


Probiotic Yeasts Inhibit Virulence of Non-*albicans* *Candida* Species

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ABSTRACT Systemic infections of *Candida* species pose a significant threat to public health. Toxicity associated with current therapies and emergence of resistant strains present major therapeutic challenges. Here, we report exploitation of the probiotic properties of two novel, food-derived yeasts, *Saccharomyces cerevisiae* (strain KTP) and *Issatchenkia occidentalis* (strain ApC), as an alternative approach to combat widespread opportunistic fungal infections. Both yeasts inhibit virulence traits such as adhesion, filamentation, and biofilm formation of several non-*albicans* *Candida* species, including *Candida tropicalis*, *Candida krusei*, *Candida glabrata*, and *Candida parapsilosis* as well as the recently identified multidrug-resistant species *Candida auris*. They inhibit adhesion to abiotic surfaces as well as cultured colon epithelial cells. Furthermore, probiotic treatment blocks the formation of biofilms of individual non-*albicans* *Candida* strains as well as mixed-culture biofilms of each non-*albicans* *Candida* strain in combination with *Candida albicans*. The probiotic yeasts attenuated non-*albicans* *Candida* infections in a live animal. *In vivo* studies using *Caenorhabditis elegans* suggest that exposure to probiotic yeasts protects nematodes from infection with non-*albicans* *Candida* strains compared to worms that were not exposed to the probiotic yeasts. Furthermore, application of probiotic yeasts postinfection with non-*albicans* *Candida* alleviated pathogenic colonization of the nematode gut. The probiotic properties of these novel yeasts are better than or comparable to those of the commercially available probiotic yeast *Saccharomyces boulardii*, which was used as a reference strain throughout this study. These results indicate that yeasts derived from food sources could serve as an effective alternative to antifungal therapy against emerging pathogenic *Candida* species.

IMPORTANCE Non-*albicans* *Candida*-associated infections have emerged as a major risk factor in the hospitalized and immunocompromised patients. Besides, antifungal-associated complications occur more frequently with these non-*albicans* *Candida* species than with *C. albicans*. Therefore, as an alternative approach to combat these widespread non-*albicans* *Candida*-associated infections, here we showed the probiotic effect of two yeasts, *Saccharomyces cerevisiae* (strain KTP) and *Issatchenkia occidentalis* (ApC), in preventing adhesion and biofilm formation of five non-*albicans* *Candida* strains, *Candida tropicalis*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis*, and *Candida auris*. The result would influence the current trend of the conversion of conventional antimicrobial therapy into beneficial probiotic microbe-associated antimicrobial treatment.

KEYWORDS probiotic yeasts, plastic adhesion assay, Caco-2 cell monolayer, mixed-species *Candida* biofilm, *Candida tropicalis*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis*, *Candida auris*, *Caenorhabditis elegans*, *Candida albicans*, biofilm

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Opportunistic invasive *Candida* infections present a major public health threat, especially in immunocompromised populations or healthy individuals with implanted medical devices (1). Impairment of immune functions permits the pathogen to penetrate the submucosal tissue of gastrointestinal tract and disseminate to the internal organs, resulting in life-threatening systemic infections (2). While the most prominent etiological agent is *Candida albicans*, other yeasts of the genus *Candida*, collectively referred to as non-*albicans* *Candida* (NAC), have also been associated with nosocomial infections (3). For example, *C. tropicalis*, *C. parapsilosis*, and *C. glabrata* are associated with 35% to 65% of all systemic *Candida* infections (4), while *C. krusei* is fluconazole resistant (5). Infection with any non-*albicans* *Candida* strain increases patient morbidity, and coinfections with *C. tropicalis* and *C. glabrata* have high (40% to 70%) mortality rates (4).

More recently, evolution of antifungal-resistant strains of *C. albicans* as well as non-*albicans* *Candida* has emerged as a critical issue (3, 6, 7). The global emergence of *Candida auris* as a multidrug-resistant fungal pathogen with high mortality rates (8) has prompted national and international surveillance programs. The antifungal agents used to treat *Candida* infections have a myriad of deleterious side effects due to their similarity to eukaryotic host cells. This has led to a growing recognition that alternative therapy for opportunistic pathogens is desirable. Food-derived probiotic yeasts present a safe and cost-effective method to keep *Candida* in check with improved health and wellness for the patient.

Microbes often exist in communities where cells attach to abiotic surfaces or host cells. The adherent cells subsequently become embedded within an extracellular matrix to form a complex ecosystem called a biofilm. Surface adhesion is usually the first step of an infection, while biofilms form a physical barrier against the drugs, directly contributing to the antifungal resistance (9–11). *Candida* biofilms on abiotic materials such as medical devices—urinary and central venous catheters, pacemakers, mechanical heart valves, joint prostheses, contact lenses—have been shown to contribute to deadly infections (12–15). Furthermore, *Candida* biofilms have been shown to damage epithelial surfaces, causing vaginitis or thrush, and in rare cases may breach the vascular endothelium and progress to endocarditis (16, 17). Therefore, methods designed to restrict adhesion and, ultimately, biofilm formation are effective therapies.

Found in many fermented foods and beverages, yeasts are an inevitable part of the daily diet of humans in most cultures. Yeast-based probiotics are especially desirable because they are naturally resistant to most antibiotics, which allows them to persist in the gastrointestinal (GI) tract during an antibiotic regimen when the bacterial microflora may be compromised. The use of *Saccharomyces cerevisiae* is particularly attractive for probiotic applications and is generally regarded as safe (GRAS) by the U.S. Food and Drug Administration (FDA) (18). Recently published preclinical and clinical studies support the use of probiotics against *C. albicans* (19), and *Saccharomyces boulardii* is already commercially available. A probiotic cocktail of yeast and bacteria in combination with prebiotics was found to reduce colonization of *C. albicans* in preteen children (20). Additionally, recent evidence suggests that vaginal administration of *S. cerevisiae* in mice significantly reduced *C. albicans* colonization during vulvovaginal candidiasis (VVC) (21). However, there is limited knowledge about the effects of probiotic yeasts on non-*albicans* *Candida* strains. Here, we tested the effects of two novel probiotic yeasts, *Saccharomyces cerevisiae* (strain KTP) and *Issatchenkia occidentalis* (strain ApC), that were derived from food sources (22). Here, we used multiple readouts, including *in vitro* and *ex vivo* assays and analyses of morphological transition and biofilm formation of four non-*albicans* *Candida* strains, *C. tropicalis*, *C. krusei*, *C. glabrata*, and *C. parapsilosis*, to demonstrate the efficacy of probiotic yeast application. We also used *Caenorhabditis elegans* as a whole-animal infection model to study the effects of exposure to probiotic yeasts. Finally, we demonstrate that the use of probiotic yeasts represents an effective method to control the multidrug-resistant species *Candida auris*.

RESULTS

We have previously reported the isolation and characterization of two probiotic yeasts, *S. cerevisiae* (strain KTP; accession no. [MH142729](#)) and *I. occidentalis* (strain ApC; accession no. [KF551991](#)) from toddy and apple cider, respectively (22). Here, we tested the probiotic potential of these yeasts against non-*albicans Candida* species *C. tropicalis*, *C. glabrata*, *C. krusei*, and *C. parapsilosis* as well as the recently identified multidrug-resistant species *C. auris*. To fully appreciate the probiotic effects of the yeasts *S. cerevisiae* and *I. occidentalis* on *C. tropicalis*, *C. glabrata*, *C. krusei*, and *C. parapsilosis*, the assays reported in this study were typically performed under three different sets of conditions: preinoculation, where treatment with probiotic yeasts was performed prior to application of the *Candida* strains; coinoculation, where probiotic yeasts and *Candida* strains were simultaneously applied; and postinoculation, where probiotic yeast treatment was performed after the application of *Candida* strains (treatment 60 and 90 min after adhesion and biofilm initiation respectively).

