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RESEARCH ARTICLE

PLASMA PROCESSES AND POLYMERS

Effects of different cold atmospheric-pressure plasma sources on the yeast *Candida glabrata*

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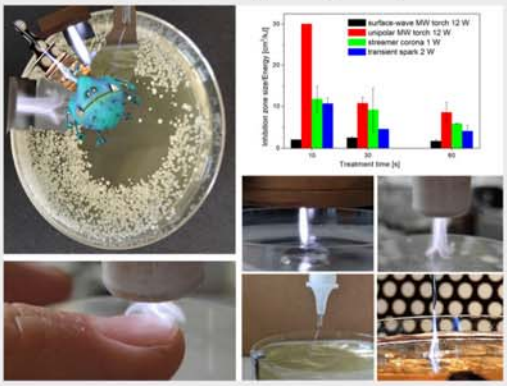
Abstract

Four different cold plasma sources were directly applied onto a 24h inoculum of *Candida glabrata* inoculated on agar plates, within the limits of in vitro experiment. Their effects were compared and evaluated with respect to the size and stability of the inhibition zones formed in the posttreatment cultivation. The results prove significant inhibitory cold atmospheric-pressure plasma effects on the yeast *C. glabrata*. The overall inhibitory effects are directly proportional to the treatment time, the applied power, and the overall functioning of the plasma source and indirectly proportional to the initial cell concentration, although this factor was less significant compared to the other examined factors. The unipolar microwave torch was found to be the most effective in the inhibition of *C. glabrata*.



KEYWORDS
antimicrobial effects, *Candida glabrata*, CAP, cold plasma, direct treatment

1 | INTRODUCTION

Cold atmospheric-pressure plasma (CAP) technology has achieved a great deal of attention in recent years thanks to its antimicrobial effects, including high efficiency against a wide range of microorganisms, ease of operation, economic simplicity, and also environmental friendliness.[1,2] Various effects and some mechanisms of bacterial inactivation and tumor cell inactivation have been explained over the last decade, but the effects and mechanisms of fungal inactivation have not yet been sufficiently investigated.[3–5] The aim of this work is to

Abbreviations: *C. glabrata*, *Candida glabrata*; CAP, cold atmospheric-pressure plasma; MW, microwave; RNS, reactive nitrogen species; RONS, reactive oxygen and nitrogen species; ROS, reactive oxygen species.

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verify and compare the effects of several cold plasma sources on the model yeast *Candida glabrata*, to establish a basic plasma setup for further studies and experiments in vivo and to expand the global knowledge of the antifungal effects of CAP. This study focuses on the effects of CAP on *C. glabrata* as a representative of nonalbicans yeast species with the intention of its inactivation to contribute to the problematic sterilization of small medical devices, as well as to find new treatment methods for superficial infections caused by *Candida*.

Candida yeasts are small oval-like cells called blastoconidia. They reproduce by budding, which means that daughter cells grow from the mother cells. Elongating daughter cells typically remain fused and form chain-like structures called pseudo-hyphae. *Candida*s are a natural part of the human microflora and belong to the group of typical opportunistic pathogens, which means that their pathogenicity depends on the overall condition of the infected organism. They can be found in their saprophytic (harmless) form as part of the physiological microflora in the oral cavity, the intestines, and the genital area, as well as on the human skin. If the host's immune system is weakened, they can turn into dangerous pathogens.[6] Various health conditions such as asthma, acquired immunodeficiency syndrome (AIDS), diabetes, cancer, organ transplantation, and long-term use of corticosteroids or antibiotics are important factors in the risk of invasive mycosis. Species from the genera *Aspergillus, Candida*, and *Cryptococcus* are the most common human pathogenic fungi responsible for a wide range of invasive and superficial infections.[7] If the cause of mycosis is yeasts of the genus *Candid*a, the infection is called candidiasis. The most common cause of candidiasis is *Candida albicans*, but recently the risk of infections caused by nonalbicans species (NAC) such as *Candida glabrata, Candida tropicalis, Candida parapsilosis*, and *Candida krusei* has rapidly increased.[8]

Infections caused by the yeasts *Candida* can be both superficial and systemic. Superficial infections with high morbidity are not life-threatening and most often cause the patient unpleasant itching or pain as a result of inflammation in the affected area. Some of the superficial candidiasis infections include mucocutaneous (mucosal) candidiasis, candidal vulvovaginitis (inflammation of the vulva and vagina) and balanitis (inflammation of the glans penis), oropharyngeal candidiasis (candidiasis of the mouth and pharynx), keratitis (inflammation of the cornea) or mycosis of the skin and nails, or internal yeast overgrowth in the gastrointestinal tract.[6] Systemic candidiasis, or the so-called invasive candidiasis, is a serious infection where the yeasts *Candida* can affect the blood, heart, brain, eyes, bones, and other parts of the body. If *Candida*s infect the bloodstream, the infection is called candidemia and is one of the most common causes of bloodstream infections in hospitalized patients in the United States.[9–11]

1.1 | *Candida glabrata*

Candida glabrata (C. glabrata) of the genus *Candida* belongs to the class *Fungi Imperfecti*, order *Moniliales*, and family *Cryptococcaceae*, and is currently considered the second most common cause of candidiasis in the world.[12] From a phylogenetic point of view, *C. glabrata* is genetically more similar to *Saccharomyces cerevisiae* than *C. albicans* precisely because of its haploid genome, which also distinguishes it from other *Candida* species. Another characteristic that distinguishes this yeast from other *Candida* species is its inability to form pseudohyphae at temperatures above 37°C.[13] This yeast appears to have relatively recently changed its lifestyle from a nonpathogenic yeast to an opportunistic pathogen, which means that when the host's immunity is weakened, it changes from a saprophytic to a pathogenic yeast and can cause candidiasis affecting various parts of the body. As in the case of the entire genus *Candida, C. glabrata* is a threat, especially for vulnerable people.[10]

1.1.1 | Pathogenicity

This high infectivity of the yeast *Candida* is mainly due to their resistance to commonly used antifungals, their ability to maintain highly proliferative and repopulating capacity through biofilm formation, the rate of adaptation and the development of resistance to new drugs, their ability to tolerate host defenses, or adaptability to long-term adverse conditions such as starvation and oxidative stress. Another strategically important ability is the production of adhesin, which is a glucan-crosslinked cell wall protein that is responsible for good adhesion to various surfaces and also triggers biofilm formation.[14] In addition to the above-mentioned factors common to the genus *Candida*, *C. glabrata* has developed many more. One of the most serious threats is its natural resistance to azole antifungals, which are the most commonly used antimycotics for the treatment of candidiasis. Recently discovered survival strategies also include macrophage avoidance, rapid escape from host immune cells, the ability to resist macrophage antimicrobial activities, and, most importantly, the use of macrophages for protective and replication processes. Resistance to antimicrobial peptides, which are small, positively charged, amphipathic molecules that act

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directly against microbes, is also important. *C. glabrata* has also found a strategy to survive under hypoxia (low oxygen concentration) by strengthening the property of biofilm formation.[15]

The methods currently used to treat candidiasis are insufficient, and their effectiveness is still declining. Therefore, there is an urgent need to find a new way to inactivate these yeasts, thus preventing the excessive use of antifungals to which the yeast has already developed a strong resistance.[8,16]

1.2 | CAP

One of the alternative methods that could be used for the inactivation of pathogenic microorganisms is CAP. CAP treatment is a nonthermal process that uses plasma (ionized gas or gas mixture) as a source of electrons, electric field, ultraviolet radiation (UV), and most importantly, reactive oxygen species (ROS), such as hydroxyl radicals, ozone, singlet oxygen or hydrogen peroxide, and reactive nitrogen species (RNS), such as nitric oxide, nitrites, nitrates, peroxynitrite, and other NO_x.[17–19]

