

Protective Effects of the Probiotic Bacterium *Streptococcus thermophilus* on *Candida albicans* Morphogenesis and a Murine Model of Oral Candidiasis

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What's Known

- Interaction between probiotic bacteria and the fungal members of oral microbiota can influence the virulence of pathogens, which is beneficial to the host.
- Probiotics such as *Lactobacillus* species and *Streptococcus salivarius* could protect immunosuppressed-mice from candidiasis.

What's New

- *Streptococcus thermophilus* successfully inhibited the growth, germination, and biofilm formation of *Candida albicans*.
- Moreover, this bacterium reduced *Candida* colonization and fungal burden on mucosal surfaces and relieved the signs and symptoms of oral candidiasis in our experimental model.

Abstract

Background: Oral candidiasis is a frequent form of candidiasis, caused by *Candida* species, in particular, *Candida albicans* (*C. albicans*). The transition of *C. albicans* from yeast to hyphae allows its attachment to epithelial cells, followed by biofilm formation, invasion, and tissue damage. Hence, we investigated the effect of *Streptococcus salivarius* subspecies thermophilus (*S thermophilus*) on the growth as well as biofilm and germ-tube formation of *C. albicans* both *in vitro* and *in vivo* in a murine model.

Methods: This experimental study was performed in the Department of Medical Mycology and Parasitology, School of Medicine, in collaboration with the Central Research Laboratory and the Comparative Biomedical Center, Shiraz University of Medical Sciences, Shiraz, Iran (2017 to 2018). The inhibitory activity of *S. thermophilus* against *Candida* species growth was evaluated using the broth microdilution method, and the inhibition of *C. albicans* biofilm formation was measured using the XTT assay. The inhibition of *C. albicans* germ-tube formation by *S. thermophilus* was evaluated using the plate assay and fluorescence microscopy. The experimental activity of the probiotic bacterium was assessed by culture and histopathological methods in six groups of five mice, comprising those treated with four concentrations of probiotics, fluconazole, and distilled water. The one-way analysis of variance, followed by a Tukey *post hoc* test, was used and a P value of less than 0.05 was considered significant.

Results: *S. thermophilus* inhibited *Candida* species growth at concentrations of 16 to 512 µg/mL. This probiotic inhibited the formation of *C. albicans* biofilms and germ tubes in a dose-dependent manner. *S. thermophilus* significantly reduced the colony-forming units in the mice receiving 30 mg/mL of this probiotic treatment compared with the control group (P=0.024). The histopathological analysis showed that *Candida* colonization was diminished in the mice following the administration of the probiotic.

Conclusion: Given the inhibitory activity of *S. thermophilus* against the growth, transition, and biofilm formation of *C. albicans*, it could be used in the management of oral candidiasis.

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Introduction

According to the definition of the World Health Organization/ Food and Agriculture Organization, probiotics are "live

microorganisms that, when administered or consumed in adequate quantities, confer health benefits on the host".¹ While the *Enterococcus*, *Streptococcus*, *Lactococcus*, *Pediococcus*, and *Saccharomyces* genera have probiotic strains, most probiotic bacteria belong to the *Lactobacillus* and *Bifidobacterium* genera.² The mechanisms of probiotic actions in the oral cavity can be classified into three groups: normalization of the oral microbiota, modulation of the immune system, and metabolic effects.³ Several studies have shown that probiotic bacteria may have a role in modulating oral fungal flora.⁴ For example, an *in vitro* study of *Lactobacilli* species showed various inhibitory activities against oral candidiasis, with *Lactobacillus rhamnosus* (*L. rhamnosus*) GG having the strongest inhibitory effect.⁵ Likewise, in a randomized clinical trial, a significant reduction in the number of *Candida* cells was observed in the cases fed with *L. reuteri*.⁶ Moreover, it has been shown that the consumption of yogurt enriched with *L. casei* and *Bifidobacterium breve* increases secretory of IgA levels in saliva and causes a significant reduction in the *Candida* species population in the oral cavity of elderlies.⁷ In an animal model study, *Streptococcus salivarius* (*S. salivarius*) K12 significantly inhibited the adhesion and invasion of *Candida albicans* (*C. albicans*) into oral mucosal surfaces, and protected the mice from oral candidiasis.⁸

Streptococcus salivarius subspecies thermophilus (*S. thermophilus*), as a part of the *Streptococcaceae* family, is a Gram-positive, fermentative, and facultative anaerobe bacterium of the viridans and lactic acid bacteria group.⁹ It belongs to the *S. salivarius* strain, which has been reported to inhibit the biofilm formation of *S. mutans*, one of the etiological factors of dental caries.¹⁰ This bacterium possesses the qualified presumption of safety (Q.P.S.), and is generally recognized as safe (G.R.A.S.) status due to a long history of safe use.¹¹ After *Lactococcus lactis*, this bacterium is considered to be the second most important starter in the dairy industry and is also known for folate production, a component involved in many metabolic reactions as a cofactor, including the biosynthesis of DNA and RNA. Furthermore, it has been reported that the consumption of this bacterium in sufficient amounts can have beneficial effects on human health such as producing antioxidants and vitamins, enhancing the immune system, and improving lactose digestion in lactose-intolerant individuals.¹¹

