operational taxonomic unit (OTU)
290 assignment of 97% sequence similarity, this yielded ~1000 OTUs in each
291 community. The non-parametric Chao1 estimator (8), suggests that for this
292 sampling effort, there are ~1200 unique 97% OTUs in each mucosa-associated
293 gut community.

294 **Antibiotic administration can result in a prolonged decrease in the**
295 **diversity of the gut microbiota**

296 In spite of the dramatic shifts in the composition of the gut microbiota
297 following administration of the amoxicillin, metronidazole and bismuth cocktail,
298 the community structure returned largely to the baseline state two weeks after
299 discontinuation of the drugs. In an additional pilot experiment, the broad-
300 spectrum cephalosporin antibiotic cefoperazone appeared to have a similar
301 dramatic effect on the microbiota of C57BL/6 IL-10$^{-/-}$ mice, but in this case there
302 were significant long-term effects on the community structure, including lower
303 overall diversity, after antibiotic recovery (data not shown).

304 To extend this initial observation, we undertook an additional antibiotic
305 administration trial employing cefoperazone in twenty female wild type C57BL/6
306 mice (Figure 1B). Five mice were maintained in a single cage on standard mouse
307 chow and sterile water (control group). The remaining 15 mice were switched to
308 water supplemented with cefoperazone (0.5 mg/mL) and after 10 days, these

309 antibiotic-treated mice were divided into three subsequent treatments. Three
310 mice were immediately euthanized to observe the effects of cefoperazone on the
311 gut microbiota. Six mice, housed three animals per cage, were returned to sterile
312 water for six weeks (isolated recovery) while another group of six mice (again
313 divided into two cages) were housed with a control mouse added to each cage
314 during the antibiotic-free period (donor recovery). The addition of the control
315 mouse allowed reinoculation of the gut microbiota via natural coprophagic
316 activity.

317 We recovered a total of 308,505 tag sequences were recovered from the
318 seventeen samples representing the animals in the three experimental groups
319 (Table S3). On average ~18,000 high quality sequence tags (23) were recovered
320 per sample. Amplification of sequence tags was not possible for the three
321 cefoperazone-treated mice that were euthanized at the end of antibiotic
322 treatment (without a drug-free recovery period). Real-time PCR targeting the 16S
323 SSU gene was used to determine to what effect the antibiotics had on overall
324 bacterial load (judged by the relative ratio of the 16S SSU signal to a genomic
325 murine target gene), and whether this could explain the inability to amplify
326 sequence tags from these animals. The bacterial load in animals treated with
327 antibiotics decreased by three orders of magnitude (average fold change 4300)
328 compared to control animals. Both groups of animals that were allowed to
329 recover from antibiotic administration for 6 weeks (isolated recovery and donor
330 recovery) had levels of bacteria that were comparable to the control animals

(average fold change 0.91 for donor recovery animals and 1.20 for isolated recovery animals).

Pairwise Bray-Curtis similarities are displayed in heatmap format (Figure 4) to allow visualization of all of the pair-wise comparisons. The bacterial community in cefoperazone-treated animals six weeks after discontinuation of the drug was distinct from that in control animals. However, the microbial community in the animals that recovered from antibiotic-administration in the presence of an untreated donor animal returned to a state that was very similar to that seen in the control animals.

When we examined the composition of the phylotypes at the level of phylum, the primary distinction was a reduction of Bacteroidetes diversity in the animals that recovered without additional input of microbes. While phylotypes assigned to Bacteroidetes comprised about 15% of the total community in the control animals and in the animals that recovered in the presence of a donor animals, they were only 0.33% of the total community in the animals that recovered in the absence of a donor.

At finer levels of taxonomic distinction, there were additional differences noted between the animals that did not have a donor animal present during the recovery phase compared to either the control animals or those that recovered with a donor (Figure 5 Table S3). At the genus level, the composition of the microbial community from controls and recovery with donor animals was quite different from the composition of the community from animals that recovered

without a donor. These data also suggest that the gut community in animals that recovered without a donor was composed of a decreased number of phylotypes.

Rarefaction curves demonstrated there were fewer phylotypes present in the microbial community from animals that recovered without a donor. Rarefaction analysis involves resampling of community survey data to generate idealized collector's curves, providing an indication of overall phylotype richness (17, 34). In addition, rarefaction can provide an estimate of the depth to which a complex community has been sampled. Rarefaction curves from the control animals and the animals that recovered in the presence of a donor overlapped, confirming that the overall diversity (phylotype richness) was similar between the two (Figure 6). Conversely, rarefaction analysis of the communities from the animals that recovered without a donor indicated that the diversity of these communities was lower than for the other two experimental groups.

