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PAPER

Inactivation of *S. mutans* dental biofilm by using Cold Atmospheric Plasma

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Abstract

S.mutans cells in oral cavity could adhere to surfaces of the teeth form the bacterial biofilm, strongly resist daily cleaning and brushing. The growth of the bacterial protected by the biofilm may lead to dental plaque formation and tooth decay. **C**old **A**tmospheric **P**lasma (**CAP**) generated from a capillary-guided corona discharge is investigated for the inactivation of *Streptococcus mutans* biofilm associated with dental caries. The plasma device was optimised to generate a 4-mm plasma plume while maintaining a temperature near to ambient. The heat generated was not susceptible to thermal damage for the biofilm, as well as the oral cavity. Through experimental evaluations, the gaseous plasma plume from the CAP with a treatment durations of up to 15 min were responsible in reducing the *S. mutans* biofilm. The highest removal was achieved after 15 min of CAP treatment. The results shown that a log-reduction of 3.08 of the cells in the *S. mutans* biofilm was killed during the treatment. The 10 min treatment demonstrated a high value of log reduction at 1.79 compared to the control well. The efficacy is found to be strongly correlated to treatment duration whereby at the minimum duration of 1 min treatment resulted a log reduction of 0.23. The outcomes of this research highlight CAP as a promising and safe approach for combating *S. mutans* biofilm. By offering a non-thermal alternative to traditional antimicrobial methods, CAP presents an opportunity to minimize harm to healthy tissues while effectively targeting biofilm.

1. Introduction

Plasma represents an ionized gas state with a mixture of ions, electrons, and neutral particles, setting it apart from the conventional states of matter such as solids, liquids, and gases. It is this unique feature of the plasma that contribute to its diverse range of properties and behaviours, rendering it a compelling subject for scientific exploration and garnering significant interest among researchers. In the laboratory, the plasma can be generated by heating a gas to extremely high temperatures, causing the gas particles to gain kinetic energy and become ionized. This ionization process may lead to the formation of plasma in different equilibrium states including thermal equilibrium and non-thermal equilibrium plasma. For non-thermal equilibrium plasma, the temperature of ions or neutral particles in the plasma are not in equilibrium with the electron temperature, which is also known as non-thermal plasma. One particular type of non-thermal plasma that can be produced at atmospheric pressure with ions and neutrals temperature close to room temperature is known as **C**old **A**tmospheric **P**lasma (**CAP**). This unique characteristic allows CAP to interact directly with biological systems without causing significant thermal damage [1], making them particularly well-suited for biomedical applications [2]. Furthermore, CAP also offers a better convenience compared to generation of plasma that necessitate the use of vacuum accessories.

In the medical field, CAP has demonstrated significant potential for various applications, including wound healing [3], inducing cancer cell death while sparing healthy cells from harm [4] and sterilization [5]. The

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antimicrobial properties of CAP have been utilized for wound disinfection, promoting faster healing and reducing the risk of infection [6]. Additionally, CAP has shown promise in selectively targeting and killing cancer cells while minimize damage to healthy cells [7], offering a potential alternative to traditional cancer therapies. Its ability to inactivate pathogens has made CAP a valuable tool for sterilizing medical equipment and surfaces. Furthermore, its potential in the medical field has emerged as an exciting area of research with promising implications across various industries, including dentistry.

Dentistry is undeniably a critical branch of healthcare. With increased focus on oral health, the industry has seen a growing interest in utilizing CAP for a wide range of treatments. This includes sterilizing dental equipment [8, 9], tackling biofilms adhered to dental surfaces [10, 11], disinfecting root canals [12, 13] and managing intraoral diseases [11]. Dental caries or easily known as tooth decay and periodontal diseases characterized by inflammation of the gum and bone, emerge as prevalent oral health concerns [14]. The excess intake of fermentable carbohydrates represents a common risk factor for both dental caries and periodontal diseases. *Streptococcus mutans* (*S. mutans*) and *Porphyromonas gingivalis* are among the key bacterial species associated with these oral diseases [15, 16]. *S. mutans*, an anaerobic, gram-positive bacterium, is commonly found in dental cavities. It has the ability to metabolize dietary sugars and produce acid as a byproduct. This acid produced can potentially lead to the demineralization of tooth enamel and resulting in the formation of cavities [17]. Furthermore, *S.mutans* and other bacteria in the oral cavity can form biofilms. Dental biofilms adhere to the tooth surface presents the potential for the formation of dental plaque which is a sticky film that is difficult to remove through regular oral hygiene practices [18]. It was reported that the colonization of *S. mutans* in the oral cavity can enhance the colonization of other species and impact polymicrobial pathogenesis, thereby increasing the risk of oral infections [19]. Treating *S. mutans* in planktonic bacteria is relatively easier than in biofilm form because biofilms are complex communities of microorganisms embedded in a self-produced extracellular matrix [20, 21]. The biofilm matrix provides a shield to the bacteria within, making them more resistant to antimicrobial agents and host immune responses, thus, contributing to the persistence of oral infections. Additionally, the presence of persister cells within biofilms, which remain dormant and highly tolerant to antibiotics [22], further contributes to the difficulty in treating *S. mutans* in this form.

Given the increasing concerns over antibiotic resistance and the limitations of the current spectrum of antimicrobial agents, there is a growing interest in exploring alternative approaches for the treatment of *S. mutans* and other dental biofilms. Cold atmospheric plasma has emerged as a prospective solution due to its antimicrobial properties and its ability to disrupt the complex structure of biofilms [23]. Many studies have shown promising results in targeting and reducing the viability of dental biofilms, including those formed by various microorganisms implicated in oral disease. In a study by Nima *et al*, CAP treatment was applied to oral biofilms dominated by *S. mutans* and the effectiveness was evaluated using **C**olony **F**orming **U**nit (**CFU**) count [11]. A reduction in bacterial viability of 38.3% was obtained, indicating a decrease in viable *S. mutans* cells after CAP treatment. Various research studies previously conducted had shown that there are significant morphological changes to the structure of the treated biofilms as compared to the untreated ones. CAP treatment can lead to the detachment of bacterial cells from the biofilm matrix, causing a breakdown of the biofilm structure. Disrupting the biofilm architecture is important as it renders the biofilm more susceptible to mechanical removal and antimicrobial agents. Lee *et al* reported a study using a cold atmospheric plasma jet produced from helium gas on *P. gingivalis* grown on titanium discs [24]. Results showed a significant decrease in *P. gingivalis* viable cell count as treatment duration increased. **S**canning **E**lectron **M**icroscope (**SEM**) images revealed that the biofilm structure on the titanium disc was destroyed after more than 3 min of treatment duration. Another study utilizing plasma jet operated with nitrogen gas irradiated on dental biofilm samples shown that the treated specimens had more hydroxyl groups and was more hydrophilic in comparison to control samples as shown by **X**-ray **P**hotoelectron **S**pectroscopy (**XPS**) and contact angle analysis [25].

