Exploring the efficacy of in-vitro low-temperature plasma treatment on single and multispecies dental cariogenic biofilms

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Abstract

The main objective of the present study was to determine how treatment with low-temperature plasma (LTP) at different exposure times affects cariogenic biofilms of single and multiple species formed by *C. albicans, L. casei*, and *S. mutans* on hydroxyapatite discs. Biofilms were treated with LTP-argon at a 10 mm distance for 30 s, 60 s, and 120 s. Chlorhexidine solution (0.12%) and NaCl (0.89%) were used as positive and negative controls, respectively. Argon flow was also used as gas flow control. Colony-forming units (CFU) recovery and confocal laser scanning microscopy (CLSM) were used to analyze biofilm viability. LTP reduced multispecies biofilms viability (log10 CFU/mL) in all the treated samples ($p < 0.0001$). For single-species biofilms, a significant reduction in all exposure times was observed for *L. casei* ($p < 0.0001$). For *C. albicans* biofilms, there was a significant decrease in LTP treatment when applied for 60 and 120 s when compared to positive and negative controls ($p < 0.0001$). LTP is a potential mechanism in the treatment of dental caries, by being an effective anti-biofilm therapy of both single and multispecies cariogenic biofilms.

1. INTRODUCTION

Dental caries of deciduous and permanent teeth is the most prevalent disease and the second-highest incidence of oral conditions in the world [1]. Caries decay is more prevalent in socioeconomically disadvantaged people [2], and even though it is a disease that can be physically and chemically controlled, studies indicate that its prevalence has not decreased in the last three decades [3]. The caries process depends on the formation of a cariogenic biofilm on the tooth surface. [4].

In a biofilm, microorganisms grow in a matrix rich in extracellular polysaccharides [5], forming a well-organized community [6] that protects against penetration of chemical agents and antimicrobial agents [7], however, resistance of the cariogenic bacteria present in this biofilm to antimicrobials may occur due to the frequent contact of the biofilm with these agents [8, 6].

Microbiologically, *Strptococcus mutans* is the most well-known pathogen in the process of caries formation and development [9, 10], frequently used to assess caries risk [11] due to its strong acid production capacity, combined with its acid tolerance. Additionally, these microorganisms have vesicles in their membranes containing proteins, extracellular DNA, and other substances capable of activating cell-to-cell communication, assisting biofilm formation and disease progression [12]. Studies have shown a low amount or absence of *S. mutans* in patients with dental caries decay [13, 14] and also in the caries-free group, indicating that other species of bacteria that are associated in the cariogenic biofilm together with *S. mutans* may also be responsible for the formation and development of caries [15, 16]. *S. mutans*, *Lactobacillus spp.* [15, 16] and *Candida albicans* [17] were also correlated with dental caries.

Gregoire et al. (2011) demonstrated through an *in vitro* study that the glycosyltransferase B (GtfB) released by *S. mutans* might be able to bind to cell surfaces of *C. albicans* through enzymatic reactions with sucrose present in the biofilm [18]. A Study has reported the mechanism of adhesion of *C. albicans* to hydroxyapatite surfaces, dentin, and even cementum [19]. Besides, the presence *of C. albicans* has

been reported in the oral mycobiome of early childhood caries and dental cavities established in an adduct [20].

For more than six decades, *Lactobacillus* sp. have been associated with tooth decay [21] and, to date, is still known as one of the main causative agents of tooth decay [22]. They are frequently isolated from sites of active and advanced caries lesions in adult and pediatric patients [23, 24, 21] but are absent in caries-free children. As they metabolize dietary sugar, they can significantly contribute to the progression of caries [24]. Both *S. mutans* and *Lactobacillus* sp. can survive in a low-pH environment [21].

Improving the current protocols for treating dental caries is urgent [25], especially after the COVID-19 pandemic caused by the new coronavirus SARS-CoV-2, as conventional restorative treatments can generate aerosols and spread bacteria and viruses [26]. In this scenario, minimally invasive dentistry (MID) is a safer alternative, using manual instruments instead of rotating equipment [27]. MID preserves healthy dental tissue and prevents damage to the pulpal dentin complex [27].

Low-temperature plasma (LTP) therapy has been presented as an innovative therapeutic tool in treating dental caries [28, 29, 30]. Plasma is the fourth state of matter, consisting of ionized fas formed by attoms and/or molecules and ions at different densities and temperatures. It is a conductive medium and responds to electric and magnetic fields [31]. Under certain conditions, LTP can be produced in the laboratory by applying an appropriate electrical stimulus, such as high voltage, microwave, radiofrequency, and noble or molecular gases, such as argon, helium, oxygen, and nitrogen [31, 32, 33]. For plasma to be considered a biologically viable energy source, it must reach a temperature of 40°C or lower [31, 33].