***S. cerevisiae* and *I. occidentalis* inhibit adhesion of non-*albicans Candida* species.** Previous studies revealed that probiotic dosage is a crucial consideration for treatment (23). Therefore, we empirically determined that 10^8 cells/ml was the effective dosage for *S. cerevisiae* or *I. occidentalis*. This effective dosage is in line with that observed for the commercially available probiotic yeast *S. boulardii*, the reference strain used in our studies. Plastic surfaces that were pretreated with 10^8 cells/ml of either of the probiotic yeast species inhibited adhesion of the non-*albicans Candida* strains by 80% to 90% compared to untreated surfaces (see Fig. S1 in the supplemental material). We and others have confirmed that inhibition of adhesion decreases in a dose-dependent manner when probiotics are applied at concentrations lower than the effective dose. When probiotic yeasts were coincubated with the *Candida* strains, adhesion of *C. krusei*, *C. glabrata*, and *C. parapsilosis* was reduced by 43% to 52% ($P < 0.05$) and that of *C. tropicalis* by 33% to 42% ($P < 0.05$) compared to the controls that were not coincubated with probiotics (Fig. 1A and B). The extent of inhibition seen with the novel probiotic yeasts was similar to the level seen with after treatment with the commercially available reference probiotic yeast *S. boulardii*, which exhibited a 43% to 53% reduction in the adhesion of *C. tropicalis*, *C. krusei*, and *C. glabrata* and a 34% reduction of adhesion of *C. parapsilosis* compared with untreated controls under identical conditions. To test whether metabolic activity was necessary for probiotic function, we exposed heat-killed probiotic yeasts to non-*albicans Candida* strains. Our results indicated that inactivation of probiotic yeasts renders them inactive, allowing the non-*albicans Candida* strains to adhere to abiotic surfaces (Fig. S2). Together, these results suggest that metabolically active probiotic yeasts are effective prophylactics. To study the effect of probiotic yeasts as a treatment, we tested whether the application of probiotic yeasts would be able to displace *Candida* strains that had already attached to the plastic surface of 96-well plates (postinoculation condition). Our results indicate that the probiotic yeasts were unable to displace *C. tropicalis* and displaced *C. krusei*, *C. glabrata*, and *C. parapsilosis* only minimally (by 5% to 25%, $P < 0.05$) compared to an untreated control population (Fig. 1C). Together, our results indicate that the novel probiotic yeasts *S. cerevisiae* and *I. occidentalis* are able to inhibit attachment of a variety of *Candida* strains, thereby preventing formation of biofilms. These novel probiotic yeasts are as effective as the commercially available probiotic yeast *S. boulardii*. These probiotics are, however, not effective once *Candida* has attached and formation of biofilms has been initiated.

***S. cerevisiae* and *I. occidentalis* inhibit biofilm formation of non-*albicans Candida* species.** Next, we wanted to test the effect of *S. cerevisiae* and *I. occidentalis* on biofilm formation in three stages of biofilm development, defined as follows: early biofilms formed after 90 min; intermediate biofilms formed after 24 h; and mature biofilms formed after 48 h. The putative probiotic yeasts *S. cerevisiae* and *I. occidentalis* (10^8 cells/ml) were applied to preformed biofilms consisting of each of the non-*albicans Candida* strains. Results were compared to those seen with untreated controls as well

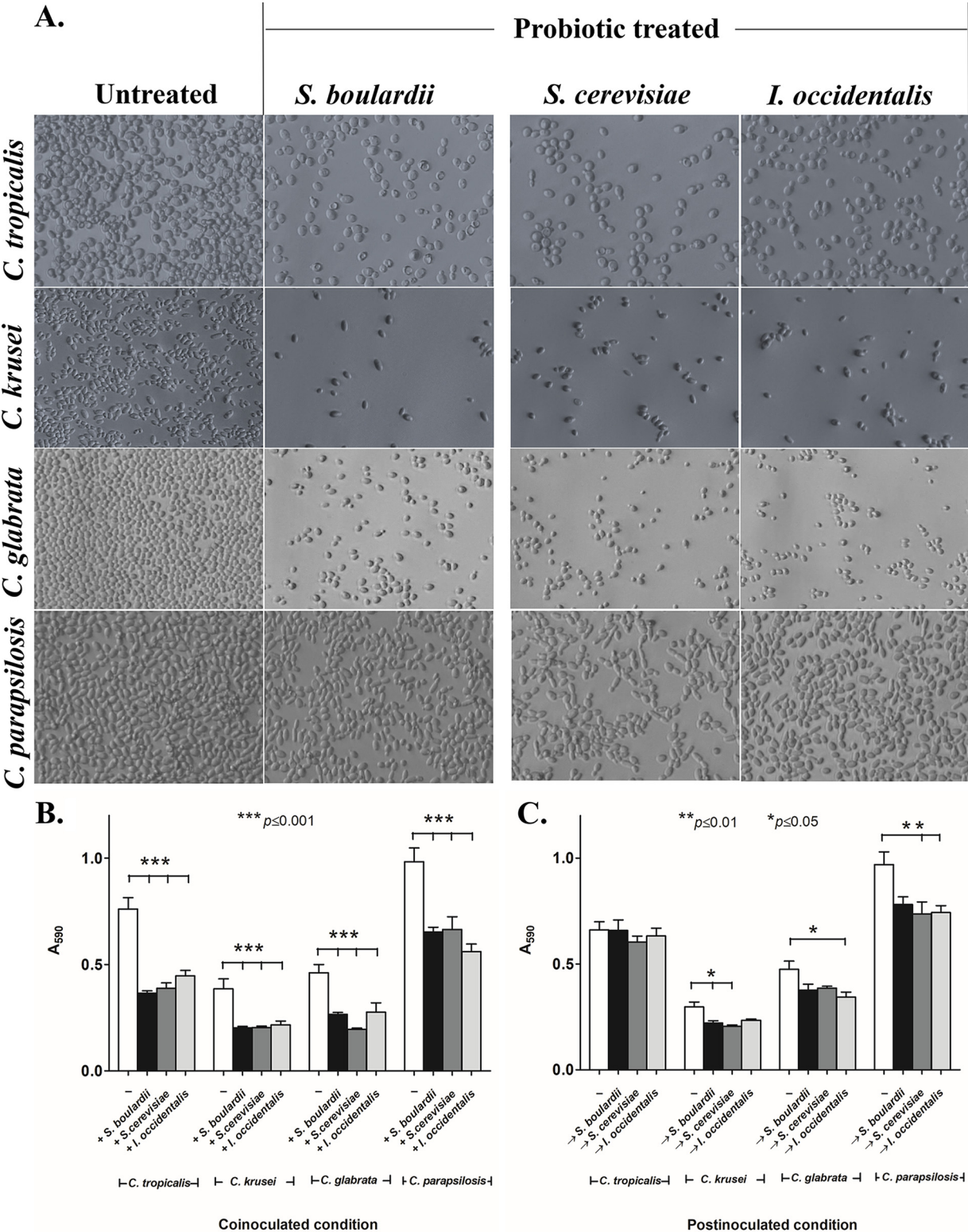


FIG 1 Probiotic treatment reduces adhesion of non-*albicans* *Candida* strains on abiotic surfaces. (A) Images show effects of potential probiotic yeasts *S. cerevisiae* and *I. occidentalis* and reference strain *S. boulardii* on adhesion of *C. tropicalis*, *C. krusei*, *C. glabrata*, and *C. parapsilosis* under coinoculation conditions. (B and C) Adhesion of non-*albicans* *Candida* strains to plastic surfaces was reduced in the presence of probiotic yeasts under coinoculation conditions (B) and postinoculation conditions (C). For the coinoculation conditions (indicated with a plus sign), probiotic yeasts and non-*albicans* *Candida* strains were incubated together for 3 h. For the postinoculation conditions (indicated with a vertical arrow), non-*albicans* *Candida* strains were applied to an abiotic surface for 60 min prior to seeding of probiotic isolates and were incubated for an additional 120 min. Crystal violet (0.5%) staining was used to quantify adhered non-*albicans* *Candida* cells on abiotic surfaces.

