Effects of a Novel Probiotic Combination on Pathogenic Bacterial-Fungal Polymicrobial Biofilms

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ABSTRACT Dysbiosis of the gut microbiome has been implicated in inflammatory bowel diseases. We have shown that levels of *Candida tropicalis*, along with those of *Escherichia coli* and *Serratia marcescens*, are significantly elevated in Crohn's disease (CD) patients. Here, we evaluated the ability of a novel probiotic to prevent and treat polymicrobial biofilms (PMB) formed by *C. tropicalis* with *E. coli* and *S. marcescens*. Since *Candida albicans* has been reported to be elevated in CD patients, we investigated the interactions of *C. albicans* with these bacterial species in biofilm formation. We determined whether the interaction between *Candida* spp. and bacteria is specific by using *Trichosporon inkin* and *Saccharomyces fibuligera* as comparators. Additionally, the effects of probiotics on *C. albicans* germination and biofilm formation were determined. To determine the ability of the probiotic to prevent or treat mature biofilms, probiotic filtrate was added to the PMB at early (prevention) and mature (treatment) phases. Biofilm thickness and architecture were assessed by confocal scanning laser microscopy. The effects of the probiotic on germination were evaluated in the presence of serum. Exposure of *C. tropicalis* PMB to probiotic filtrate reduced biofilm matrix, decreased thickness, and inhibited hyphal formation. We showed that *C. albicans* or *C. tropicalis* formed significantly thicker PMB than control biofilms, indicating that this interaction is *Candida* specific. Treatment with probiotic filtrate inhibited *C. albicans* germination and prevented/treated *C. albicans* PMB. The designed probiotic may have utility in the management of biofilm-associated gastrointestinal diseases such as Crohn's and colorectal cancer.

IMPORTANCE The effects of diversity of the gut microbiome on inflammation have centered mainly on bacterial flora. Recent research has implicated fungal species and their interactions with other organisms in the inflammatory process. New ways to restore microbial balance in the gut are being explored. Our goal was to identify beneficial probiotic strains that would antagonize these fungal and bacterial pathogens that are elevated in the inflamed gut, and which also have antibiofilm activity. Fungus-bacterium correlation analysis allowed us to identify candidate probiotic species that can antagonize microbial pathogens, which we subsequently incorporated into a novel probiotic formulation. Amylase, which is known to have some antibiofilm activity, was also added to the probiotic mixture. This novel probiotic may have utility for the management of inflammatory bowel diseases by disrupting polymicrobial biofilm formation.

KEYWORDS *Candida albicans*, *Candida tropicalis*, biofilms, probiotics

Human gastrointestinal (GI) microbiome research has primarily focused on resident bacteria. Although much has been learned, there is still much left to be discovered, especially with regard to the mycobiome (i.e., the fungal community) and its impact on

human health and disease. One GI disease whose relationship with gut fungi has been partially explored is Crohn's disease (CD), an inflammatory disease of the bowel.

Recent studies showed that the global fungal load is higher in patients with CD than healthy individuals (1). When there is a relative increase in the abundances of *Candida albicans*, *Aspergillus clavatus*, and *Cryptococcus neoformans*, the CD activity index is elevated, along with the cytokines tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), and interleukin 10 (IL-10) (2). In contrast, when higher levels of *Saccharomyces cerevisiae* and *Filobasidium uniguttulatum* are found in their gut, CD patients are in a less inflammatory state (1). Standaert-Vitse et al. (3) have shown that *C. albicans* is more abundant in patients with CD than healthy individuals, and this abundance is associated with significantly higher levels of anti-*Saccharomyces cerevisiae* antibodies (ASCA), a well-known biomarker of CD (4). Further, a significant positive correlation between *Candida tropicalis* abundance and ASCA was also observed in patients with CD.

These associations allude to the possibility that certain fungal species may be protective (e.g., *S. cerevisiae*), while others are detrimental (e.g., *C. tropicalis*). These findings provide experimental evidence that the mycobiome plays a pivotal role in CD pathogenesis. However, its role cannot be observed in isolation; rather, it must be observed through its interactions with the GI bacterial community (i.e., bacteriome).

A study by Kalan et al. (5) assessed fungus-bacterium interactions in patients with nonhealing diabetic foot ulcers and demonstrated that the mycobiome present in these wounds forms multispecies biofilms, or polymicrobial biofilms (PMB), with bacteria. *Corynebacterium* spp. were negatively correlated with *C. albicans* and *Candida parapsilosis*. *C. albicans*, however, was positively correlated with the order *Alcaligenaceae*, a group of Gram-negative proteobacteria.

Polymicrobial interactions have also been observed in patients with oral tongue cancer. Positive correlations were observed between the fungal phylum Zygomycota and the bacterial phyla *Bacteroidetes* and *Fusobacteria*. Further, the Gram-negative bacteria *Campylobacter* spp., *Fusobacterium* spp., and *Porphyromonas* spp. were negatively associated with *Emericella* spp. (sexual state of *Aspergillus nidulans*) and positively associated with the zygomycetes of *Lichtheimia* (*Absidia*) (6).

