Pre-colonization with the commensal fungus *Candida albicans* reduces murine susceptibility to *Clostridium difficile* infection

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**ABSTRACT**

*Clostridium difficile* is a major nosocomial pathogen responsible for close to half a million infections and 27,000 deaths annually in the U.S. Preceding antibiotic treatment is a major risk factor for *C. difficile* infection (CDI) leading to recognition that commensal microbes play a key role in resistance to CDI. Current antibiotic treatment of CDI is only partially successful due to a high rate of relapse. As a result, there is interest in understanding the effects of microbes on CDI susceptibility to support treatment of patients with probiotic microbes or entire microbial communities (e.g., fecal microbiota transplantation). The results reported here demonstrate that colonization with the human commensal fungus *Candida albicans* protects against lethal CDI in a murine model. Colonization with *C. albicans* did not increase the colonization resistance of the host. Rather, our findings showed that one effect of *C. albicans* colonization was to enhance a protective immune response. Mice pre-colonized with *C. albicans* expressed higher levels of IL-17A in infected tissue following *C. difficile* challenge compared to mice that were not colonized with *C. albicans*. Administration of cytokine IL-17A was demonstrated to be protective against lethal murine CDI in mice not colonized with *C. albicans*. *C. albicans* colonization was associated with changes in the abundance of some bacterial components of the gut microbiota. Therefore, *C. albicans* colonization altered the gut ecosystem, enhancing survival after *C. difficile* challenge. These findings demonstrate a new, beneficial role for *C. albicans* gut colonization.

**Introduction**

In recent years, the bacterium *Clostridium difficile* has become an increasingly significant cause of human morbidity and mortality.\(^1\) The major risk factor for infection is the use of broad-spectrum antibiotics, with elderly and immunocompromised individuals at particularly high risk for disease.\(^2\)-\(^4\) Antibiotic treatment disrupts the normally protective, resident bacterial community in the gut and leads to susceptibility to *Clostridium difficile* infection (CDI). This debilitating infection is treated with other antibiotics, all of which continue to disrupt the gut microbiota to some degree, and recurrence occurs in up to 25% of cases.\(^3\),\(^4\)

An alternative approach to CDI, fecal microbiota transplantation (FMT), relies on restoring a normal gut microbial community in a CDI patient who has experienced more than one recurrence. A randomized-controlled clinical trial and other smaller studies show success rates of 80–90% for FMT in treatment of recurrent CDI.\(^5\)-\(^7\) This approach is thus extremely promising but there is currently no consensus on the optimal source or makeup of donor microbiota. In addition, there have been instances of unintended consequences following FMT, including exacerbation of inflammatory bowel disease\(^8\),\(^9\) and unexplained weight gain.\(^10\) A deeper understanding of the effects of commensal organisms on CDI would contribute to identification of an optimal donor microbiota composition.

The study described in this communication focuses on the effects of the commensal fungus *Candida albicans* on the outcome of CDI. *C. albicans* colonizes the...
gut of most humans as a benign commensal. Huffnagle and co-workers demonstrated that, in mice, gut colonization by *C. albicans* influences the recovery of the bacterial gut community after antibiotic disruption.\(^1^1\) Therefore, *C. albicans* gut colonization might affect the susceptibility of a host to CDI following antibiotic treatment. In addition, *C. albicans* affects the immunological milieu. Gut colonization by *C. albicans* promotes accumulation of regulatory T cell populations.\(^1^2\) Cytokines such as IL-17 and IL-22 are up-regulated in the gut in response to *C. albicans* colonization.\(^1^3^–^1^6\) Monocytes exposed to *C. albicans* are reprogrammed to confer nonspecific protection from secondary infections, a process termed trained immunity.\(^1^7\) These properties of *C. albicans* led us to hypothesize that the presence of the fungus in the gut microbiota would protect an antibiotic-treated host from a *C. difficile* challenge.

The data described here show that mice pre-colonized with *C. albicans* exhibit an increased ability to survive a lethal challenge with *C. difficile*. These results highlight a new aspect of *C. albicans* biology and show that under some circumstances, the effects of *C. albicans* colonization are beneficial for the host.

