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Effect of *Streptococcus salivarius* K12 on the *In Vitro* Growth of *Candida albicans* and Its Protective Effect in an Oral Candidiasis Model

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Oral candidiasis is often accompanied by severe inflammation, resulting in a decline in the quality of life of immunosuppressed individuals and elderly people. To develop a new oral therapeutic option for candidiasis, a nonpathogenic commensal oral probiotic microorganism, *Streptococcus salivarius* K12, was evaluated for its ability to modulate *Candida albicans* growth *in vitro*, and its therapeutic activity in an experimental oral candidiasis model was tested. *In vitro* inhibition of mycelial growth of *C. albicans* was determined by plate assay and fluorescence microscopy. Addition of *S. salivarius* K12 to modified RPMI 1640 culture medium inhibited the adherence of *C. albicans* to the plastic petri dish in a dose-dependent manner. Preculture of *S. salivarius* K12 potentiated its inhibitory activity for adherence of *C. albicans*. Interestingly, *S. salivarius* K12 was not directly fungicidal but appeared to inhibit *Candida* adhesion to the substratum by preferentially binding to hyphae rather than yeast. To determine the potentially anti-infective attributes of *S. salivarius* K12 in oral candidiasis, the probiotic was administered to mice with orally induced candidiasis. Oral treatment with *S. salivarius* K12 significantly protected the mice from severe candidiasis. These findings suggest that *S. salivarius* K12 may inhibit the process of invasion of *C. albicans* into mucous surfaces or its adhesion to denture acrylic resins by mechanisms not associated with the antimicrobial activity of the bacteriocin. *S. salivarius* K12 may be useful as a probiotic as a protective tool for oral care, especially with regard to candidiasis.

The overgrowth of *Candida albicans*, which is one of the members of the oral microbial flora in a healthy human, causes pathogenic symptoms such as oral candidiasis. Oral candidiasis accompanied with severe inflammation can significantly degrade the quality of life of immunosuppressed individuals and elderly people (9). It can cause a variety of mucosal infections in the gastrointestinal, respiratory, and genital tracts and is a major cause of oral and esophageal infections (9, 23, 29). Oral candidiasis is common in patients with advanced AIDS, hyposalivation, and diabetes mellitus, those on antibiotic therapy or immunosuppressive drugs, and those who have poor oral hygiene (9, 22, 23, 29).

The probiotic strain *Streptococcus salivarius* K12 was originally isolated from the saliva of a healthy child and produces several megaplasmid-encoded bacteriocin-like inhibitory substances (BLISs), such as the lantibiotics salivaricin A and salivaricin B (11, 13, 31). It has been used commercially as a probiotic for more than a decade and has numerous studies supporting its safety (3, 4, 5). *S. salivarius* strains have been reported to inhibit the biofilm formation of *Streptococcus mutans* (13, 19, 28), and *Streptococcus salivarius* K12 has been shown to have the ability to inhibit various potentially deleterious upper respiratory tract bacteria, such as *Streptococcus pyogenes* and *Streptococcus pneumoniae* (13, 31), and decrease oral malodor (2). These properties suggest that *S. salivarius* K12 might be widely applied as a management tool for oral health applications.

C. albicans is a polymorphic yeast and grows predominantly as yeast, pseudohyphae, or hyphae (23). Mycelial growth of *C. albicans* is often observed in mucosal infection and is considered to contribute to pathogenesis by biofilm formation (22). In this study, we aimed to elucidate the potential mechanisms of *Streptococcus salivarius* K12 suppression of the mycelial growth of the *C. albicans* first by *in vitro* analysis and then by testing an experimental oral candidiasis model with mice with furry white tongues.

MATERIALS AND METHODS

***Candida albicans* and *Streptococcus salivarius*.** The *C. albicans* strain TIMM1768 was isolated clinically from the blood of a candidiasis patient and maintained at Teikyo University Institute of Medical Mycology; this strain, which was shown to induce oral candidiasis in a murine model, has been used for animal experiments (12, 14). Cultures were stored at −80°C in Sabouraud dextrose broth (Becton Dickinson, MD) containing 0.5% yeast extract (Becton Dickinson, MD) and 10% glycerol (vol/vol, final concentration) until use. Strain TIMM1768 was cultured on a Sabouraud dextrose agar plate for 18 h at 37°C, and the cells were harvested with a microspatula and suspended in RPMI 1640 medium containing 2.5% fetal calf serum (RPMI 1640 medium). The cultured *C. albicans* cells were used for *in vitro* germ tube formation, the mycelial growth experiment, and also *in vivo* oral inoculation of *Candida*.

S. salivarius K12 is a commercially available probiotic that was originally isolated from the oral cavity of a child. It was supplied as a freeze-dried powder at 2×10^{11} CFU per gram of material tested and was used with CAB K12 agar, which consisted of Columbia blood agar base (Becton Dickinson, MD), 0.5% yeast extract (Becton Dickinson, MD), 0.25% glucose, and 0.1% calcium carbonate (pH 7.3 ± 0.2).

Measurement of antimicrobial activity of bacteriocins produced by *S. salivarius* K12. To determine if the bacteriocins or other secretory molecules from *S. salivarius* K12 inhibited *C. albicans* TIMM1768, a de-

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ferred antagonism assay was employed. This was conducted essentially as described by Tagg and Bannister (25), in duplicate, using the nine bacterial indicator strains described to be positive controls for *S. salivarius* K12 bacteriocin production and also applying the *C. albicans* TIMM1768 strain. In brief, *S. salivarius* K12 was preliminarily cultured on a CAB agar (with 5% blood, 0.1% $CaCO_3$) plate to form a 1-cm-wide streak. After incubation of the plate at 37°C under 5% CO_2 for 18 to 24 h, the culture of *S. salivarius* K12 was removed from the plate using a clean microscope slide and sterilized with chloroform vapors for 30 min. The plates were aired for 30 min in an extraction hood. Indicator bacterial strains as well as *C. albicans* were then inoculated horizontally across the original but now sterile *S. salivarius* K12 streak. Plates were then reincubated for 18 h. The inhibitory effect of microbial growth was evaluated as follows: −, no inhibition of the test organism; +, inhibition of the test organism only over the primary inoculation; ++, inhibition of the test organism just beyond the primary inoculation; +++, inhibition of the test organism much beyond the primary inoculation.

