Full Length Research Paper

Bacteria response to non-thermal physical factors: A study on *Staphylococcus aureus*

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The response of *Staphylococcus aureus* germ to the action of ionizing radiation and cold plasma jet was studied in the frame of a comparative experimental research. The inactivation effect of electromagnetic ionizing radiations provided by medical X-ray device has resulted in up to 75% diminution of bacterial cell density, while for the same radiation doses provided by 6 MeV electron beam delivered by hospital radiotherapy accelerator, three times slighter effect has resulted. It was further shown that bacteria inactivation could be obtained by applying cold plasma treatment for shortened irradiation times; 100 s instead of 50 min having similar effect of about 73% efficiency on *S. aureus*, as resulted from counting the survival colony forming units. The *S. aureus* antimicrobial susceptibility was found slightly changed for X-rays but not for electron beam irradiation or cold plasma exposure.

Key words: X-ray, electron beam, plasma jet, resistance to antibiotics.

INTRODUCTION

Although known as Gram positive germ, with generally simpler structure of the cellular wall, *Staphylococcus aureus* is still considered a versatile organism provided with several virulent characteristics and resistance mechanisms, thus remaining a widely spread cause of various infectious diseases even in the modern times. Classical method for large scale disinfections is based on ultraviolet lamp utilization, ultraviolet radiations being one of the most common disinfectants for large surface areas in hospital and laboratory used since the '60 years. But the typical depth of photo-chemical reactions involving ultraviolet (UV) absorption is restricted to about one micrometer, so even for UV photons reaching the target surface, the bactericidal effect is pretty much restricted to the cells grown in first thin layer – as can be ensured by fluorescence lamps (Block, 2001).

In the last decades, the researches focused on *S.*

aureus sterilization which reinforced more because of persistent hospital infections caused by this germ subtle contamination. *S. aureus* often generates life-threatening deep seated infections like bacteremia, endocarditis and pneumonia so that the investigations dedicated to its inactivation have continuously developed and need to be continued by testing new sterilization methods (LeRouge et al., 2001). In the next paragraph, some representative studies focused on the ionizing radiation and cold plasma bactericidal effects, in spite of the well known high radio resistance of microorganisms are selectively summarized. So, Sade and Jacobs (1983) obtained lethal effects of gamma radiation on *S. aureus* based on sensitizer effects of barbital derivatives, Thayer and Boyd (1992) succeeded in sterilizing *S. aureus* from meat samples with gamma rays while Caillet et al. (2009) developed new experiments regarding the mechanisms involved in gamma rays action on *S. aureus* cells. The results showed that in spite of their relatively high radio sensitivity, bacteria can respond to irradiation action depending also on the irradiation conditions. Regarding the bioeffects of cold plasma, Ekem et al. (2006)

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succeeded in *S. aureus* inactivation using pulsed plasma discharge at atmospheric pressure which provided UV photons, ozone and active oxygen radicals while in 2009, atmospheric plasma generated between two plane electrodes with ceramic discharge barrier in air was used by Burts et al. (2009) to destroy *S. aureus* methicillin-resistant strain, responsible for many hospital infections. Considering the significant UV rays and ozone presence in the electric discharges, we mention also the experimental studies based on *S. aureus* agarized cultures responses to UV rays developed by Silva and Andrade (2003), Schrier et al. (2009) and Steinka and Kukułowicz (2007). Krishnamurthy et al. (2008) also provided indubitable proofs on the injuries due to UV action in *S. aureus*: cell wall damage, cytoplasmic membrane shrinkage, cellular content leakage, and mesosome disintegration. Kowalski et al. (1998) evidenced 99% sterilization effect of ozone against agarized cultures of *S. aureus* and other bacteria; Thanomsub et al. (2002) showed the sterilizing effect of ozone flow on *S. aureus* and other germs from water while specific interest in the sterilizing effect of ozone gas on the methicillin-resistant *S. aureus* from hospitals and dentistry cabinets was shown by several research groups like Murakami et al. (2002), Estrela et al. (2006), etc. In the next pages, we present some results provided by several types of experiments focused on *S. aureus* response to ionizing radiation and plasma jet.

