**Title:** The gut microbiota mediates clearance of *Clostridium difficile* infection independent of adaptive immunity

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JLL and VBY conceived the study. JLL, KCV, and MLJ performed experiments and analyzed the data. All authors contributed to writing the manuscript and had access to all of the data.

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**Abstract** (words, current: 247, max: 350)

**Background:** *Clostridium difficile,* a Gram-positive, anaerobic bacterium is the leading single cause of nosocomial infections in the United States. A major risk factor for *C. difficile* infection (CDI) is prior exposure to antibiotics. Antibiotics increase susceptibility to CDI by altering the membership of the microbial community enabling colonization.

The importance of the gut microbiota in providing protection from CDI is underscored by the reported 80-90% success rate of fecal microbial transplants in treating recurrent infection. Adaptive immunity, specifically humoral immunity, is also sufficient to provide protection from both acute and recurrent CDI. However the role of the adaptive immune system in modulating *C. difficile* colonization has yet to be resolved. In this study we sought to determine if adaptive immunity plays a role in decreasing *C. difficile* colonization.

**Results:** Using murine models of CDI, we found that adaptive immunity is dispensable for clearance of *C. difficile*. Furthermore, the indigenous microbial community membership before either antibiotic administration or infection can be used to predict which animal will clear the infection.

**Conclusions:** This work indicates that the indigenous gut microbiota is the main factor that limits the colonization of the GI tract by *C. difficile*. Adaptive immune response, while able to limit acute disease by limiting toxin-mediated damage, has no effect on pathogen colonization. This study also has implications for the design of preclinical studies testing the efficacy of vaccines on levels of colonization as inherent differences in the baseline community structure of animals within cages may bias findings.

**Introduction/Background:**

*Clostridium difficile,* a Gram-positive, anaerobic bacterium is the leading single cause of nosocomial infections in the United States. A major risk factor for *C. difficile* infection (CDI) is prior exposure to antibiotics. Antibiotics increase susceptibility to CDI by altering the membership of the microbial community and thus the metabolome of the gut, enabling colonization [[1](#_ENREF_1)]. Colonization with *C. difficile* can manifest in a number of clinical syndromes ranging from asymptomatic colonization, inflammatory colitis characterized by diarrhea and abdominal pain, and in severe cases death. In addition to primary infection, one in five patients treated for CDI experience recurrent disease [[2](#_ENREF_2)].

Disease is mediated by the production of two toxins, TcdA and TcdB, which are the major virulence factors for *C. difficile* [[3](#_ENREF_3)]. TcdA and TcdB are large multi-domain proteins which inactivate cellular rho-family GTPases via the addition of a glucose molecule. Inactivation of these key regulatory proteins in epithelial cells results in disruption of tight junctions, increased paracellular flow, and eventually leads to cell death [[4](#_ENREF_4)].

The importance of the gut microbiota in providing protection from CDI is underscored by the reported 80-90% success rate of fecal microbial transplants in preventing recurrent infection [[5-7](#_ENREF_5)]. Other than microbiome-mediated prevention of colonization, adaptive immunity, specifically humoral immunity, is also sufficient to provide protection from both acute and recurrent CDI likely via antibody mediated neutralization of *C. difficile* toxins A and B [[8-10](#_ENREF_8)]. However, the role of the adaptive immune system in modulating *C. difficile* colonization has yet to be resolved.

In this study we sought to determine if adaptive immunity plays a role in decreasing *C. difficile* colonization. We found that adaptive immunity is dispensable for clearance of *C. difficile*. Furthermore, the indigenous microbial community membership before either antibiotic administration or infection can be used to predict which animal will clear the infection.

**Methods**

**Animal Husbandry.** Both male and female C57BL/6 specific-pathogen-free (SPF) mice age five to twelve weeks were used in these studies. The wild type mice were from a breeding colony at the University of Michigan, originally derived from Jackson Laboratories over a decade ago. The Rag1-/- (B6.129S7-*Rag1tm1Mom*/J) micewere from a breeding colony started with mice from Jackson Laboratories in 2013.

