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Assessment of Antifungal Activity of *Saccharomyces boulardii against Candida albicans* Biofilm

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ABSTRACT

Backgrounds: The fungal pathogen *Candida albicans* is a cause of biofilm formation in patients with oropharyngeal candidiasis. *Saccharomyces boulardii* is a nonpathogenic fungal probiotic that plays an important role in preventing or treating intestinal diseases. This research aimed to determine the inhibitory effect of *S. boulardii* probiotic yeast on biofilm formation capacity of *C. albicans*, which is one of the main virulence factors.

Materials & Methods: In this study, 33 oropharyngeal samples were collected from patients with suspected oropharyngeal candidiasis (OPC). The inhibitory activity of *S. boulardii* against biofilm formation capacity of *C. albicans* was investigated by crystal violet-based staining (CVS) and MTT reduction reaction. The collected data were analyzed using student's t-test in SPSS statistical software.

Findings: In this study, the probiotic yeast *S. boulardii* reduced the pathogenicity and virulence of *C. albicans* in vitro. According to the results of CVS and MTT assays, a considerable reduction ($p< .001$) in the biomass and viability of *C. albicans* biofilms was observed after 48 hours of incubation in the presence of *S. boulardii* extract.

Conclusion: There was a significant association between *S. boulardii* extract concentration and biofilm formation in both CVS and MTT assays. Biofilm formation decreased with increasing *S. boulardii* extract concentration and incubation time in both methods compared to the control group.

Keywords: *Candida albicans, Saccharomyces boulardii*, Biofilm, Crystal violet, MTT.

CITATION LINKS

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Introduction

Biofilms are organized surface-attached groups of microorganisms surrounded by an extracellular matrix, biofilm formation is one of the main virulence factors of *Candida albicans* . *Candida* biofilms destroy epithelial surfaces and cause vaginitis and thrush or oropharyngeal candidiasis (OPC); in rare cases, they may destroy vascular endothelium and progress to endocarditis [1]. Moreover, *Candida* biofilms could be formed on living (biotic) and non-living (abiotic) surfaces (like living tissues or implanted medical appliance) and cause fatal infections in different groups of patients with immature or weakened immune systems [2]. Additionally, the presence of biofilms is associated with reduced susceptibility to antimicrobial agents. Probiotics were first recognized and widely investigated in the late 19th century by various researchers [3]. They are live bacteria and yeasts with many beneficial effects on human health when consumed in sufficient quantities [4]. *Saccharomyces boulardii* is a non-pathogenic yeast belonging to the *Saccharomycetaceae* family, which is considered as one of the most commonly used probiotics. This probiotic strain modulates pro-inflammatory gene expression, host cell signals, and the activity of enzymes and the immune system in intestinal mucosal membranes .[6 ,5]*S. boulardii* has recently been shown to reduce *C. albicans* colonization in the gut and thus inflammation in the mouse model [7]. When administered orally, live *S. boulardii* probiotic strains could inhibit *C. albicans* infection in some organs such as the liver and kidneys, blood, and mesenteric lymph nodes .[8]Nowadays, antifungal drugs such as nystatin, amphotericin B, fluconazole, itraconazole, and voriconazole are commonly used to treat oral candidiasis as the first-line treatments. Nevertheless, the adverse effects of these medications and the emergence of drug-resistant isolates highlight the need to develop alternative therapies [9]. In this regard, the use of probiotics could be considered as a favorable alternative therapeutic approach. Since *S. boulardii* has not been sufficiently studied as a bio-therapeutic agent, especially for *Candida* infections.

Objectives: This research aimed to investigate the inhibitory effect of probiotic yeast *S. boulardii* on the biofilm formation capacity of *C. albicans* isolates.

Materials and Methods

Sample collection and initial identification of isolates: In this study, 33 oropharyngeal samples were collected from patients with suspected oropharyngeal candidiasis (OPC) during a nine-month period from September 2020 to July 2021. Oropharyngeal candidiasis was confirmed by the presence of hyphal, pseudohyphal, and fungal cells in direct microscopic examination, and confirmed samples were inoculated on Sabouraud dextrose agar (SDA) containing chloramphenicol and cycloheximide for 48 hours and then cultured on CHROMagar medium.