## Results

**Prior colonization of mice with *C. albicans* reduces susceptibility to lethal challenge with *C. difficile***

We conducted a direct test of the central hypothesis that *C. albicans* pre-colonization would attenuate lethal CDI. Antibiotic treatment was used to increase the susceptibility of mice to lethal CDI using a modification of the model established by Young and co-workers (Fig. 1A).\(^1^8\) These investigators demonstrated that cefoperazone-treated mice remained susceptible to CDI for up to 6 weeks after cessation of cefoperazone treatment if given clindamycin on the day before spore challenge. Building on these key observations, we modified the procedure of Reeves, et al. to establish *C. albicans* colonization in mice prior to challenge with *C. difficile*. Briefly, C57BL/6 mice received cefoperazone in drinking water for 10 days. This antibiotic regimen was used for all mice except certain control mice, as described in Materials and Methods. On the tenth day, some mice were orally inoculated with *C. albicans*. All mice were then switched to standard water without antibiotics to permit the gut microbiota to recover from the antibiotic treatment.
for 3 weeks, in the presence or absence of *C. albicans*. Colonization over this time period was shown by plating homogenized fecal pellets collected at various times post-inoculation (Fig. S1). All mice were successfully colonized with *C. albicans* following a single inoculation.

After 3 weeks, mice received a single dose of clindamycin by intraperitoneal injection and, on the following day, were orally inoculated with approximately $4 \times 10^5$ *C. difficile* spores. *C. difficile* strain UK1, a NAP1/027/BI human epidemic strain, was used for all studies. The response of inoculated mice to the *C. difficile* challenge was monitored over 5 days. Weight loss was often evident at day 2 post-inoculation and some mice died or became moribund by day 3. There were slight differences in the timing and synchrony of deaths between experiments and more deaths were observed when higher numbers of spores were used.

In the absence of *C. albicans* pre-colonization, only 1 of 10 mice with CDI survived to day 5 (Fig. 1B), consistent with previous results. Of the 9 who succumbed to severe CDI within 5 days, 5 died, 2 became moribund and were sacrificed and 2 were sacrificed due to weight loss. The average relative weight of surviving mice on day 2 post-inoculation was $0.92 \pm 0.06$ and on day 3 post-inoculation was $0.84 \pm 0.03$ of each mouse’s pre-inoculation weight ($p < 0.00004$; paired t test, day 3 versus day of inoculation), demonstrating weight loss due to CDI. Mice injected intraperitoneally with PBS on the day before and the day after *C. difficile* challenge (as a control for cytokine treatment, see below) were also highly susceptible to lethal CDI (Fig. 1C). Only 2 of the 11 mice in this group survived to day 5 and of the other 9, 7 died, 1 was sacrificed when moribund and 1 was sacrificed due to weight loss. The relative weight of these surviving mice on day 3 post-inoculation was $0.84 \pm 0.05$ ($p < 0.00098$; paired t test), demonstrating weight loss due to CDI.

In contrast, the majority of mice pre-colonized with *C. albicans* survived challenge with *C. difficile* as compared to those not pre-colonized (Fig. 1B; $p = 0.035$; log rank test). Six of the 10 mice pre-colonized with *C. albicans* survived to day 5, while only 1 died, 1 was sacrificed when moribund and 2 were sacrificed due to weight loss. Mice pre-colonized with *C. albicans* thus showed increased survival of CDI compared to mice without *C. albicans* pre-colonization.

There was no statistically significant difference in weight loss due to CDI with or without *C. albicans* pre-colonization. For *C. albicans* pre-colonized mice infected with *C. difficile*, average relative weight was $0.91 \pm 0.05$ of their starting weight on day 2 post-*C. difficile* inoculation and $0.84 \pm 0.06$ on day 3 post-*C. difficile*-inoculation, a statistically significant difference ($p < 0.00003$; paired t test, day 3 vs day of inoculation) indicating weight loss. Relative weights for surviving mice generally increased at the end of the experiment. The average relative weight at day 5 post-inoculation was $0.96 \pm 0.07$ ($p < 0.003$, t test, relative weight on day 3 vs. day 5) consistent with recovery from infection.