Animals were housed in autoclaved filter top cages with autoclaved bedding, and water bottles. Mice were fed a standard irradiated chow (LabDiet 5LOD) and had access to food and water *ad libitum*. Cage changes were carried out in a biological safety cabinet. The frequency of cage changes varied depending on the experiment. To prevent cross-contamination between cages, hydrogen peroxide-based disinfectants in addition to frequent glove changes were utilized during all manipulation of the animals. The mice were maintained under 12-hours of light/dark cycle in facilities maintained at temperature of 72C +/- 4 degrees. Animal sample size was not determined by a statistical method. Multiple cages of animals for each treatment were used to control for possible differences in the microbiota between cages. Mice were evaluated daily for signs of disease, those determined to be moribund were euthanized by CO2 asphyxiation. Animal studies were conducted under the approval of The University of Michigan Committee on the Care and Use of Animals; husbandry was performed in an AAALAC-accredited facility.

**Spore Preparation.** Spore stocks of *C. difficile* strain 630 (ATCC BAA-1382) were prepared as previously described with the following modifications; strains were grown overnight in 5mL of Columbia broth, which was added to 40 mL of Clospore media [[1](#_ENREF_1),[11](#_ENREF_11)].

**Infections.** In experiments comparing colonization in WT and Rag1-/- mice, age and sex matched mice were co-housed for thirty-three days starting at three weeks of age and continuing through cefoperazone administration. Upon infection, animals were separated into single genotype housing.

Mice were made susceptible to infection by providing ad libitum drinking water with the addition of 0.5mg/mL cefoperazone (cat # 0219969501, MP Pharmaceuticals) in Gibco distilled water. The antibiotic water was changed every two days and was provided for 10 days. Following two days of supplying drinking water without antibiotic, mice were challenged with either spores or water (mock). *C. difficile* spores suspended in 50μL of Gibco distilled water were administered via oral gavage. The number of viable spores in each inoculum was innumerate by plating for colony forming units (CFU) per mL-1 on pre-reduced taurocholate cycloserine cefoxtin fructose agar (TCCFA). Over the course of the infection, mice were weighed routinely and stool was collected for quantitative culture. Mice were infected with between 102 and 104 CFU.

**Quantitative Culture.** Fresh voided fecal pellets were collected from each mouse into a pre-weighted sterile tube. Following collection, the tubes were reweighed and passed into an anaerobic chamber (Coy Laboratories). In the chamber, each sample was diluted 1 to 10 (w/v) using pre-reduced sterile PBS and serially diluted. 100uL of a given dilution was spread on to pre-reduced TCCFA or when appropriate TCCFA supplemented with either 2 or 6 ug/mL of erythromycin. Strain 630 is erythromycin resistant; use of erythromycin in TCCFA plates reduced background growth from other bacteria in the sample. Plates were incubated anaerobically at 37C and colonies were enumerated at 18-24 hours. Plates that were used to determine if mice were negative for *C. difficile* were held and rechecked at 48 hours.

**Splenocytes Recovery and Transfer.** Spleens from individual animals were aseptically harvested from donor mice. Following harvest, the organ was gently ground up to remove the cells from the capsule. Cells were suspended in RPMI complete media and the suspension was passed through 40um cell strainer to remove large debris. Red blood cells were lysed with RBC lysing buffer (Sigma R7757) for a few minutes. Following lysis, cells were pelleted by centrifugation at 15000 rpm for 5 minutes at 4C. Cells were enumerated manually using a haemocytometer and re-suspended in Leibovitz’s L-15 (Corning 10-045-CV) media. Recipient mice were injected with XXXXX cells in 0.25mL L-15 media into the peritoneal cavity. Mice that received vehicle were injected with 0.25mL of L-15 media only.

**Serum Collection.** Blood was collected intoSerum was collected from either from the saphenous vein for pre-treatment time points or via heart puncture at the experimental endpoint.

**Total IgG ELISA.** Total serum IgG levels were measured using the IgG (Total) Mouse Uncoated ELISA Kit (ThermoFisher Scientific Cat# 88-50400). Each sample was diluted 500-fold in assay buffer and run in duplicate with Southern Biotech TMB Stop Solution (Cat# 0412-01) used as the stop solution. Optical density values were measured at 450nm and 570nm on a VersaMax plate reader (Molecular Devices, Sunnyvale, CA) and corrected by subtracting the 570nm measurement from the 450nm measurement. A 4-Parameter Standard Curve was used to calculate sample concentration values.

