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Synergistic Action of Reactive Plasma Particles and UV Radiation to Inactivate Staphylococcus Aureus

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Abstract: The direct application of low-pressure plasma for the decontamination of microorganisms was examined herein. The inactivation efficiency was studied on a Gram-positive bacterium (*Staphylococcus aureus*) using a plasma process by means of synergistic action of reactive plasma particles and UV radiation. N_2 was added to an argon/oxygen plasma mixture in order to improve the effectiveness of *S. aureus* inactivation. It was found that the decontamination mechanism is based on both the chemical sputtering effect due to the plasma particles and the UV emission originating from the NO_γ system from NO radicals in the wavelength range 200–300 nm. The best plasma bactericidal activity was found for an N_2 percentage of roughly 10–12%. A count reduction of more than 5 log cycles in a few minutes of *S. aureus* proves the potentiality of an industrial-grade plasma reactor as a decontamination agent.

Keywords: *Staphylococcus aureus*; low-pressure plasma; bacterial inactivation

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1. Introduction

Decontamination of medical equipment is a fundamental step in health care facilities in order to assure the safety of patients. Most medical devices are made of materials that may be damaged by heat; therefore, the use of conventional sterilization methods (i.e., autoclaves) can be problematic. Ethylene oxide (EO) gas sterilization is used as an alternative method. However, institutions presently tend to avoid the use of EO because it leaves a very hazardous residue. As a consequence, many studies have been conducted in order to develop non-toxic, low-temperature sterilization methods. Low-temperature plasma technology can be regarded as a viable alternative source for microbial decontamination processes, as reported, for example, for the decontamination of surgical instruments [1]. Many plasma studies have shown that various gases such as Ar, O_2 or N_2 can have microbicidal activity against bacteria, including bacterial spores [2–12] and fungi [13]. Plasma discharges operate close to room temperature (those used for biological applications) and are generated by applying electrical energy to a gas. Energetic electrons of plasma collide with the gas molecules, producing excited species (ions and radicals) that may quickly react with microorganisms. Plasmas can be generated at both low pressure and atmospheric pressure, in direct current or alternating current [14]. Due to the different resistance levels of microorganisms against the decontamination processes, any treatment process by plasma needs a dedicated set-up, and this has certainly limited industrial development.

In the 1990s, a decontamination system using plasma entered commercial development [15]; it used hydrogen peroxide gas plasma to sanitize a wide range of instruments. However, this system has not been developed in many countries, probably due to the high process times (about an hour), comparable to that of the conventional autoclave. However, the mechanism of plasma inactivation of microorganisms is complex and not yet fully

understood. Surely the process time factor is and has been the main variable limiting industrial development, because prolonged treatments also lead to degradation (due to heating) of the treated components. Therefore, the experimental work we undertook had low process time as a constraint.

In our previous study [16], we used a low-pressure capacitively coupled plasma discharge for the inactivation of *Staphylococcus aureus*, which is one of the major pathogens responsible for community- and hospital-acquired infections [17] globally and is one of the biological indicators for the validation of a sterilization process for health care devices (UNI EN ISO 14937, 2009 [18]).

Decontamination treatments were based on the inactivation of the bacterium by means of the process of chemical sputtering involving both charged particles and plasma radicals [19]. We optimized the decontamination process by exposure of the *Staphylococcus aureus* to Ar plasma as a function of oxygen concentration. We identified an experimental condition in which the synergistic effect of plasma particles (charged particles and reactive oxygen species) which interacted (bombardment of incident ions and chemical etching of radicals) with the microorganism was maximized, and a bacterium reduction greater than 4 orders of magnitude was obtained within a few minutes. Under our experimental conditions, it was also demonstrated that the Ultraviolet (UV) effect contributed to antimicrobial activity as a minor factor for the type of gas mixture used, but we know well that photons generated in the plasma, thanks to their interaction with the microbial cells (at the level of DNA), can strongly contribute to the bactericidal effect [20]. Therefore, in the present study, in order to increase the effectiveness of the process, nitrogen was added to the plasma mixture. Although N_2 alone does not have significant effects on microorganisms [21], the major contribution of N_2 in the gas plasma comes from production of metastables of N_2 and O_2, with enhancement of UV radiation. In our study, the main source of photons in the desired spectral range was the excited NO molecules. The use of plasma processes driven in $Ar/O_2/N_2$ mixture for possible decontamination applications was previously reported by some authors [22–24]; in particular, they used low-pressure plasma discharges in an inductively coupled configuration. Even though they demonstrated significant efficiency of this process, we have not seen a real development in industrial application (or on a large scale). In light of this, we think that further experiments are worthwhile in order to obtain new evidence in this regard, providing further understanding of the decontamination mechanism in this type of plasma mixture. Therefore, the main purpose of this paper is to demonstrate that the application of an appropriate $Ar/O_2/N_2$ ternary mixture in a plasma discharge in a capacitively coupled configuration, which is the configuration most used in industrial applications, allows for a bacterium reduction of at least 5 orders of magnitude, validating this process as a possible decontamination procedure for medical devices.

2. Materials and Methods

2.1. Chemicals

Oxygen (99.998% purity), argon (99.999% purity) and nitrogen (99.995% purity) gases used in the plasma processes were obtained from Rivoira Gas Srl, Milan, Italy.