Discussion

The myriad interactions between the indigenous gastrointestinal microbiota and their mammalian host have been a focus of considerable recent scientific investigation. Studies on human subjects have the advantage of directly examining the natural community responsible for specific diseases. However, due to technical and ethical constraints of examining the human microbiota, a great deal of effort has been applied to studying model systems, in particular murine models.

Two main lines of research have provided insights about host/microbiota interactions in murine models. Studies in germ free and gnotobiotic mice have

376 demonstrated that gut bacteria can transmit signals that influence host responses
377 (20, 44). However, these are highly simplified systems where community
378 complexity is orders of magnitude lower than naturally occurring murine
379 microbiota. An alternative approach has been to study ecologic stressors shape
380 complex communities in murine model systems. In many cases antibiotics are
381 employed to alter the indigenous microbiota thus disturbing the normal, baseline
382 host/microbe interactions. Such an approach has demonstrated a role for the
383 microbiota in genetic models of murine inflammatory bowel disease (26, 33) and
384 in the modulation of glucose tolerance in mouse models of insulin resistance
385 (37). Antibiotic treatment studies have shown that antibiotic resistant bacterial
386 pathogens can exploit innate immune deficits triggered by antibiotic
387 administration (5). Antibiotic regimens have been used to demonstrate a role for
388 the indigenous microbiota in shaping physiologic responses of the gut mucosa
389 including mediating protective responses to direct epithelial injury (43) and
390 directing the differentiation of IL-17-producing T-helper cells in the mucosa of the
391 small intestine (24). Antibiotic treated mice demonstrate altered susceptibility to
392 experimental infection with pathogenic bacteria. Streptomycin treatment of mice
393 increases susceptibility to oral infection with *Salmonella enterica* serovar
394 Typhimurium (52). A recently described murine model of *Clostridium difficile*-
395 associated colitis employed pretreatment with a mixture of five antibiotics
396 followed by a single dose of clindamycin a day prior to oral challenge with *C.*
397 *difficile* (9).

398 The focus of these studies has generally been on the host response to the
399 alteration in the indigenous microbiota. In most cases the nature of the antibiotic-
400 induced changes on the microbiota were not investigated. Some studies
401 measured changes in total aerobic and anaerobic colts available bacteria
402 following antibiotic administration and in a few cases limited culture-independent
403 investigation of the microbiota was performed. An implicit assumption for these
404 studies is that genetically identical mice harbor a consistent baseline microbiota.
405 A further assumption is that the use of antibiotics would result in reproducible
406 changes in the microbiota that were responsible for the altered host responses
407 observed. This critical assumption has never been formally addressed in detail
408 until the current study.
409 It has been proposed that an adult mammal harbors a stable, “climax
410 community” in each anatomic area of the GI tract (48). Although there can be
411 individual variation in the composition of shallow phylogenetic lineages within the
412 gut microbiota, there are relatively few deep lineages, with Firmicutes and
413 Bacteroidetes generally dominant in most surveys (11, 29). These observations
414 most likely reflect the influence of a variety of ecologic and evolutionary
415 constraints on the gut microbial community (28, 29). Our results, demonstrating
416 marked similarity between the gut microbiota from individual animals, albeit
417 among individuals with identical genetic background maintained in a tightly
418 controlled environment, provide strong evidence that the gut microbial community
419 represents a stable ecosystem. This high degree of similarity also provides

420 evidence for the existence of community “assembly rules” that govern the
421 establishment and stability of these microbial consortia.
422 Perhaps the more critical assumption in experiments that involve antibiotic
423 manipulation of the indigenous gut microbiota is that drug treatment results in
424 reproducible alterations of the microbial community structure. The relative
425 stability of the indigenous microbiota has been debated. From an ecological
426 standpoint, the term stability (also commonly referred to as robustness)
427 encompasses a number of components (2, 31). One aspect is temporal stability,
428 which is the constancy of community structure over time. In addition, the term
429 resistance refers to the ability of a community to maintain a given structure in the
430 setting of a perturbation, while resilience is the ability of a community to return to
431 its baseline structure following a perturbation in community structure. In this
432 regard, if a community exhibits temporal stability, this implies the presence of
433 resistance and resilience in the community structure as one assumes that most
434 communities will experience ecologic stress at some point in time.
435 A number of studies have indicated that an individual’s gut microbiota can
436 have a relatively stable community composition over a period of months to years
437 (36, 49, 58, 63). These observations have led to the conclusion that the
438 community of microbes in the gut is relatively resistant to perturbation by various
439 ecologic stressors. Subsequent environmental influences including diet, host
440 genetics, medication use, and exposure to infectious agents can all have an
441 influence on the resultant microbial community (11). It has been reported that
442 short-term administration of antibiotics (a 7 day course of clindamycin) could

443 result in long-term changes in the structure of the fecal microbiota of humans (12,
444 25, 32). In all of these human studies, there was considerable individual baseline
445 variation in the microbiota, which made it difficult to make inter-individual
446 comparisons in the microbiota responses.