LTP is produced by a partially ionized gas after an electrical discharge [34]. LTP generated at 40ºC or less triggers the production of ROS in the gas phase from reactive oxygen species (ROS) and reactive nitrogen species (RNS), including hydroxyl radicals (OH), superoxide radicals (O^{2-}), ozone (O_3), atomic oxygen and δ singlet oxygen and RNS, including peroxynitrite [35].

This study aimed to determine the impact of LTP-argon treatment on cariogenic biofilms comprising single and multiple species of *C. albicans, Lactobacillus casei*, and *S. mutans*. Previous research has demonstrated the efficacy of LTP in reducing oral biofilms and its potential for developing new MID protocols and enhancing dental practices. The study focused on treating mono and multispecies biofilms with LTP-argon at varying exposure times.

2. RESULTS

3.1 Single species biofilm

Figure 1 shows the results for single-species biofilms formed by *C. albicans*. For the LTP-treated groups, a significant reduction in all exposure times ($p < 0.0001$) was observed when compared to negative control, and there was no statistically significant difference between the exposure to LTP-argon for 60 s

or 120 s compared to 0.12% chlorhexidine ($p > 0.05$). Both LTP treatments for 60 and 120 s showed a reduction of more than 1 $\log_{10}$ CFU/mL (1.55 and 1.90 $\log_{10}$ CFU/mL, respectively). Figure 2 shows the results obtained for single-species biofilms formed by *L. casei*. Significant reduction in all exposure times ($p < 0.0001$) was observed in all LTP-treated groups compared to negative and positive controls.

3.2 Multispecies biofilms - *C. albicans, L. casei*, and *S. mutans*

Figure 3 shows the $\log_{10}$ CFU/mL results for the multispecies biofilms formed by *C. albicans, L. casei*, and *S. mutans.* In the counts of these three microorganisms recovered from the multispecies biofilms, a significant $\log_{10}$ CFU/mL reduction was observed in LTP-treated samples in all exposure times (30, 60, and 120 s) in comparison to both negative and positive control ($p < 0.0001$). Both *C. albicans* and *L. casei* LTP-treated for 120 s groups showed a reduction of more than 2 $\log_{10}$ CFU/mL. Finally, for *S. mutans*, the LTP-argon treatments for 60 and 120 s, in addition to having similar reductions, were also greater than 2 $\log_{10}$ CFU / mL. Furthermore, 0.12% chlorhexidine treatment per 1 minute for all microorganisms recovered from the multispecies biofilms showed no statistically significant $\log_{10}$ CFU/mL reduction ($p < 0.05$) compared to the negative control.

2.3 Confocal scanning laser microscopy (CSLM)

Figure 4 presents CSLM representative images showing the morphology and structural organization of multispecies biofilm after the treatment with LTP for the highest tested exposure time (120 s) and negative control. The images show live cells stained green (Syto 9), while dead cells are stained red (Propidium Iodine). As the picture shows, samples treated with LTP for 120 s present a visually larger area covered in dead cells when compared to the negative control, which can be visualized in the overlaid images (P120 live/dead), as well as when images are separated in live or dead channels.

3. DISCUSSION

In this study, we worked with three different species of microorganisms commonly related to the composition of cariogenic biofilm on the tooth surface. Here, we were able to show that the cariogenic biofilm formed by the association of these microorganisms was significantly reduced after treatment in all plasma-treated samples in comparison to both negative and positive controls.

Our results indicate that LTP treatment reduced the viability of *C. albicans* by 1.95 $\log_{10}$ CFU/mL on the multispecies biofilm after 120 s of treatment compared to negative control. This is corroborated by a previous study, where the authors used LTP-helium at a 1.5 cm distance from the plasma tip to the sample. They observed significant viable *C. albicans* cell reduction. Approximately 2-log reduction was observed after 7.5 min of exposure. [37]. Another study that also used an LTP-helium analyzed the capacity of the LTP-helium to disrupt the biofilm matrix, the cellular structure, and the *C. albicans* viability after exposure by applying 60 s of treatment and 10 mm of distance from the plasma tip to the biofilm

surface. The results indicated that after treatment, significant $\log_{10}$ CFU/mL reductions were observed by changing the microorganism morphology compared to controls [38].

Ebrahimi-Shaghaghi et al. (2021) evaluated the effects of LTP-helium/O_2 (2%) on the growth of *C. albicans*, submitted to 90, 120, 150, 180, and 210 s of treatment. The percentage of biofilm inhibition was 31.43% after 90 s of exposure and reached 41.15% after 120 s of treatment. [39]. Similar trends were observed in the present work both for monospecies and multispecies biofilms; as the dosage of LTP increased, a more significant reduction of the biofilm was observed.