Histological analysis of cecum and colon tissues demonstrated that *C. difficile*-challenged (Cd-challenged) mice exhibited histological features of murine CDI, regardless of the presence of *C. albicans*. Regions exhibiting edema with leukocyte influx into the lamina propria (Fig. S2, panel E,F) had a patchy distribution. Eighteen sections of cecal tissue from 7 Cd-challenged mice without *C. albicans* at day 2 post-infection (with or without PBS) were examined. Each cecum section exhibited multiple regions showing edema and leukocyte influx (Fig. S2, panel E; 125 10x microscope fields examined). Ten cecal sections from 5 Cd-challenged mice with *C. albicans* pre-colonization (with or without PBS) at day 2 post-infection revealed multiple regions with edema and leukocyte influx (Fig. S2, panel F; 74 10x microscope fields examined). Eleven cecal sections from 6 control mice that were antibiotic treated but not challenged with *C. difficile*, either with or without *C. albicans* did not exhibit these features (Fig. S2, panel A,B).

Additional parameters of CDI were similar in mice with or without *C. albicans* even though *C. albicans* pre-colonized mice had increased survival of CDI. For example, the levels of *C. difficile* spores detected in the GI tracts of mice at day 2 post-*C. difficile* inoculation were similar in mice with or without pre-colonization with *C. albicans* (Fig. 2A; $p = 0.76$, t test with log transformed data). For this and subsequent experiments, mice were injected intraperitoneally with PBS on the day before and the day after *C. difficile* challenge. Regardless of pre-colonization with *C. albicans*, spore levels were lower on day 1 (Fig. S3) than on day 2 (Fig. 2A; median 5,000-fold lower on day 1 than on...
Toxin levels were also similar in infected open diamond, samples that yielded no detectable C. albicans Toxin titer (pre-colonization). Results showed that two days C. dif pre-colonization. Grey diamonds, received IL-17A and C. dif pre-colonized mice with or without C. albicans pre-colonization (p = 0.91; Mann Whitney test). Toxin activity was not detected in samples from mice that were not inoculated with C. difficile. Therefore, pre-colonization with C. albicans did not reduce the ability of C. difficile to grow and produce toxins in the mouse GI tract. Thus, the presence of C. albicans did not increase the colonization resistance of the host.

Combined, our observations indicate that mice pre-colonized with C. albicans were better able to resist lethal disease due to C. difficile despite the growth of C. difficile in the GI tract, the production of C. difficile toxin and the presence of inflammation and tissue damage.

**Altered host response to C. difficile challenge in pre-colonized mice.**

A significant fraction of hospitalized patients colonized with C. difficile do not develop symptomatic CDI. Host factors such as immunological status are therefore thought to affect the risk of CDI. Based on this and on the increased survival of the C. albicans pre-colonized mice, we hypothesized that C. albicans altered the immune response to C. difficile challenge, which contributed to resistance to lethal CDI.

We therefore measured the expression of genes encoding IL-17A, IL-22, TNF-α and IFN-γ in colonic tissue of Cd-challenged mice with or without C. albicans pre-colonization. Results showed that two days post challenge with C. difficile, mice pre-colonized with C. albicans expressed higher levels of Il17a mRNA than mice without C. albicans (Fig. 3B). In contrast, expression of the other cytokines was not significantly altered by pre-colonization with C. albicans.

The genes encoding IL-17A and IFN-γ were also expressed at higher levels in C. albicans pre-colonized mice prior to inoculation with C. difficile (Fig. 3A). These results demonstrated that the presence of C. albicans altered the host response both before and after the initiation of C. difficile infection.

These results raised the possibility that higher levels of IL-17 are protective against lethal CDI. We therefore...
hypothesized that administration of IL-17A, in the absence of C. albicans pre-colonization, would protect mice from lethal CDI. To test this hypothesis, mice were given cefoperazone antibiotic treatment for 10 days and then standard water for 20 days, as shown in Fig. 1A. The day before C. difficile challenge, mice were injected intraperitoneally with recombinant IL-17A (or PBS) and clindamycin. Mice were orally inoculated with C. difficile UK1 spores and one day later injected intraperitoneally again with IL-17A (or PBS). Survival and weight loss were monitored for up to 5 days post-C. difficile infection (Fig. 1C). Only 2 of the 11 mice that were injected with PBS survived, while 7 died, 1 was sacrificed when moribund and 1 was sacrificed due to weight loss, as noted above. In contrast, 10 of the 13 mice that were injected with IL-17A survived, while 2 died and 1 was sacrificed due to weight loss. IL-17A treated mice thus showed a significant and striking improved survival of CDI in comparison to mice that were not treated (Fig. 1C, p = 0.0019; log rank test). Average relative weight of surviving IL-17A-treated mice on day 3 post-inoculation was 0.85 ± 0.04, significantly different from the starting weight (p < 0.000009; paired t test). Also, IL-17A treatment did not significantly alter the levels of spores or C. difficile toxin in the infected mice (Fig. 2). Histological analysis of 9 cecal sections from 4 IL-17A treated, Cd-challenged mice showed multiple regions exhibiting edema with leukocyte influx (Fig. S2, panel H; 80 10x microscope fields examined) but not in uninfected mice (Fig. S2, panel D). These results demonstrated that higher levels of IL-17A enhanced survival following challenge with C. difficile spores although IL17A-treated mice had similar levels of weight loss, spore counts and inflammation due to CDI when compared to mice not treated with IL-17A.