MATERIALS AND METHODS

Microbiological material was represented by bacterial strain of *S. aureus* from standard collection (ATCC 2592). The microorganism inoculums were prepared in normal physiological sterile saline starting from stock cultures, aged of 18 h grown on agar medium, by adjusting them to a density of approximately 3.10^8 CFU/ml as was assessed using calibration curves. For the ionizing radiation (X-rays and electron beam), equal aliquots of 0.2 ml of *S. aureus* inoculum with the density of 10^7 CFU/ml were added to 10 ml volumes of Mueller-Hinton (Oxoid) nutritive broth (pH of 6.8) that was further mixed thoroughly, the final bacterial samples being adjusted to 3 ml equal volumes (in glass tubes). The same inoculation procedure was followed for plasma inactivation test except that the culture medium was molten agar with 6.8 pH from Oxoid poured into plastic Petri plates (100 mm diameter). Control samples were prepared and kept in identical conditions except that they were not irradiated. Following the radiation or cold plasma treatment, standard incubation procedure (at 37.0 ± 0.5℃ for 24 h) was further applied to the bacterial samples and control ones.

The exposure to ionizing radiation and cold plasma

Radiation delivering devices

For X-ray exposure, we used a specialized X-ray system for radiation therapy of skin lesions, SRT 100 (TOPEX), characterized by 70 kV (tension) and 10 mA (current) that provides X-ray emission of low energy; the dose rate was of 227 cGy / min, measured at a source to surface distance (SSD) of 25 cm. Four

repetitions for every exposure time were carried out by using identical glass tube samples. All glass tubes that contained 3 ml inoculated culture medium were placed in a special designed holder, with holes that allowed being irradiated from underneath. Dose calculation was accomplished at 1.5 cm depth in the sample volume, considering the curve describing the percentage depth dose (*PDD*) characterizing the used irradiation arrangement:

$D_{1.5cm} = (dD/dt)_{surface} \times PDD \times t$

Where, $D_{1.5cm}$ is given in cm; $(dD/dt)_{surface}$ - in Gy/min; *PDD* is given in %; while *t* is measured in minutes. The radiation dose, corresponding to the array of exposure times of 25 up to 100 min, with $(dD/dt)_{surface}$ of 2.27 Gy/min and *PDD* equal to 57%, ranged between 30 and 128 Gy. Each exposure was composed of consecutive 3 min of effective irradiations with 2 min pauses between them according to the technical characteristics of the hospital X-ray device.

Electron beam irradiation was carried out with 6 MeV energy electron beam produced by a particle linear accelerator type VARIAN CLINAC 2100SC. All glass tubes that contained 3 ml inoculated culture medium were placed in an adequate holder that allowed the lateral uniform irradiation. The samples were exposed to a dose rate of 240 cGy/min with doses between 31 and 128 Gy, the same dose array that in the case of X-ray irradiation was carried out also in this study.

For the megavoltage electron beam exposure, the radiation doses can be calculated also at 1.5 cm depth in the samples, using following formula (Podgorsak, 2005):

$D = (dD/dt)_{(z_{max},10)} \times RDF(A) \times PDD(1.5\ cm, A) \times t \times 0.005029$ (2),

Where, *D* is the absorbed dose in the sample; $(dD/dt)_{(z)max}$ is the dose rate at the point *z* where it reaches its maximum value on the central axis of a $10 \times 10\ cm^2$ electron beam; RDF(A) is the relative dose factor (depending on the radiation type); $PDD(1.5\ cm, A, hv)$ is the percentage depth dose at 1.5 cm depth, for a *A* field size, and *t* is the irradiation time. The parameters values were: (dD/dt) =240 cGy/min, *RDF*=1.007 and *PDD*=99.2% for the given irradiation geometry (6 MeV electron beam, $20 \times 20\ cm^2$ field size, 1.5 cm depth in the sample) which were obtained after beam calibration procedures (in accordance with dosimetric standard IAEA TRS-398) using a 3D Blue Water Phantom, a PTW Frieburg Markus flat ionizing chamber (for the electron beam) and a PTW Unidose Electrometer. To obtain the same radiation doses as in the case of X-ray exposure, the irradiation time was calculated from formula (2). Four repetitions were assured for every exposure time by using identical glass tube samples. We mention that the possibility of adjusting the radiation doses in correlation with the irradiation times was limited by the characteristics of the irradiation devices used in the experimental study.

The exposure to plasma impact (asymmetric DBD- dielectric barrier discharge) was accomplished between the two electrodes (distanced at 3.5 cm) of an experimental set-up that produces and delivers helium plasma in air, in the form of a cylindrical jet with about 1 mm diameter, by means of a pulsed voltage of 9 kV - peak to peak voltage at a frequency of 1.6 kHz (Dumitrascu et al., 2005). Four repetitions of every exposure time were carried out within the same Petri dish.

