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INACTIVATION OF GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA BY USING ARGON COLD ATMOSPHERIC PRESSURE PLASMA

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Abstract

Cold atmospheric pressure plasma (CAPP) is a residue-free alternative for decontaminating surfaces and reusable equipment when chemical disinfectants are inconvenient, such as during field work in environmental engineering and land reclamation. Here, we tested an argon CAPP plume (atmospheric pressure, low-current DC discharge) against Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa after 5–120 s exposures. Antimicrobial activity increased with treatment time: inhibition-zone areas grew from 2.83 cm^2 (5 s) to 31.40 cm^2 (120 s). P. aeruginosa was the most sensitive (31.40 cm^2 at 120 s), whereas S. aureus and E. coli reached 11.30 cm^2. Viable counts for E. coli and P. aeruginosa dropped from >10^6 to 10^3 CFU mL^{-1} after 120 s, while S. aureus showed no significant reduction at 120 s; for P. aeruginosa, counts decreased from >10^6 to 10^5 CFU mL^{-1} after 15 s. Overall, these findings support CAPP as a practical, non-chemical option for rapid decontamination of hard surfaces (e.g., tools and sampling devices) in remediation and environmental monitoring workflows.

Key words: *bacteria, cold atmospheric pressure plasma, susceptibility, surface sterilization.*

INTRODUCTION

Cold atmospheric pressure plasma (CAPP) is now widely studied as a tool for surface sterilization and disinfection, with reported uses in microbial decontamination and biomedicine (Joshi et al., 2011; Fricke et al., 2012; Mitra et al., 2014; Asenjo et al., 2015; Mai-Prochnow et al., 2016; Bourke et al., 2018; Hertwig et al., 2018; Corradini, 2020; Domonkos et al., 2021; Barjasteh et. al., 2024; Kazemi et al., 2024). Compared with chemical disinfectants-which can leave residues and raise material, environmental, and occupational issues (Whittaker et al., 2004; Singh et al., 2010) - CAPP typically requires only electrical power and a carrier gas and does not produce persistent chemical waste. Because the gas remains near room temperature, CAPP can be used on heat-sensitive materials and, in some cases, on living tissues (Fridman et al., 2008; Heinlin et al., 2010). It has also been explored for decontaminating sensitive electronics and hazardous agents (Singh et al., 2010). Many plasma sources and power-supply designs are available (Ehlbeck et al., 2011), but the dominant mechanisms of microbial inactivation are still being clarified. When the plasma interacts with air and water vapor, it generates reactive oxygen species (ROS) such as O_2^-•, H_2O_2, •OH, O_3, $^1O_2$, and atomic oxygen; together with charged particles, UV radiation, mild heating, and electric fields, these factors contribute to bacterial inactivation (Alkawareek et al., 2014).

How well CAPP works depends on practical details-such as the source design and geometry, as well as the carrier-gas composition, flow rate, and physicochemical properties (Mai-Prochnow et al., 2016; Baldanov et al., 2019). Since Laroussi first showed that CAPP can rapidly inactivate bacteria (Laroussi, 1996), subsequent studies have linked this effect mainly to reactive species and to damage of the membrane and cell wall, often accompanied by leakage of intracellular components. Differences in susceptibility between Gram-positive and Gram-negative bacteria have also been reported (Laroussi & Leipold, 2004; Deng et al., 2006;

Tipa et al., 2011; Flynn et al., 2015; Mai-Prochnow et al., 2016; Olatunde et al., 2019).
CAPP is also relevant for environmental engineering and land-reclamation work. In the field, teams often need to clean and decontaminate tools and reusable sampling or monitoring devices between locations to prevent cross-contamination (Jiang et al., 2014; Scholtz et al., 2015).
In this work, we tested a simple atmospheric-pressure CAPP source (argon carrier gas; low-current DC discharge) and measured its effects on two Gram-negative strains (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* IBB_{Po16} KT315654) and one Gram-positive strain (*Staphylococcus aureus* ATCC 25923).