In vitro assay of germ tube formation and mycelial growth of *Candida albicans*. The ability of *C. albicans* cells to undergo germ tube formation or mycelial growth with *S. salivarius* K12 was assessed as described below.

(i) Germ tube formation analysis. One hundred microliters of *C. albicans* cells was aliquoted into 96-well microtiter plates (1×10^4 CFU per well for morphological analysis, 5×10^5 CFU per well for crystal violet [CV] staining), 100-μl serial dilutions of freeze-dried *S. salivarius* K12 powder were then added to the plates, which made final concentrations of 30 mg/ml (3.0×10^9 CFU/ml) to 0.12 mg/ml (1.2×10^7 CFU/ml), and the plates were incubated at 37°C in 5% CO_2 in air for 3 h. Germ tube formation was assessed microscopically: cells were fixed with 70% ethanol and stained with CV as described by Abe et al. (1) and Kamagata-Kiyoura et al. (14).

(ii) Mycelial growth analysis. Mycelial growth analysis was carried out as described for the germ tube formation assay, except that the inoculum per well was 500 cells in 100 μl and the culture period was lengthened to 16 h. Mycelial growth of *C. albicans* cells was determined as described by Abe et al. (1). Culture medium for *in vitro* assays was composed of diluted RPMI 1640 (1:3; Sigma Chemical Co., St. Louis, MO) containing 0.8% fetal calf serum, 20 mM HEPES buffer, pH 7.2, 2 mM urea, and 10 mg/ml D-glucose with or without antibiotics (60 μg/ml of benzylpenicillin potassium [Wako, Japan] and kanamycin sulfate [Wako, Japan]), according to the nutritional requirements of *S. salivarius* (6). The planktonic cells were centrifuged, stained with 50% lactophenol blue solution (containing 1 mg/ml of methyl blue [C.I. 42780], 204 mg/ml of phenol, 247 mg/ml of lactic acid, and 502 mg/ml of glycerol; Merck, Germany) in saline, and observed by microscopy.

Yeast viability assay using fluorescence microscopy. The effect of *S. salivarius* K12 on *C. albicans* viability was detected by use of a two-color fluorescent probe (FUN1; F-7030; Molecular Probes, Eugene, OR), a live/dead yeast viability kit, and fungal surface labeling with a reagent of a third color (calcofluor white M2R; Molecular Probes, Eugene, OR). *C. albicans* and *S. salivarius* K12 were cultured as described above. In brief, *C. albicans* and *S. salivarius* K12 were combined in adequate culture medium and cultured for 1 to 3 h in a CO_2 incubator. After centrifugation at 3,000 rpm for 3 min and one-time washing with GH solution (2% glucose in 10 mM HEPES buffer, pH 7.2), the GH solution was replaced with GH solution containing 20 μM FUN1 with 5 μM calcofluor white M2R. After incubation for 30 min at room temperature, cells were observed with a fluorescence microscope (BH50; Olympus, Japan) equipped with a WU (wide range of UV excitation), (WU), WBV (wide range of blue-violet excitation), WG (wide range of green excitation), and NB (narrow range of UV excitation) filter assortment. Staining with FUN1 was observed using NB and calcofluor white WU. All images were taken as digital data with a DC200 camera (Leica, Germany), and the digital data were inserted into the IM50 program and recorded.

Murine oral candidiasis model. All animal experiments were performed in accordance with the guidelines for the care and use of animals approved by Teikyo University. The derivation of the murine oral candidiasis model has been described previously (15, 27). Six-week-old female ICR mice (Charles River Japan, Inc., Yokohama, Kanagawa, Japan) were used for all animal experiments. The mice were randomized, kept in cages housing 3 to 4 individuals, and given food and water *ad libitum*. During the experimental period, the photoperiods were adjusted to 12 h of light and 12 h of darkness daily, and the environmental temperature was maintained at 21°C. To induce an immunosuppressed condition, 100 mg of prednisolone (Mitaka Pharmaceutical Co., Japan) per kg of body weight was injected subcutaneously to mice 20 to 24 h before oral inoculation. Prior to prednisolone administration, 15 mg/ml of tetracycline hydrochloride (Takeda Shering Purau Animal Health Co., Japan) was administered in drinking water for 24 h. On the day of infection, animals were anesthetized by intramuscular injection with 14.4 mg/kg of chlorpromazine chloride in the femur, after which they were orally inoculated with 2.0×10^8 CFU/ml of *C. albicans* TIMM1768 in modified RPMI 1640 medium. Oral inoculation was performed by means of rubbing and rolling a cotton swab (baby cotton buds; Johnson & Johnson Co., Tokyo, Japan) inside all parts of the mouth. The number of *Candida* cells inoculated in the oral cavity was calculated to be 1×10^6 CFU/mouse on the basis of the difference in viable cell number adhering to the cotton swabs before and just after oral inoculation, as described by Takakura et al. (27).

Oral administration of *Streptococcus salivarius* K12. Fifty microliters of *S. salivarius* K12 solution, fluconazole (2 mg/ml), or distilled water was administered into the oral cavity of the *Candida*-inoculated mice at five time points: 24 h and 3 h before and 3, 24, and 27 h after *C. albicans* inoculation. The total numbers of mice in each group during two different trials were as follows: water control, $n = 15$; *S. salivarius* K12 at 7.5 mg/ml, $n = 7$; *S. salivarius* K12 at 15 mg/ml, $n = 12$; 30 mg/ml, $n = 15$; and fluconazole at 2 mg/ml, $n = 6$. Administration was undertaken using a rounded-top needle to spread the treatment over all parts of the mouth. An active control of 50 μl of fluconazole solution (2 mg/ml) was similarly administered.

Scoring severity of oral infection. The procedure of scoring the severity of oral infection was performed as described previously (27). Forty-eight hours after inoculation, mice were sacrificed by cervical dislocation and the severity of the lesion of the tongue was evaluated by scoring the fur coating on each tongue and the squamous disorder as follows: 0, normal; 1, fur on less than 20% of the tongue; 2, fur on more than 21% but less than 90% of the tongue; 3, fur on more than 91% of the tongue and on the squamous layer; 4, thick fur on more than 91% of the tongue and on the squamous layer (12, 27).

