Keywords
Cold atmospheric plasma (CAP); Dielectric barrier discharge (DBD); P. aeruginosa; S. epidermidis; Trichophyton rubrum.

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Inactivation of Bacterial and Fungal Pathogens by Dielectric Barrier Discharge Cold Plasma

Abstract
In the present study, antibacterial and antifungal activities of dielectric barrier discharge (DBD) cold plasma were studied against Pseudomonas aeruginosa, Staphylococcus epidermidis and Trichophyton rubrum. A high purity argon gas and a high voltage AC power supply are used to fabricate a DBD plasma jet at atmospheric pressure. Antibacterial activity was carried out at 30, 60, 90 and 120 s of exposure times. Antifungal activity against T. rubrum was studied at exposure times between 3 and 24 min. The results demonstrated a clear time-dependent antimicrobial effect. After plasma treatment for 120 s, inactivation of bacteria was 55.7 % for P. aeruginosa and 60.3 % for S. epidermidis. The viable cell counts decreased with the plasma exposure time, indicating a better inactivation of the bacteria with longer treatment times. Moreover, the DBD plasma was effective in reducing the biofilm biomass, the lowest value of optical density being recorded after 120 s of treatment. SEM showed progressive morphological damage such as surface roughness, deformation of membranes, formation of pores, disruption of structure and loss of cellular integrity particularly in S. epidermidis. The antifungal results showed a gradual inhibition of T. rubrum growth with the increase of treatment time, and no visible fungal colony was observed after 24 min of DBD plasma exposure.

INTRODUCTION

Plasma is generally regarded as the fourth state of matter and is created by imparting enough energy to a gas to ionize its constituent atoms or molecules, producing a complex mixture of free electrons, positive and negative ions, neutral particles, excited atoms and molecules, and reactive species [1]. Unlike conventional gases, plasma consists of electrically charged particles that confer unique electrical, physical and chemical properties to it, which allow it to interact with biological materials and microorganisms through electric fields, charged particles, ultraviolet radiation, and chemically reactive species [2]. Plasma is a natural state of matter in the Sun, stars, lightning, auroras and the ionosphere of the earth, but it can also be created in the laboratory in a controlled manner with the help of a number of energy sources, such as direct current, alternating current, radio frequency, microwave and pulsed electrical power [3], [4]. The laboratory plasmas are usually classified as thermal plasmas and non-thermal plasmas depending on the degree of thermal equilibrium between electrons and heavy particles [5]. Thermal plasmas are high temperature plasmas where electrons, ions and neutral particles are close to thermal equilibrium. They are used for high temperature processes like welding, cutting and thermal spraying. Non-thermal plasmas, also called cold plasmas, are characterized by energetic electrons whereas the temperature of ions and neutral particles is low, often near ambient temperature [6], [7]. This unique feature allows cold plasma to generate highly reactive chemical species without generating too much heat and thus is especially attractive for applications involving temperature-sensitive biological materials. Among the various atmospheric pressure cold plasma systems, dielectric barrier discharge (DBD) plasma has been of great interest since it can produce non-equilibrium plasma at atmospheric pressure without the need of vacuum chamber [8],[9]. A typical DBD system is composed of two electrodes separated by at least one dielectric material. The dielectric barrier restrains the discharge current and prevents the transition from a non-uniform discharge to a continuous electrical arc, resulting in the generation of many short-lived micro discharges [10],[11]. These micro discharges supply energy to excite and ionize gas molecules, resulting in the production of a wide variety of chemically and biologically active species and with the gas temperature being relatively low. The antimicrobial effect of cold DBD plasma is mainly due to the combined action of ROS, RNS, charged particles, UV radiation, electric fields and other short-lived excited species [12], [13]. The cooperative action of these plasma-generated species can then gradually disrupt the microbial cellular functions and lead to the inactivation of microorganisms. The antimicrobial effect of DBD plasma is particularly important, because microorganisms differ significantly in their cellular architecture, membrane composition, resistance mechanisms and susceptibility to oxidative stress [14]. Thus, studying the response of different bacterial and fungal species to DBD plasma is important to understand the antimicrobial potential of DBD plasma [15]. Three clinically and biologically relevant microorganisms were used in the present study: the Gram-negative bacteria Pseudomonas aeruginosa (P. aeruginosa), the Gram-positive bacteria Staphylococcus epidermidis (S. epidermidis) and the dermatophytic fungus Trichophyton rubrum (T. rubrum). The different structural and biological properties of these microorganisms provide a valuable platform to evaluate the broad-spectrum antimicrobial activity of cold plasma. P. aeruginosa is a Gram-negative opportunistic bacterium with a relatively complex outer membrane and a strong ability to adapt to environmental stresses [16]. S. epidermidis is a Gram-positive bacterium commonly found on human skin, and it is known to form biofilms, especially on surfaces and medical devices. These two species of bacteria have different structures of cell envelopes and thus provide an opportunity to study the dependence of antimicrobial action of DBD plasma on bacterial cell-wall architecture [17]. In addition to bacterial microorganisms, fungal species are also important targets for non-thermal plasma treatment. T. rubrum has a cellular organization that is structurally different from bacteria. Thus, the investigation of the response of T. rubrum beside Gram-negative and Gram-positive bacteria provides a wider evaluation of the antimicrobial efficacy of DBD cold plasma [18],[19]. This study aims to evaluate the ability of DBD cold plasma to inhibit or inactivate these microorganisms and to assess their potential as a non-thermal antimicrobial treatment. The comparison between Gram-negative bacteria, Gram-positive bacteria and dermatophytic fungi aim to provide insight into the effectiveness of the DBD plasma against microorganisms with different cellular architectures and biological characteristics and thus contribute to the development of plasma-based antimicrobial applications.

