

RESEARCH PAPER



## Detection and elimination of a novel non-toxigenic *Clostridioides difficile* strain from the microbiota of a mouse colony

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### ABSTRACT

*Clostridioides difficile* is an enteric bacterial pathogen that can cause nosocomial infection leading to debilitating colitis. The development of a murine model of *C. difficile* infection has led to fundamental discoveries in disease pathogenesis and the host immune response to infection. Recently, *C. difficile* endogenously present in the microbiota of mice has been reported and was found to complicate interpretation of mouse studies. Here, we report a novel *C. difficile* strain, named NTCD-035, isolated from the microbiota of our mouse colony. The presence of NTCD-035 in mice prior to challenge with a highly pathogenic *C. difficile* strain (VPI10463) led to significantly reduced disease severity. Phylogenetic characterization derived from whole genome sequencing and PCR ribotyping identified the isolate as a novel clade 1, ribotype 035 strain that lacks the pathogenicity locus required to produce toxins. Deficiency in toxin production along with sporulation capacity and secondary bile acid sensitivity was confirmed using *in vitro* assays. Inoculation of germ-free mice with NTCD-035 did not cause morbidity despite the strain readily colonizing the large intestine. Implementation of a culture-based screening procedure enabled the identification of mice harboring *C. difficile* in their microbiota, the establishment of a *C. difficile*-free mouse colony, and a monitoring system to prevent future contamination. Taken together, these data provide a framework for screening mice for endogenously harbored *C. difficile* and support clinical findings that demonstrate the therapeutic potential of non-toxigenic strains in preventing *C. difficile* associated disease.

**Abbreviations:** PaLoc - Pathogenicity locus, CFUs - Colony forming units, TcdA - toxin-A, TcdB - toxin-B, CdtA - binary toxin A, CdtB - binary toxin B, CdtR - binary toxin R, NTCD - non-toxigenic *C. difficile*

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### Introduction

*Clostridioides difficile*, a Gram-positive, spore-forming, obligate anaerobe, is the most common nosocomial infection in the United States, totaling approximately 365,000 infections and 13,000 deaths per year.<sup>1-3</sup> A healthy microbiota provides colonization resistance against *C. difficile*. However, perturbation of the microbiota, such as that observed following antibiotic treatment, impairs colonization resistance to create a biological niche in the lower gastrointestinal tract for *C. difficile* to grow.<sup>4,5</sup> Upon establishment of infection, *C. difficile* produces cytotoxins that are the main virulence factors that drive the majority of pathologic consequences of infection,

including intestinal epithelial damage and diarrhea.<sup>6,7</sup> Two toxin proteins, toxin-A (TcdA) and toxin-B (TcdB), as well as regulatory proteins are encoded by a ~ 20kb region termed the pathogenicity locus (PaLoc). Numerous *C. difficile* strains have been identified that lack the PaLoc. These non-toxigenic strains of *C. difficile* have the PaLoc replaced with a conserved non-coding 115 bp region and are unable to produce toxin.<sup>8-10</sup>

Epidemiological surveys of healthy volunteers have shown 4–8% of humans are carriers of *C. difficile* and about 50% of them carry non-toxigenic *C. difficile*.<sup>11</sup> Non-toxigenic strains of *C. difficile* are associated with asymptomatic colonization in humans<sup>12,13</sup> and

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correlate with lower rates of *C. difficile* associated disease.<sup>14</sup> This correlation provokes the hypothesis that non-toxicogenic *C. difficile* strains can be used therapeutically to prevent pathogenic strains from colonizing the intestine. Several studies support this hypothesis. For example, prior administration of non-toxicogenic *C. difficile* protects hamsters from pathogenic strains.<sup>15–17</sup> In human patients, a phase II clinical trial reported by Gerding et al. showed a significant reduction in recurrence of disease in patients administered non-toxicogenic spores.<sup>18</sup> Combined, these data support the therapeutic potential of non-toxicogenic *C. difficile*.

The murine model of *C. difficile* infection has been developed over the past decade as a tool to better understand the immunologic mechanisms that limit or promote pathology. However, the model presents challenges that must be resolved to conduct well-controlled studies. Gewirtz and colleagues described a latent *C. difficile* strain present in the intestinal microbiota of commercially available mice.<sup>19</sup> Further, Lawley et al. demonstrated that mice can latently carry *C. difficile*, which blooms following antibiotic treatment to induce a “super spreader” state.<sup>20</sup> These observations raise the prospect of complications in using the mouse model for *C. difficile* studies. Identification and screening for contaminating *C. difficile* in animal colonies is critical to ensure controlled studies in *C. difficile* research and is a focus of this report.

In this study, the presence of a contaminating endogenous *C. difficile* strain is identified and isolated from the gastrointestinal tract of naïve mice housed in our animal colony. We demonstrate that colonization with this endogenous *C. difficile* changes the outcome of infection with pathogenic *C. difficile*. Through *in vitro* studies, PCR ribotyping, and whole genome sequencing, we characterize this strain as a novel non-toxicogenic F035 ribotype that is a clade 1 member of the *C. difficile* species. These data demonstrate the importance of thorough animal colony screening for *C. difficile* strains and identifies a novel probiotic candidate.