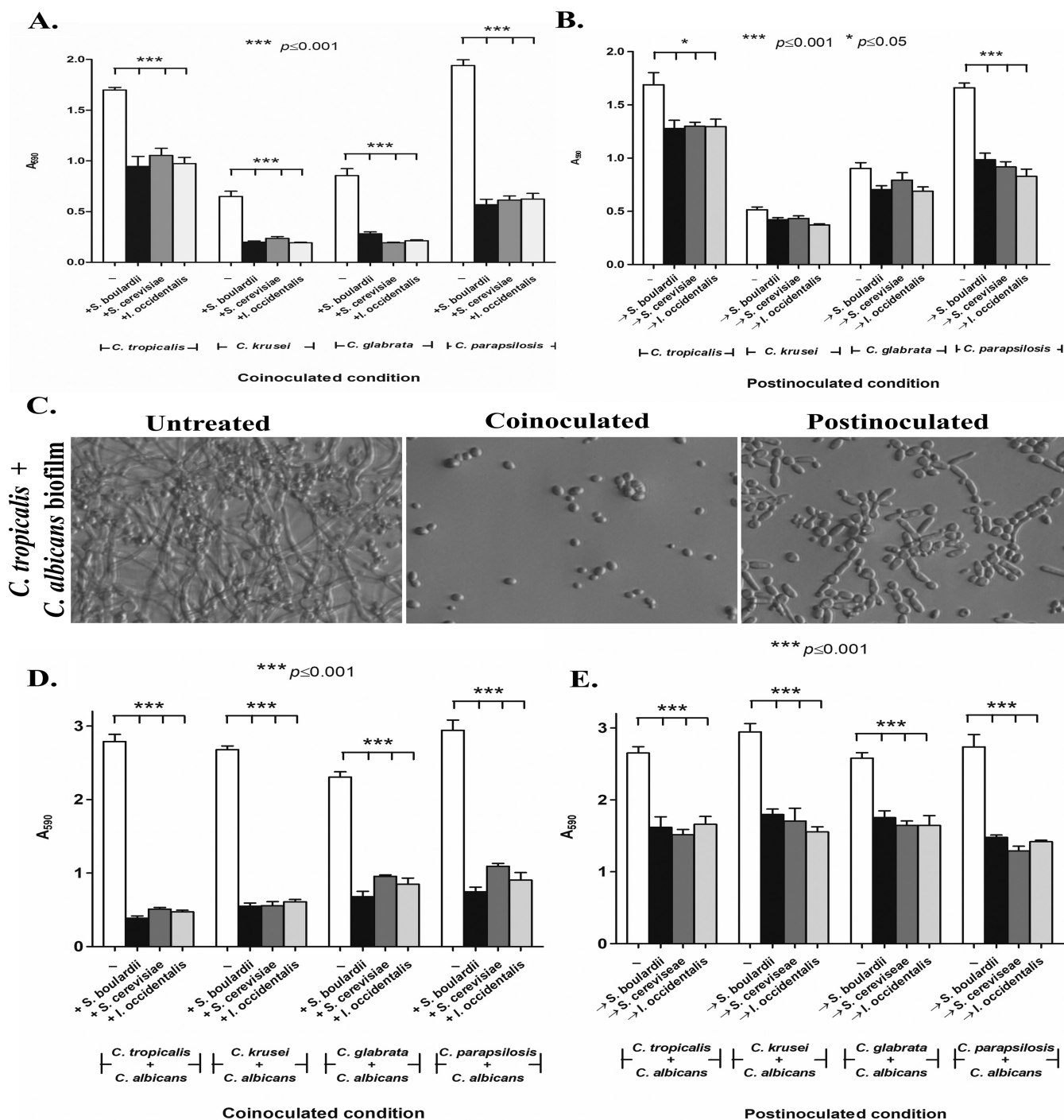


FIG 2 Probiotic yeasts *S. cerevisiae*, *I. occidentalis*, and *S. boulardii* prevented biofilm formation of non-*albicans* *Candida* species in both monoculture and mixed-culture biofilms with *C. albicans*. (A) Coinoculation of probiotics with non-*albicans* *Candida* strains for 24 h at 37°C. (B) For postinoculation of probiotics, non-*albicans* *Candida* strains were incubated for 90 min, and nonadherent cells were removed and subsequently treated with probiotic yeasts for 24 h at 37°C. Crystal violet (0.5%) staining was used to quantify the biofilm. (C) Images show the inhibitory effect of coinoculated probiotic treatment and postinoculation treatment (treatment 90 min after biofilm initiation). (D and E) Probiotic treatment inhibited mixed-culture biofilms of non-*albicans* *Candida* with *C. albicans* under conditions of coinoculation (D) or 90 min postinoculation, when biofilm formation had been initiated (E).

as treatment with the commercially available, reference probiotic strain *S. boulardii*. For all non-*albicans* *Candida* biofilms tested, probiotic yeasts inhibited further development of early biofilms compared to the intermediate and mature stages of biofilms (Fig. 2A and B). In addition, probiotic strains coinoculated with *C. krusei*, *C. glabrata*, and *C.*

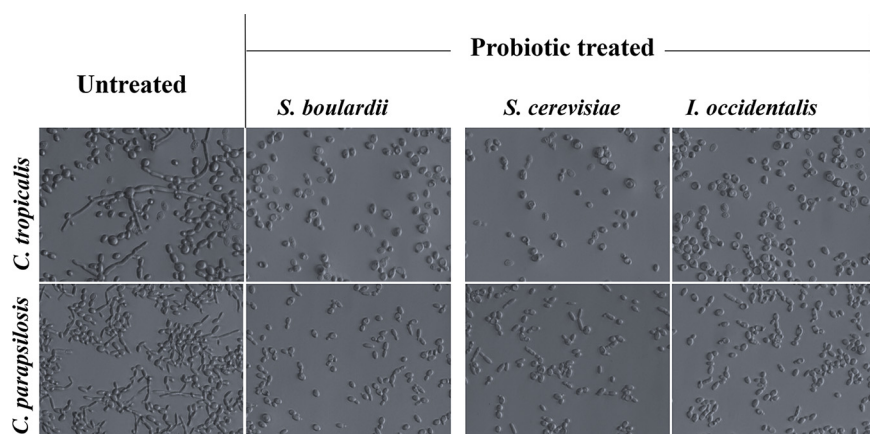


FIG 3 Probiotic yeasts *S. cerevisiae*, *I. occidentalis*, and *S. boulardii* inhibited morphological transition of *C. tropicalis* (upper row) and *C. parapsilosis* (bottom row). Probiotic yeasts and non-*albicans* *Candida* were coinoculated for 24 h, following which unattached cells were removed and photographed using a bright-field microscope.

parapsilosis exhibited 65% to 70% inhibition of biofilm formation whereas *C. tropicalis* biofilms were inhibited by 44%.

Biofilms, in nature, are found as surface-attached communities of microorganisms. Therefore, we tested the ability of the probiotic yeasts to inhibit biofilms formed by a mixed culture consisting of each of the non-*albicans* *Candida* strains in combination with *C. albicans*. Early (90-min) stages of the mixed-species biofilms showed significant (48% to 81%) reductions upon treatment with putative probiotic yeasts (Fig. 2D and E). However, intermediate (24-h) and mature (48-h) biofilms were not affected by probiotic treatment even when the duration of treatment was increased (Fig. S3). Furthermore, exposure to probiotics decreased the overall metabolic activity in the biofilm even though the biomass was not affected (Fig. S3C and D). Together, these results suggest that the putative probiotic yeasts are able to inhibit non-*albicans* *Candida* biofilms when applied early in the development.

Putative probiotics reduced filamentation of non-*albicans* *Candida* strains. Filamentation is a key virulence factor for various fungi of the *Candida* species and is positively correlated with adhesion and biofilm formation. Therefore, we wanted to test the effect of the putative probiotics *S. cerevisiae* and *I. occidentalis* on cell morphology and filamentation. Levels of hyphal development of *C. tropicalis* and *C. parapsilosis* were significantly inhibited upon treatment with probiotic yeasts at 10^8 /ml (Fig. 3). *C. krusei* and *C. glabrata* were not tested since they do not exhibit morphological transition. These results bolster the hypothesis that food-derived yeasts function as effective probiotics against pathogenic species of *Candida*.

***S. cerevisiae* and *I. occidentalis* inhibited adhesion of non-*albicans* *Candida* strains to cultured epithelial cells from human colon.** It is normal to find *Candida* in small amounts in the mouth, intestines, and skin. Overgrowth of *Candida*, however, can be problematic. In the context of the human gastrointestinal tract, *Candida* cells first attach to the epithelial cells and then invade deeper tissues. To test the ability of the putative probiotic yeasts to prevent attachment of *Candida* to human epithelial cells, we performed cell adhesion assays using monolayers of Caco-2 epithelial cells derived from human colon. The following three conditions were tested: the preinoculation condition, where Caco-2 cells were exposed to probiotics prior to exposure to non-*albicans* *Candida* strains; the coinoculation condition, where Caco-2 cells were exposed to non-*albicans* *Candida* and probiotic yeasts simultaneously; and the postinoculation condition, where Caco-2 cells were exposed to non-*albicans* *Candida* strains and then treated with probiotic yeasts. Our results indicated that adhesion to Caco-2 monolayer was inhibited by 95% to 99% under the preinoculation condition (data not shown). Under the coinoculation and postinoculation conditions, 72% to 98% of non-*albicans*

TABLE 1 Treatment with probiotic strains reduced adhesion of non-*albicans* *Candida* strains to Caco-2 cell monolayer^a

NAC species	Number of adherent probiotic cells (CFU/ml)							
	Coinoculation condition				Postinoculation condition			
	—	+ <i>S. boulardii</i>	+ <i>S. cerevisiae</i>	+ <i>I. occidentalis</i>	—	→ <i>S. boulardii</i>	→ <i>S. cerevisiae</i>	→ <i>I. occidentalis</i>
<i>C. tropicalis</i>	19,118 ± 596	401 ± 82	411 ± 85	390 ± 87	21,335 ± 314	826 ± 46	445 ± 18	498 ± 11
<i>C. krusei</i>	20,680 ± 980	550 ± 98	441 ± 82	346 ± 68	15,255 ± 731	948 ± 83	493 ± 22	1,000 ± 54
<i>C. parapsilosis</i>	1,307 ± 203	184 ± 40	186 ± 37	205 ± 17	5,504 ± 94	439 ± 45	462 ± 37	480 ± 32
<i>C. glabrata</i>	1,757 ± 168	257 ± 81	261 ± 28	171 ± 41	832 ± 33	374 ± 21	386 ± 11	424 ± 21

^aFor the coinoculation condition (+), probiotic yeasts and non-*albicans* *Candida* strains were coinoculated with monolayers of Caco-2 cells and incubated for 3 h. For the postinoculation condition (→), non-*albicans* *Candida* strains were applied on an epithelial layer of Caco-2 cells for 60 min prior to inoculation of probiotic yeasts and incubated for an additional 120 min. *Candida* chrome agar was used to assess CFU of adhered non-*albicans* *Candida* on an epithelial layer of Caco-2 cells. All values are expressed as means ± SD. All the values represent statistical significance at *P* values of <0.05 in comparison to the results determined for the untreated control group (—).

