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Fecal microbiota transplantation prevents *Candida albicans* from colonizing the gastrointestinal tract

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Abstract

Gut microbes symbiotically colonize the gastrointestinal (GI) tract, interacting with each other and their host to maintain GI tract homeostasis. Recent reports have shown that gut microbes help protect the gut from colonization by pathogenic microbes. Here, we report that commensal microbes prevent colonization of the GI tract by the pathogenic fungus, *Candida albicans*. Wild-type specific pathogen-free (SPF) mice are resistant to *C. albicans* colonization of the GI tract. However, administering certain antibiotics to SPF mice enables *C. albicans* colonization. Quantitative kinetics of commensal bacteria are inversely correlated with the number of *C. albicans* in the gut. Here, we provide further evidence that transplantation of fecal microbiota is effective in preventing *Candida* colonization of the GI tract. These data demonstrate the importance of commensal bacteria as a barrier for the GI tract surface and highlight the potential clinical applications of commensal bacteria in preventing pathogenic fungal infections.

KEYWORDS

antibiotics, *Candida albicans*, commensal bacteria, fecal microbiota transplantation

1 | INTRODUCTION

Many microbes, including bacteria, fungi, viruses, and parasites, symbiotically colonize the GI tract.[1] These microbes interact dynamically with each other and their host to maintain homeostasis. Dysbiosis, a disruption in the homeostasis of the gut microbiota, is postulated to cause various host diseases, including inflammatory bowel diseases, metabolic syndrome, cancer and infection.[1,2] Gut microbes play important roles in resisting colonization and protecting the host from infection by pathogenic microbes.[2,3] For example, commensal bacteria prevent infection by enteropathogenic bacteria such as *Listeria monocytogenes*, *Citrobacter rodentium*, *Salmonella typhimurium*, vancomycin-resistant *Enterococcus* and *Clostridium difficile*.[3–7] On the basis of the commensal bacterial characteristics that protect the host from pathogen

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Abbreviations: FMT, fecal microbiota transplantation; GI, gastrointestinal; ITS, internal transcribed spacer; PDA, potato dextrose agar; SFB, segmented filamentous bacteria; SPF, specific pathogen-free.

colonization, FMT, a therapeutic strategy using commensal bacteria, has been developed and implemented and found to eradicate *C. Difficile* infection in humans.[8]

Recent studies have shown that fungi are also major components of the human microbiota.[9,10] In healthy people, fungi reside in the oral cavity and GI and urogenital tracts; however, these fungi infect immunocompromised hosts, including patients in intensive care units, those infected with HIV, patients receiving anticancer drugs and antibiotics, and patients who have undergone allograft transplantation.[11] In addition to superficially infecting the skin or mucous membranes, fungi invade the bloodstream and disseminate to internal organs, infecting deep tissues and causing invasive candidiasis, a serious infectious disease.[11] *Candida albicans* is highly prevalent and constant in human feces, suggesting that it is a commensal fungus in the GI tract.[9,10] However, *C. albicans* colonization of the GI tract is thought to trigger invasive candidiasis.[11] Thus, the mechanism by which *C. albicans* steadily colonizes the GI tract requires identification. Previous studies have shown that multiple antibiotics affect colonization of the GI tract by *C. albicans*.[12,13] Although these reports suggest that commensal bacteria resist *C. albicans* colonization in the GI tract, detailed mechanisms and bacterial species responsible for this resistance to colonization have yet to be established.

Antifungal drug-resistant *Candida* has recently emerged as a public health problem that can be life-threatening to humans.[14] Thus, in addition to conventional antifungal drugs, alternative strategies are needed to prevent *Candida* infections. Here, we investigated the role of commensal bacteria in regulating *C. albicans* colonization in the GI tract. We used novel sequencing and PCR approaches to investigate the microbiota present during treatment with various antibiotics. We also examined the potential of commensal bacteria as therapeutic targets for *C. albicans* infections.

2 | MATERIALS AND METHODS

2.1 | Mice

Six- to ten-week-old C57BL/6 and BALB/c SPF mice were purchased from CLEA Japan (Tokyo, Japan). All animals were maintained with a gamma ray-sterilized diet, sterile water and autoclaved wood chip bedding in the experimental animal facility at the Medical Mycology Research Center. The Animal Care and Use Committee of the Chiba University approved the experiments.

