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Original Article

Protection of Mice from Oral Candidiasis by Heat-killed *Enterococcus faecalis*, possibly through its Direct Binding to *Candida albicans*

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ABSTRACT

To develop a new therapy against oral candidiasis, a commensal microorganism, *Enterococcus faecalis* was tested for its ability to modulate *Candida* growth *in vitro* and its therapeutic activities against a murine model *in vivo*. Addition of heat-killed *E. faecalis* strain EF2001 (EF2001) isolated from healthy human feces to the culture of *C. albicans* strain TIMM1768 inhibited adherence of the latter to a microtiter plate in a dose dependent manner and *Candida* cells surrounded by EF2001 were increased. To examine the protective activities of EF2001 *in vivo*, heat-killed EF2001 was applied orally before and after inoculation of *Candida* to the tongue of mice previously immunosuppressed. Two days after inoculation this inoculation, both the symptom score and CFU from swabbed-tongue were significantly reduced in the EF2001-treated animals. Histological analysis indicated that EF2001 may potentiate the accumulation of polymorphnuclear cells near a *Candida*-infected region. These results suggest that oral administration of EF2001 has protective activity against oral candidiasis and that the *in vivo* activity may be reflected by direct interaction between EF2001 and *Candida* cells *in vitro* and the potentiation of an immunostimulatory effect of EF2001.

Key words: *Candida albicans*, murine oral candidiasis, *Enterococcus faecalis*, probiotic, cross-kingdom interaction

Introduction

The opportunistic pathogen of *C. albicans*, which is one of the oral microbiota in a healthy human, may cause pathogenic symptoms such as a variety of mucosal infections in the gastrointestinal, respiratory and genital tract, and is a major cause of oral and esophageal fungal infections. This candidiasis is common in immunocompromised individuals such as the aged, patients undergoing medical treatment, or patients with advanced AIDS, fungal infection which has become a more serious clinical problem[1, 2].

Recently the cross-kingdom interactions between *Candida* and bacteria were described[3, 4]. These bacterial-fungal interactions in nature and the clinical environment affect host health and disease[3]. The interactions between *Candida* and gram-positive bacteria such as *Streptococcus*[5−8], *Staphylococcus*[7], *Lactobacillus*[9], gram-negative bacteria such as *Pseudomonas*[10], *Acinetobacter*[11], *Bukholderia cenocepacia*[12], *Escherichia coli*[13], *Salmonella enterica*[14], or yeasts[15] were reported and these phenomena were explained by direct or indirect mechanisms. The effects of the interaction can be divided into two categories instead of the impairment of host health and the probiotic effect. More recently, we reported the probiotic effect of *Streptococcus salivarius*, one of commensal oral bacteria, for experimental oral candidiasis[8], and have con-

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tinued to research the natural development of this condition.

Enterococcus faecalis is a gram-positive organism and commensal in the gastrointestinal tract, which was reported to have immuno-stimulatory or -regulatory activities resulting in an effect on host health. It was reported to have a stimulating effect of leukocyte reconstitution in cyclophosphamide-treated animals[16], and to have a prophylactic effect against experimental candidiasis in mice[17]. But this organism changes from commensal to pathogenic with its translocation from the gastrointestinal tract to the bloodstream potentially causing life-threatening infections, such as bacterial endocarditis or other systemic infection related to septicemia[18, 19].

Heat-killed *Enterococcus faecalis* derived from healthy human feces was reported to have a radiation protection effect and antitumor activity despite having been heat-killed[20, 21]. In this paper, we report that the direct contact between *E. faecalis* and *Candida* leads to inhibition of the attachment of *Candida* cells to substratum, and oral administration of this preparation has shown a therapeutic effect on an oral candidiasis model of mice. These results highlight the ability of cross-kingdom interactions to modulate host health.

Materials and Methods

Candida albicans and *Enterococcus faecalis*

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 previously been used for animal experiments[8, 22, 23]. Cultures were stored at −80℃ in Sabouraud dextrose broth (Becton Dickinson, MD, USA) containing 0.5% yeast extract (Becton Dickinson) and 10% glycerol (v/v, final concentration) until use. Strain TIMM1768 was cultured on a Sabouraud dextrose agar plate for 18h at 37℃, and the cells were harvested with a micro spatula and suspended in diluted RPMI1640 (1:3; Sigma Chemical Co., St. Louis, MO, USA) containing 0.8% heat-inactivated fetal calf serum, 20mM HEPES buffer, pH7.2, 2mM urea, and 10mg/ml D-glucose with antibiotics (60μg/ml of benzyl penicillin potassium (Wako), and Kanamycin sulfate (Wako)). The cultured *C. albicans* cells were used for *in vitro* germ tube formation, a mycelial growth experiment, and also *in vivo* oral inoculation of *Candida*.

Enterococcus faecalis EF2001 is a commercially available probiotic (Nihon BRM CO. LTD., Japan) that was originally isolated from healthy human feces. It was supplied as a heat-killed and dried powder. One gram of dried EF2001 was comparable to 7.5×10^8 CFU of cells prior to being heat-killed.