This study also significantly reduced *L. casei* in multispecies cariogenic biofilms. Another study analyzed the action of the LTP-argon brush on monospecies biofilms formed by *Lactobacillus* and *Streptococcus* on hydroxyapatite discs. Three, 9, 13, 15 and 18 s of treatment were applied. Initially, the biofilm was created by a lower concentration of inoculum (between 2.1×108 and 2.4×108 CFU/mL). After treatments, the authors observed that applying the plasma brush for just 13 s was necessary to reduce the viability of the biofilms to the point that they could not be recovered. However, when biofilms were formed at a higher concentration (between 1.7×1010 and 3.5×1010 CFU/mL), the biofilm showed greater resistance to LTP-argon and the reductions were between 1.5 and 2. 5 logs for both biofilms, respectively [40]. This study may help to understand better how LTP and its generated reactive oxygen species can act to reduce the viability of biofilms of *Lactobacillus* and *S. mutans*, two sugar metabolizers that are important in the process of caries evolution, and also to understand better how single jet that can only treat a specific point can act in comparison to LTP-brush that produces more quantity reactive species.

In a previous study carried out by our team, we analyzed the action of LTP-argon using the same plasma device commercially obtained, as well as the same parameters [29]; the argon gas flow was also used, and the effect of LTP-argon jet was analyzed on single and multispecies biofilms formed by *S. mutans, Streptococcus sanguinis*, and *Streptococcus gordonii* on top the same hydroxyapatite disk. After treatment with LTP-argon per 30 s, 60 s, and 120 s, we observed a significant reduction in viability (log10 CFU/mL) for *S. mutans* on single and muti-species biofilm [29]. This study obtained similar results for *S. mutans* on multi-species biofilm formed by *C.albicans*, *L. casei* and *S. mutans*. We observed a significant reduction in viability ($\log_{10}$ CFU/mL) for all plasma-treated samples. This allowed us to understand better the action of LTP-argon on different associations of cariogenic microorganisms present in the biofilm, especially the activity of LTP-argon on *S. mutans.*

Qing H et al. (2016) investigated the effect of LTP-argon brush treatment on the biofilm of *S. mutans* nomo-species [41] by applying a 6 mm distance from the plasma tip to the sample surface. Biofilms were treated for 1, 2, and 5 min. After just 1 minute of treatment, results show 90% biofilm reduction. Our results were important for better understanding the LTP jet since the plasma brush can treat a larger surface, producing more species of oxygen, nitrogen, and other agents. The plasma jet can only treat a specific point, which leads to less production of these agents that cause the effect [42]. Nima G et al. (2021) evaluated the action of LTP against *S. mutans* biofilms formed on sterile resin discs under anaerobic conditions. The plasma jet was applied for 30, 90, 120, and 150 s. After CFU analysis, a

significant reduction was proven at all times of treatment, using jets of LTP formed by a device similar to a brush [43]. Yang et al. (2011) observed the effectiveness of the LTP device in reducing the viability of biofilms formed by *S. mutans* and *Lactobacillus acidophilus.* The authors conclude in this study that the complete elimination of *S. mutans* took only 15 seconds and 5 minutes to eradicate *L. acidophilus* [44].

Using 0.12% chlorhexidine as a positive control was based on knowing that chlorhexidine is a gold-standard chemical substance with an antibacterial action against gram-positive and gram-negative bacteria [45]. In this study, treatment with 0.12% chlorhexidine reduced *C. albicans*, *L. casei*, and *S. mutans* $\log_{10}$ CFU/mL amount according to the exposure.

There is an urgency to develop alternative methods to contain the exponential growth of the microbial population comprised in biofilm without increasing the burden of bacterial resistance. Because it is safe, effective, non-toxic, and resistance-free [46], LTP is an optimal alternative for the microbial resistance response.

Plasmas are produced at various pressures, generally close to atmospheric pressure by high electric field intensities, which have electromagnetic radiation, ultraviolet radiation, and light in the visible spectrum, free radicals, free electrons, neutral reactive oxygen species (ROS – O, O^2●-, O_3, OH), and nitrogen (RNS – N, N_2*, NO, NO^2) that play a synergistic role in antimicrobial action [47]. LTPs are produced at the temperature of heavy species (neutral ions), which is much lower than the electron temperature and can provide energetic fluxes of ions to the substrates. This gaseous reaction at low temperatures is the main reason for using LTPs for biological interests [47]. Due to these fundamental characteristics, scientists began to innovate to update existing dentistry protocols.