2.Experimental Procedure

2.1 Experimental Setup and Sample Preparation

DBD plasma setup

The plasma (DBD) jet apparatus used in this study, shown in Figure (1), has four main components: a high-voltage AC power source (0-40 kV, 1-150 kHz), the plasma (DBD) jet, an argon gas reservoir, and a flow measurement apparatus. DBD is a plasma jet enclosed in a dielectric barrier (glass tube). The plasma discharge is created by two electrodes. The electrode arrangement is based on a ring-to-plate electrode. It shows a ring of copper which surrounds the outer surface of the Pyrex tube and the other electrode is a copper plate positioned closer to the exit of the gas. It is placed inside the glass tube and connected to the high voltage power supply through a 5 kΩ ballast resistor. This resistor is used to increase the stability of the plasma and to limit the discharge current. The secondary electrode is a grounded annular electrode surrounding the exit of the glass tube. High voltage alternating electric field is used to ionize the high purity argon gas in the glass tube to produce a stable cold plasma plume at atmospheric pressure. A flow meter controls the flow of argon to ensure consistent treatment settings and stable plasma formation.

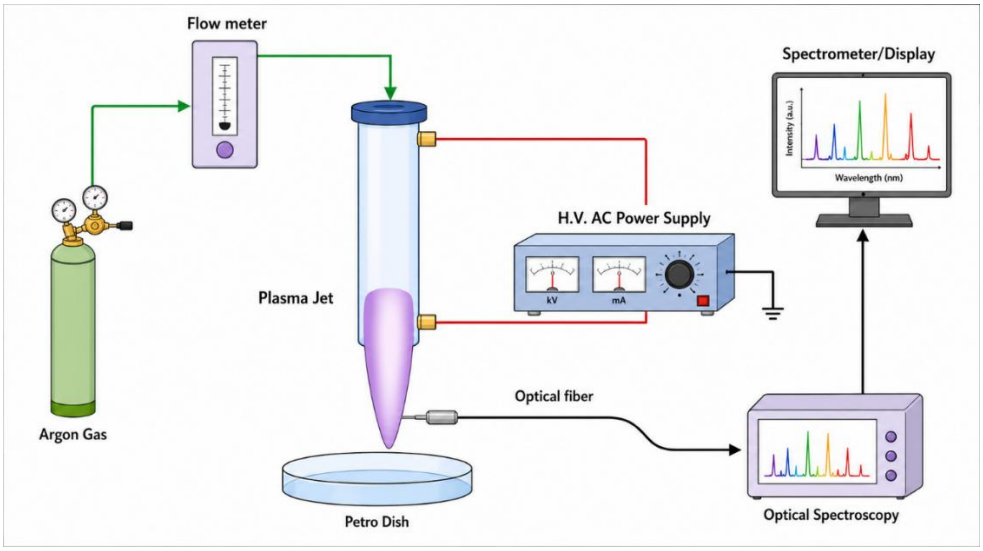

Figure 1: Plasma (DBD) system schematic diagram.