In vitro assay of germ tube formation and mycelial growth of *C. albicans*

The ability of *C. albicans* cells to undergo germ tube formation or mycelial growth with *E. faecalis* was assessed as described below. (a) Germ tube formation analysis: An aliquot of 100 μl of *C. albicans* cells was put into 96-well microtiter plates (1×10^4 CFU per well for morphological analysis and 5×10^5 CFU per well for crystal violet (CV) -staining); 100 μl serial dilutions of heat-killed and freeze-dried *E. faecalis* powder were then added to the plates to make up a final concentration of 30mg/ml to 0.12mg/ml, and the plates were incubated at 37℃ in 5% CO_2 in air for 3h. Germ tube formation was assessed microscopically: cells were fixed with 70% ethanol and stained with CV as described in the next section according to the previous report[22, 23]; (b) Mycelial growth analysis: This was carried out as described for the germ tube formation assay, except that the inoculums per well was 500 cells in 100 μl and the culture period was lengthened to 16h. Mycelial growth of *C. albicans* cells was determined as described previously[24]. Culture medium for *in vitro* assays was composed of diluted RPMI1640 (1:3; Sigma Chemical Co., St. Louis, MO, USA) containing 0.8% fetal calf serum, 20mM HEPES buffer, pH7.2, 2mM urea, and 10mg/ml D-glucose with antibiotics (60μg/ml of benzyl penicillin potassium (Wako), and Kanamycin sulfate (Wako)).

Assay of crystal violet (CV) -staining

Crystal violet (CV) -staining was performed. One hundred micro-litter of *C. albicans* suspension (for mycelial growth; 500CFU in 100 μl, for germ tube formation; 1×10^4 CFU in 100 μl) with or without heat-killed EF2001 in diluted RPMI1640 medium in 96-well microtiter plates were prepared. After incubation at 37℃ for 3 or 16h, the medium in the wells was discarded by inverting the microtiter plates. The *Candida* cells were sterilized and fixed by immersion of the plate in 70% ethanol for

2min and then the planktonic cells were washed out twice by immersing them in distilled water and the water discarded by flicking the plate. The mycelia (or germ tubes) attached to the bottom of the wells were stained by 100 μl of 0.02% CV in PBS for 20min. They were washed 3 times with water, once with 0.0625% sodium dodecyl sulfate (SDS) and twice more with water. After drying the microtiter plates, 150 μl of isopropanol containing 0.04M HCl and 50 μl of 0.25% SDS were added to the wells and mixed by a plate mixer for 2min in order to extract CV from the mycelia. The absorbance at 620nm of quintuplicate samples was measured photometrically.

Yeast viability assay using fluorescence microscopy

The effect of EF2001 on *C. albicans* viability was evaluated by a two-color fluorescent probe (FUN1; F-7030; Molecular Probes, Eugene, OR, USA), a live/dead yeast viability kit, and fungal surface labeling with a reagent of a third color (Calcofluor white M2R; Molecular Probes, Eugene, OR, USA). *C. albicans* cells were cultured with heat-killed *E. faecalis* as described above in adequate culture medium and cultured for 3h in a CO_2 incubator. After centrifugation at 3,000rpm for 3 min and one washing with GH solution (2% glucose in 10mM HEPES buffer, pH7.2), the GH solution was replaced with GH solution containing 20 μM FUN1 and 5 μM Calcofluor white M2R. After incubation for 30 min at room temperature, cells were observed with a fluorescent microscope (BH50, Olympus, Japan) equipped with an assortment of filters: WU (wide range of UV excitation), WBV (wide range of blue-violet excitation), WG (wide range of green excitation), and NB (narrow range of UV excitation). Staining of FUN1 was observed using NB and Calcofluor white WU. All images were taken as digital data with a DC200 camera (Leica, Germany), and 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[24, 25]. Six-week-old female ICR mice (Charles River Japan, Inc., 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 12h of light and 12h of darkness daily, and the environmental temperature was maintained at 23℃. To induce an immunosuppressed condition, 100mg of prednisolone (Mitaka Pharmaceutical Co., Japan) per kg of body weight was injected subcutaneously to mice 20 to 24h before oral inoculation. Prior to this administration, 15mg/ml of tetracycline hydrochloride (Takeda Shering Purau Animal Health Co., Japan) was administered in drinking water during a 24h period. On the day of infection, animals were sedated by intramuscular injection in the femoral muscle with 14.4mg/kg of chlorpromazine chloride, after which they were orally inoculated with about 2×10^8 CFU/ml of *C. albicans* TIMM1768 in diluted RPMI1640 medium. Oral inoculation was performed by 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 about 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 previously[26].

