Archives of Current Research International

Volume 24, Issue 6, Page 315-334, 2024; Article no.ACRI.120444
ISSN: 2454-7077

Effect of Nonthermal Atmospheric Plasma on *S. mutans* Biofilms

Héllen Súzany Freire Silva [a], Adália Samara Gadelha de Holanda Lima [a], Lidiany Karla Azevedo Rodrigues [b], Simone Duarte [c,d] and Iriana Carla Junqueira Zanin [e*]

[a] Postgraduate Program in Dentistry, Federal University of Ceará, Brazil.
[b] Department of Dental Clinic, Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceará, Brazil.
[c] School of Dental Medicine, University at Buffalo, USA.
[d] Department of Cariology, Operative Dentistry and Dental Public Health, Pardue University, Indianapolis, USA.
[e] Faculty of Dentistry, Federal University of Ceará, Campus Sobral, Brazil.

Authors' contributions

This work was carried out in collaboration among all authors. Authors ICJZ and SD conceived the study, wrote the protocol and managed the study analyses. Authors HSFS and ASG de HL performed the experiments, wrote the first draft of the manuscript and managed the bibliographic searches. Authors LKAR performed the statistical analysis. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/acri/2024/v24i6790

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/120444

Original Research Article

Received: 20/05/2024
Accepted: 23/07/2024
Published: 25/07/2024

**Corresponding author: Email: iriana.zanin@ufc.br;*

Cite as: *Silva, Héllen Súzany Freire, Adália Samara Gadelha de Holanda Lima, Lidiany Karla Azevedo Rodrigues, Simone Duarte, and Iriana Carla Junqueira Zanin. 2024. "Effect of Nonthermal Atmospheric Plasma on S. Mutans Biofilms". Archives of Current Research International 24 (6):315-34. https://doi.org/10.9734/acri/2024/v24i6790.*

ABSTRACT

Aims: Verify the effect of non-thermal atmospheric plasma on biofilms under different conditions.
Study Design: *In vitro* laboratory, Experimental, Randomized.
Place and Duration of Study: Federal University of Ceará, 2 years.
Methodology: Biofilms of *Streptococcus mutans* UA159 were formed on human enamel slabs, submitted to episodes of sucrose exposure for 5 days. The specimens were randomly divided in 2 different experiments; one submitted to daily plasma treatment of biofilms and other submitted to one-time treatment of the biofilms after 5 days. The samples were divided in nine groups as follows: NT (No treatment), CHX (chlorhexidine 0.12%), NaF (sodium fluoride 0.05%), ARG1 (1 minute argon flow), ARG5 (5 minutes argon flow), ARG10 (10 minutes argon flow), PLA1 (1 minute plasma jet), PLA5 (5 minutes plasma jet), PLA10 (10 minutes plasma jet).
Results: The groups CHX, ARG 1, ARG 5, ARG 10, PLA1, PLA5, PLA10 significantly reduced the bacterial viability in the daily treated biofilms ($p < 0.001$). The same results were only observed in mature biofilms submitted to one time treatment only for the groups PLA5 and PLA10 ($p < 0.005$). The results showed a reduction in soluble extracellular polysaccharides in CHX, ARG and PLA dairy treatments. The percentage of the superficial hardness loss demonstrated a significant reduction only for the CHX group ($p < 0.05$) in daily treatment. In addition, no statistically significant differences were found between the groups before ($p = 0.6978$) or after treatments ($p = 0.8904$) in the mineral profile of the specimens by Raman Spectroscopy. Also, scanning electron microscopy showed no difference in the topographic of the treated enamel surface.
Conclusion: Dairy ARG or PLA treatments were effective in reducing the number of viable bacteria and the concentration of water-soluble polysaccharide in the *S. mutans* biofilms. For the one-time treatment of mature biofilms only PLA5 and PLA10 treatments were effective. No alteration in the surface of the enamel was observed on tested conditions.

Keywords: Dental plaque; dental caries; plasma gases; streptococcus mutans; biofilm-dependent disease; tooth surface.

1. INTRODUCTION

Dental caries is a biofilm-dependent disease [1] and the only cause is dietary sugars [2]. In addition, it is the most common noncommunicable disease worldwide, according to the World Health Organization - WHO (2022) [3]. It is well established that after ingestion of fermentable sugars (glucose, sucrose or fructose), the pH in the plate falls from neutral to pH 5.0 or lower [2].

The biofilm is composed of complex structures, formed on the oral tissues, incorporated in a matrix of extracellular polysaccharides [4,5,6,7]. Bacteria in biofilms show a higher virulence compared to their planktonic counterparts [5]. Polysaccharides promote adherence and accumulation of cariogenic *Streptococcus* on the tooth surface, enhancing their pathogenic potential and contributing to the bulk and structural integrity of the matrix, besides hindering the diffusion of antimicrobial agents [8].

An estimated number of more than 700 bacterial species were found within the oral biofilm [9]. Cariogenic bacteria, such as *Streptococcus mutans*, can degrade various carbohydrates, synthesize EPS and acids, and reside in biofilms [10]. The resistance of *S. mutans* in the biofilm occurs, therefore, the EPSs produce exoenzymes, such as glucosyltransferases (Gtfs) derived from *S. mutans* present in the acquired film [11,12]. In the presence of sucrose, the Gtfs bound to different microorganisms produce large amounts of glucans *in situ* [13,14,15]. Glucans with most α1-6 bonds are called dextrans (with flexible chains) and those with α1-3 predominance are called mutanos (highly insoluble and rigid chains) [16,17], making it difficult to disorganize the biofilm.

Currently, in dentistry, new methods have been selectively killing pathogenic microorganisms present in oral biofilms [18] using technologies that do not result in the emergence of resistant strains. In this context, non-thermal atmospheric plasmas (NTAP) appear as a promising alternative treatment with several biomedical applications [19,20]. One of that is working as a potent anti-biofilm agent [21,22] by presenting antiseptic properties against many microorganisms [23] such as Candida albicans biofilms [24]. Also, plasma treatment has effect in

healing periodontal wounds [25] and is a treatment option for a variety of dermatological diseases [26].

Plasma is the fourth state of matter, consisting of a neutral ionized gas, generated from a variety of gases or gas mixtures such as argon, helium, ozone or oxygen gas. It consists of particles in permanent interaction, which include photons, electrons, positive and negative ions, atoms, free radicals and excited and unmoved molecules [27,28], which may contribute to its antibacterial properties. Reactive species are transiently generated by NATP through various pathways of collisions and dissociations. These reactive oxidant species have strong effects on the outer structures of cells. Since the cell membrane and spore coatings have been partially degraded, reactive species can damage the genetic material and the molecules within the microorganism, leading to its destruction [29,30].

NTAP have shown no adverse effects and provide evidence of the safety of the technology for various biomedical and medical applications [31,32]. Previous study shows that NTAP treatment can inhibit the formation of a matrix-rich biofilm [31,33] suggesting that it could be used in several oral care situations in which mechanical removal of the biofilm is indicated. This work advances the existing knowledge in the field by providing comprehensive evidence on the efficacy of non-thermal atmospheric plasma (NTAP) in bacterial viability and extracellular polysaccharide concentration in biofilms of *Streptococcus mutans* comparing the use of daily treatment versus one-time treatment efficacy. This study also analyzed if there are significant changes in the mineral profile of enamel or in surface topography after NTAP treatments. This is a critical observation as, if NTAP does not compromise the structural integrity of enamel while effectively reducing biofilm-related bacteria, the technique could be safe for clinical use.

2. MATERIALS AND METHODS

2.1 Experimental Design

In order to analyze the plasma effect on viability of tested biofilms, the treatments were performed in two different experimental situations: 1-treatment performed on biofilms twice a day during the five days of biofilm formation; 2-treatment performed only once on mature biofilms on the fifth day of biofilm formation. For each treatment condition, 162 human enamel specimens (4x4x2 mm) were randomly allocated into 9 groups (18 specimens per replicate). Six specimens of each group were used to microbial viability (CFU/ml) followed by RAMAN Spectroscopy and Scanning Electron Microscopy (SEM) analysis. Another six specimens were used for Extracellular Polysaccharide (EPS) measurement and three specimens were used for Confocal Microscopy analysis. To minimize the inherent bias related to microbiological procedures, three independent biological replicates were performed at different time points. The treatment conditions in which biofilms were exposed are as follow: NT (No treatment), CHX (chlorhexidine 0.12%), NaF (sodium fluoride 0.05%), ARG1 (1 minute argon flow), ARG5 (5 minutes argon flow), ARG10 (10 minutes argon flow), PLA1 (1 minute plasma jet), PLA5 (5 minutes plasma jet), PLA10 (10 minutes plasma jet).

The NTAP was generated through ionization of argon gas (Ar) at atmospheric pressure using the device Kinpen (INP, Germany) developed by Leibniz Institute for Plasma Science and Technology (INP) [20]. The device consists of a hand-held unit (170 mm in length, 20 mm in diameter and 170 g) for generation of plasma jet, a dc power supply (system power: 8Wat 220 V, 50/60 Hz), and a gas supply unit. The non-thermal atmospheric plasma jet was generated from the top of the centered electrode and expanded to the surrounding air outside the nozzle [34]. The treatment of biofilm samples was conducted at plasma tip-to-sample distance of 3 mm during the previously set times (1, 5 or 10 minutes) in continuous working mode [21], in the center of the samples. The experiment was conducted by two calibrated operators (HSFS and ASGHL) that was trained to perform the experiment in a standardized manner to minimize bias. The plasma plume emerging at the exit nozzle is about 1.5 mm in diameter. The argon gas flow was set to 5 L/min and the flow rate was controlled by a flow controller (MKS Instruments, Germany).

2.2 Specimen Preparation

For this randomized *in vitro* experiment, 324 human third molars extracted for reasons other than this research were stored in 0.01% (v/v) thymol solution at 4°C for 30 days until be used [35,36]. Teeth with fractures and cracks were excluded. Enamel specimens with 4x4x2mm were obtained using a water-cooled Diamond

discs (Extec Corp. ®, Enfield, CT, USA) and a cutting machine (Isomet Low Speed Saw, Buehler, Lake Bluff, IL, USA) [37]. The adjustment of the enamel to obtain flat specimens was done with the aid of a low-speed polishing machine (Arotec, Aropol 2v-PU, Sao Paulo, SP, Brasil) and 320 grift paper (Carbimet Paper discs, Buehler-Met ®, IL, USA) under water-cooling. Afterwards, the specimens were polished using waterproof papers (Buehler, Lake Bluff, IL, USA) 400, 600 and 1200 grift followed by felt disc with 1µm diamond paste (Buehler®, lllinois, 125 USA). The specimens were stored in humidity until be used.