This study showed significant and promising results in areas related to biofilm, specifically cariogenic biofilms. Studies also demonstrate the effect of LTP in keratinocytes, gingival fibroblasts, and reconstituted tissue [48, 38]. Future studies will be necessary to analyze the action of LTP on biofilms formed in situ. The data generated in the present work may contribute to developing LTP equipment with specific parameters for treating dental caries and developing new techniques based on minimal intervention dentistry and other forms of treatment.

Low-temperature plasma showed a significant antibiofilm effect against single and multispecies biofilms formed by *C. albicans, L. casei*, and *S. mutans*. The association of microorganisms to perform the biofilm analyzed in this work was based on studies that indicated the microorganisms that metabolize acids and are critical for colonizing the dental surface. *C. albicans* and *L. casei* were inactivated by LTP treatment in short exposure times. CFU/mL of all microorganisms studied were significantly reduced in single and multispecies biofilms. In conclusion, all tested exposure times with LTP-argon greatly affected cariogenic biofilms of single and multiple species in short exposure times. LTP-argon may be a promising therapy, capable of reducing the viability of microorganisms present in a cariogenic biofilm, thus contributing to protocols for treating and controlling dental caries in children and adults.

4. MATERIALS AND METHODS

4.1 Plasma device and parameters

LTP jet was generated with argon gas through the KINPen09™ device (Leibniz Institute for Plasma Science and Technology, INP, Germany). The device comprises a hand-held unit (170 mm in length, 20 mm in diameter, and 170 g) that generates the plasma jet, a dc power supply (system power: 8 W at 220 V, 50/60 Hz), and a gas supply unit. The LTP jet was produced from the top of the central electrode and expanded to the surrounding air outside the nozzle [29]. Biofilms formed on the top of hydroxyapatite discs were treated at a distance of 10 mm from the biofilm surface to the tip of the plasma device for 30 s, 60 s, and 120 s of exposure in continuous working mode [29]. Support developed by the laboratory collaborators and a calibrated operator trained to experiment with a consistent approach was used to guarantee the standardization of the distance between the plasma tip and the sample. The specimens were moved horizontally using sterile tweezers during LTP application to scan the whole surface. Argon gas flow was set to 5 slm, and the flow rate was controlled using a flow controller (MKS Instruments, Germany).

4.2 Microorganisms growing

Streptococcus mutans UA159 (ATCC 700610), *L. casei* (ATCC 10801), and C. *albicans* (SC 5314) were used to form mono multispecies biofilms. Mono species biofilm formation by *S. mutans* is described in Figueira LW et al. (2021) [29]. Growth curves were performed to standardize the inoculum for the biofilm, and the equivalent colony-forming units (CFU) were determined. Stock cultures were maintained at −80°C. *S. mutans* was reactivated in BD BBL™ CDC Anaerobe 5% Sheep Blood agar and incubated at 37°C for 48 hours, *L. casei was reactivated in* BHI agar (Brain Heart Infusion, Difco, Detroit, USA) and *C. albicans* was reactivated in Sabouraud dextrose cloramphenical Agar (SDA) 0.5g/L. The plates were incubated at 37°C for 72 and 24 h, respectively. *S. mutans* and *L. casei* were submitted to a captophilic (5% CO_2) environment, requiring a carbon dioxide concentration between 5–10% and approximately 15% oxygen. After that, for the formation of the pre-inoculum, ten isolated colonies were collected with the aid of a sterile loop and subsequently transferred to a falcon centrifuge tube containing 10 mL of Tryptic soy broth (TSB) supplemented with 1% glucose at 37ºC for 18 h. Then, 1 mL of the pre-inoculum was transferred to a falcon centrifuge tube containing 9 mL of TSB supplemented with 1% glucose. The same procedures were done for all microorganisms separately. The growth of microorganisms was monitored aseptically every hour (0–24 h) through subsequent absorbance readings (optical density) in a spectrophotometer using the 600 nm wavelength to ensure the reproducibility of the biofilm model.