Oral administration of *E. faecalis* (EF2001)

Fifty microliters of heat-killed EF2001 solution (5–30mg/ml), fluconazole (2mg/ml), or saline was administered in the oral cavity of the Candida-inoculated mice at five time points: 24 and 3h before and 3, 24, and 27h after *C. albicans* inoculation. The total number of mice in each group during three different trials was as follows: saline control, n = 21; EF2001 at 15mg/ml, n = 11; at 30mg/ml, n = 12; and fluconazole at 2mg/ml, n = 6. The additional number of mice used to study the precise effect of EF2001 which separated the precautionary or curing effects, was as follows: EF2001 at 15mg/ml (EF2001 was administered only 24 and 3h before inoculation and saline at the other three time points), n = 11; at 15mg/ml (EF2001 was administered only 3, 24 and 27h after inoculation and saline at the other two time points), n = 12. 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 (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[8, 23, 26]. Briefly, forty-eight hours after inoculation, mice were sacrificed by cervical dislocation and the white patches of candidiasis lesions on the tongues were evaluated by scoring as follows : 0, normal; 1, white patches on < 20% of the tongue; 2, white patches on 21 -90% of the tongue; 3, white patches on > 90% of the tongue; 4, thick white pseudomembranous like patches > 90% of the tongue.

Evaluation of the number of viable *Candida* cells on murine tongues

At 48h 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 3ml 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 microliters of each dilution was incubated on a *Candida* GS agar plate (selection medium for *Candida*; Eiken Chemical Co., Ltd., Japan) for 20h at 37℃. The number of *Candida* colonies was counted, and the total number per swab was calculated and reported as number of CFU.

Histology

For histological study, the tongues were resected at the base, fixed with phosphate-buffered 4% paraformaldehyde solution (pH7. 4) at 4℃, dehydrated by ethanol series, and embedded in paraffin in accordance with common procedure. Specimens were sectioned to an 8 μm thickness along the longitudinal centerline. Sections on the slide were deparaffinized by xylene, rehydrated by ethanol series and stained with Periodic Acid-Schiff (PAS).

Statistical analysis

Statistical analysis was performed using One way Anova with post-hoc test and Bonferroni correction. P values of < 0.05 were considered statistically significant.

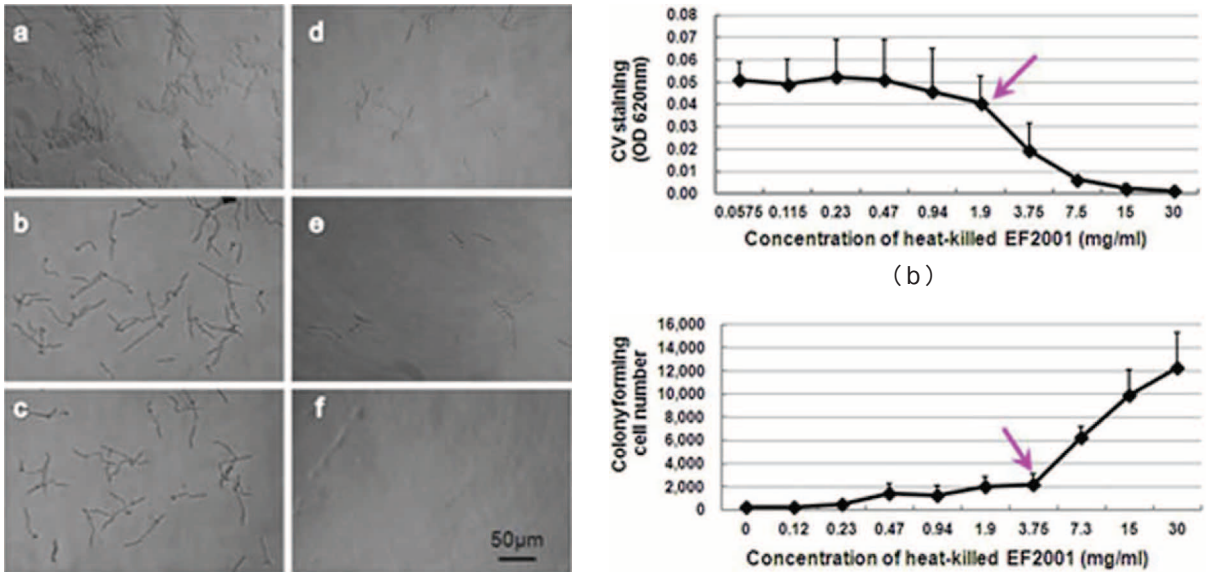
Results

Heat-killed *E. faecalis* EF2001 (EF2001) inhibited the attachment of *Candida albicans* to plastic substratum

Pathogenesis of mucosal candidiasis is considered to be due to the mycelial growth of *C. albicans*. The first step in making mycelia is germ tube formation followed by an increase of adherent capacity by hydrophobicity. We investigated the *in vitro* effects of EF2001 on the germ tube-like early hyphal formation of *C. albicans*. Figure 1Aa shows that *C. albicans* cells cultured in the control culture medium formed germ tube-like hyphae within 3h. In the experimental group where *C. albicans* was cultured in the presence of EF2001 (Fig.1A b-f) the morphological shape and size of the cells appeared almost the same as in the control experiment (Fig. 1Aa). The adherence of the mycelial form to the plastic substratum was, however, weaker and the mycelial number on the plastic bottom was dose-dependently reduced in the presence of more than 1.9mg/ml of EF2001 (Fig. 1 Ab), and the results of CV stained cells were reduced more than 1. 9mg/ml of EF2001 (Fig. 1B arrow). These results indicate that EF2001 increased the number of planktonic *Candida* cells in culture medium. The planktonic cells including unattached mycelia in the medium were centrifuged and the number of viable *C. albicans* cells was determined by colony forming cell assay (Fig. 1C). This viable number growing in planktonic form was found to increase, according to the concentration of EF2001, to more than 3.75mg/ml (Fig. 1C arrow).