2.3 Selection of Specimens

The Surface Microhardness (SMH) test was performed using a Knoop indenter with a 25g load for 5s in a Future-Tech FM Microhardness Tester coupled to the FM-ARS software (Future-Tech Corp., Tokyo, Japan). Initial SMH measurements were made in the center of the enamel surface in a row of five indentations. For selection purposes, enamel specimens with SMH values ranging from 274 and 335Knoop (305 ± 10%, respectively) hardness number were selected [38]. The selected specimens were randomically distributed in 9 groups using Excel software.

Frame 1 – Description of treatments

Code	Treatment
NT	No treatment for 10 minutes (negative control)
CHX	Chlorhexidine 0.12% for 10 minutes (anti-plaque control)
NaF	Sodium fluoride 0,05% for 10 minutes (anti caries control)
ARG1	1 minute argon flow
ARG5	5 minutes argon flow
ARG10	10 minutes argon flow
PLA1	1 minute plasma jet
PLA5	5 minutes plasma jet
PLA10	10 minutes plasma jet

2.4 Formation of the Acquired Film

Initially, a pool of stimulated human saliva was collected from 2 health donators, was diluted 1:1 v/v in adsorption buffer (KCl 50mM, KPO_4 1,0 mM, CaCl 1,0 mM, $MgCl_2$ 0,1 mM, pH = 6,5) and Phenylmethylsulphonyl fluoride - PMSF in the proportion of 1:1000 v/v. The solution was vortexed and clarified by centrifugation at 8,500 rpm, 10 minutes, 4 °C followed by vacuum filtration in a 0.22 µm pore filter (Stericup, Millipore, St Louis, MO, USA) [39]. To form the acquired enamel pellicle, the specimens were incubated for 1 hour at 37 °C in an orbital agitator (Te-145, Tecnal, Piracicaba, SP, Brasil).

2.5 *In vitro* Biofilm Formation

S. mutans inoculum was prepared from 5-8 isolated colonies inoculated in tryptone yeast-extract broth (DIFCO, Detroit, Michigan, USA) containing 1 % glucose (w/v) and incubated for 18 h at 37°C under microaerophilic conditions in partial atmosphere of 5 % of CO_2. Saliva coated enamel specimens were transferred to a 24 well-plate containing Tryptic Soy Broth and Yeast extract (TSB-YE - DIFCO, Detroit, Michigan, USA) media with 1% of sucrose inoculated with *S. mutans* inoculum and incubated as previously described. The biofilms were then formed on specimens surfaces for 120h with the culture medium being replaced at each 24 hours [39].

2.6 Biofilms Treatments

Twice-daily treatment (Experiment 1) or one time treatment (Experiment 2) formed biofilms were washed in NaCl 0.89% solution and then submitted to treatments. The biofilms of experiment 1 were exposed to the different treatments twice daily (8 a.m. and 4 p.m.) until the fifth day of the experimental period to analyze the effects of NTAP on the biofilm formation. To evaluate the effect of NTAP on mature biofilms, treatments were held only one time on the fifth day of biofilm formation. The biofilms of the groups ARG1, ARG5, ARG10, PLA1, PLA5 and PLA10 were exposed to treatment described in Frame 1 based on a previous study [40]. The specimens of the CHX [41] and NaF treatments were immersed in 2mL of the respective solution for 10 minutes and the specimens of the NT group did not pass any treatment, waiting at room temperature for 10 minutes. Next, the specimens of all groups were dipped 3 times in 1mL of saline solution (NaCl 0.89%), to remove the weakly adhered biofilm, and transferred to a new culture medium (in the case of biofilms treated daily) [39].

2.7 Bacterial Viability

The biofilms were then scraped with a sterile spatula and transferred to a sterile tube containing 5 ml of NaCl 0.89% solution. The samples were vortexed and to disperse the biofilm, 3 pulses of 15 s with a 15 s interval were

performed (on ice) at an output of 7 W (Sonifier450D, São Bernado do Campo, SP, Brazil). Ten-fold serial dilutions were carried out into a pre-weighed microtube containing 1 ml of NaCl 0.89% solution and aliquots of pure and diluted samples were plated onto blood agar and were then incubated at 37 ° C, 5% CO_2 for 48 h before enumerating the viable microorganisms. The results were expressed as colony-forming units (CFU) per milligram of biofilm [42,43].

2.8 Dry Weight

For the dry weight determination, three volumes of cold ethanol (-20°C) were added to 1 ml biofilm suspension, and the resulting precipitate was centrifuged (8,500 rpm for 10 min at 4°C). The supernatant was discarded, and the pellet was washed with cold ethanol, and then lyophilized and weighed [39].

2.9 Polysaccharide Analysis

Soluble and insoluble extracellular polysaccharides (EPS-soluble and EPS-insoluble, respectively) were analyzed. The polysaccharide content was expressed per µg. Briefly, 5 ml of the suspension was sonicated for 30-s pulses at an output of 7 W and centrifuged at 8,500 rpm for 10 min at 4°C. The supernatant was collected, and the biofilm pellet was resuspended and washed in 4 mL of milli-Q water; this procedure was repeated three times. The supernatant was used for the EPS-soluble assay and biofilm pellet was used for the EPS-insoluble. All the supernatants were pooled, three volumes of cold ethanol were added, and the resulting precipitate was collected by centrifugation and resuspended in 4 mL Milli-Q water and the total amount of carbohydrate was determined by the phenol–sulfuric acid method [44] by spectrophotometry with wavelength of 490nm. The EPS-insoluble was extracted using 1 N NaOH (1 mg biofilm dry weight/0.3 ml of 1 N NaOH) under agitation for 1 h at 37°C. The supernatant was collected by centrifugation, and the precipitate was resuspended again in 1 N NaOH; this procedure was repeated three times. The total amount of carbohydrate was also determined by the phenol–sulfuric acid method [44]. The polysaccharide content was expressed by mg of dry weight of the biofilm. The readings were performed at triplicate.

2.10 Confocal Electronic Microscopy

The enamel specimens were placed into 24-well plate, biofilms upwards, and 1 mL of Milli-Q water containing 3 µL of live/dead stain containing SYTO 9 and propidium iodide (PI) (Molecular Probes, Eugene, OR, USA) carefully added without disturbing the samples [21,45]. After incubation in the dark for 30 min, the biofilms were examined with ZEISS LM 710 confocal laser-scan (Microscopy GmbH, Oberkochen, Baden-Württemberg, Germany) at wavelengths of 488 nm equipped with a 20x lens. The resulting collections of confocal optical sections were collected ZEN 2.3 SP1 (Black) software as stacks of images. The images were subsequently analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA) to produce xy projections (the sum of pixel brightness in the z-axis). Each sample was processed and analyzed individually and five images from each biofilm were selected from randomly chosen areas [46].

2.11 Surface Microhardness Analysis

For the determination of changes in the enamel surface after exposure to the different treatments, the specimens of enamel (from experiment 1 and from experiment 2) were again subjected to the hardness test using a Knoop indenter with a 25 g load for 5 s. Afterwards the treatments SMH measurements were repeated on the right side of the five baseline indentations. Also, the percentage of surface microhardness change (%SMC) was calculated (%SMC = SMH after treatment – SMH baseline x 100/ SMH baseline) [40,47].

2.12 Raman Spectroscopy

The Raman spectra were acquired on a Raman spectrometer (Xplora HORIBA, 212 Kyoto, Japan). For excitation of the samples, it was used a laser operating at 638 nm wavelength, 1% and grid 600 in the LABSPEC6 program (HORIBA). The enamel slab sample was placed onto a plate, the laser beam was focused on the slabs surface using a microscope (OLYMPUS, Japan) with lens of 100x and numerical aperture 17mW forming a spot approximately 1 µm over the surface of the sample and three points were chosen for Raman measurement with an exposure time of 10s. The first point was in the central region of the enamel specimen, and the others were located 1 mm to the right and left of this first point [48]. The Raman system was calibrated with a silicon semiconductor using the Raman peak at 521 cm^{-1}. All spectra were taken and collected in the region from 800 to 2000 cm^{-1} and allowed a characterization of mineral content (phosphate and carbonate). To

normalize the measurements and allow their comparison, the area formed by the peaks in each analysis was calculated. Six specimens of each group of the microbiological analysis, were subjected to this test totalizing 54 specimens from each experiment. The curve was identified for each band using an OriginPro 9.0 32 bit software system (Operating System: 7, Copyright 2012 by OriginLab Corporation, Northampton: MA. USA).

2.13 Scanning Electron Microscopy (SEM)

For SEM, samples from human enamel specimens rinsed with distilled water, were vacuum dried for 24h and then mounted on a SEM stub (aluminum discs), fixed with double sided adhesive carbon tape, coded, and mounted onto aluminum stubs with Acheson silver DAG (Agar Scientific, U.K.) and then coated with a 20 nm thick layer of gold, using a Polaron SEM coating unit. The specimens were then examined using an SEM (Inspect™ S50, Jeol, Tokyo, Japan), operating at 20 kV [49] and working distance 10mm. Images were taken on magnification of 10000x in surface of enamel specimens and captured using a specific software (EDS Software for SEM, Oxford instruments). The images were analyzed visually.

2.14 Statistical Analysis

The sample size was calculated using data from a pilot study, assuming an average effect size of 0.5%, significance level of 5% and statistical power of 95%. The BioEstat5.3 software program for Windows (version 5.3, 2007, Belém, Pará, Brazil) was used. Initially, the distribution of normality was verified in the Kolmogorov-Smirnov statistical test. The mean and standard deviation (SD) of the number of surviving microorganisms for each treatment were calculated. The colony forming units (CFU) were transformed into CFU log10 with the objective of reducing the variance heterogeneity. To determine the difference between the values of the test groups with the controls, the Kruskal-Wallis non-parametric test followed by the Dunn test (multiple comparison test) was performed. The final surface microhardness data underwent linear transformation to perform one-way ANOVA variance analysis, followed by the Tukey test. In Raman spectroscopy, to standardize the measurements and allow comparison among groups, the area of the peaks was used as a parameter. Peak area averages were compared through one-way ANOVA variance analysis.