In a study by Hong *et al*, the cells within the *S. mutans* biofilm were severely damaged after treatment with atmospheric pressure plasma. However, the treatment becomes much more effective when combined with antibiotic [26]. In his recent work on the treatment effects on *P. gingivalis* biofilms, the study showed that the treatment by using argon gas, or argon + oxygen gas mixture resulted in reduction in bacterial load [27]. This study also found that the recovered biofilm exhibited increased susceptibility to amoxicillin. In other words, the non-thermal atmospheric gas plasma is a potential method to treat oral biofilms and possibly reduce the chance of bacterial reinfection. In addition to cold plasma treatment on *S. mutans* biofilms, research on biofilm formed by other bacterial species were also reported. A study by Rupf *et al*, demonstrated 3–4 log reduction for *L. casei, S. mutans, C. albicans* and *E. coli* biofilms formed on the dentine slices with plasma jet treatment [15]. CAP treatment has shown potential in targeting *E. faecalis* biofilms in root canals. A study conducted by Theinkom *et al* revealed a significant reduction of $\geqslant 5$ $\log_{10}$ in cell viability after 10 min plasma treatment on *E. faecalis* biofilms [28]. Other researchers also demonstrated the capability of CAP to effectively reduce the viability of both young and mature *E. faecalis* biofilms [29, 30]. Another research conducted by Jungbauer *et al*, on *A. actinomycetemcomitans* biofilms showed a promising result with log reduction of 4 on titanium specimen

after 120 s of cold atmospheric plasma treatment [31]. Few studies have demonstrated the inhibitory on the growth of bacteria within dental biofilms after the cold plasma treatment [32, 33]. It was reported that CAP can suppress the proliferation of bacteria, including key pathogens associated with oral infections, such as *S. mutans*, *E. faecalis*, *C. albicans*, and *P. gingivalis* [34]. By inhibiting bacterial growth, CAP can help control the progression of biofilm-associated oral diseases.

Plasma treatment has proven effective against both Gram-positive and Gram-negative bacteria. Gram-positive bacteria are characterized by a thick cell wall composed of multiple layers of peptidoglycan, but unlike Gram-negative bacteria, they lack an additional outer membrane [35]. The microbicidal efficacy of CAP is multifactorial, largely correlated with the thickness of the bacterial cell wall. The Gram-positive *Bacillus subtilis* biofilm, with a cell wall thickness of 55.4 nm has been observed to exhibit greater resistance to plasma treatment than the Gram-negative *Pseudomonas aeruginosa* biofilm, which had a cell wall thickness of only 2.4 nm [36]. Nevertheless, another independent study has demonstrated that the susceptibility of bacterial cells to plasma treatment is also influenced by the presence of an outer membrane [37].

The versatility of plasma allows for the control of the composition and concentration of reactive species, which is essential for its antimicrobial action against bacteria and biofilms. However, the mechanisms involved in microbial inactivation have not yet been fully established. Reactive nitrogen and oxygen species (RONS) produced in plasma have been shown to damage the bacterial cell membrane and disrupt cellular structures in Gram-positive bacteria [38]. Additionally, they induce membrane lipid peroxidation in Gram negative bacteria as evidenced by the research of Xiaoye *et al* [39]. Once the cell wall is broken, reactive species can diffuse into the cell interior, where they proceed to inflict additional damage on DNA and intracellular proteins through oxidative or nitrosative means [40, 41]. The oxygen atoms can break C–O, C–N, and C–C bonds in the structure of peptidoglycan [42], while nitrosative species have been implicated in protein degradation [43]. The bactericidal effect attributed to the generation of intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS) was also reported [44, 45]. Studies have shown that plasma exhibits strong antimicrobial effects against both aerobic and anaerobic bacteria [46], with ROS contributing to its efficacy in aerobic settings. Notably, even in anaerobic conditions, plasma demonstrates significant bactericidal action, underscoring the importance of direct interaction. Interestingly, while Gram-negative strains appeared slightly more susceptible to plasma treatment, this difference was not statistically significant. Furthermore, a study utilizing a plasma jet system with argon gas demonstrated effective inactivation of the *Streptococcus mutans* UA159 biofilm on human enamel slabs, significantly reducing the levels of water-soluble extracellular polysaccharides while leaving water-insoluble polysaccharides unchanged [47]. These studies underscore the significance of further exploring the application of CAP for dental treatments.

Aside from studying the efficacy of biofilm treatment by using CAP alone, there are numerous research studies conducted to demonstrate the synergistic effects of CAP when combined with conventional antimicrobial agents and mechanical debridement methods. The combination of CAP with antimicrobial agents, such as laser, air abrasion and chlorhexidine [48] or hydrogen peroxide [49] has shown enhanced antimicrobial activity against dental biofilms. Similarly, using CAP as a supplement to mechanical debridement procedures, such as scaling and root planning has also shown an improvement on biofilm removal and microbial reduction [50].

In recent years, there has been a rapid grow of interest in exploring the potential applications of plasma including air disinfection and purification [51]. Plasma technology has also been recognized as eco-friendly method for modifying the surface of silk fabrics to improve the adherence of plant extracts, thereby enhancing its microbial resistance [52]. Substantial efforts on the studies of sterilization and antimicrobial properties of CAP have provided insights, prompting further investigation into its potential application for fighting against resilient bacterial biofilms. The ability of CAP to generate various reactive species and selectively target pathogenic microorganisms has emerged as viable alternative to the conventional methods for dental disease management.