2.2 | Antibiotic treatment

Mice were given the following broad-spectrum antibiotics in their drinking water: ampicillin (1 g/L; Nacalai Tesque, Kyoto, Japan), vancomycin (0.5 g/L; Shionogi, Osaka, Japan), neomycin (1 g/L; Nacalai Tesque), metronidazole (1 g/L; Nacalai Tesque), penicillin (1.5 g/L; Meiji Seika Pharma, Tokyo, Japan), streptomycin (2 g/L; Nacalai Tesque), gentamycin (0.1 g/L; Nacalai Tesque) and colistin (1 g/L; MP Biomedicals, Santa Ana, CA, USA).[15–17] These antibiotic treatments were continued throughout the experiments. For the quantitative kinetic experiments, mice were orally administered 0.5 mg ampicillin 1 d before injection with *C. albicans*.

2.3 | Isolation of fecal bacterial DNA

Fecal bacterial DNA was isolated as previously reported with modifications.[18] Mouse feces were collected, weighed, added to tubes containing glass beads, and suspended in 500 μL of DNA extraction buffer (200 mM Tris-HCl, 20 mM EDTA, 200 mM NaCl; pH8.0), 210 μL of 10% SDS, and 500 μL of buffer-saturated phenol. The mixtures were agitated vigorously for 60 s using a FastPrep-24 5 G homogenizer (MP Biomedicals). After centrifugation at 14,000 rpm for 5 min, 400 μL of supernatant were collected into a new tube. Subsequently, 500 μL of buffer-saturated phenol and 100 μL of DNA extraction buffer were added to the suspension and the mixture vortexed vigorously. After centrifugation at 14,000 rpm for 5 min, 400 μL of supernatant was collected into a new tube. Bacterial DNA was then obtained by isopropanol precipitation. Bacterial DNA was pelleted by centrifugation at 14,000 rpm for 5 min and washed with 70% ethanol. Finally, DNA was suspended in 100 μL of Tris-EDTA buffer.

2.4 | Real-time qPCR

Fecal DNA was used to analyze the bacterial groups quantitatively. Quantitative real-time PCR was performed using a LightCycler 96 system (Roche Diagnostics, Mannheim, Germany). 16 S rRNA genes were amplified by bacterial group-specific primers. Primers were UniF340 5′-ACTCCTACGGGAGGCAGCAGT-3′ and UniR514 5′-ATTACCGCGGCTGCTGGC-3′ for total bacteria, UniF338 5′-ACTCCTACGGGAGGCAGC-3′ and C.cocR491 5′-GCTTCTTAGTCAGGTACCGTCAT-3′ for *Clostridiales*, LabF362 5′-AGCAGTAGGGAATCTTCCA-3′ and LabR677 5′-CACCGCTACACATGGAG-3′ for *Lactobacillaceae*, BactF285 5′-GGTTCTGAGAGGAAGGTCCC-3′ and UniR338 5′-GCTGCCTCCCGTAGGAGT-3′ for *Bacteroidales*, and Uni515F 5′-GTGCCAGCAGCCGCGGTAA-3′ and Ent826R 5′-GCCTCA

AGGGCACAACCTCCAAG-3′ for *Enterobacteriaceae*.[19] All reactions were performed using a KAPA SYBR Fast qPCR kit (Kapa Biosystems, Wilmington, MA, USA).

2.5 | 16 S rRNA gene amplicon sequencing

Extracted fecal bacterial DNA was used to analyze the gut microbiota composition on the basis of 16 S rRNA genes. 16 S rRNA gene amplicon sequencing was performed as per the Illumina 16 S Metagenomic Sequencing Library Preparation protocol with modifications. Briefly, the corresponding sequences were amplified using primers specific for the V3-V4 region of the 16 S rRNA genes. For the PCR reaction, KAPA HiFi DNA polymerase (Kapa Biosystems) was used and the PCR product purified using AMPure XP Beads (Beckman Coulter, Brea, CA, USA). A second PCR was performed using the purified PCR product as a template and a Nextera DNA Index kit (Illumina, San Diego, CA, USA). After purifying the second PCR products, the DNA concentration was measured using a Qubit 4 fluorometer (Thermo Fischer Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Combined samples at a concentration of 10 pM were mixed with a PhiX Control kit v3 (Illumina) and loaded onto a MiSeq Reagent Kit v2 500 cycle cartridge (Illumina) and the MiSeq sequencing platform (Illumina). The 250-bp paired-end reads were merged using PANDAseq (ver. 2.8) with the default parameters set to other than "–l 400".[20] The merged sequences were analyzed by the pick_open_reference_otus.py workflow of QIIME (ver. 1.9.1) using the default parameters.[21] The samples' alpha- and beta-diversities were calculated using the core_diversity_analyses.py workflow of QIIME.