2.2. Microorganism Decontamination System

The *Staphylococcus aureus* inactivation process was carried out in a radio-frequency (RF) capacitively coupled plasma reactor [25], as shown in Figure 1. The electrodes were 20 cm in diameter and at a distance of 6.5 cm. The containers of bacteria were arranged on the lower electrode, which was grounded. Prior to each process, the plasma chamber was evacuated to a base pressure of 1×10^{-4} Pa. The neutral atom density, n_n, was estimated using the ideal gas law ($p_n = n_n k T_g$). The temperature T_g was measured using a thermal couple (chromel–alumel, type K), and it was around 300 K for the entire process. The plasma parameters, i.e., electron density, electron temperature, electron distribution function, floating and plasma potential, were investigated using an RF-compensated cylindrical single Langmuir probe with a Tungsten tip (diameter = 0.1 mm and length = 10 mm)

positioned at 2.5 cm above the ground electrode. The density of O atoms in the chamber was estimated using a common thermocouple catalytic probe [26–29]. A high-purity nickel disc (99.8%) with a diameter of 4 mm and thickness of 0.05 mm was used. Chromel–alumel thermocouple wires with a diameter of 0.15 mm were spot welded onto the disc. The probe enabled real-time measurements with a temporal resolution of about 0.5 s. The probe was positioned at the same distances from the powered electrode where the ion concentrations were measured. The thermal load due to plasma particles was monitored by a thermocouple joined on the substrate containing the bacteria. Optical emission spectroscopy (OES) measurements were performed in order to detect the emitting species in the discharge. Measurements were carried out using a high-resolution (0.06 nm) iHR550 spectrometer coupled to a Synapse CCD camera cooled to −70 °C (HORIBA Jobin Yvon S.A.S., 16-18 rue du Canal, 91165 Longjumeau cedex, France). The emission spectra were acquired using a 1200 grooves/mm grating and an acquisition time of 0.2 s.

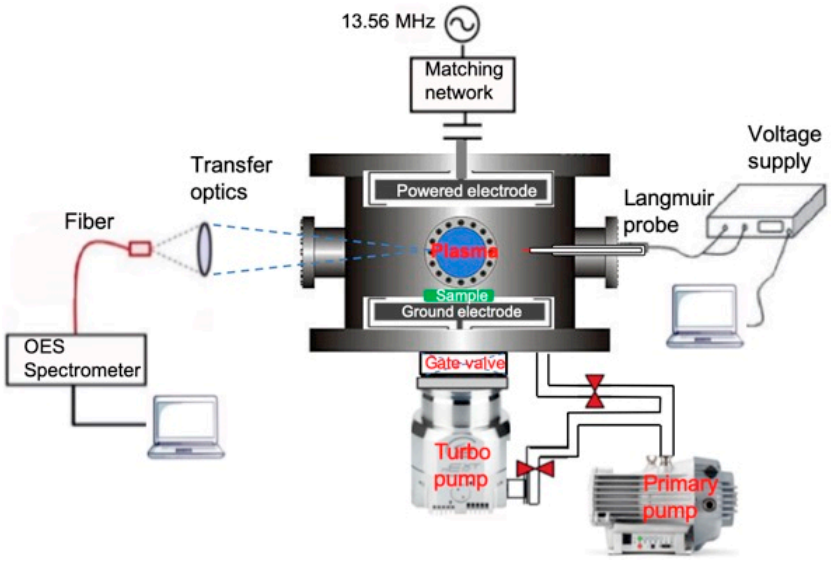

Figure 1. Schematic representation of the apparatus for plasma treatments.

2.3. Preparation of Bacterial Inoculum

Staphylococcus aureus GP32 (belonging to the CNR-ISPA collection) and the procedure used in our previous work [16,30] were adopted in this study. The bacterial strain was taken from frozen stocks and subcultured twice in Brain Heart Infusion (BHI) broth (Scharlau Microbiology, Barcelona, Spain) with aerobic incubation at 37 °C for 16–18 h before each experiment. The overnight bacterial culture was diluted 10-fold with sterile BHI and centrifuged at 14.000 rpm for 5 min to collect cell pellets with a concentration of about 7.00 $\log_{10}$ CFU. Two aliquots (5 µL each) of the cell pellet were used to inoculate each of two 12.25 cm^2 squares, previously drawn on the base of a sterile glass Petri plate. The plasma treatments were applied to different Petri plates containing the two inocula, so that they were simultaneously exposed to the same conditions and then analyzed separately. Controls consisted of inoculated and not-treated plates.

2.4. Bacterial Enumeration

After plasma treatment, a sterile cotton swab was used to recover the microbial pellet from each square. The swab was added to a tube containing quarter-strength Ringer's solution (Scharlau, Barcelona, Spain) and vortexed for 30 s. Serial decimal dilutions were prepared in Ringer's solution, and the appropriate dilutions were cultured into BHI agar (Biolife Italiana, Milano, Italy) and incubated aerobically at 37 °C for 24 h.

2.5. Statistical Analysis

All bacterium counts were repeated four times in triplicate for each treatment. The results are presented as the average number of $\log_{10}$ CFU ± standard deviation (SD).

3. Results

3.1. Analysis of Plasma Parameters

Based on our previous study [16], in which optimal plasma conditions were determined for the inactivation of *Staphylococcus aureus* with an Ar/O_2 plasma mixture (Table 1), in this experimental section, the investigations were performed in an $Ar/O_2/N_2$ ternary mixture with a fixed Ar/O_2 ratio, varying the N_2 concentration and power of the discharge, while keeping the total gas flow constant at 30 sccm. The process parameters used are summarized in Table 1.