Candidiasis, as one of the most frequent fungal infections, can manifest in various forms such as cutaneous candidiasis, onychomycosis, mucosal

involvement (oral, esophageal, gastrointestinal, and vaginal), and eventually systemic and life-threatening infections.¹² Oral candidiasis, as the most common form of this infection, is usually accompanied by severe inflammation, pain, and dysphasia and may be presented in three types: erythematous, pseudomembranous, and hyperplastic.¹³ It is more prevalent in patients with acquired immunodeficiency syndrome (AIDS), diabetes mellitus, and xerostomia, as well as in individuals wearing dentures, patients under treatment with broad-spectrum antibiotics and immunosuppressive drugs, and individuals with poor oral hygiene.¹³ *Candida* genus yeasts, the causative agents of candidiasis, are part of the normal flora of mucosal membranes and may transform into a pathogenic hyphal form under specific conditions. In most cases, the organisms isolated from the clinical cases of oral candidiasis are of the *C. albicans* complex, which consists of *C. albicans*, *C. dubliniensis*, and *C. africana*.¹⁴ Phenotypic switching and transition from yeast to the filamentous form are of the main virulence factors of *C. albicans*.¹⁵ Moreover, the ability to adhere and form a biofilm on different surfaces and to secrete degradative enzymes is an additional factor associated with candidiasis.¹⁶

Since *Candida* biofilms include a dense matrix of yeast cells and commensal bacteria, their physical proximity in biofilm structures or on mucosal surfaces makes their interactions possible through various secondary metabolites or microbial secretions.^{17, 18} In this regard, numerous investigations have demonstrated that oral bacterial flora may play a significant role in the pathogenesis of *C. albicans*.¹⁹ For instance, it was found that the coaggregation of *C. albicans* and *S. gordonii*, a normal inhabitant of the oral cavity, contributed to *C. albicans* survival and persistence through enhancing its growth, biofilm formation, and tissue invasion.²⁰ Nonetheless, an antagonistic interaction between *Candida* and *Lactobacilli* has been reported by previous studies, such that *Lactobacilli* inhibit *C. albicans* through reducing its growth, proliferation, adhesion, and hyphal formation through outcompeting for adhesion sites, secreting biosurfactants, and bacteriocin-like substances.^{21, 22} Since, a bacterial population may indirectly or directly influence the *Candida* population,²³ restoring the microbial balance by probiotic bacteria can be considered a novel therapeutic method for the prevention or even treatment of oral candidiasis.²⁴

Given the strain-dependency of probiotic properties and limited information on the interaction between *S. thermophilus* and

Candida yeasts, we investigated the effect of this probiotic bacterium on the morphogenesis and pathogenesis of *Candida* yeasts first by conducting an *in vitro* analysis and then, by conducting an experiment on a murine model of oral candidiasis.

Materials and Methods

This experimental study was performed in the Department of Medical Mycology and Parasitology, School of Medicine, in collaboration with the Central Research Laboratory and the Comparative Biomedical Center, Shiraz University of Medical Sciences (Shiraz, Iran), from September 2017 to October 2018.

Determination of the Antifungal Activity

Preparation of Microorganisms

The antifungal activities of *S. thermophilus* against several American Type Culture Collection (ATCC) and Centraal Bureau voor Schimmelcultures (CBS) strains of *Candida*, comprising *C. albicans* (CBS 562, 1905, 1912, 1949, 2730, and 5982), *C. tropicalis* (ATCC 750), *C. krusei* (ATCC 6258), *C. glabrata* (ATCC 90030), *C. parapsilosis* (ATCC 4344), and *C. dubliniensis* (CBS 8501), together with three clinical azole-resistant strains of *C. albicans*, were investigated in this study. *S. thermophilus* (PTCC 1738) was obtained from the Persian Type Culture Collection (PTCC) as a freeze-dried powder and cultivated in the de Man, Rogosa, and Sharpe (MRS) broth medium (Merck, Germany) at 37 °C in an anaerobic incubator (5% CO₂) for 24 hours. The cells were harvested by centrifugation (Labnet, Korea) at 2000×g for five minutes, and washed twice in phosphate-buffered saline (PBS; Merck, Germany) 0.8% (w/v), NaCl 0.02% (w/v) (Merck, Germany), KH₂PO₄ 0.31% (w/v) (Merck, Germany), Na₂HPO₄+12H₂O 0.02% (w/v) (Merck, Germany), and KCl pH 7.2 (Panreac, Spain). The bacterial cells were resuspended in sterile distilled water and freeze-dried for quantification purposes.⁸ Additionally, cell-free supernatants were prepared by growing *S. thermophilus* in sterile falcon tubes (SPL Life Sciences Co., South Korea) containing 10 mL of the brain heart infusion broth (Merck, Germany), and the supernatant was collected by centrifugation (Labnet, Korea) at 17500×g for 10 minutes and sterilized by passage through a 0.2-µm filter (Control Biogene, Spain).²⁵