MATERIALS AND METHODS

Experimental setup for cold argon plasma generation

Mechanical layout of the plasma source is shown in Figure 1, being similar to that described in (Stoican, 2011), except for a few minor changes. A sharp brass rod (1), 3 mm in diameter, is placed along the axis of a cavity (2), having axial symmetry, formed by two cylindrical segments, made in an aluminum block (3). One of the ends of the cavity is closed by the front disk (4), made by aluminum, having an output hole 2 mm in diameter. The plasma plume (5) is ejected through that hole, its external length being about 6-8 mm, depending on the experimental conditions. The other end of the outer cylinder is closed by a PTFE (polytetrafluoroethylene, Teflon) disk (6), which assures the mechanical support of the brass rod (1) and electrical insulation between the brass rod (1) and the aluminum block (3). An inlet (7) directed normal to the cavity axis, serves to deliver the carrier gas. The electrical discharge occurs between the brass rod (1), acting as cathode and the aluminum block (3) connected to ground, which serves as anode.
In Figure 2 the block diagram of the whole experimental setup is shown, including both the system to generate the CAPP and the biological sample holder. The electrical supply circuit for the plasma source operates according to the principle described in (Stoican, 2019). As a typical feature, the electrical discharge is initiated by a train of high voltage pulses and sustained by a common DC voltage source. A ballast resistor embedded into the DC voltage source circuit was used to keep the discharge stable and limit the discharge current, so as to obtain a CAPP with negligible thermal effect.

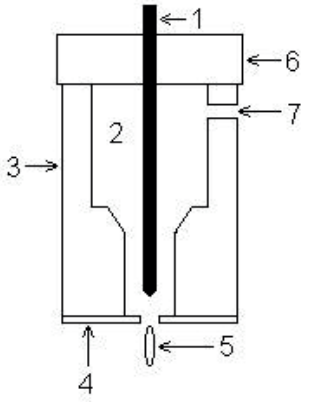


Figure 1. Plasma source mechanical layout representing a slightly modified version of that described in (Stoican, 2011) (not to scale) 1-brass rod (cathode); 2-cavity; 3-aluminum block (anode); 4-front disk with plasma plume output hole 5-plasma plume; 6-PTFE disk; 7-carrier gas input

For a short period of time, both voltages mentioned above are applied simultaneously to the discharge electrodes by means of a summing circuit. The high voltage pulses train is interrupted after the plasma plume occurs and becomes stable. Therefore, plasma is practically due to a low current, pure DC electrical discharge. According to the classification made in (Tendero et al., 2006) it operates as a current-carrying arc plasma torch.
The experimental setup is controlled by means of an electronic interface board that communicates with a personal computer through its RS-232 serial port. The electronic interface board comprises digital outputs necessary to switch on/off DC voltage and carrier gas electric valve, a digital output which provides the excitation signal for the high voltage pulses generator, and analog inputs necessary to measure voltages across terminals of a current sensor resistor Rs.
A software application allows maintaining the plasma plume for the requested period of time, monitoring voltage and current discharge during plasma plume existence, switching on/off the high voltage pulses generator, DC voltage, and carrier gas flow.

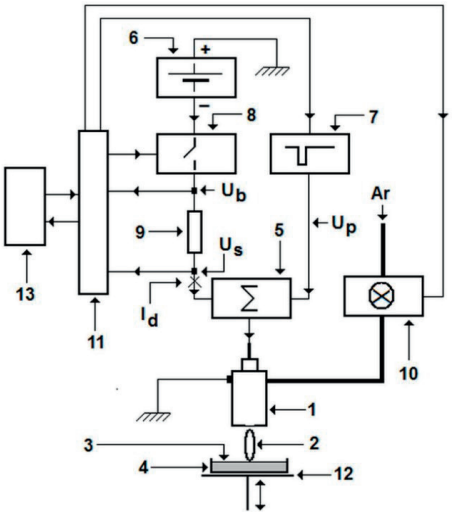


Figure 2. Experimental setup-block diagram
1-plasma source; 2-plasma plume; 3-biological sample;4-Petri plate; 5- summing circuit; 6-DC voltage source; 7-high voltage pulses generator; 8-DC high voltage switch; 9-current sensor Rs; 10-carrier gas electric valve; 11-electronic interface board; 12-metallic plate; 13-personal computer which communicates with the electronic interface board through a RS-232 serial port

The plasma plume is initiated by switching on simultaneously a high voltage pulse generator, DC voltage, and carrier gas flow. A high voltage pulses train is applied until the discharge current becomes greater than a threshold value. After the requested plasma exposure time (PET) has elapsed, the plasma plume is stopped by switching off the DC voltage and carrier gas flow. The PET is considered the time interval from the turn off of high voltage pulses train to the turn off the DC voltage. All necessary commands to perform the above operations are automatically sent by the software application to the electronic interface board.