Effect assessing

Cell density was assessed by spectro-colorimetric assay; in the case of X-ray and electron beam irradiation, measurements being
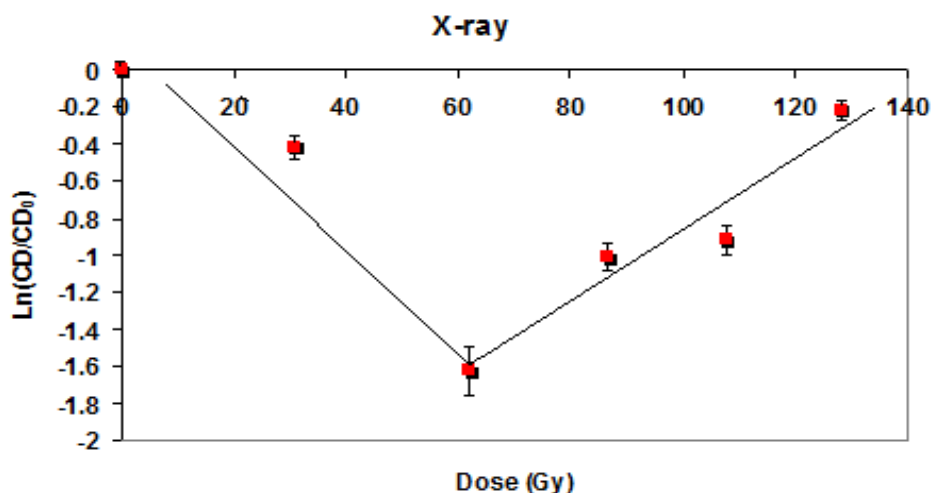
Figure 1. Linear logarithmic dependence of relative cell density (CD, cell density; CD_0, cell density in the control sample) on the radiation dose for X-ray exposure.

accomplished at 560 nm and the resulted average values and standard deviations being used for graphical representation. Shimadzu Spectrophotometer UV type 1700 Pharmaspec with quartz cells was used for the spectro-colorimetric measurements. The circular inhibition zones revealed on the agarized microbial samples after plasma treatment were observed by direct visual inspection, their diameters being measured with 1 mm precision usual ruler. The counting of colony forming units (CFU) was carried out to evaluate sterilization efficiency following cold plasma exposure.

Antimicrobial susceptibility (the method of Kirby-Bauer (1973) with tetracycline and chloramphenicol disks from Oxoid) was tested (after ionizing radiation or plasma exposure), by adding 0.2 ml inoculated liquid medium to 10 ml of agarized culture medium in sterile Petri dishes. Four repetitions were carried out within the same Petri dish for every bacterial sample. The diameter of inhibition growth areas was measured using transparent ruler with 1 mm precision.

Statistical analysis

All described experiments were repeated twice so that taking into account the four replays of every sample and control arranged in the frame of every experiment, the average values and standard deviations were computed from eight values in each case. Statistic signification was assessed according to Student t-test with the threshold of 0.05 in all experiments.

RESULTS

The effect of ionizing radiation

In the case of the X-ray exposure, the experimental data are represented in Figure 1. To obtain a bactericidal effect, several exposure times were tested, the range from 25 to 100 min being chosen; with equivalent radiation doses of 31-62-87-107-128 Gy. The average values (corresponding to four repetitions) of raw data,

that is, cell density (in millions/ml, as provided by spectral measurements at 560 nm and calibration curve) revealed non-linear response, the most remarkable decrease, with more than 75% corresponding to about 50 min irradiation time, that is, corresponding to the dose of 62 Gy. The graphical plot in linear logarithmic coordinates suggested a combination between an exponential survival curve (negative slope) and an exponential curve of cell growth (positive curve). The variations of cell density evidenced in all irradiated samples compared to the control ones were statistically significant according to t-test ($p<0.05$).

In Figure 2, the data obtained following the irradiation with accelerated electrons (6 MeV energy) are represented, the doses range being adjusted to that corresponding to the X-ray treatment.

The most significant diminution of approximately 25% ($p<0.05$) was obtained for the highest dose of 128 Gy (exposure time equal to 68 min) while lower effect, but lacking the statistical significance, was recorded for most of the other doses; slight stimulatory effect given by 16% increase in the cell density (expressed in millions/ml according to spectral measurements at 560 nm and using calibration curve) was evidenced for the lowest dose, of about 31 Gy (exposure time equal to 16 min). So, about three times lower bactericidal effect could be obtained using accelerated electrons in comparison to X-rays of the same radiation doses. The further increase of electron beam exposure time was not allowed because of the therapy device peculiarities.