In this paper, we build upon our previous research on capillary-guided corona discharge for the inactivation of bacteria in chronic wounds [53] by extending the study to target bacteria within biofilms. Our goal is to tackle more challenging scenarios, such as applying cold plasma treatment to biofilm matrices, thereby advancing the application of capillary-guided corona discharge in complex dental and medical settings. While most of the cold plasma can be easily generated below atmospheric pressure, we aimed to develop a method for producing CAP at atmospheric pressure with the use of ambient air as the feed gas of our system. Our research will focus on the efficacy of the CAP treatment by assessing the duration of treatment on *S. mutans* biofilms. By investigating the antimicrobial effects of CAP on biofilm associated with dental caries and periodontal diseases, this research will make contribution on the development of innovative approaches in dental care. Ultimately, our findings may help improve oral health outcomes and enhance patient well-being.

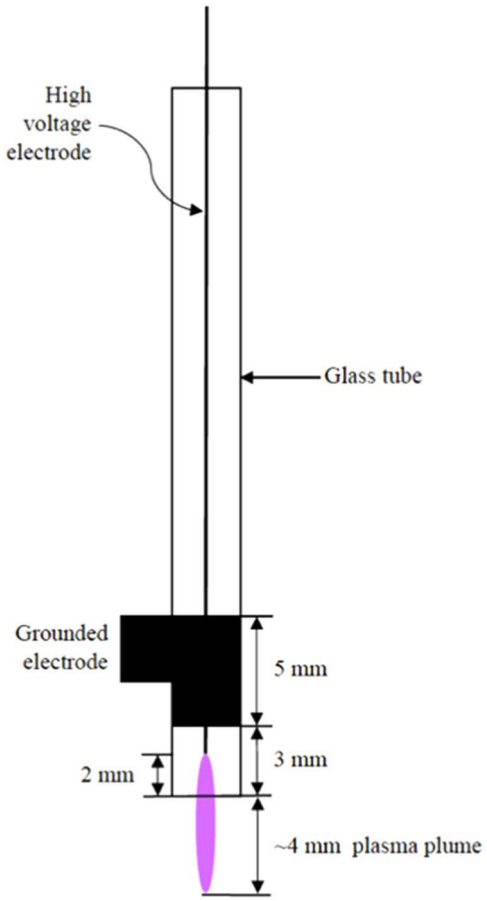


Figure 1. A schematic diagram of the plasma device.

2. Methodology

2.1. Plasma device

The cold atmospheric plasma in this study is produced through a **C**apillary-**G**uided **C**orona **D**ischarge (**CGCD**). Several experimental settings for plasma generation using capillary-guided corona discharge have been explored in previous studies, building on the extensive groundwork conducted by our research group [53, 54]. This groundwork provided valuable insights into the experimental settings used in the present study, which allow for the generation of a stable plasma discharge, making it ideal for biofilm inactivation applications. The plasma device consisted of a hollow cylindrical glass tube with an inner diameter of 1.5 mm and an outer diameter of 3 mm, enclosing a high voltage (HV) electrode where the schematic diagram is illustrated in figure 1. The grounded electrode is made from a piece of copper sheet wrapped around the glass tube. In this configuration, the cold plasma is generated within the glass tube, extended all the way to the open end with a bright violet emission. The plasma plume length extended from the open end of the glass tube was measured to be approximately 4 mm from the tip of the device.

2.2. Plasma diagnostics

The experiment was carried out to characterize the plasma electrical properties and perform the biofilm treatment. A PVM500 plasma power generator served as the power supply, providing high voltage AC to the plasma device. Voltage and current waveforms during discharge were recorded using a Tektronix P6015A High Voltage (HV) probe and a current probe, respectively, with data captured by a digital storage oscilloscope. The characterization of plasma in terms of plume length and thermal characterization were carried out. Image of the plasma plume was captured using a smartphone equipped with a high resolution 64-megapixel camera. The length of the plasma plume was determined from a calibrated length in the image. Thermal characterization was aimed to measure the temperature of the plasma plume. For this purpose, a thermometer was placed at a distance of 7 mm from end of glass tip and in contact with the plasma plume. The temperature was measured and recorded for each incremental degree of temperature rise where the variation of temperature against time of

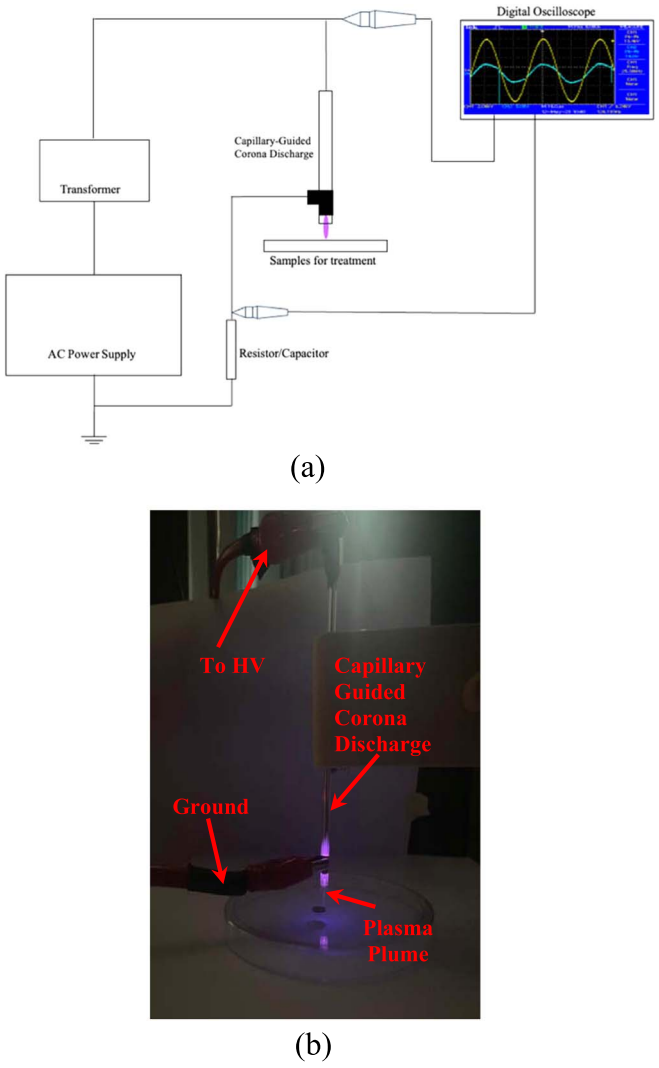


Figure 2. (a) Schematic setup for the biofilm treatment with cold atmospheric plasma; (b) Capillary-Guided Corona Discharge with plasma generation.

exposure can then be plotted. The overall schematic diagram of the experimental setup is presented in figure 2(a), while the capillary-guided corona discharge with plasma generation is depicted in figure 2(b).