447 Although human studies such as these are important, one advantage of
448 conducting murine experiments as described herein is that the ability to conduct
449 true controlled, replicate experiments. Our replicate experiments allowed us to
450 detect consistent shifts in the gut microbial community in the response to
451 antibiotic administration. The reproducibility of these changes indicates that even
452 if the influences on microbial community structure are complex and numerous,
453 the community will exhibit stereotypic responses, if ecologic stressors are
454 consistently applied. We observed reproducible shifts in the community structure
455 of the gut microbiota following antibiotics including significant alterations in both
456 the richness and distribution of 16S V6 phylotypes. The power of a deep survey
457 of diversity allowed us to demonstrate that certain low abundance phylotypes
458 present at baseline could become dominant in response to the shift in
459 environmental conditions brought about by antibiotic administration. In control
460 animals, 16S V6 tag sequences corresponding to members of the family
461 Enterobacteriaceae comprised only a small fraction of the populations (1%).
462 During administration of amoxicillin, metronidazole and bismuth (AMB), this
463 group of organisms became the most dominant phylotype, indicating that this
464 antibiotic regimen created an environment that somehow favored this taxonomic
465 group of organisms. Simple resistance to the antibiotics cannot entirely explain

466 this observation as other phylotypes were unchanged in relative abundance
467 following AMB administration, but did not undergo the remarkable relative
468 expansion during drug treatment exhibited by the Enterobacteriaceae.
469 In this case, the gut microbial community exhibited resilience as the
470 community structure shifted back towards the baseline state following cessation
471 of the AMB treatment. However, the ability of this community to recover following
472 antibiotic disturbance was not absolute. The administration of cefoperazone also
473 caused dramatic shifts in community structure, but in this case, diversity did not
474 recover even six weeks after the discontinuation of the drug. Rarefaction analysis
475 revealed a persistent, significant decrease in overall species richness in the gut
476 community following cefoperazone administration. However, the addition of an
477 untreated mouse to cages of cefoperazone-treated animals during the recovery
478 phase allowed complete restoration of diversity, presumably through natural
479 coprophagic activity. This observation indicates that cefoperazone administration
480 did not change host physiology, as exposure to a baseline microbiota resulted in
481 normalization of the community structure. Additionally, we infer that the baseline
482 community structure is “preferred” since all four animals in the cage possessed a
483 community that we not distinguish from that in untreated control animals. Since
484 the donor animal were exposed to the altered community present in the
485 cefoperazone-treated animals, it is possible that the resultant communities would
486 possess the antibiotic-altered community structure or an intermediate structure.
487 The reasons for the observed differences in community resilience are not
488 entirely clear. The ecologic disturbance mediated by cefoperazone appears to

489 have overcome the community resilience, potentially due to different spectra of
490 anti-microbial activity. Regardless of the underlying reasons for the differences
491 in observed community resilience, from an experimental standpoint it is important
492 to understand that manipulation of the indigenous gut microbiota by various
493 antibiotic regimens may result in altered community structures that persist even
494 after the antibiotic is discontinued. Whether or not the gut community returns to
495 the baseline state after perturbation can influence the conclusions that can be
496 drawn from a particular experiment.

497 The implications of long-lasting changes in community diversity following
498 antibiotic administration are several-fold. Even though the microbial composition
499 of the animals that recovered from cefoperazone treatment remained altered
500 compared to baseline, overall bacterial biomass returned to the level observed in
501 control mice. It has been postulated that functional redundancy in complex
502 microbial communities can allow an altered community to perform equivalent
503 ecosystem functions as the original community (2). Studies are ongoing to
504 determine if the specific alterations in the gut microbiota result in any significant
505 changes in gut ecosystem functioning.

506 One function of the gut microbiota that has captured our attention is that of
507 “colonization resistance”, the ability of the indigenous microbiota to prevent
508 ingress of pathogens into the gut community (16, 18, 59). Antibiotic associated
509 colitis resulting from *Clostridium difficile* infection may result from a loss of the
510 intrinsic colonization resistance of the gut microbiota (4, 38). Theoretically, the
511 administration of antibiotics could disturb the indigenous microbiota allowing *C.*