Candida strains were inhibited (*P* < 0.05) (Table 1). Interestingly, *C. glabrata* and *C. parapsilosis* showed poor adhesion to Caco-2 monolayer compared to *C. tropicalis* and *C. krusei*. These results suggest that the probiotic yeasts prevent the attachment of non-*albicans* *Candida* yeasts to cultured human epithelial cells.

Application of probiotic yeasts protects *C. elegans* from non-*albicans* *Candida* species. To further investigate the protective phenotypes of the probiotic yeasts in a live animal, we used *C. elegans* as a model host. *C. elegans* mimics key aspects of human intestinal physiology, including the presence of polarized microvillus-containing cells (24). The life span of *C. elegans* reared on a diet of probiotic yeasts (*S. cerevisiae*, *I. occidentalis*, or *S. boulardii*) was similar to that seen with those fed the standard diet of *Escherichia coli* OP50 (Fig. S4), suggesting that the probiotic treatment is benign and does not affect normal development of the worm.

Coinfection of *C. elegans* with probiotic yeast along with *C. tropicalis*, *C. krusei*, or *C. parapsilosis* exhibited a life span that was extended (by 5 to 6 days) compared to the life span seen with untreated control worms (Fig. 4). Furthermore, CFU levels of non-*albicans* *Candida* strains recovered from the gut of probiotic-treated worms were significantly decreased compared to the levels seen with the untreated group (Fig. S5). Together, these results suggest that probiotic treatment inhibits the gut colonization of non-*albicans* *Candida* strains and extends the nematode life span. In addition, we tested conditions under which the probiotic yeasts were administered after *C. elegans* worms were infected with *C. tropicalis*, *C. krusei*, or *C. parapsilosis*. The nematodes that were treated with probiotic yeasts postinfection with non-*albicans* *Candida* were able to reduce colonization, with no CFU recovered on day 5 after treatment with probiotics. We also noted that worms treated with yeasts of the genus *Saccharomyces* (both *S. cerevisiae* and *S. boulardii*) resisted pathogenic insult better than those treated with the non-*Saccharomyces* yeast *I. occidentalis*.

Effect of application of probiotic yeasts on virulence of *C. auris*. Recently, *Candida auris* has emerged as a multidrug-resistant superbug that presents a serious global health threat, especially among people with a weakened immune system (25). It attaches to abiotic surfaces, prompting hospitals to take extraordinary decontamination measures, including removal of ceiling and floor tiles, to eradicate it. It has been suggested that the recent rise in *C. auris* infections is in part due to the overuse of antimicrobial agents that wipe out competing microbes, giving drug-resistant *C. auris* a chance to overgrow. Therefore, we tested the ability of competing probiotic yeasts to inhibit adhesion of *C. auris* (obtained from the U.S. Centers for Disease Control). We tested *C. auris* representing each of the clades. Coinoculation of *C. auris* strains with probiotics resulted in inhibition of adhesion by 44% to 62% (Fig. 5A), while treatment after probiotics after *C. auris* had attached to the surface (postinoculation condition) showed a modest decrease in adhesion (34% to 40%; data not shown). We also tested the effect of probiotic treatment on mixed-species biofilms of *C. auris* and *C. albicans*, since monocultures of *C. auris* do not form rich biofilms. Our results revealed that coinoculation of *S. cerevisiae* or *I. occidentalis* inhibited mixed-species biofilms of *C. auris*

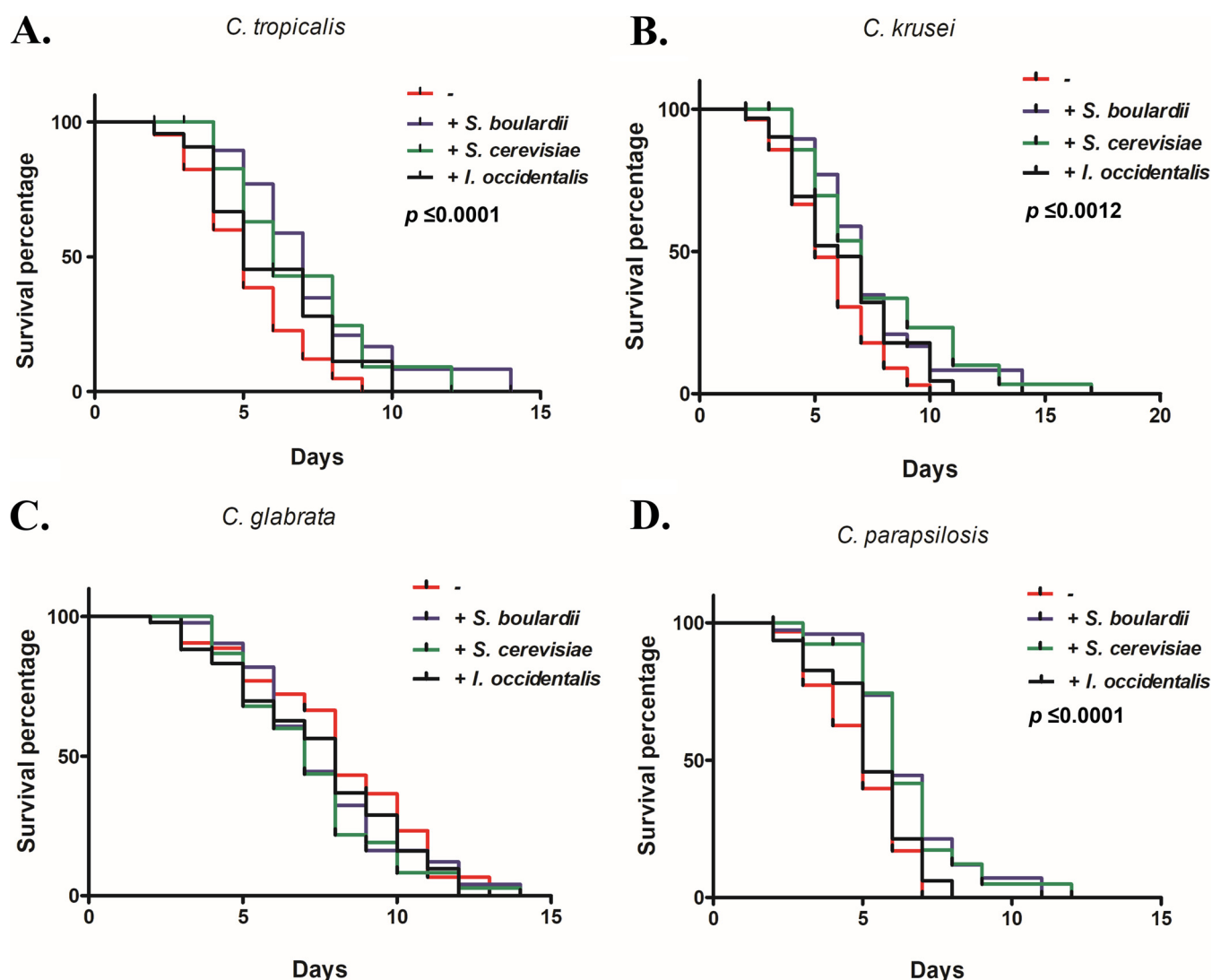


FIG 4 Simultaneous exposure of probiotic yeasts *S. boulardii*, *S. cerevisiae*, and *I. occidentalis* increased the life span of *C. tropicalis* (A), *C. krusei* (B), and *C. parapsilosis* (D) but not *C. glabrata* (C). *C. elegans* worms were fed a mixture of probiotic yeasts and non-*albicans* *Candida*. Live and dead worms were scored for survival each day and compared to worms fed a diet consisting only of non-*albicans* *Candida* (untreated group).

and *C. albicans* by 90% (Fig. 5B) and by 27% to 46% (data not shown) under the postinoculation condition. Together, these results indicate that probiotic yeasts inhibit adhesion and biofilm formation of *C. auris*.