**Effects of pre-treatments on microbiota composition.**

In humans, susceptibility to CDI is strongly affected by the state of the gut bacterial microbiota and antibiotic treatment is a major risk factor for infection.\(^2\)-\(^4\) Therefore, we analyzed the composition of the cecal bacterial microbiota in Cd-challenged mice with or without C. albicans pre-colonization and with or without additional IL-17A treatment. We did not detect a statistically significant difference in the overall microbiota composition between groups; however, there were some differences between groups for specific genera.

The cecal microbiota of mice treated with cefoperazone, colonized (or not) with C. albicans and then given clindamycin, PBS or IL-17A, and finally, C. difficile spores was analyzed. Bacterial community composition was characterized by sequencing the V4 region of the 16S rRNA gene and analyzed using QIIME.\(^22\) Principal Coordinate Analysis (PCoA) of weighted UniFrac distance for the bacterial communities in Cd-challenged mice with or without C. albicans pre-colonization and with or without additional IL-17A is shown in Fig. 4A. Permanova analysis of these results did not detect a statistically significant difference between the treatment groups (p = 0.148).

Total levels of bacteria were measured using qPCR and universal 16S rRNA primers. The total number of bacteria detected per milligram of cecum sample was not significantly different between mice that received PBS versus mice that were pre-colonized with C.

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**Figure 3.** Increased expression of Il17a in the colon of C. albicans-precolonized, Cd-challenged mice. All mice were treated with cefoperazone for 10 days and received clindamycin. Mice were euthanized on the day of challenge before receiving spores (A) or 2 days post challenge with C. difficile (B). RNA was extracted from colon tissue, converted to cDNA and expression of transcripts was measured in cDNA by qRT-PCR and was expressed in arbitrary units using GAPDH for normalization. The average for Cd-challenged mice was set to 1 for each experiment and samples were expressed relative to this value. Combined results of two experiments are shown. Each symbol represents a sample from an individual mouse and the bar indicates the geometric mean. Black diamond, received PBS but no C. albicans; open triangle, received PBS and C. albicans. p values, Mann Whitney test.
Figure 4. Bacterial microbiota of Cd-challenged mice with or without C. albicans pre-colonization or IL-17A treatment. Cecal bacterial microbiota composition from mice euthanized on day 2 post-C. difficile challenge was analyzed by Illumina sequencing of the V4 region of bacterial 16S rRNA gene. Relative abundance of bacterial taxa was determined using QIIME. (A) Results were analyzed by determining weighted UniFrac distances and performing Principal Coordinate Analysis using QIIME. Black Diamond, PBS-treated, Cd-challenged mice without C. albicans; white triangle, PBS-treated, Cd-challenged mice pre-colonized with C. albicans; grey diamond, IL-17A-treated, Cd-challenged mice without C. albicans. (B) Total levels of bacteria per cecal tip sample were measured by qPCR using eubacterial primers and normalized to milligrams of cecum sample used for DNA extraction. Symbols, as in (A); bar indicates the geometric mean. (C) Normalized abundance per mg of cecum sample (arbitrary units) for bacterial genera in the cecal microbiota of mice treated with PBS and C. difficile (black bars; n = 9); with PBS, C. albicans and C. difficile (white bars; n = 8); or with IL-17A and C. difficile (grey bars; n = 9). All genera with a statistically significant difference between at least two groups are shown. Phyla are indicated with a one letter abbreviation as follows: P = Proteobacteria, V = Verrucomicrobia, A = Actinobacteria, F = Firmicutes, B = Bacteroidetes. Column indicates the median value, bar indicates the upper quartile, brackets indicate statistically significant comparisons (**p<0.01, *p<0.05). Kruskal Wallis Test followed by Dunn’s multiple comparisons test) D) Chao1 rarefaction analysis indicates that gut microbial community diversity for C. difficile-inoculated mice is low. Diversity of each sample was calculated by averaging 10 rarefacts taken at a depth of 11,500 sequences. For comparison, community diversity in untreated mice (no antibiotics and not inoculated with C. albicans or C. difficile) is shown (Grey circle). Black diamond, PBS and C. difficile; open triangle, PBS, C. albicans and C. difficile; grey diamond, IL-17A and C. difficile. Symbol indicates mean for mice in each group, with standard error of the mean.
albicans (Fig. 4B). Cecum samples from mice that received IL-17A contained higher numbers of bacteria per milligram of cecum sample in comparison to mice receiving only PBS (Fig. 4B; geometric mean for IL-17A treated mice 5-fold higher than PBS-treated mice; p = 0.0385, ANOVA with Tukey’s multiple comparisons test, using log transformed data).

