**Title:** Protection from lethal *Clostridioides difficile* infectionvia intra-species competition for co-germinant

**Running Title:** Glycine mediated colonization resistance to CDI

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**Abstract**

*Clostridioides (Clostridium) difficile* a Gram-positive, spore-forming bacterium is the primary cause of infectious nosocomial diarrhea. Antibiotics are a major risk factor for *C. difficile* infection (CDI) as they disrupt the gut microbial community, enabling increased germination of spores and growth of vegetative *C. difficile*. To date the only single species bacterial preparation that has demonstrated efficacy in reducing recurrent CDI in humans is non-toxigenic *C. difficile.* Using multiple infection models we determined that pre-colonization with a less virulent strain is sufficient to protect from challenge with a lethal strain of *C. difficile,* surprisinglyeven in the absence of adaptive immunity. Additionally, we showed that protection is dependent high levels of colonization by the less virulent strain and that it is mediated by exclusion of the invading strain. The prevailing hypothesis in the field as has been that pre-colonization with one strain of *C. difficile* limits vegetative growth of the challenging strain. Our results challenge this model and suggest that reduction of amino acids, specifically glycine following colonization by the first strain of *C. difficile* is sufficient to decrease germination of the second strain thereby limiting colonization by the lethal strain.

**Importance**

**Introduction**

*Clostridioides (Clostridium) difficile* a Gram-positive, spore-forming bacterium is the primary cause of infectious nosocomial diarrhea ([1](#_ENREF_1), [2](#_ENREF_2)). Susceptibility to *C. difficile* infection (CDI) results from perturbations of the gut microbial community, enabling increased germination of spores and growth of vegetative *C. difficile* ([3](#_ENREF_3), [4](#_ENREF_4)). Following colonization, vegetative *C. difficile* produces toxins. The primary toxins, TcdA and TcdB, are glycosyltransferases that inactivate cellular GTPases ([5](#_ENREF_5)). Inactivation of these key cellular proteins results in damage of the colonic epithelium and inflammation manifesting as diarrhea and in severe cases, toxic megacolon or even death. Currently the principal treatment for acute CDI is the antibiotic vancomycin ([6](#_ENREF_6)). While this treatment limits vegetative *C. difficile*, this therapy further disrupts the gut microbiota potentially delaying community recovery ([7](#_ENREF_7)).

Due to the high rate of recurrent infection associated with existing treatment for CDI, alternative approaches that spare or even restore the gut microbiota have been a focus of recent work. As is the case with many toxin-mediated diseases early studies noted that generation of a humoral immune response to the toxins could be sufficient to protect against disease ([8](#_ENREF_8), [9](#_ENREF_9)). A recent clinical trial demonstrated that patients receiving monoclonal antibodies targeting the toxins were fifty percent less likely to experience recurrent disease ([10](#_ENREF_10), [11](#_ENREF_11)). Unlike antibiotics, antibody therapy prevents illness while likely sparing the microbial community. Recently, a non-toxigenic strain of *C. difficile* was demonstrated to successfully reduce the rate of recurrent CDI by approximately fifty percent ([12](#_ENREF_12)). The prevailing hypothesis is that the protection provided by the non-toxigenic strain is mediated by competitive exclusion, limiting the ability of toxigenic *C. difficile* to colonize the gut ([13](#_ENREF_13)). However this has never been conclusively demonstrated. Using a murine model of CDI we sought to address this question.

From our studies we determined that in the absence of adaptive immunity, pre-colonization with one strain of *C. difficile* is sufficient to provide protection from lethal infection with another. Using gnotobiotic mice we show that protection is mediated by limiting colonization of the highly virulent strain. Furthermore, we provide evidence that exclusion is not predicated on nutrient-based limitation of the vegetative form of the invading strain, but rather on depletion of the co-germinant glycine. This work is important, as it is the first study to identify a possible mechanism though which pre-colonization with *C difficile*, a current clinical therapy, provides protection from recurrent CDI. Furthermore, limitation of germination due to decreased levels of glycine in the gut is a novel paradigm for colonization resistance.

**Materials and Methods.**

**Animals and housing**

Both male and female mice age five to twelve weeks were used in these studies. The wild-type (WT) C57BL/6 specific-pathogen-free (SPF) mice were from a breeding colony originally derived from the Jackson Laboratory over a decade ago. The RAG1-/- (B6.129S7-*Rag1tm1Mom*/J) SPF micewere from a breeding colony started with mice from the Jackson Laboratory in 2013. Germfree RAG1-/- mice were obtained from a colony established and maintained by the University of Michigan germfree facility.

Animal husbandry was performed in an AAALAC-accredited facility. Animals were housed with autoclaved cages, 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 performed in a biological safety cabinet. To prevent cross-contamination between cages, hydrogen peroxide-based disinfectants in addition to frequent glove changes were utilized during all manipulation of SPF animals. A chlorine-based disinfectant was used during manipulation of the germfree mice. The frequency of cage changes varied depending on the experiment. All mice were maintained under a cycle of 12-hours of light/dark in facilities maintained at temperature of 72° C +/- 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. The University Committee on the Care and Use of Animals at the University of Michigan approved this study.

**Preparation of spores**

Spore stocks of *C. difficile* strains 630 (ATCC BAA-1382) and VPI 10463 (ATCC 43255) were prepared as previously described by ([3](#_ENREF_3)) with the following modifications, strains were grown overnight in 5mL of Columbia broth which was added to 40 mL of Clospore media ([14](#_ENREF_14)).

