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# Inhibitory effect of helium cold atmospheric plasma on cariogenic biofilms

Leandro Wagner Figueira [a], Ana Bessa Muniz [a], Anelise Cristina Osorio Cesar Doria [b], Thalita Mayumi Castaldelli Nishime [c], Konstantin Georgiev Kostov [d] and Cristiane Y. Koga-Ito [a]

[a]Department of Environmental Engineering and Oral Biopathology Graduate Program, Institute of Science and Technology, São Paulo State University, UNESP, São José dos Campos, São Paulo, Brazil; [b]Biotechnology and Electric Plasma Laboratory (Biotechplasma) - Research and Development Institute – IPD - Universidade Do Vale Do Paraíba. Av. Shishima Hifumi, São José dos Campos, Brazil; [c]Leibniz Institute for Plasma Science and Technology, Greifswald, Germany; [d]Department of Physics, Guaratinguetá Faculty of Engineering, São Paulo State University/ UNESP, Guaratinguetá, Brazil

**ABSTRACT**

This study aimed to determine the effects of low-temperature plasma jet produced in gas helium (LTP-helium) on cariogenic biofilms composedby *Streptococcusmutans, Streptococcus sanguinis* and *Streptococcus gordonii*, and also by the combination of *Candida albicans, Lactobacillus acidophilus* and *S. mutans*. Biofilms were treated for 1, 3, 5, and 7 minutes. A 0.12% chlorhexidine solution was used as the positive control and sterile physiologic solution was the negative control. Biofilm viability was analyzed by viable cell recovery, scanning electron microscopy, and confocal laser scanning microscopy. All assays were performed intriplicate in three independent experiments. Multispecies biofilms exposed to LTP-helium had a significant reduction in viability when compared to the negative control ($p < 0.0001$). For biofilm formedby *S. mutans, S. sanguinis*, and *S. gordonii*, LTP treatments for 5 and 7 minutes caused similar reduction of morethan 2 $\log_{10}$. Also, a significant reduction in the viability of biofilms formedby *C. albicans, L. acidophilus*, and *S. mutans* was detected ($p < 0.0001$). In conclusion, LTP-helium reduced theviability of cariogenic biofilms with different microbial compositions, which indicates that LTP-helium is a potential tool for developing new protocols for dental caries prevention and treatment.

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## Introduction

Caries is a disease caused by frequent carbohydrate intake and increased oral levels of acidogenic and aciduric species [1–4]. Host susceptibility and lack of adequate oral hygiene also influence the caries risk. The occurrence of caries is related to the presence of polymicrobial cariogenic biofilms that are formed on dental surfaces due to an imbalance in the oral microbial community [5–9]. When the concentration of acids produced by the micro-organisms is higher than the capacity of the saliva to neutralize them, demineralization of dental enamel occurs, leading to the formation and progression of caries lesions [10–13].

Sucrose is considered the most cariogenic dietary carbohydrate [14], which can be fermented by the cariogenic bacteria and is an excellent substrate for the synthesis of extracellular and intracellular polysaccharides [15–17].

*Streptococcus mitis, Streptococcus sanguinis*, and *Streptococcus gordonii* are initial colonizers [18,19]. *Streptococcus mutans, Veillonella* spp., and *Fusobacteria* spp. are involved in the late colonization [19], which initiates the development of the mature cariogenic biofilm [20, 21–23].

*Candida albicans* has been frequently associated with a synergistic relationship with *S. mutans* in the dental biofilm [24–26]. In this sense, some studies were conducted to identify, quantify, and explore the interaction between *C. albicans* and *S. mutans* and its impact on the pathogenesis of dental caries [25,27–29].

It is already known that lactobacilli interact with *S. mutans* in the biofilm during the cariogenic biofilm formation process by different mechanisms [30–34]. As reported in the literature, the pathogenesis of dental caries may be associated to *S. mutans* and lactobacilli growth, and a decrease in the diversity of other microbial species in the oral cavity [34,35].

In the last decades, the treatment of caries lesions has evolved and the minimally invasive dentistry (MID) protocols were highlighted [36]. These protocols include different treatment techniques, such as cavity preparation by laser, air abrasion, and chemo-mechanical procedures [37,38]. The atraumatic restorative technique (ART) is one of the methods aligned with MID that includes the use of manual instruments for removing carious tissue [39–41].

**CONTACT** Leandro Wagner Figueira leandrowf@live.com Department of Environmental Engineering and Oral Biopathology Graduate Program, Institute of Science and Technology, São Paulo, Brazil

Plasma is commonly considered the fourth state of matter. It is defined as an ionized gas, composed of electrons, ions, neutral particles, and molecules in the ground or excited state [42,43]. Plasmas are classified into equilibrium and non-equilibrium according to the relative temperature of electrons, ions, and neutral particles [44,45]. Non-equilibrium plasmas, also known as low-temperature plasmas (LTP) [46,47] are suitable for biomedical applications.

Some previous studies investigated the action of LTP on cariogenic biofilms. Yang et al. (2011) and Blumhagen et al. (2014) reported the high effectiveness of LTP in reducing the counts of *S. mutans* and *L. acidophillus* in monospecies biofilms after a few seconds of treatment [48,49]. Another recent study showed that polymicrobial cariogenic biofilms formed by *S. gordonii, S. mutans*, and *S. sanguinis* were significantly reduced after 30, 60, and 120 seconds of LTP exposure [50].

This study aimed to evaluate the effects of helium-LTP on cariogenic multispecies biofilms with different microbial compositions (*S. sanguinis, S. gordonii, S. mutans* and *C. albicans, L. acidophillus, S. mutans*).

## Materials and Methods

### Plasma generating device and parameters

The plasma jet was generated using the experimental setup reported in Kostov et al. (2015) [51]. It consists of a syringe-like dielectric enclosure made of Delrin, ending with a 1-mm-thick nozzle. Inside it was centered a thin Ni-Cr wire (0.45 mm), which is encapsulated in a quartz tube. The wire was connected to an AC high voltage generator (Minipuls4 from GBS, Elektronik, Dresden, Germany) capable of generating repetitive bursts, with repetition period of 1.5 ms, each with 10 sinewave voltage oscillation with an amplitude of 12.0 kV pp and frequency of 31.7 kHz. The working gas was He with 99.5% purity. The flow rate of 2.0 L minute$^{-1}$ was controlled by a rotameter. The discharge power in pulsed voltage mode (Mp) reached 1.2 W. Under these conditions, the plasma jet ejected from the device nozzle reached a length of approximately 2.5 cm. In this work the distance from the nozzle tip to the biofilm surface was set to 1.5 cm (Figure 1). It was determined by a previous optimization study [52] in a way to ensure a low degree of cytotoxicity and the best plasma action on biofilms.