512 *difficile* spores encountered in the environment to germinate and successfully
513 colonize the gut (4, 61). Although *C. difficile* infection responds to the
514 administration of specific anti-microbial therapy, including metronidazole or
515 vancomycin, recurrence following the end of *C. difficile* therapy has become an
516 increasing problem (35). In a previous study, we have provided evidence that
517 recurrent *C. difficile* infection is associated with a decrease in fecal microbial
518 diversity (7). This observation is in line with the fact that the administration of
519 stool from healthy individuals to patients with recurrent *C. difficile* can break the
520 cycle of recurrence (1, 57). The data presented here indicate that antibiotic
521 therapy of sufficient magnitude can result in an altered microbial community. It
522 remains to be determined if this can be directly correlated with a loss of
523 colonization resistance, but our findings provide evidence that antibiotic
524 administration can result in long term decreases in gut microbial diversity, which
525 in turn is associated with recurrent *C. difficile* disease.
526 As we learn more about the intricate relationship between the gut
527 microbiota and their host we may find that unintended disturbance of this
528 microbial community will have significant deleterious health effects. A more
529 complete understanding of the ecologic forces that determine the formation and
530 maintenance of microbial community structures could lead to novel ways to
531 prevent or treat diseases that result from disruptions of the gut microbiota.

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Figure legends

Figure 1. **Schemata for antibiotic administration.** A. Fifteen C57BL/6 IL-10$^{-/-}$ mice received the combination of amoxicillin, metronidazole and bismuth in their chow for 10 days while 10 animals remained on control chow. Mice were either euthanized immediately after antibiotic administration or after a 2-week period of recovery on non-medicated chow. Control animals remained on non-medicated chow for the entire experiment. B. Five animals remained on sterile water while 15 mice were treated with 0.5 mg/ml of cefoperazone in sterile drinking water for 10 days. The antibiotic-treated animals were subsequently divided into three groups. One group of 3 animals was immediately sacrificed. One group of 6 animals (divided into two cages) was returned to sterile water without antibiotics for a 6-week recovery period. A final group of 6 animals (also divided into two cages) was returned to water without antibiotics, and a non-treated control mouse was added to the cage for the 6-week recovery period. The cecae of all animals were harvested for microbial community analysis.

Figure 2. **Comparison of the microbial community composition in the cecae of antibiotic treated mice.** Over one million V6 sequence tags were retrieved and classified from cecal DNA purified from untreated mice (Control), animals that received amoxicillin, metronidazole and bismuth (AMB) in chow for 10 days (Antibiotic Treated) or AMB-treated mice that were allowed to recover on plain chow for two weeks (Recovery). The sequence tags were classified to the level

of bacterial division (phylum). The pie charts show the distribution of the pooled tags for each experimental group with the mean ± S.D. distribution of tags recovered from each individual animal is indicated.

Figure 3. **Genus level diversity of the gut communities in the cecae of control animals.** Approximately 16,000 V6 SSU hypervariable region tags were retrieved from the cecal mucosa-associated microbiota from each of three wild-type C57BL/6 mice. Pie charts show the distribution for the most prevalent taxonomically assigned tags while the percentages for the 12 most common assignments is indicated below. Bray-Curtis similarities were calculated for each pairwise comparison. The nonparametric Chao1 diversity estimator was calculated for each community based on 97% sequence similarity.

Figure 4. **Comparison of microbial communities in cefoperazone-treated animals.** Over 300,000 V6 sequence tags were retrieved from the cecae of cefoperazone treated mice that recovered from drug treatment in the presence or absence of an untreated “donor” animal. The tags were assigned a taxonomy at the level of genus and the pair-wise Bray-Curtis distance calculated for all possible comparisons. The Bray-Curtis values are presented in a heatmap fashion as a color-coded distance matrix with the most similar (Bray-Curtis similarity of 1.0) represented by blue and the most dissimilar (Bray-Curtis similarity of 0.0) represented in red. The housing of the animals is indicated and the animals that served as “donor” in each cage are marked with a star.

Figure 5 **Genus level diversity of the gut communities from cefoperazone-treated animals.** The taxonomic assignments of V6 tags from untreated animals (control), animals that recovered without an untreated animal (isolated recovery) and animals that recovered in the presence of an untreated animal (donor recovery) are shown. The pie charts show the most abundant genus level assigned tags for the pooled animals in each experimental group. The average (± S.D.) distribution of tags recovered from each individual animal is indicated below. ND = not detected.

Figure 6 **Rarefaction analysis of microbial communities from cefoperazone treated animals.** The number of assigned phylotypes as a function of the number of tags retrieved. The V6 tags from untreated animals (control), animals that recovered without an untreated animal (isolated recovery) and animals that recovered in the presence of an untreated animal (donor recovery) were used to construct rarefaction curves with an OTU definition of >97% sequence similarity.