**Preparation of autoclaved *C. difficile***

Heat-killed *C. difficile* strain 630 was made from an overnight culture grown anaerobically at 37°C in brain-heart infusion (BHI) broth supplemented with 0.01% cysteine. The culture was enumerated by platting for colony forming units (CFU) per mL-1. Following plating, the culture was removed from the chamber, spun down, and the pellet was washed and re-suspend in PBS. The solution was autoclaved to kill the vegetative cells and inactivate any spores. The heat-killed *C. difficile* solution was equivalent to 2.7x1010 per mL-1. In experiments using heat-killed *C. difficile,* mice were given 109 CFU equivalents in 0.05mL. A portion of the sample was cultured on pre-reduced cycloserine-cefoxitin-fructose agar plates containing 0.1% taurocholate (TCCFA) to confirm that the inactivation was successful.

**Infections**

In experiments using both WT and RAG1-/- SPF mice, age and sex matched mice were co-housed starting at three weeks of age for thirty-three days through antibiotic administration. Upon infection, animals were separated into single genotype housing. In one experiment, two cages of mice remained co-housed throughout the infection; no obvious difference in infection outcome was observed.

All SPF mice received the antibiotic cefoperazone (MP Pharmaceuticals, 0219969501) dissolved in Gibco distilled water at concentration of 0.5 mg/mL for either 10 or 5 days ([4](#_ENREF_4), [15](#_ENREF_15)). While mice were on antibiotics, the water was changed every two days. Following completion of antibiotics, mice were given plain Gibco distilled water for two days before challenge with either spores or water (mock). *C. difficile* spores suspended in 50-100μL of distilled water were administered via oral gavage. The number of viable spores in each inoculum was innumerate by platting for CFU per mL-1 on pre-reduced TCCFA. Over the course of the infection, mice were weighed routinely and stool was collected for quantitative culture.

In our persistent colonization model, forty-one days after primary infection mice were given an IP of clindamycin (Sigma, C5269) in sterile saline at concentration of 10 mg/kg to perturb the gut microbial community as described previously ([3](#_ENREF_3), [16](#_ENREF_16)). The next day mice were either mock challenged with water or with spores from strain VPI 10463. In the short-term infection model, mice were challenged with one strain and the following day challenged with the other. In the simultaneous co-infections, mice were challenged with different ratios of strain 630 and strain VPI 10463 within a total amount of 104 spores.

At the conclusion of the experiments mice used in dual genotype experiments were genotyped using DNA from an ear snip using primers and cycling conditions as outline by The Jackson Laboratory.

**Quantitative culture from intestinal content**

Fecal pellets or colonic content 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 6ug/mL of erythromycin. Strain 630 is erythromycin resistant; use of 2ug/mL of erythromycin in TCCFA plates reduced background growth from other bacteria in the sample while TCCFA with 6ug/mL of erythromycin enabled selection of 630 (erythromycin resistant) from VPI 10463 (erythromycin sensitive). Plates were incubated at 37°C in the anaerobic chamber and colonies were innumerate at 18-24 hours. Plates that were used to determine if mice were negative for *C. difficile* were held for 48 hours.

**Toxin activity assay**

Intestinal content was collected from each mouse into a pre-weighted sterile tube and stored at -80°C. At the start of the assay each sample was diluted 1:10 weight per volume using pre-reduced sterile PBS. Following dilution, the sample was filter sterilized through a 0.22μm filter and the activity of the toxins was assessed using a Vero cell rounding-based cytotoxicity assay as described previously ([17](#_ENREF_17)).

**Histopathology evaluation**

Mouse ceca and colon tissue were saved in histopathology cassettes and fixed in 10% formalin, followed by storage in 70% ethanol. McClinchey Histology Labs Inc (Stockbridge, MI) prepared the tissue including embedding samples in paraffin, sectioning, and generation of haematoxylin and eosin stained slides. A board certified veterinary pathologist scored the slides blinded to the experimental groups, using previously described criteria ([16](#_ENREF_16), [18](#_ENREF_18)).

**Anti-TcdA IgG ELISA**

Blood was collected from mice via saphenous vein puncture into capillary blood collection tubes. Samples were spun down and serum was stored at -80°C. Levels of IgGspecific to *C. difficile* TcdA were measured in serum from mice using a previously described ELISA protocol ([19](#_ENREF_19)) with a few modifications. Briefly, 96-well EIA plates were coated with 100μl of purified *C. difficile* TcdA (List Laboratories, cat #152C) at 1μg/mL in 0.05M sodium bicarbonate buffer pH 9.6 overnight at 4°C. Mouse serum was diluted to 1:400 in blocking buffer and serially diluted 1:3. Each sample was run in duplicate. Negative controls included pre-immune sera from the mice as well as wells serum negative wells. A positive control consisting of a monoclonal mouse anti-TcdA IgG antibody was run on each plate. The optical density at 410 nm was recorded on a VersaMax pate reader (Molecular Devices, Sunnyvale CA). The anti-Toxin A IgG titer was defined as the last dilution where both replicates had an OD410 greater than mean absorbance of all negative wells on the plate plus three times the standard deviation from that mean.

**Serum neutralizing anti-toxin antibody assay**

The serum neutralizing anti-toxin antibody assay was based on a previously described assay ([9](#_ENREF_9)). With the following modifications: the concentration of purified TcdA or TcdB (List Laboratories) that resulted in 100% cell rounding was determined empirically, using thetoxin activity assay. Vero cells were seeded at a density of 105 cells/0.075mL per well onto tissue culture treated plates. Four times the concentration of toxin that resulted in 100% cells rounding (64μg/mL for TcdA and 4μg/mL for TcdB) was incubated with serial dilutions of serum from mice. The serum toxin mixture was added to Vero cells and incubated overnight. The neutralizing titer was determined to be the last dilution with had less than 50% of round cells in the well. Each sample was run in duplicate and the results from discordant wells were averaged. Toxin only wells served as negative controls while goat anti-TcdA and B serum served as a positive control (TechLab T5015). Sera from mice before they were infected were run as an additional negative control.

**Scoring of clinical signs of disease**

Mice were scored for signs of disease based on criteria previously described by ([20](#_ENREF_20)). A member of the lab who had not been party to the experiment scored the mice before necropsy to avoid bias.