3. RESULTS AND DISCUSSION

The study demonstrates that daily treatments with NTAP significantly reduce bacterial viability in S. mutans biofilms. This finding supports the potential of NTAP as a daily therapeutic option to manage biofilms effectively. The study highlights that one-time treatments with NTAP are effective only at specific durations (5 and 10 minutes). This underscores the importance of treatment duration in achieving significant antibacterial effects, particularly in mature biofilms. Also, the study shows that NTAP treatments reduce the concentration of soluble extracellular polysaccharides in biofilms. This is crucial because these polysaccharides contribute to the structural integrity and resilience of biofilms, making them harder to eradicate. The findings indicate no significant changes in the mineral profile of enamel or surface topography after NTAP treatments. This is a critical observation as it suggests that NTAP is safe for enamel, not compromising its structural integrity while effectively reducing biofilm-related bacteria. Utilizing advanced techniques like Raman Spectroscopy and Scanning Electron Microscopy, the study provides detailed insights into the impact of NTAP on enamel and biofilms, adding robustness to the findings.

3.1 Bacterial Viability

Fig. 1 shows the results of log10 CFU/ml of *S. mutans* biofilms daily treated and the Fig. 2 shows the results of one treatment on the mature biofilms. The results showed no significant difference between the NT group and the NaF group. Daily treatment with 0.12% chlorhexidine completely inhibited the formation of *S. mutans* biofilm in the daily treatment, but as expected, did not reduce the amount of CFU in mature biofilms. The treatments with argon gas or plasma significantly reduced the viability of the bacteria during biofilm formation ($p < 0.0001$). In the mature biofilms, the reduction of *S mutans* counts was observed when plasma treatment was applied during 5 and 10 minutes ($p < 0.0001$).

The daily treatments with Non-Thermal Atmospheric Pressure Plasma (NTAP) significantly reduce bacterial viability in *Streptococcus mutans* biofilms. Studies have shown that NTAP generates reactive species

that can penetrate biofilms and effectively kill embedded bacteria without harming surrounding tissues [50].

The production of ROS, chemically reactive molecules containing oxygen, represents an essential arm of the innate immune system.Phagocytic cells, especially macrophages and neutrophils, generate an extensive amount of ROS to kill bacteria [51,52].

This study also emphasizes that one-time treatments with NTAP are effective only at specific durations (5 and 10 minutes). This has several implications. First, the efficacy of NTAP at specific durations highlights the need to optimize treatment protocols. Understanding the precise duration necessary to achieve significant antibacterial effects ensures the maximum benefit of NTAP while potentially minimizing any adverse effects. Also, targeting mature biofilms is important as they are notoriously difficult to treat due to their complex structure and resistance mechanisms. The ability of NTAP to disrupt mature biofilms at specific durations underscores its potential as a potent biofilm disruptor. Also, in clinical settings, knowing the optimal treatment duration can aid in developing standardized treatment regimens. This can lead to more predictable and reliable outcomes in managing infections related to biofilms.

This is important because biofilms, such as those formed by *S. mutans*, are particularly challenging to treat due to their protective matrix that shields bacteria from antibiotics and the host immune system. NTAP's effectiveness in reducing bacterial viability offers a promising alternative to conventional treatments. Thus, *S. mutans* is a primary contributor to dental caries. Effective daily treatment that reduces *S. mutans* viability can potentially decrease the incidence of dental caries, improving oral health outcomes. Finally, with rising concerns over antibiotic resistance, NTAP offers a non-antibiotic approach to managing bacterial infections. This can be crucial in reducing the overuse of antibiotics and the development of resistant strains.

In daily treatments, CHX was the best treatment, considered the gold anti-plaque standard, due to its antimicrobial effect and its great substantivity [41]. However, in the mature biofilm, 0.12% chlorhexidine was ineffective inactivating the mutans group bacteria even after 10 minutes of treatment. By comparing NTAP treatments with conventional antibacterial agents like chlorhexidine and sodium fluoride, the study provides a relative measure of NTAP's effectiveness. This comparison helps in understanding NTAP's potential as an alternative or adjunctive therapy in dental biofilm management.

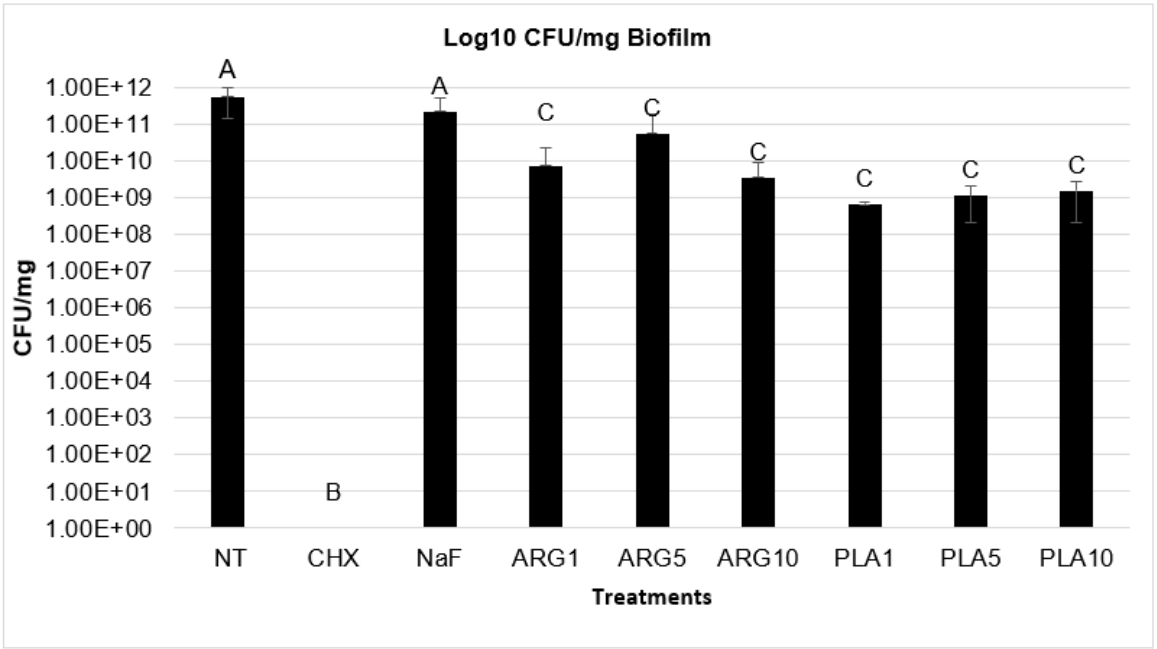


Fig. 1. Microbiological results of the daily treatment - biofilms in formation

Data represent mean values and the error bars represent the standard deviation. Different letters indicate a statistically significant difference (p <0.05) in the reduction of the Streptococcus mutans count.

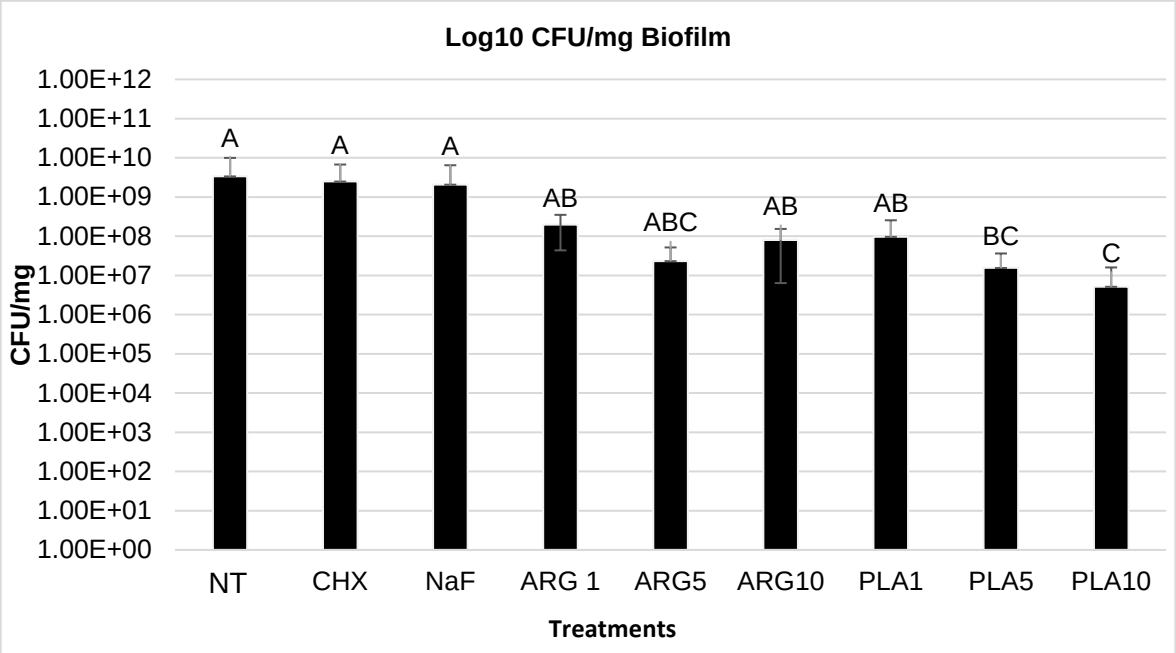


Fig. 2. Microbiological results of the one treatment - mature biofilm.
Data represent mean values and the error bars represent the standard deviation. Different letters indicate a statistically significant difference (p <0.05) in the reduction of the Streptococcus mutans count.

3.2 Biochemical Analysis - Extracellular Polysaccharides (EPS)

Fig. 3 shows the results of the analysis of the water-soluble EPS in the biofilm with daily treatment. CHX, ARG1, ARG10, PLA1, PLA5 and PLA10 groups significantly reduced the number of EPS ($p<0.05$). In the mature biofilm, a statistical increase of water-soluble polysaccharide was only observed to 0.12% CHX group ($p <0.0001$) as observed in Fig. 4. There was no statistical difference ($p = 0.5132$) among all groups when water-insoluble polysaccharide was analyzed. (Fig. 5 and 6).