More recently, we demonstrated that *C. tropicalis*, *Escherichia coli*, and *Serratia marcescens* were significantly more abundant in CD patients than in their nondiseased first-degree relatives (4). In this same study, the abundances of each of these individual microorganisms (i.e., *C. tropicalis*, *E. coli*, and *S. marcescens*) were positively associated with one another. In addition to increased abundance, when grown together, these organisms formed pathogenic PMB that were capable of initiating an inflammatory response and were significantly thicker than biofilms formed by these microorganisms individually or in pairs with one another. (i.e., *C. tropicalis/E. coli*, *C. tropicalis/S. marcescens*, or *E. coli/S. marcescens*) (4).

Since the cooperative interaction of fungi and bacteria as well as that of bacteria and bacteria in the dysbiotic state often results in biofilm formation in the gut (termed digestive plaque) and has been shown to produce harmful effects on the host, it is logical to suggest that correction of this dysbiosis through inhibition of biofilm formation may possibly benefit the host. One way to correct this dysbiosis could be through the use of probiotics. Recently, certain probiotic bacteria have been studied as a potential method to prevent opportunistic infectious diseases by stimulating the host immune system (7–9). Additionally, previous studies have reported the positive effects of probiotics in mucosal candidiasis, such as *Candida* vaginitis and vulvovaginal candidiasis, oral candidiasis, and gastrointestinal infection (10–13). To date, however, very little research has been done on the effect of probiotics in the setting of CD. Of the studies that have been conducted, most have been relatively small trials with small numbers of enrolled patients (14).

Further, it is important to remember that designing a probiotic capable of reducing intestinal dysbiosis requires the selection of appropriate microbial species that target pathogenic bacterial and fungal species elevated in gastrointestinal diseases, while at

the same time supporting beneficial ones. In order to identify such a composition, we conducted correlation analyses of bacterium-bacterium, fungus-fungus, and bacterium-fungus interactions as a prelude to the current study and were able to identify probiotic species of bacteria and fungi that are able to antagonize the pathogenic GI microbes while simultaneously supporting the beneficial ones (15). Additionally, given our previous biofilm findings, we were able to identify probiotic species that possess antibiofilm activity, which is important given the role of biofilm formation in microbial pathogenicity. Furthermore, we incorporated amylase into the probiotic mixture based on its demonstrated antibiofilm activity (16). Based on our preliminary findings, we developed a novel probiotic formulation consisting of *Saccharomyces boulardii*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, and *Bifidobacterium breve*, which contained amylase as a hydrolytic enzyme, that would prevent and treat PMB. Since in our previous publication we showed that *C. tropicalis* can form robust biofilms with *E. coli* and *S. marcescens*, and others have shown that *C. albicans* is also elevated in CD patients, we wanted to determine whether this interaction is specific to *Candida* spp. Therefore, we evaluated the ability of *C. albicans* and *C. tropicalis* to form PMB compared to *Trichosporon inkin* and *Saccharomyces fibuligera*. These two control yeast species were chosen because *T. inkin* was present equally in both CD patients and their healthy relatives, while *S. fibuligera* is a nonpathogenic yeast which has been employed as a comparator in *C. tropicalis* gut inflammation studies (17, 18). Next, we determined the ability of the designed probiotic to prevent and treat PMB formed by *C. tropicalis* and *C. albicans* in collaboration with *S. marcescens* and *E. coli*. The ability of the probiotic to inhibit *C. albicans* germination was also assessed.

RESULTS

Effects of probiotic filtrate on PMB. The antibiofilm activity of the designed probiotic was tested against PMB formed by *C. tropicalis* combined with *E. coli* and *S. marcescens*. The effects of the probiotic was assessed using confocal scanning laser microscopy (CSLM), where vertical (*xz*) sections and side views of the three-dimensional (3D)-reconstructed images were used to determine biofilm thickness and architecture. Additionally, the effect of the probiotic on the ultrastructure of PMB was assessed using scanning electron microscopy (SEM).

Probiotics prevent *Candida tropicalis* PMB. CSLM analysis demonstrated that untreated *C. tropicalis* PMB exhibited healthy biofilms composed mainly of yeast and a few hyphal structures within the extracellular matrix (Fig. 1A). SEM analysis confirmed that untreated PMB consisted of healthy yeast and hyphal structures (Fig. 1C). Furthermore, bacterial cell aggregates were clearly visible (Fig. 1C, arrow). In contrast, CSLM images of *C. tropicalis* PMB exposed to probiotic filtrate had no biofilm matrix, with few fungal cells (Fig. 1B). SEM micrographs showed that treated PMB showed an absence of matrix, very few yeast cells, and no visible bacteria (Fig. 1D).

