Effect of *Streptococcus salivarius* K12 on the *in vitro* growth of *Candida albicans* and its protective effect on oral candidiasis model

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**Running title:** Effect of probiotic *Streptococcus salivarius* K12 on murine oral candidiasis

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bacteriocin-like inhibitory substances (BLIS)
Abstract

Oral candidiasis is often accompanied with severe inflammation resulting in a decline in the quality of life of immunosuppressed individuals and elderly people. To develop a new oral therapeutic options for candidiasis, a non-pathogenic commensal oral probiotic microorganism, Streptococcus salivarius K12 was evaluated for its ability to modulate Candida albicans growth in vitro and its therapeutic activity was tested on an experimental oral candidiasis model.

In vitro inhibition of mycelial growth of C. albicans was determined by plate assay and fluorescent microscopy. Addition of S. salivarius K12 to modified RPMI1640 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 substratum by preferentially binding to hyphae rather than yeast. To determine the potentially anti-infective attributes of S. salivarius K12, to oral candidiasis infections, 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 invasion process of C.
*albicans* into the mucous surfaces or its adhesion to denture acrylic resins by mechanisms not associated with the antimicrobial bacteriocin activity. *S. salivarius* K12 may be useful as a probiotic as a protective tool for oral care especially with regards to candidiasis.
Introduction

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, hypo-salivation, 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 (BLIS) 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*. 

5
Streptococcus pneumoniae (13, 31) and decrease oral malodor (2). These properties suggest that S. salivarius K12 will be widely applied as a management tool for oral health applications.

C. albicans is 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 suppressing the mycelial growth of the C. albicans, firstly by in vitro analysis and then by testing an experimental oral candidiasis mouse model 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, USA) containing 0.5% yeast extract (Becton Dickinson, MD, USA) and 10% glycerol (v/v, final concentration) until use. Strain TIMM 1768 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 RPMI1640 medium containing 2.5% fetal calf serum (RPMI 1640 medium). The cultured C. albicans cells were used for in vitro germ tube formation, mycelial growth experiment and also for 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 x 10^{11} colony forming units (CFU) per gram of material tested using with CAB K12 agar consisted of Columbia blood agar base (Becton Dickinson, MD, USA), 0.5% yeast extract (Becton Dickinson, MD, USA), 0.25% glucose, 0.1% calcium carbonate (pH 7.3...
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 the *C. albicans* TIMM 1768, it was tested using a deferred antagonism assay. This was conducted as essentially described by Tagg and Bannister (25) in duplicate, using the nine bacterial indicator strains as described as positive controls for *S. salivarius* K12 bacteriocin production and also applying the *C. albicans* TIMM1768 strain. In brief, *S. salivarius* K12 preliminary cultured on CAB (with 5% blood, 0.1% CaCO₃), plate to form a 1 cm wide streak. After incubation of the plate at 37°C under 5% CO₂ for 18-24 hours, the culture of *S. salivarius* K12 was removed from the plate using a clean microscope slide and sterilised with chloroform vapours for 30 min. 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 hours. The inhibitory effect of microbial growth was evaluated as below: (-); denotes 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
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 follows. (a) Germ-tube formation analysis: One hundred microlitres of C. albicans cells were aliquoted into 96 well microtitre plates [1 x 10^4 CFU per well for morphological analysis, 5 x 10^5 CFU per well for crystal violet (CV) staining], 100 μl serial dilutions of freeze-dried S. salivarius K12 powder was then added the plates which made a final concentration of 30 mg/ml (3.0 x 10^9 CFU/ml) to 0.12 mg/ml (1.2 x 10^7 CFU/ml) and incubated at 37°C in 5% CO₂ 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); (b) mycelial growth analysis: this was carried out as 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 RPMI1640 (1:3 Sigma Chemical Co. St. Louis, MO, USA) containing 0.8% FCS, 20mM HEPES buffer pH7.2, 2mM urea, and 10mg/ml d-glucose with or without
antibiotics (60 μg/ml of Benzylpenicillin potassium (Wako, Japan), and Kanamycin sulfate (Wako, Japan)) according to 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 for *C. albicans*’ viability was detected by two-color fluorescent probe of FUN1 (F-7030; Molecular Probes, Eugene, OR, USA) live/dead yeast viability kit and fungal surface labeling reagent of a third color of Calcofluor™ White M2R (Molecular Probes, Eugene, OR, USA). *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 during one to three hours in CO₂ incubator. After centrifugation at 3000 rpm for 3 min and sequential one time washing with GH solution (containing 2% glucose in 10mM HEPES buffer, pH7.2) replaced the GH solution including 20μM FUN1 with 5μM Calcofluor™ White M2R. After incubation for 30 min at room temperature, cells were observed with fluorescent
microscope (BH50, Olympus, Japan) equipped with WU, WBV, WG and NB filters assortment. Staining of FUN1 was observed using NB and Calcofluor WU. All images were taken as digital data by DC200 camera (Leica, Germany) and inserted the digital data into 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) were used for all animal experiments. The mice were randomized, kept in cages housing 3 to 4 individuals, and were 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/kg of prednisolone (Mitaka Pharmaceutical Co., Japan) was injected subcutaneously to mice 20 to 24 hours 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 during 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 $2.0 \times 10^8$ CFU/ml of *C. albicans* TIMM1768 in modified RPMI1640 medium. Oral inoculation was performed by means of rubbing and rolling a cotton swab (baby cotton buds; Johnson & Johnson Co., Tokyo) inside all parts of the mouth. The number of *Candida* cells inoculated in the oral cavity was calculated to be $1 \times 10^6$ CFU/mouse based on the difference in viable cell number adhering to the cotton swabs before and just after oral inoculation according to Takakura et al. (27).