2.6 | *C. albicans* injection and detection

C. albicans (IFM 60662, ATCC 18804) obtained from the Medical Mycology Research Center of Chiba University's culture collection were routinely cultivated in potato dextrose broth at 37°C before oral administration. A total of 5×10^7 *C. albicans* were orally administered to mice receiving or not receiving antibiotics. Feces were collected at the indicated time points, diluted and plated on PDA containing chloramphenicol (0.05 g/L; Nacalai Tesque), penicillin (0.6 g/L) and streptomycin (0.167 g/L). Systemic tissues (spleen, kidney, and liver) were isolated from ampicillin-treated mice, homogenized using a TissueRuptor (Qiagen, Hilden, Germany), and plated on PDA. Colonies were counted after incubation for 2 d. Feces were collected 7 d after *C. albicans* injection to detect fungal DNA in the feces. Primer sets specific for ITS rDNA sequences, ITS1 5′-CTTGGTCATTTAGAGGAAGTAA-3′ and ITS2 5′-GC TGCGTTCTTCATCGATGC-3′, were used.[10] ITS sequences were amplified using EmeraldAmp PCR Master Mix (TaKaRa, Shiga, Japan).

2.7 | FMT and antifungal drug treatment

Mice treated with ampicillin were orally administered *C. albicans*. After *C. albicans* colonization of the feces of these mice had been confirmed by plating fecal diluents on PDA containing chloramphenicol, penicillin, and streptomycin, the mice were treated with fluconazole (0.5 g/L; LKT Laboratories, St Paul, MN, USA), 5-fluorocytosine (2 g/L; TCI Chemicals, Tokyo, Japan), and amphotericin B (0.1 g/L; Nacalai Tesque) mixed with drinking water containing ampicillin.[22,23] For the FMT, sterile water was substituted for drinking water containing ampicillin 1 d before the FMT. Donor feces were obtained from SPF C57BL/6 mice and subjected to FMT. After homogenizing one or two fecal pellets from donor mice in 1 mL of water, the supernatants were orally administered to a recipient mouse colonized with *C. albicans*. For the control mice without FMT treatment, sterile water was substituted for drinking water containing ampicillin 1 d at the same time as FMT mice. Feces were then collected from these recipient mice at the indicated time points and the *C. albicans* counted.

2.8 | Statistical analysis

Statistical analyses were conducted using Microsoft Excel and the GraphPad Prism software (San Diego, CA, USA). Results were compared using two-tailed Student's *t*-tests and Fisher's exact test. Data were plotted as means ± SD.

3 | RESULTS

3.1 | Experimental mice were resistant to *C. albicans* colonization of the gut

Based on a previous report demonstrating that commensal fungi endogenously colonize experimental mouse guts,[10] we first used culture- and DNA-based approaches to investigate whether test mice had commensal fungi in their GI tracts. In contrast to the previous report, we did not detect fungi in the feces of experimental mice maintained at our facility (Figure 1A,B). Therefore, we attempted to establish colonization by *C. albicans*, a commensal and pathogenic fungus, by administration this organism orally to fungus-free, wild-type C57BL/6 mice. We detected *C. albicans* in the feces immediately after oral administration; however, the number of fungi decreased gradually, *C. albicans* eventually becoming undetectable in these mice (Figure 1C). After failing to detect *C. albicans* in the feces,