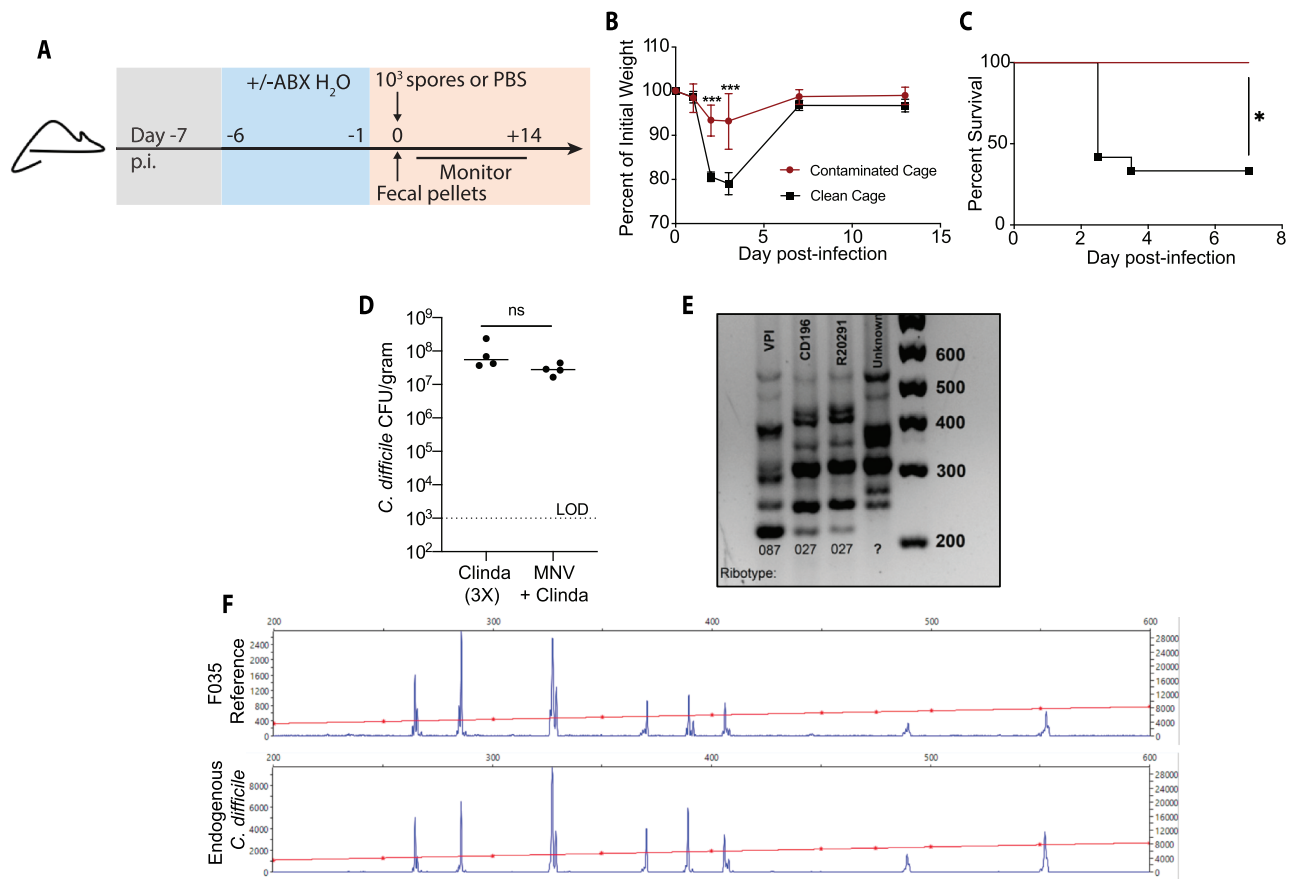
## Results

### **Endogenous *C. difficile* attenuates severe infection from a highly virulent strain of *C. difficile***

The murine model of *C. difficile* infection mimics human exposure via administration of broad-

spectrum antibiotics to predispose mice to infection followed by oral inoculation with the oxygen tolerant spore form of *C. difficile* (Figure 1(a)).<sup>21</sup> Spore Inoculation with the virulent VPI10463 strain of *C. difficile* leads to germination into vegetative cells that produce toxins in the large intestine resulting in diarrhea, weight loss, and mortality within the first 72 hours following infection.<sup>21,22</sup> Over a series of mouse experiments, we observed a cage-dependent bifurcation of disease severity in mice infected with the VPI10463 strain of *C. difficile* as determined by weight loss (Figure 1(b)) and survival (Figure 1(c)). These observations raised the possibility that variation in the endogenous microbiota from cage to cage could be driving phenotypic disease differences. Next, the microbiota of uninfected antibiotic treated control mice were screened. Unexpectedly, fecal pellets from uninfected control mice pretreated with two distinct antibiotic regimens grew bacterial colony forming units (CFU) on *C. difficile* selective agar plates (Figure 1(d)), despite the mice not exhibiting any overt disease symptoms (data not shown).

To confirm this outgrowth was *C. difficile*, bacterial DNA was isolated from a pure culture derived from a colony picked from the agar plate. The 16S rDNA gene region of the bacteria was sequenced by Sanger sequencing methodology and subsequently cross-referenced against the NCBI BLAST database. The search confirmed the bacterium to belong to the *C. difficile* species, though strain level specificity could not be determined (Suppl. Table 1). To identify the strain of *C. difficile* isolated from our animal colony, we employed PCR ribotyping, a well-characterized method of classifying *C. difficile* strains.<sup>23</sup> This technique is generally used to track outbreaks of *C. difficile* in hospitals,<sup>24–26</sup> and was adapted to identify and track our novel strain. The unknown *C. difficile* strain exhibited a banding pattern distinct from the three other strains of *C. difficile* used in our lab (CD196, VPI10463 and R20291), suggesting the strain’s origins came from an external source (Figure 1(e)). To determine the specific ribotype, fluorescent PCR ribotyping was performed and compared to a database of known *C. difficile* ribotypes as described by Martinson et al.<sup>27</sup> and was determined to be the F035 ribotype (figure 1(f)). The F035 ribotype has been reported to be non-