**Quantitative PCR**

Primer3 was used to design primers that differentiated strain VPI 10463 (IMG Genome ID: 2512047057) from strain 630 (GenBank: AM180355.1) using publically available genomes. While the primers differentiated between the strains in pure culture they also picked up other bacteria in the context of the microbiota and were only used in gnotobiotic experiments. VPI 10463 primers: Forward: 5’- TTTCACATGAGCGGACAGGC -3’, Reverse: 5’-TCCGAAGGAGGTTTCCGGTT-3’. The expected product size is 153 nucleotides and the optimal annealing temperature was empirically determined to be 56°C.

For quantitative PCR, DNA from fecal samples were diluted in ultrapure water such that such that 20 ng of DNA was added to each reaction. Each sample was run in triplicate. Samples were loaded into a Light cycler 480 multiwell plate, with FastStart Essential DNA Green master mix, and 0.5μM of each primer. The plate was sealed with optically clear sealing tape, briefly spun down, and run on the Roche LightCycler 96. The following conditions were used for qPCR: 95°C for 10 minutes, followed by thirty cycles of 95°C for 10 seconds, 56°C for 10 seconds, and 72°C for 10 seconds. A melt cycle was run at the conclusion of the amplification.

Genomic DNA from VPI 10463 from a culture with a known quantity of CFU was used to generate a standard curve. Negative controls included no template control wells in addition to dilutions of genomic DNA from strain 630.

**Preparation of cecal media**

Cecal media was prepared from SPF mice that were treated for ten-days with cefoperazone. Uninfected mice were sacrificed two (day 0) or three days (day 1) after the completion of the antibiotics. Media was also made from infected mice, following our antibiotic regime, two days after the completion of antibiotics mice were infected with strain 630 and sacrificed one day later (630 day 1). Following euthanasia, the cecum was removed and the content was squeezed into a sterile 50mL conical. The conical was spun at 3000 rpm at RT for ten minutes to separate the solid matter from the liquid portion. The liquid portion of the sample was removed and diluted 1:2 by volume in sterile PBS (pH 7.4). The sample was spun again at 3000 rpm for 5 minutes at RT; the liquid portion was then filter sterilized using filters with successively smaller pore size (from 0.8μm, to 0.45μm, and finally a 0.22μm filter). Following passage through the 0.22μm filter, the media was frozen at -80°C until use. The media was tested for sterility by inoculating into pre-reduced BHI in the anaerobic chamber; samples that gave rise to turbid growth after 48 hours were discarded.

***Ex vivo* vegetative growth**

To determine if 24 hours of strain 630 growth depletes the gut of the nutrients required for vegetative growth of strain VPI 10463 we used an *ex vivo* approach, utilizing sterile cecal media from susceptible mice. 180μL aliquots of day 0 cecal media were thawed and allowed to equilibrate in the anaerobic chamber overnight. The inoculums were prepared from an overnight culture of *C. difficile* (strains 630 and VPI 10463) grown in BHI + 0.01% cysteine. Each culture was back diluted 1:10 and incubated at 37°C for two hours. After two hours had elapsed, 500μL of the culture was spun down for four minutes in the anaerobic chamber using a mini-centrifuge. To prevent the introduction of nutrients from carry over BHI, the supernatant was removed and the tubes were spun for an additional minute. Any remaining BHI was removed and the pellets were then suspended in 500μL of sterile anaerobic PBS. Both strains were then diluted 1:100 into sterile anaerobic PBS. 20μl of this 1:100 dilution was inoculated into the cecal media. Vegetative CFU were enumerated at T=0, 24 hours by platting on CCFA (which lacks the germinant taurocholate). Additionally samples were plated on BHI + 0.01% cysteine to check for any contamination. After 24 hours, the samples were removed from the chamber; cells were pelleted by centrifugation at 2,000 g for five minutes. The supernatant was filter sterilized using a 0.22μm 96-well filter plate and the sterile flow through was passed back into the chamber to equilibrate overnight. The following day, the VPI 10463 inoculum was prepared as described earlier and inoculated into the spent cecal media. CFU were monitored by plating sample at T=0, 6, and 24 hours on CCFA and BHI+0.01% cysteine.

***Ex vivo* germination assay**

To determine if 24 hours of strain 630 growth altars the ability of VPI 10463 spores to germinate we performed an *ex vivo* germination assay based on a previously described method ([21](#_ENREF_21)) with the following modifications. Rather than intact content we used sterile cecal media from mice that were off of antibiotics for three days (day 1 cecal media) or infected with strain 630 for 24 hours (630 day 1 cecal media). 180μL aliquots of the cecal media were thawed and allowed to equilibrate in the anaerobic chamber overnight. The next day 10uL of spores from strain VPI 10463 were inoculated into the media. Controls included sterile PBS (Gibco cat#10010023), PBS + 0.1% sodium taurocholate, and PBS + 0.1% sodium taurocholate + 100mM glycine. Following inoculation, samples were incubated anaerobically at RT for 15 minutes, after which approximately half the volume was immediately plated on BHI + 0.1% Taurocholate +0.01% cysteine agar and the tubes were passed out of the chamber and placed in a water filled heat block set at 65°C for 20 minutes. The heating step kills off any vegetative cells. Additional controls included plating the spore inoculum on BHI without taurocholate to check for presence of any vegetative cells in the stock as well as heating suspensions of vegetative cells to confirm efficacy of heat killing. Following the 20 minutes incubation, tubes were passed back into the chamber and the remaining sample was plated on BHI + 0.1% Taurocholate +0.01% cysteine agar. The CFU recorded from the pre-heat plate represents the entire inoculum including remaining spores and any cells that germinated, while the post-heat plate represents only remaining spores.

**Metabolomics**

Quantification amino acid concentrations in the cecal media via targeted metabolomics was performed by the University of Michigan Medical School Metabolomics Core. Amino acids were measured using the Phenomenex EZfaast kit. Samples were extracted, semipurified, derivitized and measured by EI-GCMS using norvaline as an internal standard for normalization.