Quantitative analysis of the PMB exposed to probiotic showed a significant reduction ($P < 0.05$) in thickness compared to the untreated controls (Fig. 2A). Biofilms formed by *C. tropicalis* alone were included as an additional control and showed a similar significant reduction ($P < 0.05$) in thickness after exposure to the probiotic (Fig. 2A).

Probiotics treat *Candida tropicalis* PMB. CSLM analysis showed that untreated mature PMB exhibited robust growth and a noticeable abundance of extracellular matrix (Fig. 3A). Conversely, mature PMB exposed to probiotic showed a complete absence of the extracellular matrix, leaving only scattered yeast cells lacking any structural biofilm elements (Fig. 3B).

Quantitative analysis of the mature PMB exposed to probiotic revealed a significant reduction ($P < 0.05$) in thickness compared to untreated controls (Fig. 2B). Mature biofilms formed by *C. tropicalis* alone were included as an additional control and showed similar significant reduction ($P < 0.05$) in thickness after exposure to the probiotic (Fig. 2B).
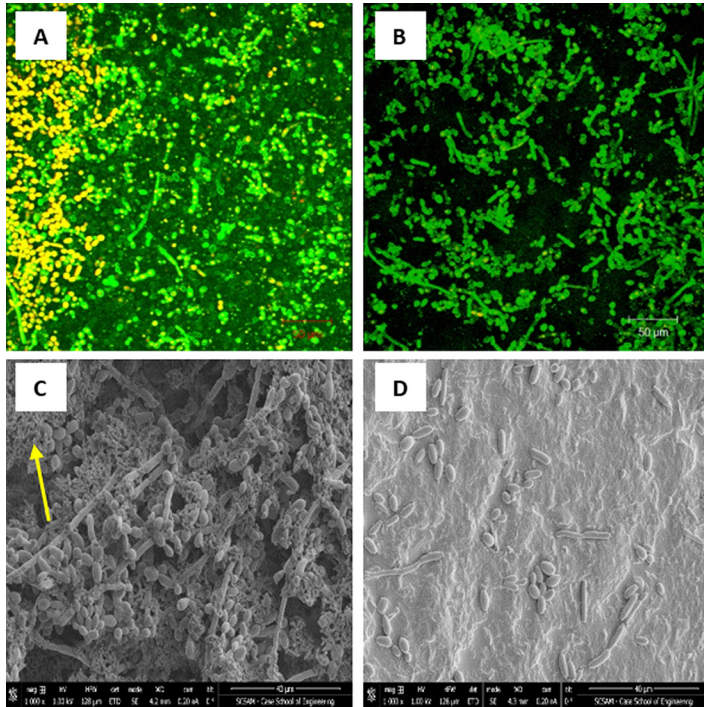
FIG 1 Effect of probiotic on prevention of *C. tropicalis*, *E. coli*, and *S. marcescens* polymicrobial biofilms (PMB). PMB were exposed to probiotic filtrate during the early biofilm phase and allowed to form biofilms. The effects were examined using CSLM and SEM. (A) CSLM micrograph of untreated PMB showing healthy biofilm composed of yeast and hyphal structures. (B) CSLM micrograph of probiotic-treated PMB showing no biofilm matrix and reduced fungal cells. (C) SEM micrograph of untreated PMB showing dense biofilm with yeast and hyphal structures, as well as bacterial aggregates (arrow). (D) SEM micrograph of probiotic-treated PMB showing absence of matrix, very few yeast cells, and no visible bacteria. CSLM magnification, ×100; SEM magnification, ×1,000.

Is the interaction between yeast and bacteria *Candida* specific? To determine whether the enhanced bacterium-yeast PMB is specific to *Candida* spp., we compared the abilities of *C. albicans* and *C. tropicalis* relative to two control yeast species (*T. inkin* and *S. fibuligera*) to form PMB when grown with *E. coli* and *S. marcescens*.

Our data showed that both *C. albicans* and *C. tropicalis* were able to form significantly thicker biofilms ($P < 0.05$) with *E. coli* and *S. marcescens* than biofilms formed by single species (Fig. 4). In contrast, no significant differences were observed between
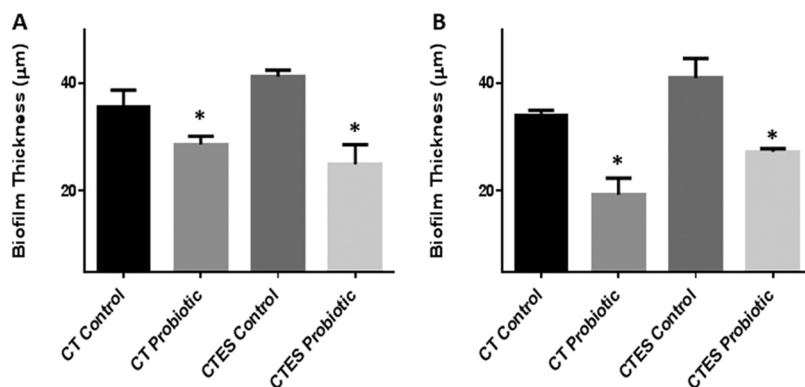