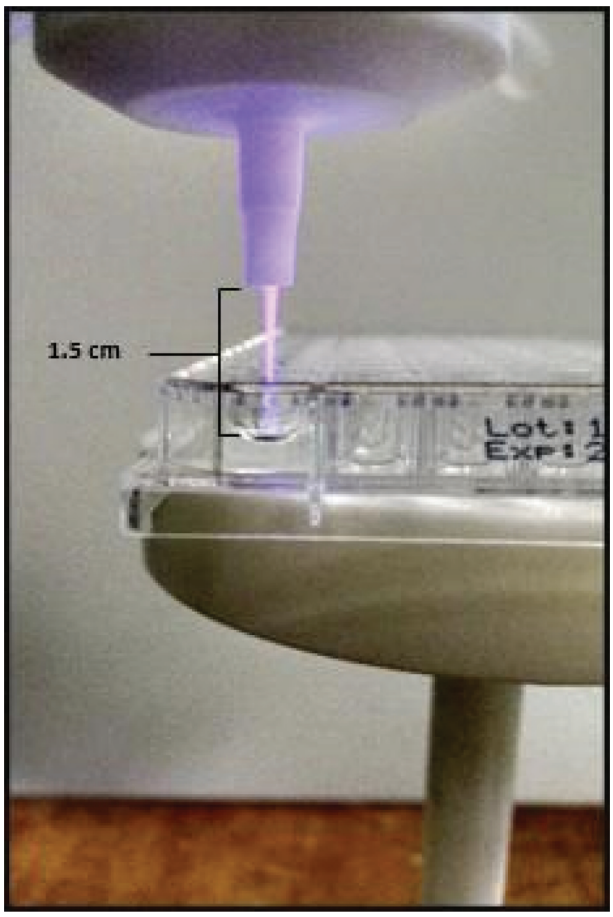


Figure 1.Low-temperature plasma (LTP) jet applied on multispecies biofilms inside a 96-well plate using a distance of 1.5 cm from the plasma outlet to the sample surface.

### Microorganisms

*Streptococcus mutans* (ATCC 35688), *Streptococcus gordonii* (ATCC 10558), *Streptococcus sanguinis* (ATCC 10556), *Lactobacillus acidophilus* (ATCC 393), and *Candida albicans* (SC 5314) were used to form multispecies biofilms. Stock cultures were maintained at −80° C and reactivated onto a specific medium. Species of *Streptococcus* and *Lactobacillus* were activated on BHI 5% sheep blood agar, and Sabouraud dextrose (SD) was used for *Candida albicans*.

The microorganisms were incubated at 37°C for 48 hours. Cultures of *Streptococcus* and *Lactobacillus* were kept in a $CO_2$ atmosphere.

A sucrose solution (20%) was prepared, and then the Tryptic Soy Broth (TSB) supplemented with 1% sucrose was also previously made and stored in a fridge (−20°C). The supplemented medium (TSB 1% sucrose) was prepared at the time of use. After that, to form the inoculum, 10 isolated colonies were collected with the aid of a sterile loop and subsequently transferred to a centrifuge tube containing 50 mL of TSB sucrose 1% and incubated at 37°C for 18 hours. The same procedures were carried out for each microorganism separately. The protocol was based on Figueira et al. (2021) [50].

### Multispecies cariogenic biofilm

Multispecies biofilms were formed by *C. albicans, L. acidophilus, S. mutans, S. gordonii, S. sanguinis*, according to Arthur et al. (2013), with modifications [53]. Two different microbial combinations were adopted. Firstly, multispecies biofilms were formed

with *S. mutans, S. gordonii*, and *S. sanguinis*. All microorganisms were activated on 5% sheep blood agar and incubated at 37°C, 5% $CO_2$ for 48 hours. Subsequently, the microorganisms were inoculated in BHI broth and incubated at 37°C, 5% $CO_2$ for 24 hours.

After the incubation period, the inoculum was centrifuged at 2,000 rpm for 5 minutes and washed twice with sterile saline solution (NaCl 0.9%). The suspensions of each microorganism were standardized at $10^7$ cells $mL^{-1}$ using a spectrophotometer (B582, Micronal, São Paulo, Brazil). After that, each microbial suspension was diluted to $8 \times 10^6$ cells $mL^{-1}$. An equal part of each suspension was deposited in a sterile tube to form a multispecies suspension and the tube was also homogenized. To form a multispecies biofilm, 40 µL of multispecies suspension and 160 µL of TSB supplemented with 1% sucrose were added to each experimental group well and the biofilms were formed into the plate surface. The plate was incubated at 37°C, 5% $CO_2$ for 48 hours and fresh culture medium was replaced after 24 h.

The same procedures were applied to form the biofilms with *C. albicans, L. acidophilus*, and *S. mutans*. Firstly, *S. mutans* was activated on 5% sheep blood agar, BHI agar for *Lactobacillus*, and SD agar for yeasts. *Lactobacillus* and *S. mutans* were incubated at 37°C, 5% $CO_2$ for 48 hours. *C. albicans* was incubated for 24 hours at 37°C. The inoculum was formed as described above. Then, the inoculum was centrifuged at 2,000 rpm for 5 minutes and washed twice with sterile saline solution (NaCl 0.9%). Each microbial suspension was standardized at $10^7$ cells $mL^{-1}$ in a spectrophotometer (B582, Micronal, São Paulo, Brazil). After that, *S. mutans* was diluted to $8 \times 10^6$ cells $mL^{-1}$ and *C. albicans* to $10^5$ cells $mL^{-1}$. The *L. acidophilus* suspension was maintained $10^7$ cells $mL^{-1}$. Equal parts of each suspension were transferred to a sterile tube to form a multispecies suspension. A volume of 40 µL of multispecies suspension and 160 µL of TSB broth supplemented with 1% sucrose were added to each well of each experimental group and the biofilms were formed into the plate surface. The plate was incubated at 37°C, 5% $CO_2$ for 48 hours and fresh culture medium was replaced after 24 h.

### Scanning electron microscopy (SEM) on multispecies biofilm

Based on the methodology proposed by de Oliveira et al. (2021) [54], standardized polystyrene specimens (5 $mm^2$) were pre-sterilized (overnight) with 2% glutaraldehyde solution, rinsed 10 times with sterile distilled water, then stored in 24-well plates and used for this experiment.

The multispecies biofilms were formed on top of those polystyrene test specimens, as previously described. The plate was incubated at 37°C, 5% $CO_2$ for 48 hours and fresh culture medium was replaced after 24 hours. Each well was washed with PBS twice, then the biofilm formed on top specimens was fixed. Glutaraldehyde solution (0.25%), paraformaldehyde (4%), and PBS (ratio per mL: 125: 400: 475 µL) were used in the fixation. After 20 minutes of fixation, the specimens contained in each well were washed with PBS and exposed to different dehydrating solutions, such as 70% alcohol (10 min), 90% alcohol (10 min), and 100% alcohol (10 and 20 min) [54]. The samples were dried at room temperature overnight. Finally, each sample fixed on the stub was metalized in vacuum with golden powder. The specimen analysis was performed in SEM (LEO 1460Vp, Carl Zeiss, USA).