Antimicrobial Susceptibility Tests

The antifungal susceptibility test was performed using the broth microdilution method

in accordance with the reference method of the Clinical and Laboratory Standards Institute (CLSI document M27-A3). To that end, 100 µL of *S. thermophilus* serial dilutions (1–512 µg/mL) were prepared in 96-well microtiter plates using the Roswell Park Memorial Institute (RPMI-1640) medium (Sigma, St. Louis, MO, USA) and then buffered with 3-morpholinopropane-1-sulfonic acid (MOPS) (Sigma, St. Louis, MO, USA). The *Candida* strains were suspended in the RPMI-1640 medium, and cell densities were adjusted to 0.5 McFarland at a 530-nm wavelength using the spectrophotometric method (1–5×10⁶ colony-forming units [CFU]/mL). The working inocula were diluted at a ratio of 1:1000 with the buffered RPMI-1640 medium. After the addition of 100 µL of the working inocula to the wells, the microtiter plates were incubated in a humid atmosphere at 37 °C for 48 hours. Uninoculated media (200 µL) were included as a sterility control (blank). Fluconazole (Sigma, St. Louis, MO, USA), ranging from 0.125 to 128 µg/mL, was used as a positive control. In addition, growth controls (media with inocula but without *S. thermophilus*) were also included. Minimum inhibitory concentrations were visually determined and reported as the lowest concentration of *S. thermophiles*, which produced a minimum of 90% growth inhibition in comparison with the growth in the control wells. Each experiment was performed in triplicate.²⁵

Determination of the Antibiofilm Activity

Biofilm Preparation and Growth

First, 100 µL of *S. thermophilus* serial dilutions (0.5 to 256 µg/mL) were prepared in 96-well microtiter plates using the RPMI-1640 medium. Standard strains of *C. albicans* (CBS 5982) and *C. dubliniensis* (CBS 8501) were cultured on Sabouraud dextrose agar (Merck, Germany) plates. After 48 hours, one colony of each yeast was transferred to Erlenmeyer flasks, containing 20 mL of the Sabouraud dextrose broth, and was incubated overnight at 37 °C on an orbital shaker at 100 rpm under aerobic conditions. The harvested yeast cells were washed twice in sterile PBS and resuspended in the RPMI-1640 medium buffered with MOPS (Sigma, St. Louis, MO, USA). The cell concentrations were adjusted to match the turbidity of a 0.5 McFarland standard at a 530-nm wavelength. After dilution at a ratio of 1:1000, 100 µL of the working inocula was added to the wells, and the microtiter plates were then incubated at 37 °C for 48 hours in a humid atmosphere. Next, 200 µL of the uninoculated medium was used as the negative control (blank), and the RPMI-1640 medium with the yeasts, but without the

probiotic bacterium, was considered the positive control.²⁶

Assessment of Biofilm Formation

The extent of biofilm formation was assayed using the (2, 3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) (XTT) reduction assay.²⁶ XTT (Sigma Chemical Co, St. Louis, USA) was prepared as a saturated solution with the final concentration of 0.5 mg/mL in Ringer's lactate (Merck, Germany). The solution was filter-sterilized with a 0.22- μ m-pore-size filter (Control Biogene, Spain), divided into aliquots, and then stored at -70 °C until further usage. Prior to each assay, the defrosted XTT stock solution was mixed with a menadione sodium bisulfite solution (10 mM prepared in distilled water, Sigma Chemical Co, St. Louis, USA) to reach the concentration of 1 μ M. After 48 hours, the biofilms were washed twice with sterile PBS to remove the non-adherent cells, and a 100- μ L aliquot of the XTT-menadione was added to each well. The plates were then incubated at 37 °C for two hours in a dark room. Finally, the colorimetric changes were measured at 570 nm using a microplate reader (BMG Labtech, Germany).²⁶

In vitro Assay of Germ-tube Formation and the Mycelial Growth of *C. albicans* and *C. dubliniensis*

Germ-tube Formation Analysis

Serial dilutions of freeze-dried *S. thermophilus* were prepared in 96-well cell-culture plates to reach concentrations of 1 to 512 μ g/mL. *C. albicans* (CBS 5982) and *C. dubliniensis* (CBS 8501) were separately added to the RPMI-1640 medium enriched with sheep serum to reach the concentration of 0.5 McFarland. Afterwards, 100 μ L of a working inoculum made by a 1:1000 dilution of yeast suspension was inoculated into each well, and the plates were incubated at 37 °C in for three hours. After staining was done with Calcofluor White (Sigma, USA), the germ-tube formation was measured under a fluorescence microscope (Olympus, USA).⁸

Mycelial Growth Analysis

The analysis of mycelial growth inhibition by *S. thermophilus* was also carried out similar to the germ-tube formation analysis, but with a longer incubation period (i.e., 24 hours).⁸