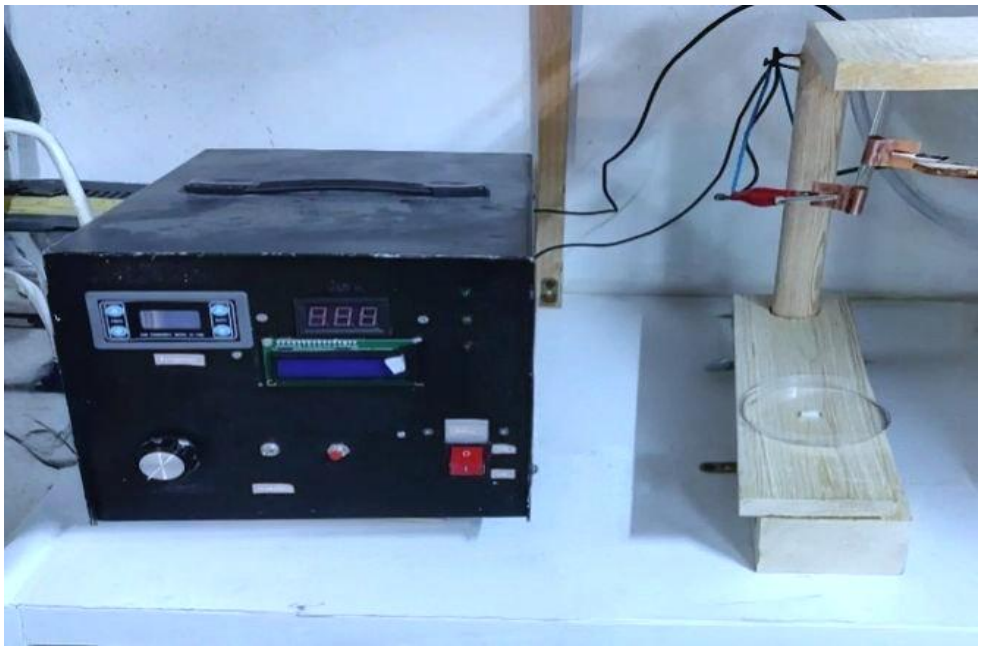

Figure 2: A Photograph Image representation of a plasma DBD apparatus.

Bacteria sample preparation

The bacterial strains used in the experiment were S. epidermidis and P. aeruginosa isolated from wounds of burn patients in the Biology Department at the College of Science, University of Al-Qadisiyah. These strains were preserved and frozen in 15% glycerol (BHIB) after subculturing and reactivation on Brain Heart Infusion Agar (BHIA). The media were autoclaved for (20 minutes) at (121°C) and (15) pounds per square inch of pressure to sterilize them. The media's pH was adjusted to (7.0 ±0.3). The media were heated up to (100°C) and fully dissolved then sterilized by Millipore filter paper (0.22 µm pore size) to maintain the solutions with heat-sensitive properties viable. Prepared media were dispensed in sterile petri dishes or tubes and incubated at 37ºC for 24 h before storage at 4°C. In addition, all glassware was sterilized by dry heat in an electric oven at 160–180°C for 2–3 h to avoid contamination. Nutrient agar plates were wrapped in Parafilm for isolation and stored at 4–8°C for short-term preservation (2-3 months) and long-term preservation (up to 1-3 years) as required.

Preparation of bacterial Lawn and antibiogram test

The Kirby-Bauer method, which uses the disk diffusion susceptibility method for antibiogram testing, was carried out in accordance with the Clinical Laboratory Standard Institute's guidelines [20]. In summary, identical pure colonies of S. epidermidis, P. aeruginosa were pre-subculture on nutrient agar (N.A.) plates at (37°C). The bacterial suspension was then resuspended and adjusted to the turbidity of McFarland standard No.0.5 (1.5x104) (CFU/ml). After making sure the inoculums were distributed evenly, the Muller Hinton Agar (MHA) plate dry surface was inoculated by streaking the swab over the whole sterile agar surface. Standard antibiotic disks (Ciprofloxacin, Amikacin, Gentamycin, Erythromycin, Methicillin, Vancomycin, Cefoxitin, Oxacillin, Ofloxacin, Ceftriaxone, Ceftazidime) were applied firmly to the agar surface of other MHA plates within 15 min of the plates

being inoculated, as in [21], [22]. Zones of inhibition for all plates (plasma exposure and standard antibiotic disks) were determined and compared to the CLSI criteria following an overnight incubation at (37°C).

Preparation of Trichophyton rubrum

Trichophyton rubrum fungal strain was used in this study to evaluate the antifungal activity of the developed dielectric barrier discharge (DBD) plasma system. The fungal isolate was cultivated on Sabouraud Dextrose Agar (SDA) medium under aseptic conditions and incubated at 28–30°C for 7–10 days to obtain well developed fungal colonies. After incubation, one of the active growing colonies was aseptically transferred to new sterile petri plates containing SDA medium. The inoculated plates were incubated in the same conditions until uniform growth of fungi was seen. These prepared cultures were then used for plasma treatment for different exposure times as per the experimental procedure described in this study.

Exposure and Treatment by DBD

Argon DBD was used to expose the bacterial petri dishes on a steel platform that was isolated and had a mechanical lifter to regulate the distance between the jetting exit source and the culture medium's surface. Four exposure times (30, 60, 90, and 120) seconds were attained for the high voltage (14 kHz) used in this study, which corresponded to a flow rate of (3 L/min) for the Argon jet gas used. Additionally, a negative control group was used for each treatment time.

2.2 Characterization Methods

Plasma DBD length measurement

The length of the plasma DBD was measured using a calibrated metric ruler, which was placed parallel to the plasma plume, as seen in Figure 3. The measurement was taken by holding the ruler near the plasma DBD jet and measuring from the end of the Pyrex tube to the visible end of the plasma plume, to minimize reading errors. The plasma DBD jet length was measured at varied voltages and gas flow rates of argon to study the influence of the operational conditions.

