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

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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 subsp. 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. While the Enterococcus, Streptococcus, Lactococcus, Pediococcus, and Saccharomyces genera have probiotic strains, most probiotic bacteria belong to the Lactobacillus and Bifidobacterium genera. 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. Several studies have shown that probiotic bacteria may have a role in modulating oral fungal flora. 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. Likewise, in a randomized clinical trial, a significant reduction in the number of Candida cells was observed in the cases fed with L. reuteri. 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 elderly people. 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.

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. 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. 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. 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.

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. 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. 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. 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. Phenotypic switching and transition from yeast to the filamentous form are of the main virulence factors of C. albicans. Moreover, the ability to adhere and form a biofilm on different surfaces and to secrete degradative enzymes is an additional factor associated with candidiasis.

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. In this regard, numerous investigations have demonstrated that oral bacterial flora may play a significant role in the pathogenesis of C. albicans. 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. Nonetheless, an antagonistic interaction between Candida and Lactobacilli has been reported by previous studies, such that Lactobacili inhibit C. albicans through reducing its growth, proliferation, adhesion, and hyphal formation through outcompeting for adhesion sites, secreting biosurfactants, and bacteriocin-like substances. Since, a bacterial population may indirectly or directly influence the Candida population, restoring the microbial balance by probiotic bacteria can be considered a novel therapeutic method for the prevention or even treatment of oral candidiasis.

Given the strain-dependency of probiotic properties and limited information on the interaction between S. thermophilus and
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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 CentraalBureau 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) in phosphate-buffered saline (PBS; Merck, Germany), and KCl pH 7.2 (Panreac, Spain). The bacterial cells were resuspended in sterile distilled water and freeze-dried for quantification purposes. 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).

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 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. thermophilus, which produced a minimum of 90% growth inhibition in comparison with the growth in the control wells. Each experiment was performed in triplicate.

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
probioctic 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-μm-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 μM. After 48 hours, the biofilms were washed twice with sterile PBS to remove the non-adherent cells, and a 100-μL 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 μg/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 μL 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 Animals, 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 2.0×10⁸ 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 1×10⁵ CFU per mouse.8

**Probiotic Treatment**

The sample size calculation was done using $n=\left\lceil 1+\frac{2C(s/d)^2}{d^2} \right\rceil$ 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.26 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 μL 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 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 the 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 formation of C. albicans and C. dubliniensis.

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

| Species          | ATCC/CBS | S. thermophilus MIC (μg/mL) | Fluconazole MIC (μg/mL) |
|------------------|----------|----------------------------|-------------------------|
| C. albicans      | CBS 562  | 128                        | 0.25                    |
| C. albicans      | CBS 1905 | 256                        | 0.25                    |
| C. albicans      | CBS 1912 | 16                         | 1                       |
| C. albicans      | CBS 1949 | 64                         | 0.5                     |
| C. albicans      | CBS 2730 | 256                        | 1                       |
| C. albicans      | CBS 5982 | 256                        | 0.25                    |
| C. glabrata      | ATCC 90030 | 256                       | 1                       |
| C. tropicalis    | ATCC 750 | 256                        | 2                       |
| C. parapsilosis  | ATCC 4344 | 32                        | 0.25                    |
| C. krusei        | ATCC 6258 | 64                        | 2                       |
| C. dubliniensis  | CBS 8501 | 64                         | 1                       |
| C. albicans      | SUMS-8808* | 512                       | 128                     |
| C. albicans      | SUMS-2302* | 256                       | 64                     |
| C. albicans      | 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: Centraal Bureau voor Schimmelcultures; SUMS: Shiraz University of Medical Science; MIC: Minimum inhibitory concentration; C. albicans: Candida Albicans

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formation and mycelial growth of \textit{C. albicans} and \textit{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 \textit{S. thermophilus} compared with the control groups (figure 3).