To test for possible differences in specific genera within the microbiota between groups, we compared the relative abundance of bacterial genera, normalized to the total level of bacteria per milligram of cecum sample. Normalization was used to account for differences in the total levels of bacteria per mg of sample. All bacterial genera with a median fraction greater than 0 in at least one of the three groups were included in order to identify the consistently observed genera. These mice had received multiple antibiotic treatments and were infected with C. albicans, and they were colonized with relatively few genera. We detected a total of 67 genera and 28 exhibited a median greater than 0 in at least one group.

The normalized abundance of the 28 genera was compared between Cd-challenged mice with or without C. albicans or IL-17A using the Kruskal-Wallis non-parametric test followed by Dunn’s multiple comparisons test (Table S1). Of the 28 genera that met the criterion for analysis, 7 exhibited a statistically significant difference between at least two of the three experimental groups (Fig. 4C and bold text within Table S1). These included the abundant genera Akkermansia sp. (phylum Verrucomicrobia) and Sutterella sp. (phylum Proteobacteria) and the relatively rare genera Bifidobacterium sp. (phylum Actinobacteria), Adlercreutzia sp. (phylum Actinobacteria), and unidentified genera within the family Comamonadaceae (phylum Proteobacteria), the family Erysipelotrichaceae (phylum Firmicutes) and the family S24-7 (phylum Bacteroidetes).

To detect a possible effect of C. albicans or IL-17A on the relative abundance of rare taxa, we measured community diversity using the Chao1 estimator (Fig. 4D). The microbiota of Cd-challenged mice exhibited relatively low diversity in comparison to mice not treated with antibiotics or microbes, consistent with previous results, and this decrease was statistically significant. However, the microbiota of mice that were more likely to survive C. difficile challenge (C. albicans precolonized or IL-17A treated) did not exhibit increased diversity in comparison to the highly susceptible, antibiotic-treated infected mice using the Chao1 or other measures (Fig. 4D; Simpson index and Shannon index shown in Fig. S4). The above results showed that the communities present in C. difficile-challenged mice with or without C. albicans or IL-17A exhibited some differences in composition and overall low diversity.

To summarize, the results of this study demonstrate that increased levels of IL-17A were protective against CDI and that C. albicans pre-colonization enhanced expression of IL-17A following C. difficile challenge. Differences in microbiota composition or amounts were detected in association with C. albicans colonization or IL-17A administration and might also play a role in protection. Thus, C. albicans pre-colonization altered the GI tract ecosystem, leading to reduced susceptibility to C. difficile challenge.

Discussion

The results of this study demonstrated significant attenuation of lethal murine CDI conferred by pre-colonization with the commensal fungus C. albicans. Previous studies have shown that bacterial microbiota influence susceptibility to CDI by affecting the metabolism of bile acids that serve as spore germinants and by increasing colonization resistance. By contrast, the protective effect of C. albicans did not alter growth of C. difficile in the GI tract or the production of C. difficile toxin. Thus, C. albicans protects against lethal murine CDI through a mechanism other than colonization resistance.