4.3 Multispecies cariogenic biofilm

Single- and multispecies biofilms composed of *C. albicans*, *L. casei*, and *S. mutans* were formed as described by Arthur et al. (2013) with modifications. S. *mutans, L. casei*, and *C. albicans* were incubated at 37°C for 48, 72, and 24 hours, respectively [36]. To prepare the pre-inoculum, the microorganisms were transferred from the agar plate to a centrifuge tube containing 10 mL of TSB broth supplemented with

1% glucose. Tubes were incubated at 37 ºC, 5% CO_2 for 18 h. Then, 1 mL of the pre-inoculum was transferred to another tube containing 9 mL of TSB supplemented with 1% sucrose, and the tubes were incubated as described in 2.2. According to the determination of the growth curve performed for each microorganism under analysis, the inoculum corresponded to 10^8 CFU/mL after the incubation time. The tubes were centrifuged, the supernatant was discarded, and 10 mL of saline was added. This same process was performed for two more times. The inoculum's microorganism concentration (108 CFU/mL) was confirmed with a spectrophotometer. Then, the *L. casei* suspensions were then concentrated to 109 CFU/mL, *C. albicans* diluted to 105 CFU/mL, and de *S. mutans* to 10^7 CFU/mL. To form multispecies biofilms, an equal part of each standardized suspension was mixed up using a vortex. For single and multispecies biofilms, 200 µL of the inoculum was added to the wells of 24-well plates containing sterile hydroxyapatite discs, plus 800 µL of TSB broth supplemented with 0.2% sucrose was added [29]. Biofilms were incubated for 48 hours (37°C, 5% CO_2). The fresh culture medium was replaced after 24 hours.

4.4 LTP treatment and biofilm processing

Single and multispecies biofilms were treated with LTP and flow control (argon gas) for 30, 60, and 120 s, as described in 2.1. Chlorhexidine (0.12%) was used as a positive control (PC), and 0.89% NaCl was used as a negative control. Both Chlorhexidine and NaCl were in contact with the samples for 1 minute. After that, the biofilms were scratched from the top of the hydroxyapatite discs using a sterile spatula and immediately diluted in 5 mL of sterile 0.89% NaCl solution. Biofilm suspension was sonicated for 10 s pulses at an output of 9 W (Fisher Scientific, Sonic Dismembrator model 100, USA) three times [29]. The same procedures were performed for each experimental group and control. The obtained suspensions were serially diluted and plated on selective agar. SDA with chloramphenicol (0.1 mg/mL) for *C. albicans*, Mitis Salivarius-Bacitracin (MSB) (0.1 unit ml^{-1}) sucrose (1%) for *S. mutans*, and Rogosa agar 1.32 mL/L glacial acetic acid were used to recover *L. casei.* After incubation at 37 ºC for 24 hours for *C. albicans* and 5% CO_2 at 37º C for 48 and 72 h for *S. mutans* and *L. casei*, respectively, the number of CFU/mL of recovered *C. albicans*, *L. casei*, *S. mutans* from the biofilm was recorded.

4.5 Confocal laser scanning microscopy (CLSM) of single- and multispecies biofilm

Multispecies biofilms composed of *C. albicans*, *L. casei*, and *S. mutans* (formed as described at 2.3) were stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit (L13152; Molecular Probes, Inc.) and incubated in the dark at room temperature for 15 minutes to allow penetration of the fluorophores inside the bacterial cells. Specimens were then washed twice with 0.89% NaCl and examined under a Leica SP8 Resonant-scanning confocal/multiphoton microscope using a Leica Fluotar VISIR 25x/0.95 water objective, with a free working distance of 2.3 mm. Serial sessions on the XYZ plane were observed.

4.6 Statistical analysis

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

Declarations

Competing Interests:

We would like to declare that no known conflicts of interest are associated with this publication, and no significant financial support for this work could have influenced its outcome.

Author Contribution

CYKI, SD, LWF: the conception and design of the studyLWF, CYKI, SD, BHDP: acquisition of data, analysis and interpretation of data. CYKI, LWF, SD, BHDP: Drafted the article and revised it critically for important intellectual content. LWF, CYKI, SD, BHDP: final approval of the version to be submitted.

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This work was conducted while the corresponding author was an Associate Professor at Indiana University School of Dentistry.

Data Availability

Data availability statment: All data are included in the manuscript, and additional information and further queries about sharing data can be directed to the corresponding author.