**Anti-*C. difficile* TcdA IgG ELISA.** Titers of serum IgGspecific to *C. difficile* TcdA (toxin A) was measured by ELISA as previously described, with the following modifications [[12](#_ENREF_12)]. Serum from Rag1-/- mice that received an adoptive transfer was diluted 1:50 in blocking buffer with subsequent serial dilutions to a final dilution of 1:12150. Serum from the wild-type mice was diluted 1:1200 in in blocking buffer with subsequent serial dilutions to a final dilution of 1:874800. Each sample was run in duplicate. Each plate had the following negative controls: all reagents except serum, all reagents except toxin and pre-immune serum if applicable. Additionally, each plate had a positive control consisting of toxin coated wells reacted with mouse TcdA monoclonal antibody clone TCC8 diluted 1:5000 in blocking buffer (antibodies-online.com). The optical density at 410nm and 650nm was recorded on a VersaMax plate reader (Molecular Devices, Sunnyvale CA). The absorbance for each sample was corrected by subtracting the OD650 reading from the OD410 reading. The anti-TcdA IgG titer for each sample was defined as the last dilution with a corrected OD410 greater than average corrected OD410 of the negative control wells plus three times the standard deviation of those wells.

**DNA Extraction.** Freshly voidedfeces were collected from each mouse into sterile 1.7mL snap cap tube. Samples utilized for quantitative culture were often also used for DNA extraction and sequencing. Samples were stored at -80C until extraction. Genomic DNA was extracted from approximately 200-300 μl of fecal sample using the MoBio Power-soil htp 96 Well DNA isolation kit (formerly MoBio, now Qiagen) on the Eppendorf EpMotion 5075 automated pipetting system according to manufacturer’s instructions.

**Sequencing.** The University of Michigan Microbial Systems Laboratory constructed amplicon libraries from extracted DNA as described previously [[13](#_ENREF_13)]. Briefly, the V4 region of the 16S rRNA gene was amplified using barcoded dual index primers as describe by Kozich et al. [[14](#_ENREF_14)]. The PCR reaction included the following: 5 μl of 4 μM stock combined primer set, 0.15 μl of Accuprime high-fidelity Taq with 2 μl of 10× Accuprime PCR II buffer (Life Technologies, #12346094), 11.85 μl of PCR-grade water, and 1 μl of template. The PCR cycling conditions were as follows: 95°C for 2 minutes, 30 cycles of 95°C for 20 s, 55°C for 15 s, and 72°C for 5 minutes, and 10 minutes at 72°C. Following construction, libraries were normalized and pooled using the SequelPrep normalization kit (Life Technologies, #A10510-01). The concentration of the pooled libraries was determined using the Kapa Biosystems library quantification kit (KapaBiosystems, #KK4854) while amplicon size was determined using the Agilent Bioanalyzer high-sensitivity DNA analysis kit (#5067-4626).  Amplicon libraries were sequenced on the Illumina MiSeq platform using the MiSeq Reagent 222 kit V2 (#MS-102-2003) (500 total cycles) with modifications for the primer set. Illumina’s protocol for library preparation was used for 2 nM libraries, with a final loading concentration of 4 pM spiked with 10 % PhiX for diversity. The raw paired-end reads of the sequences for all samples used in this study can be accessed in the Sequence Read Archive under XXXXXXXXXXXXXX.

**Sequence Curation and Analysis.** Raw sequences were curated using the mothur v.1.39.0 software package [[15](#_ENREF_15)] following the Illumina MiSeq SOP. Briefly, paired end reads were assembled into contigs and aligned to the V4 region using the SLIVA 16S rRNA sequence database (release v128) [[16](#_ENREF_16)], any sequences that failed to align were removed; sequences that were flagged as possible chimeras by UCHIME were also removed [[17](#_ENREF_17)]. Sequences were classified with a naïve Bayesian classifier [[18](#_ENREF_18)] using the Ribosomal Database Project (RDP) and clustered in to Operational Taxonomic Units (OTUs) using a 97% similarity cutoff with the Opticlust clustering algorithm [[19](#_ENREF_19)].

To reduce erroneous signal, the shared file was filtered to remove any OTU that was in less than three samples across the entire data set. The number of sequences in each sample was then rarefied to 10,000 sequences to minimize bias due to uneven sampling. The mothur implementation of LefSe (linear discriminant analysis effect size) was used to determine OTUs that differentiated IgG positive verses negative mice [[20](#_ENREF_20)]. Following curation in mothur, further data analysis and figure generation was carried out in R (v 3.3.3) using standard and loadable packages [[21](#_ENREF_21)]. The data and code for all analysis associated with this study are available at <https://github.com/jlleslie/AdaptiveImmunity_and_Clearance>.