Evaluation of antimicrobial effects of argon cold plasma on Gram-negative and Gram-positive bacteria

Three bacterial strains were used in this study: *Escherichia coli* ATCC 25922 (Minogue et al., 2014), *Staphylococcus aureus* ATCC 25923 (Treangen et al., 2014), and *Pseudomonas aeruginosa* IBB_{Po16} (KT315654, Stancu, 2018). The strains were inoculated into Luria-Bertani (LB) medium and incubated overnight at 30°C with agitation at 150 rpm.

Reagents used in this study were purchased from Merck and Sigma.

Bacterial growth inhibition assay. The bacterial cultures were adjusted to an optical density of 0.1 at 660 nm (OD_{660}), and 500 µL of each suspension was spread onto LB agar plates using the lawn culture method. After air-drying for 15 min under a laminar flow hood, the inoculated plates were exposed to the plasma plume for different time intervals (5, 7, 10, 15, 30, 60, 90, and 120 s). The distance between the agar surface and the outlet of the plasma-generating device was approximately 2 mm. Following plasma exposure, the plates were incubated overnight at 30°C, and the area of the inhibition zone (defined as the clear region on the LB agar where no bacterial growth was observed) was measured.

Quantification of bacterial survival. Bacterial strains were inoculated into LB medium and incubated overnight at 30°C with agitation at 150 rpm. Subsequently, 10 µl of bacterial suspensions adjusted to an optical density of 0.1 OD_{660} were pipetted onto the center of sterile, empty Petri plates, allowed to air-dry, and exposed to the plasma plume for varying time intervals (5, 7, 10, 15, 30, 60, 90, and 120 s). Control samples were prepared under identical conditions but were not subjected to plasma treatment. Following exposure, bacterial recovery and enumeration were performed using the Most Probable Number (MPN) method. Briefly, the bacterial cells were scrubbed from the surface of each Petri plate using sterile 0.9% saline solution. Serial dilutions were prepared, and 20 µl of aliquots of each dilution were inoculated into wells of a 96-well microplate containing 180 µl of LB medium. The microplates were incubated overnight at 30°C. Bacterial growth was assessed by adding 0.3% triphenyl tetrazolium chloride (TTC), as previously described (Stancu, 2019). The appearance of a red coloration in the wells, resulting from TTC reduction, was interpreted as a positive indication of bacterial growth.

To evaluate changes in cell membrane permeability following plasma exposure, a permeability assay (Ardelean et al., 2009) was performed. SYBR Green I and propidium homodimer served as selective fluorescent markers. The assay was conducted on chemically fixed cells (2% buffered formalin) at

5 h post-plasma exposure. SYBR Green I penetrates the membranes of all cells, while propidium homodimer, due to its larger molecule size and negative charges, penetrates only cells with severely compromised membrane integrity, which are considered non-viable (Noble & Fuhrman, 1998).

RESULTS AND DISCUSSIONS

The effects of CAPP exposure on the inactivation of three bacterial species were investigated in this study. The selected strains belong to the two principal bacterial groups: Gram-positive, represented by *S. aureus*, and Gram-negative, represented by *E. coli* and *P. aeruginosa* IBBPo16.

Details on the plasma source, its electrical supply circuit, and biological sample arrangement are presented in Figures 1 and 2. The high voltage pulses had a peak value of U_p ≈-3.9kV/60Hz (negative polarity with respect to ground), being applied for about 1-2 s in order to initiate the plasma plume. The average DC voltage U_b (having negative polarity with respect to ground) was about 0.68kV. The DC discharge current has been calculated as I_d=$|U_b - U_s|/R_s$≈13-20mA. Argon (Ar) was used as a carrier gas. The biological sample was deposited in a Petri plate placed on a metallic support below the output hole of the plasma source.