The secretome of probiotic yeasts inhibited adhesion of non-*albicans* *Candida* to abiotic surfaces. To probe the mechanism of probiotic action, we tested whether the secretome of the probiotic yeasts retained the ability to inhibit adhesion of non-*albicans* *Candida* to abiotic surfaces. A two-chamber cell culture insert was used where probiotic yeasts were maintained in the upper chamber and were separated from the lower chamber by a 0.4- μ m-pore-size membrane that allowed diffusion of small bioactive molecules to the lower chamber containing non-*albicans* *Candida* cells (Fig. 6). Our results indicate that a soluble metabolite(s) present in the secretome of probiotic yeasts was able to partially inhibit adhesion of non-*albicans* *Candida* (by 22% to 30%) compared to the untreated control. In addition, spent media buffered to neutral pH (pH 7) retained the adhesion-inhibitory effect (18% to 31%), suggesting that antiadhesion nature of probiotic yeast is likely due to a bioactive metabolite(s) and not to the acidic nature of the spent media. These results suggest that a secreted metabolite(s) of probiotic yeasts is able to inhibit the virulence of non-*albicans* *Candida*.

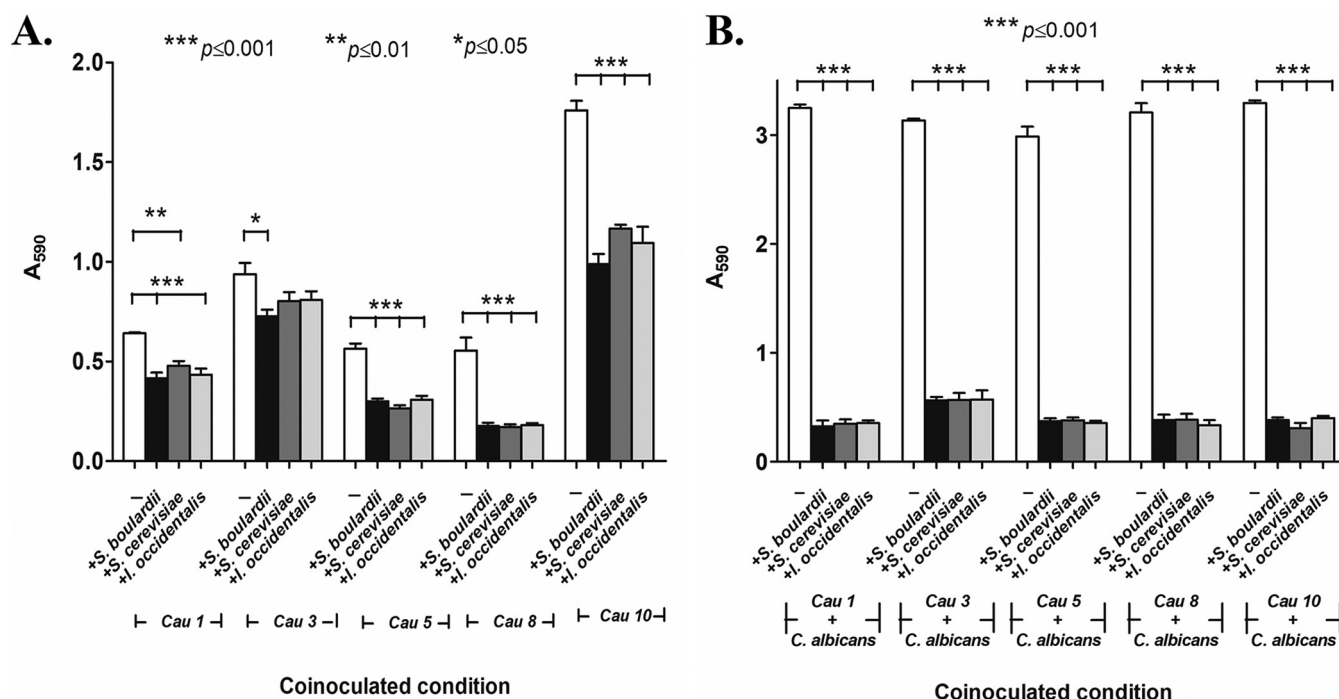


FIG 5 Effect of probiotics treatment on adhesion (A) and biofilm formation (B) of *C. auris*. Probiotic yeasts *S. cerevisiae* and *I. occidentalis* as well as reference strain *S. boulardii* and *C. auris* were coinoculated for 3 h and 24 h at 37°C. Adhesion and biofilm formation were quantified by crystal violet (0.5%) staining after unattached cells were removed by washing. (A) Coincubation of probiotic yeasts decreased adhesion of *C. auris* to abiotic surfaces. (B) Coinoculation of probiotic yeasts inhibited mixed-culture biofilms of *C. auris* with *C. albicans*.

DISCUSSION

Biofilm-related clinical complications are a major issue in the health sector. Naturally occurring biofilms are usually polymicrobial, where interaction between microbes may be synergistic or antagonistic. We and others have observed that non-*albicans* *Candida* species such as *Candida parapsilosis*, *C. pseudotropicalis*, and *C. glabrata* produced immature biofilms when grown as monocultures compared to *C. albicans* (26). Fungi of the *Candida* species have been shown to synergize with bacteria in the oral cavity for adhesion and biofilm formation (27). Likewise, *C. glabrata* relies on *C. albicans* for initial adhesion and biofilm development (28). On the other hand, *Lactobacillus* species have been shown to inhibit the initial stages of biofilm in mixed cultures (29). We used several *in vitro* and *ex vivo* tools, including analyses of adhesion to abiotic surfaces and cultured epithelial cells derived from human colon (Caco-2 cell), morphological transition, and biofilm formation, to assess effects of our potential probiotic strains against non-*albicans* *Candida* stains. Our results converge on the notion that probiotic yeasts inhibit adhesion and decrease metabolic activity of biofilms, thereby controlling growth and colonization of pathogenic *Candida*.

We used the nematode *Caenorhabditis elegans* as a live host model (30) since facets of its innate immune system are faithfully conserved in humans (31). We demonstrated that nematodes treated with probiotic yeasts are better able to withstand pathogenic insult from several non-*albicans* *Candida* species. Similarly, *Lactobacillus acidophilus* was previously shown to significantly decrease the colonization of infectious Gram-positive bacteria in *C. elegans* gut and to enhance the life span of the worm (32).

Probiotic action can be attributed to physical and/or chemical factors. Here, we provide evidence of the chemical nature of probiotic action since cell-free secretomes of probiotic yeasts retained inhibitory activity. We propose that a secondary metabolite(s) produced by the probiotic yeasts is secreted into the milieu, where it interferes with the pathogenic program of the non-*albicans* *Candida* species. Other reports have demonstrated that short-chain fatty acids or bacteriocins showed significant anti-

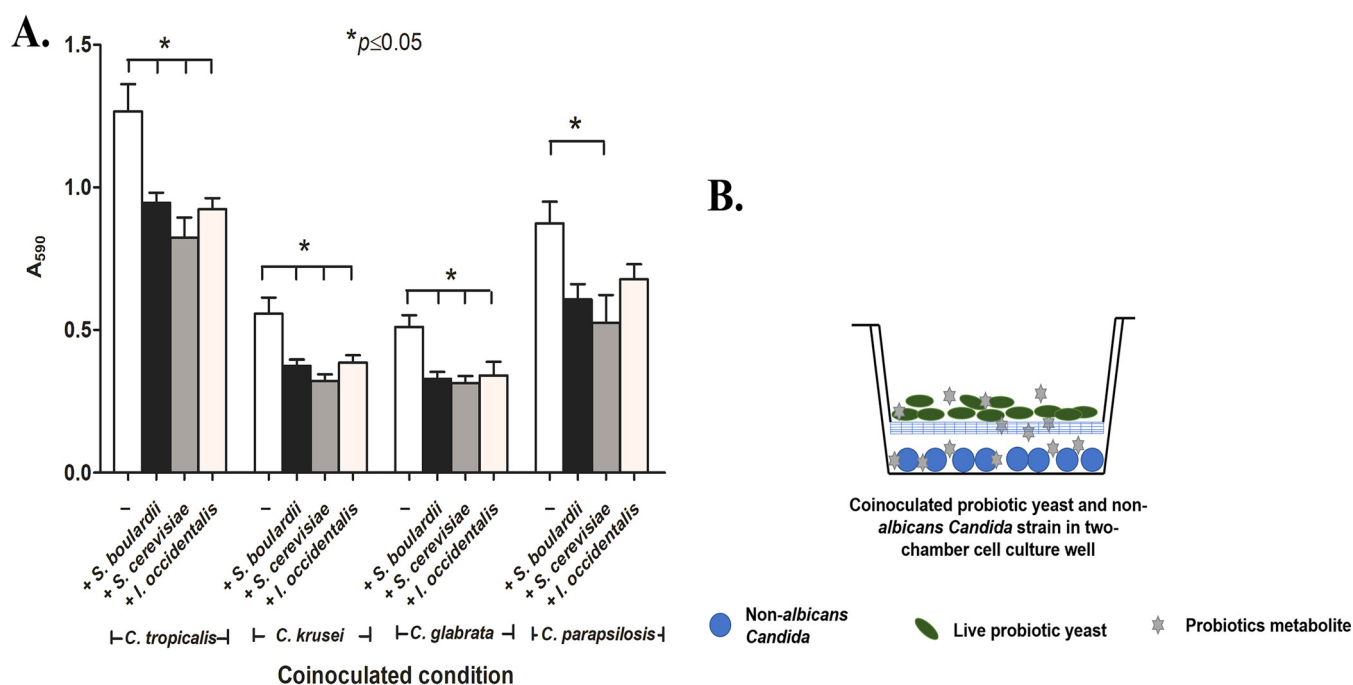


FIG 6 (A) The effect of cell-free probiotic metabolites on adhesion of non-albicans *Candida* strains was quantified using crystal violet staining. (B) Graphic representation of experimental setup where probiotic yeast were inoculated into the upper chamber of the cell insertion section and non-albicans *Candida* strains were maintained in the lower chamber; probiotic soluble metabolites freely diffused through a 0.4- μ m-pore-size membrane into and all over the media, including the lower compartment where the non-albicans *Candida* cells were inoculated.