**Oral administration of *Streptococcus salivarius* K12**

Fifty micro liters of *S. salivarius* K12 solution, fluconazole (2 mg/ml) or distilled water was administered in the oral cavity of the *Candida* inoculated mice five time points: 24 hours and 3 hours before, and 3, 24 and 27 hours after *C. albicans* inoculation. The total numbers of mice of two times trial in each group were; water control control $n=15$; *S. salivarius* K12 7.5 mg/ml $n=7$; 15 mg/ml $n=12$; 30 mg/ml $n=15$ and fluconazole 2 mg/ml $n=6$. This was undertaken using a top-rounded needle to spread over all parts of the mouth. An active control of 50 μl of fluconazole solution (2mg/ml) was similarly administered.
Scoring the 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 in less than 20%; 2, fur in more than 21% but less than 90%; 3, fur in more than 91% and on the squamous layer; 4, thick fur in more than 91% and on the squamous layer (12, 27).

Evaluation of the number of viable Candida cells on murine tongues

Uniformly between animals, cheek, tongue, and soft palate of the mice were swabbed using a cotton swab at 48 hours after inoculation 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 by a series of 20-fold and 100-fold dilutions of sterile saline. Fifty microlitres of each dilution was incubated on a Candida GS 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 colony forming units.
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 in common procedure. Specimens were sectioned at 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 data of scores were compared by the non-parametric Mann-Whitney U test. Statistical analysis of the log_{10} CFU of *C. albicans* isolated from each mouse part was compared using a Student’s t-test. 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 pathogenesis of mucosal candidiasis. The first step to make mycelia is germ tube formation followed by increase of adherent capacity by hydrophobicity. We investigated the *in vitro* effects of *S. salivarius* K12 for 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 hours. In the experimental group where *C. albicans* was cultured in the presence of *S. salivarius* K12 (Fig. 1 b-j) the morphological shape and size of the *Candida* cells appeared almost the same to control group, however, the adherence of mycelial form to plastic substratum was weaker and that the mycelial number 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 x 10⁸ CFU/ml.

Figure 2 shows the results of the number of viable *C. albicans* cells growing in planktonic form, increasing according to the concentration of *S. salivarius* K12 at more than 1.9-3.8 mg/ml (approximately 2.0-4.0 x 10⁸ 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 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 hours culture and inhibit *Candida* adherence to plastic plates, it is not clear whether these effects continue for longer periods of culture with *Candida*. Mycelial growth of *C. albicans* for 16 hours culture was quantified using Crystal violet-staining method (1), and the results were shown in Fig. 4. When *S. salivarius* K12 existed at 3.75 mg/ml (3.75 x 10^8 CFU/ml), there was no *Candida* hyphae attached to the plastic plate.

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

**Preferential binding of *S. salivarius* to hyphae of *C. albicans***

Earlier experiments indicated that *S. salivarius* K12 inhibited *C. albicans* mycelial adhesion to plastic plate and possible interactions between *S. salivarius* K12 and *C. albicans* were occurring, these were further investigated using staining techniques. *C. albicans* was cultured on PLL-coated slide glass with or without *S. salivarius* K12 for 3 hours, then they were stained by FUN1 to determine their viability by detecting metabolic activity. Slides were also stained by Calcofluor White to identify the cell wall of *Candida* which is composed of $\beta$-glucans. FUN1 staining showed the hyphae were surrounded by numerous small green particles (Fig. 5). These particles were not stained with Calcofluor White (not composed of $\beta$-glucans) but were bacterial bodies of *S. salivarius* K12. Concurrently to staining *C. albicans* with
Calcofluor White, the green and red fluorescence of FUN1 was also applied. In this system, broad green accumulates in the cytoplasm and red particles transfer and concentrate in the vacuoles in cytoplasm indicating metabolic activity. The red pigments appeared concentrated in vacuoles indicated the mycelial form of *C. albicans* were alive although they were surrounded by *S. salivarius* K12. These results suggest *C. albicans* were not killed by *S. salivarius* K12, but form some interaction with each other (Fig.5). This interaction appears to occur preferentially for mycelial form of *C. albicans* than the yeast form.