FIG 2 Effect of probiotic on *C. tropicalis*, *E. coli*, and *S. marcescens* (CTES) PMB, as well as biofilms formed by *C. tropicalis* alone. Vertical (*xz*) sections or side views of the 3D-reconstructed CSLM images were used to determine biofilm thickness. (A) Prevention of biofilm formation by probiotic. (B) Treatment of biofilms by probiotic. Exposure to probiotic led to a significant reduction in the thickness of PMB and single-species biofilms compared to the untreated controls.
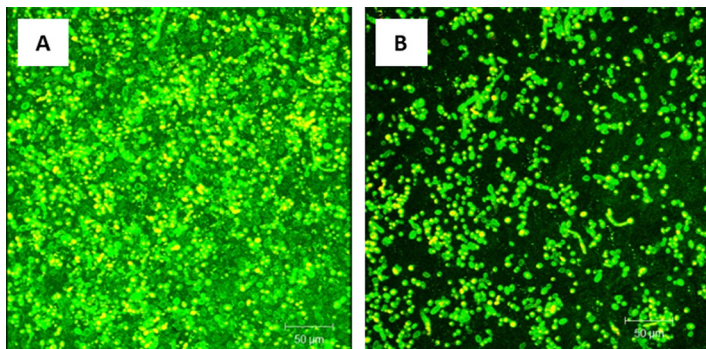
FIG 3 Effect of probiotic on treatment of *C. tropicalis*, *E. coli*, and *S. marcescens* PMB. Mature PMB were exposed to probiotic filtrate and incubated for an additional 24 h. The effects were examined using CSLM. (A) CSLM micrograph of untreated PMB showing healthy biofilm composed of yeast and hyphal structures. (B) CSLM micrograph of probiotic-treated PMB showing no biofilm matrix and reduced fungal cells.

single-species biofilms or PMB formed by *T. inkin* and *S. fibuligera* ($P > 0.05$, Fig. 4). These data suggest that the ability of *C. tropicalis* and *C. albicans* to form thick biofilms with these two bacterial pathogens is *Candida* specific.

Effect of probiotic filtrate on germ tube formation by *C. albicans*. Since germination is an important *Candida* virulence factor, we evaluated the effect of probiotic filtrate on the ability of *C. albicans* to form germ tubes. CSLM images showed that untreated *C. albicans* (Fig. 5A) formed germ tubes, while exposure to probiotic filtrate inhibited the ability of *C. albicans* to germinate (Fig. 5B). Quantitative analysis showed that there was a significant reduction in percent germ tube formation up to 2 h following exposure to probiotic filtrate compared to the untreated control ($P < 0.05$, Fig. 5C).

Probiotics prevent *Candida albicans* PMB. The effect of probiotic exposure on *C. albicans* PMB was assessed using CSLM. Our data showed that untreated *C. albicans* PMB exhibited heterogeneous biofilm architecture composed mainly of yeast cells and hyphal structures embedded within an extracellular matrix (Fig. 6A). In contrast, *C. albicans* PMB grown in the presence of probiotic filtrate showed no biofilm matrix and very few yeast cells and hyphal structures (Fig. 6B).

Quantitative analysis of *C. albicans* PMB showed a significant reduction ($P < 0.05$) in biofilm thickness when exposed to probiotic filtrate compared to the untreated controls (Fig. 7A). Biofilms formed by *C. albicans* alone were included as an additional
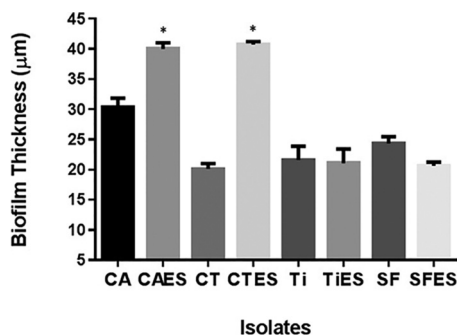