**DNA extraction**

Genomic DNA was extracted from approximately 200-300 μl of fecal sample using the MoBio PowerSoil HTP 96 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([7](#_ENREF_7)). Briefly, the V4 region of the 16S rRNA gene was amplified using barcoded dual index primers as describe by Kozich et al. ([23](#_ENREF_23)). The PCR reaction included the following: 5μl of 4μM stock combined primer set, 0.15μl of Accuprime high-fidelity Taq with 2μl of 10× Accuprime PCR II buffer (Life Technologies, #12346094), 11.85μl of PCR-grade water, and 1μl of template. The PCR cycling conditions were as follows: 95°C for 2 minutes, 30 cycles of 95°C for 20 seconds, 55°C for 15 seconds, 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 4pM 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 PRJNA388335.

**Sequence curation and analysis**

Raw sequences were curated using the mothur v.1.39.0 software package ([24](#_ENREF_24)) following the Illumina MiSeq standard operating procedure. Briefly, paired end reads were assembled into contigs and aligned to the V4 region using the SLIVA 16S rRNA sequence database (release v128) ([25](#_ENREF_25)), any sequences that failed to align were removed; sequences that were flagged as possible chimeras by UCHIME were also removed ([26](#_ENREF_26)). Sequences were classified with a naïve Bayesian classifier ([27](#_ENREF_27)) using the Ribosomal Database Project (RDP) and clustered in to Operational Taxonomic Units (OTUs) using a 97% similarity cutoff with the Opticlust clustering algorithm ([28](#_ENREF_28)).

The number of sequences in each sample was then rarefied to 9,000 sequences to minimize bias due to uneven sampling. Following curation in mothur, further data analysis and figure generation was carried out in R (v 3.3.3) using standard and loadable packages([29](#_ENREF_29)). The data and code for all analysis associated with this study are available at <https://github.com/jlleslie/Intraspecific_Competition>. 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.

**Results**

**Development of murine model of persistent *C. difficile* colonization**

The only single species bacterial preparation that has demonstrated efficacy in reducing recurrent CDI in humans is non-toxigenic *C. difficile* ([12](#_ENREF_12)). However the mechanisms by which one strain of *C. difficile* prevents colonization of another are currently unknown. To begin to address this question we developed a model of persistent *C. difficile* colonization. Mice were made susceptible to colonization via administration of the antibiotic cefoperazone. Following two days off of the antibiotic, mice were either mock challenged or challenged with *C. difficile* strain 630. Compared to mock-challenged animals, infected animals displayed significant weight loss between days four to six post-challenge (figure 1A, p<0.05). After ten days, infected mice remained highly colonized despite a significant reduction in fecal levels of *C. difficile* relative to levels on day one post-infection (figure 1 B, p<0.01). Contemporaneous with the decrease in colonization, infected animals recovered weight until they were indistinguishable from the mock-infected animals (figure 1A). Additionally, the diversity of the gut microbiota increased within the first ten days of infection corresponding to the recovery of weight and decrease in colonization (figure 1C box plots, right axis). Throughout the experiment, toxin was detected in the feces of most of the infected mice. However, toxin started to decrease by day 14 and was significantly decreased by day 33 post challenge relative to early in the infection (figure 1D). Together these data demonstrate that *C. difficile* strain 630 can persistentlycolonize wild type mice as a minority member of the gut microbiota (figure 1C, left axis).

**Pre-colonization with *C. difficile* protects mice from challenge with a highly virulent strain and doesn’t require the adaptive immune response.**

To determine if a resident strain of C*. difficile* protects mice from challenge with a second strain in the context of perturbation to the gut microbiota, we administered a second antibiotic, clindamycin, to both the colonized and un-colonized mice. Clindamycin did not result in weight loss in either group of mice (Supplemental figure 1A). However, levels of strain 630 in the colonized mice significantly increased following administration of clindamycin likely because it is resistant the antibiotic (Supplemental figure 1B, p< 0.001) ([30](#_ENREF_30)). The day after mice were given clindamycin, they were challenged with 105 CFU of strain VPI 10463 spores. Strain VPI 10463 expresses both TcdA and TcdB and is lethal in this model of infection ([16](#_ENREF_16), [31](#_ENREF_31)). To control for possible variations in the microbiota across cages, mice from 630-infected and mock-infected (naïve) cages were split into two groups (figure 2A). Approximately half the mice in a cage were challenged with VPI 10463 while the rest of the mice we placed in a new cage and mock infected. Naïve mice that had neither been infected with 630 nor VPI 10463 maintained stable weight, however naïve mice that were challenged with VPI 10463 lost a significant amount of weight and had to be euthanized (figure 2B, p<0.01). Mice that were persistently colonized with 630 did not lose weight despite being challenged with the lethal strain. In addition, 630 pre-colonized mice had significantly lower toxin titers than the naïve mice challenge with VPI 10463 (figure 2C, p<0.05). This finding was confirmed by the histopathology, as the total score of the colon was significantly less in the 630 colonized mice challenged with VPI 10463 compared to the naïve mice challenged with VPI 10463 (figure 2D p<0.01).

Since both strains express nearly identical forms of TcdA and TcdB and we observed decreased signs of disease in the primary infection after a week of first being challenged we questioned if protection might be due to the development of a humoral immune response to the toxins ([9](#_ENREF_9), [32](#_ENREF_32)). We found that mice previously infected with 630 developed a high anti-TcdA titer with a median titer of 32,400 with a portion of these antibodies being neutralizing, capable preventing TcdA mediated cell rounding (figure 2E 630 colonized vs. naïve mice p< 0.0, 2F).