Most of the analysis relied on the R package vegan [[22](#_ENREF_22)]. This includes, determining the axes for the multidimensional scaling (MDS) plots using Bray-Curtis dissimilarity calculated from sequence abundance. Additionally, vegan was used to determine significance between groups using ANOSIM, calculation of Inverse Simpson index, and Bray-Curtis dissimilarity a between samples. Final figures were modified and arranged in Adobe Illustrator CC. For the purpose of distinguishing between values that were detected at the limit of detection versus those that were undetected, all results that were not detected by a given assay were plotted at an arbitrary point below the LOD. However for statistical analysis, the value of LOD/√2 was substituted for undetected values. Wilcoxon ranked sum test was used to determine significant differences and when appropriate, reported p-values were corrected for multiple comparisons using the Benjamini–Hochberg correction.

**Random Forest Analysis.** Random Forest analysis was performed using R (v.3.2.3) using the randomForest package [[23](#_ENREF_23),[24](#_ENREF_24)]. Model parameters ntree and mtry were tuned based on the input datasets in order to achieve optimal classification without over-fitting [[25](#_ENREF_25)]. Briefly, ntree was calculated by multiplying the total number of OTUs included in the analysis by a ratio of the quantity of samples in each classification category. Additionally, mtry was defined as the square root of the number of OTUs. The significance cutoff for Mean Decease Accuracy (MDA) values was determined by the absolute value of the lowest MDA measured. Testing for significant difference in OTU relative abundance following feature selection was performed using Wilcoxon signed-rank test with Benjamini–Hochberg correction.

**Results**

**Effect of reconstitution of adaptive immunity on C*. difficile* colonization**

We sought to determine the contribution of adaptive immunity in clearance of *C. difficile*. To address this question we asked if reconstitution of adaptive immunity via transfer of splenocytes from wild-type mice into RAG1-/- mice, which are deficient in both B and T cells, is sufficient to clear *C. difficile*. Reports of immunization with various *C. difficile* antigen suggests that antibodies to these antigens may decrease colonization so we additionally asked if transfer of cells from mice immunized via natural infection with *C. difficile* might facilitate clearance [[26](#_ENREF_26),[27](#_ENREF_27)]. Splenocytes were collected from wild type donor mice that were either naïve or colonized with *C. difficile* strain 630 for three weeks (figure 1A, supplemental figure1). The development of humoral immune responses to *C. difficile* in the donor mice was confirmed by the detection of high titers of anti-TcdA IgG in the serum while uninfected mice had undetectable levels of anti-TcdA serum IgG (p < 0.01) (figure 1B). Prior to the adoptive transfer, recipient mice (RAG1-/-) were made susceptible to infection by pre-treatment with the antibiotic cefoperazone and then inoculated with *C. difficile* strain 630 spores. Three days after *C. difficile* challenge, mice received splenocytes transfer. Recipient mice were randomly assigned to one of three groups and either received splenocytes from naïve wild type donors, splenocytes from infected wild type donors or vehicle (figure 1A). To confirm the successful engraftment of the WT cells, we measured total serum IgG in the recipient mice three-weeks post transfer. The mice that received splenocytes had significantly higher levels of serum IgG three-weeks post-transfer compared to the mice that received vehicle (p <0.05) (figure 1C). Of the mice that received splenocytes, two did not develop any detectable serum IgG. There was no difference in the levels of serum IgG between the mice that received splenocytes from infected donors versus uninfected donors (p >0.05). Furthermore, we determined that we successfully transferred anti-*C. difficile* immunityas we detected anti-TcdA IgG only the serum from the mice that received splenocytes from the infected donors (p < 0.01) (figure 1D).

Following adoptive transfer, levels of *C. difficile* in the feces were monitored for three weeks. We observed clearance of *C. difficile* from mice that were housed in a single cage. However clearance of *C. difficile* did not occur in any of the animals in the two other cages within that treatment group (figure 2A). Three weeks post transfer there was no significant difference in levels of colonization in any of the groups (figure 2B). Notably, in the cage that cleared, one of the mice had undetectable levels of serum IgG while the other three mice in the cage had detectable levels. Together these results suggest that presence of adaptive immunity is not required for clearance of *C. difficile*.