FIG 4 Determination of the specificity of fungus-bacterium interactions in PMB formation. PMB formed by *C. tropicalis* (CT) or *C. albicans* (CA) with *E. coli* (E) and *S. marcescens* (S) were compared with biofilms formed by *T. inkin* (Ti) or *S. fibuligera* (SF) with *E. coli* and *S. marcescens*. PMB formed by *C. tropicalis* or *C. albicans* were significantly thicker than biofilms formed by *C. tropicalis* or *C. albicans* alone. In contrast, no significant difference was seen between PMB formed by *T. inkin* or *S. fibuligera* and biofilms formed by these yeasts alone, indicating that the fungus-bacterium interaction in PMB formation is *Candida* specific.
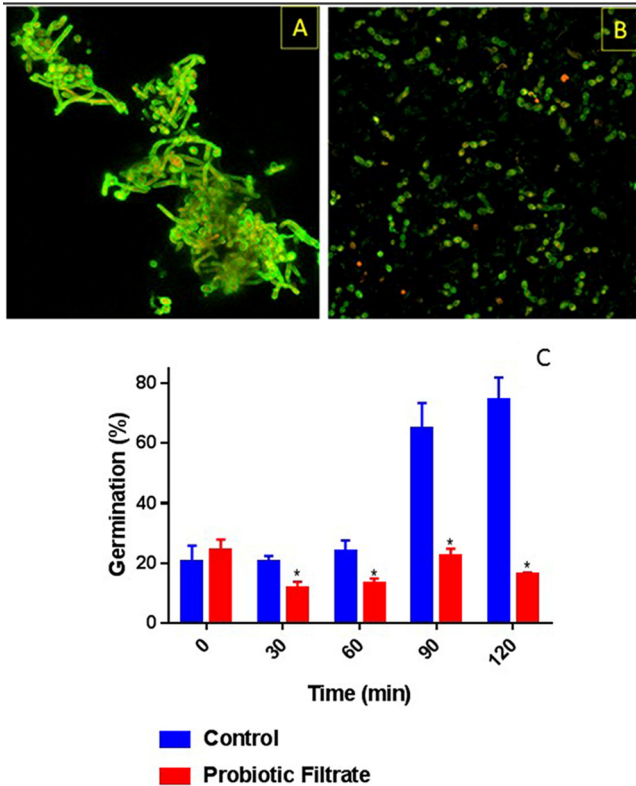
FIG 5 Effect of probiotic on germ tube formation by *C. albicans*. Ability of *C. albicans* to form germ tubes was evaluated in the presence or absence (control) of probiotic filtrate. Exposure to probiotic led to inhibition of germ tube formation by *C. albicans*. (A) CSLM image of control untreated *C. albicans* showing profuse germ tube formation. (B) CSLM image of probiotic-treated *C. albicans* showing yeast morphology only with no germ tubes. (C) Quantitative analysis of temporal germ tube formation of probiotic-treated and untreated *C. albicans* cells showing a significant reduction in percent germination.

control and showed a similar significant reduction ($P < 0.05$) in thickness following exposure to the probiotic (Fig. 7A).

Probiotics treat *Candida albicans* PMB. CSLM analysis showed that untreated mature PMB had an enriched extracellular matrix and biofilm formation (Fig. 8A). In contrast, mature PMB exposed to probiotic filtrate showed complete absence of the extracellular matrix, with few yeast cells and no visible hyphae (Fig. 8B).

FIG 6 Effect of probiotic on prevention of *C. albicans*, *E. coli*, and *S. marcescens* PMB. PMB were exposed to probiotic filtrate during the early biofilm phase and allowed to form biofilms. The effects were examined using CSLM. (A) CSLM micrograph of untreated PMB showing robust biofilm composed of yeast and hyphae. (B) CSLM micrograph of probiotic-treated PMB showing inhibition of biofilm with absence of extracellular matrix and reduced fungal elements. Magnification, ×100.
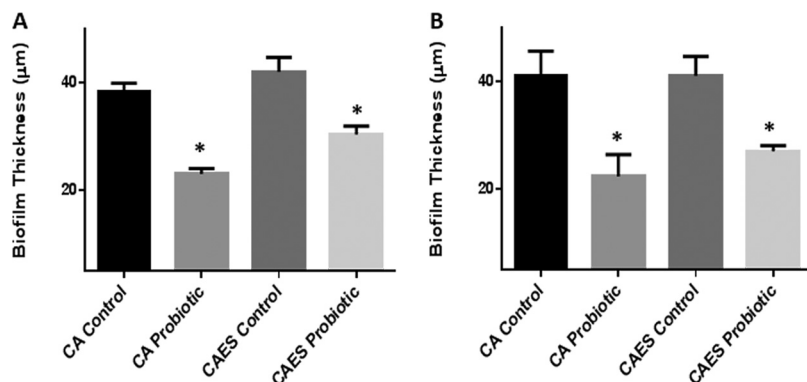
FIG 7 Effect of probiotic on *C. albicans*, *E. coli*, and *S. marcescens* PMB, as well as biofilms formed by *C. albicans* alone. Vertical (*xz*) sections or side views of the 3D-reconstructed CSLM images were used to determine biofilm thickness. (A) Prevention of biofilm formation by probiotic. (B) Treatment of biofilms by probiotic. Exposure to probiotic led to a significant reduction in the thickness of PMB and single-species biofilms compared to that in untreated controls.