Since protection was correlated with both pre-colonization and the development of an adaptive immune response to the toxins, we next tested if adaptive immunity was the sole factor preventing disease in our model by utilizing mice defective in recombination-activating gene 1 (RAG), a gene that is critical in the development of B and T cells. RAG1-/- lack the adaptive arm of their immune system ([33](#_ENREF_33)). Following forty days of colonization mice were given an IP of clindamycin and then challenged with the lethal strain VPI 10463. Surprisingly, both the RAG1-/- and WT mice pre-colonized with strain 630 were protected following the challenge, while the naïve mice of both genotypes succumbed to the infection (figure 3A and B, naïve vs. colonized p<0.05). Scoring of the colonic pathology demonstrated that both the RAG1-/- and WT mice pre-colonized with 630 had less pathology relative to the naïve mice (figure 3C, naïve vs. colonized p<0.05). These data demonstrate that in the absence of adaptive immunity pre-colonization with *C. difficile* is sufficient to protect from lethal infection with another strain. Furthermore, protection from severe disease in mice pre-colonized with 630 is not mediated by adaptive immunity.

**Protection afforded by low virulence *C. difficile* strain develops rapidly and depends on limiting colonization of the lethal strain**

Having ruled out the role of adaptive immunity in our model, we next sought to determine if protection required treatment with live*C. difficile*. Other groups have demonstrated that triggering of innate immune pathways with microbial associate molecular patterns, such TLR5 with flagella, protects against acute CDI ([34](#_ENREF_34), [35](#_ENREF_35)). Additionally in other colitis models, both viable and heat-killed probiotic strains are able to ameliorate disease via stimulation of host pathways ([36](#_ENREF_36)). Thus we tested if defense against severe CDI could be conferred by via pre-treatment of mice with a high dose of heat-killed 630. As we had not observed a role for adaptive immunity we shortened the model from forty-two days of pre-infection with strain 630 to one day. However, since we were additionally testing heat-killed vs. live colonization we included RAG1-/- mice in this experiment to confirm findings from our persistent colonization model. Susceptible mice were infected with strain 630, given the equivalent of 109 CFU of autoclaved 630, or mock infected. Twenty-four hours later they were all challenged with VPI 10463. We observed no protective effect of heat-killed 630, indicating that protection requires colonization with *C. difficile* strain 630 not merely exposure to antigen(figure 4A, p<0.05 for all comparisons where significance is indicated). In this short infection model, we also repeated our finding that adaptive immunity is not necessary for protection as both RAG1-/- and WT mice colonized with strain 630 were protected after just twenty-four hours of colonization. Mice given viable strain 630 were highly colonized with strain 630, while mice given heat-killed strain 630 or mock were not (figure 4B, for all comparisons where significance is indicated). However total levels of *C. difficile* between groups were not significantly different (figure 4C, p>0.5). These data suggested that pre-colonization with strain 630 limits levels of the lethal strain.

Since treatment with viable *C. difficile* was required for protection, we sought to determine if this was mediated via limiting levels of the more virulent strain. Using selective culturing it is only possible to differentiate strain 630 from total *C. difficile* as we were unable to identify an antibiotic that only strain VPI 10643 was resistant to. To overcome this limitation, we developed a quantitative PCR assay using primers that amplify target in VPI 10463 that is absent in strain 630 (Supplemental figures 2 A and B). While these primers are not specific to solely strain VPI 10463 when used in the context of the rest of the gut microbiota, they could be used in an infection model in germ-free mice. RAG1-/- germ-free mice were either colonized with strain 630 or left germ-free; the following day, the germ-free mice in addition to one of the groups of 630-monoassocaited mice were challenged with VPI 10463. Using this model, we were able to determine the pre-colonization with strain 630 inhibits establishment of the lethal strain, as we were unable to detect VPI 10463 genomic DNA in the mice that were co-infected despite high overall levels of *C. difficile* measured by quantitative culture (figure 4D, E, F).

Reports of patients infected with multiple strains of *C. difficile* suggest that despite our results, infection with multiple strains can occur ([37](#_ENREF_37), [38](#_ENREF_38)). When we infected mice with ratios of the two strains simultaneously we found that both strains were capable of colonizing despite starting with over a 1000x less strain 630. While both strains were detectable when co-inoculated, protection was not observed unless strain 630 was the dominant strain (figure 4G). Interestingly, when infecting with different ratios of the two strains, the strains would not colonize past a threshold of 109, suggesting a population carrying capacity for *C. difficile* in the mouse gut. Together these data demonstrate that protection requires colonization with high levels of strain 630 to prevent establishment of the second strain.

**Protection is mediated by decreased availability of the co-germinant glycine**

Others have reported that pre-colonization with one strain of *C. difficile* provides protection from challenge with a more virulent strain ([39-41](#_ENREF_39)). The prevailing hypothesis in the field is that consumption of nutrients by the first strain limits the ability of the invading strain to grow. We tested this hypothesis in an *ex vivo* assay using sterile media prepared from the cecal contents of a susceptible mice. Using this approach, we found that when vegetative *C. difficile* was inoculated into susceptible mouse cecal media, both strains displayed significant growth after twenty-four hours (Supplemental figure 3A, p< 0.01). To test if one day of colonization was sufficient to reduce the nutrients required for growth, we added vegetative VPI 10463 to filter sterilized spent culture from the experiments in Supplemental figure 3A. Spent culture media from twenty-four-hour cultures of both 630 and VPI 10463 supported another round of significant growth (figure 5A). We wondered if perhaps nutrient utilization by strain 630 was different *in vivo,* thus we additionally tested growth of strain VPI 10463 in cecal media made from mice that were infected with strain 630 for twenty-four hours. This media also supported robust growth, demonstrating that in both batch culture and *in vivo*, twenty-four hours of colonization is not sufficient to reduce the nutrients required for growth of a vegetative invading strain (Supplemental figure 3B). Additionally, since we were able to culture vegetative VPI 10463 in the spent culture of strain 630, we ruled out the role of inhibition due to secreted protects like bacteriocins or phage in this model. This was confirmed by agar overlay assays (data not shown).