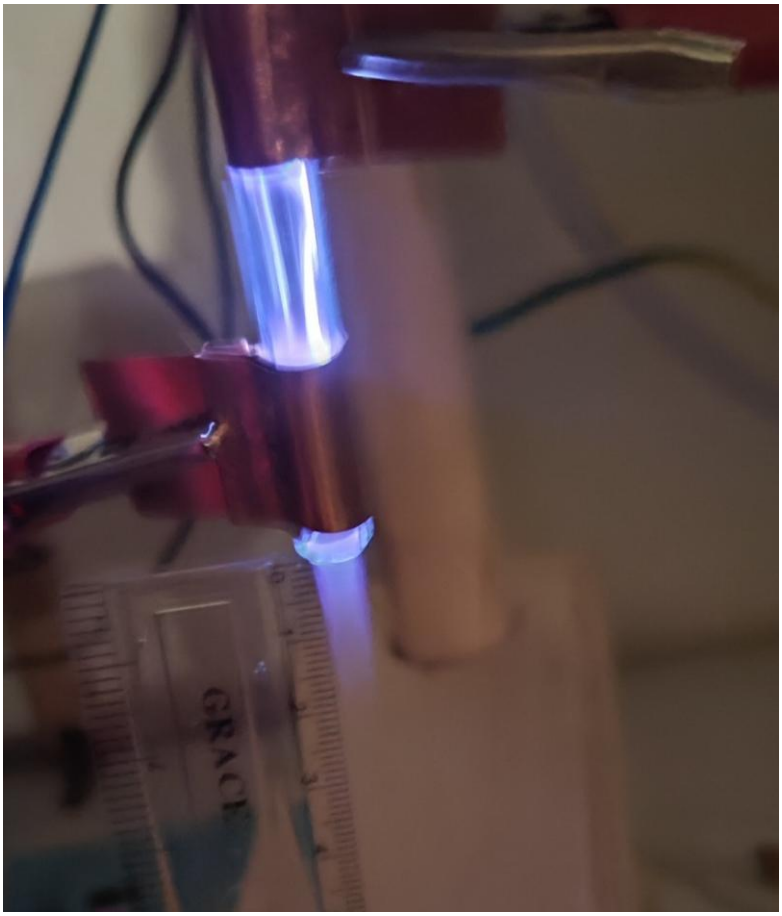

Figure 3: An empirical evaluation of the plasma DBD length via a calibrated measuring ruler.

Plasma Temperature Measurement

The temperature of the atmospheric pressure plasma DBD jet was measured with a non-contact infrared (IR) thermometer. The infrared thermometer was chosen because it allows one to make fast measurements of the temperature without disturbing the plasma discharge itself or changing its properties. During the measurements, the infrared sensor was directed to the visible plasma plume at a fixed distance from the plasma jet output, so that the measurement conditions remained constant during the experiments.

3. Results and Discussion

3.1 Effect of DBD Plasma Exposure Time on Inactivation efficacy

The antibacterial efficiency of the developed DBD plasma system was examined against bacterial species including P. aeruginosa and S. epidermidis. In all tested species, the viable bacterial cell density continuously decreased with increasing the exposure time of DBD plasma. DBD plasma showed bacterial inactivation efficiencies of (55.7%) for P. aeruginosa and (60.3%) for S. epidermidis after (120 s) of treatment by the plate-counting method. The highest antibacterial activity was observed at (120 s), showing that the prolonged plasma exposure can effectively improve the bacterial inactivation. The viable cell density of P. aeruginosa gradually decreased with the increase of DBD plasma exposure time as shown in Figure 4, confirming the time-dependent antibacterial activity of the developed plasma system. The cell density of untreated control was the highest at about (2.89×106 CFU mL-1) and decreased gradually to (2.61×106), (2.24×106), (1.75×106), and (1.28×106 CFU mL-1) after 30, 60, 90, and 120 s, respectively. The highest reduction was observed at 120 s, which means that the bacterial inactivation is highly dependent on the plasma exposure time. The reduction of viable bacterial cells observed is mainly due to the continuous production of reactive oxygen and nitrogen species (RONS), such as hydroxyl radicals (OH), atomic oxygen (O), ozone (O_3), nitric oxide (NO) and hydrogen peroxide (H_2O_2). These reactive species cause oxidative damage to bacterial cell membranes, proteins, lipids and nucleic acids. This results in membrane disruption, leakage of intracellular contents and ultimately death of the bacterial cell. With the increase of plasma exposure time, more reactive species interacted with the bacterial cells. Hence, the antibacterial activity was improved, and the number of viable colonies was reduced. A strong time-dependent antibacterial effect was observed, since the viable cell density of S. epidermidis decreased remarkably with the increasing DBD plasma exposure time.

The untreated control showed the highest cell density of about (2.87×106CFUmL-1) which gradually decreased to about (2.59×106), (2.05×106), (1.52×106) and (1.14×106CFUmL-1) after 30, 60, 90 and 120 s respectively. The most significant reduction of viable bacterial cells was observed after 120 s of plasma treatment, confirming that the longer exposure time greatly enhances the bactericidal efficiency of the developed DBD plasma system. This progressive reduction in bacterial viability is attributed to the continuous generation of reactive oxygen and nitrogen species (RONS) such as hydroxyl radicals (OH), atomic oxygen (O), ozone (O_3), nitric oxide (NO) and hydrogen peroxide (H_2O_2). These reactive species induce oxidative stress by attacking the bacterial cell wall, cytoplasmic membrane, intracellular proteins and nucleic acids, resulting in membrane disruption, leakage of intracellular components and eventual death of the bacterial cell. Thus, the total concentration of reactive species interacting with the bacterial cells increases with the plasma exposure time, and the antibacterial efficiency of the DBD plasma improves. The results obtained are in agreement with the results of [23].