The CAP components, especially the electric field and the charged and reactive species, erode ("etch") the cell wall or the plasma membrane, disrupting the chemical bonds in the molecules. The high-energy electrons or other active particles interact with the membrane molecules, causing excitation and inducing vibrational energy transfers in the cell membrane molecules. If these energy transfers exceed certain limits, ionization of membrane molecules (positive ion formation) or electron capture (negative ion formation) may occur, and thus the membrane molecules may dissociate into other organic molecules, leading to a reduction of local surface tension in the cell membrane. If this process is continuous, the pressure in the cell increases, and the cytoplasm may escape from the pores that occur near the dissociating molecules of the membrane. UV radiation and RONS (reactive oxygen and nitrogen species) can then penetrate the cell through these openings. After the entry of these reactive particles into the cell, severe symptoms of cytoplasmic deformation and mutation or damage of DNA or RNA occur, all leading to cell death.[1] The penetration of UV radiation into the cytoplasm of the cells causes a change in the molecular structure of the cytoplasm.[20] When a cell reacts with energetic UV photons, thymine dimers may form in the DNA, which inhibit the ability of the cells to replicate. However, in the case of CAP, UV(C) production is relatively low, and therefore, the energy of photons emitted from the plasma is not sufficient to induce this effect. In addition, UV photons produced by CAP are often absorbed by the surrounding atmosphere, so the sterilizing effects of plasma are, in most cases, not attributed solely to UV photons, but their synergistic effect cannot be fully refuted.[21] In the case of a direct application of the plasma discharge to the treated subject, the electric field causes the so-called electroporation (opening of pores in cell membranes due to the high electric field) as well. All these effects synergistically cause various types of damage to microbial cells leading to cell death.[18,22]

Numerous studies demonstrated a promising use of CAP in oncology,[23–25] dermatology,[26–28] and wound healing.[29,30] In general, decontamination using plasma technologies is the most investigated and discussed topic.[31–33] Some studies have already gone beyond the in vitro experiments to be performed in vivo, and some have progressed to the phase of clinical trials in real patients.[34–37]

2 | EXPERIMENTAL PART

2.1 | CAP sources

Four different CAP sources were tested for their inhibitory effects on *C. glabrata*. Their setups are discussed below.

2.1.1 | Surface-wave microwave (MW) torch

MWs generated by the solid-state generator (Sairem, GMS 200 W) at a frequency of 2.5 GHz and a power of 9–12 W are guided by a coaxial cable into the surfatron resonant cavity, where a part of the waves starts to form a standing wave and part of the waves escapes through a small slit and reaches the boundary of the dielectric (quartz capillary surrounded by air, through which the carrier gas flows). The so-called bound surface waves propagate on the interface between the air and the plasma delivering a part of their energy to argon, which is blown out of the quartz capillary, creating the plasma plume. As the wave gives a part of its energy to gas molecules, it loses some of its energy. When the energy is too low to sustain the discharge, the visible plasma plume disappears. Thus, the length of the plasma plume itself is dependent on the power of the MW supply (the energy of the MWs). The concentration of the active species is the highest at the tip of the plasma plume[26] and that is why the distance between the treated sample and the edge of the cavity (for both MW sources) was chosen in a way that the end of the plasma plume would touch the treated surface (see Table 1). The

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schematic setup and the discharge can be seen in Figure 1, and a detailed description with the diagnostics and other characteristics, including voltage, current, and reactive species analysis, can be found in Krcma et al. and Trebulova et al.[26,38]

2.1.2 | Unipolar MW torch (Surfayok)

MWs generated by the same generator as in the case of the surface-wave MW torch and a power of 9–12 W are guided by a coaxial cable into the plasma generator, where they propagate in all directions and pass their energy to argon molecules, thus ionizing them. The plasma is formed in the cavity and is blown out in the form of a single plasma beam. However, the formation of plasma beams is so fast that it is impossible for the human eye to perceive one beam after another, with irregular small projections. Instead, we perceive several filaments at once (see Figure 2). This device is legally protected and has been designed by Todor Bogdanov (Bulgaria); its description can be found in Trebulova.[39]

2.1.3 | Streamer corona

A DC-positive streamer corona discharge was generated in ambient air at atmospheric pressure between the tip of the needle electrode and the treated surface using the portable corona pen described in more detail in.[40] The tip of the needle was sticking 1 mm out of the glass tube cover (see Figure 3). Two plasma parameters were tested: (i) streamer frequency 10.5 kHz and voltage 12.8 kV (corresponding to the power 0.5 W) and (ii) frequency 13 kHz and voltage 14 kV (corresponding to the power 1 W). The distance between the needle and the agar surface was adjusted so that the end of the visible plasma plume would touch the treated surface, approximately 5 mm. For the experiments with the streamer corona and the transient spark, circles of aluminum foil were stuck to the bottom of the Petri dishes to support an approximately homogenous field in the Petri dish and ensure radially homogenous electric potential on the agar surface. This aluminum foil circle had a sticking end by which the treated sample was grounded. A complete description of this plasma device with the diagnostics and other characteristics, including voltage, current, and reactive species analysis, can be found in Sersenova et al., and Machala et al.[41,42]

2.1.4 | Transient spark

A DC-driven positive transient spark discharge was generated in ambient air at atmospheric pressure with a frequency of 1 kHz and ignition voltage of 12 kV, which corresponds to the power of ~2 W. Agar plates were also prepared with the aluminum foil with the grounded end, as described above in the case of the streamer corona discharge. The peculiarity of this discharge compared to the discharges described above is that the conductive discharge channel extends to the treated surface, which has to be grounded to close the electrical circuit (see Figure 4). Another difference from the other tested discharges is its higher

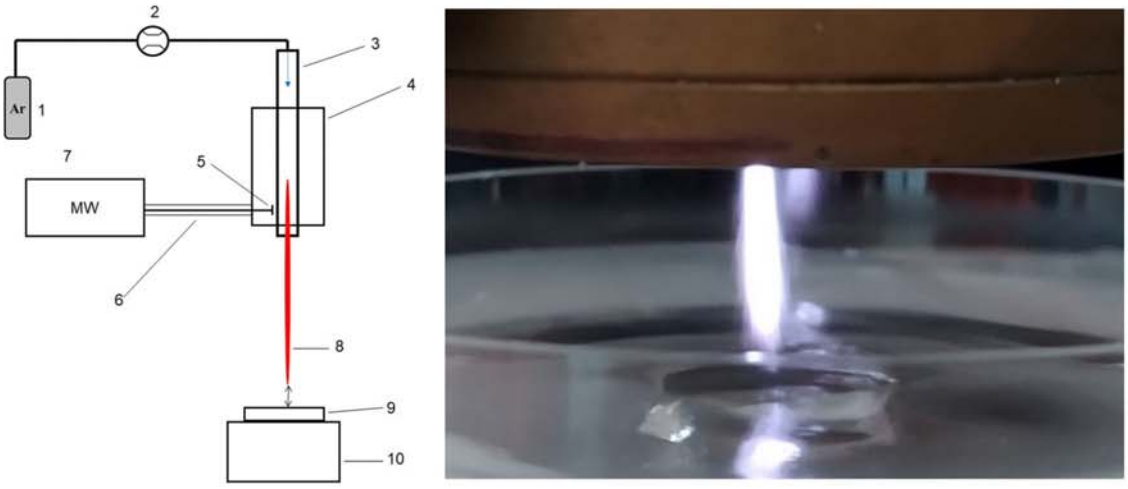

FIGURE 1 Experimental setup of the surface-wave microwave (MW) torch: 1—argon source; 2—mass flow controller; 3—quartz capillary with flowing argon; 4—resonance cavity; 5—MW antenna; 6—MW-coaxial cable; 7—MW source; 8—plasma plume; 9— *Candida glabrata* inoculated on Petri dish; 10—Petri dish holder.

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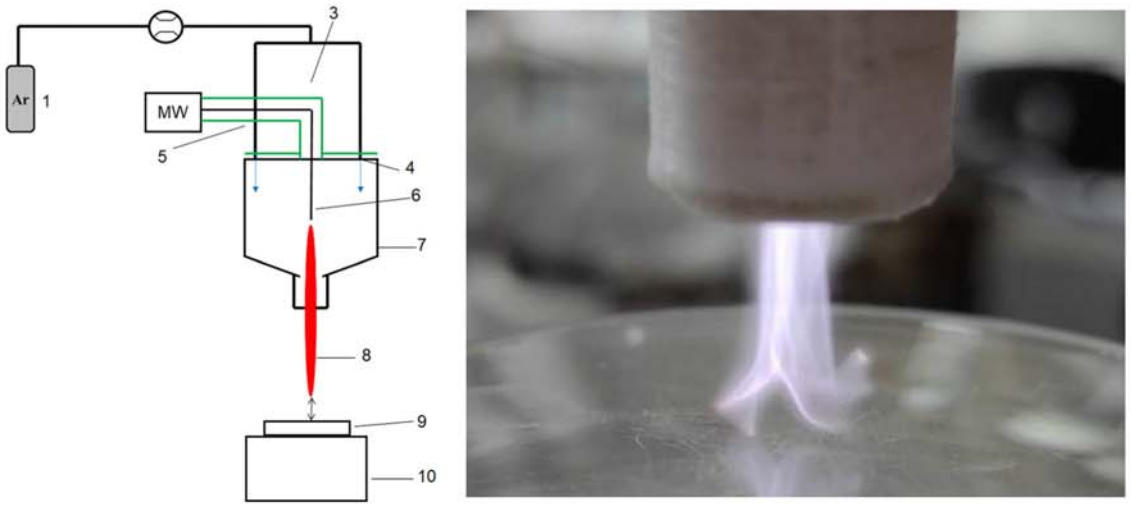

FIGURE 2 Experimental setup of the unipolar microwave (MW) torch: 1—argon source; 2—mass flow controller; 3—MW source; 4—direct argon supply; 5—MW coaxial cable; 6—free ending of the coaxial cable acting like antenna; 7—cavity, 8—plasma plume; 9—*Candida glabrata* inoculated on Petri dish; 10—Petri dish holder.