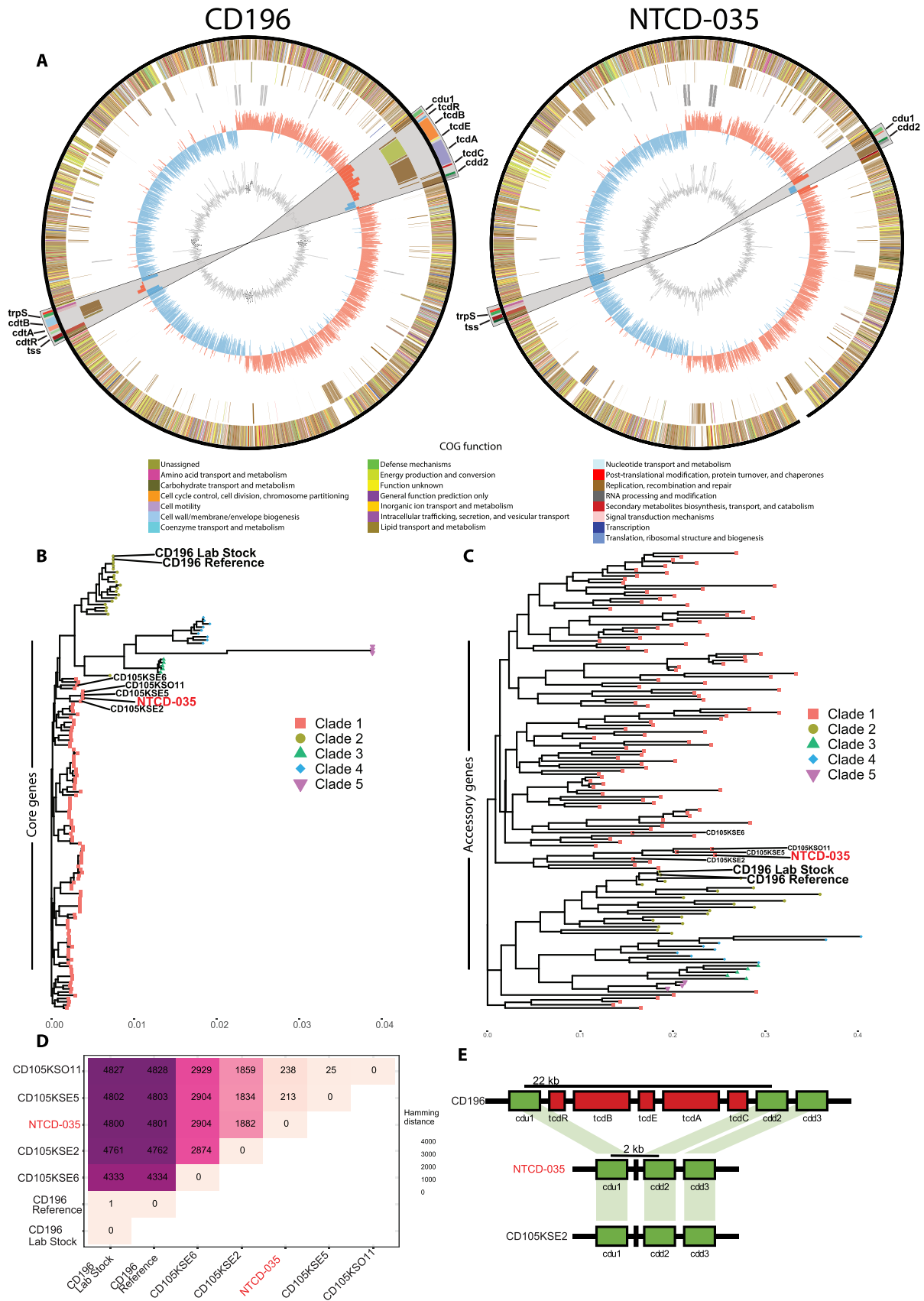
**Figure 1.** Endogenous *C. difficile* contamination attenuates severe disease in mice infected with pathogenic VPI. (a) Schematic of antibiotic (ABX) treatment and *C. difficile* infection. (b-c) Mice were infected with VPI 10463 spores. (b) Percent of initial weight from day of infection. (c) Survival of VPI infected mice contaminated with endogenous *C. difficile* and non-contaminated mice. (d) *C. difficile* burden in fecal pellets of C57BL/6 mice following a PBS mock infection. (e) PCR ribotyping of CD196, VPI10463, R20291 *C. difficile* strains and the unknown *C. difficile* strain isolated from gastrointestinal track of PBS mock infected *C. difficile* culture positive mice. (f) PCR ribotype of unknown *C. difficile* strain and reference F035 ribotype as determined by fluorescent PCR ribotyping. n = 6–8 mice/group. LOD – Limit of Detection. \* =  $p < .05$ , \*\*\* =  $p < .001$ .

toxigenic<sup>24,28</sup> and has been found on the floors and common equipment of hospitals.<sup>25</sup> Taken together, these data reveal the presence of a non-toxicogenic strain of *C. difficile* in our animal colony that was capable of attenuating disease severity in our murine *C. difficile* infection model.

### Identification of NTCD-035 as a novel strain of *C. difficile* that lacks the pathogenicity gene locus by whole genome sequencing

To determine the identity of the *C. difficile* strain isolated from our mouse colony, whole genome sequencing was performed on this strain, termed NTCD-035 (non-toxicogenic *C. difficile* ribotype 035), along with the CD196 strain as a comparative control. The complete

genomes of the two strains were annotated and compared by their core genes (genes present in all genomes) using an in-house pipeline, coreSNPs (<https://github.com/chrgu/coreSNPs>). Circular representations of the genomes were generated using the Circos software.<sup>29</sup> The sequence homology between the core genes for the two strains was 99.3%. Analysis of the complete NTCD-035 genome readily identified genes known to flank the toxin genes. However, NTCD-035 lacks toxin A, toxin B, and toxin regulatory genes that are encoded within the pathogenicity locus as well as all binary toxin gene elements (*cdtA*, *cdtB*, *cdtR*) (Figure 2(a)). These analyses demonstrate this strain lacks the genetic code to produce toxin. Next, NTCD-035 and the reference CD196 strains were compared to publicly available high-quality *C. difficile* whole genome sequences.<sup>30</sup>



**Figure 2.** Whole genome sequencing identifies endogenous *C. difficile* as a novel, non-toxicogenic clade 1 member. (a) Circos plots of CD196 and NTCD-035 chromosome contigs. From outside to inside, circles represent shared genes between isolates, unique genes to each isolate, GC skew, and GC content. Color of genes represents annotated gene COG function by Prokka. Grey region 10x zoomed in portion highlighting the region of pathogenicity locus and binary toxin genes. Genomes were rotated with respect to the origin of replication. (b) Phylogenetic analysis of core genes (c) and hierarchical clustering of the presence or absence of accessory genes of *C. difficile* isolates. (d) Hamming distance of the core genomes between selected *C. difficile* isolates. (e) Annotated segment of pathogenicity locus comparing distances between *cdv1* and *cdd2* genes in CD196, NTCD-035, and a similar phylogenetic isolate, CD105KSE2.

A phylogenetic tree comparing core genes from various *C. difficile* strains was generated (Figure 2(b)). While the sequenced CD196 strain phylogenetically matched the publicly deposited CD196 genome, NTCD-035 was distinct from any publicly deposited *C. difficile* genomes. Phylogenetic characterization based on single nucleotide variants in core genes (Figure 2(b)) and hierarchical clustering based on the presence or absence of accessory genes (Figure 2(c)) determined NTCD-035 clustered with clade 1 isolates, specifically clustering closely to CD105KS strains isolated from soil samples<sup>31,32</sup> (Figure 2(b,c)). Due to the high degree of similarity between NTCD-035 and the cohort of CD105KS strains, the genomes were compared to determine if our isolated strain was novel. Hamming distance calculated between the CD105KSE5 and NTCD-035 core genomes revealed 213 single nucleotide variants between strains, suggesting that NTCD-035 is closely related but distinct from CD105KSE5 (Figure 2(d)). In comparison, our lab's CD196 reference genome had 1 nucleotide variant difference from the publicly deposited CD196 genome, demonstrating the robustness of our whole genome sequencing methods. Presence of toxin genes outside of the pathogenicity locus had previously been described.<sup>9,33</sup> Therefore, the NTCD-035 genome was searched via blast for toxin genes (*tcdA*, *tcdB*, *tcdC*, *tcdE*, *tcdR*), which came up negative. Instead, a conserved non-coding 115 bp region was identified in the PaLoc region similar to other non-toxigenic *C. difficile* isolates.<sup>8–10</sup> This confirmed the lack of toxin genes in the NTCD-035 strain, a pattern that was repeated in the closely related CD105KS strains (Figure 2(e)). Taken together, these data demonstrate that NTCD-035 is a novel, non-toxigenic *C. difficile* strain that lacks a pathogenicity locus and binary toxin genes and clusters most closely with strains isolated from soil samples.