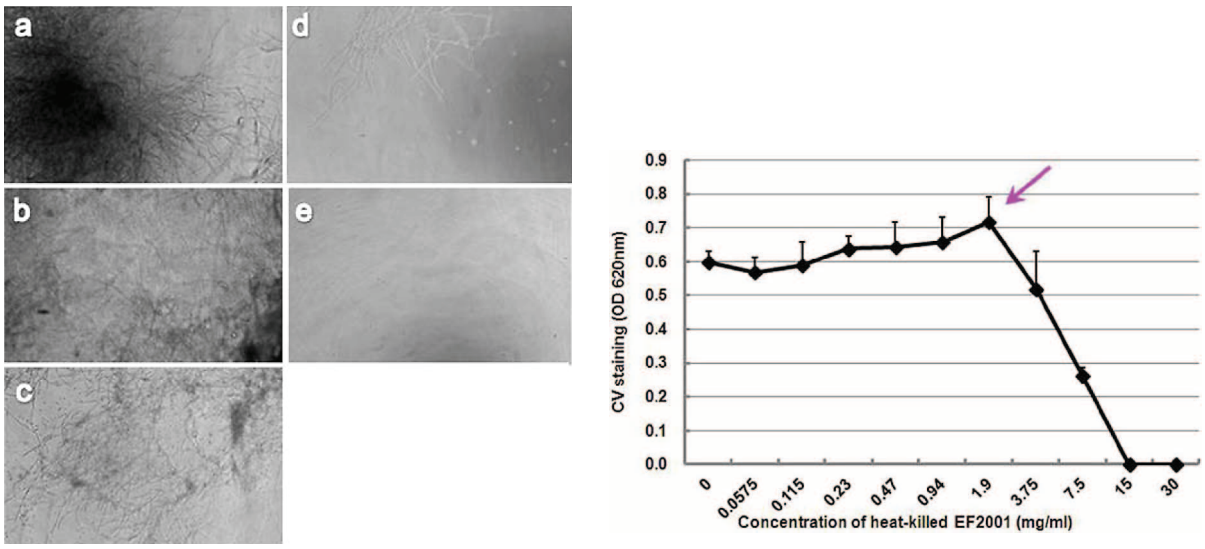
Heat-killed EF2001 effectively inhibited *C. albicans* attachment to substratum during overnight incubation

Although EF2001 was shown to bind to early mycelia of *C. albicans* at 3h culture and to inhibit the attachment of the fungi to plastic microtiter plates, it is not clear whether these effects continue for longer periods of culture with *Candida*. Mycelial growth of *C. albicans* for 16h culture was quantified using the crystal violet-staining method[24]. As shown in Fig. 2Ae, when



(a) (b) (c)

Fig. 1. Inhibitory effect of heat-killed EF2001 on early hyphal formation of *C. albicans*. *C. albicans* was cultured with different doses of EF2001 for 3h at 37℃ in 5% CO_2 in air. A; Microscopical observation of *Candida* cells attached to a microtiter plate. (a; 0, b; 1.9, c; 3.8, d; 7.5, e; 15, f; 30mg/m*l* of EF2001) B; Dose of EF2001 dependent decrease of plastic adherent *Candida* cells stained with crystal violet. C; Viable and colony forming *C. albicans* of planktonic cells in the medium after 3h culture with EF2001.



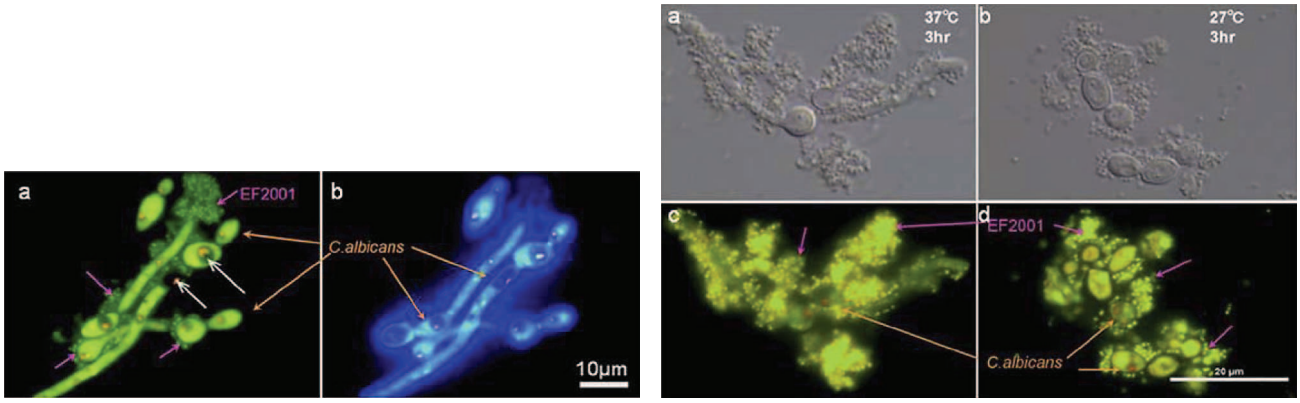
(a) (b)