**Effect of Reconstitution of Adaptive Immunity on the Fecal Microbiota**

The range in the levels of colonization we observed within each treatment group suggested that something other than adaptive immunity might be playing a role in modulating levels of *C. difficile*. The gut microbial community is crucial in protecting from initial colonization and for facilitating clearance of *C. difficile* [[28](#_ENREF_28)]. Using 16S rRNA gene amplicon sequencing we examined the gut microbial community structure of the mice over the course of the experiment. Visualization of the Bray-Curtis dissimilarity between the day one post infection communities (before the adoptive transfer) using multidimensional scaling revealed that the mice that cleared *C. difficile* had a distinct community compared to the mice that remained colonized, ANOSIM, p = 0.02, R= 0.7363 (figure 2C). This result suggests that gut microbiota rather than the adoptive transfer of splenocytes enabled clearance of *C. difficile*.

The microbiota and the immune response interact in a complex manner, whereby each can modulate the other [[29](#_ENREF_29),[30](#_ENREF_30)]. In the cefoperazone mouse model of infection, the diversity of microbiota begins to recover by two weeks follow cessation of the antibiotic [[31](#_ENREF_31)]. Therefore we asked if reconstitution of adaptive immunity altered the recovery of the community following antibiotics and infection with *C. difficile*. Our first approach sought to determine if we could detect changes in the overall community composition of the mice. We calculated the Bray-Curtis dissimilarity between each mouse’s day twenty-one sample (nineteen days after the adoptive transfer) and their pre-antibiotic sample. The closer the Bray-Curtis metric is to one, the less similar the two samples are. We hypothesized that the addition of adaptive immunity might prevent the microbiota from returning to the same structure as was observed before adoptive transfer. Thus we thought that perhaps the mice that received splenocytes might have higher Bray-Curtis dissimilarity values compared to the vehicle group. Since we were unable to confirm that we successfully restored adaptive immune function in two of mice that received splenocytes, we excluded them from the rest of analysis as our questions hinged on immune status-gut microbiota interactions. Additionally we lost the ability to calculate this metric for a couple of mice due to the lack of pre-antibiotic samples. Comparing the Bray-Curtis dissimilarity results between the three treatment groups revealed no significant differences between any of the groups (figure 3A). We also wondered if addition of adaptive immunity might alter alpha diversity so we calculated the inverse Simpson index of each fecal community at day 21-post transfer. However again we did not observe any significant differences between the treatment groups (figure 3B). This suggests that by broad metrics of community structure, the perturbation of antibiotics and infection with *C. difficile* potentially mask any trends related to reconstitution of adaptive immunity.

While we saw no significant differences in the recovery of the community structure or alpha diversity at day 21-post infection, we wondered if perhaps the abundance of only a few OTUs were altered by reconstitution of the adaptive immune system. For this analysis we grouped all of the mice that received splenocytes and developed detectable levels of serum IgG at day 26-post infection together and called them IgG positive. The mice that only received vehicle and thus had undetectable levels of serum IgG were designated the IgG negative group. Using sequence abundance from day 21 post infection samples, LefSe identified 27 OTUs with LDA values greater than 2. The ten OTUs with the highest LDA values were primarily enriched in the IgG negative mice (figure 3C). OTU 3, which is classified as Akkermansia, had the highest LDA value. This OTU was found at a lower abundance in the IgG positive mice compared to the mice IgG negative mice. Interestingly another study also observed a decrease in the abundance of Akkermansia following reconstitution of adaptive immunity via adoptive transfer of from wild-type mice into RAG1-/- [[32](#_ENREF_32)]. Since our model included pre-treatment with antibiotics and infection with *C. difficile* we were admittedly surprised to have observed the same trend. However, the identified OTUs indicate that restoration of adaptive immunity can and do alter levels of colonization of some gut bacteria taxa.  **Structure of the fecal microbiota before treatment is associated with clearance In both WT and RAG1-/- mice**