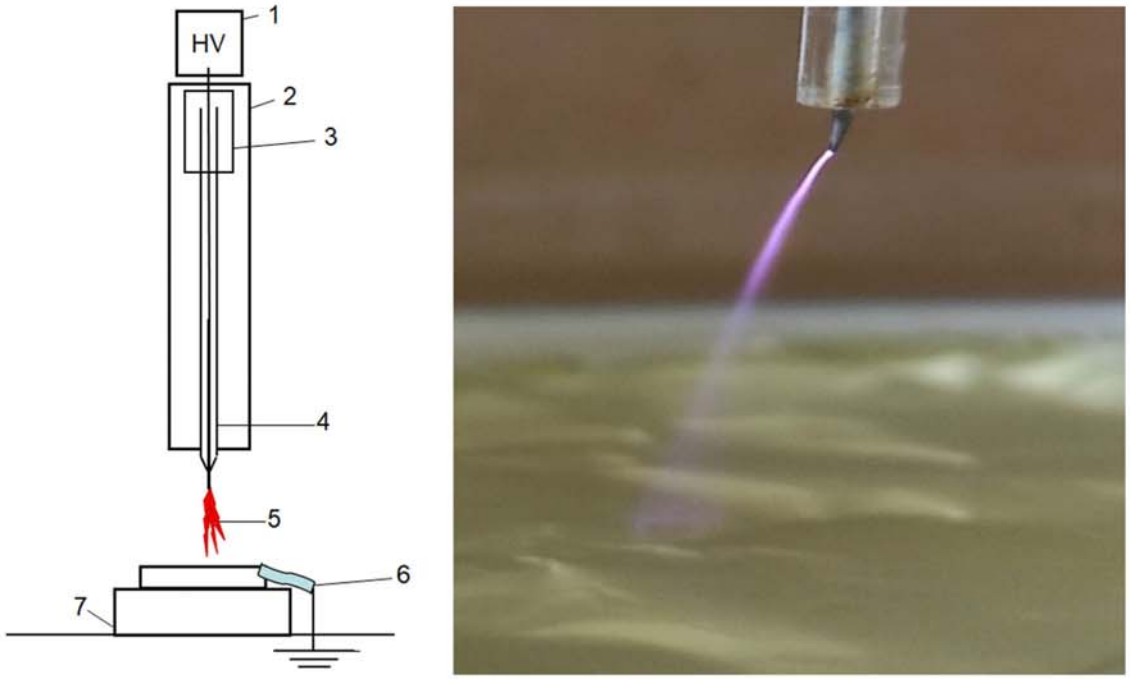

FIGURE 3 Experimental setup of the streamer corona: 1—DC high voltage power supply; 2—resistor holder; 3—protective cover; 4—syringe needle; 5—streamer corona discharge; 6—grounded Petri dish with an inoculated culture of *Candida glabrata*; 7—Petri dish holder.

energy in the spark channel, so it would not be suitable for application to the skin itself but rather to less thermally sensitive areas of the body (teeth, nails, etc.), inanimate objects, or for indirect plasma treatment. A detailed description of the transient spark with the diagnostics and other characteristics, including voltage, current, and reactive species analysis, can be found in Refs.[43–46]

2.2 | Microbiological part

For testing the antimicrobial activity of selected discharges, the yeast strain *C. glabrata* CCM 8270 was used, supplied by the Czech Collection of Microorganisms in Brno.[47] The yeast culture was grown in the YPD (Yeast extract—Peptone -Dextrose/Glucose) liquid medium for 24 h. The temperature of cultivation was 37°C.

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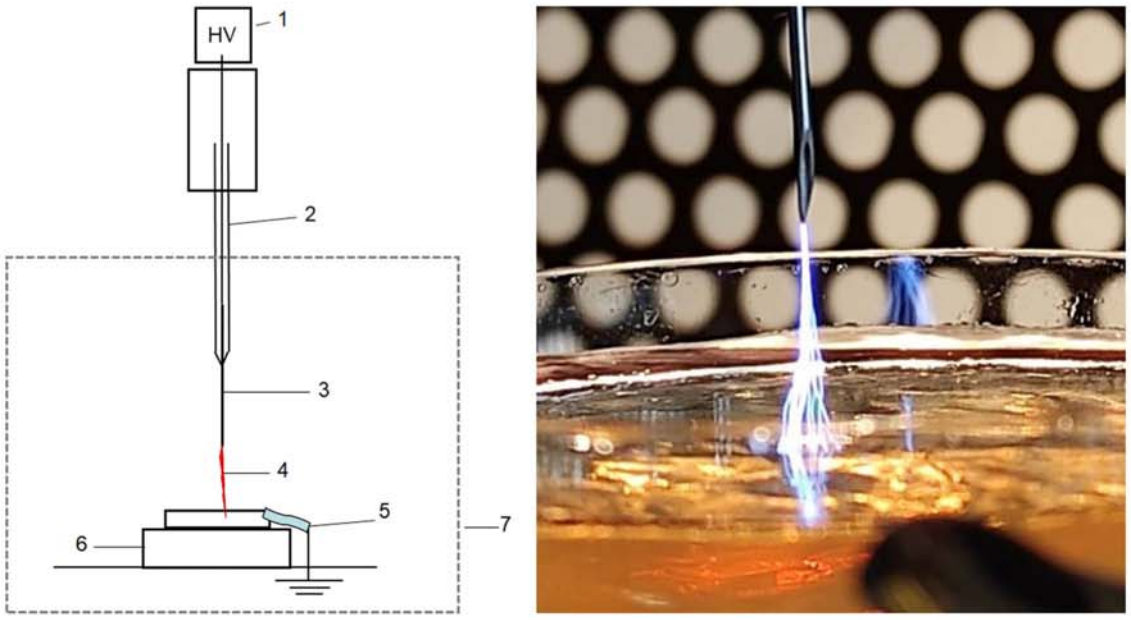

FIGURE 4 Experimental setup of the transient spark discharge: 1—DC high voltage power supply; 2—capillary, 3—syringe needle; 4—transient spark discharge; 5—grounded Petri dish with an inoculated culture of *Candida glabrata*; 6—petri dish holder; 7—faraday cage.

The prepared 24-h inoculum was diluted to the required initial cell concentrations chosen for the experiment. The concentration of 10^6 CFU/mL (colony forming units per milliliter) was chosen as the reference concentration (absorbance ~0.35), from which further dilutions were derived. For the cell concentration calculation, the experimentally determined equation of the calibration line ($y = 3.475\ x$) was used. This refers to the absorbance of the *C. glabrata* inoculum at $\lambda = 630$ nm, where x is the number of cells in 1 mL of the medium and y is the measured absorbance value, whereas, for the absorbance equal to 1, the number of cells is 10^7 CFU/mL.

Sterile YPD medium was poured evenly into plastic Petri dishes with a diameter of 52.8 mm so that the agar layer was about 2 mm. The volume of 50 μL of prepared cultures with different concentrations of *C. glabrata* was inoculated on these agar plates.

C. glabrata cultures were exposed to the plasma immediately after inoculation. Each Petri dish was treated individually, with the plasma beam oriented perpendicularly to the sample, approximately to the center of the Petri dish. At the constant gas flow and selected power, all concentrations of *C. glabrata* were exposed to the plasma for the chosen times (10 s, 30 s, 60 s). In the case of the unipolar MW torch, intermittent irradiation was also tested, and therefore, treatments of 3×10 s and 2×30 s were included. The pause (period of no contact with plasma) between the individual procedures was chosen to be as long as the treatment itself. This means that for the treatment time 3×10 s, the total exposure time was 30 s consisting of 10 s treatment—10 s pause—10 s treatment—10 s pause—10 s treatment. In the case of the 2×30 s procedure, one 30 s pause was included between two 30-s treatments. An overview of the settings of the individual devices is given in Table 1 and illustrative diagrams of the devices are shown in Figures 1–4.