Evaluation of number of viable *Candida* cells on murine tongues. At 48 h after inoculation, the cheek, tongue, and soft palate of each mouse was swabbed uniformly using a cotton swab, and the swab was used for microbiological evaluation. After swabbing, the cotton end was cut off and placed in 3 ml of sterile saline. *Candida* cells were resuspended by mixing on a vortex mixer and diluted with a series of 20-fold and 100-fold dilutions of sterile saline. Fifty microliters of each dilution was incubated on a *Candida* GS agar plate (selection medium for *Candida*; Eiken Chemical Co. Ltd., Japan) for 20 h at 37°C. The *Candida* cells were counted, and then the total numbers per swab were calculated and reported as numbers of CFU.

Histology. For histological study, the tongues were resected at the base of the tongue, fixed with 4% paraformaldehyde (pH 7.4) at 4°C, dehydrated by ethanol series, and embedded in paraffin in accordance with common procedure. Specimens were sectioned to an 8-μm thickness along the longitudinal center line. Sections on the slide were deparaffinized by xylene, rehydrated by ethanol series, and stained with periodic acid-Schiff (PAS).

Statistical analysis. The score data were compared by the nonparametric Mann-Whitney U test. Statistical analysis of the $\log_{10}$ number of CFU of *C. albicans* isolated from each mouse part was compared using a Student's *t* test.

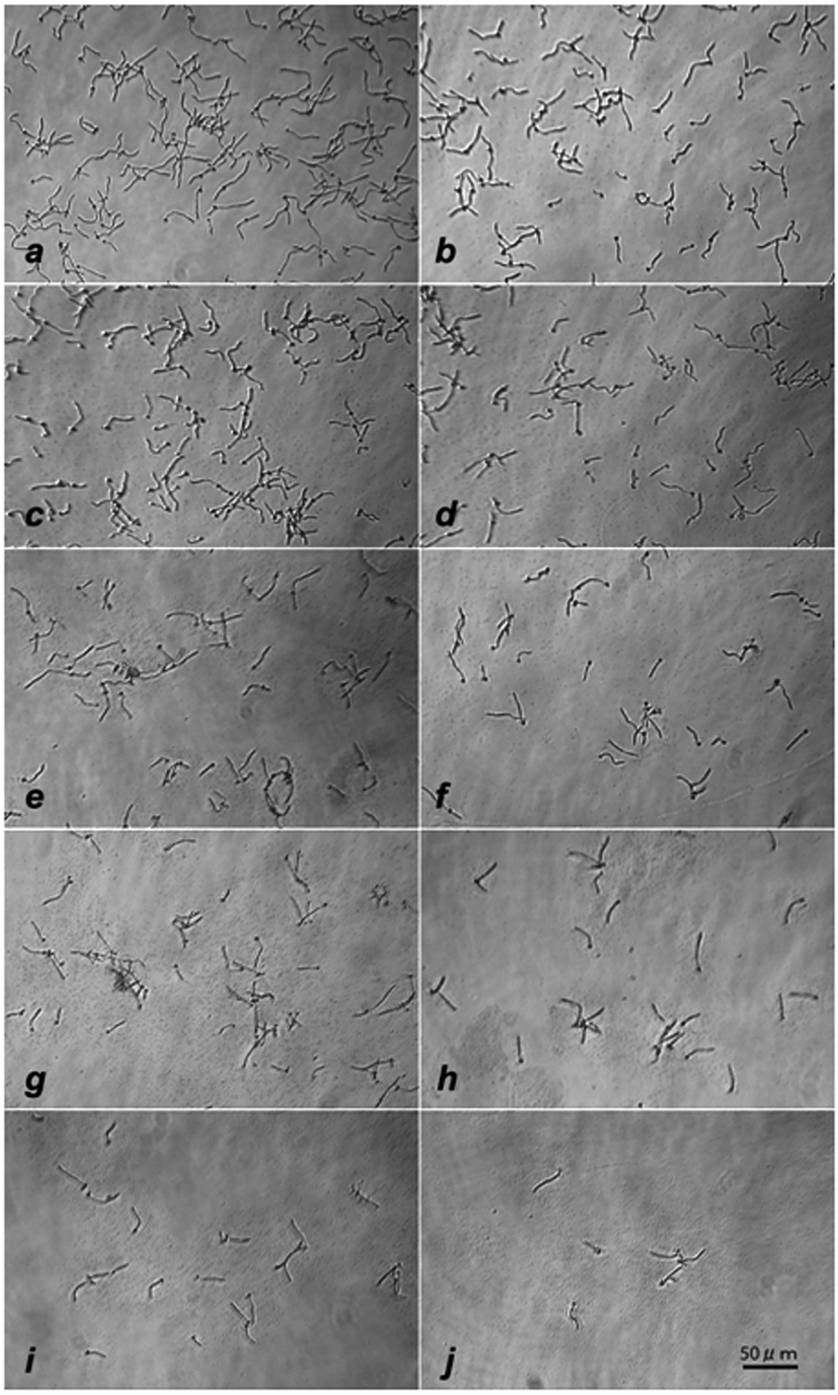

FIG 1 Inhibitory effect of BLIS K12 on germ tube formation of *C. albicans* cultured with different doses of *S. salivarius* K12 for 3 h at 37°C in 5% CO_2 in air. Starting concentrations of *Streptococcus salivarius* K12 freeze-dried material were control (a), 0.12 mg/ml (b), 0.23 mg/ml (c), 0.47 mg/ml (d), 0.94 mg/ml (e), 1.88 mg/ml (f), 3.75 mg/ml (g), 7.5 mg/ml (h), 15 mg/ml (i), and 30 mg/ml (j) (1.2×10^7 to 3×10^9 CFU/ml).

P values of <0.05 were considered statistically significant. All mean values given in the text include the standard deviation of the mean.