Fig. 2. Inhibitory effect of heat-killed EF2001 on mycelial growth of *C. albicans*. *C. albicans* was cultured with different doses of EF2001 for 16h at 37℃ in 5% CO_2 in air. A; Microscopical observation of *Candida* cells attached to a microtiter plate. (a; 0, b; 3.8, c; 7.5, d; 15, e; 30mg/m*l* of EF2001) B; ; Dose of EF2001 dependent decrease of plastic adherent *Candida* cells stained with crystal violet.

EF2001 existed at 30mg/m*l*, there were no *Candida* hyphae attached to the plastic microtiter plate and at 15mg/m*l* the rest of the hyphae attached to the microtiter plate were few in number (Fig. 2Ad). CV-staining of these remaining hyphae showed the decreased number was dependent on the increased amount of EF2001 (Fig. 2B).

EF2001 bind to both hyphal and yeast form of *C. albicans*

Earlier experiments indicated that EF2001 in-



(a) (b)

Fig. 3. Vital staining of *C. albicans* by FUN1. A; *C. albicans* was cultured with EF2001 at 37℃ for 3h and stained by FUN1 (a) and Calcofluor White M2R (b). Hyphal forms of *C. albicans* (stained with FUN1 and Calcofluor White) were surrounded by small green particles of EF2001 (stained with only FUN1). White arrows indicates the accumulated red crystals of FUN1. B; *C. albicans* was cultured with EF2001 at 37℃ (a, c) or 27℃ (b, d) for 3h, stained by FUN1 and observed by differential interference contrast microscope (a, b) and fluorescent microscope (c, d). Both hyphal and yeast forms of *C. albicans* were surrounded by EF2001 and were viable.

hibited *C. albicans* mycelial adhesion to a plastic microtiter plate and that there were possible interactions between these two types of cell; these were further investigated using staining techniques. *C. albicans* was cultured on PLL-coated cover glass with or without EF2001 for 3h, then stained by FUN1 to determine its viability by evaluating the metabolic activity. These cells were also stained with Calcofluor White to identify the cell wall of *Candida* which is composed of chitin. FUN 1 staining showed the hyphae were surrounded by numerous small green particles (Fig. 3Aa). Since these particles were not stained with Calcofluor White (not being composed of chitin, Fig. 3Ab), they were bacterial bodies of EF2001. Concurrent with the staining of *C. albicans* with Calcofluor White, the green and red fluorescence of FUN1 was also applied. In this system, broad green accumulate in the cytoplasm and red particles transferred and concentrated in the vacuoles in the cytoplasm indicating metabolic activity. The red pigments appeared concentrated (Fig. 3A, white arrows) in vacuoles, indicating that the mycelial forms of *C. albicans* were alive although they were surrounded by EF2001. *C. albicans* is ableto differentiate its morphology according to the environmental conditions; so that the same lot of *C. albicans* were divided into two different cultures at 27℃ and 37℃ including polylysine coated glass cover slips. The results of 37℃-3h culture showed the hyphal growth of *Candida* (Fig. 3Ba, c), but 27℃-3h culture showed its yeast form growth (Fig. 3b, d). The hyphal form and the yeast form of *C. albicans* were surrounded by EF2001 and alive (Fig. 3B). These results suggest *C. albicans* was not killed by heat-killed EF2001, but that the two had some form of interaction. This interaction appeared to occur with both the mycelial and yeast form of *C. albicans*.

Protection of mice from oral candidiasis by treatment with heat-killed EF2001

The effects of EF2001 on murine oral candidiasis were examined. EF2001 was orally administered to the mice 24 and 3h before and 3, 24 and 27h after *Candida* infection. The tongues of those treated mice (Fig. 4B-E) showed fewer lesions than the tongues of control mice (Fig.4A). There was also a significant decrease in fungal burden for the mice given 15mg/m*l* of EF2001 (Fig. 4C), although this was not observed to be a complete cure, in contrast to that observed with the chemotherapeutic agent, fluconazole (Fig. 4F). Figure 5A shows that EF2001 application caused a dose-dependent improvement in symptom score and fungal burden. Oral administration of 750 μg/50 μ*l* (15mg/m*l*) and 1,500 μg/50 μ*l* (30mg/m*l*) of EF2001 (symptom score = 2.3 ± 0.62, n = 11; symptom score = 1.9 ± 0.67, n = 12 each) indicated an obviously significant difference from the control saline group (score = 3.5 ± 0.8, n = 21, $P < 0.01$, Fig. 5A). And the viable cell number from the