The results reported here used an experimental mouse model to demonstrate protection against lethal CDI due to C. albicans colonization. Some evidence suggestive of a protective effect of Candida colonization against CDI has been observed in humans. Manian and Bryant found that only 10 of 60 patients with CDI (16.7%) were colonized with high levels of Candida spp., statistically significantly different from the rate in patients who tested negative for CDI (30.5%). Similarly low frequencies of Candida colonization in CDI patients were observed by Nerandzic et al. (16%) and Blanco et al. (18%). Interestingly, Blanco et al. observed that CDI patients who carried the hypervirulent C. difficile 027 ribotype were more likely than patients carrying other ribotypes to exhibit high level C. albicans colonization. This observation may reflect the association between high level Candida colonization and situations that produce higher levels
of inflammation. In another study, recent antifungal use (within 6 weeks) was found to be a risk factor for CDI. Perhaps related, administration of the probiotic yeast, *Saccharomyces boulardii*, to human patients has also shown some efficacy in ameliorating symptoms of CDI. Combined, the observations from these studies are consistent with fungal colonization being protective against CDI. However, one investigation of CDI and *Candida* colonization contradicts this showing a significant correlation between CDI and *Candida* colonization. Since the histories of the patients in the various studies are not identical, other variables may have contributed to the finding of a correlation between CDI and *Candida* colonization in the latter study. Our results are consistent with those human studies in which fungal colonization appears to protect against symptomatic CDI and support a role for *C. albicans* in altering the intestinal environment and preventing the most serious consequences of *C. difficile* infection.

Pre-colonization with *C. albicans* had multiple effects on the GI tract ecosystem. Pre-colonized mice exhibited higher IL-17A expression in the colon both before and after challenge with *C. difficile*, demonstrating that the activities of the host were altered. Further, we demonstrated that direct administration of IL-17A resulted in reduced lethality following challenge with *C. difficile* spores. Despite the strong effect of IL-17A administration seen in these studies, loss of IL-17 does not exacerbate CDI; an IL-17A IL-17F double null mutant mouse exhibits normal (or even slightly reduced) susceptibility to CDI. These results suggest that high levels of IL-17A as a result of administration can improve host resistance but that basal levels of IL-17A are dispensable.

In contrast, cytokine IL-22 is necessary for normal susceptibility to CDI; IL-22-deficient mice are highly susceptible to lethal infection by *C. difficile*. IL-27 receptor deficient mice also exhibit high susceptibility to CDI. Further, administration of either IL-27 or IL-25 increases resistance of mice to lethal CDI. These cytokines likely have related but not identical effects. In the absence of IL-17, the functions of other cytokines may compensate for the lack of IL-17.

IL-17 has a well-known role in promoting inflammation. IL-17 stimulates the recruitment of neutrophils to the site of inflammation and is involved in neutrophil recruitment during CDI. In addition, IL-17 plays a role in promoting the repair of epithelia. IL-17A deficient mice exhibit enhanced intestinal permeability and mislocalization of the tight junction protein occludin after treatment with dextran sodium sulfate (DSS). Either or both of these effects, enhancing neutrophil recruitment or repair processes, might contribute to attenuation of *C. difficile* infection.

We explored potential differences in the bacterial microbiota in the different groups of mice because alterations in bacterial microbiota increase susceptibility to CDI. Our results do not exclude a role for the bacterial microbiota in the protection from lethal CDI seen with *C. albicans* pre-colonization. However, we did not observe a major shift in gut bacterial microbiota in the different groups of mice. Pre-colonized mice did exhibit statistically significantly increased abundance of two bacterial genera, *Bifidobacterium* and *Akkermansia*, both of which are associated with beneficial effects on mammalian host health. Probiotic strains of *Bifidobacterium* sp. have been shown to decrease inflammation, protect against infection by intestinal pathogens and improve gut mucosa integrity in various mouse models. *Akkermansia* sp. are mucin-degrading bacteria and a member of the normal human gut microbiota. Gastrointestinal pre-colonization with *Akkermansia* sp. can decrease colonic inflammation and tissue damage, improve gut barrier function, and promote immune tolerance. It is possible that the increased abundance of these two bacterial genera in the *C. albicans*-pre-colonized mice might contribute to the protective effects of *C. albicans* pre-colonization against *C. difficile* infection. These genera did not exhibit increased abundance in IL-17A-treated mice; *Bifidobacterium* and *Akkermansia* may be less important for protection when IL-17A is administered.