RESULTS

Inhibition of *Candida albicans* attachment to plastic substratum by *S. salivarius* BLIS K12. Mycelial growth of *C. albicans* is considered to contribute to the pathogenesis of mucosal candidiasis. The first step to make mycelia is germ tube formation, followed by an increase of the adherent capacity by hydrophobicity. We investigated the *in vitro* effects of *S. salivarius* K12 on the germ tube formation of *C. albicans*. Figure 1a shows that *C. albicans* cells cultured in control culture medium formed germ tube-like hyphae within 3 h. In the experimental group, where *C. albicans* was cultured in the presence of *S. salivarius* K12 (Fig. 1b to j), the

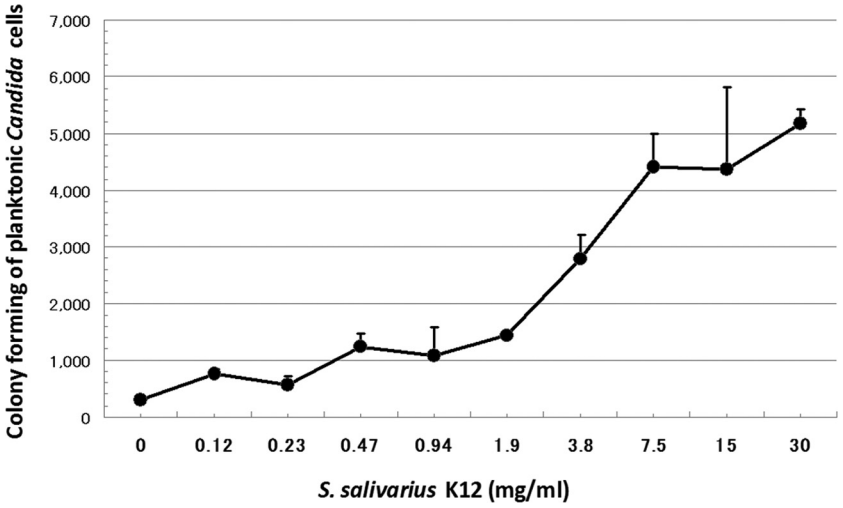

FIG 2 Number of planktonic cells after 3 h culture of *C. albicans* with various concentrations of *S. salivarius* K12. *C. albicans* was cultured with different doses of *S. salivarius* K12 for 3 h at 37°C in 5% CO_2 in air. After the cultured plate was shaken, the supernatant was collected, diluted, and seeded on a GS agar plate for determining the number of planktonic cells. The experiments were performed in duplicate.

morphological shape and size of the *Candida* cells appeared to be almost the same as those for the control group; however, the adherence of the mycelial form to the plastic substratum was weaker and the mycelial numbers on the plastic bottom were dose-dependently reduced in the presence of more than 0.94 mg/ml of freeze-dried *S. salivarius* K12 starting material, equating to approximately 1×10^8 CFU/ml.

Figure 2 shows the results of the number of viable *C. albicans* cells growing in planktonic form, which were found to increase, according to the concentration of *S. salivarius* K12, to more than 1.9 to 3.8 mg/ml (approximately 2.0×10^8 to 4.0×10^8 CFU/ml). These results indicate that *S. salivarius* K12 increased the number of planktonic *Candida* cells in culture medium. The planktonic cells, including unattached mycelia, were centrifuged and stained with lactophenol blue. Figure 3 shows that mycelial cells of *Candida* attached to and were surrounded by *S. salivarius* K12.

Effective inhibition of *C. albicans* attachment to substratum by viable *S. salivarius* K12. Although *S. salivarius* K12 was shown to bind to mycelial growth of *Candida* at 3 h of culture and inhibit *Candida* adherence to plastic plates, it is not clear whether these

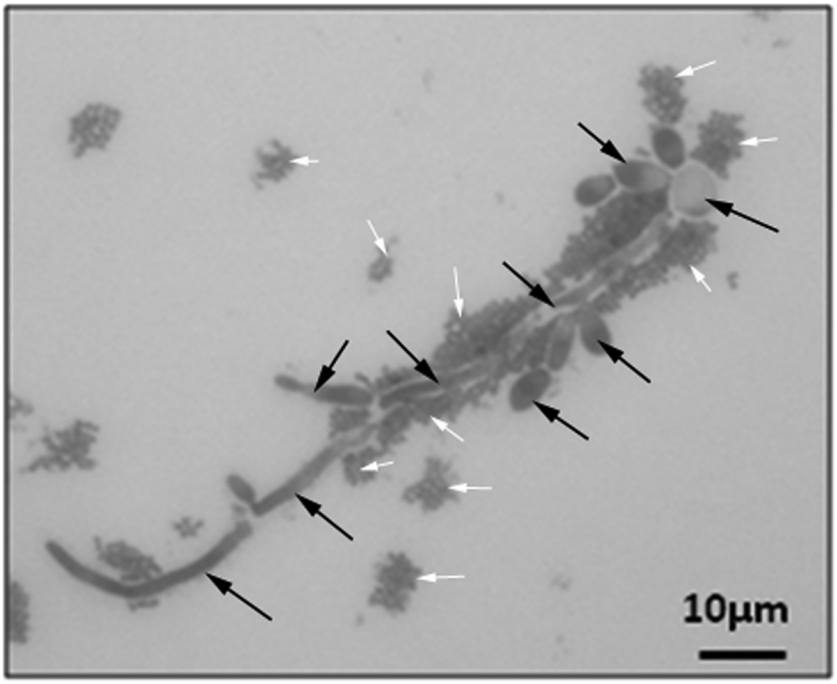

FIG 3 Microscopic observation of interaction between *S. salivarius* K12 and *Candida* planktonic cells after 3 h culture and staining with lactophenol blue. Black arrows, *Candida* cells; white arrows, *S. salivarius*.

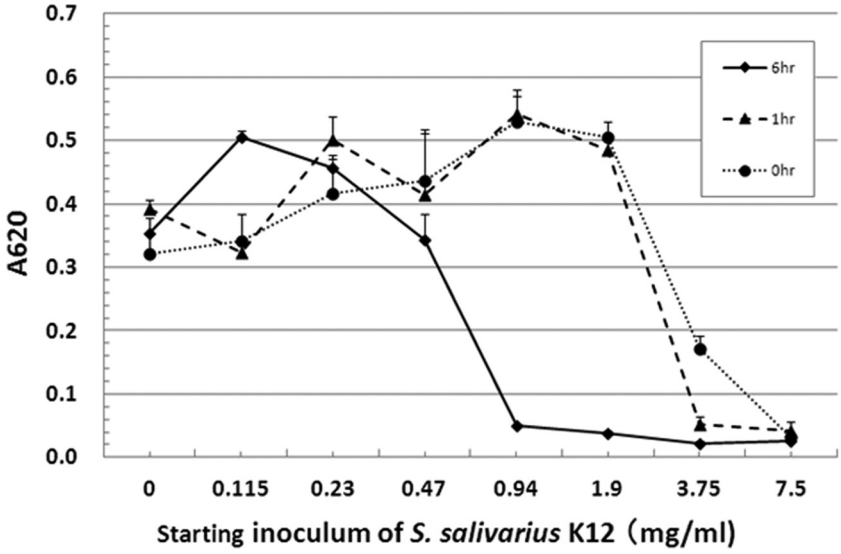

FIG 4 Inhibitory effect of *S. salivarius* K12 on hyphal growth of *C. albicans*. *S. salivarius* K12 was preliminarily cultured for 1 h or 6 h and then cultured with *C. albicans* for 3 h and stained with crystal violet. The optical absorbance at 620 nm (A620) was then detected. The experiments were performed in triplicate.

effects continue for longer periods of culture with *Candida*. Mycelial growth of *C. albicans* for 16 h of culture was quantified using the crystal violet staining method (1), and the results are shown in Fig. 4. When *S. salivarius* K12 existed at 3.75 mg/ml (3.75×10^8 CFU/ml), there were no *Candida* hyphae attached to the plastic plate.