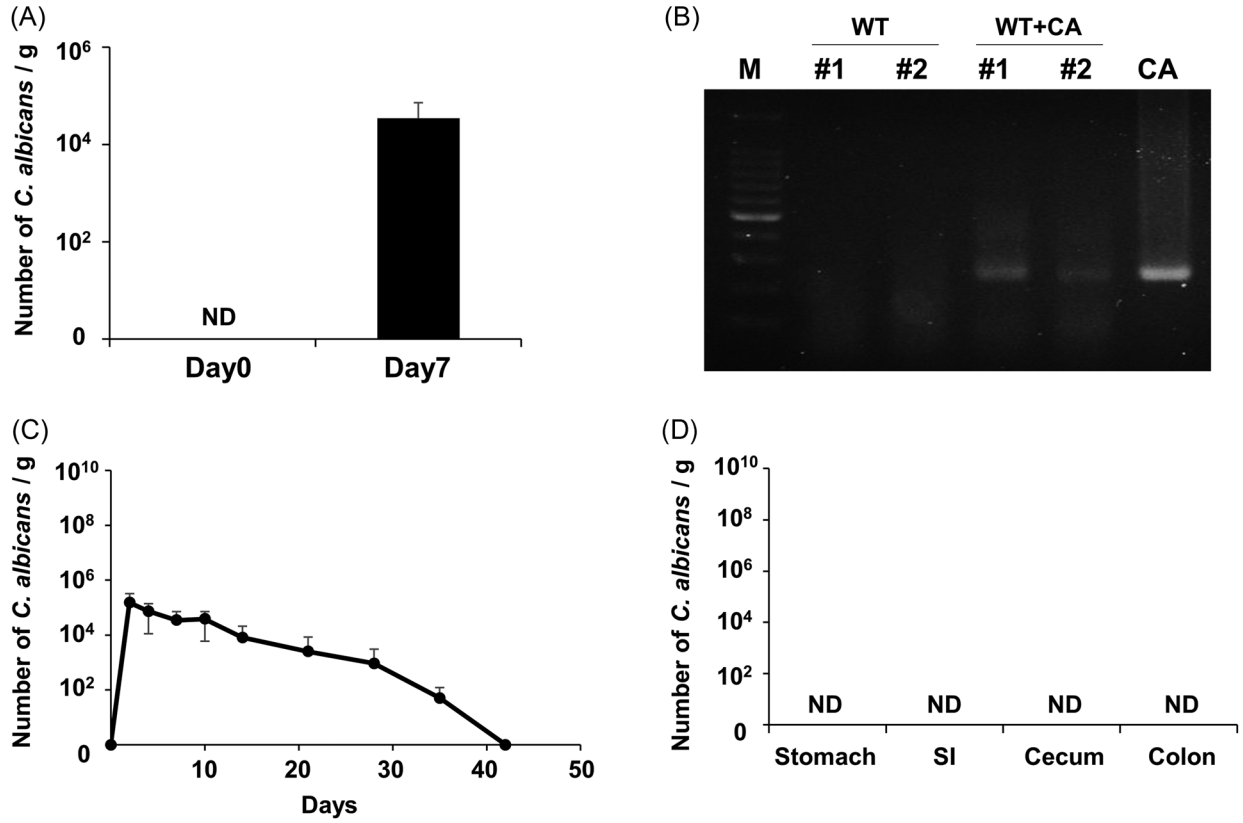

FIGURE 1 Experimental mice are resistant to *C. albicans* colonization of the GI tract. A, Culture-based quantification of fungi of feces isolated from wild-type C57BL/6 mice and mice injected with *C. albicans* ($n = 4$). Feces were collected 0 and 7 d after orally injecting *C. albicans*. B, Fungus-specific DNA was detected in feces isolated from mice injected with *C. albicans* but not in untreated C57BL/6 mice. CA, *C. albicans;* M, marker; WT, wild-type. C, *C. albicans* numbers in feces isolated from C57BL/6 mice injected with *C. albicans* were counted at the indicated time points ($n = 10$). Error bars, SD. D, *C. albicans* numbers of GI contents of mice 45 days after oral administration of *C. albicans* ($n = 3$). ND, not detected; SI, small intestine. Data from two to three independent experiments

we examined luminal contents of various parts of the GI tract (e.g., stomach, small intestine, cecum, and colon) and did not detect *C. albicans* in these contents (Fig. 1D), indicating that this organism had been completely cleared from the GI tract. We also established that BALB/c mice are resistant to *C. albicans* colonization of the GI tract (data not shown). These data indicate that wild-type mice are resistant to *C. albicans* colonization of the GI tract.

3.2 | β-lactam antibiotic treatment enables *C. albicans* to colonize the gut

We next examined why wild-type C57BL/6 mice are resistant to *C. albicans* colonization of the gut. Given that several environmental factors in murine guts can affect *Candida* colonization, we hypothesized that commensal microbes in the murine GI tract may protect against *Candida* colonization. To determine whether commensal microbes impacted *C. albicans* colonization, we treated SPF mice with multiple antibiotics. After antibiotic treatment, we collected feces from these mice and plated them on PDA for detection of *C. albicans*. We detected significant numbers of *C. albicans* colonies in the feces of mice treated with ampicillin or penicillin, both of which are β-lactam antibiotics (Figure 2A). Conversely, mice treated with other antibiotics (vancomycin, metronidazole, neomycin, colistin, streptomycin and gentamycin) had almost the same number of *C. albicans* in their feces as did the wild-type mice (Figure 2A). We next addressed whether *C. albicans* was detectable in the luminal contents of the GI tracts of ampicillin-treated mice and found significant and comparable numbers of *C. albicans* in the luminal contents from the stomach to the colon of ampicillin-treated mice, indicating that *C. albicans* had successfully colonized the GI tracts of these mice (Figure 2B). In the ampicillin-treated mice, these *C. albicans* levels were maintained for at least 3 months after administration (data not shown), indicating that *C. albicans* had indeed colonized their