Finally, *C. albicans* promotes the ability of *C. difficile* to grow in aerobic conditions in the laboratory. Thus, there may be direct effects of *C. albicans* on the growth of *C. difficile* in the GI tract. Attenuation of disease likely resulted from these multiple effects of *C. albicans* colonization on the GI tract ecosystem. Taken together, the results of this study establish a new facet of *C. albicans*-host interaction, demonstrating that this fungal organism, a common colonizer of humans, can have a
beneficial effect on the host through increased survival of CDI.

**Materials and methods**

**Strains and growth conditions**

*C. albicans* strain CKY101, a virulent strain derived from the sequenced strain SC5314, was used for all studies. For mouse inoculations, cells were grown at 37°C in YPD (1% yeast extract (BD 212750), 2% peptone (Difco 0118-17-0), 2% glucose (Sigma G8270)) for 21–24 hrs.

*C. difficile* strain UK1, a NAP1/027/BI human epidemic strain, was used for all studies. Spores were isolated as previously described except that gradient purification was omitted. For enumeration of *C. difficile* spores in extracts from mice, samples were plated on pre-reduced TCCFA plates (Taurocholate (Calbiochem 580217), cycloserine (Sigma C6880), cefoxitin (Sigma C4786), fructose (Macron Fine Chemicals 7756-12)) and incubated at 35°C for 2 days in an anaerobic chamber.

**GI colonization in mice**

Five-week-old female C57BL/6 mice (Jackson Laboratory) were co-housed in a large cage (24” x 17”). Mice were treated with antibiotic (cefoperazone (Sigma C4292), 0.5 gm/L) in drinking water for 10 days. All mice were treated with cefoperazone for 10 days except for the no antibiotics, no *C. difficile* mice used in Figs. 4D and S4.

Prior to inoculation with *C. albicans*, mice were tested and shown to be negative for cultivable fungi on YPD-SA agar medium [YPD agar plus 100 μg/ml streptomycin (Sigma S6501) and 50 μg/ml ampicillin (Sigma A9518)] incubated for 2 days at 37°C. On the 10th day of antibiotic exposure, some mice were inoculated orally with 5 × 10^7 *C. albicans* cells in 25 μl (n = 10 mice). All of the inoculated mice described in this study became colonized with *C. albicans* following a single inoculation.

All mice were transferred from the large cage to standard sized cages, housed 2 mice per cage and switched to water without cefoperazone. *C. albicans* colonization was measured over time by collecting fresh fecal pellets and plating homogenates on YPD-SA agar. Mice that were not inoculated with *C. albicans* (n = 10 mice), but had been treated with cefoperazone in the same large cage as the *C. albicans*-inoculated mice, were housed in the same room as the *C. albicans*-inoculated mice.

After 3 weeks of *C. albicans* colonization, mice were orally inoculated with *C. difficile* spores (∼3–8 × 10^5 spores per mouse). On the day before inoculation with *C. difficile*, mice were inoculated intraperitoneally with clindamycin (Sigma C5269) (10 mg/kg). Some mice were inoculated intraperitoneally with IL-17A (R&D Systems 421-ML-025, 1 μg in 100 μl PBS) or with PBS on the day before and the day after *C. difficile* inoculation. All mice used in these experiments were shown to be negative for *C. difficile* colonization prior to inoculation with *C. difficile* spores by collecting fecal pellets and plating on pre-reduced TCCFA.

Mice were weighed daily and sacrificed 5 days post-inoculation or when moribund. Mice exhibiting severe signs of illness (extreme inactivity, hunched posture, ruffled fur) were considered moribund and were sacrificed. Mice were also sacrificed if their weight loss exceeded 20%. Within one experiment (i.e., one batch of mice), some of the mice were sacrificed on day 1 or 2 post-inoculation for the various analyses while other mice were monitored for survival over 5 days. The survival data show combined results of mice from 3–4 experiments.

Relative weights were compared using the t test. Survival was compared using the log rank test. The cecum was dissected for further studies. Samples of cecum contents were plated on both YPD-SA to detect *C. albicans* and on pre-reduced TCCFA after heating at 60°C for 10 min. to detect *C. difficile*.

All experiments were done in compliance with Tufts University IACUC guidelines. *C. difficile* susceptibility experiments were performed during the winter months to avoid the occasional presence of cultivable fungi in the GI tracts prior to inoculation with *C. albicans*.