### Confocal scanning electron microscopy (CSLM)

Biofilms were stained using the Live/Dead BacLightTM Bacterial Viability kit (Invitrogen-Molecular Probes, USA) to reveal the ratio of viable (green fluorescent) and dead (red fluorescent) cells. The labeling of live and dead cells was performed with a mixture of dyes A and B. For that, biofilms were first formed on the surface of sterile 13 mm diameter coverslips, distributed in sterile 24-well plates. For each well, live and dead cell marker dye was prepared by mixing 7.5 µL of staining component A (SYTO 9) and 7.5 µL of staining component B (propidium iodide). After washing the wells 3 times with sterile PBS at 1% phosphate buffer (PBS-pH 7.4), the samples were covered with 15 µL of the reagent mixture and incubated for 20 minutes at room temperature, protected from light. A series of images were obtained in the z section using a confocal microscope (LSM 700, Zeiss).

### Treatment with LTP and biofilm processing

The methodology was based on Borges et al. (2017) with modifications [52]. After 48 hours of biofilm formation, multispecies biofilms were exposed to LTP for 1, 3, 5, and 7 minutes according to the parameters described above (section 2.1). A 0.12% chlorhexidine digluconate solution and a physiologic solution (0.9% NaCl) were used as positive and negative controls, respectively. The biofilms were recovered from each well in 200 µL of sterile 0.9% NaCl solution using a pipette tip. The content was transferred to an Eppendorf ® tube with 600 µL of sterile 0.9% NaCl solution. The suspension was sonicated for 30s at an amplitude of 40 W (Vibra Cell™, Sonics, and Materials Inc., Newtown, USA). The

same procedures were performed for each experimental group and control.

For the multispecies biofilm formed by *S. mutans, S. gordonii*, and *S. sanguinis*, the microorganisms were plated on BHI 5% sheep blood agar and the total number of microorganisms recovered were counted and converted to CFU $mL^{-1}$ [50]. For biofilms composed of *C. albicans, L. acidophilus*, and *S. mutans*, the suspensions obtained were plated on selective agar. Sabouraud dextrose agar supplemented with chloramphenicol (0.1 mg $mL^{-1}$) was used for *C. albicans* and Mitis salivarius bacitracin (MSBS) (0.1 units $ml^{-1}$) supplemented with sucrose (1%) was used for *S. mutans*. For *L. acidophilus*, suspensions were plated in Rogosa agar supplemented with glacial acetic acid (1.32 mL $L^{-1}$) using the pour plate technique. After the incubation period, the number of colonies were counted. Sabouraud agar plates were incubated for 24 hours at 37°C. The MSBS agar plates were incubated for 48 hours at 37°C and 5% at $CO_2$. Finally, Rogosa agar plates were incubated for 72 hours at 37°C and 5% $CO_2$.

### Statistical analyses

The normal distribution of data was verified by the Shapiro-Wilk test and the homogeneity of variance was checked by the Levene test ($\alpha = 0.05$). To analyze the normality and homogeneity, the IBM SPSS statistical software package (version 25) for Windows (IBM Corp., New York, NY, USA) was used. Results were presented in mean values (± standard deviation), analyzed by ANOVA and Tukey's post-hoc test, considering $\alpha = 0.05$. For this analysis, the GraphPad Prism 5.0 program was used.

To calculate the surface ratio between dead and living cells, a quantification of colored pixels (red and green) from the CSLM images was performed automatically via a python (version 3.11) code (Pillow and NumPy libraries). The calculated ratio considered the percentage of red pixels (dead cell) among all detected stained pixels (dead and living) within one image.

## Results

### *Multispecies biofilms* – *S.* gordonii, S. mutans *and* S. sanguinis

Figure 2 shows a significant $\log_{10}$ CFU $mL^{-1}$ reduction in the counts of total microorganisms (*S. gordonii, S. mutans*, and *S. sanguinis*) in relation to negative control after exposure to LTP for 1, 3, 5, and 7 minutes ($p < 0.0001$). The LTP treatments for 5 and 7 minutes showed similar effects leading to a reduction of more than 2 $\log_{10}$ CFU $mL^{-1}$ (2.546 and 2.548 $\log_{10}$ CFU $mL^{-1}$, respectively). These reductions were similar to the observed after treatment with 0.12% chlorhexidine digluconate (Chx1) for 1 minute ($p < 0.0001$). Significant reductions in biofilms viability were detected after exposure to 0.12% chlorhexidine digluconate for 1, 3, 5, and 7 minutes ($p < 0.0001$).

### *Multispecies biofilms* – C. albicans, L. acidophilus *and* S. mutans

A significant reduction in the counts of *C. albicans* recovered from the multispecies biofilm was observed after exposures to LTP for 3, 5, and

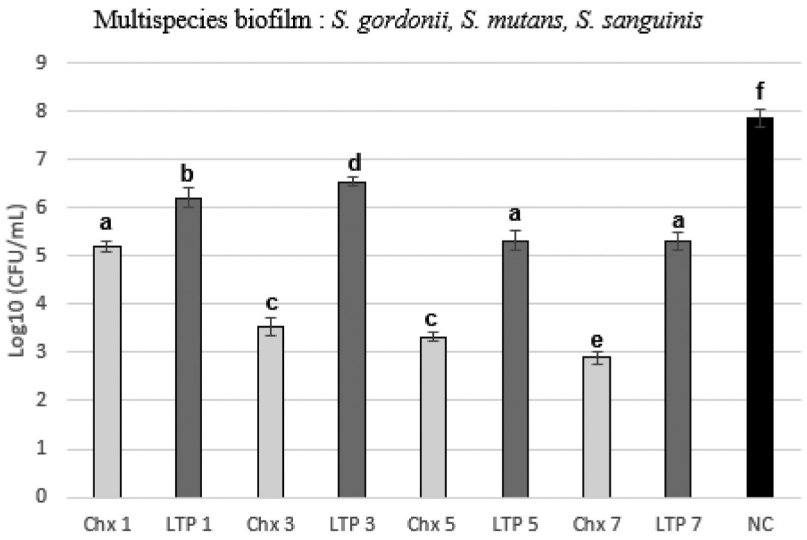


Figure 2.Mean values and standard deviations $\log_{10}$ (CFU/mL) of polymicrobial biofilms composed by *S. mutans, S. gordonii*, and *S. sanguinis* treated with low-temperature plasma (LTP) or chlorhexidine digluconate (0.12%) (Chx). Different letters indicate significant statistical difference ($n = 9$; $p \leq 0.05$; ANOVA, Tukey's Test). Chx1, Chx3, Chx5, and Chx7 (0.12% chlorhexidine digluconate treatment for 1, 3, 5, and 7 minutes), LTP1, LTP3, LTP5, and P7 (LTP treatment for 1, 3, 5 and 7 minutes), NC (negative control).