Table 1. Plasma process parameters.

-	**Ar/O_2 Binary Mixture [13]**	**$Ar/O_2/N_2$ Ternary Mixture**
RF power (W)	100–250	100–250
Power density (W/cm^2)	0.3–0.8	0.3–0.8
Process pressure (Pa)	20	20
Total gas flow (sccm)	30	30
Ar/O_2 ratio	10	8
Process time (min)	4	4
Nitrogen (%)	0	0–30

The plasma characterization by OES showed that the addition of nitrogen led to an increase in UV radiation with the appearance of NO* emission bands corresponding to the γ system. An example of such a spectrum obtained in a gas mixture of 80% Ar, 10% O_2 and 10% N_2 at 200 W, 20 Pa and $V_{bias} \approx 400$ V is depicted in Figure 2b. As shown in Figure 2, the UV region is larger in $Ar/N_2/O_2$ mixture plasmas compared with the Ar/O_2 gas mixture (with 12% O_2) studied in our previous work [16]. Overall, the addition of N_2 produced several emissions, in particular, two emission peaks at 247.9 and 258.8 nm assigned to NO_γ ($A^2\sum^+ \rightarrow X^2\Pi$) electron transition in the range 220–260 nm (marked in Figure 2b), arising from the de-excitation process of NO* bands [31] and others at 282.0, 297.7, 313.6, 315.9, 337.1 and 357.7 nm (unmarked) from the second positive systems ($C^3\Pi_u \rightarrow B^3\Pi_g$) of N_2 molecules [32–34]. Moreover, by analyzing the UV radiation in the spectral range 220–260 nm as a function of the N_2 fraction in the discharge mixture, we obtained a maximum value of UV intensity around 10–12% (Figure 3).

Since experimental studies [35] have comprehensively shown that the UV range of the NO_γ system is particularly effective towards microbes due to the fact that the radiation penetrates the outer walls of microorganisms, damaging their DNA [36], in order to achieve optimal effectiveness in decontamination, a 10% N_2 concentration in the discharge mixture was used to study the antimicrobial activity.

Regarding the plasma parameters, given that the intensity of OES spectra lines is very sensitive to change in T_e, the intensity ratio of the lines may be used as a qualitative indicator of the trend of T_e. For this purpose, taking into consideration the intensity ratio 434.8/750.4 nm, related to ionic Ar and the neutral excited species, respectively, it is evident that it remains *rather* constant against nitrogen concentration (Figure 4), and the only evidence of variation in the intensity ratio can be observed between the cases with and without N2 in the mixture, so it slightly decreases for N_2 percentage greater than 3%. Thus, it can be asserted that there was no substantial change in T_e with nitrogen dilution. This result is in accordance with the T_e measurements (Figure 5) by the Langmuir probe.

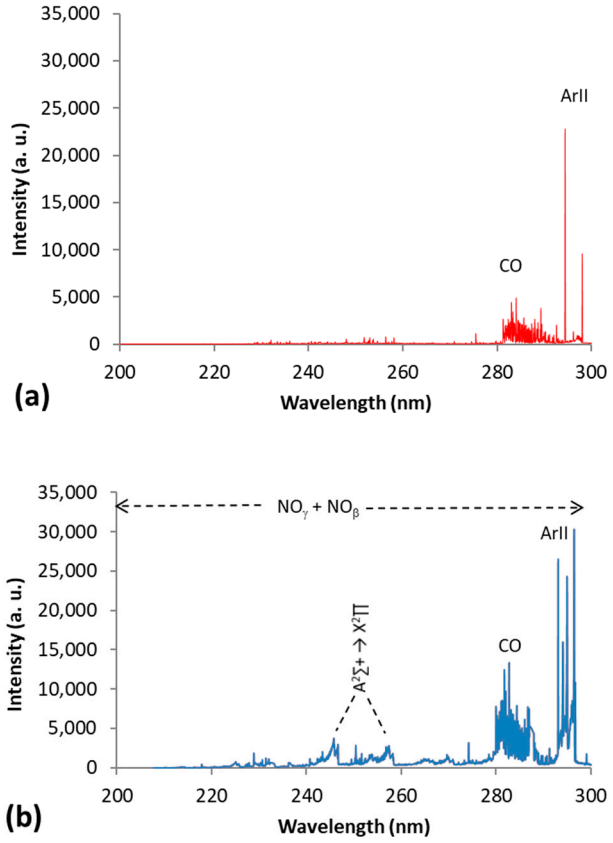

Figure 2. Optical emission spectra in the range of 200–300 nm in the case of (**a**) Ar/O_2 and (**b**) Ar/O2/N2 mixture plasmas with 10% nitrogen.

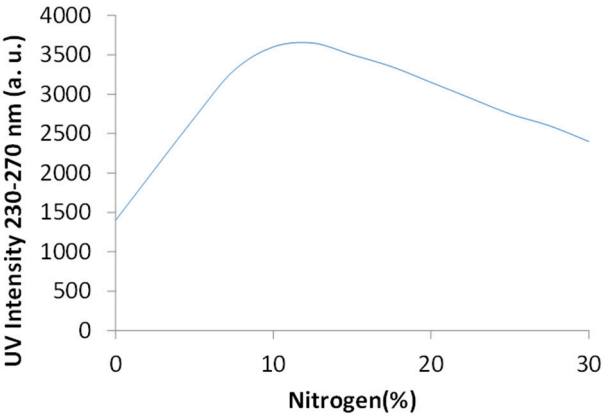

Figure 3. UV radiation intensity integrated in the spectral range 230–260 nm as a function of N_2 concentration (10 Pa, 200 W, total gas flow 30 sccm).

