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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

99% methanol was added to the wells (Sigma-Aldrich, São Paulo, Brazil). After 15 min, the supernatants were removed and the plates were air-dried.

Then, 100 µl of a 1% crystal violet (CV) solution was added to all wells. After 20 min, the residual CV solution was removed by washing with PBS. Finally, bound CV was released by adding 150 µl of 33% acetic acid (Sigma-Aldrich). The absorbance was measured at 540 nm. All steps were carried out at room temperature. The CV assay was performed as two independent experiments with n = 6 biofilms per group.

2.8. Statistical analysis

The results were compared by ANOVA and Tukey's test. Student's *t*-test was used to compare the CFU/mL results from the *in vitro* biofilm formation assay and the CV assay. All analyses were performed using the GraphPad Prism 6 Program (GraphPad Software, Inc., La Jolla, CA, USA), and a level of significance of 5% was adopted.

3. Results

We selected 22 clinical strains of *Lactobacillus* that were previously isolated from the oral cavities of caries-free subjects, including 22 strains of *L. paracasei* (1.1, 3.1, 6.2, 7.5, 8.4, 11.6, 15.8, 16.4, 17.1, 20.3, 21.4, 23.4, 24.1, 25.4, 26.1, 27.1, 28.4 and 30.1), 3 strains of *L. fermentum* (14.5, 20.4 and 31.4) and 1 strain of *L. rhamnosus* (19.3). All of the strains were screened for antibacterial activity against *S. mutans* using planktonic cultures. For this purpose, we analyzed the indirect effects of *Lactobacillus* using only the *Lactobacillus* culture filtrate that was obtained after its growth in MRS broth (*S. mutans* + *Lactobacillus* supernatant interaction group) for 12 and 24 h. As a control, we also tested monocultures of *S. mutans* (*S. mutans* + physiological solution control group). To determine whether the MRS broth of the *Lactobacillus* culture could exert an effect on *S. mutans* and interfere with the results, we included a control group consisting only of *S. mutans* and MRS broth (*S. mutans* + MRS broth control group) (data not shown). After 12 or 24 h in culture, the *S. mutans* growth was evaluated by determining the colony-forming unit numbers (CFU/mL).

All 22 *Lactobacillus* strains (100%) analyzed showed antibacterial activity against *S. mutans*. Only the *L. paracasei* 8.4, 23.4 and 24.1 strains had no inhibitory effects on *S. mutans* after 24 h in culture. We observed a reduction in the number of *S. mutans* cells in the *S. mutans* + *Lactobacillus* interaction group compared to the *S. mutans* + physiological solution control group (100% of *S. mutans* growth). The reductions in *S. mutans* growth ranged from 12 to 91.4% depending on the strain analyzed (Fig. 1).

The results showed that the MRS broth used to prepare the *Lactobacillus* supernatant did not interfere with the growth of *S. mutans*. The CFU/mL count of *S. mutans* was 10.02 ± 0.06 for the *S. mutans* + physiological solution control group and 10.04 ± 0.03 for the *S. mutans* + MRS broth control group (Student's *t*-test, $p = 0.44$). These data indicated that the anti-*S. mutans* activity of the supernatants could be attributed to metabolites produced by the *Lactobacillus* strains.

Based on the results presented in Fig. 1, we can observe that the four strains with the highest antibacterial activity against *S. mutans* were *L. paracasei* 25.4, *L. fermentum* 20.4, *L. paracasei* 20.3 and *L. paracasei* 11.6. These strains reduced *S. mutans* growth by more than 86% after 24 h in culture. Therefore, these strains were selected for the *in vitro S. mutans* biofilm studies.