The analysis of the biofilm in formation reveled that the groups CHX, ARG1 and ARG10 and PLA 1, PLA5 and PLA10 reduced water-soluble polysaccharides when compared to groups ST, NaF and ARG5 ($p<0.05$). In the mature biofilm, it was possible to observe a reduction of the soluble polysaccharides of the NTAP treated groups compared to the CHX group ($p<0.0001$). The reduction of soluble extracellular polysaccharides in biofilms by NTAP treatments is an important finding. By compromising the structural integrity and resilience of biofilms, NTAP can enhance the effectiveness of antimicrobial treatments and immune responses. This has significant implications for the management of biofilm-associated infections, offering a promising approach to overcoming one of the major challenges in treating chronic infections.

This study demonstrates that NTAP treatments decrease the concentration of soluble extracellular polysaccharides in biofilms. According to Flemming and Wingender [53], extracellular polymeric substances (EPS), including polysaccharides, are critical for the formation and stability of biofilms. They contribute to the biofilm's physical and chemical environment, affecting bacterial adhesion, protection, and nutrient retention. This is crucial because extracellular polysaccharides are key components of the biofilm matrix, providing structural integrity and protection to the bacterial community within. By reducing these polysaccharides, NTAP weakens the biofilm structure, making it more susceptible to eradication by immune responses or antimicrobial agents. Also, the resilience of biofilms to environmental stresses, including antimicrobial treatments, is largely due to the presence of these extracellular polysaccharides. They act as a physical barrier and sequester nutrients, which supports bacterial survival and growth. Reducing these components diminishes the biofilm's ability to withstand such stresses. Lower concentrations of extracellular polysaccharides can improve the penetration of

antimicrobial agents and immune cells into the biofilm. This can lead to more effective treatments, reducing the likelihood of chronic infections and associated complications.

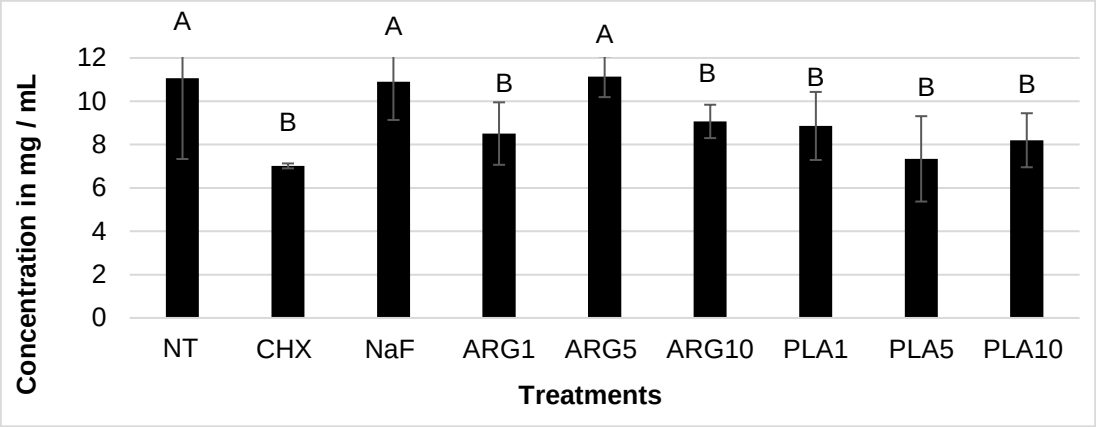


Fig. 3. Water-soluble EPS of the daily treatment - biofilms in formation

Data represent mean values and error bars represent standard deviation. Different letters indicate a statistically significant difference between the groups (p< 0.05).

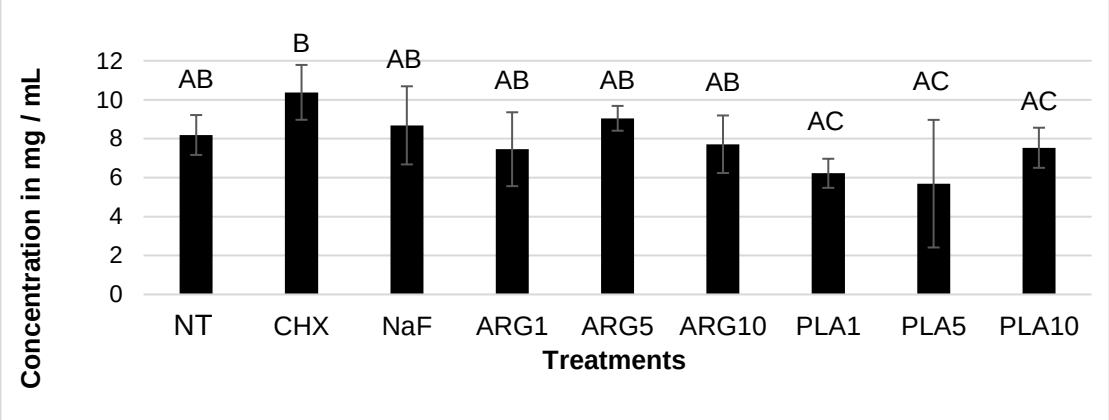


Fig. 4. Water-soluble EPS of the one treatment - mature biofilm

Data represent mean values and error bars represent standard deviation. Different letters indicate a statistically significant difference between the groups (p< 0.05).

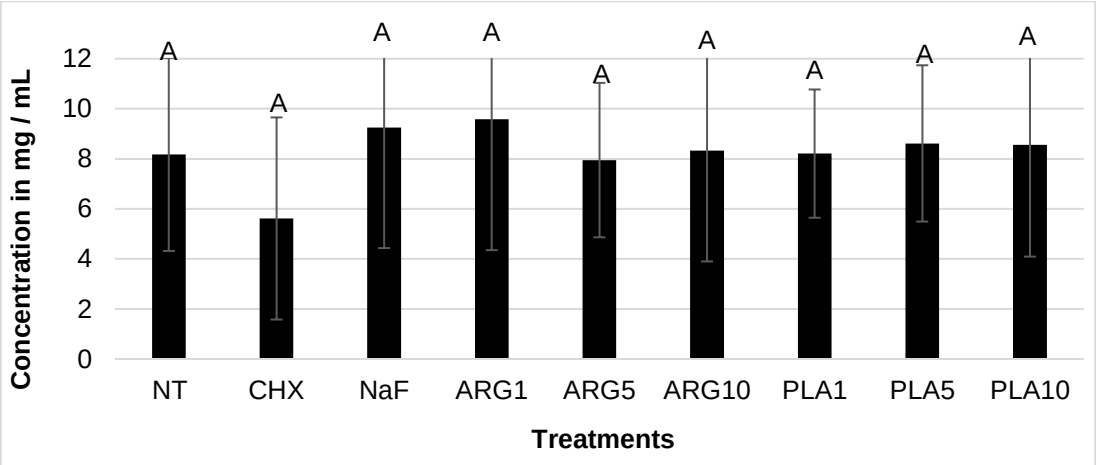


Fig. 5. Water-insoluble EPS of the daily treatment - biofilms in formation

Data represent mean values and error bars represent standard deviation. Different letters indicate a statistically significant difference between the groups (p< 0.05).

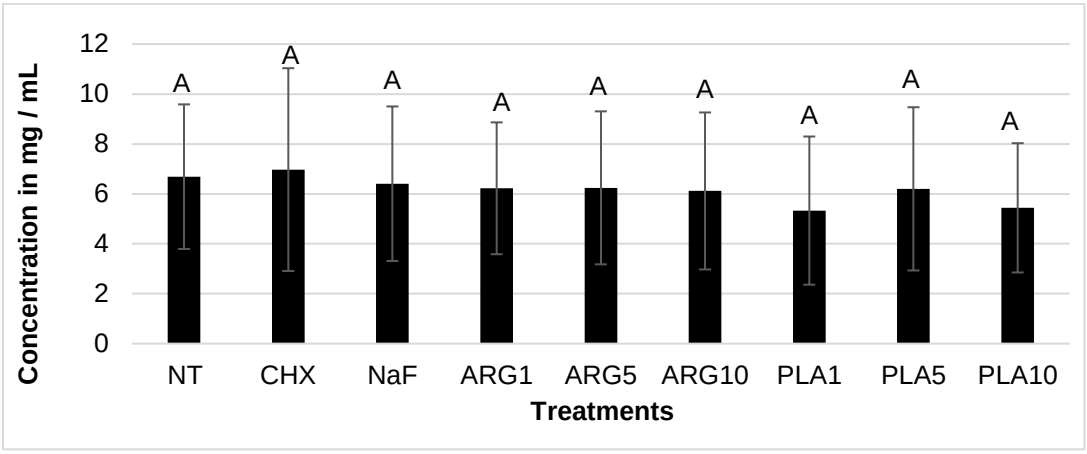


Fig. 6. Water-insoluble EPS of the one treatment - mature biofilm

Data represent mean values and error bars represent standard deviation. Different letters indicate a statistically significant difference between the groups (p< 0.05).

However, for water-insoluble EPS there was no significant difference among all groups. This may indicate that in both daily treatment (p = 0.5132) and single time treatment (p = 0.9844) NTAP has effect on prevention of the formation of EPSs with more flexible bonds of type α1-6 (dextrans). This may be explained by the α1-3 more complex structure presents in water- insoluble EPS [47] that may turn it harder to be affect by NTAP treatment.

3.3 Laser Confocal Microscopy – CLM

The representative CLM images after the treatments of the biofilms in formation are shown in Fig. 7 and of the treatments of the mature biofilms in Fig. 8. The total area of the images was 415 x 415µm, using the Plan-Apochromat 20x / 0.8 M27 lens. In the samples of the biofilms in formation, the groups NT, NaF and ARG10 showed a greater amount of areas stained in green (live). Although the effectiveness of the treatments by CLM images cannot be quantitatively compared, red (dead) stained areas can be seen more clearly in the biofilms of the CHX, PLA1 and PLA10 groups. A single treatment of chlorhexidine for 10 min on cells of mature biofilms had no significant effect on the number of live cells, while the treatments of ARG10, PLA1 and PLA10 produced characteristic images of cell death by the qualitative analysis used.