\textbf{Table 2: Candida albicans and Candida dubliniensis biofilm formation in the presence of the different concentrations of \textit{Streptococcus thermophilus}}

| Probiotic Bacterium | \textit{C. albicans} |  | \textit{C. dubliniensis} |  |
|---------------------|----------------------|----------------------|
| Proportions of 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% |

\textbf{Table 3: Germ-tube formation and mycelial growth of Candida albicans and Candida dubliniensis in the presence of the different concentrations of \textit{Streptococcus thermophilus}}

| Probiotic Bacterium (µg/mL) | Percentage of the Germ-Tube Formation and Mycelial Growth of \textit{Candida} Species |  |
|-----------------------------|----------------------------------|--------|
|                            | \textit{C. dubliniensis} | \textit{C. albicans} |
| 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% |

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

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

\textbf{Effect of \textit{S. thermophilus} on the Oral Candidiasis Model}

The therapeutic effect of \textit{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 \textit{Candida} inoculation. It appears that \textit{S. thermophilus} administration caused a dose-dependent reduction in fungal burdens and the number of viable \textit{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 \textit{Candida} cells in the oral cavity between the mice treated with 7.5 and 15 mg/mL of
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*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. Since these interactions can
influence the virulence of pathogens and the host’s immune responses, they can be beneficial or detrimental to the host. 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*. 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. platatur*, *L. paracasei*, *L. salivarius*, and *L. rhamnosus*, which might be due to the strain-dependency of the probiotic properties.

In the host environment, microorganisms are mostly found in polymeric biofilms rather than planktonic cells, and it is estimated that a considerable proportion of microbial infections involve biofilm formation. 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,
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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.35 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 immnosuppressed-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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Conflict of Interest: None declared.

References

1. Morelli L, Capurso L. FAO/WHO guidelines on probiotics: 10 years later. J Clin Gastroenterol. 2012;46 Suppl:S1-2. doi: 10.1097/MCG.0b013e318269fdd5. PubMed PMID: 22955349.
2. Chen CC, Kong MS, Lai MW, Chao HC, Chang KW, Chen SY, et al. Probiotics have clinical, microbiologic, and immunologic efficacy in acute infectious diarrhea. Pediatr Infect Dis J. 2010;29:135-8. doi: 10.1097/inf.0b013e3181b530bf. PubMed PMID: 20135748.
3. Haukioja A. Probiotics and oral health. Eur J Dent. 2010;4:348-55. PubMed PMID: 20613927; PubMed Central PMCID: PMCPMC2897872.
4. Meurman JH, Stamatova IV. Probiotics: Evidence of Oral Health Implications. Folia Med (Plovdiv). 2018;60:21-9. doi: 10.1515/folmed-2017-0080. PubMed PMID: 29668457.
5. Jiang Q, Stamatova I, Kari K, Meurman JH. Inhibitory activity in vitro of probiotic lactobacilli against oral Candida under different fermentation conditions. Benef Microbes. 2015;6:361-8. doi: 10.3920/BM2014.0054. PubMed PMID: 25380800.
6. Kraft-Bodi E, Jorgensen MR, Keller MK, Kragelund C, Twetman S. Effect of Probiotic Bacteria on Oral Candida in Frail Elderly. J Dent Res. 2015;94:181S-6S. doi: 10.1177/0022034515595950. PubMed PMID: 26202995.
7. Mendonca FH, Santos SS, Faria Ida S, Goncalves e Silva CR, Jorge AO, Leao MV. Effects of probiotic bacteria on Candida presence and IgA anti-Candida in the oral cavity of elderly. Braz Dent J. 2012;23:534-8. doi: 10.1590/s0103-64402012000500011. PubMed PMID: 23306230.
8. Ishijima SA, Hayama K, Burton JP, Reid G, \[8. doi: 10.1590/s0103-64402012000500011. PubMed PMID: 23306230.\]
Okada M, Matsushita Y, et al. Effect of Streptococcus salivarius K12 on the in vitro growth of Candida albicans and its protective effect in an oral candidiasis model. Appl Environ Microbiol. 2012;78:2190-9. doi: 10.1128/AEM.07055-11. PubMed PMID: 22267663; PubMed Central PMCID: PMC3302625.