To test if the gut microbial community is sufficient to provide clearance of *C. difficile* rather than transferring splenocytes into the mice, we co-housed RAG1-/- mice with WT mice (figure 4A). After a little over a two weeks of co-housing, the two groups consisting of both wild-type mice into RAG1-/- of mice had significantly different community structures as demonstrated by the MDS plot of the Bray-Curtis dissimilarities (ANOSIM, p = 0.042, R= 0.1756) (figure 4B). Following infection we again observed some mice cleared while others remained colonized. In this experiment the two cages were both from the same cohousing group (figure 4A,C). At the conclusion of the experiment the two cages that remained colonized had significantly higher levels of *C. difficil*e compared to the mice that cleared (figure 4C). Clearance or colonization did not track with genotype but rather co-housing group (figure 4D). These results in addition to our previous experiment demonstrate that clearance of *C. difficile* is independent of adaptive immunity.

**Random Forest feature selection identifies three OTUs crucial for classification of mice that will clear.**

We constantly observed associations between an altered structure of the gut microbiome and clearance of *C. difficile*. Specifically these differences were observed early in the experimental timeline, even before antibiotics (figure 4B). Thus we wondered if there were OTUs present in the mice before any intervention that might be used to predict if the mice would clear the infection. For this analysis, we pooled data from three independent experiments (the two described earlier and a third experiment including only WT mice) where cages of mice had spontaneously cleared *C. difficile* (figure S2). We utilized Random Forest for feature selection to identify OTUs that could classify mice as “cleared” or “colonized” based on their pre-intervention microbiota. Using the pre-treatment community we could classify mice with 76.9% accuracy. Nine out of the top ten OTUs that most contributed to classification were from the Firmicutes phylum (figure 5A and B). Three OTUs in particular (OTUs 52, 93, and 26) ranked highest in their ability to discriminate between the groups. Therefore we tested if those three OTUs alone were sufficient to classify the mice. Generating a new Random Forest model using only those three OTUs, we found that the out of bag error did not increase.

**Discussion**

In this study we asked if adaptive immunity was required for clearance for the gastrointestinal pathogen *C. difficile*. Results from multiple experimental models lead us to conclude that adaptive immunity is dispensable for clearance of *C. difficile* in mice. Our results show that reconstitution of adaptive immunity can alter the abundance of some bacteria in the gut however it does impact levels of *C. difficile* colonization. This finding is in contrast to the paradigm observed in other gastrointestinal infections. For example infection with the attaching effacing pathogen *Citrobacter rodentium*, provides a framework by which the adaptive immunity plays a role in clearance [[33](#_ENREF_33)][[34](#_ENREF_34)]. Currently the role of attachment to the host epithelium in *C. difficile* pathogenesis is unresolved. In addition to the potential direct effects adaptive immunity may have on the bacterium itself, it is known that there is a complex interaction loop between the microbiota and adaptive immunity. Both the innate and adaptive arms of the immune system regulate membership of the gut microbial community while the gut microbiota in turn modulates the immune system via the production of metabolites and/or MAMPs.

Based on our repeated observations that altered communities early in the experimental timeline were associated with clearance of *C. difficile* we used Random Forest to eventually identify just three OTUs that discriminate between mice that clear vs. remain colonized with 76.47% accuracy. Previous work using a similar approach identified OTUs present on the day of challenge that were predictive of levels of colonization, however we are the first group to look at how the gut microbiota of mice before any treatment might affect the outcome of C*. difficile* infection [[35](#_ENREF_35)]. Of three OTUs we identified, two belonged to the family *Lachnospiraceae.* Our group has previously observed that high levels of *Lachnospiraceae* is associated with protection from severe disease in a murine model of CDI [[36](#_ENREF_36)]. Furthermore we have also reported that mono-association of germ-free mice with a single *Lachnospiraceae* isolate partially restored colonization resistance [[37](#_ENREF_37)]. It is tempting to speculate multiple *Lachnospiraceae* isolates might be able to fully restore colonization resistance. However, it remains to be seen if the same mechanisms, which prevent initial colonization of *C. difficile*, play a role in clearance of *C. difficile.* Our results suggest that community resilience is inherent to the structure of the community. Additionally it suggests the possibility of predicting who will be most at risk for persistent colonization before they undergo therapy (reach). Finally, the findings from this study have implications for the design of future preclinical studies testing the efficacy of vaccines or other manipulations of adaptive immunity on levels of colonization as “cage effects” or inherent differences in the baseline community structure of animals within cages may bias results.

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