An Experimental Model of Oral Candidiasis Inducing Oral Candidiasis

All the animal experiments in the current study were carried out in accordance with the principles of the Declaration of Helsinki and the Guide for the Care and Use of Laboratory

Animal, approved by Shiraz University of Medical Sciences (IR.SUMS.REC.1396.S591).²⁷ Thirty female BALB/c mice (age=6 weeks and weight=22–25 g) were used for this experimental study. The mice were randomized (simple randomization) and kept in cages housing five animals in pathogen-free conditions. The animals were fed with autoclave-sterilized dried food and water during the experiments. The photoperiods were adjusted to the 12-hour light/dark cycle, and the environmental temperature was maintained at 26 °C. Oral candidiasis was induced in keeping with a study by Ishijima and colleagues,⁸ with some modifications. In brief, 15 mg/mL of tetracycline hydrochloride (Hakim Pharmaceutical Company, Iran) was administered through drinking water to the mice for 24 hours. Next, an immunosuppressed condition was induced via a subcutaneous injection of 100 mg/kg of prednisolone (Hakim Pharmaceutical Company, Iran) 24 hours prior to the oral inoculation of *C. albicans*. Subsequently, the oral cavity of the animals was inoculated with a cotton swab (Talaye Teb Azma Company, Iran) soaked in 2.0×10^8 CFU/mL of *C. albicans* (CBS1912). Based on the difference in the number of yeasts grown from the swabs before and after the inoculation, the number of yeasts in the oral cavity of the mice was estimated to be 1×10^6 CFU per mouse.⁸

Probiotic Treatment

The sample size calculation was done using $n=1+2C(s/d)^2$ formula, where s is the standard deviation, d is the difference to be detected, and c is a constant dependent on the values of the significance level and the power selected.²⁸ According to the results of the study by Ishijima and colleagues⁸ and the pilot study, the standard deviation of the variables and the magnitude of difference were considered to be 0.3 and 0.7, respectively. With a power of 90% and a significance level of 5% ($C=10.51$), the sample size was calculated to be comprised of five mice in each group and 30 mice for the whole study. The mice were divided into three groups of probiotics ($n=20$), fluconazole ($n=5$), and negative control ($n=5$). In the probiotic group, 50 μ L of *S. thermophilus* in different concentrations (7.5, 15, 30, and 60 mg/mL) was administered into the oral cavity at five-time points of 24 and 3 hours before and 3, 24, and 27 hours after *C. albicans* inoculation. Moreover, the same volume of fluconazole (Sigma; 2 mg/mL) and distilled water was orally administered to the fluconazole and control groups, respectively. The animals were sacrificed 48 hours after the inoculation for further experiments.

Evaluating the Number of Viable *Candida* Cells

The oral cavity (i.e., the cheek, tongue, and soft palate) was completely swabbed 48 hours after the inoculation, using a fine-tipped cotton swab (Talaye Teb Azma Company, Iran). The cotton end was cut off and placed in a falcon tube, containing 3 mL of sterile saline. Then, the cells were suspended using a vortex mixer (Behdad, Iran), and 50 µL of each sample diluted with a series of 20-fold and 100-fold was cultured on a Sabouraud dextrose agar plate for 24 hours at 37 °C. Finally, the *Candida* cells were counted, and the CFU per swab was reported as the number of the log₁₀ CFU of *Candida* per swab.

Histopathological Evaluation

The tongues of the mice were excised, fixed in 4% paraformaldehyde (pH 7.4), dehydrated by ethanol series, and embedded in paraffin. The yielded paraffin blocks were then sectioned along the longitudinal centerline (5-µm thickness). The corresponded slides were deparaffinized by xylene, rehydrated by ethanol series, and finally stained with periodic acid Schiff (PAS) and hematoxylin and eosin (H&E). Finally, the slides were evaluated by a pathologist to detect any infection.⁸

Statistical Analysis

The data were analyzed with the SPSS software, version 25, (IBM, Chicago, USA). The values from the *in vitro* studies were reported as the mean±SD of the three independent experiments. The statistical analyses between the mice treated with different concentrations of *S. thermophilus* and the control group were done using the one-way analysis of variance (ANOVA) test, followed by the Tukey honestly significant difference (HSD) *post hoc* test.

A P value of less than 0.05 was considered statistically significant.

Results

Antifungal Activities of the Probiotic Bacterium

The potential of *S. thermophilus* to inhibit *Candida* species growth was tested using the broth microdilution method. Our results showed that *S. thermophilus* exhibited inhibitory activity against selected standard strains of *Candida* species at concentrations ranging from 16 to 512 µg/mL (geometric mean=141.32 µg/mL). Moreover, this probiotic bacterium inhibited the growth of azole-resistant clinical strains at concentrations of 256 to 512 µg/mL (table 1).

Biofilm-formation Inhibition

Our findings showed that in the presence of 256 µg/mL of *S. thermophilus*, the biofilm formation of *C. albicans* and *C. dubliniensis* was inhibited by 68.21% and 62.02%, respectively (figure 1). Indeed, *S. thermophilus* inhibited *C. albicans* and *C. dubliniensis* biofilm formation in a concentration-dependent manner as reflected by a low absorbance reading when compared with the untreated control (table 2).