Concerning the Langmuir probe measurements (Figure 5), the analysis showed that the T_e tended, in general, to decrease with N_2 addition. This trend can be attributed to a higher electron collision cross section of nitrogen compared to argon and, consequently, to the decrease in the mean energy of the electrons, due to the decrease in the acceleration time of the electrons by the electric field, with a consequent reduction in T_e. As already known from other plasma studies, a significant result of the decontamination process is most likely connected not only with the behavior of the density of radical species produced in the plasma but also with the variation in the plasma density. As shown in Figure 4, N_2 addition into the Ar/O_2 discharge mixture leads to a plasma density decrease. This

is caused by the electron's energy dissipation via various channels such as ionization, excitation and recombination [37]. The variation in the mean electron energy can also be seen from the development of the discharge voltage; in fact, the effect of adding N_2 leads to an increase in the voltage required to sustain the discharge (Figure 6). This trend is mainly attributable to electronic energy losses due to the excitation of vibrational and electronic levels of molecules.

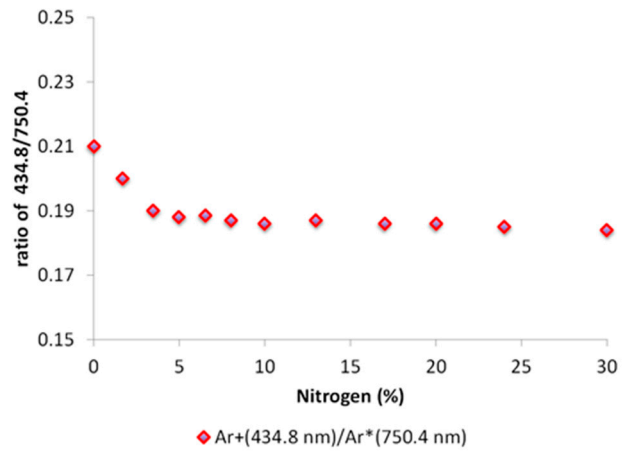

Figure 4. Influence of the N_2 concentration upon the intensity ratio of the 750.4 nm atomic Ar to the 434.8 nm ionic Ar.

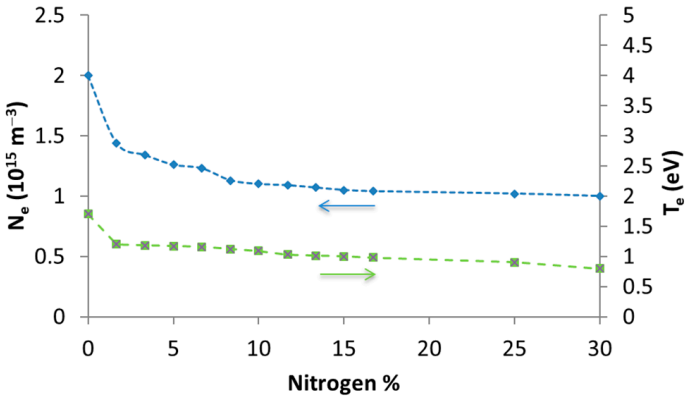

Figure 5. Electron temperature and electron density measurements as a function of N_2 concentration (20 Pa, 200 W, total gas flow 30 sccm).

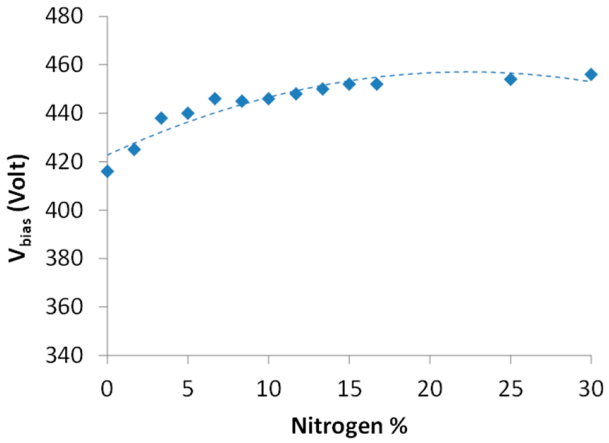

Figure 6. Discharge voltage as a function of N_2 concentration (20 Pa, 200 W, total gas flow 30 sccm).

3.2. Antimicrobial Activity

In order to exclude a possible contribution to the inactivation of microorganisms due to thermal effects produced by plasma particle interaction with the substrate [38], with the help of a thermocouple on the sample, the temperature was evaluated as a function of time (Figure 7). The results show that the temperature of the sample always remained below 60 °C for treatment time up to four minutes and power up to 200 W, and at these temperatures, the thermal effects on microorganisms may be considered negligible [39,40].