Quantitative analysis of mature PMB showed a significant reduction ($P < 0.05$) in biofilm thickness when exposed to probiotic filtrate compared to the untreated controls (Fig. 7B). Mature biofilms formed by *C. albicans* alone were included as an additional control and showed a similar significant reduction ($P < 0.05$) in thickness after exposure to the probiotic (Fig. 7B).

DISCUSSION

In this study, we demonstrated that the designed probiotic possesses antibiofilm activity against PMB formed by *C. albicans* or *C. tropicalis* when combined with *E. coli* and *S. marcescens*. Specifically, when exposed to the probiotic filtrate, *C. albicans* and *C. tropicalis* PMB had an altered architecture and morphology and were significantly reduced in thickness in both the early and mature phases, indicating that the filtrate is able to prevent and treat such biofilms. Additionally, we showed that the interaction between bacteria and yeast is *Candida* specific. Furthermore, incubation of *C. albicans* with the probiotic filtrate inhibited *C. albicans* germination, an important *Candida* virulence factor (19, 20). Germination is also an important process in *Candida* biofilm formation, providing dense hyphal elements present in mature biofilms (21–24).

Since the probiotic filtrate possessed antibiofilm activity, we postulate that it is highly likely that its effects are mediated by secretory product(s) (e.g., a metabolite and/or an enzyme). In this regard, Murzyn et al. (25) provided experimental evidence showing that *S. boulardii* secretes fatty acids and 2-phenylethanol, including capric acid
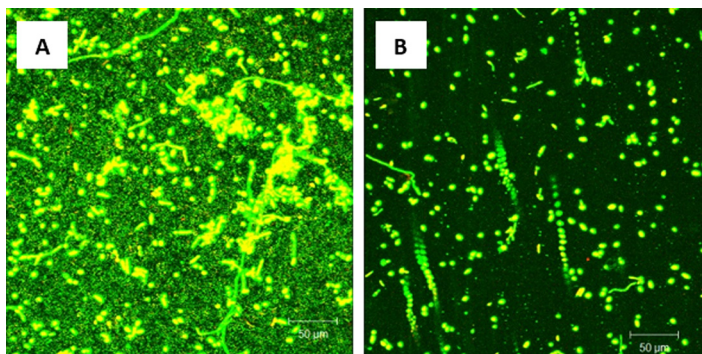

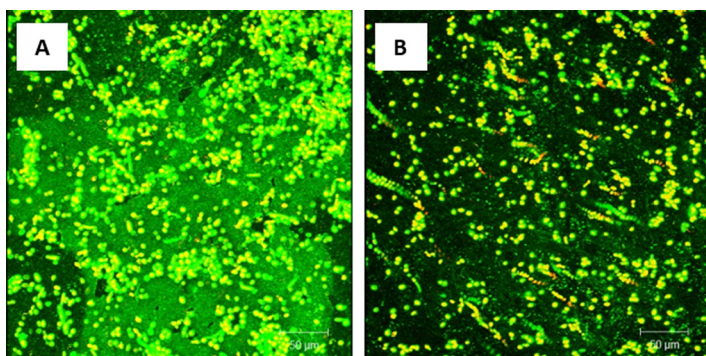**FIG 8** Effect of probiotic on treatment of *C. albicans*, *E. coli*, and *S. marcescens* PMB. Mature PMB were exposed to probiotic filtrate and incubated for an additional 24 h. The effects were examined using CSLM. (A) CSLM micrograph of untreated PMB showing enriched biofilm extracellular matrix. (B) CSLM micrograph of probiotic-treated PMB showing absence of biofilm matrix and reduced fungal cells.

($C_{10:0}$). This compound was shown to be the most effective in inhibiting candidal pathogenicity determinants, including the yeast-to-hyphal morphological transition, adhesion, and biofilm formation. Additionally, these authors showed that capric acid also reduced the expression of *C. albicans* genes implicated in its virulence, such as *HWP1* (formation of hyphae), *CSH1* (adhesion properties), and *INO1* (required for the synthesis of the virulence factor phospholipomannan). It is also likely that other metabolites secreted by *S. boulardii* may contribute to the ability of our probiotic to interfere with the virulence factors of these pathogens.

In addition to the yeast *S. boulardii*, the developed probiotic contains bacterial strains of *L. acidophilus*, *L. rhamnosus*, and *B. breve*. Metabolites released by *Lactobacillus* species, such as sodium butyrate, have been shown to inhibit biofilm formation, to potentiate the effect of antifungal agents, and to suppress *C. albicans* filamentation, thereby reducing fungal pathogenicity (26). Studying similar interactions between yeast and bacteria, Morales et al. (27) demonstrated that phenazines produced by *Pseudomonas aeruginosa* can modulate the metabolism of *C. albicans*. Although the presence of low concentrations of these substances permitted the growth of *C. albicans*, it affected biofilm formation and inhibited the yeast-to-hypha transition. Noverr and Huffnagle (28) investigated the effects of live cultures, culture supernatants, and dead cultures of probiotic bacteria on the morphogenesis of *C. albicans*. The authors observed that supernatants obtained from 2-h cultures of these bacteria inhibited germ tube formation in *C. albicans*, and the addition of 24-h cultures completely inhibited germination, suggesting that the accumulation of a soluble compound in the culture supernatant is responsible for this inhibition.