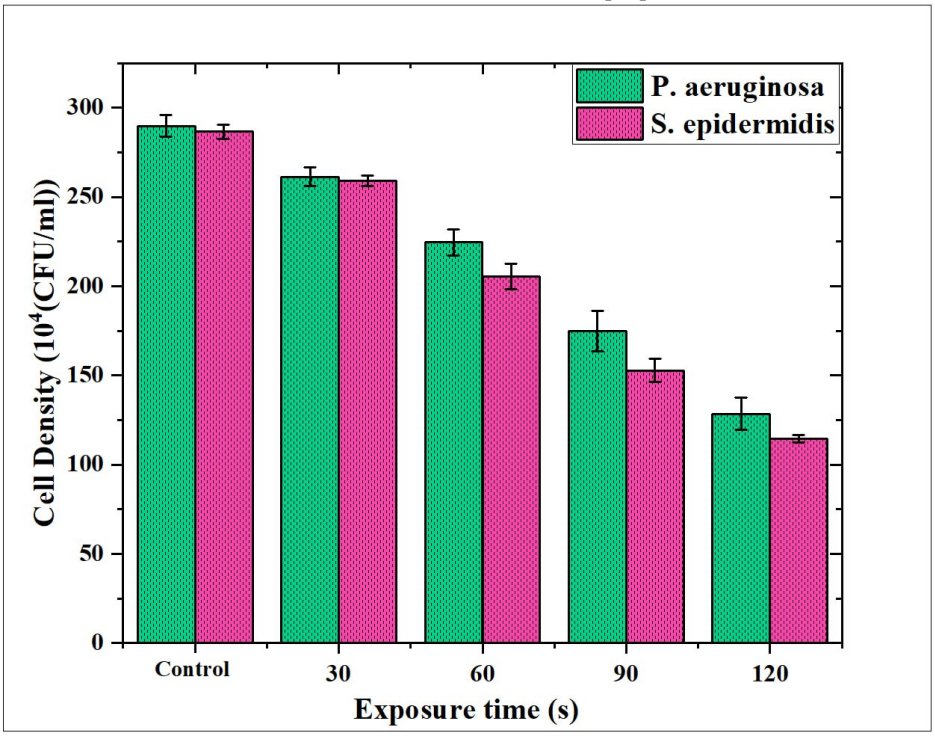

Figure 4: Effect of DBD plasma exposure time on the cell density of the P. aeruginosa and S. epidermidis determined by the plate-counting method. are mean ±standard error of the mean; n = 3. ** p < 0.05 when compared to controls.

3.2 Effect of DBD Plasma on Biofilm Formation and Removal

The biofilm formation was quantified by the spectrophotometric assay of crystal violet (CV), which is one of the most common methods to evaluate bacterial biofilm biomass. This method is based on the ability of crystal violet to bind the extracellular polymeric substances (EPS) and bacterial cells that form the biofilm matrix. The excess dye is washed off after staining, and the remaining bound crystal violet is solubilized for

measurement of absorbance on a microplate spectrophotometer at the appropriate wavelength. The optical density (OD) measured is directly proportional to the total biofilm biomass. Thus, a lower OD value indicates less biofilm formation or more biofilm removal. The inhibitory and disruptive ability of the fabricated DBD plasma system on bacterial biofilms was investigated by treating four clinically relevant bacterial species, P. aeruginosa and S. epidermidis for 30, 60, 90 and 120 s respectively. The results are shown in Figure 5, comparing optical density of untreated and plasma-treated biofilms. Figure 5 presents a gradual decrease in biofilm biomass of all bacterial species with increasing exposure time of DBD plasma. The untreated control groups had the highest optical density values in all cases, suggesting the formation of mature and well-developed biofilms. Instead, plasma treatment significantly decreased the optical density of all studied microorganisms indicating that DBD plasma effectively suppressed biofilm formation and enhanced the breakdown of already formed biofilm matrix. The reduction was more pronounced with increasing treatment time and the lowest optical density values were observed after 120 s of plasma exposure. The observed reduction of biofilm biomass is due to the interaction of plasma-produced reactive oxygen and nitrogen species (RONS), charged particles and ultraviolet radiation with the extracellular polymeric substances (EPS) that confer structural stability to bacterial biofilms. These reactive plasma species oxidize polysaccharides, proteins, lipids, and extracellular DNA in the EPS matrix, weakening bacterial adhesion to the substrate and leading to disruption of biofilm architecture. The damage to the protective matrix makes bacterial cells more susceptible to oxidative damage leading to increased bacterial inactivation. From the spectrophotometric results, the developed DBD plasma system showed excellent antibiofilm activity against Gram-negative and Gram-positive bacteria this result agrees with [24].