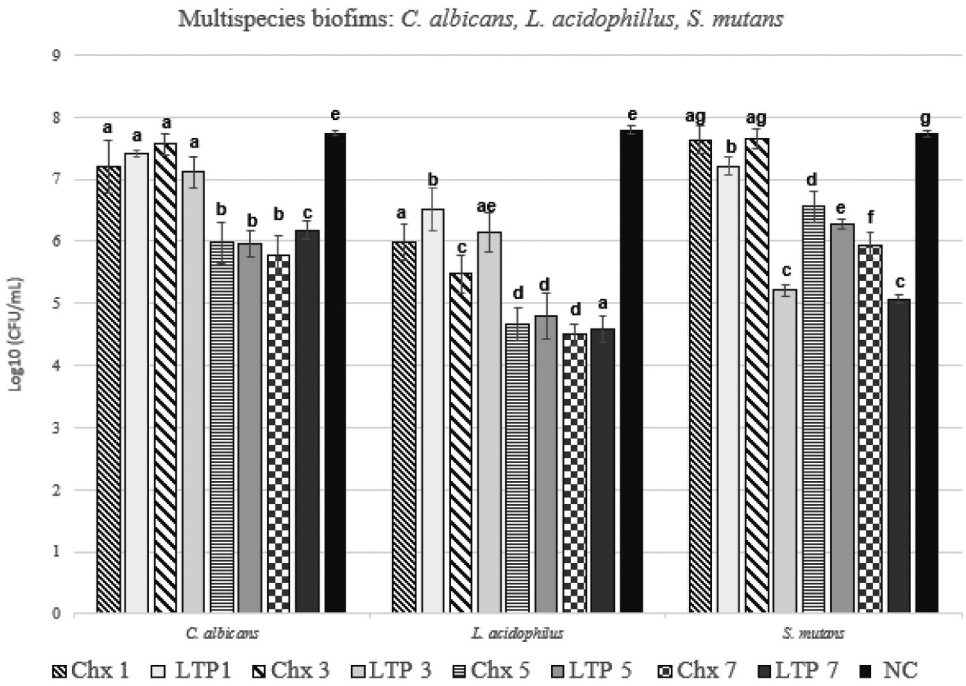


**Figure 3.**Mean values and standard deviations $\log_{10}$ (CFU/mL) of polymicrobial biofilms composed by *C. albicans, L. acidophilus*, and *S. mutans* treated with LTP or chlorhexidine digluconate (0.12%) (Chx). Different letters indicate significant statistical differences (n = 9; p ≤ 0.05; ANOVA, Tukey's Test). Chx1, Chx3, Chx5, and Chx7 (0,12% chlorhexidine digluconate treatment for 1, 3, 5, and 7 minutes), LTP1, LTP3, LTP5, and LTP7 (LTP treatment for 1, 3, 5, and 7 minutes), NC (negative control).

7 minutes when compared to the negative control ($p < 0.0001$) (Figure 3). The exposure to 0.12% chlorhexidine digluconate for the same periods also showed statistically significant cell counts reduction ($p < 0.05$) in relation to the negative control.

Similarly, significant reduction in *L. acidophilus* counts were detected after exposures to LTP for 1, 3, 5, and 7 minutes ($p < 0.0001$). The same results were observed after exposure to 0.12% chlorhexidine digluconate ($p < 0.05$). The cell count reductions after exposure to 0.12% chlorhexidine digluconate (Chx5 and Chx7) and LTP (P5 and P7) were similar.

The exposures to LTP for 1 and 3 minutes lead to 1.3 and 1.6 log10 CFU $mL^{-1}$ reduction, respectively. When the exposure durations were increased to 5 and 7 minutes, reductions higher than 2 $\log_{10}$ CFU $mL^{-1}$ were detected.

For *S. mutans* counts recovered from the multispecies biofilm, significant reduction in viable cell counts was detected after the exposure to LTP for 1, 3, 5 and 7 minutes. Treatment with chlorhexidine digluconate (0.12%) for 1 and 3 minutes (Chx1 and Chx3) showed statistically similar results when compared to the negative control ($p = 0.196$ and $p = 0.137$, respectively). However, the exposures for 5 and 7 minutes was significantly different when compared to the negative control ($p = 1.183$ and $p = 1.795$, respectively).

### Scanning electron micrography (SEM) images

The SEM images showed the effect of LTP on biofilms formed by *C. albicans, L. acidophilus*, and *S. mutans* (Figure 4a-c). Figure 4a presents the biofilm with no applied treatment and an intact surface can be observed. However, when comparing Figure 4a with Figure 4b (LTP treatment for 7 min), the damage caused by the LTP jet and its reactive oxygen species to the biofilm structure can be easily observed. The aggression caused by Chx 7 min of treatment on the biofilms is also visible when comparing Figures 4a,c. The damage caused by LTP was better observed or greater when compared with damages caused by Chx. Besides that, Figure 4 also shows the effect on biofilm formed by *S. gordonii, S. mutans*, and *S. sanguinis* (D, E, and F). A similar scenario was observed for this biofilm. Figure 4d represents the intact biofilm and no aggression is observed, once no treatment was applied. When comparing Figure 4d with Figure 4e (LTP treatment for 7 min) the damage caused by the LTP jet and its reactive oxygen species to the biofilm structure can also be observed. The aggression of Chx after 7 min (Figure 4f) of treatment can also be clearly observed when compared with Figure 4d. Here, the damage caused by LTP was also better observed when compared with the one caused by Chx.

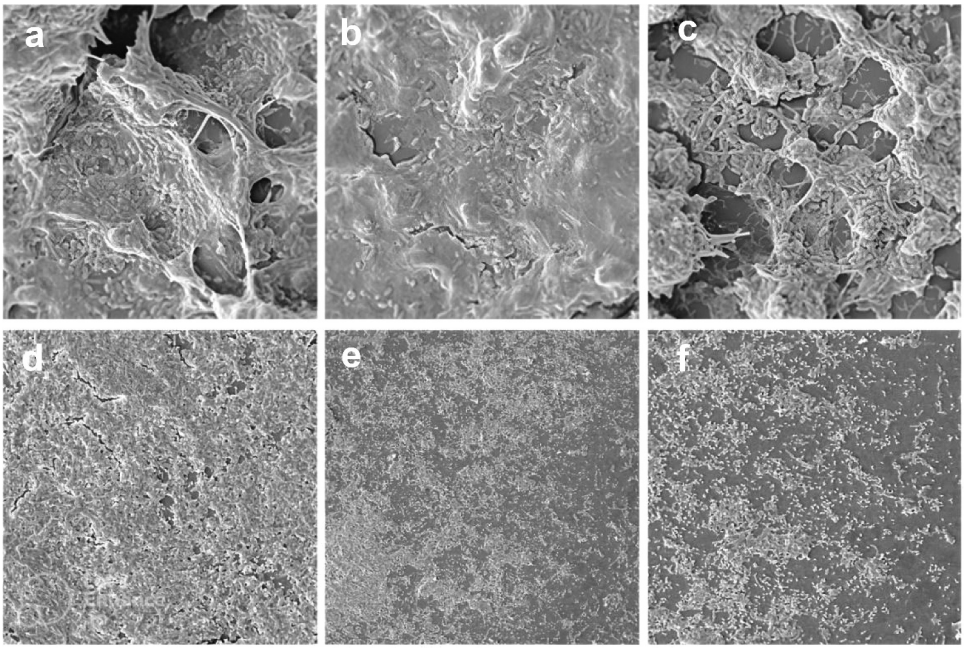


Figure 4. Scanning electron micrograph images of polimicrobial cariogenic biofilms formed by different microorganism's association *C. albicans, L. acidoppillus*, and *S. mutans* (a, b, and c); and *S. gordonii, S. mutans*, and *S. sanguinis* (d,e,f) exposed or not to LTP (×2000). Cariogenic biofilm formed by *C. albicans, L. acidophillus*, and *S. mutans*: negative control (A), LTP 7 minutes (b), and chlorhexidine digluconate (0.12%) (c), 7 minutes of treatment. Cariogenic biofilm formed by *S. gordonii, S. mutans*, and *S. sanguinis*: negative control (d), LTP 7 minutes (E), and chlorhexidine digluconate (0.12%) (f), 7 minutes of treatment.