Preparing the extract of *S. boulardii*: The dry fungal extract from YNB (yeast nitrogen base, Sigma, Germany) culture of *S. boulardii* was prepared as previously characterized [10] with some modifications. For this purpose, freeze-dried probiotic fungal isolates of *Saccharomyces cerevisiae* var. *boulardii* CNCM I-745 (Yomogi®, Mutaflor Co., Australia) were employed [11]. Freeze-dried fungal isolates were added directly to RPMI 1640 (Gibco, Thermo Fisher Scientific, USA) medium complemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 µL/mL), and streptomycin (100 µL/mL) (Gibco, Thermo Fisher Scientific, USA) and then subjected to incubation at 37 °C for 24 hrs [11].

A single colony of *S. boulardii* isolates was selected and added to 5 mL of both YNB medium (pH=5.5) supplemented with 2% D-glucose as well as PDB medium (potato

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dextrose broth, Ibresco, Iran); the culture media were incubated at 30 °C for 18 hrs. Afterwards the culture media were injected to 250 mL of YNB/PDB medium and re incubated at 30 °C for 24 hrs. Then about one liter of the 24-hour cultures of *S. boulardii* were centrifuged at 3000 × g for 10 min; supernatants were collected, filtered using a 0.22-mm paper filter (Sartorius, Germany), and sterilized. The extraction of supernatants was performed using (v/v) ethyl acetate solvent (one-fifth) for 3 hrs so that the solvent was changed every 30 min. The solvent was eliminated using a rotary evaporator. Eventually, dry masses were prepared from both YNB and PDB cultures of *S. boulardii.* The extracted residues were then adjusted to the final concentration using methanol and employed as stock solutions. The concentration of methanol in the stock solutions was lower than 1%.

Preparing *C. albicans* cell suspension: Cell suspension of *C. albicans* isolates was prepared as described previously [12] with a few changes. Briefly, a single colony of *C. albicans* isolates was added to SDB culture medium (Sabouraud dextrose broth) and incubated at 35 °C for 24 hrs. *C. albicans* cell suspension was normalized to 0.5 McFarland turbidity standard in RPMI 1640 with an optical density (OD) of 0.12 - 0.15 at 530 nm comparable to 15- × 10^6 CFU/mL. To obtain an inoculum suspension corresponding to 1 × 10^3 CFU/mL, the prepared cell suspension was mixed and diluted with RPMI 1640.

Antifungal activity of *S. boulardii* extract: The extract of *S. boulardii* was assessed in terms of its antifungal activity against *C. albicans* strains according to the microbroth dilution reference method provided by the Clinical and Laboratory Standards Institute (CLSI) (M27-A4-S4) [8]. To prepare the desired concentrations of *S. boulardii* extract (2 to 1024 mg/mL), serial dilutions were prepared from the stock solution in 10.4 g of RPMI 1640 dissolved in 900 mL of distilled water containing MOPS buffer (Sigma, St Louis, MO, USA) and 2% D-glucose. About 1 mL of fungal suspension (10^6 cells of *C. albicans* / mL of RPMI-1640) was added into a 96-well microplate and then subjected to incubation at 30 °C for 48 hrs. Fungal growth in the wells was then visually monitored.

Positive control was composed of fungal suspension plus SDB medium, and negative control was composed of *S. boulardii* extract plus SDB medium; incubation conditions were the same for all culture media. The minimum inhibitory concentration (MIC) was determined as the lowest concentration exhibiting no visible fungal growth. This experiment was performed in triplicate for all the isolates.

S. boulardii* effect on biofilms formation in *C. albicans

Biofilm formation: Biofilm formation test was performed in vitro as described previously [13]. Briefly, *C. albicans* cells (10^6 cell/mL) in RPMI-1640 medium as a fungal suspension along with 100 µL of double concentrations of *S. boulardii* extract (25 to 400 mg) were added into a 96-well tissue culture plate. Fungal suspension plus 1 mL of fluconazole (64 µg/mL) was used as a positive control, and fungal suspension alone was used as a negative control. Incubation conditions were the same for all culture media (at 32 °C for 48 hrs). This experiment was performed in triplicate for all the isolates. After 48 hours of incubation, the culture media were removed, and the wells were gently washed three times in sterile PBS (phosphate-buffered saline), and biofilms formed in the 96-well, flat-bottomed, polystyrene, sterile culture plate were evaluated as follows.

Quantification of biofilm formation: The ability of *C. albicans* clinical isolates to form biofilm was evaluated by crystal violet staining (CVS) method (Merck, Germany) and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction reaction as

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described previously [14].

