**Influence of plasma characteristics on the efficacy of Cold Atmospheric Plasma (CAP) for inactivation of *Listeria monocytogenes* and *Salmonella* Typhimurium biofilms**

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# ABSTRACT

The biofilm mode of growth protects bacterial cells from currently applied disinfection methods for abiotic (food) contact surfaces. Therefore, new inactivation technologies such as Cold Atmospheric Plasma (CAP) should be considered. However, the influence of different plasma characteristics on the CAP efficacy for biofilm inactivation is underinvestigated. In this research, the influence of (i) the applied plasma configuration (Dielectric Barrier Discharge (DBD) and Surface Barrier Discharge (SBD)), (ii) the oxygen level of the gas flow (He + 0.0/0.5/1.0 (v/v) % O2), and (iii) the plasma intensity (13.88, 17.88, and 21.88 V input voltage) on the CAP efficacy for inactivation of *L. monocytogenes* and *S.* Typhimurium biofilms was investigated. In general, (i) the DBD had the highest inactivation efficacy, (ii) the highest log10-reductions were obtained while using 0.0 (v/v) % O2, and (iii) increasing the input voltage resulted in an increased efficacy. At these specific conditions, 3.5 log10-reductions were obtained.

**Keywords:** Cold Atmospheric Plasma (CAP), biofilms, *Listeria monocytogenes, Salmonella* Typhimurium*,* predictive modelling,sub-lethal injury

# 1. INTRODUCTION

Food safety is an important aspect to take into account during all steps of the production process (e.g., during harvesting or packaging) since the occurrence of chemical, physical, and microbiological contaminants in food products can be a real threat for human health. With respect to microbiological safety, the occurrence of foodborne pathogens should be avoided and/or controlled to avoid foodborne illnesses (Martinović 2016). Within the European Union (EU), bacterial species account for the highest number of foodborne illnesses. Salmonellosis (mainly caused by *Salmonella*Typhimurium) has a high notification rate (21.2/100,000 capita) and a fatality rate of 0.24%. On the contrary, listeriosis (caused by *Listeria monocytogenes*) has a relatively low notification rate (0.46/100,000 capita), but a very high fatality rate of 17.7% (EFSA and ECDC 2017). Consequently, contamination of food products with these two pathogenic bacteria should be avoided and/or controlled by taking appropriate actions.

For a food processing plant, abiotic contact surfaces are a primary area of concern as contamination source. It has become clear that (pathogenic) bacteria, such as *Listeria monocytogenes*, *Salmonella* *enterica* and *Escherichia coli*, grow predominantly as biofilms on these surfaces, rather than as planktonic cells or colonies, which makes them more resistant (Marriott & Gravani 2006). Biofilms are functional consortiums of microorganisms attached to a surface and the cells are embedded in a matrix of self-produced extracellular polymeric substances (EPS) (Kumar & Anand 1998). The biofilm mode of growth protects them from currently applied surface disinfection methods (i.e., the use of (hot) water and antimicrobial agents, combined with a mechanical action such as the use of brushes (Gómez-López 2012)) due to different biofilm-specific properties. The EPS matrix has several protective functions, i.e., it (i) can retain water and nutrients, (ii) keeps the cells attached to the surface, and (iii) limits the diffusion of antimicrobial agents into the 3-dimensional biofilm structure. Consequently, biofilm-associated cells are more resistant towards harsh environments such as low concentrations of available nutrients, osmotic stress, and shear forces (Kumar & Anand 1998). Biofilm-associated cells can also undergo physiological changes, i.e., they become metabolically dormant cells with a reduced growth rate and a decreased consumption of nutrients and oxygen. Since antimicrobial agents are far more effective towards actively growing cells (such as planktonic cells), these dormant cells cannot get (completely) inactivated using antimicrobial agents such as chlorine or hydrogen peroxide (Kumar & Anand 1998; Gómez-López 2012). As a result, more efficient technologies for inactivation of biofilms need to be considered.

Cold Atmospheric Plasma (CAP) is one of those promising technologies as log10-reductions up to 4 log10 CFU/sample have been reported in literature for *L. monocytogenes* and *S.*Typhimurium biofilms (300 s of CAP treatment, 80 kV, ambient air) (Ziuzina et al. 2015). Plasma is the fourth state of matter and can be created by addition of energy to a gas. As a result, an ionized gas, consisting of ions, photons, free electrons, and activated neutral species (excited and radical), is formed (Banu et al. 2012; Fernández & Thompson 2012). Plasma can be generated in different ways, e.g., by using an electric discharge in a gas, at room temperature and at atmospheric pressure. This specific plasma type (i.e., CAP) has some important advantages: (i) it is fast, (ii) it can be created at a low temperature, (iii) plasma components fade out immediately after treatment, and (iv) cells can be inactivated by multiple inactivation mechanisms (Banu et al. 2012; Fernández & Thompson 2012). However, research is still required to fully assess the efficacy of the CAP technology when applied for inactivation of biofilms.