### *Confocal scanning electron microscopy (CSLM) imagens*

Figure 5 presents the confocal scanning laser microscopy (CSLM) representative images, which show the morphology and structural organization of multi-species biofilm after treatment with LTP for 7 minutes. CSLM images show the morphology and structural organization of multispecies biofilms formed by *S. mutans, S. gordonii*, and *S. sanguinis* (A, B, and C) and *C. albicans, L. casei*, and *S. mutans* (D, E, and F) after treatment of biofilms by LTP-helium or chlorhexidine (0.12%).

Figure 5 shows the images of live cells stained green, whereas dead cells are stained red. The images obtained are of biofilms formed on the surface of sterile 13 mm diameter coverslips. Samples treated for 7 minutes with LTP-helium or also with chlorhexidine (0.12%) (Chx) showed a higher number of dead cells (stained in red) when visually compared with the negative control (NC) (higher number of living cells that are stained green).

An evaluation of the surface ratio between dead and living cells was carried out using the confocal microscopy images in Figure 5. The analysis was performed by quantifying the amount of red and green pixels and calculating the percentage equivalent to dead cells among all detected stained pixels (red plus green). This approach was preferred given the high amount of built cell clusters and overlap of cells that can be observed in Figure 5, hampering the identification of single cells and increasing the associated measurement uncertainty. For multispecies biofilms formed by *S. mutans, S. gordonii*, and *S. sanguinis* (A, B, and C), the evaluation resulted in an average surface dead ratio of 1.30% ± 0.22% for the negative control and up to 99.24% ± 0.35% and 98.54% ± 0.26% for multispecies biofilms treated with Chx7 and LTP for 7 minutes, respectively. The results for multispecies biofilms formed by *C. albicans, L. casei*, and *S. mutans* (D, E, and F), the evaluation resulted in an average surface dead ratio of 0.83% ± 0.23% for the negative control and up to 91.81% ± 0.39% and 88.54% ± 0.26% for multispecies biofilms treated with Chx7 and LTP for 7 minutes, respectively.

## Discussion

The results obtained in this work, suggest that helium LTP has the potential to reduce the viability of cariogenic biofilms.

Caries is caused by a highly organized polymicrobial structure biofilm that is embedded in a matrix formed by extracellular exopolysaccharides (EPS) [55,56]. In this study, the biofilms were formed by 5 different microbial species that are commonly found in cariogenic biofilms.

The viability of polymicrobial biofilms formed by *S. gordonii, S. mutans* and *S. sanguinis* reduced significantly after helium-LTP treatment compared to the negative control. This finding is in accordance

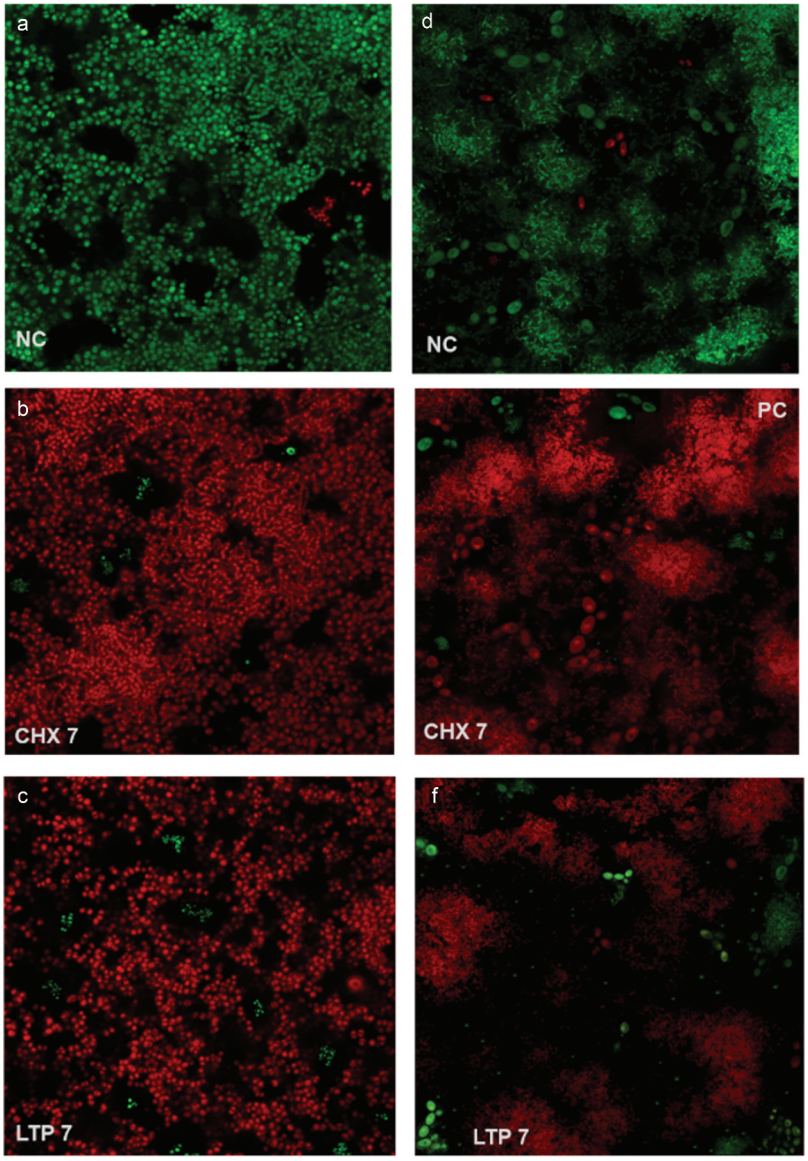


**Figure 5.**Confocal Scanning Laser Microscopy (CSLM) of cariogenic multispecies biofilms exposed to LTP-helium and 0.12% chlorhexidine (CHX 7) both for 7 min. The images represent the morphology and structural organization of the multispecies biofilm, negative control (NC), chlorhexidine (0.12%) (CHX 7), and exposure to LTP-helium for 7 min (LTP 7). Live cells are stained green, and non-viable cells are stained red.

to a previous study that tested the action of a commercially available LTP device (kINPen09™ – Leibniz Institute for Plasma Science and Technology, INP, Greifswald, Germany) [50] and observed inhibitory effects on single and multispecies biofilms formed by *S. mutans, S. sanguinis* and *S. gordonii* formed on hydroxyapatite disk. In this previous study, the exposure periods were 30, 60 and 120 seconds, but the working gas was argon and the parameters of operation of the devices were different.