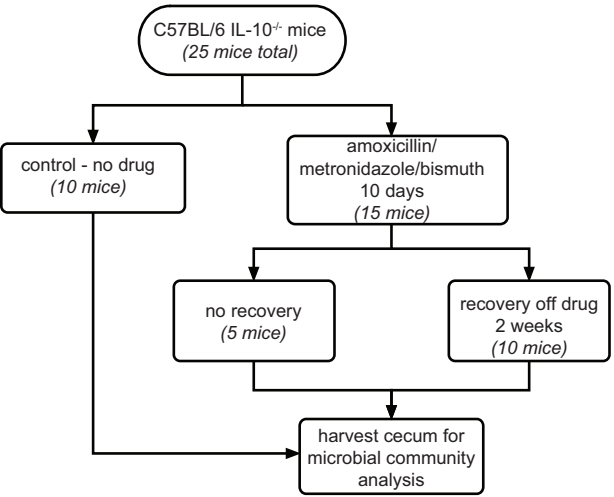
Table 1 Bray-Curtis similarities comparing communities from AMB-treated mice

	Control	Recover	Treated
Control (n=9)	0.765 ± 0.014[a1]		
Recover (n=11)	0.756 ± 0.008[a]	0.749 ± 0.011[a]	
Treated (n=2)	0.212 ± 0.019[b]	0.205 ± 0.018[b]	0.845 ± 0.083[a]

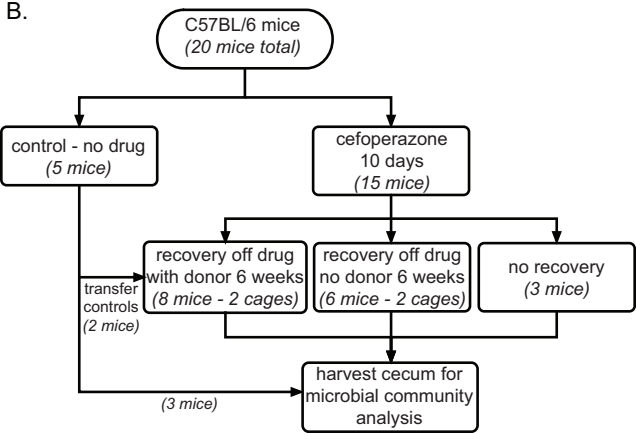
[1] average ± S.D.

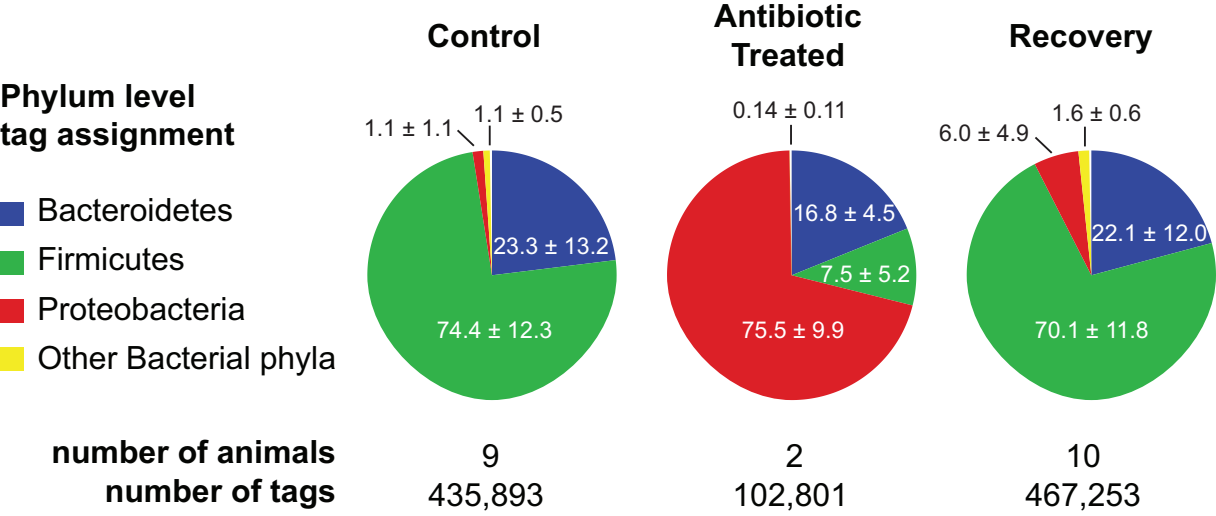
[a,b] values not connected by the same letter are significantly different. Groups were compared by ANOVA with significance set at $p < 0.05$ by Tukey Kramer.