Microscopic Evaluation of Germ-tube Formation and Mycelial Growth

In our study, 70.00% of *C. albicans* and 55.00% of *C. dubliniensis* cells produced germ tubes in serum-enriched RPMI-1640 medium as the control group, while in the presence of 512 µg/mL of *S. thermophilus*, only 16.00% of *C. albicans* and 10.00% of *C. dubliniensis* cells formed germ tubes (table 3). Our results demonstrated that in a concentration-dependent manner, *S. thermophilus* inhibited the germ-tube

Table 1: Antifungal activities of *Streptococcus thermophilus* determined via the broth microdilution method (CLSI-M27-A3)

Species	ATCC/CBS	<i>S. thermophilus</i> MIC (µg/mL)	Fluconazole MIC (µg/mL)
<i>C. albicans</i>	CBS 562	128	0.25
<i>C. albicans</i>	CBS 1905	256	0.25
<i>C. albicans</i>	CBS 1912	16	1
<i>C. albicans</i>	CBS1949	64	0.5
<i>C. albicans</i>	CBS 2730	256	1
<i>C. albicans</i>	CBS 5982	256	0.25
<i>C. glabrata</i>	ATCC 90030	256	1
<i>C. tropicalis</i>	ATCC 750	256	2
<i>C. parapsilosis</i>	ATCC 4344	32	0.25
<i>C. krusei</i>	ATCC 6258	64	2
<i>C. dubliniensis</i>	CBS 8501	64	1
<i>C. albicans</i>	SUMS-8808*	512	128
<i>C. albicans</i>	SUMS-2302*	256	64
<i>C. albicans</i>	SUMS-625*	512	128

*The azole-resistant clinical strains; CLSI-M27-A3: Clinical and Laboratory Standards Institute reference method (document M27-A3); ATCC: American Type Culture Collection; CBS: CentraalBureau voor Schimmelcultures; SUMS: Shiraz University of Medical Science; MIC: Minimum inhibitory concentration; *C. albicans*: *Candida Albicans*

Table 2: *Candida albicans* and *Candida dubliniensis* biofilm formation in the presence of the different concentrations of *Streptococcus thermophilus*

Probiotic Bacterium Concentration (µg/mL)	C. albicans		C. dubliniensis	
	Optical Density (mean±SD)	Formation (%)	Optical Density (mean±SD)	Formation (%)
0.0	0.195±0.003	100.00%	0.179±0.003	100.00%
0.5	0.181±0.004	92.82%	0.156±0.004	87.15%
1	0.145±0.003	74.36%	0.140±0.004	78.21%
2	0.140±0.001	71.79%	0.130±0.003	72.62%
4	0.128±0.004	65.64%	0.123±0.002	68.71%
8	0.119±0.002	61.02%	0.113±0.004	63.12%
16	0.107±0.001	54.87%	0.108±0.003	60.33%
32	0.104±0.003	53.33%	0.089±0.003	49.72%
64	0.086±0.003	44.1%	0.082±0.003	45.81%
128	0.08±0.001	41.02%	0.078±0.002	43.57%
256	0.062±0.003	31.79%	0.068±0.003	37.98%

Table 3: Germ-tube formation and mycelial growth of *Candida albicans* and *Candida dubliniensis* in the presence of the different concentrations of *Streptococcus thermophilus*

Probiotic Bacterium (µg/mL)	Percentage of the Germ-Tube Formation and Mycelial Growth of <i>Candida</i> Species	
	<i>C. dubliniensis</i>	<i>C. albicans</i>
0	55.00%	70.00%
1	50.00%	66.00%
2	41.00%	58.00%
4	26.00%	42.00%
8	25.00%	36.00%
16	21.00%	32.00%
32	20.00%	30.00%
64	19.00%	28.00%
128	18.00%	26.00%
256	14.00%	18.00%
512	10.00%	16.00%

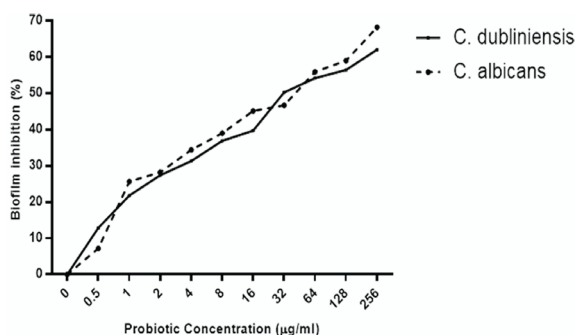


Figure 1: The figure shows that the formation of *Candida albicans* (*C. albicans*) and *C. dubliniensis* biofilms were inhibited by the different concentrations of *Streptococcus thermophilus*.