The *S. mutans* biofilms were formed on hydroxyapatite discs, and after incubation times of 24 and 48 h, the biofilms were analyzed by counting the CFU/mL. In these experiments, we found a strong inhibitory activity of *Lactobacillus* supernatant on *S. mutans* cells for all time points tested. For all of the *Lactobacillus* supernatants tested (11.6, 20.4, 20.3 and 25.4), there were statistically significant differences between the *S. mutans* + physiological solution control group and the *S. mutans* + *Lactobacillus* supernatant interaction group (Fig. 2). *L. paracasei* strain 25.4 presented the largest reduction (time points: 24 h - 3.73 log and 48 h 3.49 log) in the number of viable *S. mutans* cells determined by the CFU count. These findings indicated that these *Lactobacillus* strains release bioactive substances that can inhibit *S. mutans* growth and biofilm formation. Due to the great clinical importance of mature *S. mutans* biofilms in caries formation, and as we demonstrated the efficiency of the *Lactobacillus* supernatants in the early and late stages of *S. mutans* biofilm formation, we decided to evaluate the interference of the pH levels of these supernatants on the biofilms, and the total biomass and SEM analysis were performed only on biofilms formed after 48 h of incubation.

To investigate whether the effects of *Lactobacillus* supernatants on *S. mutans* were associated with pH variations, we monitored the pH values of the culture media at the same time point as for the biofilm growth (48 h). For all of the experimental groups, the pH values decreased from 7.2 (initial biofilm pH) to between 4 and 5, and no major pH variations were observed at the different times of biofilm growth (Fig. 3). Since the pH values in the *S. mutans* + PBS control group were similar to the *S. mutans* + *Lactobacillus* supernatant interaction groups, we concluded that pH variation is not a crucial factor in the inhibitory activity of *Lactobacillus* supernatants on *S. mutans* biofilms.

The biofilms formed were also evaluated by SEM, and with this approach, we can observe mature biofilm formation on hydroxyapatite discs. The *S. mutans* cells observed in the biofilms had different growth features depending on the experimental group. The biofilms formed by *S. mutans* in the absence of *Lactobacillus* supernatant were characterized by the presence of numerous bacterial cells and formation of an extracellular matrix (Fig. 4A and B). In the "*S. mutans* + *Lactobacillus* supernatant interaction group", we verified a reduction in the number

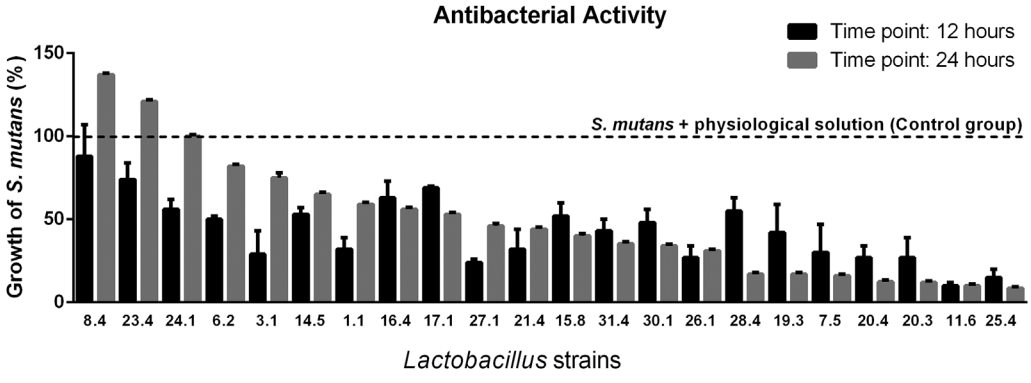

Fig. 1. Growth percentages of *S. mutans* obtained by counting the CFU/mL in the antibacterial activity *in vitro* test for 12 and 24 h in the presence of *Lactobacillus* supernatants. The supernatants of 22 *Lactobacillus* strains were tested: 22 strains of *L. paracasei* (1.1, 3.1, 6.2, 7.5, 8.4, 11.6, 15.8, 16.4, 17.1, 20.3, 21.4, 23.4, 24.1, 25.4, 26.1, 27.1, 28.4 and 30.1), 3 strains of *L. fermentum* (14.5, 20.4 and 31.4) and 1 strain of *L. rhamnosus* (19.3). The graph shows the percentage of *S. mutans* growth in the "*S. mutans* + *Lactobacillus* supernatant interaction group (gray and black bars)" in relation to the "*S. mutans* + physiological solution control group (—)" (100% of *S. mutans growth*).

