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Inhibitory effect of probiotic *Lactobacillus* supernatants from the oral cavity on *Streptococcus mutans* biofilms

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ABSTRACT

Probiotics can release bioactive substances that can inhibit the growth and biofilm formation of pathogenic microorganisms such as *Streptococcus mutans*. In this context, we evaluated whether the supernatants of *Lactobacillus* strains isolated from caries-free subjects can inhibit *S. mutans*, one of the most important bacteria for dental caries. First, the supernatants of 22 *Lactobacillus* strains were screened for antibacterial activity against *S. mutans* in planktonic cultures. All 22 *Lactobacillus* strains studied (100%) showed antibacterial activity. Thereafter, the *Lactobacillus* strains with the greatest reductions in the planktonic *S. mutans* cultures were tested on biofilms. The *L. fermentum* 20.4, *L. paracasei* 11.6, *L. paracasei* 20.3 and *L. paracasei* 25.4 strains could significantly reduce the number of *S. mutans* cells in biofilms formed in hydroxyapatite ($p < 0.05$). This reduction was also confirmed by scanning electron microscopy analysis and was not caused by the decreased pH value in the medium ($p > 0.05$). In addition, the supernatants of these probiotic strains could also reduce the total biomass of *S. mutans* biofilms ($p < 0.05$). In conclusion, most of the *Lactobacillus* strains tested have some antibacterial activity against *S. mutans*. *L. fermentum* 20.4, *L. paracasei* 11.6, *L. paracasei* 20.3 and *L. paracasei* 25.4 produce bioactive substances that caused a significant reduction in *S. mutans* biofilms.

1. Introduction

The term “probiotic” is used to describe live microorganisms that have beneficial effects on human health when administered in adequate amounts [1–4]. Some strains of the bacterial genera *Lactobacillus* and *Bifidobacterium* have been widely used as probiotics in several foods and dietary supplements to improve gastrointestinal health [5–7]. However, little is known about the effects of these strains on common oral infections, such as dental caries [8,9]. Previous studies suggested that consumption of dietary products containing probiotic lactobacilli reduces the number of *Streptococcus mutans* cells in saliva [8–10]. However, it is known that *Lactobacillus* spp. are acidogenic bacteria that can participate in the progression of dental caries [11]. Recently, it has been suggested that there are differences among various strains with respect to their ability to produce acid and that not all *Lactobacillus* strains have a caries-inducing effect [12].

Based on these observations, several authors have investigated the effects of certain *Lactobacillus* strains commonly used as probiotics in dietary products on the development of dental caries [10,13,14]. Söderling et al. [13] compared the effects of four probiotic *Lactobacillus* strains (*Lactobacillus rhamnosus* GG, *Lactobacillus plantarum* 229v, *Lactobacillus reuteri* SD2112 and *L. reuteri* PTA5289) on *S. mutans* biofilm formation. All of the *Lactobacillus* strains inhibited *S. mutans* biofilm formation on glass surfaces, although *L. plantarum* and *L. reuteri* PTA5289 showed a weaker inhibitory effect compared to *L. reuteri* SD2112 and *L. rhamnosus*. Marttinem et al. [14] also verified that *L. reuteri* ATCC PTA5289 could interfere with the adhesion of *S. mutans* to hydroxyapatite discs and inhibited biofilm formation. Lin et al. [10] studied other probiotic *Lactobacillus* strains, including *Lactobacillus casei* Shirota, *Lactobacillus casei* LC01, *Lactobacillus plantarum* ST-III, *Lactobacillus paracasei* Lpc-37 and *Lactobacillus rhamnosus* HN001. All five *Lactobacillus* strains inhibited *S. mutans* growth and biofilm formation

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on glass surfaces, but the effects depended on the *Lactobacillus* strains used.

These studies suggested that several strains used in dietary products can inhibit *S. mutans*, showing potential for their use as probiotics to prevent dental caries [10,13,14]. However, most commercially available probiotic strains were isolated from human or animal gastrointestinal tracts [5,11]. Thus, their beneficial effects for preventing dental caries and other oral diseases may be questionable [8,15]. The identification of new *Lactobacillus* strains from the oral cavity that can inhibit *S. mutans* is essential for the development of the use of probiotics for the prevention of dental caries. Our study was based on the hypothesis that the oral cavities of healthy individuals may harbor some beneficial *Lactobacillus* strains that can release bioactive substances with inhibitory activities against oral pathogens.

Most of the studies cited above focused on the use of living cells of probiotics; however, the effects of *Lactobacillus* supernatants from the oral cavity on *S. mutans* biofilms have been studied very little. Thus, in order to understand the effects of exometabolites produced by *Lactobacillus* and to explore the possible application of the secondary metabolites on oral biofilms in the future, in this study, we evaluated the antibiofilm effects of cell-free supernatants of *Lactobacillus* strains from the oral cavity on *S. mutans*.

2. Materials and methods

2.1. Microorganisms and growth conditions

In this study, we used *Streptococcus mutans* UA159 and 22 clinical strains of *Lactobacillus* that were previously isolated from the oral cavities of caries-free subjects, which were identified by an API 50 CHL system (BioMérieux, France) and confirmed by PCR. These *Lactobacillus* strains showed great potential to inhibit *C. albicans* strains from the oral cavity in *vitro* and to negatively modulate virulence gene expression [16]. This study was approved by the Human Research Ethics Committee of the Institute of Science and Technology, Univ Estadual Paulista/UNESP (protocol number 754.634).