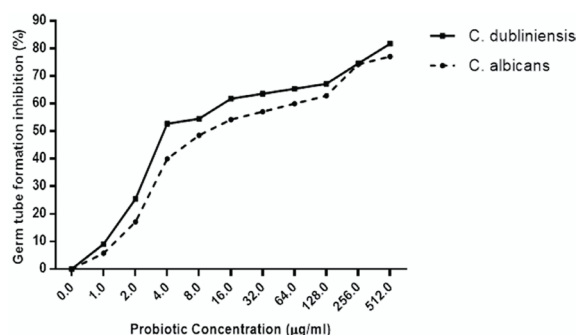


Figure 2: The figure shows that the germ-tube formation and mycelium growth of *Candida albicans* and *Candida dubliniensis* were inhibited by the different concentrations of *Streptococcus thermophilus*.

formation and mycelial growth of *C. albicans* and *C. dubliniensis* up to 77.14% and 81.81%, respectively (figure 2). Calcofluor White staining under fluorescence microscopy also showed significant inhibition against the germ-tube formation and mycelial growth in the presence of 512 µg/mL of *S. thermophilus* compared with the control groups (figure 3).

Effect of *S. thermophilus* on the Oral Candidiasis Model

The therapeutic effect of *S. thermophilus*

was examined in a murine model of oral candidiasis through the oral administration of the probiotic bacterium at five-time points before and after *Candida* inoculation. It appears that *S. thermophilus* administration caused a dose-dependent reduction in fungal burdens and the number of viable *Candida* cells in the oral cavity. The one-way ANOVA, followed by the Tukey HSD *post hoc* tests, showed that there were no differences in the number of *Candida* cells in the oral cavity between the mice treated with 7.5 and 15 mg/mL of

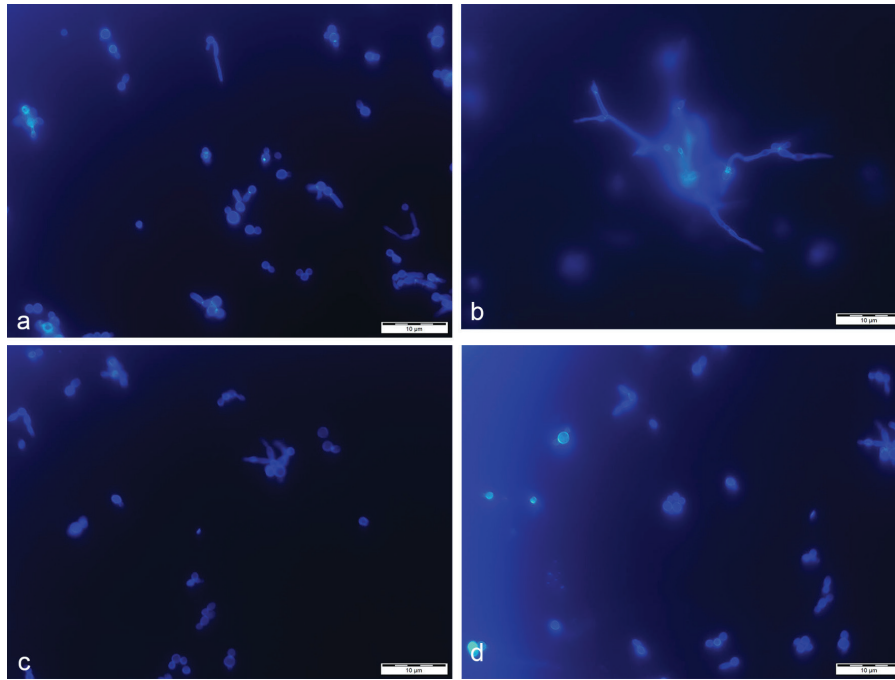


Figure 3: The figures show the formation of the hyphae and mycelium of untreated *Candida albicans* in (a and b) the serum-enriched Roswell Park Memorial Institute (RPMI)-1640 medium (c and d) the serum-enriched RPMI-1640 medium containing 512 µg/mL of *Streptococcus thermophilus*.

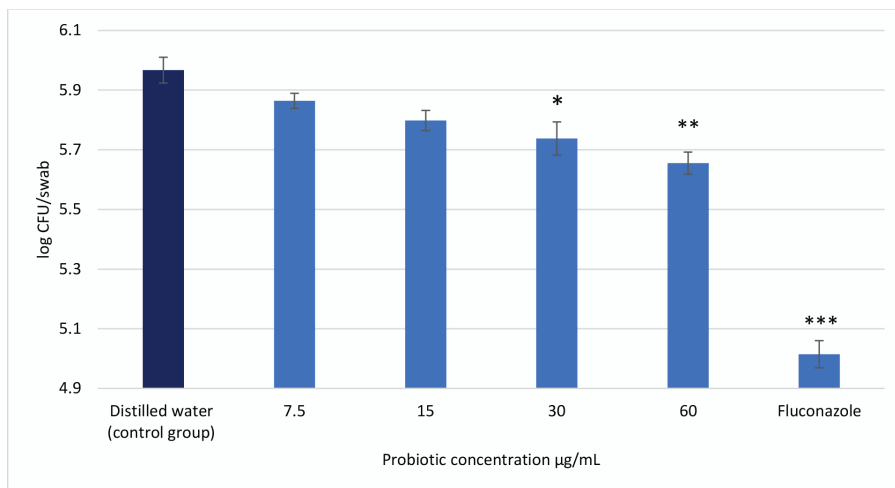


Figure 4: The figure shows the fungal burden (\log_{10} CFU/swab) of *Candida* on the oral cavity of the mice treated with the different concentrations of *Streptococcus thermophilus*. *, ** and *** show significant differences ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively) in comparison with the controls, as determined via the one-way analysis of variance followed by the Tukey honestly significant difference *post hoc* test. CFU: Colony-forming unit