9 Hols P, Hancy F, Fontaine L, Grossoir B, Prozzi D, Leblond-Bourget N, et al. New insights in the molecular biology and physiology of Streptococcus thermophilus revealed by comparative genomics. FEMS Microbiol Rev. 2005;29:435-63. doi: 10.1016/j.femsre.2005.04.008. PubMed PMID: 16125007.

10 Ogawa A, Furukawa S, Fujita S, Mitobe J, Kawarai T, Narisawa N, et al. Inhibition of Streptococcus mutans biofilm formation by Streptococcus salivarius FruA. Appl Environ Microbiol. 2011;77:2190-9. doi: 10.1128/AEM.07055-11. PubMed PMID: 22267663; PubMed Central PMCID: PMC3302625.

11 Iyer R, Tomar S, Maheswari TU, Singh R. Streptococcus thermophilus strains: Multifunctional lactic acid bacteria. International Dairy Journal. 2010;20:133-41. doi: 10.1016/j.idairyj.2009.10.005.

12 Mehta M, Dave M. Candidiasis-review of risk factor. IJCR. 2018;10:67838-40.

13 Manik A, Bahl R. A review on oral candidal infection. Journal of Advanced Medical and Dental Sciences Research. 2017;5:54.

14 Sharifynia S, Badali H, Sharifi Sorkherizi M, Shidfar MR, Hadian A, Shahrokhi S, et al. In Vitro Antifungal Susceptibility Profiles of Candida albicans Complex Isolated from Patients with Respiratory Infections. Acta Med Iran. 2016;54:376-81. PubMed PMID: 27306344.

15 Soll DR. The role of phenotypic switching in the basic biology and pathogenesis of Candida albicans. J Oral Microbiol. 2014;6. doi: 10.3402/jom.v6i6.22993. PubMed PMID: 24455104; PubMed Central PMCID: PMC3895265.

16 Tsui C, Kong EF, Jabra-Rizk MA. Pathogenesis of Candida albicans biofilm. Pathog Dis. 2016;74:ftw018. doi: 10.1093/femsdp/ftw018. PubMed PMID: 26960943; PubMed Central PMCID: PMCPMC5975230.

17 Kolenbrander PE, Palmer RJ, Jr., Periasamy S, Jakubovics NS. Oral multispecies biofilm development and the key role of cell-cell distance. Nat Rev Microbiol. 2010;8:471-80. doi: 10.1038/nrmicro2381. PubMed PMID: 20514044.

18 Dongari-Bagtzoglou A, Kashleva H, Dwivedi P, Diaz P, Vasilakos J. Characterization of mucosal Candida albicans biofilms. PLoS One. 2009;4:e7967. doi: 10.1371/journal.pone.0007967. PubMed PMID: 19956771; PubMed Central PMCID: PMC2776351.

19 Diaz PI, Strausbaugh LD, Dongari-Bagtzoglou A. Fungal-bacterial interactions and their relevance to oral health: linking the clinic and the bench. Front Cell Infect Microbiol. 2014;4:101. doi: 10.3389/fcimb.2014.00101. PubMed PMID: 25120959; PubMed Central PMCID: PMC4114182.

20 Bamford CV, d’Mello A, Nobbs AH, Dutton LC, Vickerman MM, Jenkinson HF. Streptococcus gordonii modulates Candida albicans biofilm formation through intergeneric communication. Infect Immun. 2009;77:3696-704. doi: 10.1128/IAI.00438-09. PubMed PMID: 19528215; PubMed Central PMCID: PMC2737996.

21 Boris S, Barbos C. Role played by lactobacilli in controlling the population of vaginal pathogens. Microbes Infect. 2000;2:543-6. doi: 10.1016/s1286-4579(00)00313-0. PubMed PMID: 10865199.