Hong et al. (2016) investigated the effect of argon plasma treatment on *S. mutans* monospecies biofilms using an LTP brush. Argon flow and 4 mm of distance from the plasma tip to the sample were applied. The effect of LTP brush was analyzed on *S. mutans* biofilms formed on the surfaces of hydroxyapatite discs. After 1 minute of LTP brush treatment, a 90% reduction in biofilm viability was detected. Compared with the present study in which a different plasma source and LTP jet instead of LTP brush, our results were also significant [57]. Firstly, the plasma brush can treat a larger surface [57]. Secondly, the distance used in our study was bigger (15 mm), and lastly, the plasma jet can treat just a specific point, penetrating less than LTP brush [58] and also possibly forming less reactive oxygen species [59] compared with the plasma brush.

Another study that investigated the action of LTP argon/oxygen (distance of 5 mm from the plasma tip to the sample) on dual-species biofilms formed by *S. mutans* and *S. sanguinis* in polystyrene plates. After 120 seconds exposure time, viable cells reduced by 99% when compared with the negative control [60]. The total of viable cells in this study was lower than in our study, but the device, gases, and parameters used were different. A mix of different gases was used

and the distance from LTP tip to the sample was smaller.

Our investigation showed a significant reduction in total viable cells in all exposure times on multispecies biofilm formed by *S. mutans, S. gordonii*, and *S. sanguinis*. The longest exposure times (5 and 7 minutes) showed a reduction of more than 2 logs of $\log_{10}$ CFU/mL. Hirano et al. (2019) also analyzed the effect of a LTP prototype device on biofilm formed by *S. mutans* [61]. The distance between the LTP tip to biofilm surfaces was 1, 3, and 5 mm. The treatment times were 1, 3, 5, and 7 minutes. The treatment with LTP caused a significant reduction starting from 3 minutes of treatment. Our results showed a reduction of more than 2 $\log_{10}$. The comparison with the results reported by [61] with *S. mutans* monospecies biofilm, suggests the significance of our results since a multispecies biofilm is considered more resistant to anti-biofilm therapies [62]. Also, a greater distance from the LTP tip to the biofilm surface was adopted in our study, and this can be interesting to clinical application.

All microorganisms on polymicrobial biofilms formed by *C. albicans, L. acidophilus*, and *S. mutans* were significantly reduced after plasma exposure compared with the negative control. For *C. albicans*, in a study where the authors also used helium as the working gas, the biofilm viability had 2-log reduction from 5 minutes of exposure [52]. Our results corroborate with these previous observations with similar *C. abicans* reduction (approximately 2-log) in the multispecies biofilm after 5 and 7 minutes of treatment.

Another study analyzed the capacity of the kINPen09 to disrupt *C. albicans* cell structure using argon gas and LTP applying 10 mm distance from the tip of the plasma outlet to the biofilm surface. The authors reported, after LTP-treatment for 60 seconds, significant viability reduction in $\log_{10}$ CFU $mL^{-1}$ and changes in microbial morphology compared with controls [63].

The viability of *L. acidophilus* on multispecies cariogenic biofilm was also significantly reduced. Lactobacilli interact with *S. mutans* by different mechanisms [64] during the cariogenic biofilm formation process [33,34,65]. Carious process installation on the dental surface may be associated with the growth of *S. mutans* and lactobacilli and also with a decrease in the diversity of other species that can colonize the oral cavity [35,36,66]. Blumhagen et al. (2014) analyzed the action of argon plasma brush on *L. acidophilus* and *S. mutans* biofilms. The LTP treatment was performed with approximately 5 mm from the nozzle tip to sample surface for 3, 9, 12, 13, 15, and 18 seconds. Thirteen seconds of exposure time eliminated all the bacteria, when the biofilm was formed by low concentration of the inoculum, for both *L. acidophilus* and *S. mutans*. However, when the biofilms were formed by a high concentration of the inoculum, the biofilms showed higher resistance to LTP, and the reductions resulted in 2 log and 2.5 log for *L. acidophilus* and *S. mutans*, respectively. Compared with our study, they used different gas, smaller distance, and other parameters that could have contributed to their results [67]. In the present study, a significant reduction of 3.0 and 3.2 $\log_{10}$ was detected after 5 and 7 minutes exposure, respectively. Besides that, in our study, the equipment that generates plasma was able to form just a single plasma jet different from the plasma brush used by them. Nevertheless, our study showed higher log reduction, for both *L. acidophilus* and *S. mutans*.

In this study, we compared the LTP biofilm treatments with 0.12% chlorhexidine digluconate that is considered the gold-standard with wide antimicrobial spectrum [68]. In this sense, we can observe that treatment with chlorhexidine reduced the number of viable cells according to the exposure time. The antimicrobial effect was directly correlated to the period of exposition, corroborating the good effect of chlorhexidine on mature biofilm [69].

The choice of the combinations of microorganisms (*S. sanguinis, S. gordonii*, and *S. mutans* and *C. albicans, L. acidophilus*, and *S. mutans*) was based on the primary colonization on the surface of the teeth and on the potential of these microorganisms to metabolize dietary sugars, transforming the microbiota that normally resides in the oral cavity into an acidogenic, aciduric, and cariogenic population [70].

Some questions remain unanswered and the limitations of the basic research cannot clarify them. In our research, 96 wells plate was used as a substrate, which does not replicate natural teeth and the complex environment of the mouth, like the interactions between dental plaque with the salivary flow, pH, temperature, friction and interaction with other species of microorganisms.

In the future, better clarification of the LTP effect on other multispecies cariogenic biofilms formed *in situ* and *in vivo* will be necessary. Also, the effect of LTP on the activity of microbial virulence factors has not been investigated yet. The effect of LTP on the surrounding tissues and on restorative materials must be investigated as well. However, the results obtained in this work, which analyzed the effect of LTP on cariogenic biofilms, can contribute to the development of an *in situ* study in the future.

## Conclusion

Low-temperature helium plasma jet showed a significant inhibitory effect against multispecies cariogenic biofilms formed by two different associations of species: *S. mutans, S. gordonii*, and *S. sanguinis*; and *C. albicans, L. acidophilus*, and *S. mutans*.

The viability of biofilms formed by *S. mutans, S. gordonii*, and *S. sanguinis* was significantly reduced by 3 minutes of plasma treatment. When biofilms composed by *C. albicans, L. acidophillus*, and *S. mutans* were exposed to LTP, significant reduction in the viabilities of *L. acidophillus* and *S. mutans* were detected.

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## Author contributions

CYKI, LWF, KK: conception and design of the study. LWF, CYKI, BA, ACOCD, TN: acquisition of data, analysis, and interpretation of data. CYKI, LWF, KK: drafting the article and critical revision for important intellectual content. LWF, KK, CYKI, final approval of the version for submission.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

## Data availability statement

All data generated or analyzed during this study are included in this article and its supplementary material. Further inquiries can be directed to the corresponding author.

## Featured Application

Short exposure to low-temperature plasma is an effective way to inhibit multi-species biofilms related to dental caries. This is a promising technology that can be applied to new protocols development for dental caries prevention and treatment.

## Statements ethical

'Not applicable' for studies not involving humans or animals. All experimental studies on Low-Temperature plasma were complied with relevant institutional, national, and international guidelines and legislation".