Owing to structural differences between Gram-positive and Gram-negative bacteria, distinct responses to plasma exposure are anticipated among strains belonging to these groups. In view of the potential applications of CAPP in surface sterilization, argon was selected as the carrier gas, and three bacterial strains were chosen based on their opportunistic and pathogenic characteristics, which represent a potential risk to human health. Previous studies (Tipa et al., 2011; Baldanov et al., 2019) have demonstrated that argon exhibits higher bacterial inactivation efficiency compared with other carrier gases (e.g., helium), thereby justifying its use in the present study.

Several long-living ROS, such as O_2^{-}•, H_2O_2, and •OH, are capable of diffusing beyond the plasma plume, thereby affecting an area larger than the plasma jet diameter. The distance between the jet nozzle and the treated sample is a crucial parameter, as increasing this distance may lead to attenuation of photons and reactive species, resulting in reduced fluxes reaching the surface and consequently diminishing the antimicrobial efficacy. This aspect is particularly relevant because bacterial inactivation is predominantly mediated by ROS. Additionally, the intensity of UV radiation decreases proportionally to the square of the distance from the source (Lackmann & Badow, 2014).

To address this limitation, all treatments were performed at an approximate distance of 2 mm, corresponding to the condition in which the plasma plume directly contacts the surface and spreads across it, thereby facilitating the transport of reactive species to adjacent regions.

The inhibition zone area as a function of treatment time for each bacterial strain is presented in Figure 3a. According to our results (Figure 4), increasing the PET led to a progressive enlargement of the inhibition zone for all three tested strains, ranging from a minimum of 2.83 cm^2 (after 5 s) to a maximum of 31.40 cm^2 (after 120 s). The response of *S. aureus* followed a similar trend to that of *E. coli*, with no major differences observed between the two strains; both reached a maximum inhibition zone of 11.30 cm^2 after 120 s of exposure. In contrast, *P. aeruginosa* IBB_{Po16} exhibited higher sensitivity to plasma treatment, showing a maximum inhibition zone of 31.40 cm^2 after 120s.

Tetrazolium salts are widely used to assess the viability of bacterial cultures. As reported (Moussa et al., 2013), the TTC-reducing activity of a culture is directly proportional to the number of metabolically active cells within the bacterial population. Figure 3b and Figure 5 present the relationship between PET and the number of colony-forming units per milliliter (CFU mL^{-1}).

S. aureus exhibited the highest resilience to plasma treatment, with no significant differences observed between the control sample and those exposed to 120 s of cold plasma. In contrast, *E. coli* and *P. aeruginosa* IBB_{Po16} exhibited slightly higher sensitivity, showing a reduction in viable counts from $>10^6$ to 10^3 CFU mL^{-1} after 120 s of exposure. Compared with *S. aureus* and *E. coli*, plasma treatment exerted a more pronounced effect on *P. aeruginosa* IBB_{Po16}. Within 15 s of plasma

exposure, a CFU reduction from $>10^6$ to 10^5 CFU mL^{-1} was achieved. Overall, the results indicate that increasing PET leads to a progressive reduction in the cell viability in both Gram-positive and Gram-negative bacteria.

The permeability assay revealed a decrease in the number of viable bacterial cells (Figure 3c, green fluorescence) and a corresponding increase in the proportion of non-viable cells (Figure 3c, orange fluorescence) after 60 s of cold plasma exposure compared with the untreated control. Before plasma exposure, the proportion of non-viable bacterial cells was low relative to the total cell population. Following 60 s of treatment, a marked increase in non-viable cells was detected, with the majority of cells exhibiting membrane permeabilization and positive homodimer staining.