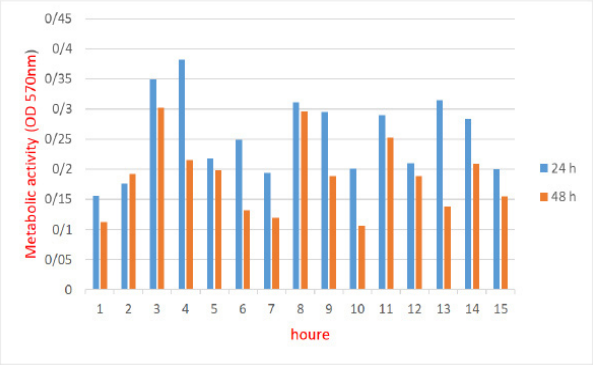
For CVS assays, the wells were stained with CV solution for 15 minutes after washing with PBS. The supernatants were completely removed, and the microplates were air-dried overnight. Then in order to dissolve the CV in the microplates, 200 µL of ethanol solvent (95%) was applied for 20 min. For each sample, 100 µL of the solution was evaluated on a spectrophotometer by recording the absorbance at 570 nm [14]. The minimum inhibitory concentration of the extract against biofilm formation capacity of *C. albicans* was evaluated using colony counting technique. For MTT assays, 50 mL of MTT solution (a stock solution containing 5 mg of MTT diluted in 1 mL of pre-warmed 0.15 M PBS before use) was poured into the wells and incubated at 37 °C for 2 hrs while protected from light. The supernatants were then completely removed, and 150 µL of 100% DMSO solvent was used for 10 minutes in the dark to dissolve the MTT in the microplates. For each sample, 80 µL of the solution was evaluated on a spectrophotometer by recording the absorbance at 570 nm [15].

Statistical analysis: All the obtained data were examined in SPSS software Ver. 25 (SPSS Inc, USA, Chicago) by employing student's t-test to evaluate between-group statistical differences. A *p* value of less than 0.05 was considered as a meaningful difference.

Findings

Overall, 15 out of 33 *Candida* species isolated from OPC patients were determined to be *C. albicans* (45.4%) as the prominent species, and the *non-albicans Candida* species isolated from the samples were as follows: *C. tropicalis* (7 of 33, 21.2%), *C. glabrata* (6 of 33, 18.1%), and *C. krusei* (5 of 33, 15.1%). Mixed infection with *C. albicans* and *C. krusei* was observed in one patient (1 of 33, 3%). The dry masses gained from 1 liter of YNB and PDB cultures were 23.5 and 59.5 mg, respectively. According to the results of microbroth dilution assay, MIC of *S. boulardii* dry extract was determined in the range of 2 to 1024 mg/mL, and significant differences were observed at concentrations of 16 mg/mL (MIC 50) and 32 mg/mL (MIC 90) compared to the positive control group (fluconazole) ($p< .05$). Based on the results of CVS and MTT experiments, the dry extract of *S. boulardii* significantly inhibited the biomass and viability of *C. albicans* biofilms after 48 hours of incubation. The viability and biomass of biofilms were reduced by 99 and 37% in the treated groups, respectively, in comparison with the control group ($p< .05$). The results were analyzed in SPSS software by employing multifactorial ANOVA (analysis of variance and T test) and statistical t-test.

A.



B.

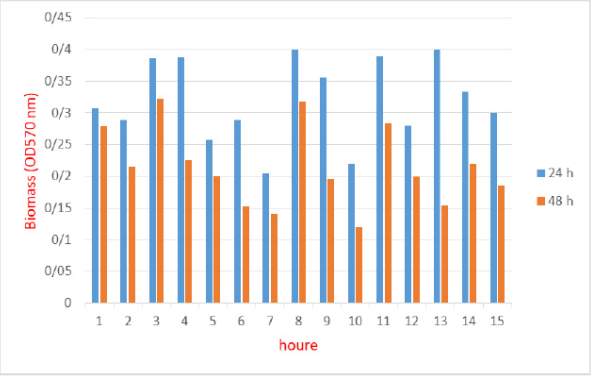


Figure 1) Quantification of *C. albicans* biofilms in the presence of MIC 50 of *S. boulardii* extract using two different methods: MTT reduction assay (A) and crystal violet staining (B). *: $P < .05$