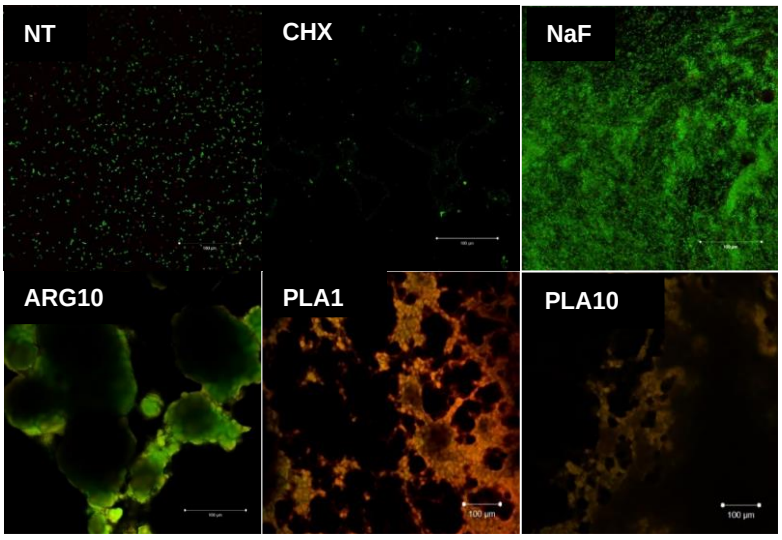


Fig. 7. Confocal Microscopy images of the daily treatment - biofilms in formation

Treatments indicated in the image by group code.

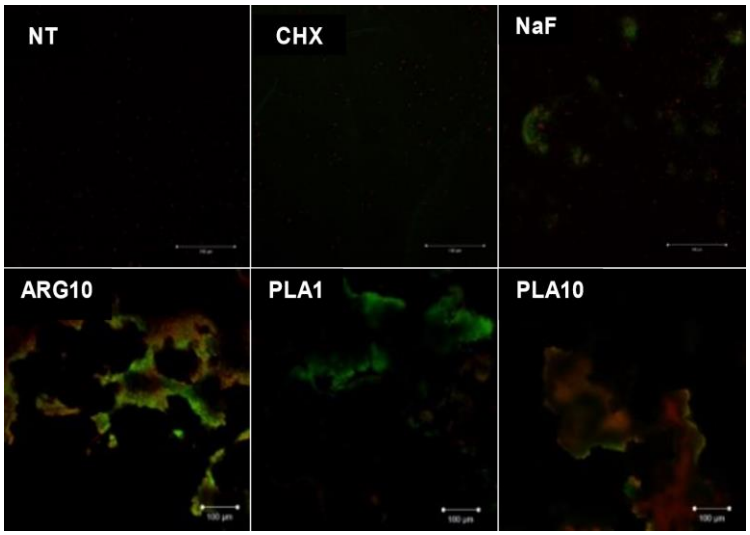


Fig. 8. Confocal microscopy images of the one treatment - mature biofilm
Treatments indicated in the image by group code.

Although this is a qualitative analysis, biofilm images exposed to NTAP treatment for 1 and 10 minutes seems to have large reddish areas indicating cell death populations. The images also suggest differences in viability in PLA10 treatment as found in the quantitative analysis performed. A study by [44] also showed reduction in bacterial viability for the plasma group. The SEM scanning results showed that the NTAP jet inactivated C. albicans biofilm mainly via disrupting cell envelopes and then leading the release of cellular components, thus resulting in loss of cell viability [24].

3.4 Surface Microhardness (SMH)

Regarding the microhardness, all the specimens started the experiment in similar conditions, and all suffered mineral loss by the biofilm model, confirming that it is an effective cariogenic model under tested conditions. In the daily treatment biofilme, only the group treated with chlorhexidine (0.12%) was able to reduce the percentage of loss of surface hardness (% LSH), according to Table 1. In the mature biofilm treated at the end of experimental period, there was no statistically significant differences of% LSH were observed between groups ($p = 0.2207$), as can be seen in Table 2. Fig. 9 shows the initial and final indentations of after biofilm formation and daily treatments.

Human enamel demineralization was observed after 5 days of plaque accumulation at high frequency of exposure to sugar. The same result was found by [54] when they conducted an experiment using a continuous flow biofilm model acting as an artificial mouth. The reduction in the superficial microhardness of the enamel was observed due to the cariogenic challenge to which the enamel specimens were submitted. In the biofilm treated daily, the treatment with NTAP or argon gas result in similar mineral loss percentage to those treated with NaF. The group treated with 0.12% chlorhexidine obtained the lowest mineral loss, as it completely prevented the formation of biofilm on the enamel surface. In the group treated once after 5 days of biofilm formation. the different tested treatments did not affect the enamel hardness in the tested conditions. Loss of surface hardness is considered a very sensitive method of assessing early lesion of caries and [47] suggest that it takes about 3 to 4 days for dental plaque to show its cariogenicity on the dental substrate. Thus, the percentage of hardness loss resulting in our study resembles the results found by [54]. These results indicated that plasma treatment did not induce damage to the surface of the enamel, although the enamels had demonstrated alterations due cariogenic challenge.

The NTAP treatments do not significantly alter the mineral profile of enamel or surface topography for several reasons. First, enamel is the hardest tissue in the human body and essential for protecting teeth against decay and wear. The fact that NTAP does not compromise enamel's mineral content or surface integrity ensures that it is safe for dental applications. This is particularly important for maintaining the long-term health and functionality of teeth. Thus,

treatments should ideally be effective against pathogens without causing harm to the tooth structure. NTAP's ability to reduce biofilm-related bacteria without altering the enamel's mineral profile or surface topography makes it a non-destructive treatment option. This can lead to better patient outcomes and increased acceptance of NTAP as a therapeutic modality. Maintaining the natural appearance and functionality of enamel is crucial. Treatments that alter enamel's structure can lead to issues such as increased sensitivity, susceptibility to decay, and aesthetic concerns. The preservation of enamel integrity by NTAP supports its use in cosmetic and restorative dentistry.

3.5 Raman Spectroscopy

The mean Raman spectra in the 800 to 2000 cm^{-1} region of the enamel specimens of the daily treatment, are shown in Fig. 10 and the spectra of the treatment of mature biofilm groups are shown in Fig. 11. For this purpose, phosphate (958 cm^{-1}) and carbonate (1070 cm^{-1}) measurements at three points in the block were performed. No significant difference between the groups in both daily ($p = 0.6978$) and one final treatment ($p = 0.8904$) was observed.

Micro-Raman spectroscopy has already been applied in the field of dental research to investigate dental hard tissue. The reading at three different points allows us to compare all treatments statistically. In this study, no significant difference was observed in the mean area under the analyzed peaks for phosphate and carbonate ($v = 958cm^{-1}$ and 1070 cm^{-1}, respectively), differing from another study [47], which found a decrease in the same organic matrix bands for enamel.

3.6 Scanning Electron Microscopy (SEM)

The qualitative analysis of the SEM showed that, in the daily treatment as well as the treatment after the biofilm maturation, the surface of the dental enamel under the treated biofilm was not

Table 1. Surface microhardness analysis of enamel blocks treated daily according to treatments (mean ± SD)

Treatment	Surface Microhardness		%LSH
	Initial	Last	
NT	328,66 ± 43,18 a	13,64 ± 5,14c	-95,92 ± 1,02A
CHX	300,66 ± 17,89 a	123,18 ± 107,85b	-57,54 ± 39,56B
NaF	296,66 ± 6,02 a	46,25 ± 15,52c	-84,47 ± 4,88A
ARG1	289,33 ± 9,07 a	29,76 ± 26,50c	-89,47 ± 8,85A
ARG5	309,00 ± 10,58 a	19,25 ± 9,17c	-93,69 ± 3,24A
ARG10	308,00 ± 12,48 a	16,77 ± 12,28c	-94,46 ± 4,21A
PLA1	306,66 ± 11,01 a	48,98 ± 9,26c	-83,94 ± 3,61A
PLA5	304,66 ± 18,71 a	55,29 ± 31,60c	-81,72 ± 10,85A
PLA10	319,00 ± 22,60 a	42,12 ± 24,96c	-86,86 ± 7,30A

Different letters indicate statistically significant differences ($p < 0.05$). Capital letters compare the initial readings with the endings; lowercase letters compare the groups in the same period. LSH = loss of surface hardness.

Table 2. Surface microhardness analysis of treated enamel blocks of the mature biofilm, according to treatments (mean ± SD)

Treatment	Surface Microhardness		%LSH
	Initial	Last	
NT	316,4 ±6,59a	14,9±0,46b	-92,1±0,24A
CHX	306,3±29,80a	21,4±11,78b	-93,1±3,62A
NaF	298,3 ±22,57a	71,9±34,51b	-75,6±12,4A
ARG1	300,4 ±22,59a	46,8±21,34b	-84,0±7,98A
ARG5	286,7±10,29a	28,9±16,98b	-89,8±6,01A
ARG10	305,3±16,70a	19,6±3,14bb	-90,6±5,26A
PLA1	308,3±14,8a	31,6±8,33b	-89,6±6,01A
PLA5	317,8±26,88a	83,6±96,73b	-71,8±34,38A
PLA10	314,07±16,51a	33,4±35,61b	-89,5±11,12A

Different letters indicate statistically significant differences ($p < 0.05$). Capital letters compare the initial readings with the endings; lowercase letters compare the groups in the same period. LSH = loss of surface hardness.

significantly altered by the argon and plasma treatments. A smooth and homogeneous surface in the 10000x increase can be observed in Fig. 12 (daily treatment) and in Fig. 13 (treatment mature biofilm).

In this study, no visible changes were observed by the SEM in the enamel surface structure among specimens submitted to the different treatments [49]. Also, it was not observed any significant alteration in the surface of the tooth even after 10 minutes of exposure to low temperature plasma which confirms that NTAP does not alter the structure of the enamel [49], an important aspect to be considered when using it in *vivo* [20]. Thus, the results demonstrated that the treatment with NTAP was effective in reducing the viability of S. mutans inserted in forming and mature biofilms without damaging the enamel surface under tested conditions, being a promising technique in the control of oral biofilms.