S. salivarius K12 was obtained as a lyophilized ingredient. To determine the effect of the probiotic in an active culture, different inoculum or dose sizes were tested to see if hyphal growth and the subsequent adherence ability were inhibited. When *S. salivarius* K12 was preliminarily grown for 6 h, it appeared to enhance the inhibition of *Candida* adherence; with concentrations as low as 0.94 mg/ml (approximately 1×10^8 CFU/ml), *S. salivarius* K12 completely inhibited adherence of *Candida* (Fig. 4). Shorter incubation periods with higher inoculum doses also appeared to greatly affect hyphal growth and adhesion. In contrast, when *Candida* was preliminarily grown for 3 h, the inhibitory effect of *S. salivarius* K12 at concentrations as low as 15 mg/ml (1.5×10^9 CFU/ml; data not shown) on *Candida* adherence after an additional 3 h culture appeared to decrease.

Preferential binding of *S. salivarius* to hyphae of *C. albicans*. Earlier experiments indicated that *S. salivarius* K12 inhibited *C. albicans* mycelial adhesion to plastic plates and that possible interactions between *S. salivarius* K12 and *C. albicans* were occurring; these findings were further investigated using staining techniques. *C. albicans* was cultured on a poly-L-lysine-coated glass slide with or without *S. salivarius* K12 for 3 h and then stained by FUN1 to determine its viability by detecting metabolic activity. Slides were also stained by calcofluor white to identify the cell wall of *Candida*, which is composed of β-glucans. FUN1 staining showed that the hyphae were surrounded by numerous small green particles (Fig. 5). These particles were not stained with calcofluor white (and thus not composed of β-glucans) and were bacterial bodies of *S. salivarius* K12. Concurrently with staining of *C. albicans* with calcofluor white, the green and red fluorescence of FUN1 was also applied. In this system, green fluorescence accumulates throughout the cytoplasm and red particles transfer and concentrate in the vacuoles in the cytoplasm, indicating metabolic activity. The red pigments appeared to be concentrated in vacuoles, which indicated that the mycelial form of *C. albicans* was alive, although it was surrounded by *S. salivarius* K12. These results suggest that *C. albicans* is not killed by *S. salivarius* K12 but that *C. albicans* and *S. salivarius* K12 form some sort of interaction with each other (Fig. 5). This interaction appears to occur preferentially for the mycelial form of *C. albicans* rather than the yeast form.

No susceptibility of *Streptococcus salivarius* K12 bacteriocins to *Candida albicans* TIMM1768. To examine the possibility that *Streptococcus salivarius* K12 bacteriocins interfere with the growth of *C. albicans*, the susceptibility of *C. albicans* to *S. salivarius* K12 was tested by the deferred antagonism test (11, 25, 26). *S. salivarius* K12 inhibited all of the bacteria which were used as indicators of bacteriocin inhibitory activity but not the *C. albicans* strain, when tested in duplicate, as shown in Table 1. To further confirm a lack of bacteriocin inhibitory activity on the *Candida* strain, a simultaneous antagonism test was also performed in the liquid RPMI 1640 medium used in the other experiments described above, and again, no activity of live or heat-killed supernatants against *C. albicans* was shown (data not shown).

Effect of treatment with oral *S. salivarius* K12 on oral candidiasis model. The effects of *S. salivarius* K12 on murine oral candidiasis were examined. *S. salivarius* K12 was orally administrated to the mice at 24 and 3 h before and 3, 24, and 27 h after *Candida* inoculation. It appeared that *S. salivarius* K12 application caused a dose-dependent improvement in symptom score and fungal burden (Fig. 6). Although the oral administration of 7.5×10^8 CFU/ml of *S. salivarius* K12 (score = 3.4 ± 0.79, $n = 7$) resulted in no significant difference in symptom score from that for the saline-treated control group (score = 3.3 ± 0.88, $n = 15$), oral administration of 1.5×10^9 CFU/ml and 3×10^9 CFU/ml of *S. salivarius* K12 (scores = 2.0 ± 0.74 [$n = 12$] and 2.3 ± 0.62 [$n = 15$], respectively) resulted in an obviously significant difference from the saline-treated control group ($P < 0.01$) (Fig. 6).

The tongues of mice administered *S. salivarius* K12 showed fewer lesions than the tongues of the control mice (Fig. 7A and B).