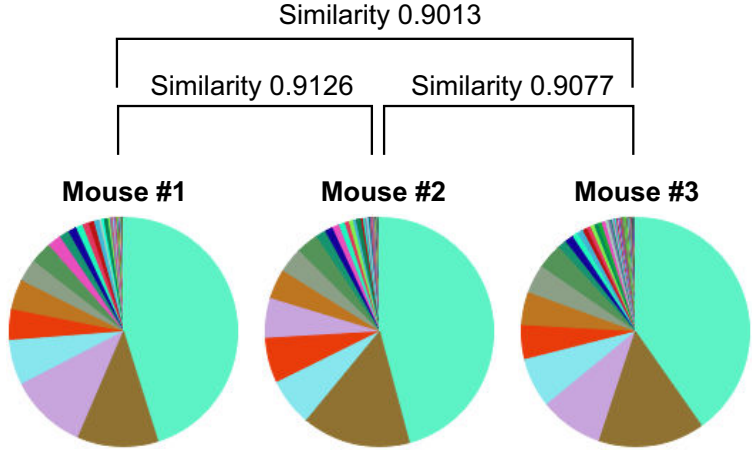
A.


C57BL/6 IL-10-/- mice
(25 mice total)
control - no drug
(10 mice)
amoxicillin/
metronidazole/bismuth
10 days
(15 mice)
no recovery
(5 mice)
recovery off drug
2 weeks
(10 mice)
harvest cecum for
microbial community
analysis

B.

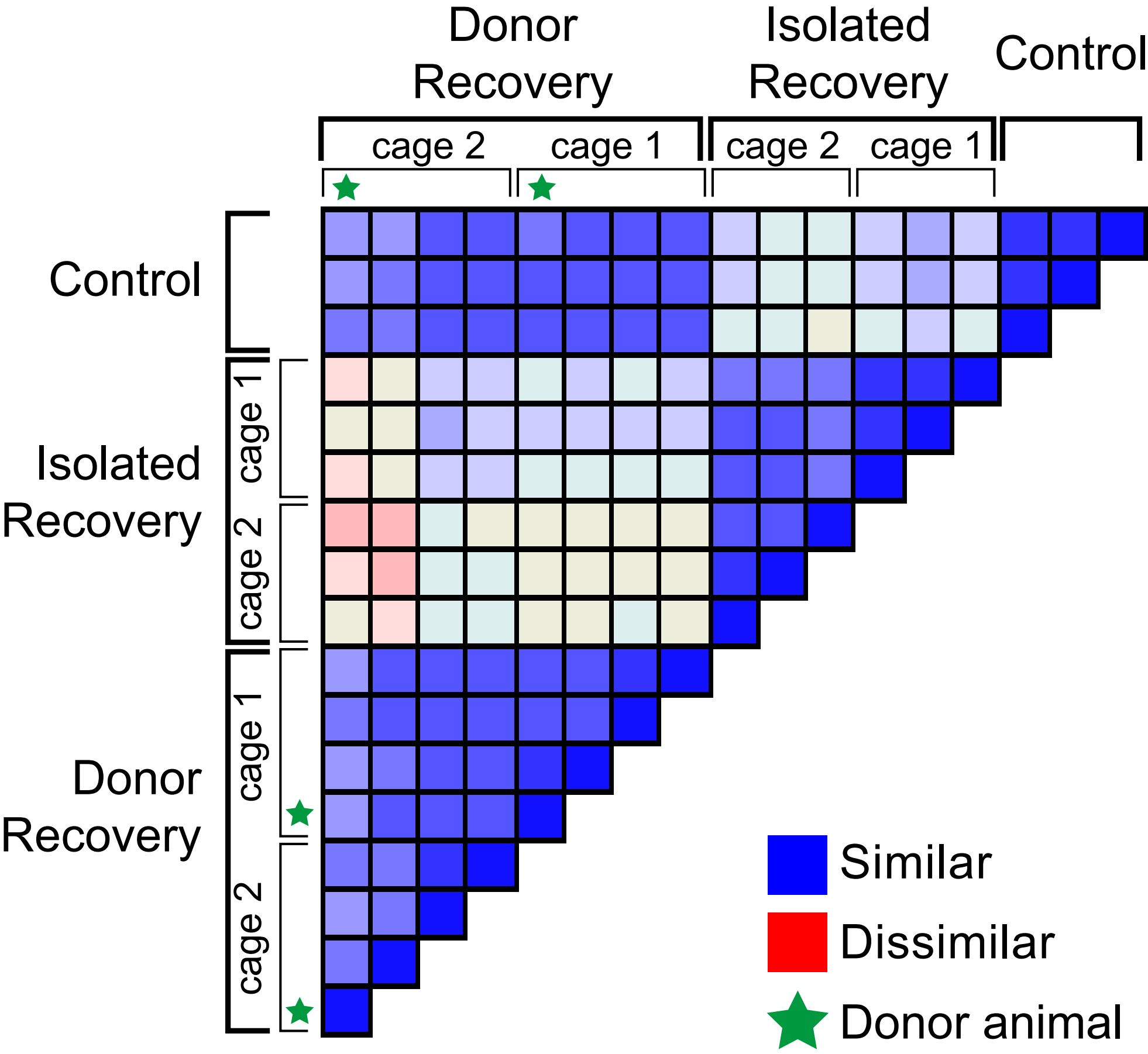

C57BL/6 mice
(20 mice total)
control - no drug
(5 mice)
cefoperazone
10 days
(15 mice)
recovery off drug
with donor 6 weeks
(8 mice - 2 cages)
recovery off drug
no donor 6 weeks
(6 mice - 2 cages)
no recovery
(3 mice)
transfer
controls
(2 mice)
(3 mice)
harvest cecum for
microbial community
analysis