It is believed that the primary infectious form of *C. difficile* is the not the vegetative cell but rather the environmentally stable spore ([42](#_ENREF_42)). Recently groups have demonstrated that *C. difficile* colonization can be decreased via reduction of germination ([43](#_ENREF_43), [44](#_ENREF_44)). To date, the primary focus of studies evaluating germination of *C. difficile* spores have focused on bile acids, specifically the primary bile acid taurocholate ([45](#_ENREF_45), [46](#_ENREF_46)). In the cefoperazone-treated murine model, we have previously observed that susceptible mice have significantly increased levels of the bile acid taurocholate and that this is associated with enhanced germination ([47](#_ENREF_47)). Since strain 630 cannot metabolize taurocholate we felt it was unlikely that pre-colonization with strain 630 result in decreased taurocholate so we looked for other factors that may prevent colonization with a second strain ([48](#_ENREF_48)).

Our group has also previously shown that compared to susceptible mice, amino acids like glycine are significantly decreased following colonization (22). *C. difficile* can ferment glycine in a paired reaction to enhance growth in addition utilizing it as a co-germinant ([49-51](#_ENREF_49)). Using targeted metabolomics, we measured levels of amino acids including glycine in cecal media made from mice that had been off cefoperazone for three days (D1 CM) compared to mice that have been off of cefoperazone for two days followed by infection with *C. difficile* for twenty-four hours (C diff D1 CM) (5C). We observed that twenty-four hours of colonization with strain 630 (or a non-toxigenic strain) results in a significant reduction of glycine relative to mock challenged susceptible animals (figure 5B, supplemental figure 4). As we had already ruled out limitation of vegetative growth, we asked if a decrease in glycine in mice colonized with strain 630 altered germination of VPI 10463 spores. PBS with both taurocholate and glycine was used as a positive control while PBS alone and PBS with taurocholate were used as negative controls. Germination was assessed by incubation of spores in a given condition for 15 minutes followed by heat-treatment to kill any cells that germinated. If germination occurred, then the post-heat CFU should be lower than the pre-heat amount; if there is minimal germination, the spores will survive heating and levels should remain constant between the pre and post time points.

Incubation of spores in PBS or PBS + taurocholate resulted minimal changes between the pre and post levels. However, heating the spores incubated in PBS+ taurocholate + 100mM glycine resulted in a significant reduction in CFU, indicating robust germination (figure 5C). A similar result was observed in D1 CD suggesting this media also supports germination. However when spores were incubated in 630 D1 CM there was no significant difference in levels of pre and post CFU, suggesting that 630 D1 CM does not support robust germination. To show that this was due to decreased levels of glycine we were able to restore germination with the addition of 100mM glycine to 630 D1 CM. Together these results demonstrate that pre-colonization with 630 reduces levels of glycine in the gut leading to a reduction in the ability of a second strain to germinate and colonize.

**Discussion**

The role of the gut microbiota in limiting colonization by *C. difficile* has been appreciated for over three decades, however how the microbiota provides colonization resistance remains to be fully elucidated ([52-54](#_ENREF_52)). Many studies have focused on a top down approach to identify and build defined consortia that confer the same protection as the intact community ([43](#_ENREF_43), [44](#_ENREF_44), [55](#_ENREF_55)). We took an alternative approach and built off the observation that administration of a single bacterium (non-toxigenic *C. difficile*) limited colonization([12](#_ENREF_12), [41](#_ENREF_41)). We sought to determine how pre-colonization with one strain of *C. difficile* protects from infection with another. We hypothesized that protection was a result of the both intraspecific bacterial competition and the development of host immunity to *C. diffic*ile antigen. To evaluate this we utilized two well-characterized lab strains that despite being differentially virulent in our mouse model express nearly identical forms of both TcdA and TcdB ([15](#_ENREF_15), [32](#_ENREF_32)).

Using multiple infection models we determined that pre-colonization with a less virulent strain is sufficient to protect from challenge with a lethal strain of *C. difficile,* surprisinglyeven in the absence of adaptive immunity. Additionally we showed that protection is dependent high levels of colonization by the less virulent strain and that it is mediated by exclusion of the invading strain. The prevailing theory in the field as has been that pre-colonization with one strain of *C. difficile* limits vegetative growth of the challenging strain. Our results challenge this model, as twenty-four hours of growth by one strain is not sufficient to deplete nutrients such that it limited vegetative growth of a second strain.

While other bacterial therapies for *C. difficile* infection such as fecal microbial transplants can lead to clearance of *C. difficile* from the gut ([7](#_ENREF_7), [55](#_ENREF_55)) we were unable use strain 630 to “treat” mice already colonized with the lethal strain. Recently another group reported that a different toxigenic but low virulence strain of *C. difficile* could both protect and treat infection with VPI 10463 ([56](#_ENREF_56)). This highlights an important consideration when designing bacterial based therapies for treatment of CDI, as unique strains have different relative fitness.

The major finding from this study is that reduction of glycine following colonization with one strain of *C. difficile* is sufficient to decrease germination of the second strain. This finding provides a novel target for the development of therapeutics that aim to restore colonization resistance.