2.3. Growth and treatment of *S. mutan* biofilm

The microtiter plate method was used to investigate the effect of plasma treatment on *S. mutans* ATCC 25175 biofilm. This method has been widely adopted and is a reliable method for providing quantitative and reproducible measurements of biofilm biomass [55]. The microtiter plate method has been essentially used for assessing the impact of various treatments on *S. mutans* biofilm integrity [56, 57]. The *S. mutans* ATCC 25175 was first revived on a Tryptic Soy Agar (TSA) plate. After incubation at 37 °C for 16–18 h, a fresh culture of *S. mutans* was used to prepare bacterial inoculum. 1 ml of **T**ryptic **S**oy **B**roth (**TSB**) supplemented with 0.5% glucose was aliquoted into a 5 ml sterile cell culture tube. A sterile cotton swab was moisturized with TSB and a single colony from the bacterial culture plate was picked. The bacterial cells were suspended in TSB by softly pressing and rotating the swab tip on the wall of the tube. The turbidity of the inoculum was adjusted to a McFarland standard of $0.5 \pm 0.02/0.03$ (equivalent to approximately 1.5×10^8 CFU ml^{-1}), with at least three readings taken to ensure consistency. A fixed volume of 100 µl of the adjusted inoculum (0.5 McFarland) was then aliquoted into each well of a sterile non-treated 96-well flat-bottom cell culture plate (Nest Biotechnology Co., Ltd, Jiangsu, China), in triplicate. Sterile distilled water (dH_2O) was added to the adjacent wells to retain moisture and the culture plate was incubated at 37 °C for 48 h to allow biofilm formation. The plate was checked daily to ensure the inoculum was not desiccated. Three wells filled with sterile TSB (supplemented with 0.5% glucose) served as sterility controls.

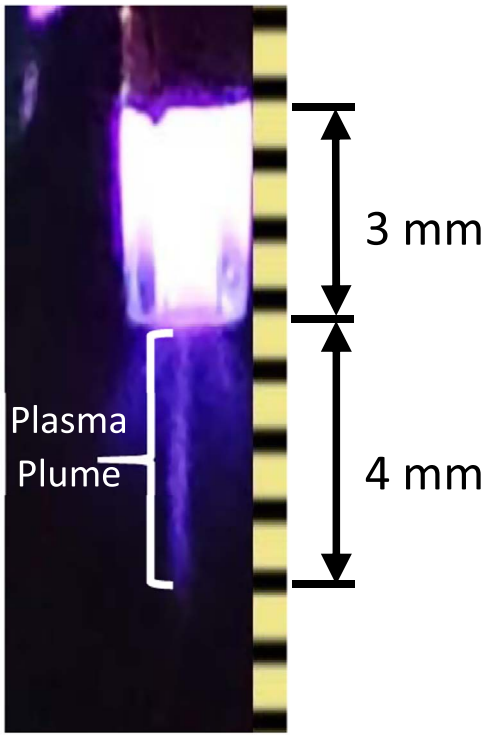


Figure 3. A plasma plume image of 4 mm in length extended from the end of the glass tip.

After the 48 h incubation period, each well was washed three times with 200 μl of sterile **P**hosphate-**B**uffered **S**aline (**PBS**) and drained before the CAP treatment. The biofilm was treated with CAP in triplicates for different durations of 0 (control) minutes, 1, 6, 10 and 15 min to assess the effectiveness of plasma irradiation as a potential anti-biofilm strategy. A group of three wells with *S. mutans* biofilms without plasma treatment served as growth control. The treated biofilm was then subjected to post-treatment procedures to determine the viability of the cells within the *S. mutans* biofilm based on viable cell count method. The viable cell count method was performed to determine the number of **C**olony **F**orming **U**nit (**CFU**). The sessile cells were dislodged from both treated and untreated biofilms using sonication at a moderate ultrasonic wave strength for 5 min (40 kHz at 0.21 W cm^{-2}) in an ultrasonic water bath (PowerSonic 410, Hwashin, Seoul, Korea). The bacterial cell suspensions were then serially diluted in sterile PBS. Next, 100 μl of selected dilutions were plated on TSA plates in duplicates by using the spread plating technique. The inoculated TSA plates were incubated at 37 °C for 24 h. Subsequently, the number of colonies on each plate within the countable range (30–300 CFUs) were counted and the results were tabulated and further analyzed.

The log reduction of the biofilm has been determined to characterize the efficacy of the plasma treatment. Log reduction is a measure of the decrease in the number of cells or pathogens after a treatment or disinfection process. Log reduction is given by:

$$\text{Log reduction} = \log_{10}(A/B)$$

Where:

A is the number of viable cells before treatment

B is the number of viable cells after treatment

3. Results and discussions

Prior to the biofilm treatment procedures, a careful selection of suitable configurations for the capillary-guided corona discharge was conducted to identify the setup that could maintain a stable plasma state suitable for biofilm inactivation studies. Through systematic adjustments and parameter tuning, setting the applied voltage to achieve a peak-to-peak discharge voltage of approximately 13.6 kV and a frequency around 25 kHz has resulted in the production of a 4 mm plasma plume as shown in figure 3. The selection of a suitable setup is a crucial step in ensuring the reliability and reproducibility of subsequent experiments targeting at biofilm eradication.

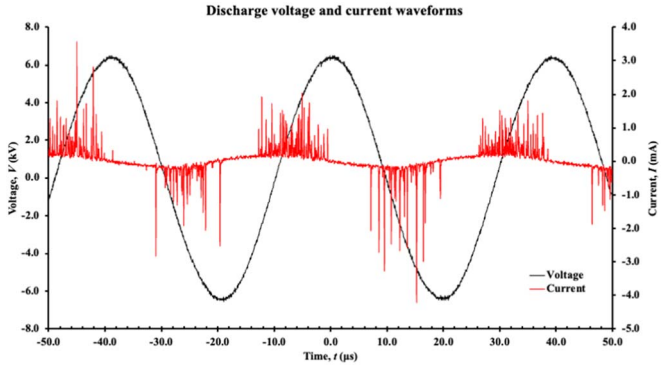


Figure 4. Discharge voltage and current signal of the plasma discharge.