The experiments were performed in a way to prevent the temperature rise of the treated surface from exceeding the temperature tolerance of the human skin.[48–52] Based on our previous results obtained by the thermal camera, the temperature of the treated object did not exceed 40°C in the case of MW discharges.[53] As the power of the streamer corona was much lower than the power used with the MW discharges, we did not perform complementary thermal camera measurements on the agar plates for the streamer corona. In the case of the transient spark, the discharge was not intended for direct application on the skin, but for the treatment of water or other nonliving surfaces; thus, there is no need to worry about temperature tolerance.

The samples treated with the selected discharges were subsequently cultivated in a thermostat for 48 h at a temperature of 37°C. All measurements were replicated twice, and control samples (without the plasma treatment) were evaluated for each concentration. Specific experiments were performed in a number of parallel measurements (multiplicates).

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TABLE 1 Conditions used for measurements with selected cold atmospheric-pressure plasma sources.

Parameters	Surface-wave microwave (MW) torch		Unipolar MW torch		Streamer corona		Transient spark
Mass flow rate (Slm)	5		5		-		-
Working gas	Argon 4.6		Argon 4.6		Ambient air		Ambient air
Power (W)	9	12	9	2	0.5	1	2
Distance of the cavity edge/needle from the agar surface (mm)	4	6	5	7	5	5	10
Treatment time (s)	10, 30, 60, 120, 300		10, 30, 60, 3 ×10, 2 ×30		10, 30, 60		10, 30, 60

All Petri dishes were photographed after 24 and 48 h. Then, the size of inhibition zones was determined using Microsoft PowerPoint, in which the corresponding ellipse or circle, the dimensions of which were recorded, was drawn across each zone and the corresponding dish. Subsequently, the areas of individual zones and dishes were calculated from the recorded dimensions, which were converted to the actual dimensions of the Petri dishes with a diameter of 5.28 cm. Results obtained by the application of individual discharges were also compared for their efficiencies using the software Statistica.

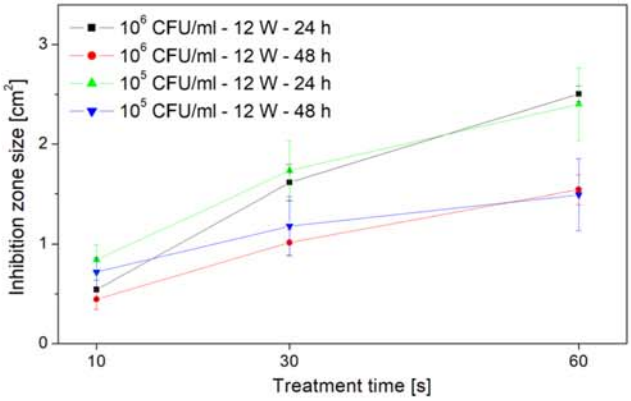

FIGURE 5 Dependence of inhibitory effects on the treatment time for two different concentrations of *Candida glabrata* 10^6 CFU/mL, 10^5 CFU/mL, cultivated for 24 and 48 h at 37°C, respectively.

3 | RESULTS AND DISCUSSION

3.1 | Surface-wave MW torch

3.1.1 | Impact of the treatment time

The results of the measurements with the surface-wave MW torch show that even after a short exposure to the plasma (10 s), the formation of inhibition zones can be observed, the size of which increases with increasing treatment time (see Figure 5). However, after this short treatment, the zones are not large enough to be considered effective for inhibiting the yeast cells, as was observed for bacterial cells.[54] Therefore, treatments under 30 s had been eliminated as a part of the process optimization. Treatments over 60 s may potentially cause discomfort in sensitive individuals due to thermal irritation, mainly if a higher power is used. Also, the long holds may not be applicable to restless patients (children and animals), so they were excluded from further experiments with the application to living subjects. Treatments lasting more than 60 s could advantageously be applied to inanimate objects and for sterilization of small thermally stable instruments, materials, and medical tools.

3.1.2 | Impact of the power

A similar trend applies to the power used. The use of 12 W resulted in larger inhibition zones than the use of 9 W. It has also been found that with the use of 12 W, half of the treatment time is sufficient to create an equally large inhibition zone compared to the use of 9 W. Both tested powers are highly efficient, but due to the higher energy, the treatment with the power of 12 W is more energy demanding, and thus, temperature tolerance needs to be taken into account for specific applications.

3.1.3 | Impact of the initial cell concentration

By observing the effect of the initial cell concentration, it was found that with a smaller number of cells, and thus their less cohesion, larger and more stable inhibition zones are formed, but this factor is the least significant compared to the other factors examined. The discharge

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was tested on concentrations of 10^7–10^4 CFU/mL. As the best results were obtained for the concentration of 10^5–10^6 CFU/mL, these concentrations were used as the initial cell concentrations for further measurements (see Figure 6).

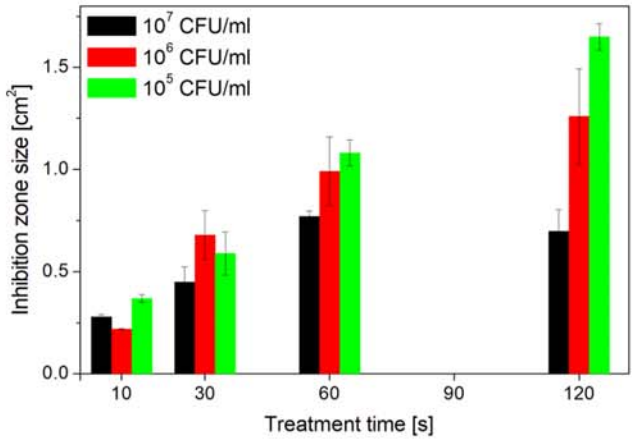

FIGURE 6 The inhibitory effects of the surface-wave microwave (MW) torch with the power of 12 W on three different concentrations of *Candida glabrata*: 10^7 CFU/mL, 10^6 CFU/mL, and 10^5 CFU/mL, after 48 h cultivation at 24°C.

After 48 h of posttreatment cultivation at 37°C, surviving cells within the individual inhibition zones could be clearly observed. Figure 7 shows that the difference in the size of the inhibition zones for 10^6 and 10^5 CFU/mL is almost negligible, but the efficiency of the inhibition is not the same. At a concentration of 10^6 CFU/mL, relatively large inhibition zones were formed, but after 48 h of cultivation, it was clear that decontamination within the zone was far from 100%, and surviving colonies could be observed inside the zones. Neither at the concentration of 10^5 CFU/mL was there a 100% inhibition and formation of completely clear zones, but the incidence of surviving colonies within the inhibition zones was much lower than at the higher concentrations.

The stability of the inhibition zones after 48 h was fairly good. The size of the inhibition zones decreased very little after 48 h. This change in the zone size was mainly due to the proliferation of vital cells within the inhibition zone or the colonization of the cells from the edges of the inhibition zone. Distinct inhibition zones with occasional colonies within the zone could have been observed even after 1 week of cultivation. A detailed study on this issue was published recently.[38]