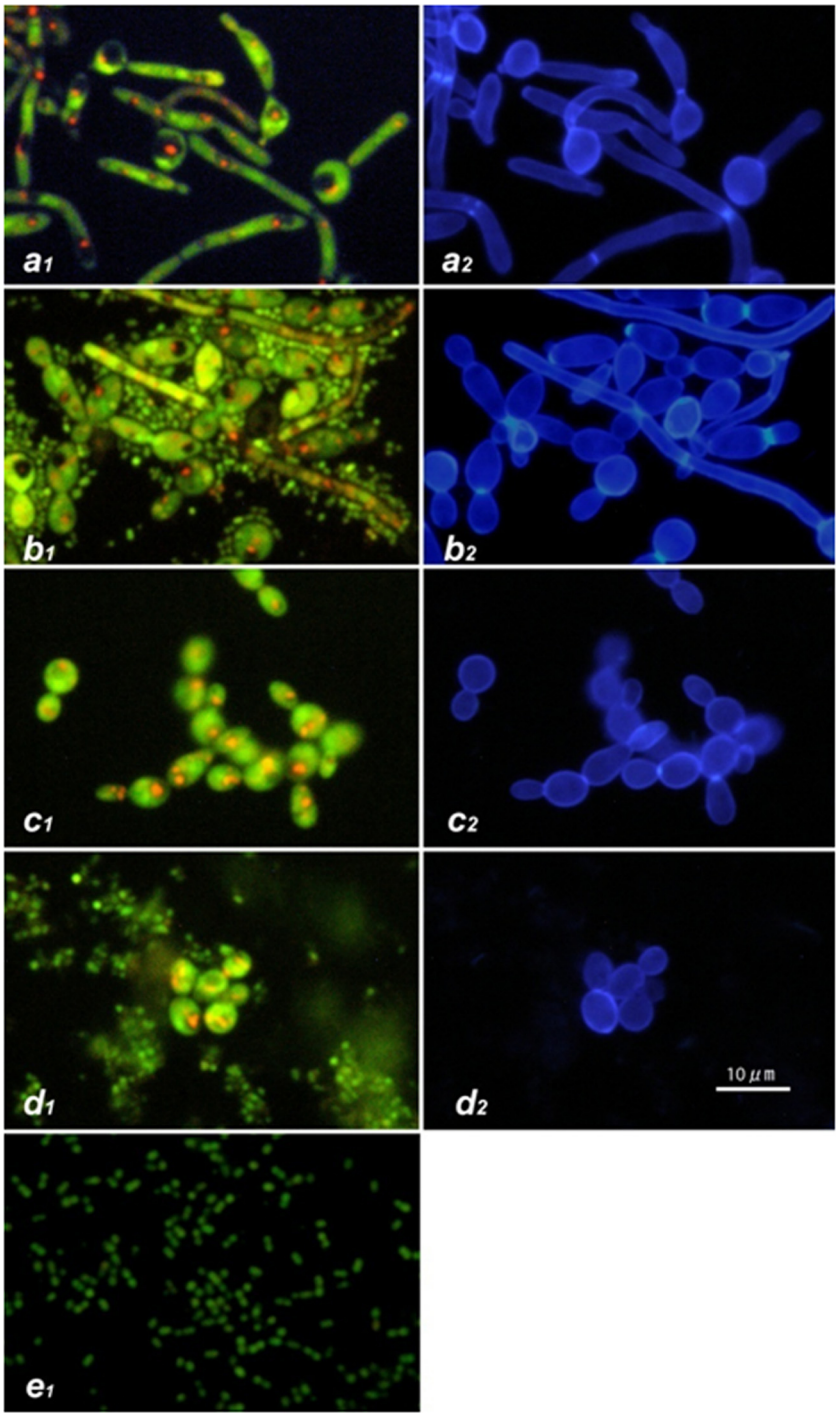

FIG 5 Vital staining of *C. albicans* by FUN1 (1) and calcofluor white M2R (2). *C. albicans* was cultured with or without *S. salivarius* K12 at 30°C or 37°C for 3 h. (a) Hyphal form of control culture at 37°C; (b) hyphal form of culture at 37°C with *S. salivarius* K12; (c) yeast form of control culture at 30°C; (d) yeast form of culture with *S. salivarius* K12 at 30°C; (e) *S. salivarius* K12.

Even though there was no total eradication, as observed in the control using fluconazole, in the mice that were administered *S. salivarius* K12 at 30 mg/ml (3×10^9 CFU/ml), there was a significant decrease in fungal burden compared to that for the untreated control. The reduced pathogenicity of *C. albicans* cells when mice were given *S. salivarius* K12 was illustrated by the histopathology of tongue sections (Fig. 8A and B). Fewer PAS-staining mycelial elements were found to invade the oral epithelium of tongues treated with *S. salivarius* K12 than the tongues in the control group.

DISCUSSION

The *in vitro* culture experiments that were conducted showed that *S. salivarius* K12 bound directly to *Candida* cells and inhibited the adherence of *Candida* cells to the plastic petri dish. We also report that *S. salivarius* K12 had a protective effect against *Candida* inva-

TABLE 1 Detection of inhibitory properties of *S. salivarius* K12 against test organisms using deferred antagonism testing

Test organism	K12 deferred antagonism[a]
Candida albicans TIMM1768	−
Micrococcus luteus T-18	+++
Streptococcus pyogenes FF22, M type 52, T pattern 3/13	+++
Streptococcus anginosus T-29	+++
Streptococcus uberis ATCC 27958	+++
Streptococcus pyogenes 71-679, M type 4, T pattern 4	+++
Lactococcus lactis T-21	+++
Streptococcus pyogenes 71-698, M type 28	+++
Streptococcus pyogenes W-1, T pattern 6	+++
Streptococcus agalactiae T-148	+++

[a] −, no inhibition of the test organism; +++, inhibition of the test organism much beyond the primary inoculation.

sion, indicated by the results obtained with the *in vivo* experimental model of oral candidiasis.

The results of *in vitro* culture experiments showed that *S. salivarius* K12 bound *Candida* cells at both the stage of germ tube formation and the stage of mycelial expansion. When *S. salivarius* K12 was preliminarily cultured aerobically in a low-ionic-strength medium with properties like those of saliva, the absorption at 620 nm increased, binding to *Candida* cells was enhanced, and the adherence of *Candida* cells to the petri dish was further inhibited.

The planktonic cells from mixed culture of *S. salivarius* K12 and *Candida* cells proportionally increased according to the concentration of *S. salivarius* K12 in the medium. The planktonic cells were composed of the mycelial form of *Candida* cells and appeared to be surrounded by *S. salivarius*, which may decrease its adhesive ability and pathogenic potential. To analyze the cross-kingdom interaction of *Candida* cells and *S. salivarius* in detail, vital staining with FUN1 for viability check and calcofluor white for yeast body analysis was undertaken, and it was confirmed that