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## ORCID

Leandro Wagner Figueira http://orcid.org/0000-0002-8504-2183
Ana Bessa Muniz http://orcid.org/0000-0003-4414-9854
Anelise Cristina Osorio Cesar Doria http://orcid.org/0000-0001-8533-420X
Thalita Mayumi Castaldelli Nishime http://orcid.org/0000-0003-2844-3156
Konstantin Georgiev Kostov http://orcid.org/0000-0002-9821-8088
Cristiane Y. Koga-Ito http://orcid.org/0000-0002-2416-2173

## References

[1] Kassebaum NJ, Smith AGC, Bernabé E, et al. Global, regional, and national prevalence, incidence, and disability-adjusted life years for oral conditions for 195 countries, 1990–2015: a systematic analysis for the global burden of diseases, injuries, and risk factors. J Dent Res. 2017;96(4):380–387.
[2] Tinanoff N, Baez RJ, Diaz Guillory C, et al. Early childhood caries epidemiology, aetiology, risk assessment, societal burden, management, education, and policy: global perspective. Int J Paediatr Dentistr. 2019;29(3):238–248.
[3] Pitts N, Baez R, Diaz-Guallory C, et al. Early childhood caries: IAPD Bangkok declaration. Int J Paediatr Dent. 2019;29:384–386.
[4] Rosier BT, Marsh PD, Mira A. Resilience of the oral microbiota in health: mechanisms that prevent dysbiosis. J Dent Res. 2018;97(4):371–380.
[5] Pitts NB, Zero DT, Marsh PD, et al. Dental caries. Rev Nat Rev Dis Primers. 2017;3:17030.
[6] Takahashi N, Nyvad B. The role of bacteria in the caries process. J Dent Res. 2011;90:294–303.
[7] Chen X, Daliri E, Banan-Mwine K, et al. Microbial etiology and prevention of dental caries: exploiting natural products to inhibit cariogenic biofilms. Pathogens. 2020;9(7):569.
[8] Nguyen PT, Nguyen TT, Bui DC, et al. Exopolysaccharide production by lactic acid bacteria: the manipulation of environmental stresses for industrial applications. AIMS Microbiol. 2020;6(4):451–469.
[9] Du Q, Fu M, Zhou Y, et al. Sucrose promotes caries progression by disrupting the microecological balance in oral biofilms: an in vitro study Sci Rep. 2020;10:2961.
[10] Xu X, Chen F, Huang Z, et al. Meeting report: a close look at oral biofilms and microbiomes. Int J Oral Sci. 2018;10(3):28.
[11] Hoare A, Marsh PD, Diaz PI. Ecological therapeutic opportunities for oral diseases. Microbiol Spectr. 2017;5(4):10.1128.
[12] Takahashi N, Nyvad B. The role of bacteria in the caries process: ecological perspectives. J Dent Res. 2011;90(3):294–303.
[13] Marsh PD. Dental plaque as a biofilm and a microbial community – implications for health and disease. BMC Oral Health. 2006;6(S1).
[14] Du Q, Fu M, Zhou Y, et al. Sucrose promotes caries progression by disrupting the microecological balance in oral biofilms: an in vitro study. Sci Rep. 2020;10(1):2961.
[15] Leme AFP, Koo H, Bellato CM, et al. The role of sucrose in cariogenic dental biofilm formation—new insight. J Dent Res. 2006;85(10):878–887.
[16] Cai J-N, Jung J-E, Dang M-H, et al. Functional relationship between sucrose and a cariogenic biofilm formation. PLoS One. 2016;11(6):e0157184.