S. thermophilus and the untreated mice ($P=0.618$ and $P=0.147$, respectively). Nevertheless, the oral application of 30 and 60 mg/mL of *S. thermophilus* resulted in significant differences in the number of *Candida* cells in comparison with the untreated group ($P=0.024$ and $P=0.002$, respectively) (figure 4). The regulatory effect of *S. thermophilus* on *C. albicans* pathogenicity was illustrated by the histopathological analysis of mice tongues. Whereas, the oral inoculation of 7.5 and 15 mg/mL of *S. thermophilus* resulted in no differences in tissue invasion and fungal burden compared with the untreated control group, the administration of 30 and 60 mg/mL of

this probiotic bacterium resulted in the reduction of mycelial elements and invasion into the oral epithelium of tongues in comparison with the animals fed with distilled water (figure 5).

Discussion

In the present study, *S. thermophilus* inhibited the growth, germination, and biofilm formation of *C. albicans* and reduced *Candida* colonization and fungal burden on oral mucosal surfaces. These inhibitory effects might be related to the interaction between *Candida* and other members of oral microbiota.¹⁹ Since these interactions can

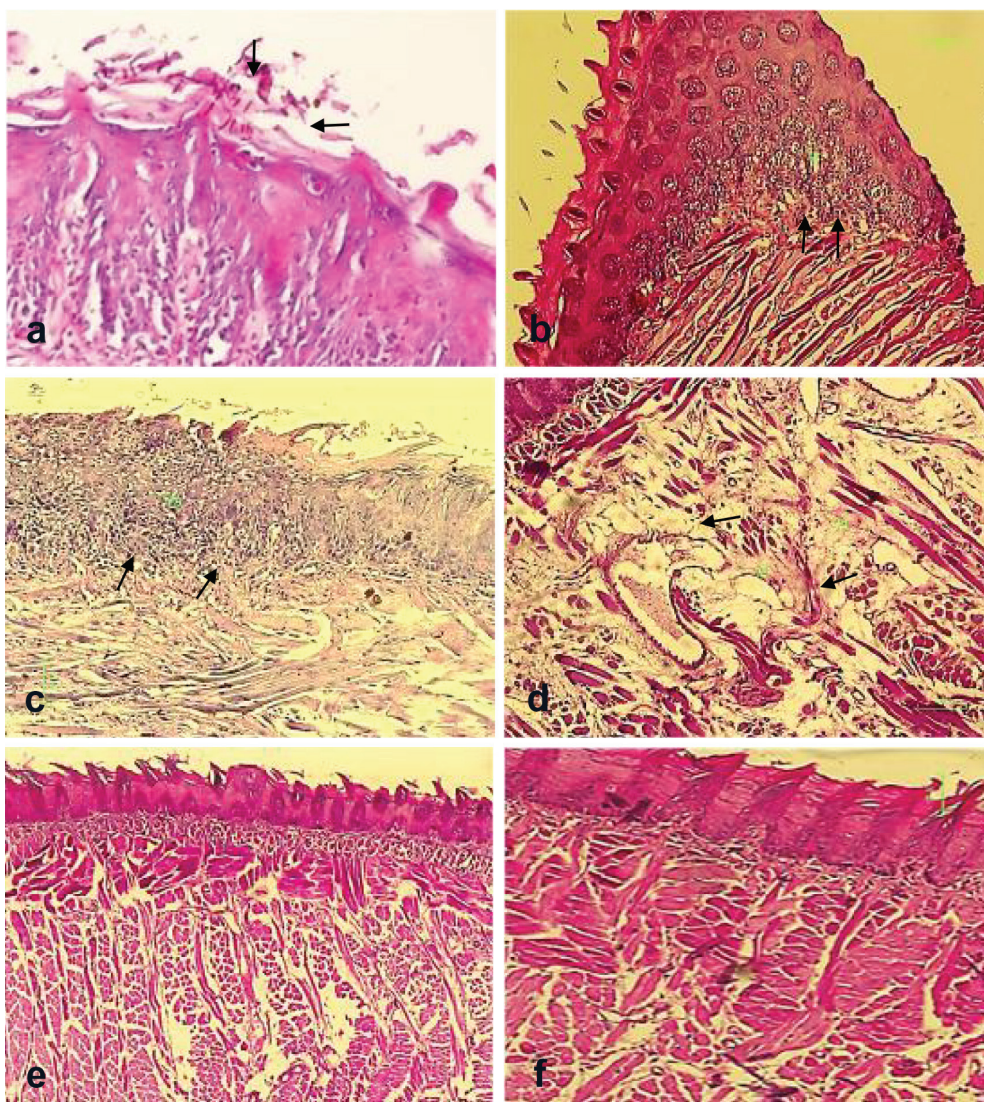


Figure 5: (a and b): The untreated control group showed severe inflammation, lymphatic dilation, and basal epithelial degeneration (arrows in 5b) with the presence of *Candida albicans* (arrows in 5a) dispersed in the tissue (a: periodic acid Schiff [PAS], 40×, b: hematoxylin and eosin [H&E], 100×). (c): The mice treated with 7.5 mg/mL of *Streptococcus thermophilus* showed severe inflammation and degeneration (arrows in 5c) with the presence of *Candida albicans* dispersed in the tissue (H&E, 100×). (d): The group treated with 15 mg/mL of *Streptococcus thermophilus* exhibited moderate inflammation, lymphatic dilation, vessel congestion (arrows in 5d), and edema with the presence of *Candida albicans* (H&E, 100×). (e): However, the mice treated with 30 mg/mL of *Streptococcus thermophilus* manifested mild inflammation with the presence of a few *C. albicans* (H&E, 100×). (f): Additionally, the group treated with 60 mg/mL of *Streptococcus thermophilus* demonstrated tissue healing with a significant decrease in the number of *C. albicans* and the amount of tissue inflammation (H&E, 100×).

influence the virulence of pathogens and the host's immune responses, they can be beneficial or detrimental to the host.¹⁹ As this bacterium inhibited the growth of azole-resistant strains, its mechanism of action might be different from those of azole drugs. These findings are in line with other studies indicating that probiotic bacteria exhibit inhibitory activities against the growth of yeasts including *C. albicans*.²⁹⁻³¹ Nonetheless, due to the differences in experimented probiotic bacterium strains and the methods of determining the antimicrobial susceptibility, different minimum inhibitory concentrations have been reported. Our results differed from those reported by Koll and colleagues, who found no

antifungal activity against *Candida* species by *L.* species, including *L. platarum*, *L. paracasei*, *L. salivarius*, and *L. rhamnosus*, which might be due to the strain-dependency of the probiotic properties.³²