Another factor that plays a role in filamentation is the pH of the medium. In the case of *C. albicans*, pH serves as a strong signal for morphological differentiation. Alkaline conditions favor the growth of hyphal forms over yeast forms (29), a factor which may in turn facilitate biofilm formation. Thus, the production of lactic acid and other organic acids by *Lactobacillus* spp., which can substantially decrease the pH, may play an important role in fungal growth (30). Köhler et al. (31) investigated the potential of *L. rhamnosus* and *Lactobacillus reuteri* to control *C. albicans*. The reduction in cell viability was greater when *C. albicans* was incubated with the *Lactobacillus* culture filtrate in de Man-Rogosa-Sharpe (MRS) broth at pH 4.5 compared to *C. albicans* alone control. Furthermore, the incubation of *C. albicans* in MRS broth alone did not reduce cell viability. Evidence supporting this contention is derived from the findings that a clear difference exists in *C. albicans* response following treatment with purified capric acid and extract from *S. boulardii*. Further research is necessary to characterize the metabolites involved in the modulation of microbial virulence factors.

Our findings showing that our novel probiotic formulation significantly inhibited biofilm formation are in agreement with the findings of others. Krasowska et al. (32) showed that *C. albicans* adhesion and biofilm formation are significantly inhibited by exposure to both *S. boulardii* cells and filtrate. Additionally, an *in vitro* study by Ribeiro et al. (33) showed that *L. rhamnosus* cells and supernatant can both reduce *C. albicans* biofilm formation, filamentation, and gene expression of adhesion (*ALS3* and *HWP1*). Amylase has also been shown to have antibiofilm activities. In one study by Taraszkiewicz et al. (34), amylase was able to disrupt biofilms and biofilm formation via biofilm-enzyme degradation.

The antibiofilm activity of our probiotic has significant health implications given that biofilms are increasingly being recognized as primary contributors to host infections and certain GI diseases (e.g., Crohn's disease and colorectal cancer) (35). According to the World Health Organization, biofilms are implicated in 65 to 80% of microbe-based diseases. In the lining of the gut, epithelial cells are covered by a mucus layer that provides protection against adherence and invasion by pathogenic organisms (36). Several studies have described the role of biofilm formation in the inflammatory processes of bowel diseases and have identified adherent-invasive *E. coli* (AIEC) strains as having a greater capacity for biofilm formation than nonadherent strains. Not only were these phenotypes more able to form biofilms, but they were also able to replicate

within macrophages (37). According to Martinez-Medina et al. (38), 65.4% of *E. coli* strains capable of producing moderate to strong biofilms were also able to adhere to and invade the epithelial lining of the gut, while 74.4% of strains that produced weak biofilms were unable to do so. These adherence and invasion properties have also been demonstrated in other human intestinal pathogens, including species of *Yersinia*, *Shigella*, *Salmonella*, *Campylobacter*, and *Aeromonas*, though these do not normally cause chronic inflammation (36). Colorectal cancer is another disease entity that was recently linked to PMB, with PMB proposed as a trigger of procarcinogenic inflammatory responses. In this regard, Dejea et al. (39) identified biofilms composed predominantly of *E. coli* and *Bacteroides fragilis* in patients with familial adenomatous polyposis. Thus, the availability of a probiotic mixture that is able to prevent and treat PMB may have utility in managing these diseases.

MATERIALS AND METHODS

Strains and culture media. The clinical strains used belonged to *Candida albicans* (strain SC5314), *C. tropicalis* and *E. coli* (both isolated from the fecal sample of a CD patient), *Serratia marcescens*, *Trichosporon inkin*, and *Saccharomyces fibuligera*. The following probiotic strains were purchased from the American Type Culture Collection: *Saccharomyces boulardii* ATCC MYA-796, *Lactobacillus acidophilus* ATCC 43121, *Bifidobacterium breve* ATCC 15701, and *L. rhamnosus* ATCC 39595. Yeast nitrogen base (YNB) and brain heart infusion (BHI) media were purchased from Becton, Dickinson and Company (Sparks, MD). Hanks' buffered saline solution (HBSS), phosphate-buffered saline (PBS), and fetal bovine serum (FBS) were purchased from Mediatech, Inc. (Manassas, VA).