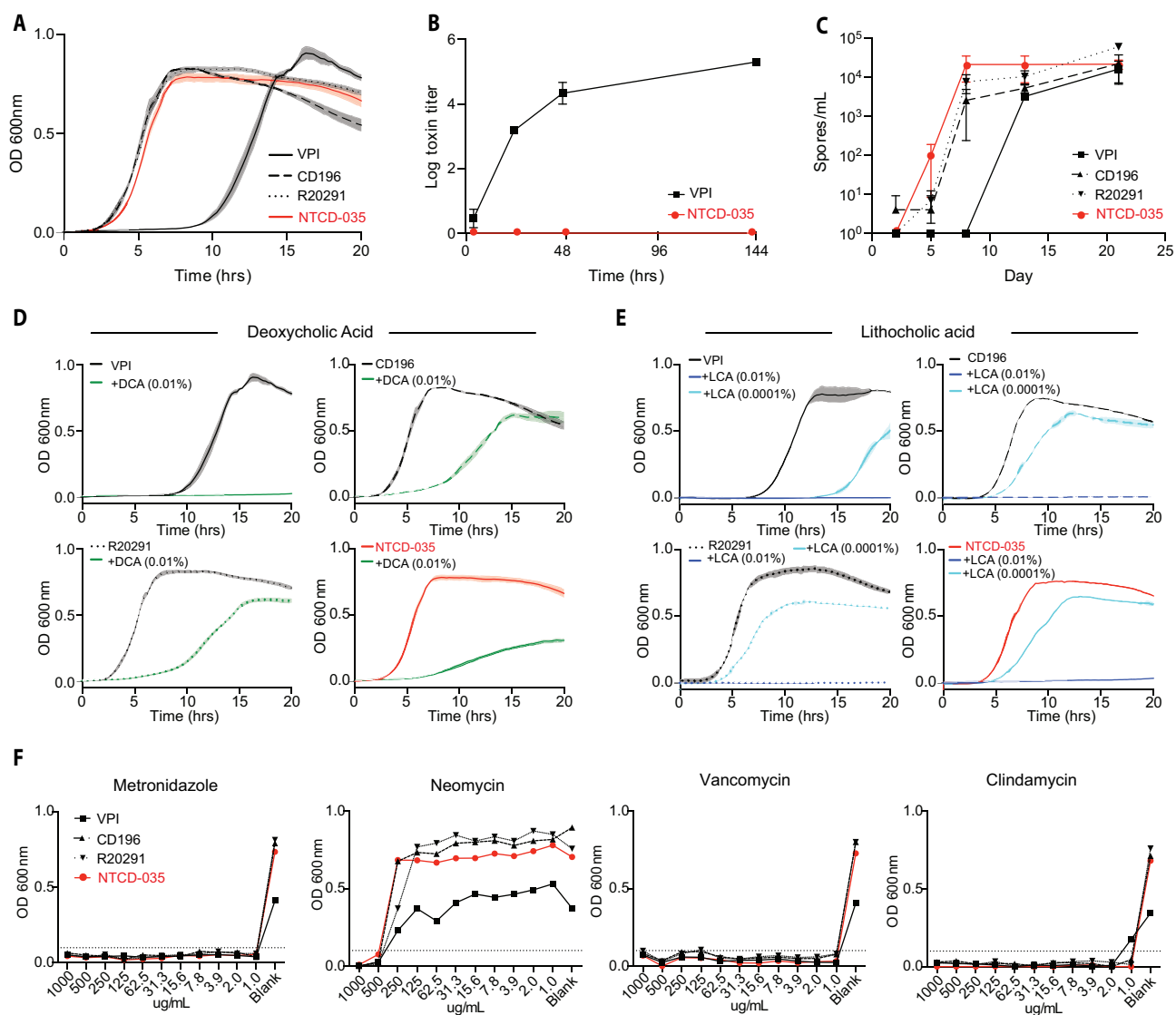
### **Endogenous *C. difficile* strain NTCD-035 sporulates but does not produce toxin**

Next, aspects of *C. difficile* biology were characterized for NTCD-035 and compared to three other pathogenic *C. difficile* strains (VPI10463, CD196, and R20291) as reference standards. First, the *in vitro* growth rate of NTCD-035 was found to be comparable to the CD196 and R20291 strains (Figure 3(a)). Second, the absence of the PaLoc in the genome and the inability of NTCD-035 to produce toxin was functionally confirmed. Toxin

production of the endogenous strain was quantified and compared to a well-characterized high toxin producing strain (VPI10463) using a cell-based cytotoxicity assay. VPI10463 culture supernatant had high concentrations of toxin that induced cell rounding after a 10,000-fold dilution of the culture supernatant. In contrast, undiluted culture supernatant from NTCD-035 did not cause morphological changes to treated Vero cells, functionally confirming the strain's inability to produce toxin proteins (Figure 3(b)). Third, the capacity and rate of sporulation was assessed. NTCD-035 generated spores at a similar rate to CD196 and R20291, while VPI10463 generated spores at a slower rate than other strains, consistent with Akeurlund et. al.<sup>34</sup> (Figure 3(c)). Fourth, susceptibility of each strain to secondary bile acids was assessed by culturing vegetative *C. difficile* in the presence of deoxycholic acid or lithocholic acid. Secondary bile acids are essential for colonization resistance to *C. difficile* infection, as they have been shown to inhibit *C. difficile* growth,<sup>35–38</sup> as well as inhibit *C. difficile* germination.<sup>39–41</sup> NTCD-035 exhibited a secondary bile acid sensitivity profile similar to the CD196 and R20291 strains (Figure 3(d,e)). Finally, antibiotic sensitivity of NTCD-035 to the specific antibiotic cocktail used in our murine infection system (metronidazole, neomycin, vancomycin, or clindamycin) was tested and compared to lab strains. No difference in the antibiotic sensitivity profile was observed in the four *C. difficile* strains tested. NTCD-035 did not grow in the presence of metronidazole, vancomycin, or clindamycin, while maintaining resistance against neomycin (figure 3(f)). Taken together, our *in vitro* characterization studies suggest that the isolated strain is physiologically similar to the CD196 and R20291 strains in terms of growth rate, sporulation rate, secondary bile acid and antibiotic sensitivity, but it lacks the capacity to produce functional toxin.