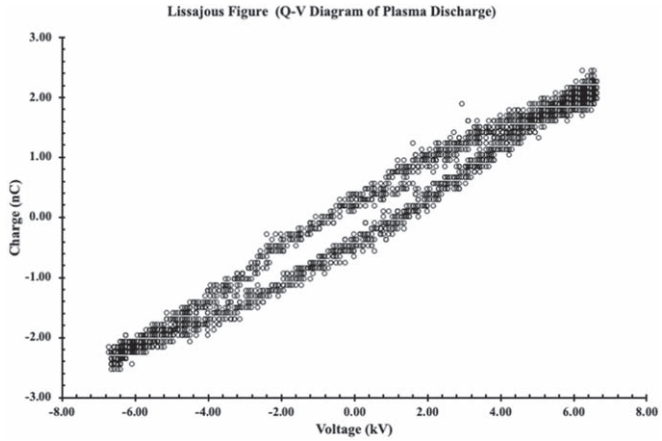


Figure 5. Lissajous figure of the plasma discharge.

Typical discharge voltage and discharge current signal of the plasma discharge is shown in figure 4. Multiple spikes in the discharge current were observed during each complete cycle of the supply voltage. These spikes imply the manifestation of a filamentary discharge mechanism in plasma formation. Each of the current spikes has the pulse duration of about 120 ns. In the current experimental setup, it is inferred that at an applied voltage of 13.6 kV in the presence of ambient air, the ionization of ambient gas leads to the predominance of filamentary discharge mode over glow discharge mode. Charge and voltage measurement of the discharge was also performed to obtain the Lissajous figure. Figure 5 illustrates the corresponding Lissajous figure of figure 4. From the Lissajous figure, the energy dissipated of the plasma system is estimated as 1.6 μJ. The power dissipated was then determined to be about 40 mW as calculated from the multiplication of the energy dissipated and frequency of the discharge. Power dissipation measurement provides a quantitative assessment of the plasma discharge conditions. In other words, this measurement allows for the evaluation of how different configurations and operational parameters affect the power used relative to the level of biofilm inactivation achieved. This enables comparisons across various studies and helps identify the most effective conditions for biofilm treatment.

In order to delineate the impact of cold plasma treatment on biofilm inactivation, it was imperative to discern between the direct effect of plasma exposure (treatment duration) and secondary influences such as the heating effect resulting from the plasma. Hence, a meticulous approach was adopted to measure the temperature of the cold plasma prior to biofilm inactivation experiments. This precautionary step is thus allowed for a more nuanced analysis of the experimental results. The temperature of the cold plasma was observed to increase gradually over time until reaching equilibrium as illustrated in figure 6. Please note that the time zero on the graph is established when the temperature reaches 31 °C. Subsequently, the time taken for each incremental increase of 1 °C is recorded. The direct temperature measurement at a distance of 7 mm from the glass tip shown that the plasma plume reached thermal equilibrium after approximately 75 s, maintaining a temperature of 37 °C. This temperature was within the optimum range for the growth of *S. mutans* biofilm and did not exceed the threshold where it would cause the biofilm to be killed. This was supported by a previous studies on the

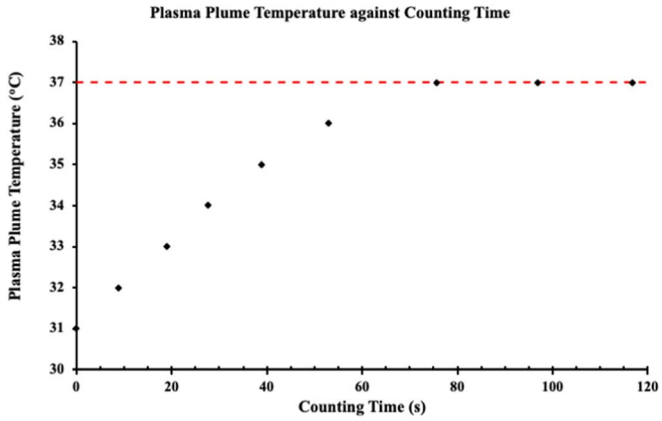


Figure 6. Variation of plasma plume temperature with counting time.

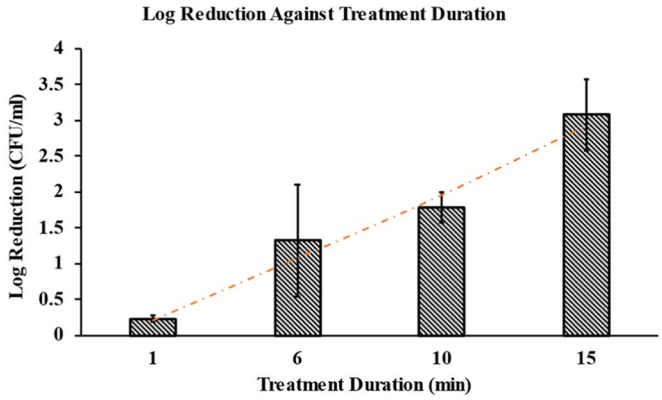


Figure 7. Log reduction against treatment duration.

optimal temperature range for *S. mutans* biofilm growth [58, 59]. In other words, the inactivation of biofilm is attributed to the effect of plasma instead of thermal effect.

The viable cell count method was performed to determine the number of colonies forming unit (CFU) indicating the efficacy of plasma treatment. Figure 7 showed the log reduction at different treatment duration. 0-min time of treatment refers to the control set. It is interesting to note that consistent increasing pattern of log reduction can be observed from 1 to 15 min of CAP treatment. In other words, higher log reduction can be obtained at increasing treatment duration. Highest log reduction was obtained at longest treatment duration of 15 min with log reduction of 3.08 in the cells of the *S. mutans* biofilm. Notably, even a 10 min treatment demonstrated a high value for log reduction, at 1.79 compared to the control sample. This implies that CAP treatment is effective in reducing the viable cells in the biofilm even in shorter durations. Comparatively, a significant increase in log reduction from 0.23 after 1 min of treatment to 3.08 after 15 min, indicating an almost thirteenfold increase in log reduction. A careful analysis suggests a potential proportional relationship between treatment duration and log reduction in inactivating the biofilm, which was further validated through statistical analysis.

The Pearson's coefficient, r in the correlation analysis was found to be 0.95. The result shows a positive correlation between the treatment duration and log reduction where there is a continuous and steady reduction in the number of viable cells from the treated samples as compared to the untreated samples. Hence, the values for log reduction are directly proportional to the treatment duration. In other words, the longest treatment duration of 15 min had shown the highest log reduction than any other parameters in the study. Analysis of variance (ANOVA) was also performed to find the r^2 and P values. From the calculation, it was found that the value of r^2 is 0.91 and the P value of 0.01. The reduction of viability of bacterial cells as the treatment duration increases is said to be significant ($P < 0.05$).