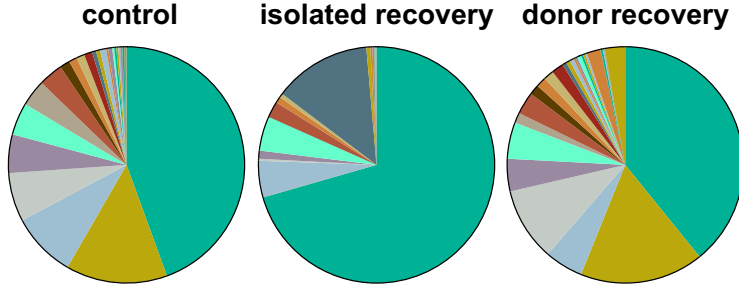

Control
Antibiotic Treated
Recovery
Phylum level tag assignment
Bacteroidetes
Firmicutes
Proteobacteria
Other Bacterial phyla
1.1 ± 1.1
1.1 ± 0.5
23.3 ± 13.2
74.4 ± 12.3
0.14 ± 0.11
16.8 ± 4.5
7.5 ± 5.2
75.5 ± 9.9
6.0 ± 4.9
1.6 ± 0.6
22.1 ± 12.0
70.1 ± 11.8
number of animals
number of tags
9
435,893
2
102,801
10
467,253



	Mouse #1	Mouse #2	Mouse #3
Total tags	16490	16172	15932
Unique tags	967	928	1038
Chao estimate	1183	1095	1399
Chao 95% c.i.	1128-1257	1049-1158	1314-1510

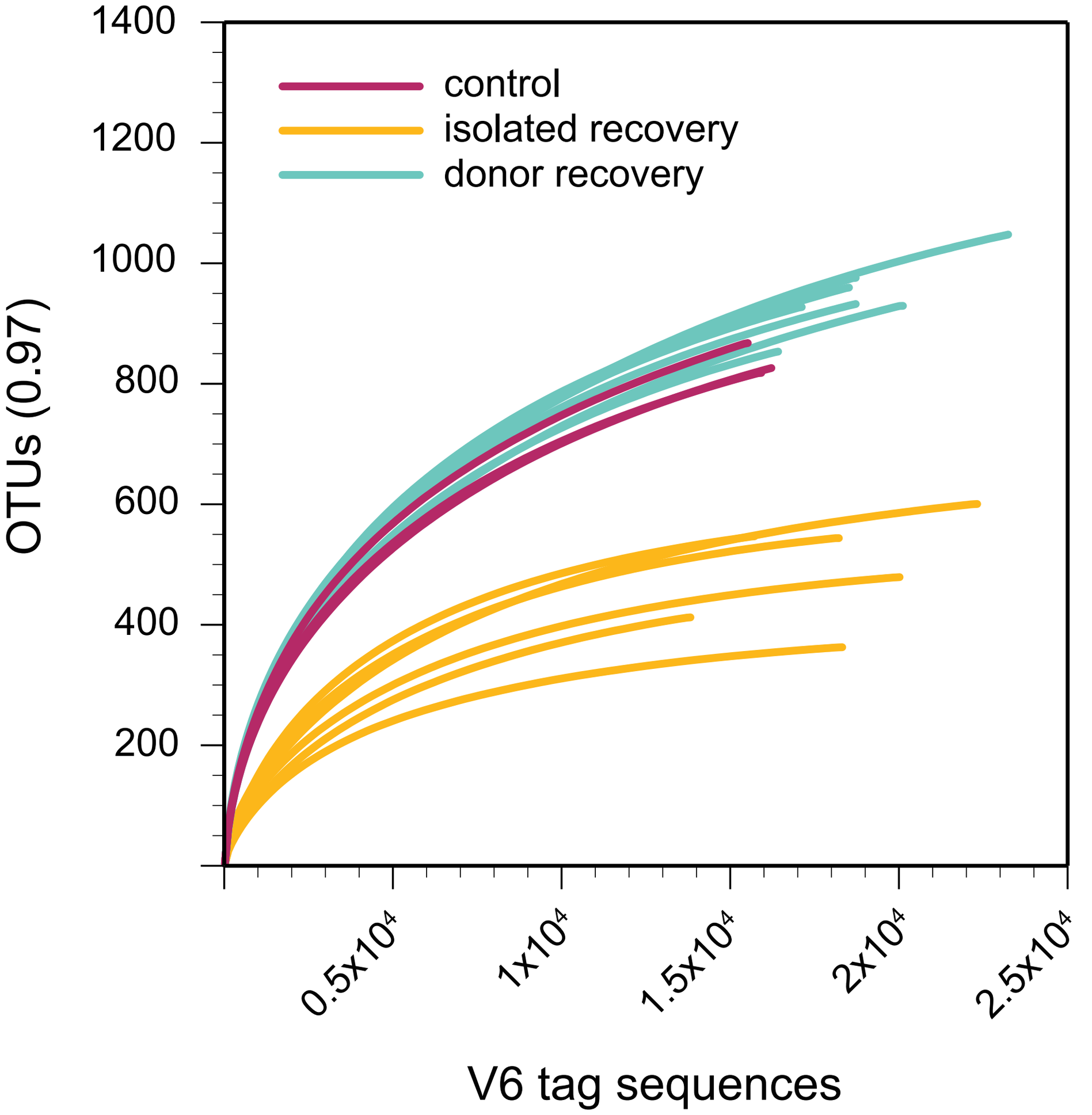
Genus level tag assignment	**Percentage of total community**		
Firmicutes Lachnospiraceae NA	43.8	47.9	43.3
Bacteroidetes Porphyromonadaceae NA	11.4	15.2	14.9
Proteobacteria Pseudomonadaceae Pseudomonas	10.8	6.3	8.9
Firmicutes NA	9.5	8.7	6.6
Firmicutes Ruminococcaceae NA	7.6	5.5	7.0
Firmicutes Ruminococcaceae Paillibacter	4.5	4.8	4.9
Bacteria NA	2.8	3.3	3.7
Firmicutes ErysipelotichaceaeTuricibacter	1.9	0.9	0.5
Firmicutes Clostridiaceae NA	0.8	0.8	0.4
Firmicutes NA Bryantella	0.8	0.8	0.8
Firmictues Rumunococcaceae Ruminococcus	0.8	0.3	1.1
Firmicutes Eubacteriaceae Anaerovorax	0.6	0.5	0.5