Plasma inactivating effect

Following the exposure to plasma discharge jet, the bacteria growth appeared to be inhibited within circular
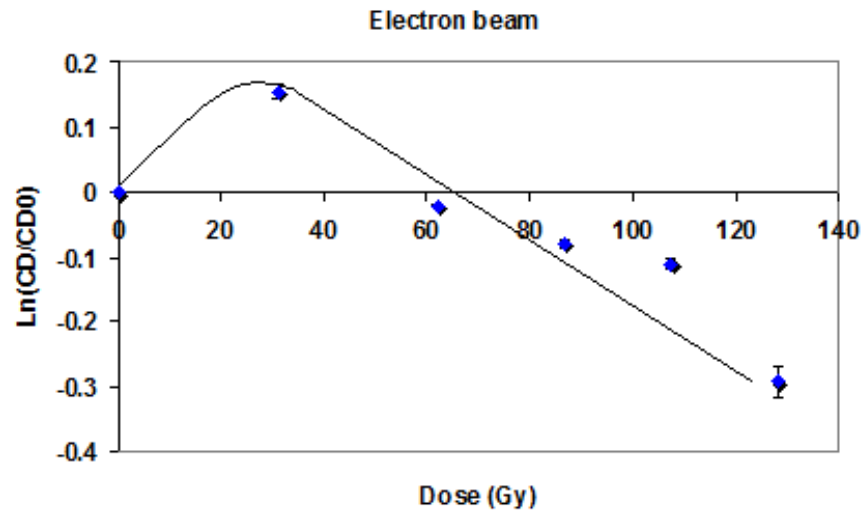
Figure 2. Linear logarithmic dependence of relative cell density (CD - cell density; CD_0 –cell density in the control sample) on the radiation dose for electron beam exposure.
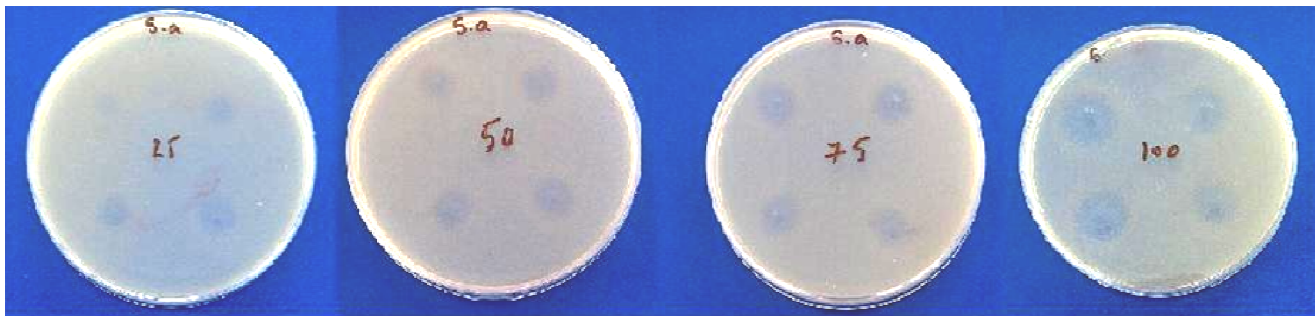

Figure 3. The zones of bacteria growth inactivation following exposure to atmospheric plasma jet for exposure times of 25, 50, 75, 100 s.

areas that increased with the time exposure increasing, as can be seen from Figure 3.

So, the cold plasma effect resulted in up to 16 mm diameter of the inactivation area, for the longest exposure time, of 100 s, tested in this experiment (longer exposure being limited by the generator particularities). Standard deviations were of 8.5 to 9.5%. All differences between the control, non-exposed Petri dishes, and those allowed to interact with plasma jet were statistically significant as was assessed by applying the t-test ($p<0.05$). Linear function was found to be the mathematical approximation that has fitted with highest correlation coefficient, the relationship between the diameter of the bacteria inactivation area and the exposure time; the correlation coefficient being $R^2=0.971$ (Figure 4).

CFU counting carried out within the growth inhibition areas compared to the background of non-exposed cultures have resulted in values of up to 27% corresponding to cca. 73% sterilization efficiency for 100

s plasma treatment; no mathematical correlation with the exposure time could be established.