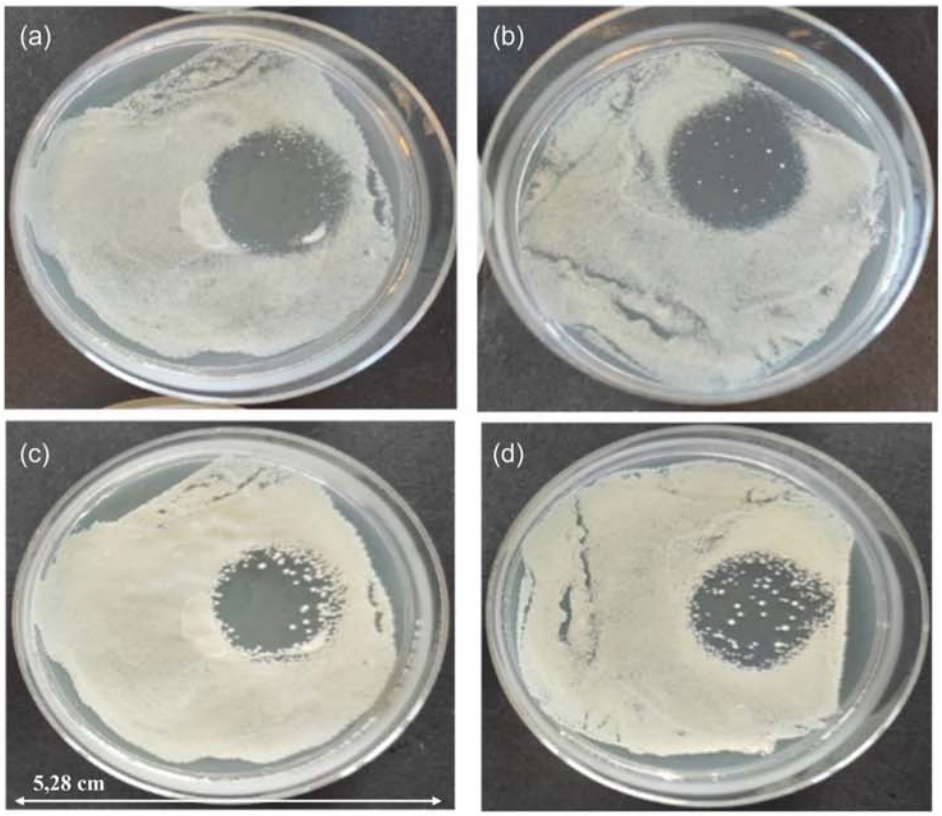

FIGURE 7 Photographs of inhibition zones created by the surface-wave microwave (MW) torch with 12 W power for 60 s on *Candida glabrata* with concentrations of 10^5 CFU/mL (a, c) and 10^6 CFU/mL (b, d) after 24 h (a, b) and 48 h (c, d) cultivation at 37°C.

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3.2 | Unipolar MW torch (surfayok)

The resulting inhibition zones were much larger compared to the zones created by other discharges tested in this work. Using this discharge, it is obvious that even treatment times as short as 10 s are characterized by high inhibitory efficiency and can be used for sufficient inhibition of *C. glabrata*. However, it is still the case that with a longer treatment time, the efficiency increased sharply, and therefore, when possible, it is better to choose the longer time with better effects.

As with all discharges, in the case of the power, we observed a direct ratio between the applied power and the size of the inhibition zone and, conversely, an indirect ratio when examining the effect of the initial cell concentration. Using the power of 12 W, the exposure time of 60 s, and the concentration of 10^6 CFU/mL, approximately 30% inhibition efficiency was achieved within the area of the Petri dish with a diameter of 5.28 cm and a total area of 21.88 cm^2, 50% efficiency for the concentration of 10^5 CFU/mL, and 90% efficiency was observed for the concentration of 10^4 CFU/mL, as the inhibition zone with a few remaining colonies was created over the entire area of the Petri dish. In contrast to the surface-wave MW torch, decontamination within the inhibition zones was more efficient as the number of cells surviving the treatment was much smaller, and thus, their overgrowth from the inside or from the edges is negligible.

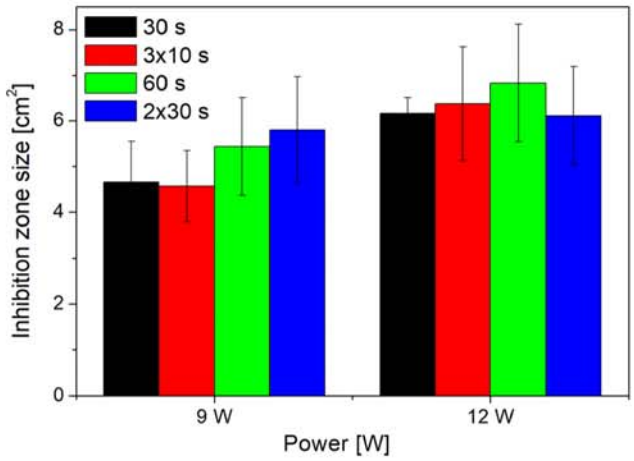

FIGURE 8 Histogram comparing intermittent irradiation and single uninterrupted treatment with the unipolar microwave (MW) torch (power of 9 W and 12 W) on the *Candida glabrata* concentration of 10^5 CFU/mL after 24 h cultivation at 37°C.

Concentrations of 10^9, 10^8, and 10^7 CFU/mL were also used to study the effect of the initial cell concentration, but no inhibitory effects in the form of inhibition zones were observed. This may be caused by too many cells present in the system, some of which were deactivated, but the number of cells was still large and formed a layer over the entire surface of the dish. In some cases, inhibition zones were also formed, but their incidence was very low, and therefore, their sizes were not recorded. Further experiments were based on this experiment, where individual dilutions were below 10^6 CFU/mL.

Favorable results have been observed in the case of intermittent irradiation, and thus the sum of shorter treatments with short breaks can give similar results as in the case of one longer uninterrupted treatment (see Figures 8 and 9). This fact is particularly advantageous for the application to the skin or other living tissues since the skin can cool down during a short break between

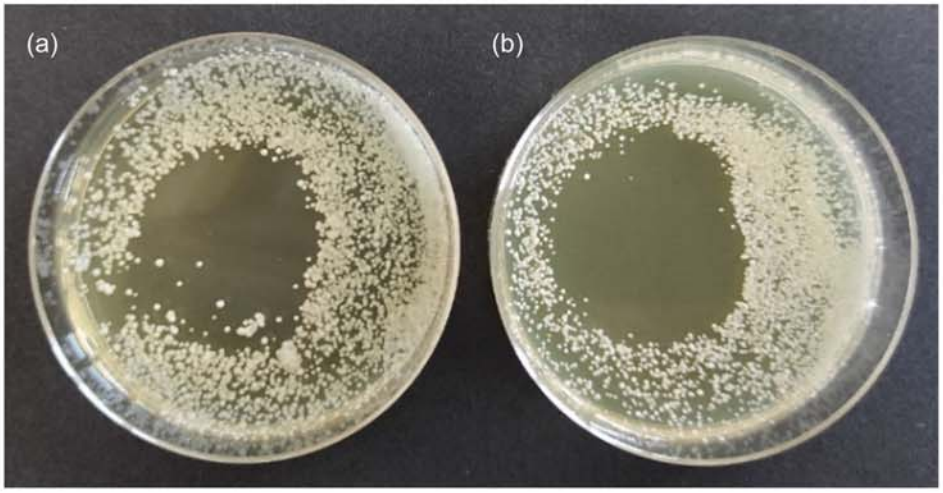

FIGURE 9 Inhibition zones created after 60 s (a) and 2 × 30 s exposure (b) to the unipolar microwave (MW) torch with 12 W power on the concentration 10^5 CFU/mL, after 24 h cultivation at 37°C.

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treatments, and therefore, it is possible to use even higher power with better efficiency and treatment times that would not otherwise be possible due to local overheating. It is also advantageous for the application on animals, children, and other restless patients or when aimed at more thermally sensitive skin areas.

3.3 | Streamer corona

The results correlate with the above-mentioned trends obtained for the MW plasma sources. In the case of the streamer corona discharge, a great advantage is its extremely low power, which allows safe application for longer treatment times. Inhibition zones after the treatment up to 30 s can be considered negligible because their area is in square millimeters. The zones created after 30 s of treatment correspond to 10 s of treatment using the surface-wave MW torch. Therefore, we still consider this treatment time to be relatively short for sufficient yeast inhibition. Treatment times greater than 60 s should then be used for a successful procedure (see Figure 10). With this discharge, it would also be appropriate to test treatment times between 60 and 300 s or more, as examined by the surface-wave MW torch, with the proviso that in the case of the streamer corona, these times could be applied to living tissues without the risk of local overheating.

Concerning two investigated powers (0.5 W and 1 W), the discharge with the higher power of 1 W is clearly more efficient for yeast inactivation. The 0.5 W power was chosen from the previous experiments on bacteria,[55,56] but usage of higher power is recommended to inhibit yeast growth if the temperature tolerance allows.

By examining the effect of the initial concentration, the general trend applicable to all discharges was verified, indicating a higher discharge efficiency at lower cell concentrations (see Figures 11 and 12). The concentrations of 10^3–10^6 CFU/mL were selected for this discharge. Inhibition zones were determined for concentrations of 10^4 –10^6 CFU/mL. The zones at the concentration of 10^4 CFU/mL were unobservable for the short treatment (10 s) due to the occurrence of individual colonies separated by free agar and the impossibility of locating the inhibition zone. The concentration of 10^3 CFU/mL was also tested, but this could not be evaluated by the inhibition zone size method due to the impossible localization of the inhibition zones among individual colonies.