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Figures

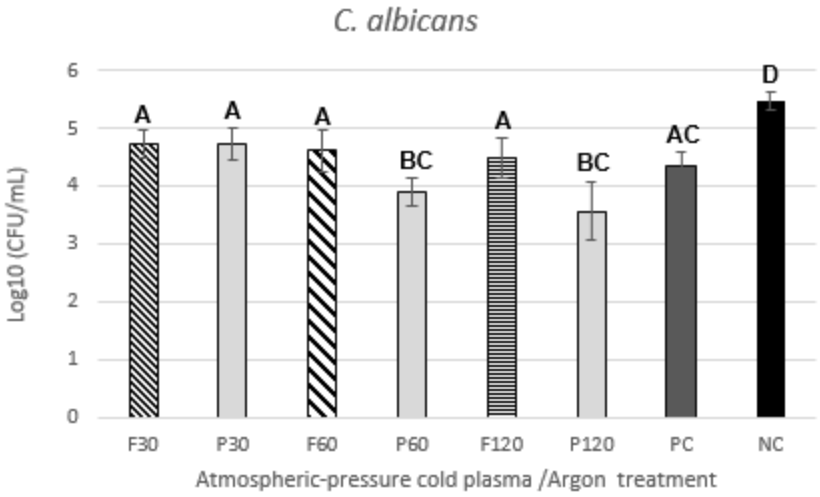

Figure 1

Mean values and standard deviations $\log_{10}$ (CFU/mL) of single-species biofilms composed by *C. albicans* treated with LTP-argon 0.12% chlorhexidine digluconate (positive control) or 0.89% saline solution (negative control). *values equal to zero. Different letters indicate significant statistical differences (n= 9; $P \leq 0.05$; ANOVA, Tukey's Test). F30, F60, and F120 (argon gas – flow control treatment for 30, 60, and 120 s); P30, P60, and P120 (LTP-argon treatment for 30, 60, and 120 s), PC (positive control) and NC (negative control).