It is interesting to compare the efficacy of plasma treatment with standard disinfectants, such as chlorhexidine, highlighting the effectiveness of plasma in similar contexts. Chlorhexidine (CHX) is recognized

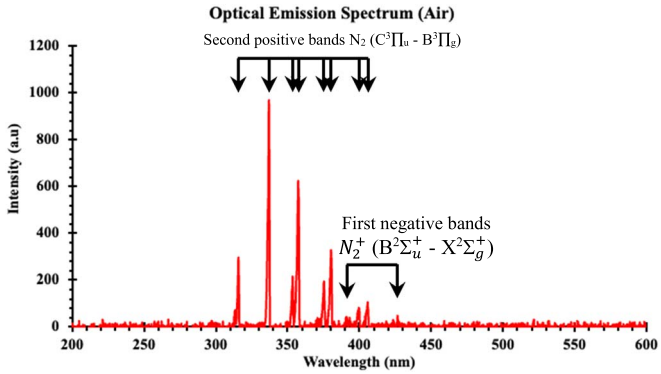


Figure 8. Typical optical emission spectrum of plasma discharge in atmospheric air.

as the gold standard for inhibiting plaque formation due to its antimicrobial properties and strong substantivity [60]. In that study, the authors compared and evaluated the effectiveness of twice-daily treatments over five days versus a single treatment on mature *S. mutans* biofilms that were five days old, using both chlorhexidine and cold plasma [47]. The results showed that daily treatment with 0.12% chlorhexidine for 10 min completely inhibited the formation of *S. mutans* biofilm. However, there was no obvious log reduction in the colony-forming units (CFUs) of mature biofilms. In contrast, cold plasma treatment proved effective not only against younger biofilms but also significantly reduced bacterial viability in five-day-old mature biofilms. This suggests that mature biofilms are particularly challenging to treat due to their complex structure and resistance mechanisms. However, cold plasma has the potential to effectively disrupt these mature biofilms.

The findings also emphasize the need for optimal treatment duration when developing standardized protocols in clinical settings, allowing for more predictable and reliable outcomes in managing infections related to biofilms. In addition, cold plasma treatment is unlikely to result in the development of antibiotic-resistant strains of bacteria with repeated use of non-thermal atmospheric plasma, due to its nonspecific and multi-target mechanism of action [43, 61]. This highlights the advantages of employing advanced green technologies, such as cold plasma, for biofilm control. Unlike antiseptic methods such as chlorhexidine, which can contribute to antimicrobial resistance and environmental pollution [62], this dry treatment approach offers an effective and environmentally friendly alternative, serving as a complementary concept for antiseptic treatment.

Moreover, antiseptics was demonstrated to enhance pathogenic characteristics [63], increase resistance to antiseptics [64], and exhibit cytotoxic effects on host cells [65]. Research suggests that antiseptics alone are inadequate for treating oral biofilms. For instance, a short treatment with 0.12% chlorhexidine (CHX)—a concentration commonly used in oral care products—results in only temporary reduction in viable cell concentration [66]. Therefore, a combination of treatments may be necessary to improve overall treatment efficacy. It was demonstrated in a study that complete sterilization of *P. aeruginosa* biofilms can be achieved after combinatorial treatment using plasma and chlorhexidine (CHX) [67].

We believe that the reactive species generated from this plasma discharge likely interact with the biofilm, leading to generation of further reactive species that contribute to the inactivation of bacteria within the biofilm. This phenomenon is supported by a study on the intracellular generation of reactive species in bacteria exposed to low-power atmospheric pressure cold plasma [45]. To further elucidate this, we have conducted optical emission spectrum measurements using the Ocean Optics HR4000 high-resolution spectrometer to identify various reactive species generated during plasma discharge in ambient air. A typical optical emission spectrum from 200 to 600 nm during plasma discharge, as depicted in figure 8, was analyzed. The identification of atomic lines in the emission spectrum was carried out using NIST data [68]. It is clearly observed that the emission spectrum is dominated by N_2 peaks, particularly with prominent second positive bands of N_2 ($C^3\Pi_u - B^3\Pi_g$) followed by a relatively lower intensity of the first negative bands of N_2^+ ($B^2\Sigma_u^+ - X^2\Sigma_g^+$). This indicates that the primary emitting species are excited nitrogen molecules and molecular ions. This is aligned with expectations for non-thermal plasma discharge in ambient air under atmospheric conditions. In fact, the emission spectra resemble to those of pure nitrogen discharge as reported in previous studies [69]. This similarity supports our findings and highlights the consistent behaviour of nitrogen species in plasma environments.

The lines emission of the second positive bands of N_2 arises from transitions between energy levels of 11.1 eV to 7.39 eV, while the first negative bands of N_2^+ results from transitions between 18.7 eV to 15.6 eV. The relatively lower emission intensity of the peaks for the first negative bands of N_2^+ ($B^2\Sigma_u^+ - X^2\Sigma_g^+$) implies that the plasma discharge produces fewer fraction of electrons with the 18.7 eV energy necessary for the transition. This may be explained as follows: The line emissions from molecular nitrogen species primarily occurs at spectral

transitions involving low vibrational levels of excited electronic states [70]. This is attributed to the vibrational relaxation of molecules in vibrationally excited state under the given experimental conditions. Furthermore, the upper electronic levels of all emitting excited nitrogen molecules are quite high, with excitation energies exceeding 11 eV. In fact, the energies of the excited levels of molecular nitrogen ions are even higher, with a minimum value of 18.7 eV. As a result, the population of excited levels of molecular nitrogen ions is lower compared to that of excited nitrogen molecules. This suggests that there are fewer electrons available to facilitate transitions to the excited levels of molecular nitrogen ions. It is reported that the population of the upper state $N_2(C^3\prod_u)$ is due to several excitation and quenching processes, including electron impact excitation from the molecular ground state ($X^1\Sigma_g^+$) and the first metastable state ($A^3\Sigma_u^+$), as well as associative excitation, pooling reactions, energy transfer between collisional partners, and Penning excitation [70]. While, the generation of $N_2^+(B^2\ \Sigma_u^+)$ occurs primarily through direct impact ionization by high-energy electrons [71].