DISCUSSION

Regarding the inhibitory effect of ionizing radiation, the raw data evidenced that relative cell density (the cell density in the irradiated samples/cell density in the control samples) in the X-ray irradiated bacteria cultures (Figure 1) exhibited progressive diminution, with 35 and 75% after 31 Gy and respectively 60 Gy X-ray dose administration corresponding to 25 min and respectively 50 min irradiation. Standard deviation was of 11% in average. The cell density has appeared increased progressively in the samples irradiated for longer time durations; for 65, 85 and 100 min, corresponding to 87, 108 and 128 Gy but still remained under the control sample level.
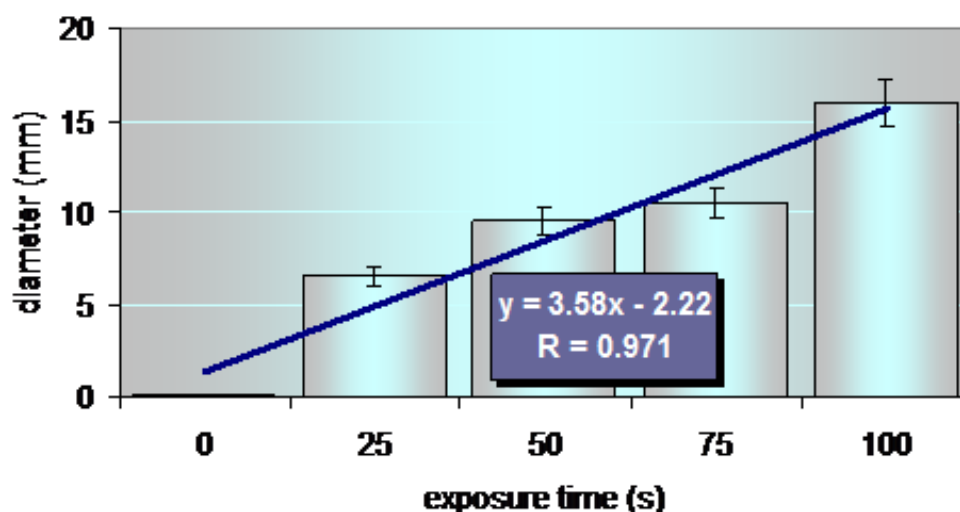
Figure 4. Correlation between inhibition growth diameter and plasma exposure time; *x*-exposure time; *y*-inhibition growth diameter; *R*- correlation coefficient (computed in MsExcell).

For the explanation of the progressive decrease of cell density to the increase of the exposure time up to 50 min (62 Gy), the dominancy of lethal effects should be taken into account, caused mainly by DNA injuries, while for further increase of the exposure time up to 100 min (128 Gy), the physiological recovery could be supposed to occur successfully as well as growth stimulation mechanism activation in the irradiated but surviving cells. This recovery could be favored by the irradiation protocol that involved alternative pauses between radiation exposures so that during irradiation pauses, DNA repair mechanisms could be stimulated and cell accommodation to the radiation impact could occur with higher probability; this being consistent with cell density increase for longer exposure time durations.

The hospital that was chosen (that used X-ray device) was motivated by the possibility to get an insight into the putative bacterial side effects of patient treatment with radiation considering the possible presence of *S. aureus* germs on the skin or within the irradiated internal organs. In this respect, the test of *S. aureus* resistant to antibiotics (Figure 5) was accomplished too, as mentioned earlier.

The diameter of the inhibition growth area that resulted from the antibiotic diffusion appears to be generally increased, with up to 15%, in the case of the bacterial samples exposed to X-ray doses (31 to 128 Gy) corresponding to exposure times ranging from 25 to 100 min, which is consistent with the slight diminishing of *S. aureus* resistance to antibiotic. This is visible for tetracycline better than for chloramphenicol, where the variations corresponding to the exposed samples comparatively to the control ones are similar to standard deviation values thus having no statistic signification

(according to *t*-test). The two chosen antibiotics have broad activity spectrum due to their inhibitory effect on protein synthesis in bacterial cells. Tetracyclines are still widely used in chemotherapy and also the use of chloramphenicol is re-considered at present time because of the global problem of advancing bacterial resistance to newer drugs (Falagas et al., 2008).