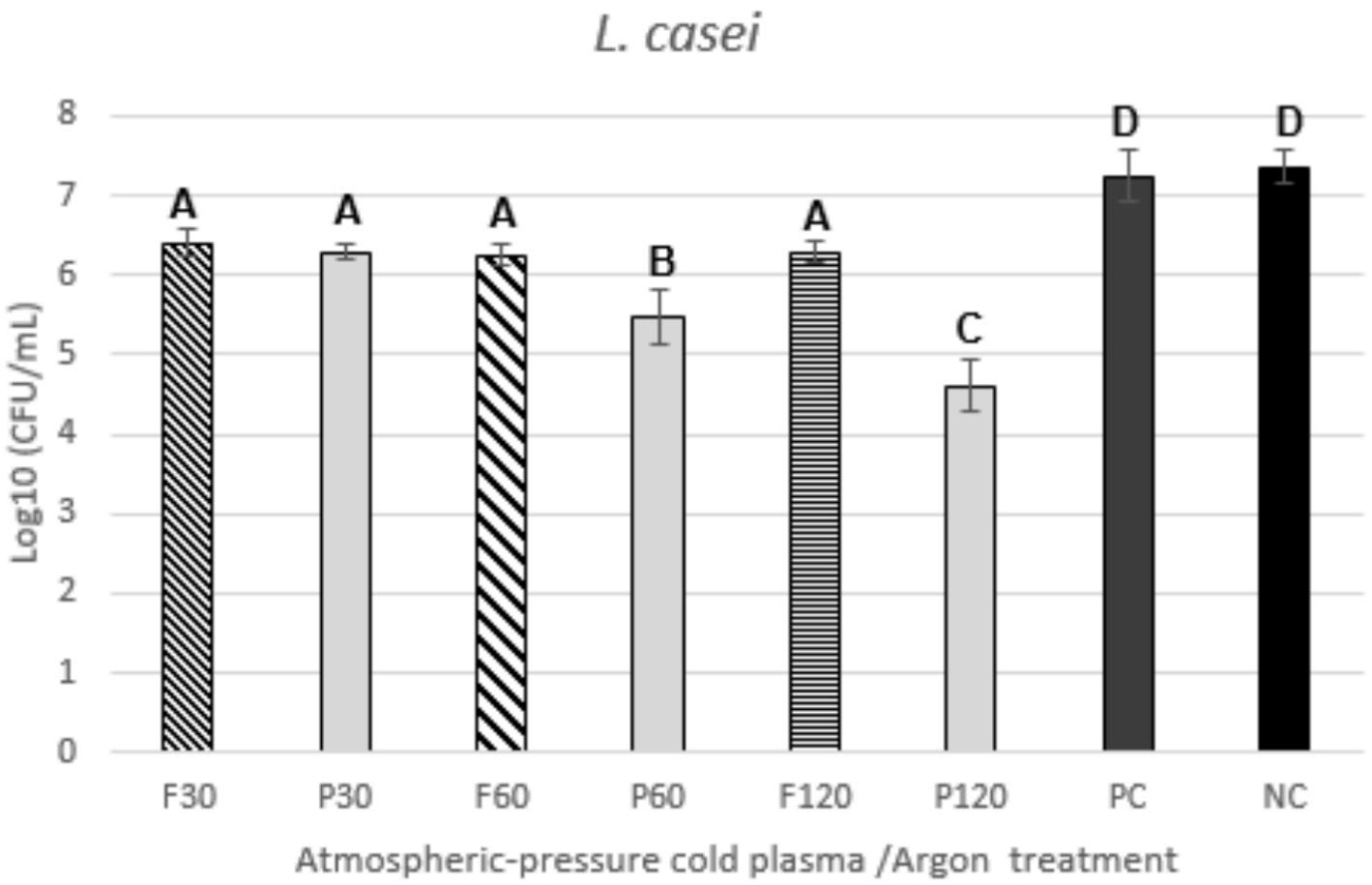

Figure 2

Mean values and standard deviations log10 (CFU/mL) of single-species biofilms composed by *L. casei* treated with LTP-argon, 0.12% chlorhexidine digluconate (positive control) or 0.89% saline solution (negative control). *values equal to zero. Different letters indicate significant statistical differences (n= 9; $P \leq 0.05$; ANOVA, Tukey's Test). F30, F60, and F120 (argon gas–flow control treatment for 30, 60, and 120 s); P30, P60, and P120 (LTP-argon treatment for 30,60 and 120 s), PC (positive control) and NC (negative control).