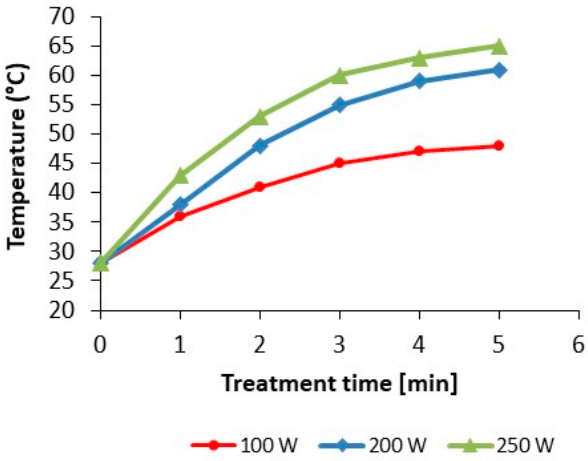

Figure 7. Temperature of the sample as a function of the treatment time for $Ar/O_2/N_2$ (10% N_2) plasmas at a pressure of 20 Pa and power in the range 100–250 W.

Based on the results obtained from previous characterizations, in order to have a strong UV radiation intensity, we used 10% nitrogen in the plasma mixture (Figure 3), at a fixed pressure of 20 Pa. The plasma antibacterial activity towards *S. aureus* (Figure 8) was tested as a function of power up to 200 W (0.6 W/cm^2) for a process time of 4 min. The control concentration was in the range of 7.1–7.5 $\log_{10}$ CFU throughout the experiment. The bacteria were also exposed to vacuum only under the same experimental conditions in order to exclude other possible inactivation contributions in addition to the plasma. An irrelevant count reduction (in the range of 0.1–0.2 $\log_{10}$ CFU $\pm$ 0.5) compared to the control (7.2 $\log_{10}$ CFU) was found. The comparison of Ar/O_2 [15] and $Ar/O_2/N_2$ discharges revealed that the N_2 addition led to an increase in antimicrobial activity of about 0.5 $\log_{10}$ CFU. Considering that above 200 W (Figure 7) there is a thermal effect (which may also lead to a microbial reduction of up to two orders), the best result of bacterial inactivation that we obtained in this experimental session was at 200 W (0.6 W/cm^2). In this condition, the plasma density (Figure 5) remained quite high, i.e., an Ar^+ ion flux density of $j_{Ar+} = 1.2 \times 10^{18}$ m^{-2} s^{-1} (calculated from the Bohm criterion [41]), while the neutral atom density was 10^{21} m^{-3}. The O density, evaluated by catalytic probe, was found to be of the order of 2×10^{19} m^{-3}. Regarding the latter plasma process, in which a 5.5 log average bacterial reduction was obtained, we would make the point that in a couple of trials, no visible growth of the microbial pellet collected from glass Petri plates after plasma treatment was observed. This result suggests that those operating conditions allowed for achieving the minimum bactericidal concentration, although the same result was not confirmed by the corresponding triplicate sample. In the same trials, a 6 log reduction was obtained, meeting the requirements for sterilization validations. It is therefore necessary to optimize procedures to ensure reproducibility and repeatability over time.

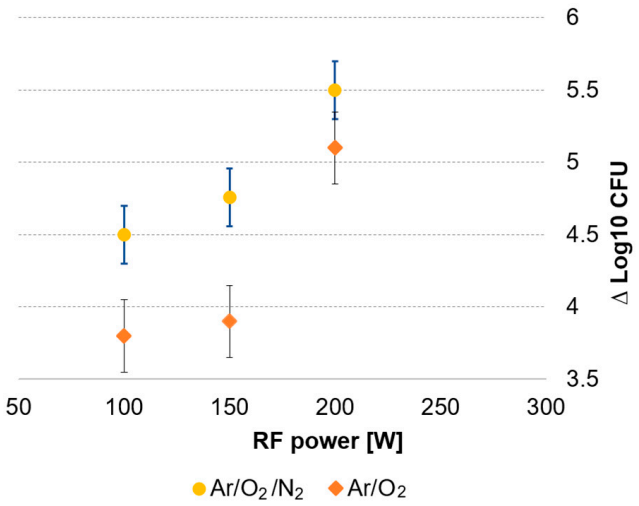

Figure 8. Reduction in *S. aureus* versus the RF power. Error bars are related to the standard deviation of a set of four measurements.

4. Discussion

This experimental study enabled us to develop a bacterium decontamination process by means of a low-pressure capacitively coupled plasma device. Petri plates containing the bacteria positioned on the ground electrode (Figure 1) were exposed to plasma discharge for a few minutes. In this case study, the main focus was on the inactivation of Gram-positive bacteria. This is because in the scientific literature, it is demonstrated that Gram-positive bacteria show higher resistance to plasma treatment than Gram-negative bacteria [42,43]. This effect can be correlated with the cell envelope: the Gram-positive bacteria possess a thicker (20–80 nm) cell wall than Gram-negative bacteria, which have a relatively thin (<10 nm) layer of cell wall. These differences confer different properties to the cell, particularly in terms of responses to external stresses. *Staphylococcus aureus* was chosen as a representative of non-sporulating Gram-positive bacteria and is included in the list of biological indicators suggested by the UNI EN ISO 14,937 (2009) directive to be used for assessing the microbicidal effectiveness of a sterilizing agent [18]. As already demonstrated [44,45], bacteria can be inactivated using a wide range of gases, in particular, plasma discharges containing oxygen in the mixture, exploiting the etching/sputtering effects. In addition to these effects, in the experiments performed in this study, inhibition of biological activity was realized through an increase in UV radiation produced by the addition of N_2 to the Ar/O_2 plasma. The results confirmed that the synergistic effect of the ions/radicals and UV radiation lead to an increase in decontamination action.