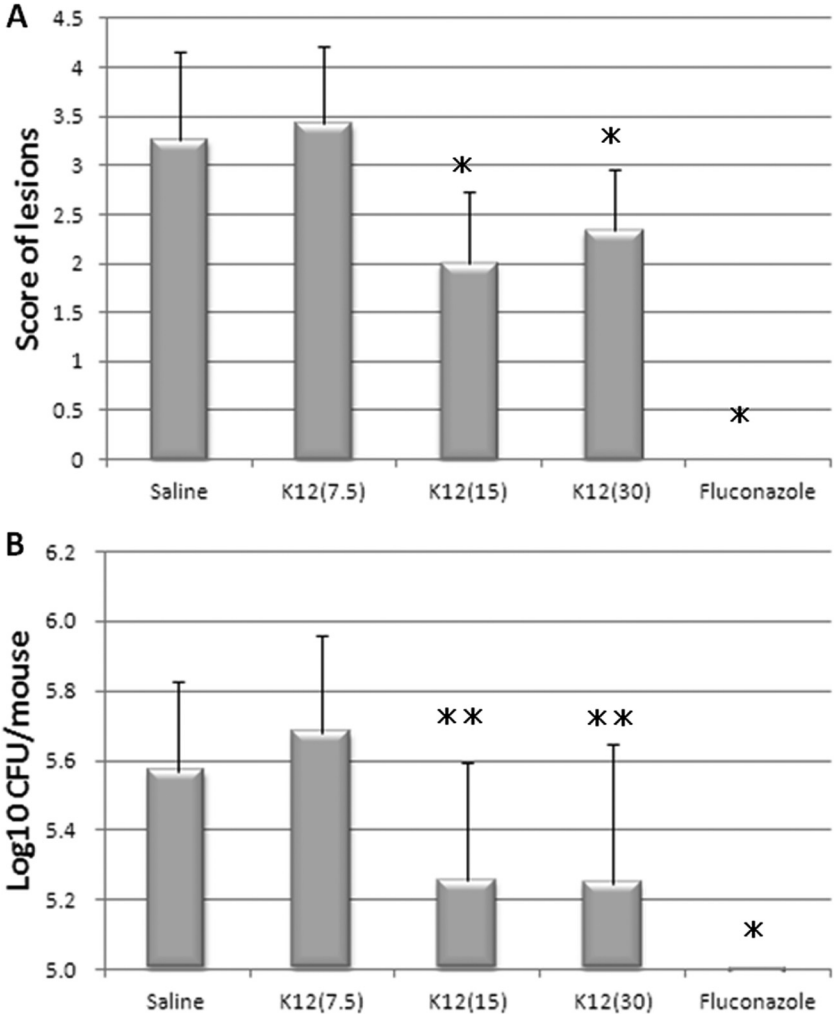

FIG 6 Effect of *S. salivarius* K12 on the symptom score (A) and fungal burden (B) in the murine model of oral candidiasis. Groups of immunosuppressed mice (control, $n = 15$; *S. salivarius* K12 at 7.5 mg/ml, $n = 7$; *S. salivarius* K12 at 15 mg/ml, $n = 12$; *S. salivarius* K12 at 30 mg/ml, $n = 15$; fluconazole at 2 mg/ml, $n = 6$) were inoculated with *C. albicans* TIMM1768, and *S. salivarius* K12 was administered as described in Materials and Methods. Symptom scores (A) and fungal burden (B) were assessed after 48 h, as described in Materials and Methods. * and **, significant differences ($P < 0.01$ and $P < 0.05$, respectively) with no *S. salivarius* K12 (control), as determined using Student's *t* test.

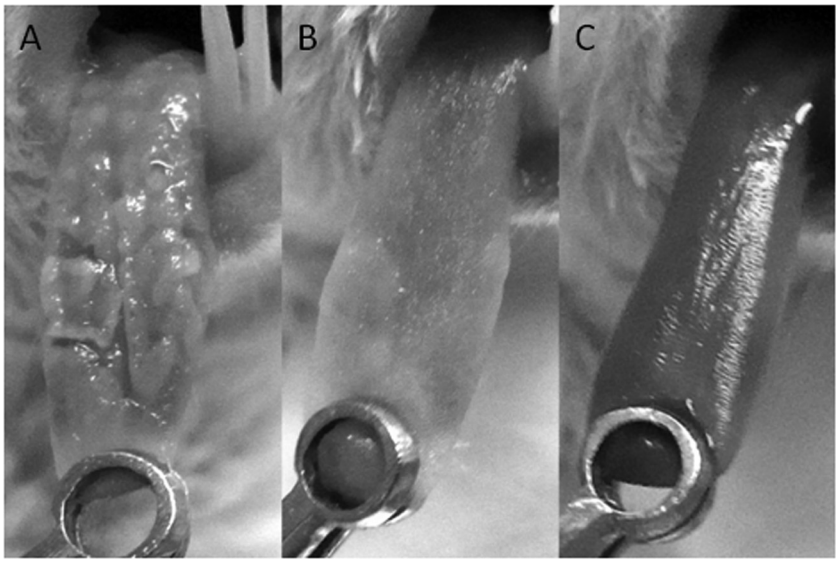

FIG 7 Typical images of tongues from mice inoculated with *C. albicans* TIMM1768. (A) control; (B) *S. salivarius* K12 (30 mg/ml; 3×10^9 CFU/ml); (C) fluconazole (2 mg/ml).

Candida cells were surrounded by *S. salivarius*. Interestingly, almost all of these were the mycelial form, and the staining indicated that they were viable. These findings, i.e., an increase in the number of planktonic *Candida* cells able to form colonies with the presence of *S. salivarius* K12 in the medium, were in agreement with the results observed in Fig. 2. This interaction was kinetically observed from 30 min to 6 h, and the results showed that more than 30 min was needed for adhesion and 1 h was enough for interaction (data not shown).

The results of tests of the *in vivo* effects of *S. salivarius* K12 against murine experimental oral candidiasis indicated that *S. salivarius* K12 had the ability to protect against severe fungal infection in a dose-dependent manner in the model used. The symptom scoring of mouse tongues and histological studies of their fungal burdens indicated the appearance of an infection less severe than that in the control group. However, those in the fluconazole group showed no symptoms of infection after treatment. Further studies may ascertain whether the *S. salivarius* K12 treatment over a longer period of time will reduce the infection.

The protective or therapeutic efficacy against oral candidiasis was evaluated, as multiple possible probiotic mechanisms were thought to be involved. These mechanisms include not only the fungicidal effect or the inhibitory effect on germ tube formation but also blocking of the attachment of mycelium to the host epithelial cells. Additionally, the reduction of *C. albicans* attachment to artificial dentition or acrylic resin was previously presumed to be an important mechanism for infection prevention (9). Saliva, which commonly contains *S. salivarius*, also has defensive effects and may play a key role in the process. Previous studies showed that adhesion of *C. albicans* germ tubes to polystyrene is decreased by saliva, whereas *C. albicans* yeast cell adhesion to the same material is enhanced (8, 14). One may postulate that the possible action of *S. salivarius* K12 *in vivo* might involve the latter type of effect, whereby the rolled up *Candida* mycelial form prevents adhesion to mucosal surfaces of the oral cavity, resulting in the fungus then traveling harmlessly through to the esophagus and beyond. This was an interesting result, in that previous studies of the antimicrobial activity of *S. salivarius* K12 against bacteria indicated that activity resulted from its bacteriocin production, since the percolation liquid in the culture, including bacteriocins from *S. salivarius* K12, did not appear to affect *C. albicans* when tested here, as shown in Table 1.