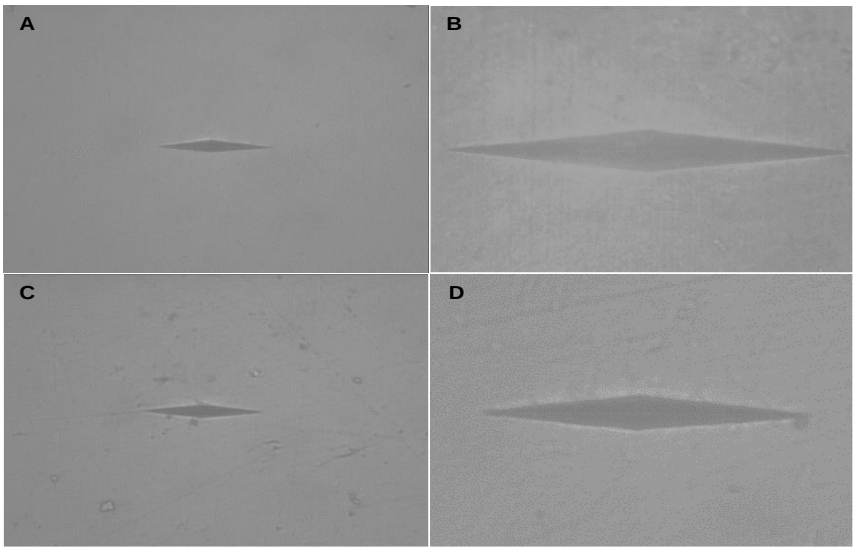
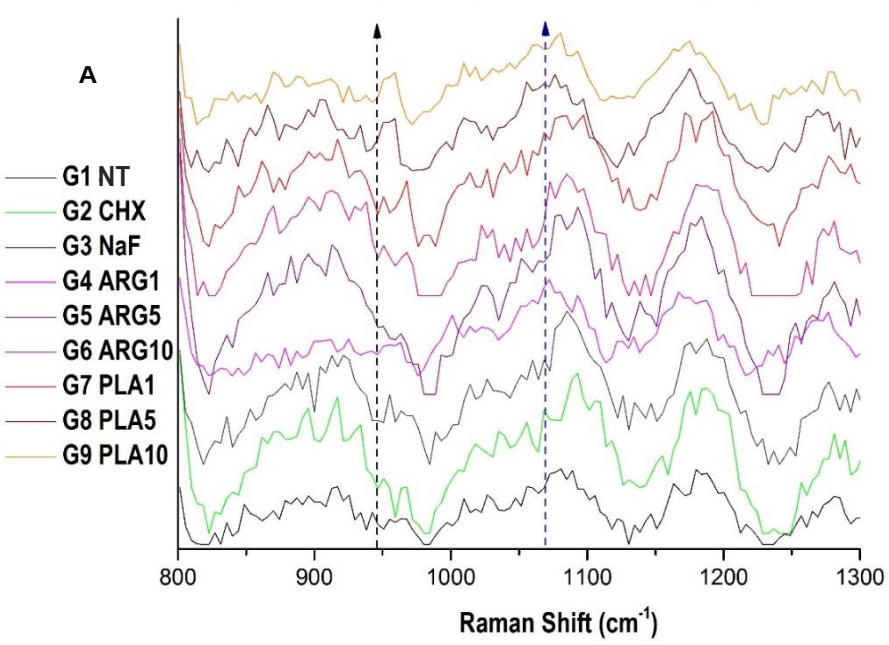


Fig. 9. Surface Microhardness

Images of the longitudinal microhardness of the enamel surface, where A and B represent the initial and final indentation of the daily treatment, respectively, while the C and D images represent the initial and final indentation of mature biofilm treatment.



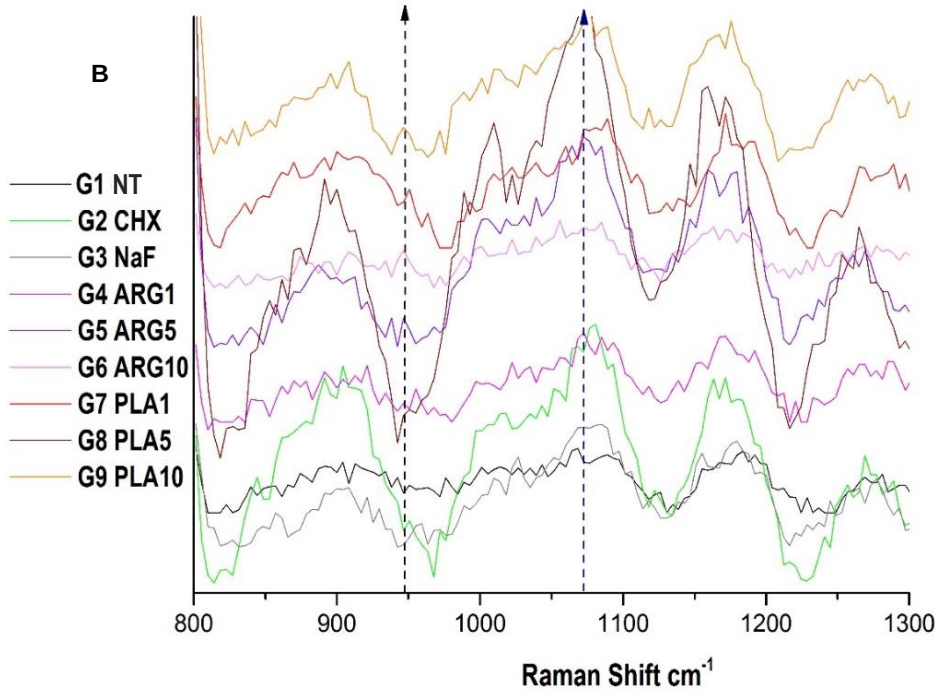
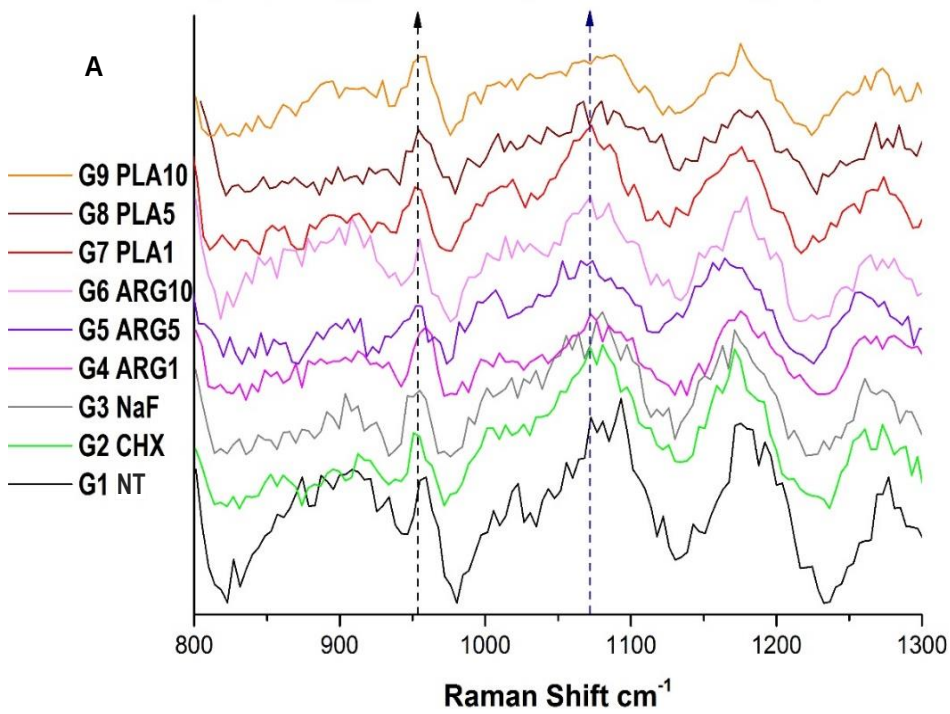


Fig. 10. Raman Spectroscopy results of the daily treatment - biofilms in formation

Raman Spectroscopy graphs showing that there was no difference between the peaks of the groups treated daily before (A) and after (B) treatments. NT - black lines, CHX - green lines, NaF - gray lines, ARG1 - pink lines pink, ARG5 - purple lines, ARG10 - light pink lines, PLA1 - red lines, PLA5 - brown lines and PLA10 - orange lines; Blue arrow = 958cm^{-1} Peak; Black arrow = 1070cm^{-1} peak.



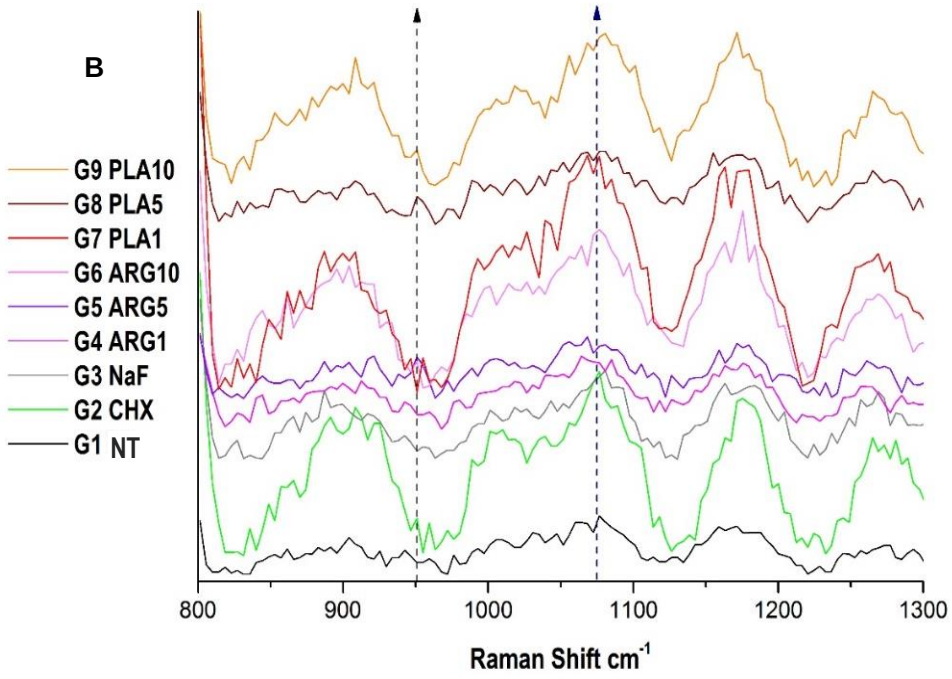


Fig. 11. Raman Spectroscopy results of the one treatment - mature biofilm

Raman spectroscopy graphs showing that there was no difference between the peaks of the groups before (A) and after (B) of the treatments of the mature biofilm. NT - black lines, CHX - green lines, NaF - gray lines, ARG1 - pink lines pink, ARG5 - purple lines, ARG10 - light pink lines, PLA1 - red lines, PLA5 - brown lines and PLA10 - orange lines; Blue arrow = 958cm^{-1} Peak; Black arrow = 1070cm^{-1} peak.

