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Inhibition activity of *Lactobacilli* supernatant against fungal-bacterial multispecies biofilms on silicone

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ABSTRACT

Fungal-bacterial multispecies biofilms play a major role in failure of medical silicone devices, such as voice prostheses in laryngectomiy. In this study, we determined the effect of *Lactobacilli* supernatant (cell free) on mixed biofilm formation of fungi and bacteria on silicone *in vitro*. *Lactobacilli* supernatant inhibited the adhesion (90 min) of mixed fungi and bacteria species with an efficiency of > 90%. Mixed biofilm formation and the metabolic activity of the biofilms were inhibited by 72.23% and 58.36% by *Lactobacilli* supernatant. The examination using confocal laser scanning microscopy and scanning electron microscopy confirmed that *Lactobacilli* supernatant inhibited the growth of mixed biofilm and damaged the cells. Moreover, *Lactobacilli* supernatant also inhibited *Candida* yeast–to-hyphal transition. Therefore, *Lactobacilli* supernatant may serve as a possible antibiofilm agent to limit biofilm formation on voice prostheses.

1. Introduction

Microbial biofilms are three-dimensional structured microbial communities, which are found on the surfaces of the medical devices, for example, voice prostheses [1,2]. As a standard method for voice rehabilitation after total laryngectomy, the voice prosthesis is inserted in a surgically created tracheoesophageal fistula [3]. However, the biofilm on the surface of the prosthesis increases the risk of infection and limits its life time [4].

These biofilms are well-defined as microbial multispecies communities of different bacterial and fungal species. The main fungal species identified are *Candida albicans* and *Candida tropicalis*. Several bacterial members of the skin flora of the host and commensal oral also have been detected, such as *Staphylococcus, Streptococcus, Rothia dentocariosa* and *Candida* spp. [5–8]. Inter-kingdom co-operations such as increased cell-surface adhesion and colonization and enhanced resistance to antimicrobials have been reported [9,10]. Therefore, new therapy methods for biofilms on medical devices should be investigated.

Probiotics are described as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO 2001). Several food products based on probiotics have been used for nutritional or therapeutic purposes [11,12]. In recent years, the effects of probiotics on microorganisms and biofilms have been investigated, which has proven the ability of probiotics, such as *Lactobacilli*, to inhibit several bacterial or fungal pathogens growth and biofilm formation *in vitro* [13–15]. However, most of the work reported only focused on the effect of probiotics on single species biofilm, which is different from the multispecies biofilm.

In our previous work, the mixed fungal-bacterial biofilms were grown on silicone, which is the mainly used material for voice prostheses [1]. In order to understand the mechanism of the antibiofilm effect of exometabolites produced by probiotic *Lactobacillus* and explore the application of the secondary metabolites in supernatants for biofilm treatment in future, in this study, the activities of *Lactobacilli* supernatant (cell free) on the mixed biofilm biomass and cell viability were investigated. Scanning electron microscopy and confocal laser scanning microscopy were used to determine the biofilm structure and live/dead cells of biofilm.

2. Materials and methods

2.1. Bacterial strains

The fungal and bacterial pathogens used in this study were *Candida albicans*, *Candida tropicalis, Streptococcus salivarius, R. dentocariosa, Staphylococcus epidermidis*, which were clinical isolated by the Department of Otorhinolaryngology, Division of Phoniatrics-Logopedics, Medical University of Vienna. Dysfunctional voice

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prostheses due to biofilm formation were explanted from the tracheoesophageal fistulas and processed within 24 h. The prostheses were vortexes in 5 ml PBS for 3 min, the microbial specimen was isolated and stored in −80 °C, and thawed before use. *Lactobacillus rhamnosus* was obtained from the Department of Microbiology, Medical University of Vienna.

2.2. The cell free supernatant preparation

L. rhamnosus were inoculated anaerobically (Anaerobic jar, Sigma-Aldrich, Austria) at 37 °C for 24 h at 150 rpm in brain heart infusion broth (BHI, Sigma-Aldrich, Austria). The cell-free supernatant was centrifuged at 10000 rpm for 10 min to remove all cells and filter-sterilized with 0.2 μM pore-size syringe filter (Sarstedt AG & Co, Germany).

2.3. Growth of mixed biofilms on silicone plates

Mixed biofilms on silicone platelets were performed according to our previous work [16]. In brief, 3-mm-diameter medical grade silicone platelets (Websinger, Austria) were placed into each well of 96-wells microtiter plates. Microbes (bacteria and *Candida*) were grown overnight and diluted with BHI media to OD_{600} = 0.01. The fungal and bacterial suspensions were mixed equally. 200 μl mixed suspension was added to each well. Non-adherent cells were removed by washing the wells with PBS after 90-min-adhesion phase. The microtiter plates were again incubated at 37 °C for 48 h.