The resulting microbial inactivation effects for S. aureus decreased in the order of 5.5 log reduction (Figure 8).

Evidence of synergistic effects (ions, radicals, and UV radiation) is demonstrated by the fact that, despite a decrease in density and temperature observed with respect to the Ar/O_2 mixture, a greater bacterial reduction (at least 0.5 log) was obtained with N_2 addition. This result can be explained by considering the greater amount of UV radiation in the case of the $Ar/O_2/N_2$ mixture (Figure 2).

The findings reported here highlight the importance of the gas mixture used in order to obtain a significant bactericidal effect, which could be of interest for real applications. In particular, under our experimental conditions, in order to have high bacterial inactivation, an optimal N_2 concentration in the range of 10–12% was used, at a pressure of 20 Pa, treatment time of 4 min, RF power of 200 W (0.6 W/cm^2), Ar^+ ion flux density of approximately 1.2×10^{18} m^{-2} s^{-1} and O_2 concentration of 8–10%, with O density in the range of 2×10^{19} m^{-3}. Regarding these process parameters that we have indicated, we believe it is important to mention that the obtained results were optimized for S. aureus;

therefore, the treatment of other microorganisms requires a dedicated study to optimize the process parameters [46]. Although this last statement may seem like a downside, on the contrary, we think that the tuneability of plasma processes provides the most added value; indeed, the plasma can be tuned ad hoc for any kind of microorganism.

5. Conclusions

Although the effects of plasma processes on microorganisms have been reported, the mechanism of action of plasmas still requires further study. This experimental work confirmed the ability of plasma components against S. aureus, and a better understanding of the role of gas species in decontamination was achieved. In particular, the role of N_2 addition in Ar/O_2 plasma in order to obtain an efficient bacterium decontamination process was investigated. N_2 injection improved the effectiveness of S. aureus inactivation due to the UV radiation component. In general, it can be concluded that the synergistic effects of plasma components could play important roles in increasing the effectiveness of the inactivation of microorganisms. The experimental data obtained and, principally, the low process time (a few minutes) would potentially contribute to expanding the applicability of this technology, in particular, for scaling up in real applications. As regards sterilization systems for medical devices, where sterilization is the term for a > 6 log reduction in the viable cells of a microorganism, the reduction obtained in this work (5.5 log) cannot be classified as complete sterilization. In any case, the outcomes will be useful for the consequent development of a validated low-pressure plasma sterilization procedure for medical devices according to UNI EN ISO 14,937 (2009). Further, tests will be also necessary to assess the antibacterial efficiency of the developed plasma process against other microorganisms (e.g., other Gram-positive bacteria, Gram negative bacteria, sporulating bacteria and yeasts) associated with nosocomial infections.

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References

1. Niemira, B.A. Cold Plasma Decontamination of Foods. *Annu. Rev. Food Sci. Technol.* **2012**, *3*, 125–142. [CrossRef]
2. *Gas Plasma Sterilization in Microbiology: Theory, Applications, Pitfalls and New Perspectives*; Caister Academic Press: Norfolk, UK, 2016; ISBN 978-1-910190-25-8.
3. Dobrynin, D.; Friedman, G.; Fridman, A.; Starikovskiy, A. Inactivation of Bacteria Using Dc Corona Discharge: Role of Ions and Humidity. *New J. Phys.* **2011**, *13*, 103033. [CrossRef]
4. Eto, H.; Ono, Y.; Ogino, A.; Nagatsu, M. Low-Temperature Sterilization of Wrapped Materials Using Flexible Sheet-Type Dielectric Barrier Discharge. *Appl. Phys. Lett.* **2008**, *93*, 221502. [CrossRef]
5. Rossi, F.; Kylián, O.; Rauscher, H.; Gilliland, D.; Sirghi, L. Use of a Low-Pressure Plasma Discharge for the Decontamination and Sterilization of Medical Devices. *Pure Appl. Chem.* **2008**, *80*, 1939–1951. [CrossRef]
6. Stapelmann, K.; Fiebrandt, M.; Raguse, M.; Awakowicz, P.; Reitz, G.; Moeller, R. Utilization of Low-Pressure Plasma to Inactivate Bacterial Spores on Stainless Steel Screws. *Astrobiology* **2013**, *13*, 597–606. [CrossRef] [PubMed]
7. Shintani, H.; Sakudo, A.; Burke, P.; McDONNELL, G. Gas Plasma Sterilization of Microorganisms and Mechanisms of Action. *Exp. Ther. Med.* **2010**, *1*, 731–738. [CrossRef]
8. Park, J.Y.; Park, S.; Choe, W.; Yong, H.I.; Jo, C.; Kim, K. Plasma-Functionalized Solution: A Potent Antimicrobial Agent for Biomedical Applications from Antibacterial Therapeutics to Biomaterial Surface Engineering. *ACS Appl. Mater. Interfaces* **2017**, *9*, 43470–43477. [CrossRef] [PubMed]