2.2. Preparation of microbial inocula and culture filtrates

S. mutans was grown in Brain Heart Infusion broth (BHI broth, Himedia, Mumbai, India), and the *Lactobacillus* strains were grown in DeMan-Rogosa-Sharpe broth (MRS broth, Difco, Detroit, USA) at 37 °C for 48 h (5% CO_2). The microbial cells in culture were centrifuged, and the pellets were washed twice with 0.85% NaCl (Labimpex, São Paulo, Brazil). The cell suspensions were adjusted to 10^8 cells/mL using a spectrophotometer (B582, Micronal, Brazil).

To prepare culture filtrates of the *Lactobacillus* strains, 1 mL of a 10^8 cells/mL *Lactobacillus* culture (previously prepared) was added to MRS broth and was incubated at 37 °C for 24 h (5% CO_2). Next, the culture was centrifuged and the supernatant was filtered through a 0.22-μm filter (MFS, Dublin, USA).

2.3. Antibacterial activity of Lactobacillus supernatants against S. mutans in planktonic cultures

The antibacterial activity of the *Lactobacillus* strains against *S. mutans* in planktonic cultures was assessed according to the methodology described by Lin et al. [10] with some modifications. Standardized *S. mutans* and *Lactobacillus* cell suspensions were prepared as described above. Next, 250 μL of a *S. mutans* suspension and 250 μL of the *Lactobacillus* supernatant were mixed with 1.5 mL of BHI broth. In the control group, the microbial suspension of *S. mutans* was cultured with a physiological solution. All of the cultures were incubated at 37 °C for 12 and 24 h (5% CO_2). After incubation, the cultures were diluted and plated on Mitis Salivarius Agar (Difco, Detroit, USA) supplemented with bacitracin (Sigma-Aldrich, St. Paul, Brazil, 0.2 IU/mL) and 15% sucrose (MSBS) for *S. mutans* growth. The plates were incubated at 37 °C for 48 h (5% CO_2), and the number of colony-forming units (CFU/mL) was determined. This assay was performed as three independent experiments with four replicates per group.

2.4. Antibacterial activity of Lactobacillus supernatants on S. mutans counts in biofilms

To form biofilms, we used hydroxyapatite discs (5 mm diameter x 2 mm thick) purchased from Clarkson Chromatography Products, Inc. (South Williamsport, USA) following the methodology described by Marttinen et al. [14] with modifications. The sterilized discs were placed in 24-well culture plates (Kasvi, Curitiba, Brazil) containing 1.8 mL of a mixture composed of 70% saliva and 30% BHI broth supplemented with 0.3% glucose and 67 mmol/L Sörensen's buffer (pH 7.2). A 225-μL aliquot of a standardized suspension of *S. mutans* was added, and the plates were incubated at 37 °C for 1 h (5% CO_2) to promote the initial adhesion of *S. mutans* onto the discs. Next, 225 μL of *Lactobacillus* supernatant was added. In the control group, the microbial suspensions of *S. mutans* were cultivated with physiological solution. For this experiment, *S. mutans* cells were counted in biofilms formed in 24 h and in 48 h.

After incubation (24 or 48 h), the discs were washed 3 times and transferred to a tube containing 1 mL of a NaCl solution. The biofilms formed were detached using an ultrasonic homogenizer (Sonopuls HD 2200, Bandelin Electronic) at 7 W for 30 s. The suspensions were serially diluted and plated on MSBS agar to determine the number of CFU/mL. The biofilm experiments were performed in three independent experiments on different days with four biofilms per group. For the biofilm group formed in 48 h, after 24 h of incubation, the discs were washed 3 times with a NaCl solution and transferred into a fresh medium mixture composed of 1.8 mL of 70% saliva and 30% BHI broth supplemented with 0.15% glucose and 0.15% sucrose.

2.5. Measurement of pH values

The pH values of the media were tested during the biofilm formation under the same conditions as the biofilm assay described above. After 48 h of incubation in the 24-well culture plates, the supernatants from each well were collected, and the pH values were measured using a pH meter (Mettler, Toledo, Ohio, USA). Four wells were measured per group, and the experiment was done at three different times.

2.6. Analysis of biofilms by scanning electron microscopy (SEM)

In this experiment, we used hydroxyapatite discs (5 mm diameter x 2 mm thick), and the biofilms were formed as mentioned above. After biofilm formation, the specimens were fixed in 1 mL of 2.5% glutaraldehyde for 1 h. The specimens were then dehydrated in an increasing ethanol concentration series (10, 25, 50, 75 and 90%) for 20 min each, followed by immersion in 100% alcohol for 1 h. The plates were kept in an oven at 37 °C for 24 h to permit total drying of the specimens.

After drying, the specimens were transferred to aluminum stubs and sputter coated with gold for 160 s at 40 mA (Denton Vacuum Desk II, Denton Vacuum LLC, Moorestown, NJ, USA). The specimens were examined and imaged using a JEOL JSM-5600 scanning electron microscope (JEOL USA, Inc., Peabody, MA, USA) at the Institute of Science and Technology, UNESP – Univ Estadual Paulista. These experiments were performed at two different times with n = 3 biofilms per group.

2.7. Antibacterial activity of Lactobacillus supernatants on S. mutans biofilm biomass

After biofilm formation, the biofilm biomass was quantified utilizing an assay previously described by Rossoni et al. [16] and Peeters et al. [17], with modifications. For fixation of the biofilms, 100 μl of