Candida activity for various *Candida* species (19, 33–35). Probiotics may also pose a physical barrier by binding surface proteins that promote pathogen attachment or compete for limited nutrients. We showed that metabolically inactive probiotic yeasts retained minimal inhibitory effect, suggesting that the live probiotic cells and their metabolites likely act synergistically. Prior studies using various *in vitro* and *in vivo* models have also revealed synergistic mechanisms that involve immune simulation and competitive binding (21, 36–38).

Small molecules (such as filastatin, farnesic acid, and gymnemic acids) and hydrophilic or antibody-coated compounds have been proposed as biotherapeutic agents but are associated with significant safety concerns (39–42). Therefore, probiotics such as *S. cerevisiae* and *I. occidentalis* have the potential to inhibit key virulence traits of the most common non-albicans *Candida* species, i.e., *C. tropicalis*, *C. krusei*, *C. glabrata*, and *C. parapsilosis*. To meet the growing need for treatment options for biofilm-associated clinical complications, these food-derived yeasts represent a safe and attractive alternative to conventional treatment for *Candida* infections.

MATERIALS AND METHODS

Chemicals, yeast strains, and growth conditions. Standard yeast culture conditions were performed as described previously by Guthrie and Fink (43). Medium and/or medium components RPMI 1640, Dulbecco's modified Eagle medium (DMEM), and fetal bovine serum (FBS) and chemicals such as MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] were obtained from Sigma-Aldrich.

Saccharomyces cerevisiae (strain KTP; accession number [MH142729](#)) and *Issatchenkia occidentalis* (ApC; accession number [KF551991](#)) were isolated from toddy and fermented apple juice, respectively. The commercially available *Saccharomyces cerevisiae* var. *boulardii* (NCDC363) strain obtained from National Collection Centre for Dairy Cultures, India, was used as a reference strain in the study. The non-albicans *Candida* species *C. tropicalis* (MYA 3404), *Candida krusei* (44), *C. glabrata* (44), and *C. parapsilosis* (CDC317) were used in the study. The *C. albicans* strain (SC5314) was used for mixed-culture biofilm studies with non-albicans *Candida* stains. All strains were cultured in yeast extract-peptone-dextrose (YPD) media at 30°C for 24 h. RPMI 1640 containing L-glutamine, phenol red, 0.2% glucose, and 0.165 M MOPS (morpholinepropanesulfonic acid) buffer without sodium bicarbonate was used to test for plastic adhesion and biofilm formation.

Assays to monitor adhesion of non-*albicans* *Candida* species. (i) *In vitro* plastic adhesion assay and biofilm formation assay.

In order to investigate probiotic effects on non-*albicans* *Candida* adhesion, we used a published protocol (45, 46) with minor modifications. Briefly, 10^7 cells/ml of any *Candida* strain was treated with 10^8 cells/ml of *S. cerevisiae*, *I. occidentalis*, or the reference strain of *S. boulardii*. In order to understand the limitations and effects of the probiotic treatment against non-*albicans* *Candida* strain, the assays were performed under preinoculation, coinoculation, and postinoculation conditions. First, a preexposure condition where probiotic yeast were inoculated into 96-well plates for 60 min (representing a doubling time of approximately 1) was applied. After 60 min, non-*albicans* *Candida* strains were introduced and incubated for an additional 120 min at 37°C with mild shaking (90 rpm). Second, the three probiotic strains *S. boulardii*, *S. cerevisiae*, and *I. occidentalis* were coinoculated with non-*albicans* *Candida* strains and incubated for 3 h. Third, a postinoculation condition where the non-*albicans* *Candida* strains were inoculated in the 96-well microtiter plates for 60 min was applied. Then, test probiotic isolates *S. cerevisiae* and *I. occidentalis* or cells of the reference strain *S. boulardii* were seeded on the adhered non-*albicans* *Candida* cells and incubated further 120 min under the conditions mentioned above. After treatment, plates were washed three times with phosphate-buffered saline (PBS; pH 7.4) to remove nonadherent cells. Finally, plates were air-dried and incubated with 50 μ l of 0.5% crystal violet for 45 min, followed by washing with PBS to remove the excess crystal violet. The stained cells were destained with 95% (vol/vol) ethanol, and absorbance was measured at 590 nm to analyze the number of adhered cells.

(ii) *In vitro* biofilm formation assay. The effect of probiotic yeasts was tested on biofilms initiated as monocultures of non-*albicans* *Candida* species or as a mixed-culture biofilm with *C. albicans*. Furthermore, biofilms were tested at the early, intermediate, and mature stages of development. Non-*albicans* *Candida* strains were coinoculated with yeast (*S. cerevisiae*, *I. occidentalis*, or reference strain *S. boulardii*) for 24 h at 37°C. Under the postinoculation condition, non-*albicans* *Candida* strains were incubated for 90 min, and nonadherent cells were removed by PBS washing. Subsequently, probiotic yeasts (10^8 /ml) were treated for 24 h at 37°C. These two experiments were considered to represent the initial stage of biofilm formation. Intermediate and mature biofilm treatments were conducted after 24 and 48 h of growth of non-*albicans* *Candida* biofilm, respectively (47). Similar treatments were performed for biofilms consisting of mixed *Candida* strains where *C. albicans* was used along with individual non-*albicans* *Candida* strains. The metabolic activity of biofilm was checked by MTT assays (48).

(iii) *Ex vivo* adhesion assay in Caco-2 cell monolayers. Adhesion to epithelial cells is a prerequisite for *Candida* to invade deeper tissues. To assess adhesion to live cells, we employed an *ex vivo* system using a Caco-2 cell monolayer (obtained from the National Centre for Cell Sciences [NCCS], India). The Caco-2 cells (5×10^4 cells/ml) were seeded into a 96-well microtiter plate and incubated at 37°C in a 5% CO₂ incubator for 20 days to produce a monolayer. The monolayer was further treated with probiotic isolates and non-*albicans* *Candida* strains under preinoculation, coinoculation, and postinoculation conditions, as explained above. Nonadherent cells were removed by washing with PBS (pH 7.4). Adhered cells were harvested using trypsin-EDTA (0.25% [wt/vol] trypsin and 0.02% [wt/vol] EDTA) treatment for 5 min at 37°C. Non-*albicans* *Candida* CFU levels were calculated on *Candida* chrome agar plates.

(iv) *In vivo* *Caenorhabditis elegans* infection assay. Eggs were harvested from six to eight worms reared on a Nematode growth media (NGM) agar plate containing *E. coli* OP50 and were incubated at 20°C for 3 days, and 40 to 50 harvested eggs were transferred to a fresh NGM plate that contained *E. coli* OP 50. The assay was conducted under coculture and postexposure conditions. For the coinoculation condition, 40 to 50 larval stage 3 (L3) and L4 worms were transferred into NGM plates containing probiotic yeasts and non-*albicans* *Candida* (10^6 cells/20 μ l as the test inoculum) and non-*albicans* *Candida* (10^6 cells/20 μ l as control) lawns, and the live and dead worms were counted each day using a dissection microscope. For the postinoculation condition, worms were exposed to non-*albicans* *Candida* species for 2 days (10^6 cells/20 μ l) and then transferred into a probiotic lawn (10^6 cells/20 μ l). Probiotic-treated non-*albicans* *Candida*-infected worms were compared with the control (non-*albicans* *Candida*-infected worms transferred into a OP50 lawn). Each day, dead and live worms were counted manually (49).

(v) Non-*albicans* *Candida* colonization assay. Averages of 5 to 6 worms grown under the coinoculation condition or the postinoculation condition or both were used in the study. Briefly, the worms were washed four times with PBS. The washed worms were then resuspended in 100 μ l PBS buffer and crushed using a pellet pestle. Finally, the appropriate dilution of the sample was transferred into *Candida* chrome agar to differentiate non-*albicans* *Candida* strains from probiotic yeast isolates. Finally, levels of colonized non-*albicans* *Candida* cells were expressed in CFU per milliliter.