9. Ikawa, S.; Tani, A.; Nakashima, Y.; Kitano, K. Physicochemical Properties of Bactericidal Plasma-Treated Water. *J. Phys. D Appl. Phys.* **2016**, *49*, 425401. [CrossRef]
10. Vandervoort, K.G.; Brelles-Mariño, G. Plasma-Mediated Inactivation of Pseudomonas Aeruginosa Biofilms Grown on Borosilicate Surfaces under Continuous Culture System. *PLoS ONE* **2014**, *9*, e108512. [CrossRef]
11. Sakudo, A.; Yagyu, Y.; Onodera, T. Disinfection and Sterilization Using Plasma Technology: Fundamentals and Future Perspectives for Biological Applications. *Int. J. Mol. Sci.* **2019**, *20*, 5216. [CrossRef]
12. Neogi, S. Inactivation Characteristics of Bacteria in Capacitively Coupled Argon Plasma. *IEEE Trans. Plasma Sci.* **2009**, *37*, 2347–2352. [CrossRef]
13. Soušková, H.; Scholtz, V.; Julák, J.; Savická, D. The fungal spores survival under the low-temperature plasma. In *Plasma for Bio-Decontamination, Medicine and Food Security (NATO Science for Peace and Security Series A: Chemistry and Biology)*; Machala, Z., Hensel, K., Akishev, Y., Eds.; Springer: New York, NY, USA, 2012; pp. 57–66.
14. Fridman, A. *Plasma Chemistry*; Cambridge University Press: Cambridge, UK, 2012.
15. Jacobs, P.; Kowatsch, R. Sterrad Sterilization System: A new technology for instrument sterilization. *Endosc. Surg. Allied Technol.* **1993**, *1*, 57–58.
16. Vassallo, E.; Pedroni, M.; Silvetti, T.; Morandi, S.; Brasca, M. Inactivation of Staphylococcus Aureus by the Synergistic Action of Charged and Reactive Plasma Particles. *Plasma Sci. Technol.* **2020**, *22*, 085504. [CrossRef]
17. Klein, S.; Hannesen, J.; Zanger, P.; Heeg, K.; Boutin, S.; Nurjadi, D. Entry of Panton–Valentine Leukocidin-Positive Methicillin-Resistant Staphylococcus Aureus into the Hospital: Prevalence and Population Structure in Heidelberg, Germany 2015–2018. *Sci. Rep.* **2020**, *10*, 13243. [CrossRef]
18. UNI EN ISO ISO14937:2009; Sterilization of Health Care Products—General Requirements for Characterization of Sterilizing Agent and the Development, Validation and Routine Control of a Sterilization Process for Medical Devices. International Organization for Standardization: Geneva, Switzerland, 2009.
19. Raballand, V.; Benedikt, J.; Wunderlich, J.; von Keudell, A. Inactivation of *Bacillus Atrophaeus* and of *Aspergillus Niger* Using Beams of Argon Ions, of Oxygen Molecules and of Oxygen Atoms. *J. Phys. D Appl. Phys.* **2008**, *41*, 115207. [CrossRef]
20. Privat-Maldonado, A.; O'Connell, D.; Welch, E.; Vann, R.; van der Woude, M.W. Spatial Dependence of DNA Damage in Bacteria Due to Low-Temperature Plasma Application as Assessed at the Single Cell Level. *Sci. Rep.* **2016**, *6*, 35646. [CrossRef]
21. Rossi, F.; Kylián, O.; Hasiwa, M. Decontamination of Surfaces by Low Pressure Plasma Discharges. *Plasma Process. Polym.* **2006**, *3*, 431–442. [CrossRef]
22. Stapelmann, K.; Kylián, O.; Denis, B.; Rossi, F. On the Application of Inductively Coupled Plasma Discharges Sustained in Ar/O_2/N_2 Ternary Mixture for Sterilization and Decontamination of Medical Instruments. *J. Phys. D Appl. Phys.* **2008**, *41*, 192005. [CrossRef]
23. Kylián, O.; Rossi, F. Sterilization and Decontamination of Medical Instruments by Low-Pressure Plasma Discharges: Application of Ar/O_2/N_2 Ternary Mixture. *J. Phys. D Appl. Phys.* **2009**, *42*, 085207. [CrossRef]
24. Kylián, O.; Denis, B.; Stapelmann, K.; Ruiz, A.; Rauscher, H.; Rossi, F. Characterization of a Low-Pressure Inductively Coupled Plasma Discharge Sustained in Ar/O2/N2 Ternary Mixtures and Evaluation of Its Effect on Erosion of Biological Samples. *Plasma Processes Polym.* **2011**, *8*, 1137–1145. [CrossRef]
25. Koenig, H.R.; Maissel, L.I. Application of RF Discharges to Sputtering. *IBM J. Res. Dev.* **1970**, *14*, 168–171. [CrossRef]
26. Zaplotnik, R.; Vesel, A.; Mozetic, M. A Fiber Optic Catalytic Sensor for Neutral Atom Measurements in Oxygen Plasma. *Sensors* **2012**, *12*, 3857–3867. [CrossRef]
27. Lazović, S.; Puač, N.; Spasić, K.; Malović, G.; Cvelbar, U.; Mozetič, M.; Radetić, M.; Petrović, Z.L. Plasma Properties in a Large-Volume, Cylindrical and Asymmetric Radio-Frequency Capacitively Coupled Industrial-Prototype Reactor. *J. Phys. D Appl. Phys.* **2013**, *46*, 075201. [CrossRef]
28. Primc, G.; Mozetič, M. Neutral Reactive Gaseous Species in Reactors Suitable for Plasma Surface Engineering. *Surf. Coat. Technol.* **2019**, *376*, 15–20. [CrossRef]
29. Kamenetskikh, A.; Gavrilov, N.; Krivoshapko, S.; Tretnikov, P. Application of the Catalytic Probe Method for Measuring the Concentration of Oxygen Atoms in Ar/O_2 Plasma of a Low-Pressure Arc. *Plasma Sources Sci. Technol.* **2021**, *30*, 015004. [CrossRef]
30. Morandi, S.; Brasca, M.; Andrighetto, C.; Lombardi, A.; Lodi, R. Phenotypic and genotypic characterization of *Staphylococcus aureus* strains from Italian dairy products. *Int. J. Microbiol.* **2009**, *2009*, 501362. [CrossRef]
31. Sharma, M.; Austin, J.M.; Glumac, N.G.; Massa, L. NO and OH Spectroscopic Vibrational Temperature Measurements in a Post-Shock Relaxation Region. *AIAA J.* **2010**, *48*, 1434–1443. [CrossRef]
32. Khan, F.U.; Rehman, N.U.; Naseer, S.; Naveed, M.A.; Qayyum, A.; Khattak, N.A.D.; Zakaullah, M. Diagnostic of 13.56 MHz RF Sustained Ar–N_2 Plasma by Optical Emission Spectroscopy. *Eur. Phys. J. Appl. Phys.* **2009**, *45*, 11002. [CrossRef]
33. Yuan, Q.; Ren, P.; Liu, S.; Wang, J.; Yin, G. The Optical Emission Spectroscopy of Nitrogen Plasma Driven by the 94.92 MHz/13.56 MHz Dual-Frequency. *Phys. Lett. A* **2020**, *384*, 126367. [CrossRef]
34. Pearse, R.W.B.; Gaydon, A.G. *Identification of Molecular Spectra*, 4th ed.; Chapman and Hall: London, UK; Wiley: New York, NY, USA, 1976.
35. Pintassilgo, C.D.; Kutasi, K.; Loureiro, J. Modelling of a Low-Pressure N_2 –O_2 Discharge and Post-Discharge Reactor for Plasma Sterilization. *Plasma Sources Sci. Technol.* **2007**, *16*, S115–S122. [CrossRef]