The data representing *S. aureus* response to 6 MeV electrons (Figure 2) suggested exponential type survival curve of peculiar kind or sigmoidal type curve with "positive" shoulder, due to the slight increase of the relative cell density for 31 Gy. It seems that different action mechanisms underline the behavior recorded for electron beam exposure compared to the case of X-ray irradiation for the same dose array, which could be expected considering the corpuscular nature of the electron beam and the shorter penetration depth. In both situations, DNA damage and partial repair could occur as well as the stimulation of cell division, but they were evidenced for different absorbed doses – for the lowest dose in the case of accelerated electrons but for highest doses in the case of X-ray irradiation. The antimicrobial susceptibility tested against tetracycline, and chloramfenicol revealed no significant variations in none of the electron irradiated samples compared to non-irradiated controls (data not shown).

Low temperature plasma exposure resulted in evident inhibitory effect on *S. aureus* agarized cultures (Figure 3) for much shorter exposure times, about 73% sterilization efficiency being obtained for 100 s exposed samples, comparable to the 75% efficacy of exposure to X-rays for 50 min.

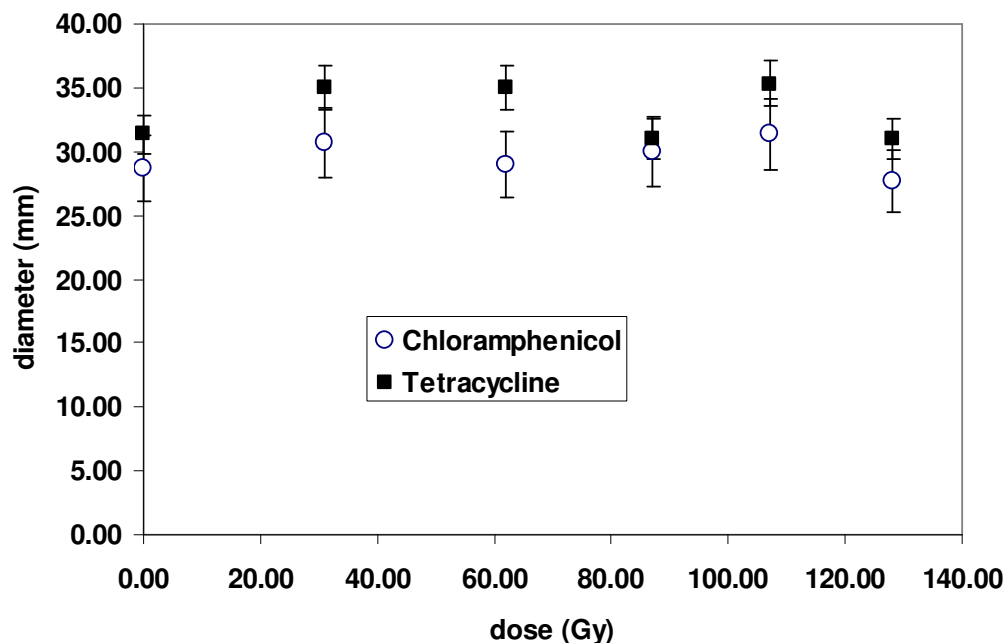The antimicrobial susceptibility of the survived bacteria colonies tested against tetracycline and chloramfenicol

Figure 5. *S. aureus* resistance to antibiotics before and following the exposure to X-rays.

revealed no significant changes compared to controls (data not shown).

All plasma main components could be involved in the biological effect induced into the bacterial cells, either directly or indirectly: UV radiation and electromagnetic field, accelerated electrons and ions, ozone molecules and neutral molecular radicals. The collisions with plasma accelerated particles and the absorption of ozone are considered as the principal causes of bacteria inactivation capable to destabilize sub-cellular structures by breaking intermolecular bonds within the membranes as well as within the biomolecules, DNA damage being mostly probable (Ekem et al., 2006). So, regarding the results of the present experiment, we believe that the lethal effects revealed in the *S. aureus* cultures are due mainly to the molecular damages caused by accelerated particles from plasma jet while the UV-generated ozone could also have some remarkable contribution to the biomolecules oxidation.

The main result is that plasma sterilization could be applied successfully for reducing bacteria loading level as alternative method to other sterilization factors causing more damaging for laboratory utensils that do not support high temperatures or chemical reagents action; *S. aureus* inactivation by short time plasma treatment (of about 100 s) seems to be equivalent with the bactericidal effect of ionizing radiation utilization for about 50 min. Nevertheless, the treatment time with cold plasma is shorter also than those required by conventional sterilization methods based on ethylene oxide or dry heat

supply (Kikuchi et al., 2011).

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