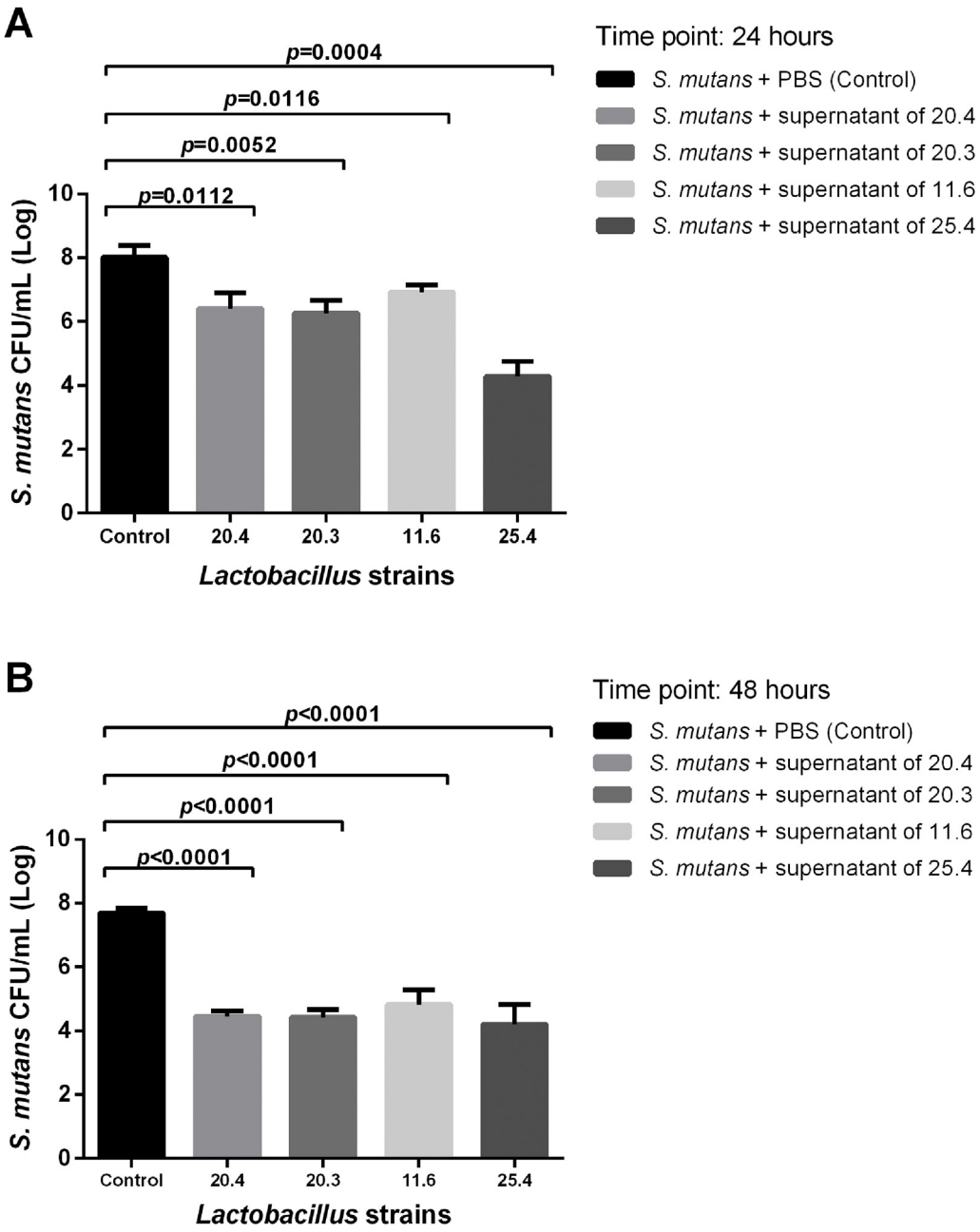

Fig. 2. Means and standard deviations of the *S. mutans* counts (CFU/mL Log10) in 24- (A) and 48-h (B) biofilm experiments. Four *Lactobacillus* strains were analyzed: *L. paracasei* 11.6, *L. fermentum* 20.4, *L. paracasei* 20.3 and *L. paracasei* 25.4. The biofilms were formed with the addition of *Lactobacillus* supernatant 1 h after the initial adherence of *S. mutans* on the hydroxyapatite discs. For each experimental condition, the following groups were analyzed: "*S. mutans* + physiological solution control group" and "*S. mutans* + *Lactobacillus* supernatant interaction group". For Student's *t*-test, the differences were considered significant at $p \leq 0.05$.

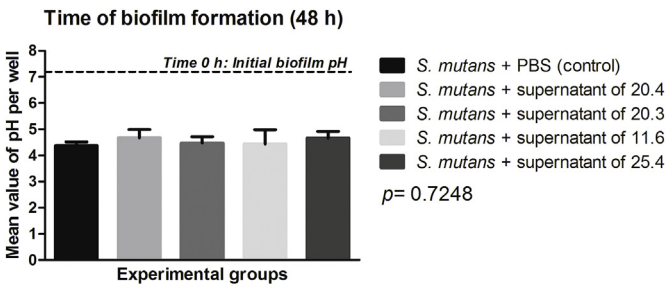

Fig. 3. Means and standard deviations of pH values obtained in the biofilm formation assay. For each *Lactobacillus* supernatant studied, the following groups were analyzed: "*S. mutans* + physiological solution control group" and "*S. mutans* + *Lactobacillus* supernatant interaction group".

of *S. mutans* cells and less extracellular matrix formation compared to the control group (Fig. 4C–F). Therefore, SEM images confirmed the results obtained from the CFU count, showing that the supernatants of *Lactobacillus* isolates influenced *S. mutans* viability and reduced the total biofilm biomass.

In order to confirm that the *Lactobacillus* supernatants also influence the extracellular matrix formation of *S. mutans* biofilms, the total biomass was quantified by the colorimetric assay using CV. Prioritizing the *Lactobacillus* supernatants that reduced *S. mutans* growth most significantly in the biofilm, we also used the following four strains: *L. paracasei* 11.6, *L. paracasei* 20.3, *L. fermentum* 20.4 and *L. paracasei* 25.4. The biofilms formed by *S. mutans* in the presence of *Lactobacillus* supernatants exhibited a significant reduction compared to the *S. mutans* control group (Fig. 5).