The stability of these zones follows the general trend that the zones decreased in size after 48 h due to the small overgrowth from the edges, but even after 1 week, they were still observable.

3.4 | Transient spark

The transient spark discharge is quite different from the other tested discharges. As it is a discharge with higher energy in the spark pulse, it is not aimed to be applied directly to the living skin. In this experiment, the discharge was tested with the intention of sterilizing inanimate objects.

The sizes of the inhibition zones created by this discharge are comparable to the sizes of the zones created by the streamer corona, but their stability over time is higher than that of any other tested discharge.

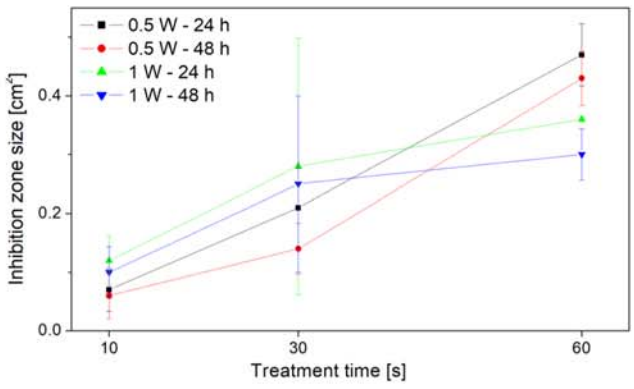

FIGURE 10 Dependence of the inhibitory effects of the streamer corona with the power of 0.5 W and 1 W on the treatment time for the *Candida glabrata* concentration of 10^6 CFU/mL, cultivated for 24 and 48 h at 37°C, respectively.

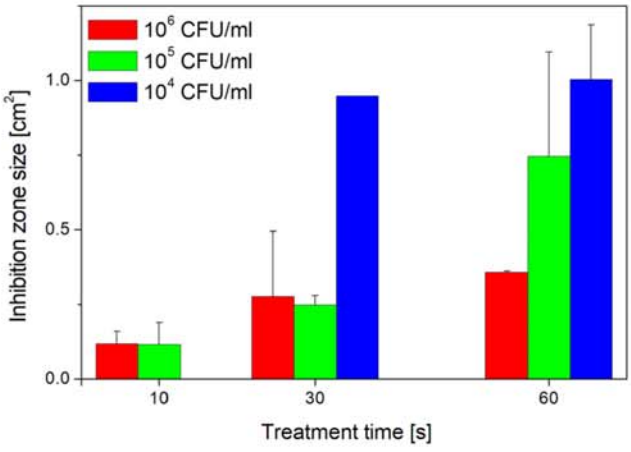

FIGURE 11 Dependence of inhibitory effects of the streamer corona with the power of 1 W on the treatment time for three concentrations of *Candida glabrata* 10^6 CFU/mL, 10^5 CFU/mL, and 10^4 CFU/mL, after 24 h cultivation at 37°C.

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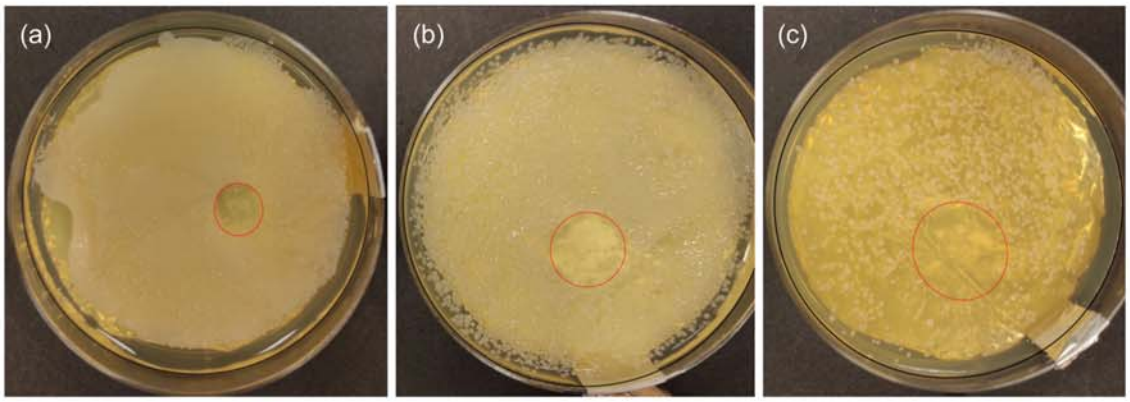

FIGURE 12 Photographs depicting inhibition zones created after 60 s exposure of the streamer corona with the power of 1 W on three concentrations of *Candida glabrata* 10^6 CFU/mL (a), 10^5 CFU/mL (b), and 10^4 CFU/mL (c), after 24 h cultivation at 37°C; inhibition zones circled in red.

Minimal or no overgrowth was observed in the zones created by the transient spark discharge, either after 48 h or even after 1 week of cultivation (see Figure 13). However, the size of the zones at treatment times of 10 s and 30 s is small, and therefore it would be appropriate to choose times around 60 s for the sterilization of inanimate objects. Longer exposure times would need to be examined. In the case of thermolabile or fragile objects, it is not possible to use this discharge because its higher energy, as well as pressure waves hitting the surface of the object, could damage the object's surface. In Figure 14, small depressions caused by the pressure waves and evaporation of the agar can be seen. This agar depression was observed even after the short treatment (10 s) and was deepening with the increasing treatment time, whereas even the caramelization of the agar surface was observed with prolonged treatment time (more than 60 s).

Concerning the effect of the initial cell concentration, it is clear that for the concentrations 10^5 and 10^6 CFU/mL, the inhibition zones were almost the same in size, but at the concentration of 10^4 CFU/mL, higher efficiency was observed (see Figure 14). The measurements with the concentration of 10^4 CFU/mL were relatively nonstandard because of the above-mentioned problem with precise size determination of the small inhibition zones in smaller concentrations. With these lower concentrations, the differences in the number of cells in the same volume become more important as the variance that cannot be noticed in high concentrations (due to the formation of a complete layer on the surface) comes into play. Thus, parallel measurements (multiplicates) showed results in a wider range of values, as can be seen in the standard deviations. Nevertheless, the general trend is still applicable; thus, the initial cell concentration does not have a significant impact on the inhibitory effects of CAP.

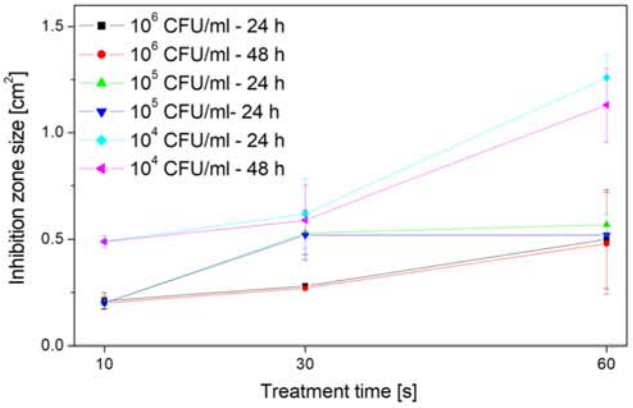

FIGURE 13 Dependence of inhibitory effects of the transient spark with the power of 2 W on the treatment time for three concentrations of *Candida glabrata* 10^6 CFU/mL, 10^5 CFU/mL, and 10^4 CFU/mL cultivated for 24 and 48 h at 37°C, respectively.

3.5 | Overall comparison

Three factors and their influence on the size of the inhibition zones and their stability in time were observed: treatment time, power, and initial cell concentration. These three parameters and their optimization are discussed below and will hopefully lead to further experiments with a higher number of parallel measurements, as well as in vivo tests (see Table 2). A summary of achieved results for the concentration 10^6 CFU/mL can be found below in Tables 3 and 4.