2.4. Antiadhesion activity of supernatant

Mixed cell suspensions were prepared as described above. 150 μl mixed suspension was added to each well containing silicone platelet together with 100 μl *L. rhamnosus* supernatant. In control group, equal volumes of mixed suspension and fresh BHI media were added. After 90 min, the silicone platelets were washed with PBS. The adhesion of cells on the silicone was evaluated by Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD, USA), which is based on bioreduction of 2-(2- methoxy- 4- nitrophenyl)- 3- (4- nitrophenyl)- 5- (2,4- disulfophenyl)- 2H-tetrazolium (WST-8). The amount of water-soluble formazan dye produced is proportional to the number of microbes adhered to the silicone surface [17].

2.5. Antibiofilm activity of supernatant

Mixed cell suspensions were prepared as described above. 100 μl (high concentration, HC) or 50 μl (low concentration, LC) Lactobacillus supernatant were added to each well of microtiter plates. 150 μl mixed cell suspensions were also added. In each well the total volume was adjusted to 250 μl using BHI. In control groups, the wells were added to BHI and the mixed cell suspensions without adding Lactobacillus supernatant.

The quantification of biofilm formation was performed by the crystal violet (CV) method. After incubation at 37 °C for 48 h, the microtiter plates were washed with PBS and stained with 250 μl 1% crystal violet for 15 min. The excess of stain was removed and 250 μl of 30% acetic acid was added to each well. The absorbance at 570 nm was measured.

The cell viability inside the biofilm was assessed by CCK-8 method. After incubation at 37 °C for 48 h, the microtiter plates were washed with PBS. 25 μl of the CCK-8 solution was added to each well of the plate. The microtiter plates were incubated for 2 h with protection from light. The absorbance was measured at 450 nm using a microplate reader.

2.6. Effect of supernatant on Candida yeast–to-hyphal transition

Candida yeast–to-hyphal transition was carried out according to Cruz et al. [18]. *Candida* cells (OD_{600} 0.1) were incubated with or without *L. rhamnosus* supernatant in YPD with 10% fetal bovine serum (FBS). After 4 h, hyphal morphology was observed by microscopy.

2.7. Scanning electron microscopy (SEM)

Mixed biofilms on silicone plates were fixed with 3% glutaraldehyde overnight, and then chemically-dried with hexamethyldisilazane. The platelets were sputter coated with gold and visualized with SEM (JSM 6310, Jeol Ltd, Akishima, Tokyo, Japan) at an acceleration voltage of 15 kV.

2.8. Confocal scanning laser microscopy (CLSM)

Live/dead analysis with CSLM was performed as previously described [16]. In brief, mixed biofilms on silicone with or without *L. rhamnosus* supernatant were formed and washed with PBS as described above, and then stained with Live/Dead® BacLight™ Bacterial Viability and Counting Kit (L34856, Invitrogen) following the manufacturer's instructions.

2.9. Statistical analysis

Data were expressed as mean values ± standard deviations (SD) of triplicate from three independent experiments. Statistical analysis was performed by *t*-test and a value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Antiadhesion of Lactobacillus supernatant

First, we determined the *L. rhamnosus* supernatant capable of inhibiting mixed species adhesion to silicone. It was observed that supernatant showed significant anti-adhesion activity against the mixed species (Fig. 1). 91.02% adhesion was prevented by *L. rhamnosus* supernatant.

3.2. Inhibitive effect of Lactobacillus supernatant on biofilm formation

Lactobacillus supernatant was found to significantly reduce the biofilm formation on silicone. As shown in Fig. 2, supernatant reduced

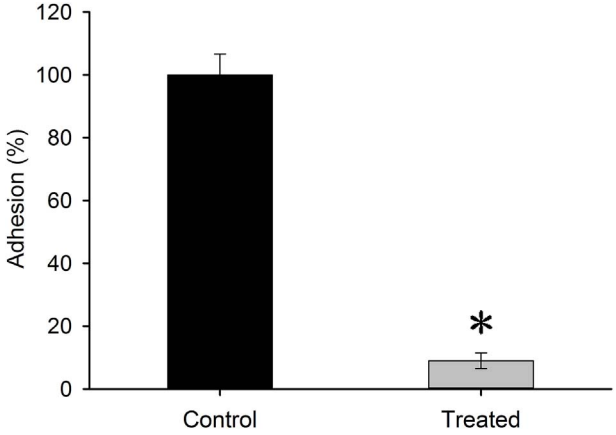

Fig. 1. Inhibition of cell-surface initial interaction by *L. rhamnosus* supernatant. The results shown represent the means and standard deviations (error bars) of three independent experiments, *$p < 0.05$ for comparison between the untreated and supernatant-treated groups.