Mechanism of probiotic effects on non-*albicans* *Candida* strains. (i) Two-chamber cell insert assay. A dual-chamber cell insert apparatus with the chambers separated by a 0.4- μ m-pore-size filter was used to evaluate the effect of the probiotic secretome on non-*albicans* *Candida* strain adhesion. Briefly, a total of 10^8 cells/ml of *S. cerevisiae*, *I. occidentalis*, or *S. boulardii* was used to inoculate the upper chamber of the cell insert compartment, and 10^7 cells/ml of non-*albicans* *Candida* strain were maintained in the lower chamber of the cell insert apparatus for 24 h with mild shaking. After incubation, the lower plates containing adhered non-*albicans* *Candida* strains were washed three times with phosphate-buffered saline (PBS; pH 7.4) to remove nonadherent non-*albicans* *Candida* cells followed by staining with the 0.5% crystal violet mentioned earlier. Finally, absorbance was measured at 590 nm to analyze the number of attached cells.

Preparation of media for cell-free conditions. Yeasts *S. cerevisiae*, *I. occidentalis*, and *S. boulardii* (10^7 cells/ml) were inoculated into Synthetic Complete (SC) media and incubated at 30°C in 150 rpm for 3 days. Cell pellets were removed by centrifugation, and the supernatant was subjected to buffer

neutralization and filter sterilization and was concentrated by lyophilization. The resulting medium, containing presumed bioactive molecules, was supplemented with SC media (at a ratio of 2:10) to replenish nutrients and was used in adhesion assays as described above.

Statistical analysis. Variations in treatments were compared using one-way analysis of variance (ANOVA) followed by *post hoc* analysis using Tukey's *t* test at a significance level of $P = <0.05$. Results were expressed as means \pm standard deviations (SD). Analyses were performed with GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Kaplan-Meier statistical analysis tools were used for the *C. elegans* survival assay.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mBio.02307-19>.

FIG S1, TIF file, 1 MB.

FIG S2, TIF file, 2.3 MB.

FIG S3, TIF file, 3.4 MB.

FIG S4, TIF file, 2 MB.

FIG S5, TIF file, 3.1 MB.

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We declare that we have no conflicts of interest.

REFERENCES

- Nobile CJ, Johnson AD. 2015. *Candida albicans* biofilms and human disease. *Annu Rev Microbiol* 69:71–92. <https://doi.org/10.1146/annurev-micro-091014-104330>.
- Miranda LN, van der Heijden IM, Costa SF, Sousa AP, Sienna RA, Gobara S, Santos CR, Lobo RD, Pessoa VP, Levin AS. 2009. *Candida* colonisation as a source for candidaemia. *J Hosp Infect* 72:9–16. <https://doi.org/10.1016/j.jhin.2009.02.009>.
- Rao B. 2008. Emerging pathogens of the *Candida* species. In Sandai D (ed), *Candida albicans*. IntechOpen, London, United Kingdom. <https://doi.org/10.5772/intechopen.80378>.
- Krcmery V, Barnes AJ. 2002. Non-*albicans* *Candida* spp. causing fungaemia: pathogenicity and antifungal resistance. *J Hosp Infect* 50:243–260. <https://doi.org/10.1053/jhin.2001.1151>.
- Cuomo CA, Shea T, Yang B, Rao R, Forche A. 2017. Whole genome sequence of the heterozygous clinical isolate *Candida krusei* 81-B-5. *G3* (Bethesda) 7:2883–2889. <https://doi.org/10.1534/g3.117.043547>.
- Tortorano AM, Rigoni AL, Biraghi E, Prigitano A, Viviani MA, FIMUA-ECMM Candidaemia Study Group. 2003. The European Confederation of Medical Mycology (ECMM) survey of candidaemia in Italy: antifungal susceptibility patterns of 261 non-*albicans* *Candida* isolates from blood. *J Antimicrob Chemother* 52:679–682. <https://doi.org/10.1093/jac/dkg393>.
- Lockhart SR, Iqbal N, Cleveland AA, Farley MM, Harrison LH, Bolden CB, Baughman W, Stein B, Hollick R, Park BJ, Chiller T. 2012. Species identification and antifungal susceptibility testing of candida bloodstream isolates from population-based surveillance studies in two US cities from 2008 to 2011. *J Clin Microbiol* 50:3435–3442. <https://doi.org/10.1128/JCM.01283-12>.
- Ben-Ami R, Berman J, Novikov A, Bash E, Shachor-Meyouhas Y, Zakin S, Maor Y, Tarabia J, Schechner V, Adler A, Finn T. 2017. Multidrug-resistant *Candida haemulonii* and *C. auris*, Tel Aviv, Israel. *Emerg Infect Dis* 23:195.
- Rosenberg A, Ene IV, Bibi M, Zakin S, Segal ES, Ziv N, Dahan AM, Colombo AL, Bennett RJ, Berman J. 2018. Antifungal tolerance is a subpopulation effect distinct from resistance and is associated with persistent candidemia. *Nat Commun* 9:2470. <https://doi.org/10.1038/s41467-018-04926-x>.
- Zarnowski R, Sanchez H, Covelli AS, Dominguez E, Jaromin A, Bernhardt J, Mitchell KF, Heiss C, Azadi P, Mitchell A, Andes DR. 2018. *Candida albicans* biofilm-induced vesicles confer drug resistance through matrix biogenesis. *PLoS Biol* 16:e2006872. <https://doi.org/10.1371/journal.pbio.2006872>.
- Dominguez E, Zarnowski R, Sanchez H, Covelli AS, Westler WM, Azadi P, Nett J, Mitchell AP, Andes DR. 2018. Conservation and divergence in the *Candida* species biofilm matrix mannan-glucan complex structure, function, and genetic control. *mBio* 9:e00451-18. <https://doi.org/10.1128/mBio.00451-18>.
- Kuhn DM, Chandra J, Mukherjee PK, Ghannoum MA. 2002. Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infect Immun* 70:878–888. <https://doi.org/10.1128/iai.70.2.878-888.2002>.
- Rajendran R, Sherry L, Deshpande A, Johnson EM, Hanson MF, Williams C, Munro CA, Jones BL, Ramage G. 2016. A prospective surveillance study of candidaemia: epidemiology, risk factors, antifungal treatment and outcome in hospitalized patients. *Front Microbiol* 7:915. <https://doi.org/10.3389/fmicb.2016.00915>.
- Finkel JS, Mitchell AP. 2011. Genetic control of *Candida albicans* biofilm development. *Nat Rev Microbiol* 9:109–118. <https://doi.org/10.1038/nrmicro2475>.
- Desai JV, Mitchell AP, Andes DR. 1 October 2014, posting date. Fungal biofilms, drug resistance, and recurrent infection. *Cold Spring Harb Perspect Med* <https://doi.org/10.1101/cshperspect.a019729>.
- Donlan RM, Costerton JW. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 15:167–193. <https://doi.org/10.1128/cmr.15.2.167-193.2002>.
- Sobel JD, Faro S, Force RW, Foxman B, Ledger WJ, Nyirjesy PR, Reed BD, Summers PR. 1998. Vulvovaginal candidiasis: epidemiologic, diagnostic, and therapeutic considerations. *Am J Obstet Gynecol* 178:203–211. [https://doi.org/10.1016/s0002-9378\(98\)80001-x](https://doi.org/10.1016/s0002-9378(98)80001-x).
- Takayama K, Suye S, Kuroda K, Ueda M, Kitaguchi T, Tsuchiyama K, Fukuda T, Chen W, Mulchandani A. 2006. Surface display of organophosphorus hydrolase on *Saccharomyces cerevisiae*. *Biotechnol Prog* 22: 939–943. <https://doi.org/10.1021/bp060107b>.
- Murzyn A, Krasowska A, Stefanowicz P, Dziadkowiec D, Łukaszewicz M.