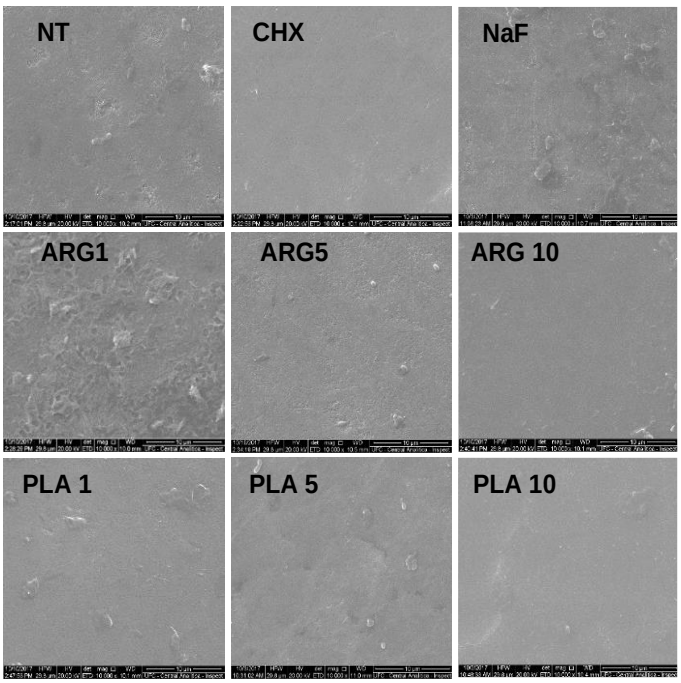


Fig. 12. SEM results of the daily treatment - biofilms in formation

MEV of the human enamel blocks treated daily according to the treatments NT, CHX, NaF, ARG 1, ARG5 and ARG 10, PLA1, PLA5 and PLA10 in the increase of x10000.

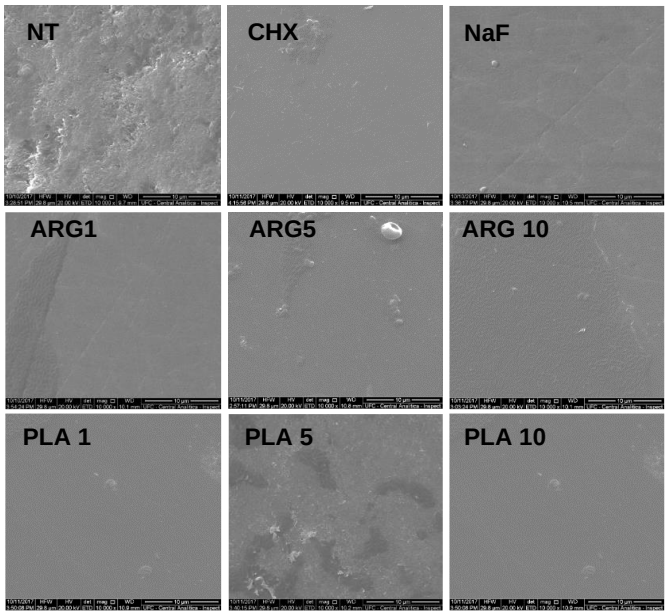


Fig. 13. SEM results of the one treatment - mature biofilm
MEV of the human enamel blocks treated daily according to the treatments NT, CHX, NaF, ARG 1, ARG5 and ARG 10, PLA1, PLA5 and PLA10 in the increase of x10000.

4. CONCLUSION

A daily treatment with NTAP during the formation of *Streptococcus mutans* biofilms as well as a single treatment on mature biofilm *seems* to be effective in reducing the bacterial viability and the water-soluble extracellular polysaccharides production without altering the water-insoluble polysaccharides amount. NTAP do not alter the biochemical properties of the enamel under the biofilms, do not affect its topographic surface and is not effective in reducing mineral loss during cariogenic challenger under tested conditions. Overall, this study not only confirms the antibacterial efficacy of NTAP but also sets the groundwork for optimizing treatment protocols and durations. It offers a promising alternative to traditional chemical agents in managing dental biofilms without damaging the enamel, thereby advancing the field of dental microbiology and therapeutic interventions.

The utilization of advanced techniques like Raman Spectroscopy and Scanning Electron Microscopy (SEM) provides significant insights and adds robustness to the study's findings. Raman Spectroscopy and SEM offer high-resolution, detailed insights into the composition and structure of materials. Their use in this study allows for precise evaluation of NTAP's effects on enamel and biofilms, ensuring that the findings are accurate and reliable. These advanced techniques validate the safety of NTAP by confirming that it does not alter the enamel's mineral profile or surface topography. This scientific rigor strengthens the credibility of the study's conclusion. By employing these techniques, the study can better understand how NTAP interacts with enamel and biofilms at a molecular and structural level.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

CONSENT

All those responsible for the tooth donor centers signed a tooth donation term. A copy of the written term is available for review by the Editorial office/Chief Editor/Editorial Board members of this journal.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of

Helsinki. CAAE: 80206617.7.0000.5054 according to the resolution of the CNS 466 of 12/12/2012.

ACKNOWLEDGEMENTS

Coordination for the Improvement of Higher Education Personnel (CAPES) for the master's scholarship granted and for the investment in research (Process nº 88881.030383/2013-01 of the CSF-PVE-S Program).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Liu Y, Daniel SG, Kim HE, Koo H, Korostoff J, Teles F, Bittinger K, Hwang G. Addition of cariogenic pathogens to complex oral microflora drives significant changes in biofilm compositions and functionalities. Microbiome. 2023;11(1):1-17. DOI: http://dx.doi.org/10.1186/s40168-023-01561-7.
2. Sheiham A, James WPT. Diet and dental caries. J Dent Res. 2015;94(10):1341-7. DOI:http://dx.doi.org/10.1177/0022034515590377.
3. World Health Organization. Global oral health status report: towards universal health coverage for oral health by 2030. Geneva: WHO; 2022. Available:https://iris.who.int/handle/10665/364538.5.
4. Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. Biofilms: an emergent form of bacterial life. Nat Rev Microbiol. 2016;14(9):563-75. DOI:http://dx.doi.org/10.1038/nrmicro.2016.94.
5. Kuang X, Chen V, Xu X. Novel approaches to the control of oral microbial biofilms. Biomed Res Int. 2018;2018:1-13. DOI:http://dx.doi.org/10.1155/2018/6498932.
6. Duarte S, Panariello BHD. Comprehensive biomedical applications of low temperature plasmas. Arch Biochem Biophys. 2020;693:108560. DOI:http://dx.doi.org/10.1016/j.abb.2020.108560.
7. Nima G, Harth-Chu E, Hiers RD, Pecorari VGA, Dyer DW, Khajotia SS, Giannini M, Florez FL. Antibacterial efficacy of non-thermal atmospheric plasma against Streptococcus mutans biofilm grown on the surfaces of restorative resin composites. Sci Rep. 2021;11(1):1-12. DOI: http://dx.doi.org/10.1038/s41598-021-03192-0.
8. Sharma D, Misba L, Khan AU. Antibiotics versus biofilm: an emerging battleground in microbial communities. Antimicrob Resist Infect Control. 2019;8(1):1-10. DOI: http://dx.doi.org/10.1186/s13756-019-0533-3.
9. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the Normal Bacterial Flora of the Oral Cavity. Journal of Clinical Microbiology. 2005;43(11):5721–32.
10. Lemos JA, Palmer SR, Zeng L, Wen ZT, Kajfasz JK, Freires IA, Abranches J, Brady LJ. The biology of Streptococcus mutans. Microbiol Spectr. 2019;7(1). DOI:http://dx.doi.org/10.1128/microbiolspec.gpp3-0051-2018.
11. Moye ZD, Zeng L, Burne RA. Fueling the caries process: carbohydrate metabolism and gene regulation by Streptococcus mutans. J Oral Microbiol. 2014;6(1):24878. DOI:http://dx.doi.org/10.3402/jom.v6.24878
12. Zhang Q, Ma Q, Wang Y, Wu H, Zou J. Molecular mechanisms of inhibiting glucosyltransferases for biofilm formation in Streptococcus mutans. Int J Oral Sci. 2021;13(1). DOI: http://dx.doi.org/10.1038/s41368-021-00137-1.
13. Bowen WH, Koo H. Biology of Streptococcus mutans-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. Caries Res. 2011;45(1):69-86. DOI: http://dx.doi.org/10.1159/000324598.
14. Kirsch J, Hannig C, Pötschke S, Basche S, Bowen WH, Rupf S, Trautmann S, Umanskaya N, Hannig M. Enzymology and ultrastructure of the in situ pellicle in caries-active and caries-inactive patients. Caries Res. 2017;51(2):109-18. DOI: http://dx.doi.org/10.1159/000452226.
15. Bowen WH, Burne RA, Wu H, Koo H. Oral biofilms: pathogens, matrix, and polymicrobial interactions in microenvironments. Trends Microbiol. 2018;26(3):229-42. DOI:http://dx.doi.org/10.1016/j.tim.2017.09.008.
16. Goree J, Liu B, Drake D, Stoffels E. Killing of S. mutans bacteria using a plasma

needle at atmospheric pressure. IEEE Trans Plasma Sci. 2006;34(4):1317-24. DOI:http://dx.doi.org/10.1109/TPS.2006.878431