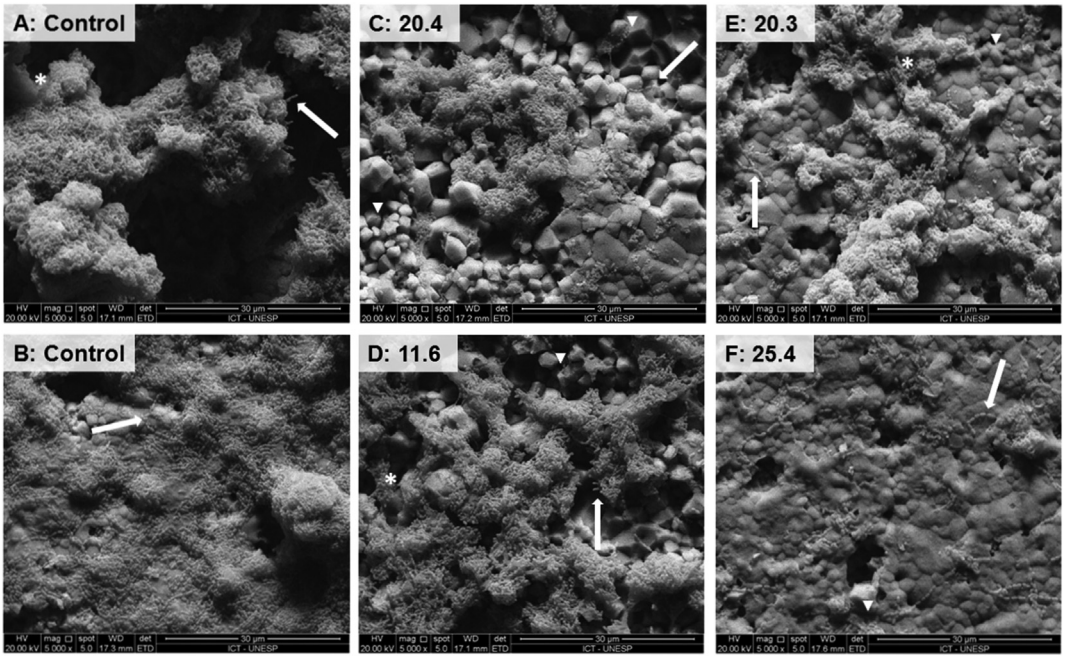

Fig. 4. SEM of biofilms formed *in vitro*. **A.** and **B.** Control group of *S. mutans* + physiological solution; the presence of numerous bacterial cells and formation of extracellular matrix is verified; **C.** Group interaction of *S. mutans* + supernatant of *L. fermentum* 20.4; **D.** Group interaction of *S. mutans* + supernatant of *L. paracasei* 11.6; **E.** Group interaction of *S. mutans* + supernatant of *L. paracasei* 20.3; **F.** Group interaction of *S. mutans* + supernatant of *L. paracasei* 25.4. In all of the interaction biofilms, it was possible to observe a reduction in the number of *S. mutans* cells compared to the control group. *S. mutans* cells (arrow), extracellular matrix (asterisk) and hydroxyapatite crystals (filled inverted triangle) are highlighted in the SEM. Magnification: 5000X.

Considering the results observed from the CV assay, we confirm the inhibitory effect of probiotic *Lactobacillus* supernatants from the oral cavity on *S. mutans* biofilm. In addition, this antibiofilm effect can be attributed to the reduction in the number of *S. mutans* cells and the lower extracellular matrix formation. *L. paracasei* strain 25.4 is a potential probiotic candidate for clinical studies focused on the prevention of dental caries.

4. Discussion

In recent decades, the interest in using probiotics to prevent oral infectious diseases has grown significantly [18–20]. Certain *Lactobacillus* strains have shown a potential ability to specifically interfere with oral ecology by inhibiting pathogenic microorganisms, such as *S. mutans* [10,11,21–23]. Therefore, the use of probiotic lactobacilli seems to be a promising method for controlling dental caries [24]. Based on these observations, the aim of our study was to identify new *Lactobacillus* strains and to test whether only its supernatant, with their associated active metabolites, can be safely used as prophylactic agents in the oral cavity to inhibit *S. mutans* growth and biofilm formation. The use of the supernatant alone is safer than using live *Lactobacillus* cells in relation to caries formation because some strains of this bacterial genus can metabolize sucrose, co-aggregate with *S. mutans* and are often tolerant toward the use of fluoride [25,26].

It has been suggested that individuals who have never had dental caries may harbor *Lactobacillus* strains in their oral cavities that contribute to a healthy microbiota and to the control of dental caries [11,27]. Thus, we screened 22 supernatants from different *Lactobacillus* strains isolated from the oral cavities of caries-free subjects, including several strains of *L. paracasei, L. rhamnosus* and *L. fermentum*. Using *in*

