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Inhibitory effects of *Lactobacillus rhamnosus* and *Lactobacillus casei* on *Candida* biofilm of denture surface

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ABSTRACT

Objective: Candida albicans biofilm is associated with denture-related stomatitis and oral candidiasis of elderly. Probiotics are beneficial bacteria and have antibacterial activity against pathogenic bacteria. The purpose of this study was to investigate the antifungal activity of various probiotics against *C. albicans* and the inhibitory effects of probiotics on *Candida* biofilm on the denture surface.
Design: The spent culture media of various probiotics were investigated the antifungal efficacy against *C. albicans*. *Candida* biofilm was formed on a denture base resin and was then treated with *Lactobacillus rhamnosus* and *Lactobacillus casei*. Also, the biofilms of *L. rhamnosus* and *L. casei* were formed and were sequentially treated with *C. albicans*. Colony-forming units of *C. albicans* on the denture surface were counted after spreading on agar plate. The denture base resin was treated with the spent culture media for 30 days, after which the denture surface roughness was analyzed with an atomic force microscope.
Results: L. rhamnosus and *L. casei* exhibited stronger antifungal activity than other probiotics. The spent culture medium of *L. rhamnosus* and *L. casei* exhibited the antifungal activity against blastoconidia and biofilm of *C. albicans*. *L. rhamnosus* and *L. casei* showed the antifungal activity against *Candida* biofilm, and the biofilm of *L. rhamnosus* and *L. casei* inhibited formation of *Candida* biofilm on denture surface. Neither of the probiotics affected the surface roughness of the denture base resin.
Conclusions: L. rhamnosus and L. casei may be the ideal probiotics for the prevention and treatment of denture-related stomatitis.

1. Introduction

Candida albicans is a diploid fungus that has characteristic of the yeast type and the hyphal type. (Gow, van de Veerdonk, Brown, & Netea, 2012) *C. albicans* is the cause of a fungal infection in the oral cavity. In elderly and in immunocompromised patients, oral candidiasis is a common problem (de Souza et al., 2009). Furthermore, Candida-associated denture stomatitis is the most frequent manifestation of oral candidiasis among denture wearers (Radovic et al., 2014). Infection of *C. albicans* is initiated by the formation of a biofilm, which is a community of heterogeneous fungal forms, such as hyphae, pseudohyphae, and blastoconidia, and their biofilm was embedded in an extracellular matrix containing polysaccharides, proteins and nucleic acids (Seneviratne, Jin, & Samaranayake, 2008). *C. albicans* biofilm on the denture surface has been also observed to coexist with blastoconidia, hyphae, and oral bacteria (Webb, Thomas, Willcox, Harty, & Knox, 1998). As compared with the blastoconidia, hyphal *C. albicans* in the biofilm expresses various infection-associated genes (Wilson et al., 2009) and penetrates the host tissue by means of hydrolytic extracellular enzymes and cell wall proteins (Gow et al., 2012; Tsang et al., 2007). Also, the fungus in the extracellular matrix-embedded biofilm are more resistant to antimicrobial agents due to the barrier function of extracellular matrix (Al-Fattani and Douglas, 2004; Douglas, 2003). Therefore, denture cleansers cannot totally remove the *Candida* biofilm from the denture surface. In addition, the misuse of such cleansers can result in gastric ulcers, allergic reactions, and burns (Ingram, Bosse, & Baldwin, 2008). Also, the long-term use of denture cleansers leads to physical change of denture as surface roughness and increase the formation of *Candida* biofilm (Izumida et al., 2014).

Probiotics are beneficial bacteria to host and have antimicrobial activity through producing bacteriocin (Gillor, Etzion, & Riley,

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2008; Hardy, Harris, Lyon, Beal, & Foey, 2013; Meurman and Stamatova, 2007). They also help to improve the immune system in immunocompromised patients (Ashraf and Shah, 2014; Mandal, Silva, & Franco, 2014). *Lactobacillus* species are representative probiotics that are widely used in the production of fermented dairy products. Furthermore, *Lactobacillus* spp. are known to play an potent role in the maintenance of human health by stimulating a natural immunity (Gill and Prasad, 2008) and have an inhibitory effect on the cariogenic biofilm (Lee and Kim, 2014). Also, some *Lactobacillus spp* showed antifungal activity against *Aspergillus fumigatus*, *Penicillium* spp and *Fusarium* spp (Gerbaldo, Barberis, Pascual, Dalcero, & Barberis, 2012; Ilavenil et al., 2015). Because of these characteristics, the current study investigated the antifungal activity of lactobacilli against *C. albicans* and their antifungal effect on *C. albicans* biofilm on denture base resins. We also evaluated the changes in surface roughness of the denture base resin as a result of long-term colonization by lactobacilli.

2. Materials and methods

2.1. Microbial strain and culture

C. albicans ATCC 10231 was used in this study and cultivated in tryptic soy broth (TSB) (BD Biosciences, San Jose, CA, USA). *Lactobacillus acidophilus* ATCC 4356, *L. casei* ATCC 334, *L. rhamnosus* GG (ATCC 53103), and *Bifidobacterium breve* ATCC 15700 were cultivated in de Man Rogosa Sharpe broth (MRS) (BD Biosciences), and *Streptococcus thermophilus* ATCC 19258 and *Streptococcus salivarius* K12 were cultured in brain heart infusion (BHI) (BD Biosciences) broth. All bacteria and fungi were cultured at 37 °C in aerobic conditions. Also, to generate *Candida* biofilm of hyphal type, *C. albicans* was cultivated in Ham's F-12 medium (HyClone, Logan, UT, USA) at 37 °C in a 5% CO_2 condition.