### **NTCD-035 lacks pathogenicity in acutely susceptible germ-free mice**

Gnotobiotic (germ-free) mice are acutely susceptible to *C. difficile* infection and present with high morbidity and mortality.<sup>42</sup> Therefore, germ-free mice were used to test whether NTCD-035 caused any pathology in mice. Germ-free mice were infected with spores from either NTCD-035 or VPI10463 as a positive control and were subsequently monitored for colonization, weight loss,

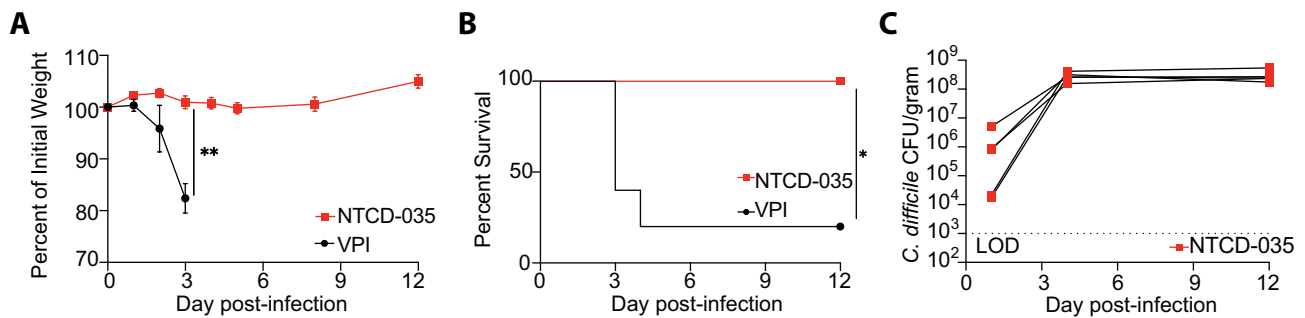


**Figure 3.** Physiological characteristics of NTCD-035 *C. difficile* is similar to those of CD196 and R20291 except for the capacity to produce toxin. (a) Growth curves of *C. difficile* strains as assessed by optical density at 600 nm. (b) Toxin production as assessed by Vero cell assay. *C. difficile* was cultured anaerobically in BHIS broth at 37°C. Aliquots were taken from liquid culture at various time points and assayed for toxin activity. (c) Sporulation rates of *C. difficile* strains. *C. difficile* was cultured anaerobically in BHIS broth at 37°C. Aliquots were taken at various time points, heat killed at 65°C for 60 minutes and anaerobically plated on BHISTA plates to quantify the number of spores. (d-e) Growth curves of *C. difficile* strains as assessed by optical density at 600 nm (d) in the presence of deoxycholic acid, (e) or lithocholic acid. (f) Antibiotic sensitivity assay assessing *C. difficile* sensitivity to metronidazole, neomycin, vancomycin, and clindamycin

and mortality. VPI10463 infection resulted in rapid weight loss (Figure 4(a)) and a high mortality rate (Figure 4(b)) within 72 hours post infection. In contrast, mice inoculated with NTCD-035 exhibited no weight loss (Figure 4(a)) and no mortality following infection (Figure 4(b)) despite exhibiting high, persistent *C. difficile* burden in fecal pellets following inoculation (Figure 4(c)). These data demonstrate that NTCD-035 is avirulent even in acutely susceptible germ-free mice.

### Mouse contact tracing determined *C. difficile* penetrance in the mouse colony and enabled subsequent elimination

To determine the extent of penetrance of the NTCD-035 strain in the resident microbiota of our mouse colony, an epidemiological survey of the university's animal housing facilities was conducted. First, a screening protocol was established for identification of mice contaminated with *C. difficile* (Figure 5



**Figure 4.** NTCD-035 lacks pathogenicity in germ free mice. (a-c) Germfree C57BL/6 mice were mono-colonized with either the VPI10463 or NTCD-035 spores by oral gavage. (a) Percent of initial weight of infected groups from day of infection. (b) Survival of mice infected with different strains. (c) *C. difficile* burden in fecal pellets collected from infected mice.  $n = 5$  mice/group. LOD – Limit of Detection. \* =  $p < .05$ , \*\* =  $p < .01$ .