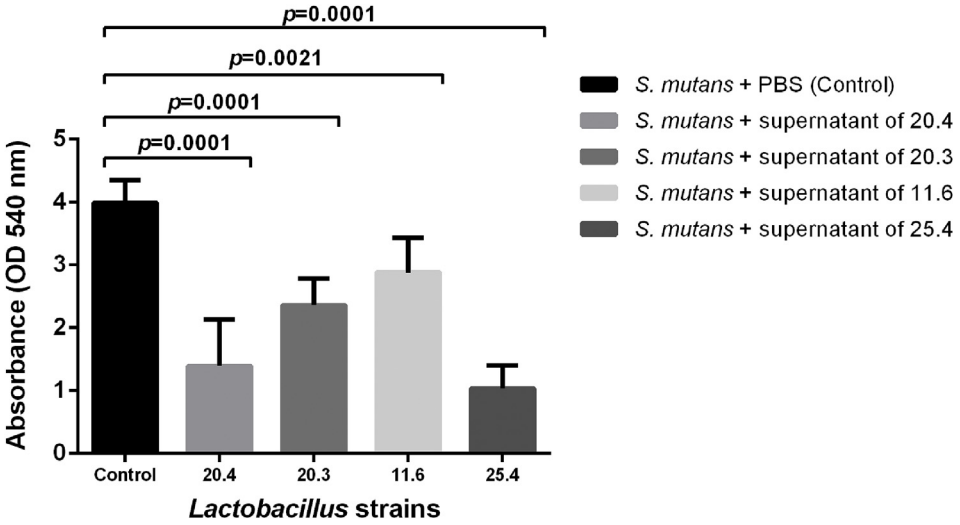

Fig. 5. Evaluation of the biomass of *S. mutans* biofilms. Means and standard deviations of the absorbance values of the control group biofilms ("*S. mutans* + physiological solution control group") and in the groups with *Lactobacillus* supernatants "*S. mutans* + *Lactobacillus* supernatant interaction group". Four *Lactobacillus* strains were analyzed: *L. paracasei* 11.6, *L. fermentum* 20.4, *L. paracasei* 20.3 and *L. paracasei* 25.4. For Student's *t*-test, differences were considered significant at $p \leq 0.05$.

vitro planktonic cultures, we identified supernatants of four strains (3 strains of *L. paracasei* and 1 of *L. fermentum*) capable of limiting *S. mutans* growth by more than 86%.

Our findings agree with the study of Simark-Mattsson et al. [11] in which they isolated *Lactobacillus* strains from caries-free subjects and from individuals with active caries. The antimicrobial activities of these strains against *S. mutans* were evaluated using agar overlay interference tests. The authors observed that the *Lactobacillus* strains isolated from caries-free subjects had higher inhibitory activities against *S. mutans* than did the *Lactobacillus* strains isolated from individuals with active caries. Among the *Lactobacillus* strains isolated from caries-free subjects, 23 could completely inhibit the growth of *S. mutans*, and the species with the highest antibacterial activities included *L. paracasei*, *L. plantarum* and *L. rhamnosus*.

Next, we used *in vitro* biofilm models to determine whether the antibacterial activities of the assayed *Lactobacillus* supernatants act upon *S. mutans* cells organized in biofilms. The use of biofilm models to study the effects of antibacterial agents against *S. mutans* is very important, since the cariogenic activity of *S. mutans* is largely attributed to its ability to adhere to teeth and to create a molecular scaffold of glucan polysaccharides on the tooth surfaces [28]. Several laboratories have developed *in vitro* biofilm models that can mimic the oral cavity environment [29]. In the present study, we evaluated two biofilm methods: the first was to form biofilms on hydroxyapatite discs to simulate the tooth surface that were placed in contact with artificial saliva to induce a salivary pellicle formation [30], and the second approach was to form biofilms at the bottom of wells to evaluate the total biomass. Using the first biofilm method, we verified that the *Lactobacillus* supernatants could interfere with *S. mutans* biofilms, leading to a reduction in their numbers of CFU/mL. These results validated the hypothesis that the antimicrobial activity of *Lactobacillus* supernatants against oral pathogens in biofilms can be attributed to the production of bioactive substances [31,32].