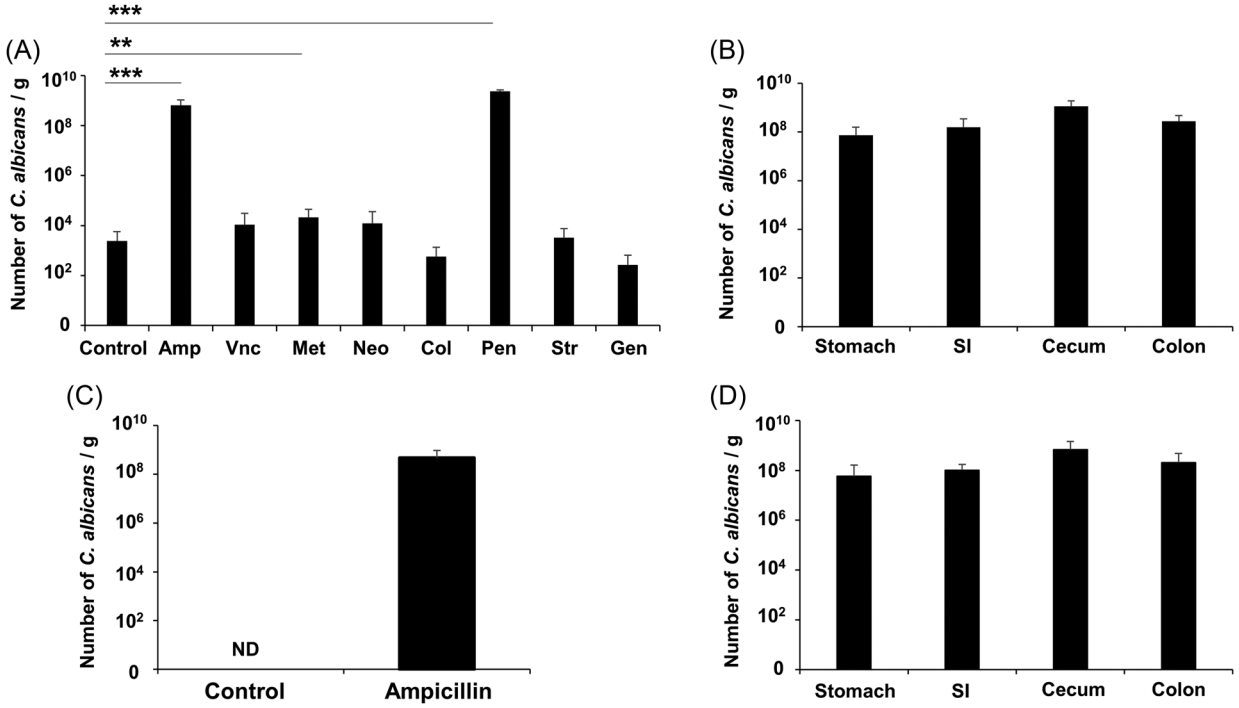

FIGURE 2 *C. albicans* colonizes ampicillin- and penicillin-treated mice (A) C57BL/6 mice were treated with or not treated with the indicated antibiotics. *C. albicans* in feces were counted 28 days after oral injection ($n = 5$). Amp, ampicillin; Col, colistin; Gen, gentamycin; Met, metronidazole; Neo, neomycin; Pen, penicillin; Str, streptomycin; Vnc, vancomycin; WT: wild-type. B, *C. albicans* numbers of GI contents of C57BL/6 mice treated with ampicillin ($n = 5$). C, *C. albicans* numbers of feces were counted 28 d after oral injection into BALB/c mice treated with or without ampicillin ($n = 6$). ND: not detected. D, *C. albicans* numbers in GI contents from ampicillin-treated BALB/c mice (n = 6). SI: small intestine. Error bars, SD. $^{***}P < 0.001$, $^{**}P < 0.01$ according to Student's *t*-test. Data are representative of two independent experiments

GI tracts. We also found that *C. albicans* colonized the GI tracts of ampicillin-treated BALB/c mice as they did the C57BL/6 mice (Figure 2C,D).

3.3 | Ampicillin treatment changes the commensal microbiota composition in mice

We next examined the bacterial compositions of ampicillin-treated and -untreated mouse microbiota. We isolated feces from these mice and analyzed their bacterial compositions based on 16 S rRNA gene amplicon sequencing. Gut microbiota compositions and bacterial diversity changed after ampicillin treatment (Figure 3A,B,C; Supplementary Figure 1, 2; and Supplementary Table 1). We also examined the bacterial compositions of the microbiota of other antibiotic-treated mice (Supplementary Figures 3, 4, 5; and Supplementary Table 1). Each antibiotic induced specific dysbiosis of fecal microbiota. We further investigated the quantity of major bacterial genera in the feces of ampicillin-treated mice by qPCR. Quantitative analysis showed that there were very much fewer *Lactobacillaceae*, *Bacteroidales*, *Clostridiales* and *Enterobacteriaceae* in the ampicillin-treated mice (Figure 3D).