3.5.1 | Initial cell concentration

The wide range of initial yeast cell concentrations between 10^3 and 10^9 CFU/mL was examined to determine the effect of the initial concentration. At higher

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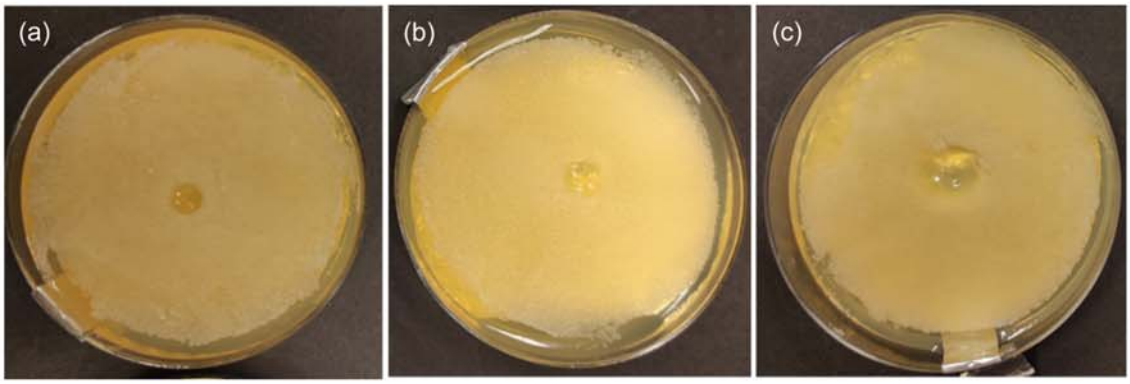

FIGURE 14 Inhibition zones created after 10 s (a), 30 s (b), and 60 s (c) exposure of the transient spark with the power of 2 W on the concentration 10^6 CFU/mL of *Candida glabrata*, after 48 h cultivation at 37°C.

TABLE 2 Summary of optimized parameters for each tested discharge.

Optimized parameters	Surface-wave microwave (MW) torch	Unipolar MW torch	Streamer corona	Transient spark
Treatment time (s)	30–60	30–60	60 or more	60 or more
Power (W)	9–12	9–12	1	2
Initial cell concentration (CFU/mL)	10^5, 10^6	10^5, 10^6	10^5, 10^6	10^4, 10^6, 10^8
Cultivation temperature (°C)	37			
Data gathering	24 h, 48 h, 7 days			

concentrations (10^7–10^9 CFU/mL), the inhibitory effects were very difficult to determine and evaluate because either the yeast culture had outgrown the inhibition zone or the number of cells was so large that the inhibition zone was not even formed.

At the low concentrations, a series of problems appeared. The inhibitory effects of some discharges (unipolar MW torch) were too high, and inhibition zones were formed over the whole Petri dish, so the zone size could not be measured. At concentrations of 10^3–10^4 CFU/mL, individual colonies are formed on the agar surface, of which there are too many cells to be counted but on the other hand, too few to accurately locate the inhibition zone. In the case of very low concentrations (below 10^3 CFU/mL) used for the plate count, the number of cells in the same volume varied, which did not allow their comparison. With the low cell concentrations, it was difficult to obtain comparable and repeatable results as the differences got much greater with a smaller number of cells.

Thus, the initial cell concentration within the range of 10^5–10^6 CFU/mL and a method of the inhibition zone sizing was selected for simple and quick testing of the inhibitory effects of CAP on the yeast *C. glabrata*, the results of which were the best evaluable and repeatable. The area of the inhibition zones created by the CAP varied slightly for the different initial cell concentrations. This was mainly because, at high concentrations, the cells were very close to each other, whereas at low concentrations, the cells were spread on a larger surface area. In some cases, there was almost no difference in the inhibition zone size but a remarkable difference in the number of colonies within the zone that survived the plasma treatment. This leads to an assumption that the number of cells successfully inhibited by the plasma treatment was very similar. These results are very beneficial because we can summarize that the CAP treatment exhibits similar efficiency for a wider range of cell concentrations and can thus be used for a variety of applications.

3.5.2 | Discharge power

As part of the optimization of the applied power, it is necessary to characterize each discharge separately, and it is not possible to generalize this criterion. Based on the characteristics of the individual discharges, it is possible

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to determine the maximum power for a given application. For medical applications, it is necessary to take into account the temperature limits of tolerance of the living organisms and to adapt the discharge parameters to the treated object, which means that the gas temperature must be within the range of its temperature tolerance. For an average human being, the temperature should not overcome 45°C if applied for a prolonged period of time. This tolerance range can vary from person to person depending on age, health, skin sensitivity, and other factors, including the environment, relative humidity, and so on.[48,49,51,52]

In the case of the MW discharges (the surface-wave and the unipolar MW torches), it is best to use the power of 9 W or 12 W, depending on the specific application. The power of 12 W was chosen as the maximum power for medical applications, the measurable temperature of which, in prolonged treatment (above 60 s), borders on the temperature tolerance of living organisms. For more sensitive patients and sensitive skin areas, it would be appropriate to choose a lower power of 9 W, with a lower heating capacity, resulting in lower inhibitory effects but avoiding overheating of the treated surface.

In the case of the streamer corona, the applied power was very small (0.5 W and 1 W), which was reflected in its low temperature and the possibility of prolonging the treatment time. For yeast inhibition, it is more appropriate to choose a higher power of 1 W ($U = 14$ kV) due to the more efficient inactivation of the more complex structure of the yeast cell compared to bacterial cells. In this case, the power is so low that there is no risk of reaching the limit of the temperature tolerance, and therefore, it is not necessary to select the lower power 0.5 W ($U = 12.8$ kV) that was sufficient for bacterial inactivation in previous studies.[55,56]

The transient spark discharge is quite specific, operating at a relatively low mean power (2 W) but with repetitive hot spark discharge filaments. As such, it is not aimed for the direct treatment of surface infections in living organisms, but rather as an effective sterilization method of inanimate objects or the treatment of liquids used for indirect application of plasma.[57,58] In all cases, for future in vivo or clinical applications, it is advisable to adhere to the recommended settings and temperature tolerance limits and consult with medical doctors.

3.5.3 | Treatment time

The treatment time and the total length of the procedure both depend on the power used. In the case of MW discharges, the treatment time between 30 and 60 s was chosen to be optimal for sufficient results. Treatment times below 30 s were excluded due to insufficient inhibitory effects on yeast (compared to bacteria in which significant inhibitory effects can be observed after very short exposure times[26,59,60]). Treatment times over 60 s were excluded mainly for temperature and comfort reasons because, as mentioned above, a prolonged application of the MW discharge with the higher power could cause local overheating of the surface.

The ability of the treated subject to stay in one position during the treatment time also plays an important role here. In applications to different parts of the human/animal body, the age and mental state of the patient needs to be taken into consideration. For example, children and animals are usually reluctant to remain in one place without movement for a longer time. For these cases, it would be appropriate to choose shorter times with short breaks (intermittent irradiation), which would sum up to achieve the overall effect. This intermittent irradiation was tested in the unipolar MW discharge only, but it can be concluded that when using this discharge, the intermittent irradiation showed effects comparable to a single long hold. In our experiments, treatment times of 3×10 s and 2×30 s were used, which corresponds to the chosen 30–60 s treatment mentioned above. This fact is very advantageous for the application to the sensitive skin, as the long hold of the plasma device in one place would not be pleasant for a patient, and a short break between treatments would allow the treated area to cool down before the subsequent plasma application. This would also allow us to use the higher power for repeated short periods of time instead of one longer hold with smaller power, which would avoid local overheating, shorten the exposure time, and achieve antimycotic effects that would not be otherwise possible with the single treatment due to the temperature limits or other complications mentioned above.

In the case of the streamer corona, it is possible to use the treatment times longer than 60 s due to the low power and, thus, low heating capacity, but this option has not been explored yet and is, therefore, a perspective for future measurements. When using the transient spark discharge, it is possible to use the time of 60 s and theoretically even higher in the sterilization of small nonliving objects, but the object should be relatively mechanically and thermally resistant due to the higher discharge temperature and pressure waves arising from the contact of the discharge with the treated surface.

3.5.4 | Post-treatment cultivation

Concerning the posttreatment cultivation, the temperature of 37°C was chosen as the temperature

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corresponding to the human body temperature. To monitor the stability of the inhibition zones, the observation at 24 h, 48 h, and 1 week after the treatment was established. The optimized parameters of the individual discharges are listed in Table 2.