**No susceptibility of Streptococcus salivarius K12 bacteriocins to Candida albicans**

**TIMM 1768**

To examine the possibility for *Streptococcus salivarius* K12 bacteriocins to interfere 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 that lack of bacteriocin inhibitory activity on the *Candida* strain a simultaneous antagonism test was also performed on the liquid RPMI media used in the other experiments.
described and again showed no activity with live or heat-killed supernatants to C. albicans (Data not shown).

Effect of treatment with oral S. salivarius K12 on the oral candidasis model

The effects of S. salivarius K12 on murine oral candidiasis was examined. S. salivarius K12 was orally administrated to the mice 24 and 3 hours before and 3, 24 and 27 hours 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 x 10^8 CFU/ml of S. salivarius K12 (score=3.4±0.79, n=7) resulted in no significant difference in symptom score from control saline group (score=3.3±0.88, n=15), oral administration of 1.5 x 10^9 CFU/ml and 3 x 10^9 CFU/ml of S. salivarius K12 (score=2.0±0.74, n=12 and score=2.3±0.62, n=15 each) indicated obviously significant difference from control saline group. (P<0.01, Fig. 6)

There on the tongues of mice administered S. salivarius K12 there were less lesions observed than compared to the tongues of the control mice (Fig. 7A and B). 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 x 10^9 CFU/ml), there was
a significant decrease in fungal burden compared to the untreated control. The improved pathogenicity of *C. albicans* cells when mice were given *S. salivarius* K12 was illustrated by the histopathology of tongue sections (Fig. 8A and B). There were less PAS-staining mycelial elements invading the oral epithelium of tongues treated with *S. salivarius* K12 than with those in the control group.
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. And we also report that *S. salivarius* K12 had protective effect against *Candida* invasion indicated by the results of in vivo experimental model of oral candidiasis.

The results of in vitro culture experiments showed that *S. salivarius* K12 bound *Candida* cells at the both stage of germ tube formation and mycelial expansion. When *S. salivarius* K12 was preliminarily aerobically cultured in a low ionic strength medium with properties like saliva, absorption at 620nm increased, the binding effect to *Candida* cells was enhanced and 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 mycelial form of *Candida* cells and appeared to be surrounded by the *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 of FUN1 for viability check and Calcofluor White
for yeast body analysis was undertaken and it was confirmed that Candida cells surrounded by S. salivarius. Interestingly, these were almost of all mycelial form and the stain indicated that they were viable. These findings were agreement with the results observed in Fig. 2, i.e. increased in the number of planktonic Candida cells able to form colonies by the presence of S. salivarius K12 in medium. This interaction was kinetically observed from 30 min to 6 hours and the results showed more than 30 min needed for adhesion and one hour was enough for interaction. (Data not shown)

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

The protective or therapeutic efficacy for oral candidiasis was evaluated as the multiple possible probiotic mechanisms were thought to be involved, not only the fungicidal effect or the inhibitory effect of germ tube formation but also blocking the attachment of mycelium to the host epithelial cells. Additionally, the reduction of C. albicans’
attachment to the artificial dentition or acrylic resin was 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 show that adhesion of *C. albicans* germ tubes to polystyrene is decreased by saliva whereas *C. albicans* yeast cells 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 oral cavity resulting in the fungus then travelling harmlessly through to the esophagus and beyond. This was an interesting result in that the previous studies against bacteria had indicated that antimicrobial activity of *S. salivarius* K12 was resulted from its bacteriocin production since the percolation liquid in the culture including bacteriocins from *S. salivarius* K12 did not appear to affect the *C. albicans* when tested here as shown in Table1.

This is the first report that the direct interaction between bacteria and *Candida* inducing 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 focussed upon direct antimicrobial interactions or through chemical mechanisms such as quorum-sensing molecules or terpenoids (10, 17, 18). Despite the abundance of
bacterial–fungal 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 as polymicrobial composition and may include bacteria and fungi. These complex microbial consortia are also usually structured into biofilms which increase 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 deliterious than by a monoculture alone, such as S. mutans and Candida which have been reported to produce a mixed-biofilm formation and to make candidiasis more severe (20).