3.4 | Numbers of ccommensal bacteria and *C. albicans* in the gut are inversely correlated

In addition to specific bacterial groups, there were dramatically fewer total commensal bacteria in the ampicillin-treated mice (Figure 4A), indicating that ampicillin treatment induces dysbiosis in murine GI tracts. We next investigated the quantitative kinetics of commensal bacteria and *C. albicans* in the gut after ampicillin treatment. A single oral administration of ampicillin drastically reduced commensal bacterial numbers; however, this effect was transient. Luminal bacterial numbers recovered within 4 d of injecting ampicillin (Figure 4B). To examine the relationship between *C. albicans* numbers and commensal bacteria, we orally injected ampicillin into mice and then administered *C. albicans*. *C. albicans* numbers increased greatly immediately after these injections and were maintained for 4 d. Numbers of *C. albicans* subsequently decreased to colonization levels comparable to those of the ampicillin-untreated mice by 7 d after *C. albicans* injection (Figure 4C). These data suggest that commensal bacterial colonization is inversely correlated with *C. albicans* in the GI tract and that commensal bacteria likely inhibit *C. albicans* colonization.

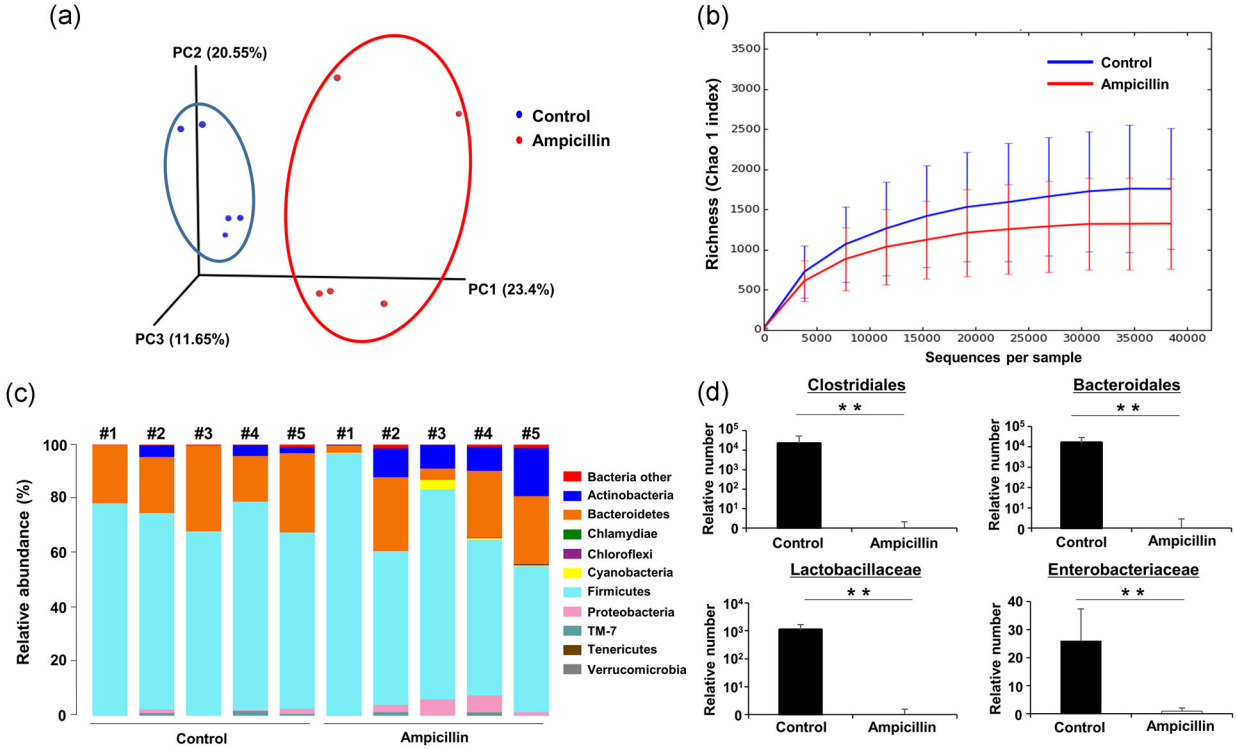

FIGURE 3 Dysbiosis was induced in ampicillin-treated mice (A), (B), (C) Based on 16 S rRNA genes in feces from ampicillin-treated and -untreated mice. A, PCoA Plots of unweighted UniFrac distances. B, alpha refraction curve. C, ratios of bacterial phyla are represented. D, Quantitative real-time qPCR analysis of bacterial groups in feces isolated from mice treated or not treated with ampicillin ($n = 5$). Error bars, SD. $^{**}P < 0.01$ according to Student's *t*-test. Data are representative of three independent experiments