The antimicrobial effect is believed to be attributed to the generation of reactive species, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), by the plasma. Studies by other researcher have shown that these reactive species can penetrate the biofilm matrix, disrupt bacterial cell membranes, and damage genetic material, leading to microbial inactivation [72, 73]. A recent 2024 study on cold atmospheric pressure plasma jet for hospital surface decontamination reported that radical reactive oxygen and nitrogen species (RONS), such as OH·, along with non-radical RONS including OI, NO^+, OH^+, $N_2O_3^-$, NO_3^-, and NO_2^-, contribute to antimicrobial effects and may also induce changes in surface properties [74]. Since most of the reactive species generated in this plasma discharge originate from nitrogen, we speculate that nitrosative species are likely involved which may lead to the inactivation of the biofilm through protein degradation [43].

Nevertheless, it is important to note that the efficacy of CAP treatment can vary depending on multitude of factor including the treatment parameters and the characteristics of the biofilm being targeted as this will affect the penetration and interaction of plasma species with bacterial cells within the biofilm. The *S. mutans ATCC* 25175 tested in this study had a weak to moderate biofilm-forming ability. Unlike the strong biofilm formers or multi-species dental biofilms that are known to be more resilient to CAP treatment, sufficient inactivation of the mono-species *S. mutans* biofilm in this study was achieved within a short duration [75]. The thick peptidoglycan cell wall of this Gram-positive *S. mutans* could have provided a protective effect by preventing the entry of reactive species from the plasma [36]. However, the absence of an outer membrane and a relatively thin extracellular matrix could have rendered the *S. mutans ATCC25175* biofilm more susceptible to the CAP treatment in this study.

To enhance our understanding and the effectiveness of atmospheric pressure plasmas, it is essential to characterize the quantities of reactive species they produce. Optical Emission Spectroscopy (OES) diagnostics is a useful tool to analyze the presence of reactive species in plasma, albeit it has limitations in providing direct quantitative analysis. To interpret the correlation between the intensity of emissions from reactive species and plasma conditions, the correlation between emission intensity and applied voltage was examined. Typical optical emission spectra at applied voltages of 9–12 kV are shown in figure 9.

The emission intensity is used as an indicator to infer the relative population of reactive species in the plasma discharge, serving as an indirect measure of their concentration, though it is not intended to replace direct quantitative analysis. It is worth to note the highest peak in each spectrum. The intensity of this peak increases with the applied voltage. This peak corresponds to the second positive bands of N_2, indicating that at higher applied voltages, more energy is transferred to the plasma, resulting in the production of more reactive species. A careful analysis of the N_2 peaks at various applied voltages demonstrates that the intensity change is nearly proportional to the applied voltage, indirectly suggesting that higher applied voltages result in a greater concentration of reactive species. Additionally, the OES spectrum is beyond its capability to measure certain reactive oxygen species, which are known to be present in air plasma discharges. Nevertheless, there is no denying that air plasma discharges contain reactive oxygen species, as has been reported in the literature [20, 51].

Quantifying the reactive species in plasma discharge presents significant challenges; however, absorption spectroscopy has been recognized as a useful method for the *in situ* detection and quantification of reactive oxygen and nitrogen species (RONS) [76]. While these limitations are acknowledged, the optical emission spectroscopy (OES) data provide valuable initial insights into the reactive species present in the plasma discharge, thereby establishing a solid foundation for the future diagnostic advancements aimed at their absolute measurement.

It is important to compare our findings with the recent results from [47]. In our study, we utilized a capillary-guided corona discharge system with ambient air, without airflow, to inactivate *Streptococcus mutans* biofilms on agar. Our results demonstrated that the efficacy of inactivation increased with treatment time, as indicated by colony-forming unit (CFU) counts. Notably, plasma temperature measurements revealed that the temperature did not exceed the threshold necessary to cause thermal damage to the biofilm, emphasizing that the effectiveness of our method on mature biofilms is attributed to plasma action rather than thermal impact. In contrast, [47] employed a plasma jet system with argon gas and airflow to treat *Streptococcus mutans* UA159

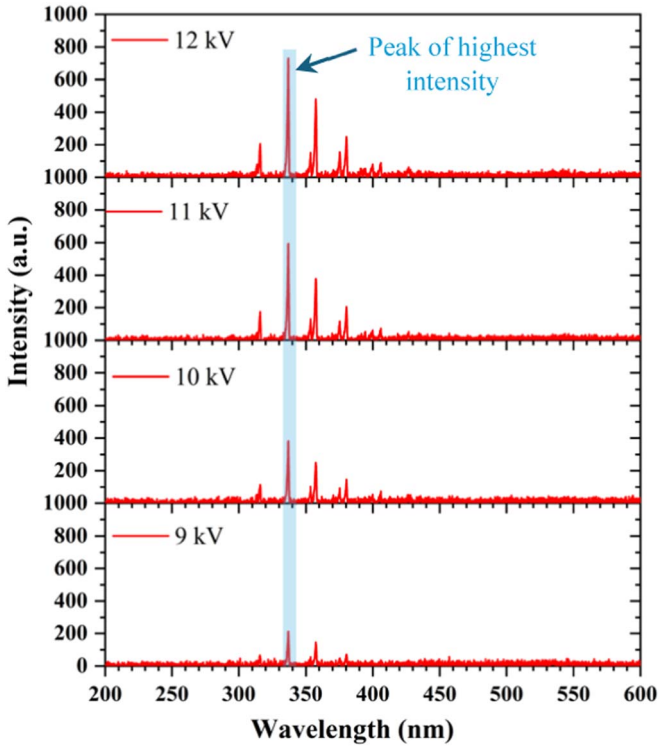


Figure 9. Typical optical emission spectra of plasma discharge in atmospheric air at different applied voltage.

biofilm formed on human enamel slabs. Their findings indicated that the plasma jet with argon airflow effectively inactivated mature biofilms and reduced the concentration of water-soluble extracellular polysaccharides, while leaving the levels of water-insoluble polysaccharides unchanged. The study shows that plasma treatment neither impacts the biochemical properties of the enamel beneath the biofilms nor affects the enamel's topographic surface as confirmed by scanning electron microscopy (SEM), making it a beneficial non-destructive treatment modality in dental setting.