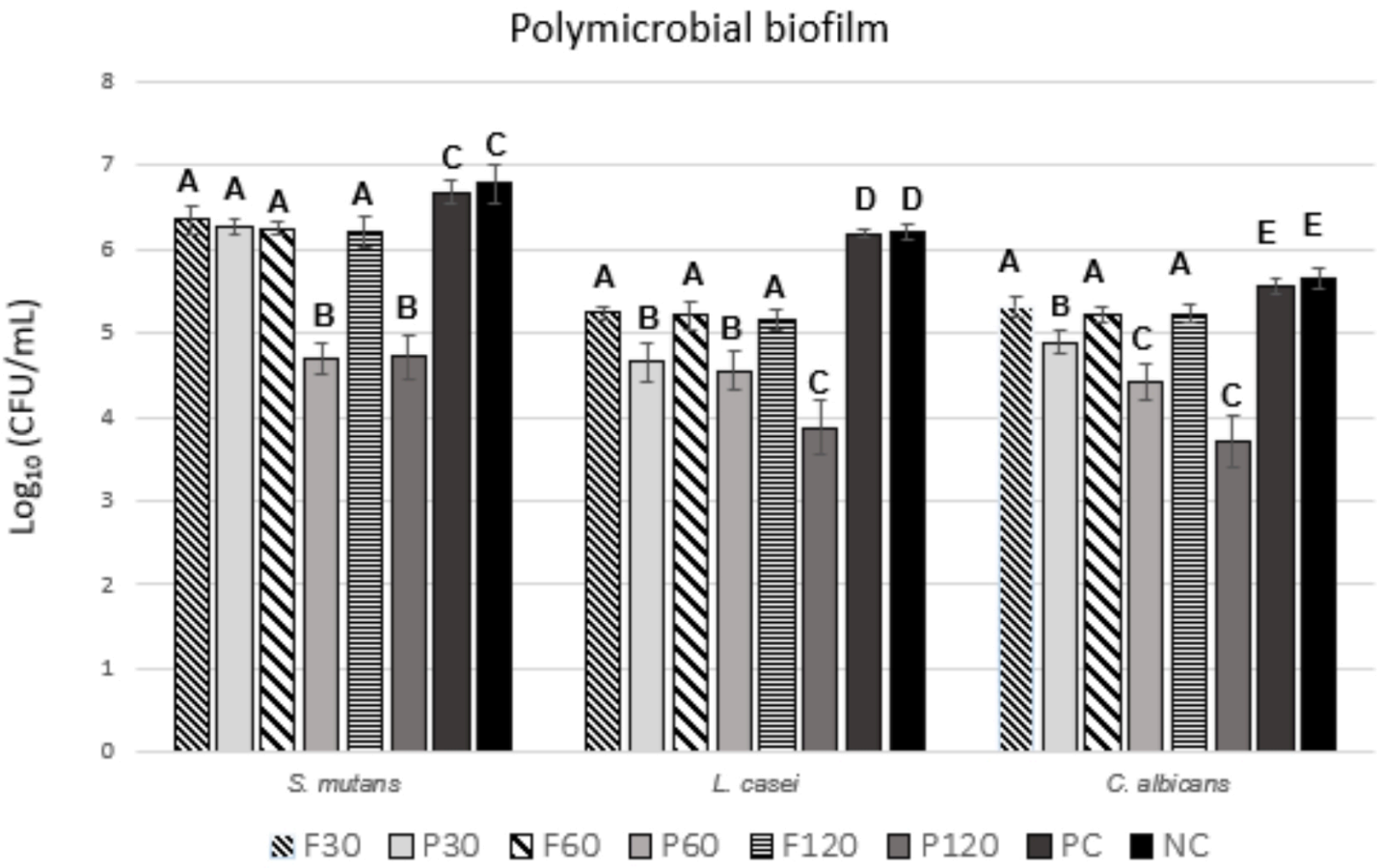

Figure 3

Mean values and standard deviations log10 (CFU/mL) of polymicrobial biofilms composed by *Candida albicans, Lactobacillus casei,*and *Streptococcus mutans* treated with LTP-argon, 0.12% chlorhexidine digluconate (positive control) or 0.89% saline solution (negative control). *values equal to zero. Different letters indicate significant statistical differences (n = 9; $P \leq 0.05$; ANOVA, Tukey's Test). F30, F60, and F120 (argon gas–flow control treatment for 30. 60, and 120 s); P30, P60, and P120 (LTP-argon treatment for 30, 60, and 120 s); PC (positive control) and NC (negative control).

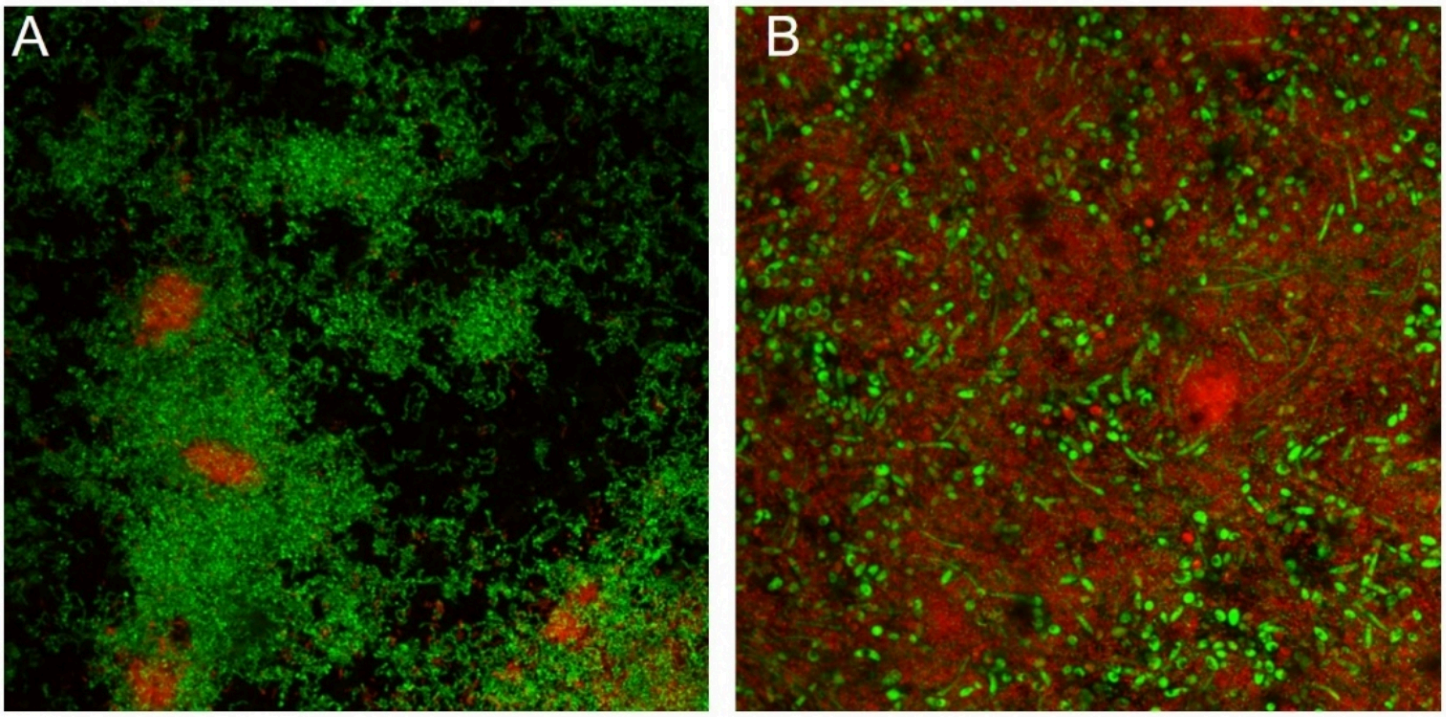

Figure 4

Confocal laser scanning microscopy of multispecies biofilm formed by *Streptococcus mutans, Streptococcus gordonii, and Streptococcus sanguinis* in the negative control group and LTP LTP-argon (120 s) group; 25 × zoom. Biofilm stained with Live/Dead BacLight Viability kit. Live cells are in fluorescent green, and dead cells are in fluorescent red. Live and Dead (live/dead) cells mixed in fluorescent green and red.