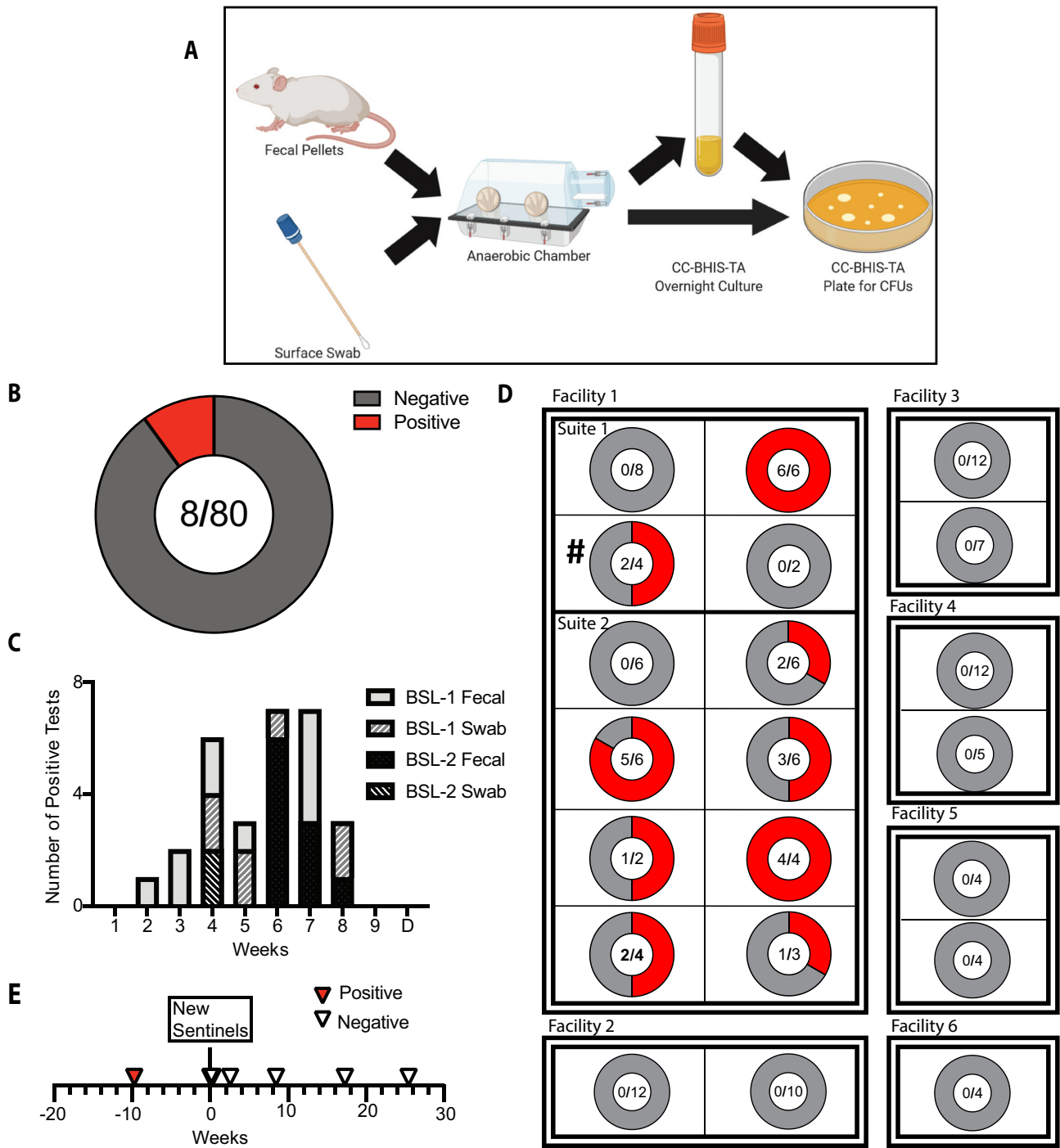
(a). Fecal pellets from mice were incubated overnight in *C. difficile* selective media and plated the following day on *C. difficile* selective agar plates. Eighty mouse cages were screened, and 10% of the cages tested positive for *C. difficile* (Figure 5(b)). No trend in mouse genotype or origin of mouse vendor was observed (data not shown). This culture-based enrichment method was capable of detecting *C. difficile* in fecal pellets that went undetected by direct plating methods or PCR amplification of fecal bacterial DNA. These observations suggest that NTCD-035 is a low abundance member of the mouse's intestinal microbial community and blooms following antibiotic perturbation. In addition to collection of fecal pellets, the animal changing hood, vivarium floor, water spigots, and cage rack surfaces were swabbed with a sterile cotton swab, brought into anaerobic conditions, and streaked on *C. difficile* selective plates. After consecutive weeks of positive tests, a thorough reorganization and decontamination of the facility room was initiated, which successfully removed the spores from the environment (Figure 5(c)).

To determine the breadth of *C. difficile* penetration, sentinel mice were screened from selected rodent rooms within our lab's mouse facility and from six other mouse facilities across the institution. Sentinel mice are housed in the soiled bedding from stock cages of that particular room and therefore will be exposed to any *C. difficile* spores harbored by mice in that room due to the coprophagic nature of mice. The feces of approximately half of the sentinel mice tested within our facility were positive for *C. difficile*. However, the feces of sentinel mice from all other animal facilities tested

negative (Figure 5(d)). This data indicated that the *C. difficile* presence in the mouse colony was localized, and that the origin of *C. difficile* was not from an outside mouse vendor as the other facilities with negative sentinel mice shared common vendors. Following euthanasia of cages that tested positive for *C. difficile* and decontamination of the mouse room, new *C. difficile* negative sentinel mice were established in our lab's mouse colony. These sentinels remained negative over several months of consistent monitoring (Figure 5(e)). Combined, these epidemiologic methods provide a working template for detecting latent *C. difficile* in a mouse colony and steps for subsequent elimination.

## Discussion

Establishment of murine models of *C. difficile* infection have led to mechanistic insights into the host response to infection. Examples of such insights include identifying inflammasome signaling<sup>43</sup> and innate lymphoid cells<sup>22,44</sup> as critical mediators coordinating the early host response following toxin-mediated epithelial damage. Such advances are dependent on maintaining a consistent murine model of *C. difficile* infection. This report demonstrates that the presence of non-toxicogenic *C. difficile* in the microbiota of mice prior to challenge by toxicogenic strains mitigates disease, which skews results of murine *C. difficile* studies. This report also highlights the importance of screening protocols in the vivarium and serves as a guide for identification and control of an outgrowth in mouse colonies.



**Figure 5.** Mouse contact tracing protocol identifies and eliminates the outgrowth of NTCD-035 in the mouse colony. (a) Schematic of screening protocol established. (b) Total cages tested for NTCD-035 in the lab’s mouse breeding room. (c) Number of positive tests per week. ‘D’ denotes decontamination of rodent rooms. (d) *C. difficile* screen of sentinel mice from representative rooms and suites across campus. ‘#’ denotes the original rodent room identified with NTCD-035 positive mice. (e) *C. difficile* screen of sentinel mice over time. ‘New sentinels’ indicates the timepoint when naïve sentinel mice were introduced in the room without soiled bedding transfer from previous sentinel cage

The data presented here demonstrates that the NTCD-035 strain can readily colonize mice but does not cause pathology even in monocolonized germ-free mice, which are highly susceptible to

*C. difficile* infection.<sup>42</sup> Further, NTCD-035 was able to protect mice from subsequent pathogenic *C. difficile* infection. Previous findings provide insight into potential mechanisms of protection



by NTCD-035 via colonization resistance (e.g. niche competition) or immunologic mechanisms. Protection through colonization resistance can be explained by the “founder effect” phenomenon, where the first colonizing strain of one bacterial species metabolically outcompetes subsequent strains for space and resources.<sup>45,46</sup> Thus, pre-colonization of a host by non-toxigenic *C. difficile* could prevent the outgrowth of subsequent pathogenic strains by consuming shared nutrients and occupying the same niche required for the growth and colonization of *C. difficile*. This concept is supported by data from Wilson and Sheagren who found that non-toxigenic *C. difficile* had to be administered prior to toxigenic *C. difficile* challenge to be protective<sup>15</sup> as well as by data that demonstrated that non-toxigenic *C. difficile* needs to be viable at the time of toxigenic *C. difficile* infection to provide protection.<sup>47</sup> Additionally, Etienne-Mesmin et al. showed their *C. difficile* strain (LEM1) could outcompete VPI10463 when co-colonized<sup>19</sup> providing evidence of protection through metabolic competition. Alternatively, the NTCD-035 strain could indirectly prevent infection with a toxigenic strain by educating the immune system’s response. NTCD-035 does not encode toxin genes, and thus an immune response to toxin proteins could not be elicited. However, shared *C. difficile* surface proteins have been tested for immunogenic properties and have been found to elicit robust antibody responses that can protect mice against subsequent infections.<sup>48</sup> However, in hamster models, previous exposure to non-toxigenic *C. difficile* was not sufficient to protect hamsters from secondary toxigenic *C. difficile* infection suggesting the immune response elicited to non-toxigenic *C. difficile* was not protective.<sup>47</sup> The magnitude and protective capacity of the immune response elicited in humans in response to non-toxigenic *C. difficile* remains to be determined. Future studies investigating the mechanism of NTCD-035 mediated protection will be needed to delineate between these two possible direct and indirect pathways.