Streptococcus mutans is known to synthesize extracellular polysaccharides and acids, which reside in biofilms [77]. These polysaccharides promote the 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 [78]. Additionally, they hinder the diffusion of antimicrobial agents. Therefore, the elimination or reduction of extracellular polysaccharides is essential for achieving effective inactivation of biofilms. While both studies aim to inactivate *S. mutans* biofilms, our methodologies differ in terms of plasma generation techniques and substrates used. Our focus on capillary-guided corona discharge without airflow allows for a distinct inactivation mechanism, which is supported by our measurements. This comparison highlights the versatility and potential applications of different plasma systems for biofilm inactivation, suggesting that distinct mechanisms may contribute to their respective efficacies.

The capillary-guided corona discharge operated with ambient atmospheric air for plasma generation in this work presents an advantageous and novel approach that provide a cost-effectiveness by eliminating the requirement for specialized gases, thereby making the setup more accessible and financially feasible. This has the potential to reduce the financial burden for both dental practitioners and patients. Furthermore, this study possesses the potential as non-invasive and targeted antimicrobial strategies for oral health since *S. mutans* is a significant contributor to dental caries whereby effective treatment options would definitely help in preventing tooth decay. This research established the clinical significance of cold atmospheric plasma as an effective and safe approach to combat *S. mutans* biofilm which demonstrated the potential to improve oral health outcomes. It offers a non-thermal alternative to conventional antimicrobial methods, contributing to enhanced patient comfort and potentially minimizing damage to healthy tissues. However, it is important to acknowledge certain limitations. The variability in CAP devices, treatment parameters, biofilm characteristics, and experimental conditions across different studies can introduce some degree of variability in the outcomes. That is to say, standardized protocols and comparative analyses are essential for obtaining reliable and comparable data.

While the biofilm inactivation effect is primarily attributed to the plasma reactive species, the potential influence of the electric field cannot be ruled out, as previous studies have reported that electric fields may also have bactericidal effect [79]. The significance of this aspect is acknowledged, warranting future studies for

diagnostic purposes in a separate investigation that will require a meticulous approach to thoroughly assess the impact of the electric field—without plasma generation—on biofilm sample. This investigation could help differentiate the effects of the electric field's presence during plasma discharge on biofilm inactivation. Further research shall be conducted to test out the ability of device in eradicating different bacterial species and not limited to *S. mutans* biofilm, as well as variations on the feed gas supplied to the device to test out on different bacterial species. With comprehensive understanding for the intricate processes involved in plasma treatment, the outcome shall provide a valuable insight for future research and potential applications in clinical settings.

4. Conclusion

In this work, capillary-guided corona discharge operated under atmospheric pressure generated a cold plasma plume in air extended to about 4 mm from the aperture of the CGCD device. The plasma plume was utilized as a treatment modality for the inactivation of biofilm for up to 15 min. The results were compared to the untreated control sample and clearly indicated a significant log reduction of 1.79 in *S. mutans* biofilm after 10 min of the cold atmospheric plasma treatment. A consistent trend of increasing efficacy with longer treatment duration has been observed which implied that the plasma treatment shows promise for potentially achieving effective inactivation of *S. mutans* biofilm. Notably, after 15 min of plasma treatment, a marked log reduction of 3.08 in the viable cells within the biofilm was achieved. Even with a short duration of just 1 min, a log reduction of 0.23 in viable cells compared to the control sample was obtained.

The thermal characterization of the cold plasma confirmed that the gas temperature maintained a temperature range at approximately 37 °C which is equivalent to human body temperature and hence does not cause damage to healthy tissue. This ensures that the treatment environment has minimal thermal effect on the *S. mutans* biofilm, ensuring that the biofilm inactivation effect was not due to thermal damage. Instead, it is the impact of the plasma's effects that leads to the inactivation of the biofilm. This reflects the importance of a thorough understanding of the correlation between the mechanisms of plasma action on biofilm inactivation which leads to optimization of the treatment efficacy and moreover tailor it to specific biofilm types.

The power dissipation measurements indicate the energy required to sustain the plasma discharge. The applied high voltage is associated with power dissipation, which can influence the concentration of reactive species and the overall characteristics of the plasma. In this work, we attribute the effects of biofilm inactivation primarily to the reactive species produced by the plasma discharge, while not excluding the possibility that the electric field generated by the high voltage may also play a role, which warrants further investigation.

The observed inactivation effect is believed to be attributed to the reactive species generated by the plasma discharge. Emission peaks corresponding to reactive nitrogen species (RNS) were observed in the OES spectrum (figure 8), providing direct evidence of their presence. While the detection of reactive oxygen species (ROS) was beyond the capability of the current spectrometer, there is no denying that air plasma discharges contain ROS, as reported in the literature [20, 51]. This implies that the role of ROS in the inactivation process cannot be ruled out. Therefore, the inactivation of the biofilm in this study is believed to be primarily driven by plasma mechanisms, rather than thermal effects, as evidenced by the plasma plume temperature measurements (figure 6).

The findings highlight **C**old **A**tmospheric **P**lasma (**CAP**) produced from a capillary-guided corona discharge as a promising and safe method for combating *S. mutans* biofilm, offering a feasible alternative to conventional antimicrobial approaches. This non-thermal treatment modality holds promising clinical significance, as it minimizes potential harm to surrounding healthy tissues and provides a novel avenue for biofilm control. A limitation of the current study is the use of the microtiter plate method, which, while effective for high-throughput analysis and quantitative measurements, does not fully replicate the complex surface and environmental conditions of human teeth. Nonetheless, the data obtained in this study contribute to the further characterization of the antimicrobial properties of capillary-guided corona discharge generated from ambient air in a non-airflow mode. Future studies should focus on utilizing more advanced biofilm models that closely simulate human dental surfaces, e.g., biofilms grown on artificial substrates, such as hydroxyapatite discs or enamel-like materials. These models would offer more realistic insights into how CAP impacts *S. mutans* biofilms in dental environments. Additionally, future research should delve into elucidating the precise mechanisms by which CAP eliminates *S. mutans* biofilm, optimizing treatment parameters to maximize efficacy, assessing long-term effects on biofilm regrowth, and evaluating the compatibility of CAP with other dental procedures. By advancing our understanding and application of CAP, we can potentially revolutionize biofilm management in dental and medical settings, leading to improved oral health outcomes and enhanced patient care.

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Data availability statement

The data cannot be made publicly available upon publication because they are not available in a format that is sufficiently accessible or reusable by other researchers. The data that support the findings of this study are available upon reasonable request from the authors.

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