Growth of probiotic strains. To evaluate the ability of the four probiotic strains to inhibit biofilms, each was grown alone in a mixture of YNB and BHI broth and incubated at 37°C for 24 h (*B. breve* was grown under anaerobic conditions). Cultures were subsequently centrifuged at 3,000 × g for 5 min, and their supernatants (filtrate) were decanted and filter sterilized using 0.22-μm-pore-size filters. Filtrates from the different probiotic strains were combined in equal amounts, to which amylase was added at a concentration of 1 mg/ml and stored at −20°C until use.

Ability of the probiotic filtrate to prevent PMB formation. To determine the ability of the probiotic strains to prevent PMB formation, suspensions of 1×10^7 cells from overnight cultures of *C. albicans* or *C. tropicalis* mixed with *E. coli* and *S. marcescens* were added to 12-well plates containing silicone elastomer (SE) discs (previously soaked in fetal bovine serum for 24 h) and allowed to adhere to the surface for 90 min. Following this early phase, the discs were rinsed twice with PBS to remove any nonadhered cells and placed in a new 12-well plate containing 4 ml of probiotic filtrate or YNB and BHI media for controls. Discs were then incubated at 37°C for an additional 24 h. Growth rate studies have previously shown that these probiotic strains did not attain lag phase until 48 h or longer, indicating that the filtrate would not be nutrient deficient at 24 h, a factor which may interfere with biofilm disruption in and of itself.

Ability of the probiotic filtrate to treat mature PMB. Suspensions of 1×10^7 cells from overnight cultures of *C. albicans* or *C. tropicalis* mixed with *E. coli* and *S. marcescens* were added to 12-well plates containing SE discs (previously soaked in FBS for 24 h) and allowed to adhere to the surface for 90 min. Discs were subsequently transferred to fresh media, and biofilms were allowed to mature for an additional 24 h. Following the maturation phase, the discs were rinsed twice with PBS to remove any nonadhered cells and placed in a new 12-well plate containing 4 ml of probiotic filtrate or YNB plus BHI media for controls. Discs were then incubated at 37°C for an additional 24 h.

Discs from both the prevention and treatment experiments were processed either for CSLM or SEM. For CSLM, discs were stained with concanavalin A Alexa Fluor 488 conjugate and FUN-1, as previously described (40). Discs processed for SEM were transferred to 2% glutaraldehyde for 24 h at 4°C. Following fixation, the discs were rinsed in 0.1 M sodium cacodylate buffer three times for 10 min each and placed in 1% osmium tetroxide for 1 h at 4°C. After the secondary fixation in osmium tetroxide, discs were rinsed again in 0.1 M sodium cacodylate buffer three times for 10 min each and placed in uranyl acetate overnight at 4°C. Discs were removed and rinsed twice in sterile water for 5 min and then passed through a gradual ethanol dehydration process using 25, 50, 75, 95, and 100% ethanol. Once air-dried, the samples were placed in a desiccator for 48 h to complete the dehydration process. The dehydrated samples were sputter-coated with palladium for 60 s and viewed with the Helios NanoLab 650 scanning electron microscope.

Determination of the specificity in PMB formation. In order to determine whether the ability to form PMB is specific to *Candida* spp., we formed biofilms containing either *C. tropicalis*, *C. albicans*, *T. inkin*, or *S. fibuligera* mixed with *E. coli* and *S. marcescens*. Biofilms were assessed using confocal scanning laser microscopy (CSLM) as described previously by our group (41). Biofilm thickness by CSLM was measured using a multitrack (dual-channel) mode, acquiring images at regular optical intervals across the depth of the biofilms to create z-stacks. In addition, a series of optical sections were captured in the *xy* plane throughout the biofilm specimen. LSM510 Browser microscope software was used to conceive 3D-reconstructed top-down images. Experiments were performed in triplicate, and the results are expressed as the mean ± standard deviation (SD).

Effect of probiotic filtrate on *Candida* germination. Since the ability of *Candida* spp. to germinate is a virulence determinant, we evaluated the effect of the probiotic strains to inhibit germ tube formation.

Briefly, *C. albicans* cells were grown in YNB medium for 18 to 20 h, washed three times with HBSS, and adjusted to a concentration of 5×10^5 blastospores/ml in fetal bovine serum. Probiotic filtrate was next added to the cell suspension, and tubes were incubated at 37°C for 2 h. Cells with no probiotic filtrate were used as untreated controls. At time zero and at 30-min intervals for 2 h, tubes were removed and vortexed, and 10 μl of the cell suspension was transferred from each time point to a hemocytometer to count the cells with germ tubes. The percentage of germinated cells (defined as a germ tube length greater than or equal to the diameter of a blastospore) per total number of cells was calculated for each time point. Additionally, cells at each time point were observed with CSLM.

Statistical analyses. Statistical analyses for all data were performed using the GraphPad Prism 6 software. Probiotic-treated groups were compared to control untreated groups using unpaired *t* tests. A *P* value of <0.05 was considered significant.

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