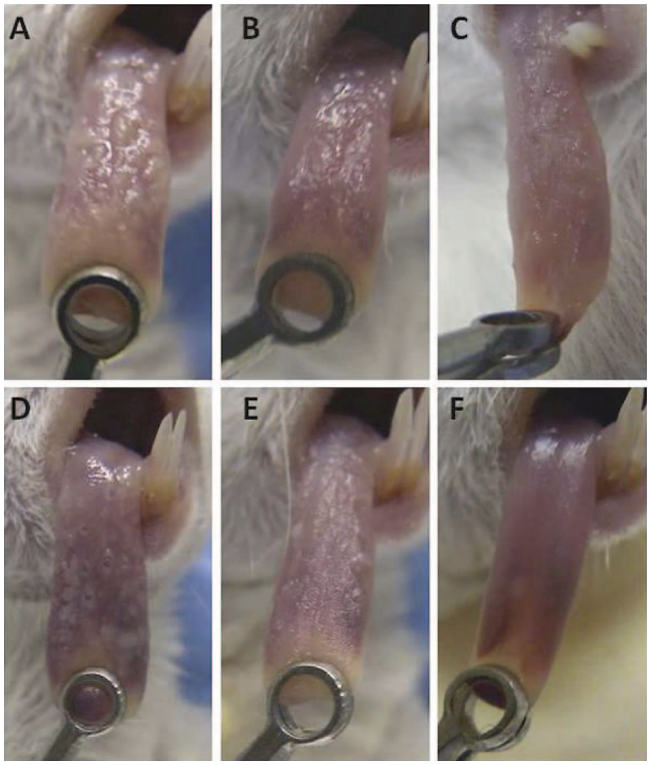


Fig. 4. Typical images of tongues from mice inoculated with *C. albicans* TIMM1768. A; 0mg/m*l*, B; 5mg/m*l*, C; 15mg/m*l* of EF2001 treated 5 times before and after inoculation of *Candida*, D; 15mg/m*l* of EF2001 treated only before inoculation of *Candida*, E; 15mg/m*l* of EF2001 treated only after inoculation of *Candida*, F; Fluconazole (2mg/m*l*) treated 5 times before and after inoculation of *Candida*.

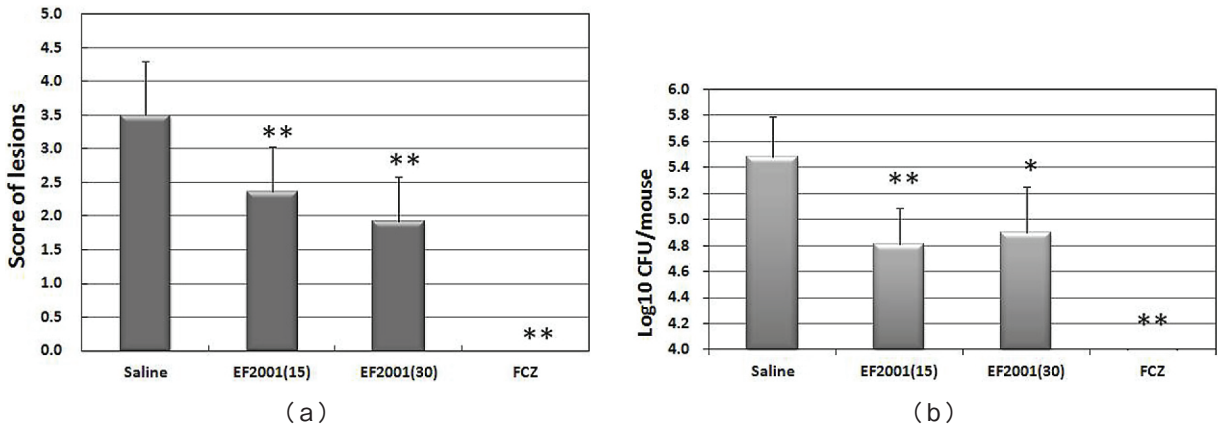


Fig. 5. Effect of EF2001 on the (A) symptom score and (B) fungal burden in the murine model of oral candidiasis. Groups of immunosuppressed mice (control n = 21; EF2001 15mg/m*l* n = 11; 30mg/m*l* n = 12; Fluconazole 2mg/m*l* n = 6) were inoculated with *C. albicans* TIMM1768, and EF2001 was administered as described in Methods. Symptom scores (A) and fungal burden (B) were assessed after 48h as described in Methods. ** and * denote significant differences ($P < 0.01$ and $P < 0.05$ each) with no EF2001 control, as determined using One way Anova with post-hoc test.

tongues administered 750 μg/50 μ*l* (15mg/m*l*) and 1,500 μg/50 μ*l* (30mg/m*l*) of EF2001 (log10CFU = 4.81 ± 0.28, n = 11 and log10CFU = 4.90 ± 0.35, n = 8 each) indicated a statistically significant drop in both doses in comparison with control (log10CFU = 5.48 ± 0.31, n = 21, $P < 0.01$, $P < 0.05$ each, Fig. 5B).