17. Molina M, Cioci G, Moulis C, Séverac E, Remaud-Siméon M. Bacterial α-glucan and branching sucrases from GH70 family: discovery, structure-function relationship studies and engineering. Microorganisms. 2021;9(8):1607. DOI:http://dx.doi.org/10.3390/microorganisms9081607.
18. Sladek REJ, Filoche SK, Sissons CH, Stoffels E. Treatment of Streptococcus mutans biofilms with a nonthermal atmospheric plasma. Lett Appl Microbiol. 2007;45(3):318-23. DOI:http://dx.doi.org/10.1111/j.1472-765X.2007.02194.x.
19. Koban I, Geisel MH, Holtfreter B, Jablonowski L, Hübner NO, Matthes R, Masur K, Weltmann KD, Kramer A, Kocher T. Synergistic effects of nonthermal plasma and disinfecting agents against dental biofilms in vitro. ISRN Dent. 2013;2013:1-10. DOI:http://dx.doi.org/10.1155/2013/573262
20. Partecke LI, Evert K, Haugk J, Doering F, Normann L, Diedrich S, Weiss FU, Evert M, Huebner NO, Guenther C. Tissue tolerable plasma (TTP) induces apoptosis in pancreatic cancer cells in vitro and in vivo. BMC Cancer. 2012;12(1):1-10. DOI: http://dx.doi.org/10.1186/1471-2407-12-473.
21. Delben JA, Murata RM, Wei X, Castro ML, Assunção WG, Silva NR, Duarte S. Low-temperature plasma: an effective approach against Candida albicans biofilm. Plasma Med. 2014;4(1-4):231-44. DOI:http://dx.doi.org/10.1615/PlasmaMed.2014012361.
22. Duarte S, Panariello BHD. Comprehensive biomedical applications of low temperature plasmas. Arch Biochem Biophys. 2020 Oct;693:108560. DOI:http://dx.doi.org/10.1016/j.abb.2020.108560.
23. Moreau M, Orange N, Feuilloley MGJ. Non-thermal plasma technologies: new tools for bio-decontamination. Biotechnol Adv. 2008;26(6):610-7. DOI:http://dx.doi.org/10.1016/j.biotechadv.2008.08.001.
24. Pu QK, Liu SJ, Huang H, Xiong JF, Zhang L, Fang Z, Wang C. [Sterilization effect of an atmospheric low temperature plasma jet on Candida albicans biofilm]. Sichuan Da Xue Xue Bao Yi Xue Ban. 2019;50(3):339-43. PMID: 31631600.
25. Kleineidam B, Nokhbehsaim M, Deschner J, Wahl G. Effect of cold plasma on periodontal wound healing—an in vitro study. Clin Oral Investig. 2018;23(4):1941-50. DOI: http://dx.doi.org/10.1007/s00784-018-2643-3.
26. Bernhardt T, Semmler ML, Schäfer M, Bekeschus S, Emmert S, Boeckmann L. Plasma medicine: applications of cold atmospheric pressure plasma in dermatology. Oxid Med Cell Longev. 2019;2019:1-10. DOI:http://dx.doi.org/10.1155/2019/3873928.
27. Yan D, Sherman JH, Keidar M. Cold atmospheric plasma, a novel promising anti-cancer treatment modality. Oncotarget. 2016;8(9). DOI:http://dx.doi.org/10.18632/oncotarget.13304.
28. Hoffmann C, Berganza C, Zhang J. Cold atmospheric plasma: methods of production and application in dentistry and oncology. Med Gas Res. 2013;3(21):1-15. DOI: http://dx.doi.org/10.1186/2045-9912-3-21.
29. Scholtz V, Pazlarova J, Souskova H, Khun J, Julak J. Nonthermal plasma—a tool for decontamination and disinfection. Biotechnol Adv. 2015;33(6 Pt 2):1108-19. DOI:http://dx.doi.org/10.1016/j.biotechadv.2015.01.002.
30. Fluhr JW, et al. In vivo skin treatment with tissue-tolerable plasma influences skin physiology and antioxidant profile in human stratum corneum. Exp Dermatol. 2012;21(2):130-4. DOI:http://dx.doi.org/10.1111/j.1600-0625.2011.01411.x.
31. Duarte S, Kuo SP, Murata RM, Chen CY, Saxena D, Huang KJ, Popovic S. Air plasma effect on dental disinfection. Phys Plasmas. 2011;18:073503. DOI: http://dx.doi.org/10.1063/1.3606486.
32. Carreiro AFP, Delben JA, Guedes S, Silveira EJD, Janal MN, Vergani CE, Pushalkar S, Duarte S. Low-temperature plasma on peri-implant–related biofilm and gingival tissue. J Periodontol. 2018; 90(5):507-15. DOI:http://dx.doi.org/10.1002/jper.18-0366.

33. Koo H, Falsetta ML, Klein MI. The exopolysaccharide matrix. J Dent Res. 2013;92(12):1065-73. DOI:http://dx.doi.org/10.1177/0022034513504218.
34. Koban I, Holtfreter B, Hübner NO, Matthes R, Sietmann R, Kindel E, Weltmann KD, Welk A, Kramer A, Kocher T. Antimicrobial efficacy of non-thermal plasma in comparison to chlorhexidine against dental biofilms on titanium discs in vitro - proof of principle experiment. J Clin Periodontol. 2011;38(10):956-65. DOI:http://dx.doi.org/10.1111/j.1600-051X.2011.01740.x.
35. White DJ, Featherstone JDB. A longitudinal microhardness analysis of fluoride dentifrice effects on lesion progression in vitro. Caries Res. 1987;21(6):502-12. DOI: http://dx.doi.org/10.1159/000261059.
36. Cury JA, Rebelo MAB, Cury AAdB, Derbyshire MTVC, Tabchoury CPM. Biochemical composition and cariogenicity of dental plaque formed in the presence of sucrose or glucose and fructose. Caries Res. 2000;34(6):491-7. DOI: http://dx.doi.org/10.1159/000016629.
37. Melo MAS, de-Paula DM, Lima JPM, Borges FMC, Steiner-Oliveira C, Nobre-dos-Santos M, Zanin ICJ, Barros EB, Rodrigues LKA. In vitro photodynamic antimicrobial chemotherapy in dentine contaminated by cariogenic bacteria. Laser Phys. 2010;20(6):1504-13. DOI:http://dx.doi.org/10.1134/S1054660X10110174.
38. Meredith N, Sheriff M, Setchell DJ, Swanson SAV. Measurement of the microhardness and Young's modulus of human enamel and dentine using an indentation technique. Arch Oral Biol. 1996;41(6):539-45. DOI:http://dx.doi.org/10.1016/0003-9969(96)00020-9.
39. Duarte S, Gregoire S, Singh AP, Vorsa N, Schaich K, Bowen WH, Koo H. Inhibitory effects of cranberry polyphenols on formation and acidogenicity of Streptococcus mutans biofilms. FEMS Microbiol Lett. 2006;257(1):50-6. DOI:http://dx.doi.org/10.1111/j.1574-6968.2006.00147.x.
40. Alves KSS. Efeito do plasma de baixa temperatura e pressão no biofilme oral formado sobre esmalte em um modelo in situ [dissertação]. Fortaleza: Universidade Federal do Ceará; 2016. Available:http://www.repositorio.ufc.br/handle/riufc/20128. Acesso em: 10 jul. 2023.
41. Batista NC, et al. Evaluation of periodontal indices in young adults submitted to chlorhexidine 0.12% mouthwash: a randomized clinical trial. Rev Odontol UNESP. 2021;50. DOI:http://dx.doi.org/10.1590/1807-2577.04521.
42. Teixeira AH, Pereira ES, Rodrigues LKA, Saxena D, Duarte S, Zanin ICJ. Effect of photodynamic antimicrobial chemotherapy on in vitro and in situ biofilms. Caries Res. 2012;46(6):549-54. DOI: http://dx.doi.org/10.1159/000341190.
43. Aragão MGB, et al. Comparative effect of two red lights on Streptococcus mutans biofilms and assessment of temperature variances in human teeth during in vitro photodynamic antimicrobial chemotherapy. Photobiomodul Photomed Laser Surg. 2019;37(1):31-7. DOI:http://dx.doi.org/10.1089/photob.2018.4511.
44. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem. 1956;28(3):350-6. DOI:http://dx.doi.org/10.1021/ac60111a017
45. Du T, Ma J, Yang P, Xiong Z, Lu X, Cao Y. Evaluation of antibacterial effects by atmospheric pressure nonequilibrium plasmas against Enterococcus faecalis biofilms in vitro. J Endod. 2012;38(4):545-9. DOI:http://dx.doi.org/10.1016/j.joen.2011.10.021.
46. Zanin ICJ, et al. Susceptibility of Streptococcus mutans biofilms to photodynamic therapy: an in vitro study. J Antimicrob Chemother. 2005;56(2):324-30. DOI: http://dx.doi.org/10.1093/jac/dki232.
47. Tenuta LMA, Lima JEO, Cardoso CL, Tabchoury CPM, Cury JA. Effect of plaque accumulation and salivary factors on enamel demineralization and plaque composition in situ. Pesqui Odontol Bras. 2003;17(4):326-31. DOI:http://dx.doi.org/10.1590/s1517-74912003000400006.
48. Alves RHP. Efeitos do plasma de baixa temperatura sobre os substratos de esmalte e dentina [dissertação]. Fortaleza: Universidade Federal do Ceará; 2015. Available:https://repositorio.ufc.br/bitstream/riufc/16972/1/2015_dis_rhpalves.pdf. Acesso em: 03 jul. 2023.

49. Kovalová Z, Zahoran M, Zahoranová A, Machala Z. Streptococci biofilm decontamination on teeth by low-temperature air plasma of DC corona discharges. J Phys D Appl Phys. 2014;47(22):224014. DOI:http://dx.doi.org/10.1088/0022-3727/47/22/224014.
50. Rupf S, Lehmann A, Hannig M, Schäfer B, Schubert A, Feldmann U, et al. Killing of adherent oral microbes by a non-thermal atmospheric plasma jet. Journal of Medical Microbiology. 2010;59(2):206–12. Available:https://doi.org/10.1099/jmm.0.013714-0.
51. Czesnikiewicz-Guzik M, Konturek S, Loster B, et al. Melatonin and its role in oxidative stress related dis- eases of oral cavity. J Physiol Pharmacol. 2007;58 (Suppl 3):5–19.
52. Hong Y, Zeng J, Wang X, Drlica K, Zhao X. Post-stress bacterial cell death mediated by reactive oxygen species. Proceedings of the National Academy of Sciences [Internet]. 2019;116(20):10064–71. Available:https://www.pnas.org/content/116/20/10064.
53. Flemming HC, Wingender J. The biofilm matrix. Nature reviews Microbiology [Internet]. 2010;8(9):623–33. Available:https://www.nature.com/articles/nrmicro2415.
54. Park BG, Kim KS, Jung KH, Jung SB. Effect of atmospheric-pressure plasma treatment on the adhesion characteristics of screen-printed Ag nanoparticles on polyimide. J Nanosci Nanotechnol. 2014;14(12):9448-53. DOI:http://dx.doi.org/10.1166/jnn.2014.10169.

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/120444