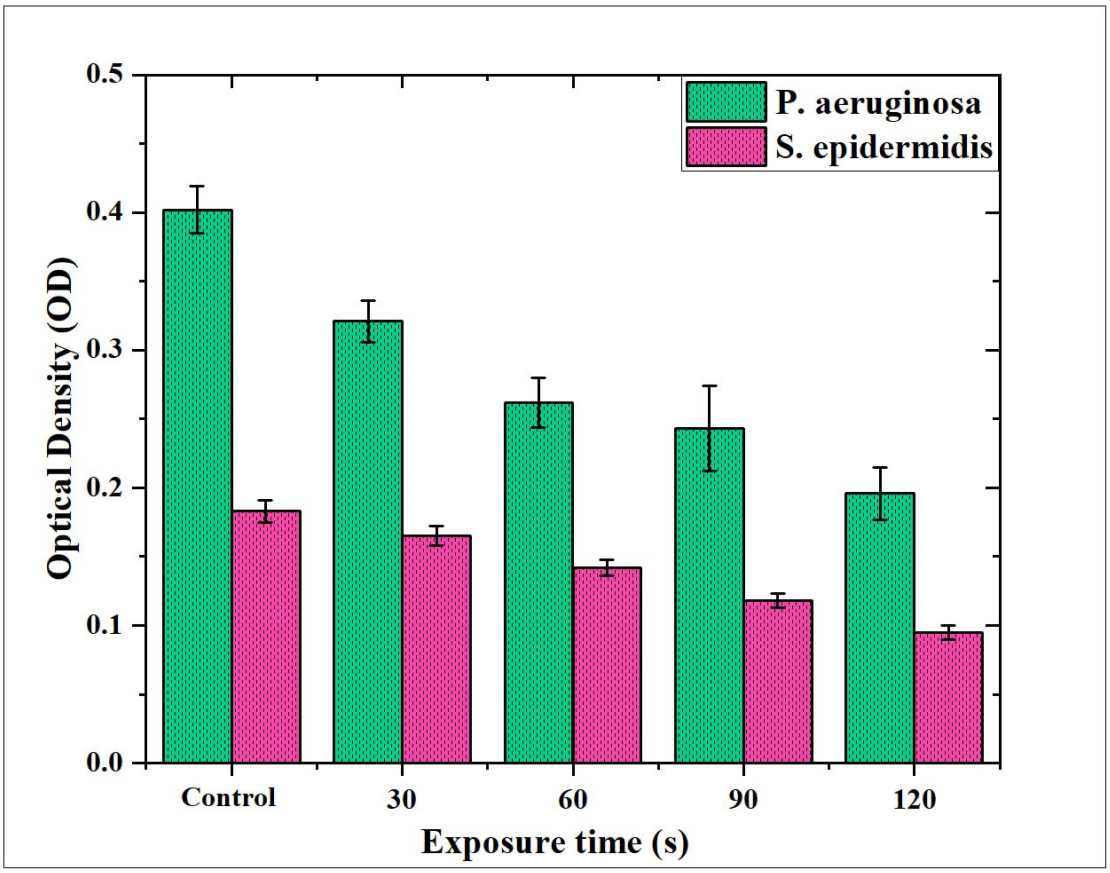

Figure 5: Effect of DBD plasma exposure time (0, 30, 60, 90, and 120 s) on the biofilm biomass of P. aeruginosa and S. epidermidis.

3.3 Effect of DBD Plasma on Morphological

The morphological modifications induced by dielectric barrier discharge (DBD) plasma treatment were studied by scanning electron microscopy (SEM) and the antibacterial activity of the developed plasma system was further verified. SEM allows the direct observation of the ultrastructure of bacterial surfaces and thus the establishment of a relationship between bacterial inactivation and structural damage. The morphological changes of P. aeruginosa and S. epidermidis after treatment with DBD plasma for 0, 30, 60, 90 and 120 s are shown in Figs 6-7. As shown in Figure 6, the P. aeruginosa showed the untreated cells elongated rod-shaped morphology with smooth surfaces and intact membranes. Progressive membrane roughening and localized surface damage were observed after (30) and (60 s) of plasma exposure. After (90 s) of treatment, the membrane pores became obviously deformed and enlarged. The (120 s) DBD plasma treatment caused extensive disruption of the bacterial membrane but many bacterial cells retained elongated morphology indicating that P. aeruginosa was more resistant to plasma treatment than the other bacterial species studied