A further question regarding the therapeutic effect of EF2001 on candidiasis was whether it has a prophylactic or a curative effect. To clarify this

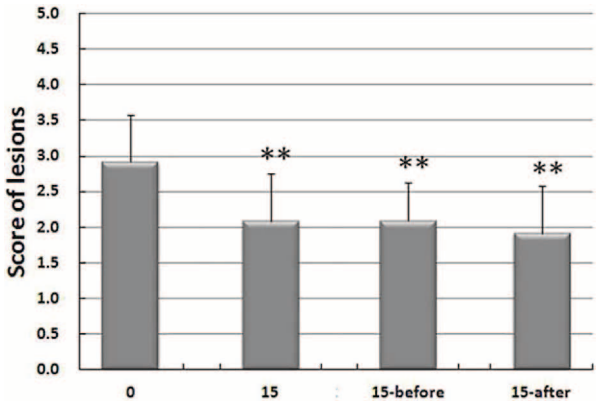


Fig. 6. Comparison between precautional and curative effects of EF2001 on candidiasis. Fifteen mg/m*l* EF2001 was administered in the oral cavity of the *Candida*-inoculated mice five times, 15mg/m*l* was administered in the oral cavity two times before *Candida*-inoculation (indicated as 15-before), or 15mg/m*l* was administered in the oral cavity three times after *Candida*-inoculation (indicated as 15 after). ** denotes significant differences ($P < 0.01$) with no EF2001 control, as determined using One way Anova with post-hoc test.

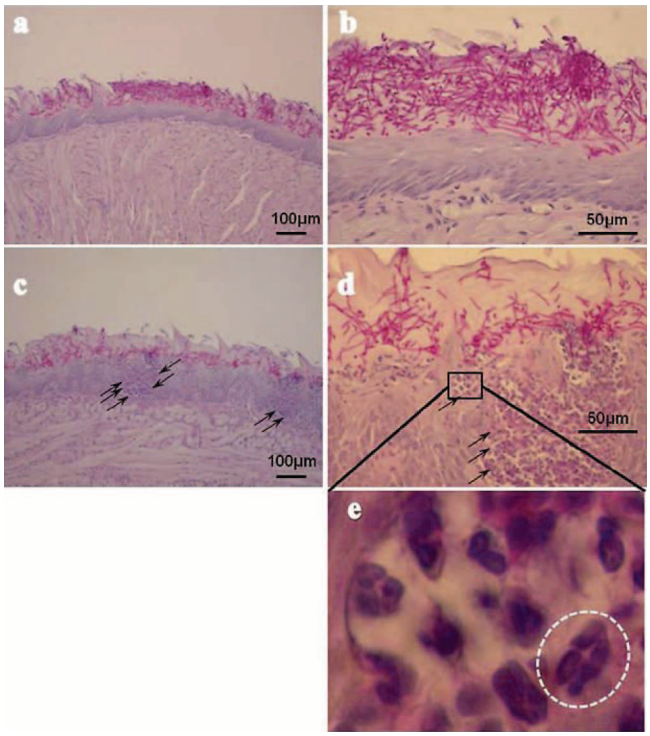


Fig. 7. Histology of longitudinal formalin-fixed-paraffin-embedded (FFPE) sections of mouse tongues inoculated with TIMM1768. a, b; representative control mouse, c-e; representative mouse given EF2001 (15mg/m*l* 5 times). Sections were stained with Periodic Acid-Schiff. Highly magnified images (b, d and e). The infiltrated cells (arrows) into the dorsal epithelium were magnified and circled by a white dotted line (e).

we conducted an additional *in vivo* experiment including two groups of mice orally administered EF2001 (15mg/m*l*); one group was only administered this at the points 24 and 3h before inoculation of *Candida* (Fig. 4D and Fig. 6, 15-before), and the other group only administered 3, 24 and 27h after inoculation (Fig. 4E and Fig. 6, 15-after). The results of the symptom score in Fig. 6 indicate there was a therapeutic effect of EF2001 both before and after the *Candida* inoculation within this short 3-day experiment.

The reduced pathogenicity of *C. albicans* cells when mice were given EF2001 was illustrated by the histopathology of tongue sections (Fig. 7). There were fewer PAS-stained mycelial elements invading the oral epithelium of tongues treated with EF2001 (Fig. 7c, d) than invading control tongues (Fig. 7a, b) although some mycelia remained between filiform papilla. The *Candida* cells were removed to some extent, but histological features indicated intense immunological inflammation by lymphoid cells which infiltrated the

dorsal epithelium when EF2001 was administered. These infiltrating cells (Fig. 7c-d, arrows) were magnified and are circled by a white dotted line (Fig. 7e); they were counter-stained by HE and have a polymorphic nuclear shape indicating polymorphnuclear cell infiltration.

Discussion

Here we report a therapeutic effect of EF2001 on mucosal candidiasis according to cross-kingdom interaction involving the direct binding between *Enterococcus* and *Candida* cells.