Donor Recovery
Isolated Recovery
Control
cage 2
cage 1
cage 2
cage 1
Control
Isolated Recovery
cage 1
cage 2
Donor Recovery
cage 1
cage 2
Similar
Dissimilar
Donor animal



	control	isolated recovery	donor recovery
number of animals	3	6	8
average intragroup similarity	0.91 ± 0.02	0.84 ± 0.06	0.85 ± 0.06

Genus level tag assignment	**Average percentage (±S.D.)**		
Bacteroidetes Lachnospiraceae NA	47 ± 2.4	73 ± 3.8	46 ± 7.8
Bacteroidetes Porphyromonadaceae NA	15 ± 2.3	0.1 ± .24	18 ± 4.1
Proteobacteria Pseudomonadaceae Pseudomonas	8.9 ± 2.8	5.5 ± 3.1	6.1 ± 3.3
Firmicutes Ruminococcaceae NA	8.1 ± 0.8	1.4 ± 1.3	11 ± 2.5
Firmicutes NA NA	7.9 ± 1.8	6.2 ± 5.9	4.9 ± 0.9
Firmicutes Ruminococcaceae Papillibacter	5.0 ± 0.3	4.1 ± 3.8	6.0 ± 1.0
Bacteria NA NA	3.5 ± .52	0 (ND)	1.2 ± 0.62
Firmicutes Erysipleotrichaceae Turicibacter	1.2 ± .72	0.5 ± 0.6	1.3 ± 0.3
Firmicutes Ruminococcaceae Ruminococcus	0.91 ± 0.27	0 (ND)	0.8 ± 0.34
Firmicutes NA Bryantella	0.83 ± 0.045	0.8 ± 1.2	0.6 ± 0.17
Firmicutes Eubacteriaceae Anaerovorax	0.54 ± 0.51	0 (ND)	0.39 ± 0.09
Firmicutes Burkholderiaceae Ralstonia	0.4 ± 0.14	0.23 ± 0.2	0.33 ± 0.24
Firmicutes Clostridiaceae Clostridium	0.3 ± 0.18	8.6 ± 5.4	0.45 ± 0.28
Firmicutes Erysiplotrichaceae Allobaculum	0.07 ± 0.02	0.023 ± 0.056	3.1 ± 2.0
Firmicutes Clostridia NA	0.53 ± 0.30	0 (ND)	0.35 ± 0.10


control
isolated recovery
donor recovery
OTUs (0.97)
1400
1200
1000
800
600
400
200
0.5x10⁴
1x10⁴
1.5x10⁴
2x10⁴
2.5x10⁴
V6 tag sequences