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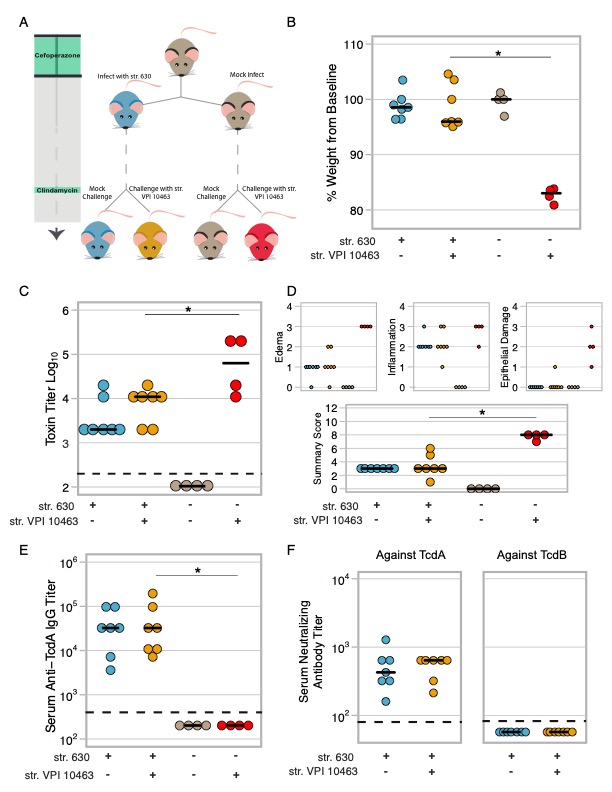
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**Main Text Figures**



**Figure 1: Murine model of persistent *C. difficile* colonization**

**A.** Change in weight relative to day of infection in infected and mock challenged mice. Points represent median weight; bars are the upper and lower quartiles. Infected mice are colored blue (n=14) while data from mock-infected animals are shown in tan (n=8). Following correction for multiple comparisons, weight loss in infected mice was only significantly different than mock-challenged mice on days 4, 5, and 6 post-infection, p< 0.05. **B.** *C. difficile* colonization over time as determined by quantitative culture. Colonization significantly decreased and remained significantly lower by day 10 post-infection relative to day 1 post-infection 40 (n=14), p< 0.01. The hashed line represents the limit of detection of 100 CFU/g feces. **C.** Relative abundance of OTU 4 (*C. difficile*) over the course of the experiment (blue line) is plotted on the left axis while Shannon diversity of the infected mice over the course of the experiment is plotted on the right axis (black box plots). **D.** Fecal toxin activity remains detectable throughout the experiment. Toxin titer on day 33 and day 40 are significantly different from day 1 post-infection levels (n=14), p<0.05. Statistical significance for all data was calculated using a Wilcoxon test with a Benjamin- Hochberg correction for multiple comparisons. The hashed line represents the limit of detection (LOD) for each assay, for visual clarity samples that were below the limit of detection were plotted below the line. However, for statistical analysis, the value of LOD/√2 was substituted for undetected values.



**Figure 2: Mice pre-colonized with *C. difficile* strain 630 are protected from challenge with a lethal strain A.** Schematic of experimental conditions. Colors corresponding to treatment groups are carried throughout the figure. **B.** Change in weight at time of necropsy relative to weight on day of challenge. Mice colonized with *C. difficile* strain 630 and challenged with the lethal strain (VPI 10463) (n=4) are protected from weight loss whereas mice that had no exposure to *C. difficile* strain 630 experienced significant weight loss (n=4), p< 0.01. **C.** Toxin titer from intestinal content from mice in panel A as measured by Vero cell rounding assay. Mice colonized with *C. difficile* strain 630 and then challenged with *C. difficile* strain VPI 10463 have a lower toxin titer relative to naïve mice challenged with VPI 10463, p< 0.05. **D.** Histopathology from colons of the mice in panel A. Small panels depict scores for each component that makes up the summary score. Summary score of VPI challenged 630 colonized vs. VPI challenged naive mice p< 0.01. **E.** Titer of serum IgG against TcdA at conclusion of experiment as measured by ELISA. Limit of detection was a titer of 400, p< 0.01. **F.** Neutralizing titer of serum against TcdA or TcdB. For all data statistical significance between the *C. difficile* strain VPI 10463 challenged strain 630-colonized and VPI 10463 challenged naive mice was determined by Wilcoxon test. The hashed line represents the limit of detection for each assay, for visual clarity samples that were below the limit of detection were plotted below the line.



**Figure 3: RAG1-/- mice pre-colonized with *C. difficile* are protected from challenge with a lethal strain of *C. difficile***

**A.** Change in weight at time of necropsy relative to weight on day of challenge. Both WT and RAG1-/- mice colonized with strain 630 and then challenged with the highly virulent strain VPI 10463 are protected from weight loss whereas mice that had no exposure to strain 630 experienced significant weight loss, VPI challenged 630 colonized RAG1-/- vs. VPI challenged naive RAG1-/- p<0.05, VPI challenged 630 colonized WT vs. VPI challenged naive WT p<0.01, no statistical difference was detected in comparisons of the same treatment between the two genotypes. **B.** Toxin titer from intestinal content of mice in figure A as measured by Vero cell rounding assay. Both WT and RAG1-/- mice colonized with strain 630 and then challenged with VPI 10463 have a lower toxin titer relative to naïve mice challenged with VPI 10463, 630 colonized RAG1-/- vs. naive RAG1-/- p<0.05, 630 colonized WT vs. naive WT p<0.001. Statistical significance was calculated using a Wilcoxon test. Limit of detection was 2.3, however for visual clarity samples with an undetected toxin titer were plotted below the limit of detection. **C.** Histopathology scoring of the colons of mice in A. Small panels depict scores for each component that makes up the summary score. Summary scores of VPI challenged 630 colonized RAG1-/- vs. VPI challenged naive RAG1-/- p<0.05, VPI challenged 630 colonized WT vs. VPI challenged naive WT p<0.001. For all panels, statistical significance was calculated using a Wilcoxon test with a Benjamini-Hochberg correction for multiple comparisons. Data are from two independently run experiments with multiple cages per each treatment group. Each point represents a mouse.