3.5 | FMT prevents *C. albicans* colonization of murine GI tracts

We next investigated whether commensal bacteria have therapeutic potential against *C. albicans* colonization of the GI tract. Because FMT is a commensal, bacteria-based, therapeutic approach to treating pathogenic bacterial infections in the gut,[8] we attempted FMT in *C. albicans*-colonized mice. After the FMT, *C. albicans* numbers in the feces were drastically and immediately reduced compared with mice that had not undergone FMT (Figure 4D). We found that total numbers of commensal bacteria were restored 4 d after FMT (Figure 4E). To compare the effects of FMT and conventional antifungal drugs against *C. albicans* colonization of the GI tract, we treated *C. albicans*-colonized mice with the antifungal reagents, fluconazole, 5-fluorocytosine and amphotericin B. Amphotericin B depleted *C. albicans* in the gut, whereas fluconazole and 5-fluorocytosine did not (Figure 4F), indicating that FMT prevents *C. albicans* from colonizing the murine GI tract.

4 | DISCUSSION

In this study, we showed that murine GI microbiota contain potential therapeutic targets for *C. albicans* colonization of the GI tract. Wild-type experimental mice were resistant to *C. albicans* colonization in the GI tract, whereas *C. albicans* successfully colonized the GI tracts of mice treated with antibiotics such as ampicillin, inducing dysbiosis in the gut microbiota. FMT prevented *C. albicans* from colonizing the GI tract. These data highlight that murine commensal bacteria function as barriers and play roles in protecting the host from *C. albicans* colonization.

The mechanism by which commensal bacteria inhibit *C. albicans* colonization remains unclear. Several studies have shown that the protective effects of commensal bacteria against pathogenic bacteria are mediated by differentiation and activation of host innate and acquired immune cells in the gut.[18,24] Commensal bacteria, especially SFB, induce Th17 cells, which protect against *Candida* infection by producing IL-17A and subsequently activating neutrophils.[25] However, SFB and Th17 cells are unlikely to affect *C. albicans* colonization of the gut, because vancomycin treatment—which effectively eliminates SFB and Th17 cells—reportedly prevents *C. albicans* colonization of the gut[24,26] (Figure 2A). Further studies are necessary to determine whether a commensal bacterial-immune cell-axis affects *C. albicans* colonization of the gut.

Another possible mechanism is that commensal bacteria directly protect against *C. albicans* colonization of the GI