36. Bjelland, S. Mutagenicity, Toxicity and Repair of DNA Base Damage Induced by Oxidation. *Mutat. Res./Fundam. Mol. Mech. Mutagenesis* **2003**, *531*, 37–80. [CrossRef] [PubMed]
37. Rhee, J.K.; Kim, D.B.; Moon, S.Y.; Choe, W. Change of the Argon-Based Atmospheric Pressure Large Area Plasma Characteristics by the Helium and Oxygen Gas Mixing. *Thin Solid Film.* **2007**, *515*, 4909–4912. [CrossRef]
38. Cvelbar, U.; Vujoševič, D.; Vratnica, Z.; Mozetič, M. The Influence of Substrate Material on Bacteria Sterilization in an Oxygen Plasma Glow Discharge. *J. Phys. D Appl. Phys.* **2006**, *39*, 3487–3493. [CrossRef]
39. Pflug, I.J.; Holkomb, G.R.; Gómez, M.M. Principles of the Thermal Destruction of Microorganisms. In *Disinfection, Sterilization, and Preservation*; Block, S.S., Ed.; Lippincott Williams Wilkins: Philadelphia, PA, USA, 2001.
40. Pijls, B.G.; Sanders, I.M.J.G.; Kuijper, E.J.; Nelissen, R.G.H.H. Synergy between Induction Heating, Antibiotics, and *N* -Acetylcysteine Eradicates *Staphylococcus Aureus* from Biofilm. *Int. J. Hyperth.* **2020**, *37*, 130–136. [CrossRef] [PubMed]
41. Bohm, D.; Burhop, E.H.S.; Massey, H.S.W. The Use of Probes for Plasma Exploration in Strong Magnetic Fields. In *Guthrie A and Waker1ing R K The Characteristics of Electrical Discharges in Magnetic Fields*; McGraw-Hill: New York, NY, USA, 1949.
42. Weltmann, K.D.; Fricke, K.; Stieber, M.; Brandenburg, R.; von Woedtke, T.; Schnabel, U. New nonthermal atmospheric pressure plasma sources for decontamination of human extremities. *IEEE Trans. Plasma Sci.* **2012**, *40*, 2963–2969. [CrossRef]
43. Mai-Prochnow, A.; Clauson, M.; Hong, J.; Murphy, A.B. Gram positive and Gram-negative bacteria differ in their sensitivity to cold plasma. *Sci. Rep.* **2016**, *6*, 38610. [CrossRef]
44. Bourke, P.; Ziuzina, D.; Han, L.; Cullen, P.J.; Gilmore, B.F. Microbiological interactions with cold plasma. *J. Appl. Microbiol.* **2017**, *123*, 308–324. [CrossRef]
45. Yang, X.; Chang, X.; Tei, R.; Nagatsu, M. Effect of excited nitrogen atoms on inactivation of spore-forming microorganisms in low pressure N_2/O_2 surface-wave plasma. *J. Phys. D Appl. Phys.* **2016**, *49*, 235205. [CrossRef]
46. Lerouge, S.; Wertheimer, M.R.; Yahia, L. Plasma Sterilization: A Review of Parameters, Mechanisms, and Limitations. *Plasmas Polym.* **2001**, *6*, 175–188. [CrossRef]