2010. Capric acid secreted by *S. boulardii* inhibits *C. albicans* filamentous growth, adhesion and biofilm formation. PLoS One 5:e12050. <https://doi.org/10.1371/journal.pone.0012050>.
20. Kumar S, Bansal A, Chakrabarti A, Singhi S. 2013. Evaluation of efficacy of probiotics in prevention of *Candida* colonization in a PICU—a randomized controlled trial. Crit Care Med 41:565–572. <https://doi.org/10.1097/CCM.0b013e31826a409c>.
 21. Pericolini E, Gabrielli E, Ballet N, Sabbatini S, Roselletti E, Cayzele Decherf A, Pelerin F, Luciano E, Perito S, Justen P, Vecchiarelli A. 2017. Therapeutic activity of a *Saccharomyces cerevisiae*-based probiotic and inactivated whole yeast on vaginal candidiasis. Virulence 8:74–90. <https://doi.org/10.1080/21505594.2016.1213937>.
 22. Lohith K, Anu-Appaiah KA. 2018. Antagonistic effect of *Saccharomyces cerevisiae* KTP and *Issatchenkia occidentalis* ApC on hyphal development and adhesion of *Candida albicans*. Med Mycol 56:1023–1032. <https://doi.org/10.1093/mmy/myx156>.
 23. Williams NT. 2010. Probiotics. Am J Health Syst Pharm 67:449–458. <https://doi.org/10.2146/ajhp090168>.
 24. Jiang H, Wang D. 2018. The microbial zoo in the *C. elegans* intestine: bacteria, fungi and viruses. Viruses 10:e85. <https://doi.org/10.3390/v10020085>.
 25. Lockhart SR, Etienne KA, Vallabhaneni S, Farooqi J, Chowdhary A, Govender NP, Colombo AL, Calvo B, Cuomo CA, Desjardins CA, Berkow EL, Castanheira M, Magobo RE, Jabeen K, Asghar RJ, Meis JF, Jackson B, Chiller T, Litvintseva AP. 2017. Simultaneous emergence of multidrug-resistant *Candida auris* on 3 continents confirmed by whole-genome sequencing and epidemiological analyses. Clin Infect Dis 64:134–140. <https://doi.org/10.1093/cid/ciw691>.
 26. Hawser SP, Douglas LJ. 1994. Biofilm formation by *Candida* species on the surface of catheter materials in vitro. Infect Immun 62:915–921.
 27. Shirliff ME, Krom BP, Meijering RA, Peters BM, Zhu J, Scheper MA, Harris ML, Jabra-Rizk MA. 2009. Farnesol-induced apoptosis in *Candida albicans*. Antimicrob Agents Chemother 53:2392–2401. <https://doi.org/10.1128/AAC.01551-08>.
 28. Tati S, Davidow P, McCall A, Hwang-Wong E, Rojas IG, Cormack B, Edgerton M. 2016. *Candida glabrata* binding to *Candida albicans* hyphae enables its development in oropharyngeal candidiasis. PLoS Pathog 12:e1005522. <https://doi.org/10.1371/journal.ppat.1005522>.
 29. Matsubara VH, Wang Y, Bandara H, Mayer MPA, Samaranayake LP. 2016. Probiotic lactobacilli inhibit early stages of *Candida albicans* biofilm development by reducing their growth, cell adhesion, and filamentation. Appl Microbiol Biotechnol 100:6415–6426. <https://doi.org/10.1007/s00253-016-7527-3>.
 30. Marsh EK, May RC. 2012. *Caenorhabditis elegans*, a model organism for investigating immunity. Appl Environ Microbiol 78:2075–2081. <https://doi.org/10.1128/AEM.07486-11>.
 31. Elkabti A, Issi L, Rao R. 2018. *Caenorhabditis elegans* as a model host to monitor the *Candida* infection process. J Fungi (Basel) 4:123. <https://doi.org/10.3390/jof4040123>.
 32. Clark LC, Hodgkin J. 2014. Commensals, probiotics and pathogens in the *Caenorhabditis elegans* model. Cell Microbiol 16:27–38. <https://doi.org/10.1111/cmi.12234>.
 33. Graham CE, Cruz MR, Garsin DA, Lorenz MC. 2017. *Enterococcus faecalis* bacteriocin EntV inhibits hyphal morphogenesis, biofilm formation, and virulence of *Candida albicans*. Proc Natl Acad Sci U S A 114:4507–4512. <https://doi.org/10.1073/pnas.1620432114>.
 34. Phaopongthai J, Wiyakrutta S, Meksuriyen D, Sriubolmas N, Suwanborirux K. 2013. Azole-synergistic anti-candidal activity of altenusin, a biphenyl metabolite of the endophytic fungus *Alternaria alternata* isolated from *Terminalia chebula* Retz. J Microbiol 51:821–828. <https://doi.org/10.1007/s12275-013-3189-3>.
 35. Hager CL, Isham N, Schrom KP, Chandra J, McCormick T, Miyagi M, Ghannoum MA. 2019. Effects of a novel probiotic combination on pathogenic bacterial-fungal polymicrobial biofilms. mBio 10:e00338-19. <https://doi.org/10.1128/mBio.00338-19>.
 36. Tareb R, Bernardeau M, Gueguen M, Vernoux JP. 2013. In vitro characterization of aggregation and adhesion properties of viable and heat-killed forms of two probiotic *Lactobacillus* strains and interaction with foodborne zoonotic bacteria, especially *Campylobacter jejuni*. J Med Microbiol 62:637–649. <https://doi.org/10.1099/jmm.0.049965-0>.
 37. Rizzo A, Losacco A, Carratelli CR. 2013. *Lactobacillus crispatus* modulates epithelial cell defense against *Candida albicans* through Toll-like receptors 2 and 4, interleukin 8 and human beta-defensins 2 and 3. Immunol Lett 156:102–109. <https://doi.org/10.1016/j.imlet.2013.08.013>.
 38. Saegusa S, Totsuka M, Kaminogawa S, Hosoi T. 2009. *Saccharomyces cerevisiae* and *Candida albicans* stimulate cytokine secretion from human neutrophil-like HL-60 cells differentiated with retinoic acid or dimethylsulfoxide. Biosci Biotechnol Biochem 73:2600–2608. <https://doi.org/10.1271/bbb.90410>.
 39. Fazly A, Jain C, Dehner AC, Issi L, Lilly EA, Ali A, Cao H, Fidel PL, Jr, Rao RP, Kaufman PD. 2013. Chemical screening identifies filastatin, a small molecule inhibitor of *Candida albicans* adhesion, morphogenesis, and pathogenesis. Proc Natl Acad Sci U S A 110:13594–13599. <https://doi.org/10.1073/pnas.1305982110>.
 40. VEDIYAPPAN G, Dumontet V, Pellissier F, d'Enfert C. 2013. Gymnemic acids inhibit hyphal growth and virulence in *Candida albicans*. PLoS One 8:e74189. <https://doi.org/10.1371/journal.pone.0074189>.
 41. Kagan S, Jabbour A, Sionov E, Alquntar AA, Steinberg D, Srebnik M, Nir-Paz R, Weiss A, Polacheck I. 2014. Anti-*Candida albicans* biofilm effect of novel heterocyclic compounds. J Antimicrob Chemother 69:416–427. <https://doi.org/10.1093/jac/dkt365>.
 42. Hoque J, Yadav V, Prakash RG, Sanyal K, Halder J. 2019. Dual-function polymer-silver nanocomposites for rapid killing of microbes and inhibiting biofilms. ACS Biomater Sci Eng 5:81–91. <https://doi.org/10.1021/acsbiomaterials.8b00239>.
 43. Guthrie CF, Fink GR. 2002. Guide to yeast genetics and molecular and cell biology. California Academic Press, Millbrae, CA.
 44. Butler G, Rasmussen MD, Lin MF, Santos MAS, Sakthikumar S, Munro CA, Rheinbay E, Grabherr M, Forche A, Reedy JL, Agrafioti I, Arnaud MB, Bates S, Brown AJP, Brunke S, Costanzo MC, Fitzpatrick DA, de Groot PWJ, Harris D, Hoyer LL, Hube B, Klis FM, Kodira C, Lennard N, Logue ME, Martin R, Neiman AM, Nikolaou E, Quail MA, Quinn J, Santos MC, Schmitzberger FF, Sherlock G, Shah P, Silverstein KAT, Skrzypek MS, Soll D, Staggs R, Stansfield I, Stumpf MPH, Sudbery PE, Srikantha T, Zeng Q, Berman J, Berriman M, Heitman J, Gow NAR, Lorenz MC, Birren BW, Kellis M, Cuomo CA. 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. Nature 459:657–662. <https://doi.org/10.1038/nature08064>.
 45. Lohse MB, Gulati M, Arevalo AV, Fishburn A, Johnson AD, Nobile CJ. 24 April 2017, posting date. Assessment and optimizations of *Candida albicans* in vitro biofilm assays. Antimicrob Agents Chemother <https://doi.org/10.1128/AAC.02749-16>.
 46. Parolin C, Marangoni A, Laghi L, Foschi C, Nahui Palomino RA, Calonghi N, Cevenini R, Vitali B. 2015. Isolation of vaginal *Lactobacilli* and characterization of anti-candida activity. PLoS One 10:e0131220. <https://doi.org/10.1371/journal.pone.0131220>.
 47. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. 2001. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. J Bacteriol 183:5385–5394. <https://doi.org/10.1128/jb.183.18.5385-5394.2001>.
 48. Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63. [https://doi.org/10.1016/0022-1759\(83\)90303-4](https://doi.org/10.1016/0022-1759(83)90303-4).
 49. Jain C, Yun M, Politz SM, Rao RP. 2009. A pathogenesis assay using *Saccharomyces cerevisiae* and *Caenorhabditis elegans* reveals novel roles for yeast AP-1, Yap1, and host dual oxidase BLI-3 in fungal pathogenesis. Eukaryot Cell 8:1218–1227. <https://doi.org/10.1128/EC.00367-08>.