Recently, Krzyściak et al. [33] evaluated the anti-cariogenic effects of *Lactobacillus salivarius* CECT5713 by limiting *S. mutans* and *C. albicans* growth and biofilm mass in a double-species biofilm model. The authors found that *L. salivarius* inhibited the cariogenic biofilm formation of *C. albicans* and *S. mutans*. Under the influence of the probiotic, the biofilm mass and the number of *S. mutans* colonies in the biofilm were decreased (about 0.15 log reduction). These results corroborate the reduction of *S. mutans* described in the present study (3.73 log for *L. paracasei* 25.4).

To assess the influence of the acids produced by *Lactobacillus* strains on *S. mutans*, which consequently lower the pH of the supernatant, we measured the pH values of biofilm formation of each *Lactobacillus* supernatant. All of the *Lactobacillus* strains had similar acidogenic activities; therefore, no major pH variations were observed at the different times of biofilm growth compared to the control group. However, some previous studies demonstrated that the acidic environment provided by *Lactobacillus* strains can directly affect their antibacterial activities against *S. mutans* [10,22]. Keller et al. [22] evaluated the ability of commercial probiotic lactobacilli to co-aggregate and to inhibit the growth of *S. mutans*. The selected lactobacilli showed co-aggregation activity and inhibited the growth of *S. mutans* according to variations in pH. Therefore, the role of the acids produced by probiotic strains on *S. mutans* growth is still unclear. In addition to acids, the production of antimicrobial compounds by probiotic bacteria has also been widely discussed. Previous studies showed that *Lactobacillus* strains produce bacteriolytic enzymes, bacteriocins and biosurfactants that can inhibit pathogenic microorganisms [34–36]. Some bacteriocins produced by lactobacilli have been purified and extensively studied, such as gasserin produced by *L. gasseri* EV1461 [34], fermecin produced by *L. fermentum* SD11 [35], and paracin 1.7 produced by *L paracasei* HD1-7 [36].

The biofilms were also evaluated by SEM analysis in which *S. mutans* is strongly adherent on hydroxyapatite discs. In addition, it was possible to verify a reduction in the number of *S. mutans* cells when the control biofilm was compared to the biofilms exposed to the *Lactobacillus* supernatants. According to the SEM analysis, *L. paracasei* 25.4 (the strain with the greatest reduction in CFU/mL count) reduced the adhesion of *S. mutans* cells to the discs, and this effect probably caused the reduction in the CFU count. The images obtained in this study are in agreement with Wasfi et al. [37], who investigated whether *L. casei* (ATCC 393), *L. reuteri* (ATCC 23272), *L. plantarum* (ATCC 14917) or *L. salivarius* (ATCC 11741) inhibit *S. mutans* biofilms. In their SEM images, the authors observed changes in the extracellular matrix, fewer bacteria and smaller microcolonies in biofilms formed during coculture of *S. mutans* and *Lactobacillus*.

Using the second biofilm evaluation method, we quantified the differences in the total biomass of the biofilms among the groups, and in this approach, the biofilms were stained with CV. The biofilms formed by *S. mutans* in the presence of *Lactobacillus* supernatants exhibited a significant reduction in biomass compared to the control group that lacked *Lactobacillus* supernatant. All of the supernatants tested significantly reduced the biomass, and *L. paracasei* 25.4 was the strain with the highest capacity to affect *S. mutans* biofilms. Ahn et al. [38] also demonstrated that *L. plantarum* lipoteichoic acid could inhibit the biofilm formation of *S. mutans* on polystyrene plates stained with CV and on hydroxyapatite discs.

5. Conclusion

In conclusion, this study showed that most *Lactobacillus* strains isolated from the oral cavities of caries-free subjects could release bioactive substances that inhibit the growth of *S. mutans* in planktonic cultures. The strains *L. paracasei* 11.6, *L. paracasei* 25.4, *L. fermentum* 20.4 and *L. paracasei* 20.3 presented antibiofilm activity against *S. mutans* in different methods of biofilm quantification. The identification of these *Lactobacillus* strains, which naturally inhabit the oral cavity and show antimicrobial activity against *S. mutans,* contributes to the development of new probiotic agents to prevent dental caries.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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