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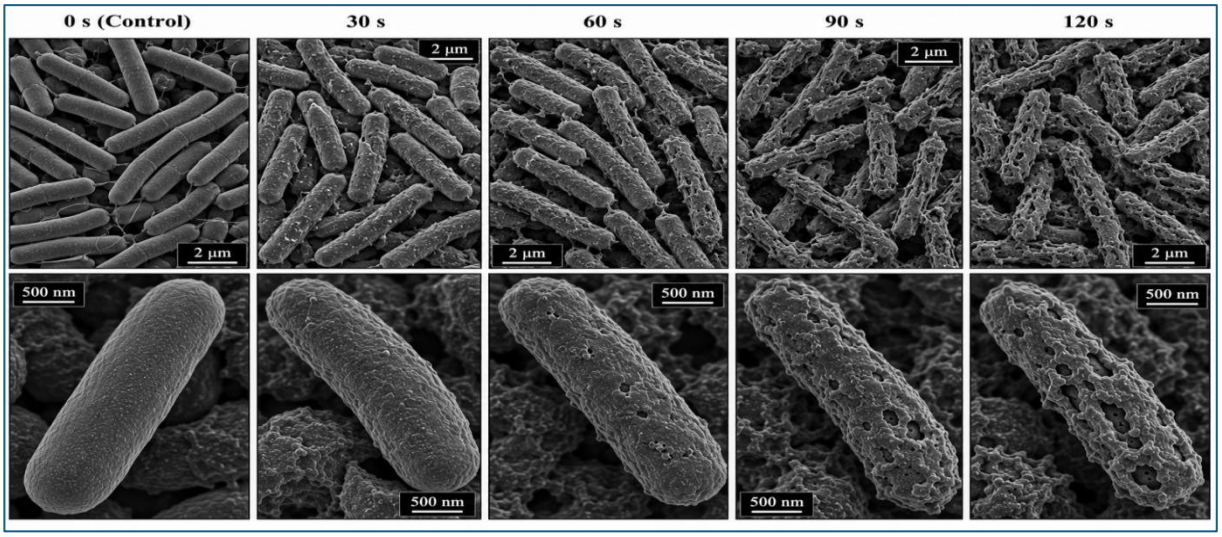

Figure 6: Scanning electron microscopy (SEM) images showing the morphological changes of P. aeruginosa following DBD plasma treatment for 0, 30, 60, 90, and 120 s.

The morphological changes observed for S. epidermidis are shown in Figure 7. Untreated cells exhibited the typical spherical morphology with smooth and well-defined surfaces. Plasma exposure led to a gradual deterioration of the bacterial structure. After (30 s), some membrane roughening was observed. Treatment for (60 s) and (90 s) showed increasing surface irregularities, membrane deformation and localized collapse. The destructive effect of prolonged DBD plasma treatment was confirmed by structural disruption, fragmentation and loss of cellular integrity in many bacterial cells after (120 s). Comparison of the SEM images reveals that DBD plasma produced morphological damage in time-dependent manner in Gram-negative and Gram-positive bacteria. While all bacterial species exhibited progressive structural damage with increasing treatment time, severity of damage varied among the investigated microorganisms. S. epidermidis exhibited maximum morphological destruction at 120 s of treatment, while P. aeruginosa displayed relatively better structural integrity although membrane damage was apparent. This trend is in good agreement with viable cell count, inhibition-zone and plasma-to-sample distance experiments confirming [24],[25].

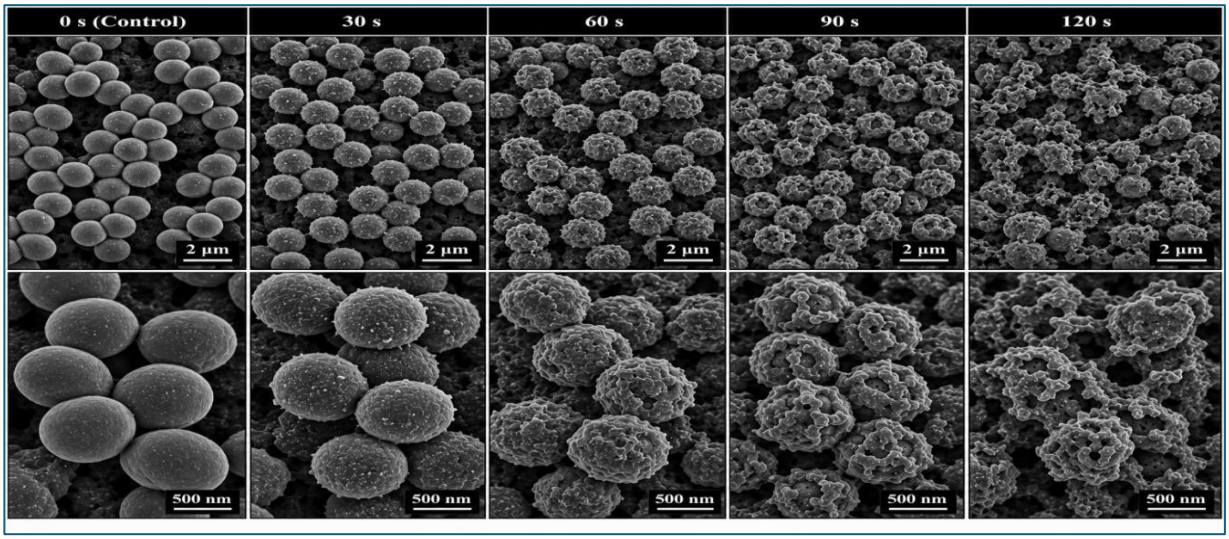

Figure 7: Scanning electron microscopy (SEM) images showing the morphological changes of S. epidermidis following DBD plasma treatment for 0, 30, 60, 90, and 120 s.