The *in vitro* culture experiments showed that EF2001 bound directly to hyphae, pseudohyphae, or the yeast form of *Candida* cells and inhibited the adherence of *Candida* to plastic plates. This direct binding of EF2001 to *Candida* cells was shown at both the stage of *Candida* growth, which is germ tube formation (3h culture in this paper) and mycelial expansion (16h culture in this paper), and also binding to the yeast form of *Candida*. The number of planktonic cells recovered from *Candida* culture with heat-killed EF2001 and proportionally increased according to the concentration of EF2001 in the medium. The planktonic cells were composed of the mycelial form of the *Candida* cells and appeared to be surrounded by EF2001 (data not shown). EF2001 was heat-killed and a dried powder, meaning that it was quite unlikely to have metabolic products which could affect the *Candida* cells. These findings suggest that the surface structure of *Enterococcus* cells and *Candida* cells may be involved in the binding. Direct cross-kingdom interaction between *Candida* and the oral bacteria *Streptococcus salivarius* K12 was studied recently[8]; such direct contact between these cells was needed to inhibit oral candidiasis, but, in contrast to this current work, living *Streptococcus salivarius* K12 was used and the bacteria did not induce host immune reaction as a tolerant bacteria against a host immune system[27].

One of the adhesion molecules of *Enterococcus faecalis*, ACE (adhesion to collagen of *E. faecalis*) has been reported[28], but whether ACE mediates these adhesive interactions between EF2001 and *Candida* cells is not known. At the same time, *Candida* hyphae express ALS (agglutinin-like sequence) family proteins, and *Streptococcus gordonii* cells bound to *C. albicans* hyphae in one of this protein family members in an Als3-dependent manner[29]. The original function of ALS proteins of *Candida* might be adherence to the host epithelial cells but the example of *S. gordonii* binding *Candida* cells suggested the possibility of ALS to help bacferial-fungal adherence. And there are simple charge effects or binding through presumed lectin-like substance remained to be elucidated for bacterial-fungal interaction. We do not believe there is a possibility of EF2001 binding to *Candida* in an ALS-dependent manner at this time. The molecular mechanisms of binding remain to be clarified.

Bacterial-fungal interactions have been reported in the natural and/or clinical environment and do affect host health conditions[3, 30]. The interactions between *Candida* and gram-positive or gram-negative bacteria have been reported as we previously stated in this paper. *Enterococcus faecalis* is one of the commensal bacteria from the gut, but depending on whether or not it is in an immune-compromised host it changes from commensal to pathogenic and induces nosocomial infections resulting in high mortality[18, 19]. We determined whether the effect of EF2001 was deteriorative or recuperative for candidiasis using *in vivo* experiments of a mouse model. Recovery from experimental murine oral candidiasis was achieved with oral treatment by EF2001; the symptom scores of tongue and CFU of tongue-surface swabbing decreased significantly. This recuperative effect could not be analyzed as to whether it was prophylactic or therapeutic in the two experimental procedures, nor could the EF2001 treatment administered prior to *Candida* inoculation or afterward; with both procedures the symptoms of oral candidiasis were inhibited to much the same degree.

Candida infection and virulence are attributed to the attachment of *Candida* to mucosal surfaces. On this occasion morphological change from yeast-to-hypha occurs (tissue penetration is more easily achieved by fungi growing in the form of hyphae as opposed to yeast) and there is invasion into deeper tissue, however, the precise mechanisms during this mucosal infection are not clear. Once *Candida* penetrates the tissue, it secretes proteinases and phospholipases causing damage to the host tissue, destroying immunoglobulins, binding complement-proteins and avoiding destruction by the host immune system. Removing *Candida* hyphae before deep penetration to host tissue may be an efficient and benign

way to prevent candidiasis. An increasing number of research papers have reported the protective action of probiotic bacteria against *Candida* infections, but the exact mechanism remains unclear[31].

In this paper EF2001 enhanced the host defense system as shown by the histochemical analysis of inoculated *Candida* and EF2001 treated murine tongue. There was reduced *Candida* mycelium on the tongue surface and significant infiltration of polymorphnuclear cells into the dorsal epithelium, which is the first aid of the host defense systems under the epithelium, while the filamentous *Candida* had grown dramatically over the filiform papilla and suffusively in the control group of mice. No significant infiltration of lymphoid cells was observed, however, because of the immunosuppression by steroids which continued until 48h after their inoculation. In fact, EF2001 enhanced the TNF-α production of macrophage[32] and we used the same lot during all experiments of this paper which induced the production of a considerable degree of TNF-α of macrophage and is comparable to the amount induced by immunostimulant OK432 (personal communication from Ms. Kazumi Hayama). This TNF-α inducing effect of EF2001 agreed with the histochemical analysis and with various reports on the immunomodulating activities of EF2001 and other strains of *Enterococcus faecalis* in the literature[16, 20, 33].

Probiotic treatment such as that with EF2001 would be a useful tool for prophylaxis of fungal infection for an immunocompromised patient who was worried about the side effects of azole antifungal treatment. We hope that EF2001 could be developed as a successful example of the use of probiotics to influence the cross-kingdom interaction between bacteria and fungus which led to therapeutic effect in future.

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