2.2. Antifungal activity of probiotics against C. albicans

The antifungal activity of various probiotics against *C. albicans* was examined with the spent culture medium of the probiotics according to Kirby-Bauer disk diffusion susceptibility test. The antifungal efficacy of the probiotics was analyzed and compared by measuring inhibition zone of *C. albicans* growth on agar plate. Also, minimum inhibitory concentration (MIC) of *L. casei* and *L. rhamnosus* against *C. albicans* was examined by a modified microplate method according to the methods of Clinical and Laboratory Standards Institute (CLSI). Briefly, 180 μl of fresh TSB was dispensed in each well of a 96-well polystyrene plate (SPL Life Sciences, Gyeonggi, Korea). After collecting the spent culture medium of each probiotic, 180 μl of this medium was added to the first row of the TSB-dispensed plate. Two-fold serial dilutions were performed with a multi-channel micropipette. The concentration of *C. albicans* was measured by Bacterial Counting chamber (Marienfeld-Superior, Lauda-Königshofen, Germany) and then diluted to 1×10^6 cells/ml with fresh TSB. The fungal suspensions (20 μl) were inoculated into each well of the prepared plate. The plates were incubated at 37 °C in an aerobic incubator for 24 h, and the optical density was then measured at a wavelength of 600 nm by a microplate reader (Biotek, Winooski, VT, USA).

2.3. Analysis of inhibitory mechanism of probiotics against C. albicans

In order to investigate *C. albicans* growth according to pH, the initial pH of TSB was adjusted with intervals of 0.5, from pH 7 to pH 3.5, by adding lactic acid (Sigma Aldrich, San Jose, CA, USA), and *C. albicans* was then inoculated into each tube containing TSB. The growth of *C. albicans* was measured by the optical density at a wavelength of 600 nm by a spectrophotometer. Also, the spent culture medium of *L. rhamnosus* and *L. casei* was treated with proteinase K (250 μg/ml) for 1 h at 50 °C to inactivate antifungal peptides, and then heated for 30 min 90 °C to inactivate proteinase K. The antifungal activity of the prepared spent culture medium was investigated.

2.4. Preparation of denture base resin

The specimens (Ø20 × 3 mm each) of polymethylmethacrylate (PMMA) material as denture base resin (Lucitone 199, Dentsply International, York, PA, USA) were prepared for formation of the *Candida* biofilm. The disk-shaped wax template was flasked with Type III dental stone (Snow Rock Dental Stone, DK Mungyo, Gimhae, Korea) to obtain a pattern for specimens. Acrylic resin specimens were processed according to the manufacturer's instructions. The flask was submerged in water at 74 °C for 90 min and at 99 °C for 30 min. After deflasking, PMMA disks were equivalent in size. Disks were placed in distilled water at room temperature until used.

2.5. Formation of Candida biofilm on denture base resin

The denture base resins were placed into a 12-well polystyrene plate (SPL LifeSciences), and 2 ml of Ham's F-12 medium was then dispensed. *C. albicans* was inoculated into the prepared 12-well plate, and the plates were incubated at 37 °C in a 5% CO_2 incubator for 72 h. The medium was changed every day by using fresh Ham's F-12 medium. After washing the denture base resins with phosphate buffered saline (PBS) (pH 7.2), *Candida* biofilm-formed denture was transferred to the 12-well plate, which included 2 ml of TSB in the presence or absence of *L. rhamnosus* and *L. casei* (1×10^7 cells/ml). The plates were then incubated at 37 °C for 12 h. The denture base resins were washed three times with PBS to remove non-adherent fungus and were subjected to mechanical disruption by a scraper, two-fold serial dilution, and inoculated onto a tryptic soy agar (TSA) plate to count *C. albicans* in the biofilm. The agar plates were incubated at 37 °C for 36 h aerobically, and the CFU were counted. Also, the formation of *Candida* biofilm was investigated when probiotics first colonized on denture base resin. *L. rhamnosus* and *L. casei* were formed biofilm on denture base resin using TSB. Thereafter, *C. albicans* was cultivated in Ham's F-12 medium with the probiotic biofilm-formed denture base resin. The denture base resins were washed three times with PBS, and the biofilm on the denture base resin was disrupted by a scraper. The bacterial suspension was diluted two-fold serially and inoculated onto a TSA plate. The agar plates were incubated at 37 °C for 36 h aerobically, and the CFU of *C. albicans* were counted.

2.6. Roughness measurement

To investigate the effect of probiotics on their physical properties, the denture base resins were soaked in the spent culture medium of *L. rhamnosus* or *L. casei* at 37 °C during the day and in tap-water at room temperature overnight. This procedure was repeated for 1 month. The surface roughness of dental base resin was assessed by using the stylus and optical based methods on atomic force microscopy (SPM-9700, Shimadzu, Kyoto, Japan).

2.7. Statistical analysis

Statistically significant differences between the untreated control and the probiotic-treated samples were analyzed by Kruskal-Wallis test and Mann-Whitney *U*-test by IBM SPSS Statistics ver. 22 software (IBM, Armonk, NY, USA). *P*-values less than 0.05 were considered statistically significant.