This report identified a novel, non-toxigenic ribotype 035 strain of *C. difficile*, termed NTCD-035, in our mouse colony’s microbiota. Two potential sources of this strain are external mouse vendors or exposure to environmental contamination

from the surrounding community. Etienne-Mesmin et. al identified a novel strain of *C. difficile* that was a minor member of the mouse microbiota from mouse vendors and bloomed following antibiotic treatment.<sup>19</sup> Subsequent observations from several laboratories support the concept of commercial mouse vendors as potential reservoirs for endogenous *C. difficile* (personal communications). In this report, however, commercial mouse vendors were likely not the source because sentinel mice from six different facilities that share common mouse vendors all tested negative for *C. difficile*. Alternatively, environmental sources are a reservoir for *C. difficile*. For instance, shoe soles commonly test positive for *C. difficile* spores, and the spores can be carried from outdoors to the floors of households and hospitals.<sup>49,50</sup> This is of particular interest, given that our isolated strain of *C. difficile* phylogenetically clustered closely with soil isolates based on whole genome sequence analysis. Further, mouse rooms within our lab’s vivarium that share common personnel and foot traffic patterns also had sentinel mice test positive. Last, NTCD-035 is distinct from all strains used in our laboratory. These pieces of evidence lead us to hypothesize that NTCD-035 is an external environmental isolate introduced into the animal colony though the precise source could not be confirmed in this study.

NTCD-035 in the microbiota of our animal colony was identified and successfully eliminated by modifying our mouse handling, cleaning and screening protocol. After initial screening of feces, *C. difficile* negative mice were transferred to a decontaminated clean mouse room (verified by negative swab test) to establish an endogenous *C. difficile* free mouse colony. In addition to conventional infection control measures in place throughout UPenn vivaria, such as sterile bedding, food, and use of personal protective equipment (PPE) as have been described by Best et al.,<sup>51</sup> supplementary measures were established to prevent future contamination. These measure include use of a rapid-acting sporicidal cleaner, individual autoclaved water bottles for each cage, and disposable surface cover sheets when handling mice in a biosafety cabinet. These increased infection control measures collectively reduced the risk of contamination from *C. difficile* as evidenced by sentinel

mice consistently testing negative following vivarium decontamination.

The use of non-toxicogenic *C. difficile* spores to prevent recurrent *C. difficile* infection has shown promising results in the laboratory and clinical settings.<sup>52</sup> Hamster studies support the idea that administration of non-toxicogenic spores to patients most at risk (i.e. patients on antibiotics) will yield the greatest therapeutic benefit.<sup>53</sup> In a phase II clinical trial, patients that experience either an initial episode or first recurrence of *C. difficile* associated disease were administered non-toxicogenic *C. difficile* M3 spores and had a lower relapse rate than placebo administered patients.<sup>18</sup> Furthermore, administration of non-toxicogenic *C. difficile* had no major adverse effects when given to healthy subjects with or without antibiotics.<sup>54</sup> Collectively, the data presented in this report support these clinical findings for the use of non-toxicogenic *C. difficile* in prevention of pathogenic *C. difficile* infection and provides evidence for a probiotic based therapeutic approach that has the potential to prevent *C. difficile* associated disease in high-risk patient populations.

## Methods

### Mice

C57BL/6 mice were purchased from the Jackson Laboratory and maintained in cages of five mice per cage. All mouse strains used were derived from a C57BL/6 background. All mice were bred and maintained in sterile autoclaved cages, bedding and food under specific pathogen-free conditions and were kept on a grain-based diet (Labdiet 5010, 0001326) at the University of Pennsylvania. Technicians and researchers donned a gown, surgical mask, hairnet, gloves, and shoe covers prior to entry into the animal room and mice were exclusively handled under a laminar flow hood. For gnotobiotic experiments, germfree C57BL/6 mice bred and maintained in a sterile isolator were orally gavaged with *C. difficile* spores and were transferred to a sterile cage and kept in a biosafety cabinet for the duration of experiment. Mice were provided with autoclaved water ad libitum from water bottles. Sex and age-matched controls were used in all

experiments according to institutional guidelines for animal care. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

### Antibiotic pretreatment and *C. difficile* infection

Two to four month old mice were administered drinking water supplemented with neomycin (MilliporeSigma, N1142) (0.25 g/L), metronidazole (MilliporeSigma, M1547) (0.25 g/L), and vancomycin (Novaplus, NDC 0409–6510–01) (0.25 g/L) for seventy-two hours, and then replaced with normal water for the duration of the experiment. Forty-eight hours following cessation of antibiotic water, mice received 200 µg of clindamycin (Sigma, PHR1159) by intraperitoneal injection. Twenty-four hours later, mice received approximately 1,000 *C. difficile* spores (VPI10463 strain ATCC #43255, or NTCD-035) via oral gavage. After infection, mice were monitored for fecal *C. difficile* burden and for weight loss. For experiments with clindamycin only, 200ug of clindamycin was administered i.p. every twenty-four hours for three days and fecal pellets were taken twenty-four hours after the last dose of clindamycin.

### *C. difficile* quantification

Fresh fecal pellets were collected from experimental mice, placed on ice, and transferred into an anaerobic chamber. Feces were resuspended in deoxygenated PBS, and ten-fold serial dilutions were plated on *C. difficile* selective plates consisting of BHI supplemented with yeast extract, taurocholate (Sigma, T4009), L-cysteine (Sigma, C7352), D-cycloserine (MilliporeSigma, C6880) and cefoxitin (MilliporeSigma, C4786) and grown at 37°C in an anaerobic chamber (Coylabs) overnight.<sup>55</sup> Colony forming units were enumerated and data presented as CFUs per gram of feces.

### Sanger sequencing

To determine the identity of the bacteria colonies, fecal pellets from contaminated mice were plated out on *C. difficile* selective plates. Individual colonies were restreaked, picked and grown in *C. difficile* selective broth. DNA was isolated after

overnight broth culture using Qiagen DNeasy PowerSoil kit (Qiagen, 12888) according to manufacturer's instructions. PCR amplification of the 16S rDNA gene was performed on purified bacterial DNA (primer sequences listed in supplemental table 2), and Sanger sequencing was performed on PCR amplified gene products. Sequence readouts were trimmed using SnapGene Viewer software with a nucleotide base quality score cutoff of 50. Trimmed sequence reads were queried using the NCBI BLAST database.