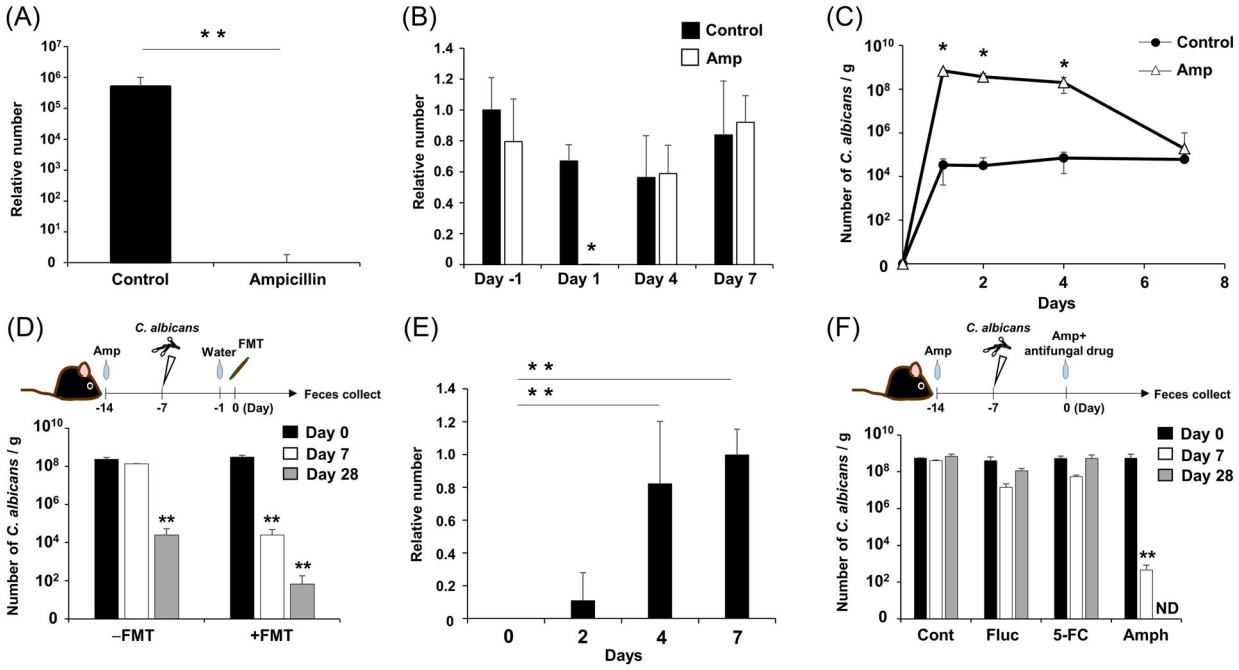

FIGURE 4 FMT prevents *C. albicans* from entering the GI tract (A) Real-time qPCR analysis of total bacteria in feces isolated from mice treated or not treated with ampicillin ($n = 5$). B, Relative numbers of total fecal bacteria were analyzed using real-time qPCR before and after injecting ampicillin ($n = 5$). C, *C. albicans* counts of feces from ampicillin-treated or -untreated mice ($n = 5$). D, *C. albicans*-colonized mice received water or FMT after cessation of ampicillin treatment. *C. albicans* counts of feces from mice at 0, 7 and 28 d after administration ($n = 3$). E, Relative numbers of total fecal bacteria from mice transferred with fecal microbiota were examined at the indicated time points ($n = 3$). F, *C. albicans*-colonized mice treated or not treated with antifungal drugs. *C. albicans* counts of feces from mice at 0, 7 and 28 d after administration (n = 3). Amph, amphotericin B;Cont, control; Fluc, fluconazole; 5-FC, 5-fluorocytosine; ND, not detected. Error bars, SD. $^{*}P < 0.05$, $^{**}P < 0.01$ according to Student's *t*-test. Data are representative of two independent experiments

tract. Commensal bacteria may compete with *C. albicans* for oxygen, pH and nutrients in this environmental niche. Commensal bacteria produce various metabolites in the gut lumen that may impact *C. albicans* colonization and proliferation.

Although we investigated the microbiota in mice treated with multiple antibiotics in this study, the specific bacterial species that protect against colonization by *C. albicans* of the GI tract are still unknown. *C. albicans* colonized the GI tracts of mice treated with ampicillin and penicillin, both of which dramatically reduced the number of total and specific commensal bacteria (Figures 3D, 4A; data not shown). In contrast, mice treated with other antibiotics that induce dysbiosis (Supplementary Figures 3, 4, 5; and Supplementary Table 1) were resistant to *C. albicans* colonization (Figure 2A), suggesting that several bacterial species may be involved in protection against *C. albicans* colonization. We also found by 16 s rRNA sequence analysis that vancomycin-treated mice predominantly harbor *Lactobacillaceae* (Supplementary Figure 6; and Supplementary Table 1). Taken together with the data that vancomycin-treated mice are resistant to *C. albicans* colonization, these data suggest that *Lactobacillaceae* are one of the types of commensal bacteria responsible for protection against *C. albicans* colonization in vivo. Indeed, a previous study showed that *Lactobacillus* inhibits expression of virulence factors of *C. albicans* in vitro.[27] The specific kinds of bacteria and detailed mechanism of *Lactobacillus* protecting against *C. albicans* colonization of the GI tract require investigation in future studies.

Because commensal bacteria affect *C. albicans* colonization, the intestinal environment that influences the commensal population may be critical for *C. albicans* colonization of the GI tract. The factors that determine *C. albicans* colonization, including environmental factors such as food, water and bedding, and how these factors affect the commensal bacterial composition and subsequent *C. albicans* colonization of the GI tract remain unclear. Indeed, several fungi, including *Saccharomyces*, *Trichosporon* and *Candida* species, have been found to colonize endogenously, even in SPF mice bred in another experimental facility.[10] Conversely, other groups have reported fungus-free SPF mice.[28]

C. albicans colonization of the GI tract is considered a trigger for invasive candidiasis.[11] However, *C. albicans* were minimally detectable in the systemic tissues, even in ampicillin-treated *C. albicans*-colonized mice (Supplementary Figure 7 and Supplementary Table 2). Conversely, many *C. albicans* were detected in the systemic tissues after i.v. injection, indicating that *C. albicans* can reach systemic

compartments via the bloodstream (Supplementary Figure 3 and Supplementary Table 2). These data suggest that the host's surface barrier system, particularly the gut epithelial cells and immune system, may prevent *C. albicans* from infiltrating blood vessels and that commensal bacteria may be important to the surface barriers preventing *C. albicans* infections. In addition to life-threatening systemic fungal infections in immunocompromised patients, fungal colonization of the GI tract exacerbates allergic airway inflammation.[22] Thus, controlling fungal colonization may be an effective strategy for regulating both infections and inflammation and allergies. Targeted modification of commensal bacteria may provide novel approaches for controlling these diseases.

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DISCLOSURE

The authors declare that they have no conflicts of interest regarding this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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