3.4 Effect of DBD Plasma on Trichophyton rubrum

The antifungal activity of the developed dielectric barrier discharge (DBD) plasma system was investigated by exposing the T. rubrum cultures to DBD plasma for 0, 3, 6, 9, 12, 15, 18, 21 and 24 min. The antifungal activity was evaluated by measuring the visible changes of fungal colony morphology and radial growth as shown in Figure 8. As shown in Figure, the untreated control (0 min) had a dense cotton-like colony with abundant aerial mycelia and vigorous fungal growth, indicating a normal development of T. rubrum. The colony was compact in structure with a well-developed network of hyphae covering a large part of the culture medium. A significant reduction in the colony diameter and the density of the aerial mycelia was

detected after 3 min of DBD plasma treatment. Plasma treatment was detected to suppress fungal growth since active fungal growth was still seen but the colony was more compact than the untreated control. The fungal growth was further reduced by increasing the treatment time to (6min). The colony was smaller and more compact, showing clear inhibition of radial growth compared with the control. At (9 min) inhibition was significantly greater as colony diameter started to decrease and the development of fungi was markedly suppressed. After (12 min) of plasma treatment only small compact fungal colony was left in center of the Petri dish indicating that the mycelial growth was strongly inhibited. After (15 min) exposure the colonies were reduced in size again, and after (18 min) the fungal biomass was drastically reduced, leaving only a very small residual colony.

At (21 min), the fungal growth was significantly inhibited, and only a minute fungal fragment was observed on the culture medium. After (24 min) of DBD plasma treatment, no visible fungal colony was observed, indicating complete inhibition and excellent antifungal efficacy of the developed plasma system against Trichophyton rubrum. The gradual inhibition of the fungal growth in Figure 8 is mainly caused by the interaction of plasma-generated reactive oxygen and nitrogen species (RONS), charged particles, ultraviolet photons and transient electric fields with the fungal cell wall and plasma membrane. Reactive plasma components lead to oxidative degradation of cell-wall polysaccharides, membrane lipids, proteins and intracellular biomolecules, leading to disruption of membrane integrity, inhibition of hyphal extension and ultimately fungal cell death. With increasing plasma exposure time, the cumulative oxidative stress gradually suppressed the growth of fungi and resulted in complete inhibition after (24 min). The observed antifungal activity is in good agreement with [26].

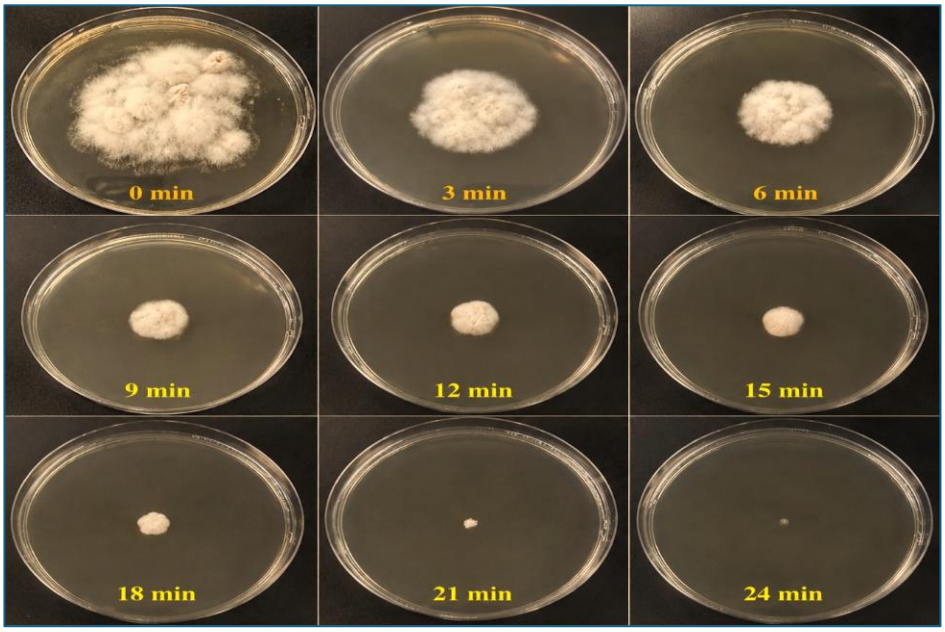

Figure 8: Effect of DBD plasma exposure time (0, 3, 6, 9, 12, 15, 18, 21, and 24 min) on the growth of T. rubrum.

4. Conclusion

The present study demonstrated that atmospheric-pressure dielectric barrier discharge (DBD) cold plasma was effective as a non-thermal antimicrobial treatment against Pseudomonas aeruginosa, Staphylococcus epidermidis and Trichophyton rubrum. The results clearly showed that antimicrobial efficiency was strongly dependent on the plasma exposure time. The bacterial strains exhibited a progressive reduction in the viable cell density with an increase of treatment time from 30 to 120 s, reaching maximum inactivation efficiencies of 55.7% for P. aeruginosa and 60.3% for S. epidermidis after 120 s of treatment. The anti-biofilm experiments also demonstrated that DBD plasma was effective in reducing bacterial biomass.

Conflicts of interest

The authors declare that they have no known competing commercial interests or personal relationships that could have appeared to influence the work reported in this paper.

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