22 Forster TM, Mogavero S, Drager A, Graf K, Polke M, Jacobsen ID, et al. Enemies and brothers in arms: Candida albicans and gram-positive bacteria. Cell Microbiol. 2016;18:1709-15. doi: 10.1111/cmi.12657. PubMed PMID: 27552083.

23 Mukherjee PK, Chandra J, Retuerto M, Sikaroodi M, Brown RE, Jurevic R, et al. Oral mycobiome analysis of HIV-infected patients: identification of Pichia as an antagonist of opportunistic fungi. PLoS Pathog. 2014;10:e1003996. doi: 10.1371/journal.ppat.1003996. PubMed PMID: 24626467; PubMed Central PMCID: PMC3953492.

24 Devine DA, Marsh PD. Prospects for the development of probiotics and prebiotics for oral applications. J Oral Microbiol. 2009;1. doi: 10.3402/jom.v1i0.1949. PubMed PMID: 21523212; PubMed Central PMCID: PMC3077007.

25 Kohler GA, Assefa S, Reid G. Probiotic interference of Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14 with the opportunistic fungal pathogen Candida albicans. Infect Dis Obstet Gynecol. 2012;2012:636474. doi: 10.1155/2012/636474. PubMed PMID: 22811591; PubMed Central PMCID: PMC3395238.

26 Ujanoey S, Chandra J, Faddoul F, Chane M, Wang J, Tafouri L, et al. In vitro effect of over-the-counter probiotics on the ability of Candida albicans to form biofilm on denture strips. J Dent Hyg. 2014;88:183-9. PubMed PMID: 24935148.
Inhibitory effects of S. thermophilus on C. albicans

27 Association WM. WMA Declaration of Helsinki-Ethical principles for medical research involving human subjects. 2016.
28 Dell RB, Holleran S, Ramakrishnan R. Sample size determination. ILAR J. 2002;43:207-13. doi: 10.1093/ilar.43.4.207. PubMed PMID: 12391396; PubMed Central PMCID: PMCPMC3275906.
29 Meurman JH, Stamatova I. Probiotics: contributions to oral health. Oral Dis. 2007;13:443-51. doi: 10.1111/j.1601-0825.2007.01386.x. PubMed PMID: 17714346.
30 Biyari S, Fozouni L. The inhibitory effect of probiotic bacteria against drug-resistant Candida species isolated from the oral cavity of the elderly. Shiraz E-Medical Journal. 2018;19:e62026. doi: 10.5812/semj.62026.
31 Rodrigues LR, Teixeira JA, van der Mei HC, Oliveira R. Isolation and partial characterization of a biosurfactant produced by Streptococcus thermophilus A. Colloids Surf B Biointerfaces. 2006;53:105-12. doi: 10.1016/j.colsurfb.2006.08.009. PubMed PMID: 16987640.
32 Koll P, Mandar R, Marcotte H, Leibur E, Mikelsaar M, Hammarstrom L. Characterization of oral lactobacilli as potential probiotics for oral health. Oral Microbiol Immunol. 2008;23:139-47. doi: 10.1111/j.1399-302X.2007.00402.x. PubMed PMID: 18279182.
33 Shirtliff ME, Peters BM, Jabra-Rizk MA. Cross-kingdom interactions: Candida albicans and bacteria. FEMS Microbiol Lett. 2009;299:1-8. doi: 10.1111/j.1574-6968.2009.01668.x. PubMed PMID: 19552706; PubMed Central PMCID: PMCPMC4406406.
34 Tang H, Ren J, Yuan J, Zeng B, Wei H. An in vitro assessment of inhibitory effect of 16 strains of probiotics on the germination of Candida albicans. African Journal of Microbiology Research. 2010;4:1251-6.
35 Elahi S, Pang G, Ashman R, Clancy R. Enhanced clearance of Candida albicans from the oral cavities of mice following oral administration of Lactobacillus acidophilus. Clin Exp Immunol. 2005;141:29-36. doi: 10.1111/j.1365-2249.2005.02811.x. PubMed PMID: 15958067; PubMed Central PMCID: PMCPMC1809411.