Exposure times of 30 s, 60 s, and the concentration of 10^6 CFU/mL were selected for the global comparison of individual discharges (see Table 2). Overall, all discharges were effective against *C. glabrata*. If we compare the chosen CAP sources for efficiency at given treatment times, from a statistical point of view, the inhibitory effects of corona discharge and transient spark discharge are almost the same. The surface-wave MW torch also differs relatively little from these two mentioned discharges. However, a very remarkable statistical difference ($p = 0.00035$) can be observed in the case of the unipolar MW torch. When considering only the treatment time of 60 s, from a statistical point of view, there is a significant difference ($p = 0.00028$) between the unipolar MW torch and the other studied discharges. These results are quite understandable, given the power used for each discharge. The larger the observable plasma plume, the better the inhibitory effects. This could lead to a misassumption that the overall inhibitory effects strongly depend on the visible size of the discharge, but in fact, the real reason is the concentration of active particles produced by the discharge. With higher power, more active species playing a major role in microbial inhibition are produced, thus resulting in greater inhibitory effects.

TABLE 3 Summary of achieved results for the chosen concentration of *Candida glabrata* 10^6 CFU/mL.

Inhibition zone size (cm^2)							
Treatment time (s)	Surface-wave microwave (MW) torch		Unipolar MW torch		Streamer corona		Transient spark
	9 (W)	12 (W)	9 (W)	12 (W)	0.5 (W)	1 (W)	2 (W)
24 h							
10	0.16	0.25	2.33	3.61	0.07	0.12	0.21
30	0.38	0.93	2.81	3.90	0.21	0.28	0.28
60	1.03	1.27	3.70	6.20	0.47	0.36	0.50
48 h							
10	0.13	0.22	2.26	2.84	0.06	0.10	0.20
30	0.24	0.68	2.70	3.85	0.14	0.25	0.27
60	0.57	0.99	3.52	5.72	0.43	0.30	0.48

TABLE 4 Summary of achieved results for the chosen concentration of *Candida glabrata* 10^6 CFU/mL considering the energy of each cold atmospheric-pressure plasma source.

Inhibition zone size/Energy (cm^2/kJ)							
Treatment time (s)	Surface-wave microwave (MW) torch		Unipolar MW torch		Streamer corona		Transient spark
	9 (W)	12 (W)	9 (W)	12 (W)	0.5 (W)	1 (W)	2 (W)
24 h							
10	1.81	2.09	25.93	30.05	13.60	11.81	10.72
30	1.40	2.59	10.40	10.83	14.21	9.22	4.69
60	1.90	1.76	6.86	8.61	15.63	5.98	4.20
48 h							
10	1.45	1.84	25.08	23.68	11.18	10.47	10.23
30	0.88	1.90	10.00	10.70	9.47	8.36	4.58
60	1.06	1.37	6.52	7.94	14.28	5.05	4.02

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However, if we take into consideration the power of the compared discharges and calculate the deposited energy, that is, when discussing the specific efficiency normalized to the deposited energy, we observe another trend. Through this lens, the surface-wave MW torch seems to be the least efficient. The streamer corona and the transient spark show similar efficiency, and the unipolar MW torch still stays the most efficient. From the comparison of the chosen discharges in their inhibition efficiencies at chosen treatment times, as well as for their specific inhibition efficiencies, it can be stated that the unipolar MW torch with the power of 12 W exhibits the greatest inhibitory effects (see Figures 15 and 16).

The stability of the inhibition zones is also an important factor to be mentioned. After 48 h, it is possible to observe the surviving colonies and gradual overgrowth of the inhibition zones due to the proliferation of the vital cells from the edges and inside the zone. However, for all discharges, the zones were clearly visible even after a week of cultivation. When comparing the stability of the inhibition zones created by tested discharges, the most stable zones were formed by the transient spark, followed by the streamer corona and the unipolar MW torch. The lowest stability of the inhibition zones was observed in the case of the surface-wave MW torch.

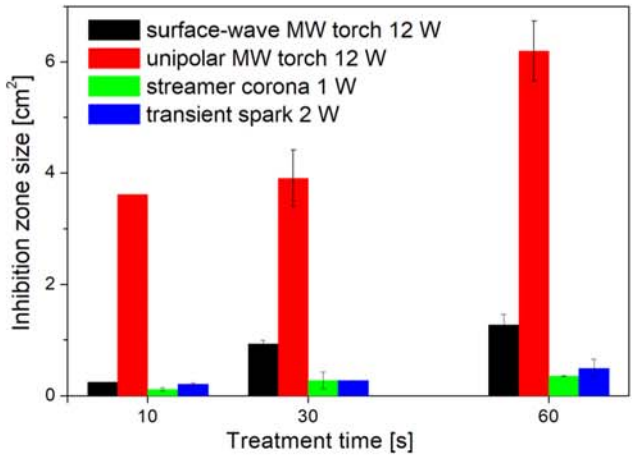

FIGURE 15 Comparison of the tested discharges in the inhibition efficiency of *Candida glabrata* concentration 10^6 CFU/mL, after 24-h cultivation.

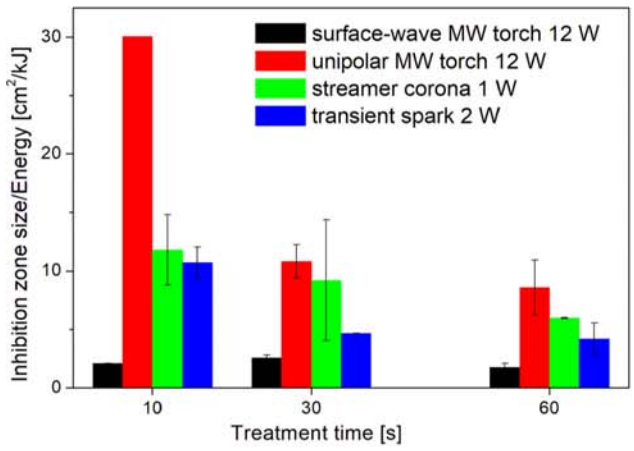

FIGURE 16 Comparison of the tested discharges in the specific inhibition efficiency of *Candida glabrata* concentration 10^6 CFU/mL, after 24-h cultivation.

4 | CONCLUSION

The aim of this work was to study the effects of various CAP sources (surface-wave MW torch, unipolar MW torch, streamer corona, and transient spark) on the yeast *C. glabrata* to examine the effects of individual discharges on the selected microorganism cultivated on agar plates, and to find the most suitable way for its inhibition, as well as to optimize the plasma setup for further measurements. The overall comparison of the investigated discharges showed that the most effective discharge for yeast inactivation is the unipolar MW torch. From the viewpoint of the inhibition zone stability over time up to 7 days, the transient spark discharge created the most stable zones.

From the previous experiments and studies, the three basic parameters were selected for different CAP sources comparison: treatment time, power, and initial concentration of the cells, which have a major impact on the CAP inhibitory effects. In all experiments, the general trend was confirmed: the directly proportional dependency applies to the treatment time and the applied power. With longer treatment times and greater power, better inhibitory effects can be achieved. Very little indirect dependency of the inhibition efficiency on the initial cell concentration was observed, and thus better inhibitory effects were achieved with a smaller number of cells. In all cases, the treatment time and the applied power had a much higher impact compared to the initial cell concentration. This is a positive fact when taking into consideration the variety of applications, as the same treatment time and power can be used for a wide range of concentrations with similar efficiency.

5 | PERSPECTIVES

The tested CAP sources proved their capability to efficiently decontaminate surfaces contaminated by the yeast cells at relatively low plasma power requirements

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and short treatment times, allowing for in vivo and even potential clinical applications in human/animal wound healing and dermatology. As mentioned in the introduction, superficial candidiasis affects mainly the oral cavity and the genitals, so the use of plasma torches or plasma jets seems very promising, as the infected area could be directly treated by the plasma. For this application, another work was published focusing on several *Candida* species, which also proves the great potential of CAP to be used in the treatment of mycosis.[61] But it is important to mention that none of these studies were conducted on biofilms, in which the *Candida* yeasts are found when the infection is developed. The formation of biofilms introduces another level of difficulty to the treatment and makes the microbes much more resistant compared to their planktonic forms. Thus, the treatment of biofilms would need to be studied first before application to real infection.

In the case of systemic candidiasis, the CAP could serve as a prevention step. As the most endangered by systemic candidiasis are hospitalized patients, one of the critical reasons for the development of invasive candidiasis is the insertion of infected venous catheters that are then left in the body for longer time periods. The use of different plasma sources, in the case of catheter sources with capillary design,[62,63] would be helpful to successfully decontaminate the catheter or other problematically sterilizable devices to prevent the development of infection in hospitalized patients. Other CAP sources like the transient spark may be beneficial for the preparation of plasma-treated water, which could also serve as a disinfectant[64] either for surfaces or other medical tools, potentially even for washing the surface infection before direct plasma treatment by another CAP source.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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