[17] Hwang G, Liu Y, Kim D, et al. Simultaneous spatiotemporal mapping of in situ pH and bacterial activity within an intact 3D microcolony structure. Sci Rep. 2016;6(1):32841.
[18] Margarita S, Quintana C, Sjostrom IPD, et al. Microbiota de los ecosistemas de la cavidad bucal Microbiota of oral cavity ecosystems. Rev Cubana Estomatol. 2017;54(1):84–99.
[19] Mark Welch JL, Rossetti BJ, Rieken CW, et al. Biogeography of a human oral microbiome at the micron scale. Proc Natl Acad Sci U S A. 2016;113(6): E791–800.
[20] Zhu B, Macleod LC, Kitten T, et al. *Streptococcus sanguinis* biofilm formation & interaction with oral pathogens. Future Microbiol. 2018;13(8):915–932.
[21] Cui G, Li P, Wu R, et al. *Streptococcus mutans* membrane vesicles inhibit the biofilm formation of *Streptococcus gordonii* and *Streptococcus sanguinis*. AMB Express. 2022;12(1):154.
[22] Nobbs AH, Zhang Y, Khammanivong A, et al. Streptococcus gordonii Hsa environmentally constrains competitive binding by streptococcus sanguinis to saliva-coated hydroxyapatite. J Bacteriol. 2007;189 (8):3106–3114.
[23] Kalweit SF, Elder BL, Taylor PF. Antibodies that bind to fimbriae block adhesion of *Streptococcus sanguis* to saliva-coated hydroxyapatite. Inf Immun. 1985;48 (3):617–624.
[24] Ellepola K, Liu Y, Cao T, et al. Bacterial GtfB augments *Candida albicans* accumulation in cross-kingdom biofilms. J Dent Res. 2017;96(10):1129–1135.
[25] Bachtiar EW, Bachtiar BM. Relationship between *Candida albicans* and *Streptococcus mutans* in early childhood caries, evaluated by quantitative PCR. F1000 Res. 2018;7:1645.
[26] Li Y, Huang S, Du J, et al. Current and prospective therapeutic strategies: tackling *Candida albicans* and *Streptococcus mutans* cross-kingdom biofilm. Front Cell Infect Microbiol. 2023;13:1106231.
[27] Falsetta ML, Klein MI, Colonne PM, et al. Symbiotic relationship between streptococcus mutans and *Candida albicans* synergizes virulence of plaque biofilms in vivo. Infect Immun. 2014;82(5):1968–1981.
[28] Kim H-E, Liu Y, Dhall A, et al. Synergism of *Streptococcus mutans* and *Candida albicans* reinforces biofilm maturation and acidogenicity in saliva: an *in vitro* study. Front Cell Infect Microbiol. 2021;10:623980.
[29] Hoshino T, Fujiwara T. The findings of glucosyltransferase enzymes derived from oral streptococci. Jap Dent Sci Rev. 2022;58:328–335.
[30] Savabi O, Kazemi M, Kamali S, et al. Effects of biosurfactant produced by Lactobacillus casei on gtfB, gtfC, and ftf gene expression level in *S. mutans* by real-time RT-PCR. Adv Biomed Res. 2014;3(1):231.
[31] Wen ZT, Huang X, Elle[ K, et al. Lactobacilli and human dental caries: more than mechanical retention. Microbiology (Reading). 2022;168(6):001196.
[32] Zhang J, Xu X. Research progress in the relationship between lactobacillus and dental caries. Sichuan Da Xue Xue Bao Yi Xue Ban. 2022;53(5):929–934.
[33] Caufield PW, Schön CN, Saraithong P, et al. Oral lactobacilli and dental caries: a model for niche adaptation in humans. J Dent Res. 2015;94(9_suppl):110S–8S.
[34] Sounah SA, Madfa AA. Correlation between dental caries experience and the level of Streptococcus mutans and lactobacilli in saliva and carious teeth in a Yemeni adult population. BMC Res Notes. 2020;13(1):112.
[35] Caufield PW, Dasanayake AP, Li Y, et al. Natural history of streptococcus sanguinis in the oral cavity of infants: evidence for a discrete window of infectivity. Inf Immun. 2000;68(7):4018–4023.
[36] Showkat N, Singh G, Singla K, et al. Minimal invasive dentistry: literature review. Curr Med Res Opin. 2020;3(9):631–636.
[37] Liu S, Wu T, Zhou X, et al. Nicotine is a risk factor for dental caries: an *in vivo* study. J Dent Sci. 2018;13:30–36.
[38] Subramaniam P, Nandan N. Effect of xylitol, sodium fluoride and triclosan containing mouth rinse on *Streptococcus mutans*. Contemp Clin Dent. 2011;2:287–290.
[39] Zero DT. Dentifrices, mouthwashes, and remineralization/caries arrestment strategies. BMC Oral Health. 2006;6(Suppl 1):S9.
[40] Wijesekera RO. Historical overview of the cinnamon industry. CRC Crit Rev Food Sci Nutr. 1978;10:1–30.
[41] Coelho CS, Fedechen MC, Volpini RC, et al. Evolution of the dental technique of atraumatic restorative treatment. Res Soc Dev. 2020;9(3): e74932439.
[42] Neyts EC, Brault P. Molecular dynamics simulations for plasma-surface interactions. Plasma Process Polym. 2016;14:1–2.
[43] Kong MG, Kroesen G, Morfill G, et al. Plasma medicine: an introductory review. New J Phys. 2009;11:115012.
[44] Thomas S, Mozetič M, Cvelbar U, et al. Non-thermal plasma technology for polymeric materials: applications in composites, nanostructured materials, and biomedical fields. eBook: Elsevier;2019.
[45] Alemán C, Fabregat G, Armelin E, et al. Plasma surface modification of polymers for sensor applications. J Mater Chem B. 2018;6:6515–6533.
[46] Domonkos M, Ižák T, Varga M, et al. Diamond nucleation and growth on horizontally and vertically aligned Si substrates at low pressure in a linear antenna microwave plasma system. Diam Relat Mater. 2018;82:41–49.
[47] Ollegott K, Wirth P, Oberste-Beulmann C, et al. Fundamental properties and applications of dielectric barrier discharges in plasma-catalytic processes at atmospheric pressure. Chem Ing Tech. 2020;92:1542–1558.
[48] Yang JB, Chen Q, Yu H, et al. Oral bacterial deactivation using a low-temperature atmospheric argon plasma brush. J Dent. 2011;39(1):48–56.
[49] Blumhagen A, Singh P, Mustapha A, et al. Plasma deactivation of oral bacteria seeded on hydroxyapatite disks as tooth enamel analogue. Am J Dent. 2014;27 (2):84–90.
[50] Figueira LW, Panariello BH, Koga-ito CY, et al. Low-temperature plasma as an approach for inhibiting a multi-species cariogenic biofilm. Appl Sci. 2021;11 (2):570.
[51] Kostov KG, Machida M, Prysiazhnyi V, et al. Transfer of a cold atmospheric pressure plasma jet through a long flexible plastic tube. Plasma Sources Sci Technol. 2015;24(2):025038.
[52] Borges AC, Nishime TMC, Kostov KG, et al. Cold atmospheric pressure plasma jet modulates *Candida albicans* virulence traits. Clin Plasma Med. 2017;7 (5):9–15.
[53] Arthur RA, Waeiss RA, Hara AT, et al. A defined-multispecies microbial model for studying enamel caries development. Caries Res. 2013;47(4):318–324.

[54] de Oliveira MA, da C Vegian MR, Brighenti FL, et al. Antibiofilm effects of *Thymus vulgaris* and *Hyptis spicigera* essential oils on cariogenic bacteria. Future Microbiol. 2021;16:241–255.
[55] Bowen WH, Koo H. Biology of *Streptococcus mutans* derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. Caries Res. 2011;45:69–86.
[56] Rezende G, Arthur RA, Lamers ML, et al. Structural organization of dental biofilm formed in situ in the presence of sucrose associated to maltodextrin. Braz Dent. 2019;30(1):36–41.
[57] Hong Q, Dong X, Chen M, et al. Disinfection of *Streptococcus mutans* biofilm by a non-thermal atmospheric plasma brush. Jpn J Appl Phys. 2016;55 (7S2):07LG02.
[58] Xiong Z, Du T, Lu X, et al. How deep can plasma penetrate into a biofilm? Appl Phys Lett. 2011;98(22):221503.
[59] Laroussi M. Low-temperature plasma jet for biomedical applications: a review. Plasma Sci IEEE Trans. 2015;43(3):703–712.
[60] Liu T, Wu L, Babu JP, et al. Effects of atmospheric non-thermal argon/oxygen plasma on biofilm viability and hydrophobicity of oral bacteria. Am J Dent. 2017;30:52–56.
[61] Hirano Y, Hayashi M, Tamura M, et al. Singlet oxygen generated by a new nonthermal atmospheric pressure air plasma device exerts a bactericidal effect on oral pathogens. J Oral Sci. 2019;61:521–525.
[62] Li Q, Liu L, Guo A, et al. Formation of multispecies biofilms and their resistance to disinfectants in food processing environments: a review. J Food Prot. 2021;84(12):2071–2083.
[63] Delben JA, Zago CE, Tyhovych N, et al. Effect of atmospheric-pressure cold plasma on pathogenic oral biofilms and in vitro reconstituted oral epithelium. Plos One. 2016;11(5):e0155427.
[64] Savabi O, Nejatidanesh F, Bagheri KP, et al. Prevention of crosscontamination risk by disinfection of irreversible hydrocolloid impression materials with ozonated water. Int J Prev Med. 2018;9(1):37.
[65] Wen ZT, Huang X, Ellepola K, et al. Lactobacilli and human dental caries: more than mechanical retention. Microbiology (Reading). 2022;168(6):001196.
[66] Li Y, Ge Y, Saxena D, et al. Genetic profiling of the oral microbiota associated with severe early-childhood caries. J Clin Microbiol. 2007;45(1):81–87.
[67] Blumhagen A, Singh P, Mustapha A, et al. Plasma deactivation of oral bacteria seeded on hydroxyapatite disks as tooth enamel analogue. Am J Dent. 2014;27(2):84–90.
[68] Nayak SU, Kumari A, Rajendran V, et al. Comparative evaluation of efficacy of chlorhexidine and herbal mouthwash as a preprocedural rinse in reducing dental aerosols: a microbiological study. Int J Dent. 2020;4:1–6
[69] Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol. 2010;8:623–633.
[70] Schwendicke F, Frencken JE, Bjørndal L, et al. Managing carious lesions: consensus recommendations on carious tissue removal. Adv Dent Res. 2016;28(2):58–67.