This is the first report that the direct interaction between bacteria and *Candida* induces a protective effect against oral candidiasis in an animal model and *in vitro* assay systems. Previous studies of the cross-kingdom interaction of bacteria and fungi have focused upon direct antimicrobial interactions or interactions through chemical mechanisms, such as quorum-sensing molecules or terpenoids (10, 17, 18). Despite the abundance of bacterium-fungus interactions in nature and the clinical environment, very little is known about the molecular mechanisms underlying these interactions and their importance to human health (7, 16, 19, 21, 24). Human microbial infections are often found to be polymicrobial in composition and may include bacteria and fungi. These complex microbial consortiums are also usually structured into biofilms, which have increased resistance against antimicrobials, enhanced colonization, and enhanced interspecies antagonism (7, 16). There are examples in the literature where polymicrobial combinations of opportunistic pathogens are thought to be much more deleterious than monoculture alone, such as *S. mutans* and *Candida*, which have been reported to produce a mixed biofilm and to make candidiasis more severe (20).

While there are various reports on the antibacterial activities of *S. salivarius* K12 and other strains with bacteriocin action in the literature (28, 30, 31), *Candida albicans* was not directly inhibited by bacteriocin action, and it appears that yeast cell-to-bacterial cell contact may be required. In this study, *S. salivarius* K12 directly interacted with *Candida*, as demonstrated by *in vitro* assays, and also showed a protective effect in a murine model of *Candida* infection. *S. salivarius* K12 appeared to inhibit the colonization of *Candida* by both direct and indirect mechanisms. It is not known if these properties are unique to this particular strain of *S. sali-*

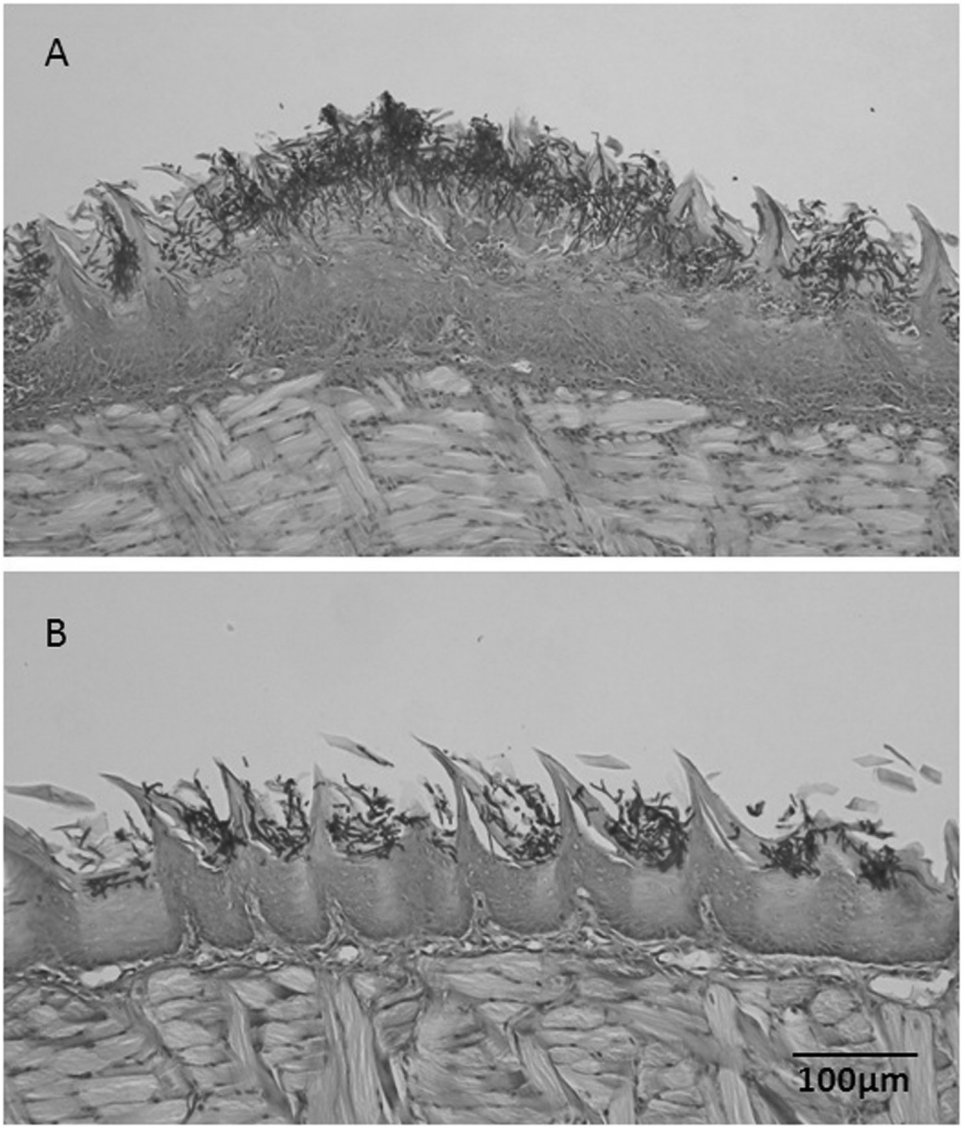

FIG 8 Histology of longitudinal formalin-fixed paraffin-embedded (FFPE) sections of mouse tongues inoculated with *C. albicans* TIMM1768. (A) Representative control mouse; (B) representative mouse given *S. salivarius* K12 (30 mg/ml; 3×10^9 CFU/ml). Sections were stained with periodic acid-Schiff.

varius or to the species in general; however, this strain has a history of safe use, and a human clinical study is warranted. The data obtained in this study suggest that the use of *S. salivarius* K12 as an oral probiotic for the prevention or treatment of oral candidiasis may have merit and warrants further clinical investigations. The mechanisms of the therapeutic effect of *S. salivarius* K12 against oral candidiasis will be studied in detail in future experiments.

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