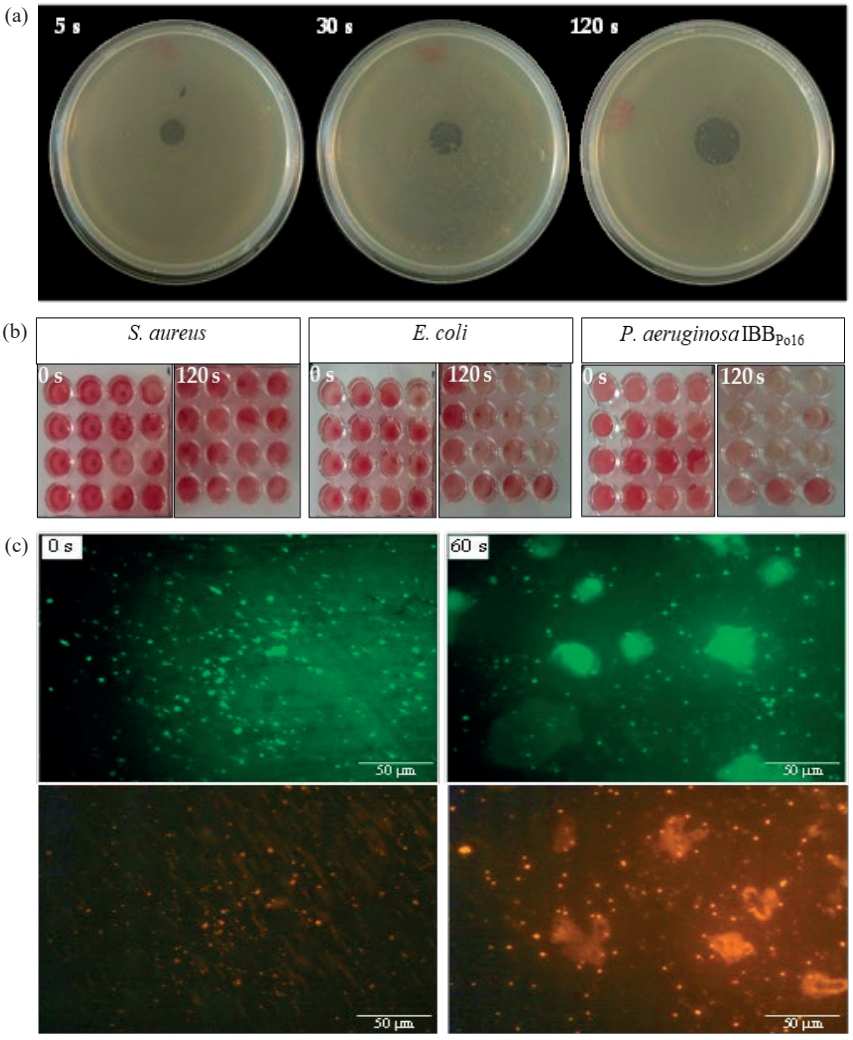
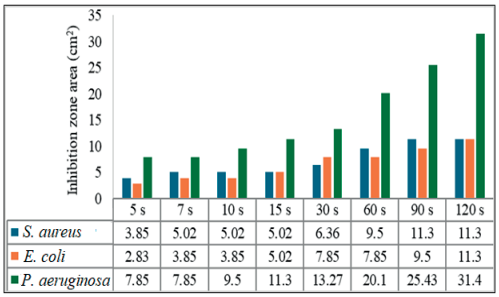
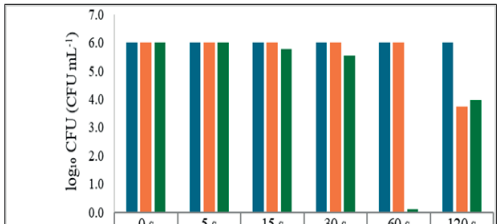


Figure 3. Antimicrobial effects of argon cold plasma on Gram-negative and Gram-positive bacteria. (a) Bacterial growth inhibition after plasma exposure. (b) Quantification of bacterial survival after plasma exposure. (c) Permeability fluorescence assay before (0 s) and after (60 s) plasma exposure of bacterial cells.