**Microbiota analysis**

The distal portion of the cecum, including contents, was dissected from mice two days post-inoculation with *C. difficile*. For comparison, ceca from control, untreated mice that did not receive antibiotic treatment and were not inoculated with microbes were collected. Mice from 4–5 different experiments were
analyzed. The mucosa was scraped using a glass slide and the scraped material plus the luminal contents were combined in PBS. The sample was centrifuged at 16,100 rcf in a refrigerated Eppendorf microcentrifuge for 5 min. and the pellet was weighed and frozen at -80°C. Microbial DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen 51504) with an additional beadbeating step. Briefly, cecum samples were lysed by beadbeating in Qiagen lysis buffer ASL, and the lysate was treated with InhibitEX tablets followed by enzymatic digestion with proteinase K (20mg/ml) and RNaseA (1mg/ml) and column DNA purification.

Libraries were prepared from each sample and sequenced as described. Briefly, PCR amplification of the V4 region of the 16S rRNA gene was performed with primers that included adapters for Illumina sequencing and twelve base barcodes. Two hundred fifty bp paired-end sequencing was performed using an Illumina MiSeq. Base calling was performed using CASAVA 1.8 and the resulting fastq files were used as input for downstream analysis using QIIME (1.8.0). Briefly, the paired-end reads from the fastq files were joined, barcodes extracted and then demultiplexed. The operational taxonomic units (OTUs) were determined using a closed reference approach by aligning reads to the Greengenes Database (version 13_8) at 99% identity. The Greengenes phylogenetic tree was used to define the phylogenetic relationship between OTUs. The resultant OTU tables contained the relative abundance of bacterial taxa in each sample. These tables were used to calculate overall diversity within each sample. To compare the composition and diversity of samples to each other taking into account phylogenetic relatedness, the OTU tables were used to calculate the weighted UniFrac distance matrix, which was summarized with Principal Coordinate Analysis. Permanova analysis was performed using Qime.

Total levels of bacteria per cecal tip sample were measured by qPCR using eubacterial primers (Table S2). qPCR reactions were conducted using SYBR Green PCR Master Mix (Applied Biosystems 4364346) and a LightCycler 480 II (Roche) instrument. Normalized abundance of bacterial genera was calculated by multiplying the fraction of total reads for a genus by the total level of bacteria per mg of cecum sample (in arbitrary units).

Histology

Cecum tissue was dissected from mice sacrificed prior to or two days post-inoculation with C. difficile. Tissue was fixed in buffered formalin and processed for staining with Hematoxylin and Eosin (H & E) by the Tufts Animal Histology Core facility (http://sites.tufts.edu/histopath/animal-histology-core/).

C. difficile toxin assay

Cecum contents from mice sacrificed one or two days post-inoculation with C. difficile or fecal pellets collected one day post-inoculation were weighed and diluted with 10 times the volume of PBS. Serial threefold dilutions were made in DMEM (Corning Cell Gro MT10-013CV) and samples were applied to monolayers of mouse embryonic fibroblast cells (MEF) cells grown in DMEM with 10% heat-inactivated Fetal Bovine Serum (Atlanta Biologicals S11150). Cells were incubated for 24 hours at 37°C and 5% CO₂ and scored visually at 10X magnification for cell rounding. Toxin titer is defined as the inverse of the greatest dilution that resulted in 100% cell rounding.

Cytokine gene expression

For measurement of cytokine gene expression, mice were sacrificed prior to or two days post-inoculation with C. difficile and colon tissue was frozen in RNA-Later (Invitrogen AM7021) at -80°C. RNA was purified with Trizol (Invitrogen 15596026) extraction and column purification, using the Ambion Purelink RNA mini kit (Invitrogen 12183018A). DNA was eliminated on-column DNase treatment. cDNA preparation with Superscript III (Invitrogen 18080051) was performed using manufacturer’s protocol. qRT-PCR reactions were performed as described above for qPCR. Triplicate samples were measured; controls lacking template did not yield products. Standard curves were generated and all results normalized to the level of GAPDH expression in each sample. Primers are listed in Table S2.

Disclosure statement

The authors have no conflicts of interest to declare. The funders of this research had no role in study design, data collection and interpretation, or the decision to submit the work for publication.
Disclosure of potential conflicts of interest

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