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Table 1) Biofilm formation assay by MTT and CVS methods

	24 h Mean+SD	48 h Mean+SD	N	P Value
MTT_{50}	0.260.61+	0.18+0.62	15	.00
MTT_{90}	0.13+0.32	0.10+0.008	15	.00
CV_{50}	0.31+0.63	0.21+0.62	15	.00
CV_{90}	0.15+0.33	0.14+0.32	15	.00

Discussion

In immunocompromised patients, OPC is one of the most important opportunistic fungal infections, which could progress to an invasive form and affect any system of the human body [16, 17]. In recent years, the incidence and mortality rate of OPC has increased [18-20]. Although *C. albicans* is part of the microbiota of the gastrointestinal and reproductive tracts, it is considered as the most common cause of candidiasis [21, 22]. *C. albicans* is found in the oral cavity of about 75% of the population [22]. Nowadays, due to the increased resistance of *C. albicans* strains to existing antifungal agents, the use of probiotics in the treatment of *Candida* infections has been considered [23]. *S. boulardii* is a probiotic fungal agent currently used to treat antibiotic-associated diarrhea, traveler's diarrhea, and inflammatory bowel diseases (IBD)[24]. This probiotic fungal strain prevents *C. albicans* adhesion and biofilm formation by secreting capric acid [25]. *S. boulardii* also reduces *C. albicans* colonization and invasive infections in newborns and prevents its translocation in the GI tract [26]. In this study, the most common species isolated were *C. albicans* and *C. tropicalis*. Similarly, previous studies have reported that the most common species responsible for OPC are *C. albicans* and *C. dubliensis* [16]. Salehi et al. (2020) found that the most prevalent *Candida* species in hospitalized patients with OPC was *C. albicans* [27]. In the present study, the dry masses gained from 1 liter of YNB and PDB cultures were greater than those reported in similar studies [23]. The dry masses gained from YNB as well as PDB cultures in the present study indicated that *S. boulardii* extract had fungicidal and inhibitory activities against *C. albicans*. Krasowska et al. (2009) showed the suppressive effect of *S. boulardii* extract on hyphal and pseudohyphal constructions and found that live cells and extract of *S. boulardi* reduced adhesion and inhibited biofilm formation of *C. albicans* on polyester sheets [23]. Salehi et al. (2018) indicated the beneficial effects of *S. boulardii* extract on modifying antifungal resistance and inhibiting virulence factors of *C. albicans* [10]. In the current research, the ability of *C. albicans* clinical isolates to form biofilm was investigated in 96-well polystyrene plates in presence of *S. boulardii* extract using two methods: CVS and MTT. The MTT tetrazolium assay is a common method used to assess the metabolic activity of mammalian cell lines, bacteria, and fungi. The test is based on the reduction of yellow tetrazolium to insoluble purple formazan by enzymes, which could be measured by spectrophotometry [28]. The current study results indicated that the biomass and viability of *C. albicans* biofilms were reduced in the presence of *S. boulardii* extract after 48 hours compared to the first 24 hours and the control group ($p< .05$). Kunyeit et al. (2019) showed that probiotic yeasts decreased the metabolic activity of *albicans* and *non-albicans Candida* biofilms [29]. Murzyn et al. (2010) described that *S. boulardii* extract reduced the expression of genes associated with *C. albicans* virulence,

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including HWP1, INO1, and CSH1 [25]. Hager et al. (2019) indicated that treatment with probiotic filtrate inhibited germination and biofilm formation in *C. albicans* [30]. The outcomes of the present study are consistent with the findings of similar studies in this field.

Conclusion

In this study, there was a significant association between *S. boulardii* extract concentration and biofilm formation in both CVS and MTT assays. Biofilm formation decreased with increasing the concentration of *S. boulardii* extract and incubation time in both methods compared to the control group ($p < .05$); therefore, it could be used in the treatment of oropharyngeal candidiasis.

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Conflicts of interest: The authors declare that they have no competing interests in this investigation.

Authors Contribution: Conceptualization: SZ, YMH, KR; Data curation and formal analysis: SZ, YMH, KR; Funding acquisition :YMH; Investigation: SZ, YMH, KR; Methodology:SZ, YMH, KR; Project administration: YMH, KR; Resources and software: SZ, YMH, KR; Supervision: YMH; Writing of the original draft: SZ, KR; Writing review and editing: SZ, KR.

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