While there are various reports on the antibacterial activities of S. salivarius K12 and other strains in the literature with bacteriocin action (28, 30, 31), Candida albicans was not directly inhibited by bacteriocin action and it appears that yeast to bacterial cell contact maybe required. In this study, S. salivarius K12 directly interacted with Candida 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. salivarius or to the species in general, however this
strain has a safe history of use and a human clinical study is warranted. This data obtained in this study suggests that the use of *S. salivarius* K12 as an oral probiotic may have merit for its use in the prevention or treatment of oral candidasis and warrants further clinical investigations. And the mechanisms of therapeutic effect of *S. salivarius* K12 against oral candidiasis will be studied in future experiment in detail.
Acknowledgments

One of contributing authors Dr. Burton was a member of the finders of *S. salivarius* K12 and made the company BLIS Technologies Ltd. (BLIS means *S. salivarius* bacteriocin-like inhibitory substances) in Innovation Center of Otago University. And Mr. Mastushita is a member of acting branch of BLIS Technologies Ltd. in Japan.
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Legend of Figures

Figure 1 Inhibitory effect of BLIS K12 for germ tube formation of \textit{C. albicans} cultured with different dose of \textit{S. salivarius} K12 for 3 hours at 37°C in 5% CO$_2$ in air. Starting concentrations of \textit{Streptococcus salivarius} K12 freeze-dried material: a; control, b; 0.12 mg/ml, c; 0.23 mg/ml, d; 0.47 mg/ml, e; 0.94 mg/ml, f; 1.88 mg/ml, g; 3.75 mg/ml, h; 7.5 mg/ml, i; 15 mg/ml, j; 30 mg/ml (1.2 \times 10^7$–$3 \times 10^9$ CFU/ml).

Figure 2 Number of planktonic cells after 3h-culture of \textit{C. albicans} in various concentration of \textit{S. salivarius} K12. \textit{C. albicans} were cultured with different dose of \textit{S. salivarius} K12 for 3 hours at 37°C, in 5% CO$_2$ in air. After shaking the cultured plate, supernatant were collected, diluted and seeded on the GS agar for determining the number of planktonic cells. The experiments were performed in duplicate.

Figure 3 Microscopical observation of interaction between \textit{S. salivarius} K12 and \textit{Candida} planktonic cells after 3 hours culture and stained with lactophenol blue. Black arrows indicate \textit{Candida} cells and white arrows indicate \textit{S. salivarius}.

Figure 4 Inhibitory effect of \textit{S. salivarius} K12 for hyphal growth of \textit{C. albicans}. \textit{S. salivarius} K12 was preliminary cultured for 1 hour or 6 hours, and after cultured with \textit{C. albicans} for 3 hours, stained with crystal violet and detected the optical absorbance at 620nm. The experiments were performed in triplicate.
**Figure 5** Vital staining of *C. albicans* by FUN1 and Calcofluor White M2R. *C. albicans* were cultured with or without *S. salivarius* K12 at 30°C or 37°C for 3 hours. (a; hyphal form of control culture at 37°C, b; hyphal form culture at 37°C with *S. salivarius* K12, c; yeast form of control culture at 30°C, d; yeast form culture with *S. salivarius* K12 at 30°C, e; *S. salivarius* K12.)

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

**Figure 7** Typical images of tongues from mice inoculated with *C. albicans* TIMM1768. A; control, B; *S. salivarius* K12 (30mg/ml; $3 \times 10^9$ CFU/ml), C; Fluconazole (2mg/ml).

**Figure 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 (30mg/ml; 3×10⁹ CFU/ml).

Sections were stained with Periodic Acid-Schiff.

**Table 1** Detection of inhibitory properties of *S. salivarius K12* against test organisms using deferred antagonism testing.
Figure 1
Figure 4
Figure 6

A

Score of lesions

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B

Log_{10} CFU/mouse

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>K12(7.5)</th>
<th>K12(15)</th>
<th>K12(30)</th>
<th>Fluconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 7
Table 1 Detection of inhibitory properties of *S. salivarius* K12 against test organisms using deferred antagonism testing

<table>
<thead>
<tr>
<th>Test organism</th>
<th>K12 deferred antagonism</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em> TIMM 1768</td>
<td>-</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em> T-18</td>
<td>+++</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em> FF22, M-type 52, T-pattern 3/13</td>
<td>+++</td>
</tr>
<tr>
<td><em>Streptococcus anginosus</em> T-29</td>
<td>+++</td>
</tr>
<tr>
<td><em>Streptococcus uberis</em> ATCC 27958</td>
<td>+++</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em> 71-679, M-type 4, T-pattern 4</td>
<td>+++</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> T-21</td>
<td>+++</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em> 71-698, M-type 28</td>
<td>+++</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em> W-1, T-pattern 6</td>
<td>+++</td>
</tr>
<tr>
<td><em>Streptococcus agalactiae</em> T-148</td>
<td>+++</td>
</tr>
</tbody>
</table>

For deferred antagonism: - denotes 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.