### PCR ribotyping

PCR ribotyping was performed as previously described.<sup>56</sup> DNA was isolated from either fecal pellets or from liquid culture using Qiagen DNeasy PowerSoil kit. Following DNA isolation, PCR ribotyping of the 16S gene region was performed (primer sequences listed in supplemental table 2), and PCR products were run on an agarose gel. Fluorescent PCR ribotyping was performed using a protocol as previously described.<sup>27,57</sup>

### Whole genome sequencing

High molecular weight DNA was extracted from vegetative cells grown overnight in BHIS broth. DNA was isolated by phenol-chloroform extraction as previously described.<sup>58</sup> Long-read libraries were prepared by using the SQK-RBK004 version of the Rapid Barcoding Kit (Oxford Nanopore Technologies, Oxford, UK) and sequenced on the Oxford Nanopore MinION using a FLO-MIN106 flow cell. Short-read libraries were prepared using the Nextera DNA Flex Library Prep Kit (Illumina, San Diego, CA) and were sequenced on the HiSeq 2500 using 2 × 125 bp chemistry. The short reads were quality controlled using the Sunbeam<sup>59</sup> while the long reads were processed with our in-house pipeline, Nanoflow (<https://github.com/zhaoc1/nanoflow>). Nanoflow uses the Albacore command line tool to basecall and demultiplex the fast5 files to fastq files. Porechop and Filtrlong were used to trim the adapter sequences and subsample to down to 500 Mbps of the highest quality reads (<https://github.com/rrwick/Porechop>, <https://github.com/rrwick/filtrlong>). Hybrid assembly was performed via Unicycler in Nanoflow. Unicycler uses Spades to generate short

read assembly and scaffolds them with a long read assembly generated from Canu and Nanopolish<sup>60,61</sup> (<https://github.com/jts/nanopolish>). CheckM was used to check the quality of draft genomes.<sup>62</sup> Phylogenetic analysis of *C. difficile* strains was generated with an in-house pipeline, coreSNPs (<https://github.com/chrgu/coreSNPs>). CoreSNPs uses Prokka to annotate the draft genomes and Roary to analyze the pangenome.<sup>63,64</sup> Sequences of the core genes were extracted and concatenated to determine the number of single nucleotide variations (SNVs) between genomes via Hamming distances using an in-house R script. Phylogenetic trees and hierarchical clustering trees were generated by FastTree2.<sup>65</sup> 111 reference genomes were gathered from the RefSeq database and 29 clinical isolates from Dr. Fredric Bushman lab's database.

### Toxin assay

*In vitro* toxin production was determined using a cell-based cytotoxicity assay modified from previously described protocols.<sup>66</sup> *C. difficile* strains were incubated in deoxygenated BHIS broth and aliquots were taken at various time points after inoculation. Culture supernatant was spun down and filter sterilized (22µm) prior to the assay. Vero cells were seeded in a 96 well plate and were incubated at 37°C for 24 hours. Culture supernatant or culture supernatant plus anti-toxin neutralizing antibody (TechLab, T1000) was added at tenfold serial dilutions and incubated for another 24 hours. Cells were then observed for cell rounding.

### Sporulation assay

*C. difficile* sporulation was assessed by growing strains in CC-BHIS broth. Aliquots were taken at various timepoints post inoculation and incubated at 65°C for an hour to heat kill vegetative cells. Heat killed culture was then plated by tenfold serial dilutions on CC-BHIS-TA plates to measure spores by colony forming units.

### Growth curves and secondary bile acid inhibition

*C. difficile* growth and secondary bile acid inhibition was assessed as previously described.<sup>37</sup> Broth culture of each strain were grown overnight in BHI media supplemented with yeast extract and L-cystine. The

following day, 1% v/v overnight starter culture was added to fresh BHIS media. For analysis of secondary bile acid inhibition, lithocholic acid (Sigma, L6250) or deoxycholic acid (Sigma, D6750) was added to the BHIS media. Deoxycholic acid was dissolved directly into BHIS media while lithocholic acid was first dissolved into sterile DMSO before being diluted 1:1000 into BHIS media. Culture was added to a 96 well plate in a one to one ratio with either BHIS media or BHIS containing secondary bile acids. The plate was incubated at 37°C under anaerobic conditions for 20 hours and growth was assessed by measuring the optical density at 600 nm (OD 600).

### Antibiotic sensitivity assay

Assay was modified from previously described protocol.<sup>67</sup> An individual colony of each *C. difficile* strain was grown overnight in BHIS. The culture was diluted 1:1000 and allowed to grow to mid-log phase. This culture was then diluted 1:1000 and plated 1:1 in test BHIS broth plus or minus antibiotics ranging in concentration from 1 mg/mL to 1 µg/mL desired concentration. Plate was incubated overnight at 37°C for 24 hours. Growth was measured on a spectrometer plate reader (OD600).

### Facility and mouse screening for *C. difficile*

For facility screening, the animal changing hood, floor, automated cage water system spigots, and cage racks surfaces were swabbed with a sterile cotton swab, brought into anaerobic conditions, and streaked on *C. difficile* selective plates. Plates were observed after 24 hours to identify growth of *C. difficile*. For mouse screening, fresh fecal pellets were collected, placed on ice, and transferred into an anaerobic chamber. Fecal pellets were resuspended in deoxygenated *C. difficile* selective broth (CCBHISTA) to enrich for *C. difficile*. After overnight culture, tenfold dilutions were plated on *C. difficile* selective agar plates to identify outgrowth of *C. difficile*.

### Decontamination of the vivarium

After identification of a contaminating *C. difficile* strain, a decontamination protocol of the mouse room was implemented. First, mice were screened for endogenous *C. difficile* in their feces, as described

above. Following a negative result, cages were transferred into a separate empty animal room. All surfaces in the room were disinfected with 10% sodium hypochlorite. The cage racks in the new room were autoclaved and the automated water system spigots removed and replaced with individually autoclaved water bottles for each cage. Use of sporicidal cleaner (Clorox Fuzion Cleaner) was implemented for cleaning the biosafety cabinet used to change mouse cages and cart surfaces and wheels brought into the room. Finally, new mouse handling protocols included placing disposable absorbent sheets (Thomas Scientific, 1157M64) on the metal surface of the biosafety cabinet when opening the cages in the hood to reduce the chance of cross contamination between users.

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### Disclosure of Potential Conflicts of Interest

The authors report no conflicts of interest.

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