**Figure 4: *C. difficile* strain 630 protects by limiting colonization of the lethal strain.**

**A.** Change in weight at time of necropsy relative to weight on day of challenge in mice pre-treated with viable strain 630, heat-killed strain 630 or water (mock). All mice were infected with strain VPI 10463 one day following pre-treatment. Both RAG1-/- or WT mice given viable strain 630 did not lose weight following challenge with strain VPI 10463 when compared to mice who received heat-killed strain 630 or water (mock), p<0.05 for all comparisons shown. **B**. Levels of strain 630 in mice at conclusion of experiment (630 is erythromycin resistant while VPI 10463 is sensitive to the antibiotic). Colonization by strain 630 was significantly different in mice given viable 630 compared to mice given heat-killed 630 or mock. RAG1-/- mice given 630 vs. heat-treated 630, p < 0.05 or vs. mock, p < 0.05. WT mice given 630 vs. heat-treated 630, < 0.05 or vs. mock, p < 0.05. There was no significant difference between colonization in the mock vs. heat-killed 630 treatments for either genotype. LOD is 100 CFU/g feces; undetected samples were plotted below the LOD for visual clarity. **C.** Total levels of *C. difficile* colonization at conclusion of experiment. There was no significant difference in total *C. difficile* colonization between any of the groups, p>0.7. **D.** CFU equivalents of strain VPI 10463 in gnotobiotic mice as determined by qPCR. Mice pre-colonized with *C. difficile* strain 630 have undetectable levels of strain VPI 10463 using this assay, LOD is 1.39 x104 CFU, levels of VPI 10463 in 630 pre-colonized mice vs. VPI 10463 only mice p < 0.01. **E**. CFU/gram of feces of *C. difficile* strain 630 in gnotobiotic across groups as determined by selective quantitative culture Mice only challenged with VPI 10463 were not colonized with strain 630. LOD is 1000 CFU, p < 0.05. **F.** Total CFU/gram of feces of *C. difficile* in gnotobiotic as determined by quantitative culture. **G.** Mice challenged simultaneously with both strains can be colonized by both strains. Left axis represents Log10 CFU of total *C. difficile* (closed circle) or strain 630 two days post challenge (open circle). Right axis depicts percent of baseline weight two days post challenge (triangle). Each different inoculum ratio was given to one cage of five mice. Points represent the median value for each treatment while the bars represent the upper and lower quartiles. For all panels in the figure, squares represent RAG1-/- mice while circles represent wild-type (WT) mice. For the data included in each figure, statistical significance was calculated using a Wilcoxon test and corrected for multiple comparisons with a Benjamini- Hochberg correction. The hashed line represents the limit of detection for each assay, for visual clarity samples that were below the limit of detection were plotted below the line.



**Figure 5: Colonization with *C. difficile* strain 630 significantly reduces the co-germinant glycine in cecal contents, leading to reduced germination of invading strain.**

**A**. Spent cecal media from 24hrs growth of str. 630 *ex vivo* supports robust growth of strain VPI 10463. Filter sterilized spent cecal media was inoculated with vegetative strain VPI 10463 and colonization was monitored by quantitative culture at time zero and six hours. Cecal media that grew str .630 a support robust growth of strain VPI 10463. Strain VPI 10463 colonization at t =0 vs. t= 6 hours in 630 spent culture, p< 0.05. Strain VPI 10463 colonization at t =0 vs. t= 6 hours in fresh media, p< 0.01. Strain VPI 10463 colonization at t =0 vs. t= 6 hours in VPI spent culture media, p< 0.01. Data represent two independent experiments using separate batches of cecal media (pooled from at least 6 mice for each batch) run in at least quadruplicates. **B.** Concentration of glycine in cecal media made from cecal contents of mice one-day post infection with *C. difficile* (*C. difficile* D1 CM) compared to cecal media made from mock challenged mice (D1 CM). Data represent values from two separate batches of cecal media per group. **C.** *Ex vivo* germination of VPI 10463 spores in cecal media from panel B. PBS and PBS + 0.1% sodium taurocholate served as negative controls while PBS supplemented with 0.1% sodium taurocholate and 100mM glycine served as a positive control. Dark grey box plots represent both the vegetative and any remaining spores before heating while the light grey box plots represent the heat-resistant spores.

Statistical significance for all comparisons was calculated using a Wilcoxon test with Benjamini- Hochberg correction when appropriate.

**Supplemental Figures**

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**Supplemental Figure 1: Effect of clindamycin on weight and colonization levels**

**A**. Change in weight from the day mice were given clindamycin to the following day. Mock-infected mice (or naïve animals) are a reference point. There was not a significant difference between the infected or mock-infected mice following administration of clindamycin, p > 0.05. **B.** *C. difficile* colonization in infected animals one day prior to administration of clindamycin and one day following. Clindamycin significantly increases levels of C. difficile strain 630 in infected mice, p < 0.001. For the data included in each figure, statistical significance was calculated using a Wilcoxon test.



**Supplemental Figure 2: Validation of primers for VPI 10463. A.** Plot of Cq (or crossing-threshold) vs. dilution of genomic DNA from VPI 10463, R2 = 0.9929. **B.** The resulting PCR products formed one melt peak.



**Supplemental Figure 3: *Ex Vivo* growth in Cecal Media.**

**A.** Sterile cecal filtrate from susceptible mice (day 0) was inoculated with vegetative cells of *C. difficile* strain 630, vehicle, or vegetative cells of *C. difficile* strain VPI 10463. Levels of colonization were monitored by quantitative culture at time zero and twenty-four hours. Cecal media supports robust growth of both strains, p< 0.001. Data from A two independent experiments using separate batches of cecal media run in at least quadruplicates. **B**. Growth of *C. difficile* strain VPI 10463 in cecal media made from content from mice infected for twenty-four hours with *C. difficile* strain 630. Strain VPI 10463 colonization at t =0 vs. t= 24 hours, p= 0.02857. Statistical significance for all comparisons was calculated using a Wicoxon test.

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**Supplemental Figure 4: Targeted metabolomics measuring amino acids in D1 and 630D1 cecal media.** Concentration of amino acids in cecal media (uM). Statistical significance for all comparisons was calculated using a t-test.