	5 s	7 s	10 s	15 s	30 s	60 s	90 s	120 s
S. aureus	3.85	5.02	5.02	5.02	6.36	9.5	11.3	11.3
E. coli	2.83	3.85	3.85	5.02	7.85	7.85	9.5	11.3
P. aeruginosa	7.85	7.85	9.5	11.3	13.27	20.1	25.43	31.4

Figure 4. Inhibition zone areas for the tested Gram-positive and Gram-negative bacteria after plasma exposure (5-120 s)



	0 s	5 s	15 s	30 s	60 s	120 s
S. aureus	$>10^6$	$>10^6$	$>10^6$	$>10^6$	$>10^6$	$>10^6$
E. coli	$>10^6$	$>10^6$	$>10^6$	$>10^6$	$>10^6$	5.5×10^3
P. aeruginosa	$>10^6$	1.2×10^6	6.0×10^5	3.5×10^5	1.3×10^5	9.5×10^3

Figure 5. Gram-positive and Gram-negative bacterial cell viability after plasma exposure (5-120 s)

It should be noted that membrane permeabilization detected by fluorescence staining does not necessarily correspond to an immediate loss of reproductive capacity. While CFU enumeration reflects the ability of bacterial cells to recover and proliferate under nutrient-rich conditions, fluorescence-based assays evaluate membrane integrity at a specific time point. Therefore, the apparent differences between the permeability assay and the viability data (Figure 3b) may indicate the presence of sublethally injured cells within the treated population. Such cells may exhibit increased membrane permeability without complete loss of culturability. Further time-resolved investigations would be required to determine whether plasma-induced membrane alterations are fully irreversible or represent transient physiological responses.

Fluorescent staining methods capable of discriminating between cells with intact membranes (classified as viable) and those with severely compromised membranes (classified as non-viable) have previously been employed to evaluate bacterial responses following CAPP treatment (Deng et al., 2006; Waskow et al., 2018; Lee et al., 2019; Olatunde et al., 2019). These studies reported findings consistent with the results of the present investigation.

All tested strains exhibited a linear increase in inhibition zone area with no evidence of saturation even after 120 s of plasma exposure. Among the strains investigated, *S. aureus* presented the highest resistance to plasma treatment, followed by *E. coli* and *P. aeruginosa* IBB_{Po16}. These results are consistent with the known structural difference between Gram-positive and Gram-negative bacteria, particularly regarding cell wall architecture. Of the three strains, *S. aureus* showed significantly higher resistance after 120 s of plasma treatment, suggesting that the increased thickness and structural complexity of the Gram-positive cell wall contribute substantially to plasma-mediated resistance.

Cold plasma has been hypothesized to target multiple cellular components, including the cell membrane, cell wall, DNA, and intracellular proteins. The ROS are capable of breaking structurally critical bonds within peptidoglycan (e.g., C–O, C–N linkages), potentially leading to cell wall damage in Gram-positive bacteria (Misra et al., 2016). In Gram-negative bacteria, ROS primarily induce lipid peroxidation of the outer membrane (Joshi et al., 2011), compromising membrane integrity. This cascade of oxidative events promotes the leakage of intracellular constituents, such as nucleic acids and proteins, ultimately resulting in cell death. The increased susceptibility of Gram-negative bacteria compared to Gram-positive bacteria may also be attributed to the higher porosity of the outer membrane, which facilitates ROS penetration and enhances oxidative damage (Mai-Prochnow et al., 2016). These findings are consistent with our results, as *P. aeruginosa* IBB_{Po16} and *E. coli* proved greater sensitivity to plasma exposure than *S. aureus*.

CONCLUSIONS

The antimicrobial effect of CAPP using argon as the carrier gas was evaluated against *S. aureus*, *E. coli*, and *P. aeruginosa* IBB_{Po16}. *S. aureus* exhibited the highest resistance, while the Gram-negative strains showed greater susceptibility. Bacterial response was strain-

dependent and probably influenced by differences in cell wall architecture. Although plasma exposure reduced viable cell counts, complete inactivation was not achieved even after 120 s, indicating that longer exposure times are required for effective surface sterilization.

These findings demonstrate that CAPP represents a promising approach for surface sterilization and decontamination. Under extended exposure conditions, CAPP may support applications in environmental decontamination and surface treatment technologies.

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