In the host environment, microorganisms are mostly found in polymicrobial biofilms rather than planktonic cells, and it is estimated that a considerable proportion of microbial infections involve biofilm formation.³³ Furthermore, biofilm-embedded microorganisms tend to exhibit increased resistance to antifungal drugs and host immune defense mechanisms. As the complex structure of the biofilm allows closer proximity for interspecies cell-cell cross-talk,

it is possible that these interactions affect *C. albicans* biofilm formation and pathogenesis.³³ In this regard, our results showed that the biofilm formation of *C. albicans* and *C. dubliniensis* was inhibited in the presence of *S. thermophilus* in a concentration-dependent manner. Our findings are also supported by other previous studies, which demonstrated that probiotics such as *S. thermophilus* and *L.* species, as well as their biosurfactants, inhibit *Candida* biofilm formation.^{21, 26, 31}

According to previous studies, germ-tube formation contributes to *Candida* adhesion and invasion into the tissue.³⁴ Therefore, as a virulence factor, the germ-tube formation of *C. albicans* and *C. dubliniensis* were measured in the presence of different concentrations of *S. thermophilus* and the results showed a dose-dependent inhibition by the probiotic bacterium, which is in agreement with the report of Ishijima and colleagues.⁸ In addition to the *in vitro* experiments, our experimental murine model of oral candidiasis showed that feeding *S. thermophilus* to *Candida*-infected mice reduced the fungal burden and tissue invasion of *C. albicans*. When compared with the control group, the number of viable *Candida* cells in the oral cavity and tissue inflammation was reduced significantly, which is in agreement with our *in vitro* results. These findings are supported by previous reports, which showed that *L. acidophilus* and *S. salivarius* K12 protected immunosuppressed-mice from candidiasis.^{8, 35}

Regarding the limitations, we restricted our investigation to the short-term effects of this bacterium on *Candida* yeasts, and did not assess the long-term effects. Additionally, we were not able to separately assess the effects of *S. thermophilus* secretory products on the pathogenesis of *Candida* yeasts.

Conclusion

The obtained data from the current study suggest that *S. thermophilus* can reduce *Candida* colonization and fungal burden on mucosal surfaces, and relieve the signs and symptoms of oral candidiasis. Considering the role of *S. thermophilus* in strengthening the immune system and its anti-caries and anti-*candida* effects, it can be assumed that this probiotic bacterium has the potential to sustain a healthy oral microbiota. Further studies are still needed to clarify the effects of the secreted biosurfactant and byproducts of this bacterium on the adhesion, morphogenesis, and pathogenesis of *Candida* yeasts. Moreover, studies on the expression of various genes

involved in the growth, adhesion, transition, and biofilm formation of *Candida* yeasts treated with this bacterium are recommended.

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