Important to investigate in this regard is the influence of different plasma characteristics on the CAP inactivation efficacy. In literature, different plasma characteristics have been reported to have an influence on this inactivation efficacy, e.g., the set-up (electrode configuration) and exposure mode, the operating gas (flow rate, pressure, and composition), the treatment time, the distance to the sample, the frequency, and the plasma intensity (Lerouge et al. 2001; Deng et al. 2007; Ehlbeck et al. 2011; Fernández & Thompson 2012; Han et al. 2014; Smet et al. 2017). For the characteristics studied in this research, i.e., (i) the electrode configuration, (ii) the gas composition, and (iii) the intensity (input voltage), main findings (for planktonic cells) have been summarized by Lerouge et al. (2001): (i) the geometry of the plasma electrode determines which species reach the sample, i.e., samples in direct contact with the plasma are more effectively inactivated than samples that are only treated with the so-called plasma afterglow, (ii) addition of oxygen to the feed gas increases the efficacy of the plasma treatment, and (iii) an increased plasma intensity results in a higher density of reactive species.

It is important to understand the (combined) influence of these parameters on the CAP efficacy to improve the inactivation of biofilms, which is still missing in literature. In addition, the possible induction of sub-lethally injured cells following CAP treatment should be investigated. Sub-lethally injured cells are damaged (e.g., they have a damaged cell membrane), but they are able to recover if they re-encounter optimal environmental conditions. As a result, they may cause public health concerns (Noriega et al. 2013). Since sub-lethal injury of bacterial cells has been reported already following treatment with currently applied (thermal) inactivation technologies (Wuytack et al. 2003; Wang et al. 2017), it should be investigated as well for novel technologies such as CAP in order to be able to comment on a possible application of this technology for inactivation of biofilms grown on abiotic surfaces.

Therefore, the goal of this research was to obtain knowledge regarding the (combined) influence of (i) the applied electrode configuration, (ii) the oxygen level of the gas flow, and (iii) the plasma intensity on the CAP efficacy for inactivation of model biofilms developed by *L. monocytogenes* (Gram positive) and *S.* Typhimurium (Gram negative). The influence of these characteristics has been determined as function of time, i.e., for each combination of characteristics, different treatment times (0-30 min) were applied. Sub-lethal injury was assessed for all experimental conditions and predictive models were implemented to better understand the impact of the plasma characteristics on the CAP inactivation efficacy for pathogenic biofilms.

# 2. MATERIALS AND METHODS

## 2.1. Experimental design

For both microorganisms, a strongly adherent and mature model biofilm was used to investigate the influence of three important plasma characteristics on the inactivation efficacy of CAP. Two different electrode configurations were investigated, i.e., the Dielectric Barrier Discharge (DBD) and the Surface Barrier Discharge (SBD). For each of the experiments, a gas mixture consisting of helium and oxygen (He + O2) was used. However, the oxygen level of the mixture was varied, i.e., oxygen levels of 0.0, 0.5, and 1.0 (v/v) % have been investigated. Finally, the influence of the plasma intensity has been examined as well, i.e., input voltages of 13.88, 17.88, and 21.88 V were applied. For each combination of plasma characteristics, the influence on the CAP inactivation efficacy has been determined as function of the treatment time (0, 1, 2, 5, 7.5, 10, 15, 20, 25, and 30 min). Two biological replicates were used for each time point, except for 0 min. For the untreated biofilms, a data set of 36 samples (general and selective medium) was used for each intensity (input voltage) and each microorganism.

## 2.2. Microorganism, pre-culture conditions, and model biofilm development

In this research, *L. monocytogenes* LMG23775 and *S.*Typhimurium LMG14933, both acquired from the BCCM/LMG bacteria collection of Ghent University in Belgium, were used. For both species, stationary phase pre-cultures (approximately 109 CFU/ml) were obtained according to the protocol of Govaert et al. (2018).

These pre-cultures were used to develop model biofilms for *L. monocytogenes* and *S.* Typhimurium according to the optimal biofilm formation conditions determined in previously mentioned research (Govaert et al. 2018). Small Petri dishes made out of polystyrene (50 mm diameter, 9 mm height, Simport, Canada) were used as abiotic surfaces and each Petri dish was inoculated with 1.2 ml of inoculum. This inoculum (approximately 107 CFU/ml) was prepared by making a 100-fold dilution of the stationary phase pre-culture. BHI and 20-fold diluted TSB were used as dilution medium for *L. monocytogenes* and *S.*Typhimurium, respectively. After inoculation, Petri dishes were closed and gently shaken to make sure the inoculum covered the entire surface. Finally, Petri dishes were incubated for 24 h at 30 (*L. monocytogenes*) or 25°C (*S.*Typhimurium), which were the optimal biofilm formation temperatures determined in the research of Govaert et al. (2018). In that research, confocal laser scanning microscopy was used to visualize the biofilm formation as function of the incubation time. Based on the number of captured slices and their thickness, the biofilm thicknesses following 24 h of incubation can be estimated as approximately 15 µm (*L. monocytogenes*) and 25 µm (*S.* Typhimurium).

## 2.3. CAP equipment and biofilm inactivation procedure

In this research, a DBD and SBD electrode configuration were used, which are both illustrated in Figure 1. For both configurations, the discharge is generated between two electrodes (DBD electrode diameter = 5.5 cm; SBD electrode diameter = 5.0 cm), covered by a dielectric layer (diameter 7.5 cm) and incorporated in an enclosure. In case of the DBD, the electrode gap was set at 0.8 cm and the sample was placed between the electrodes prior to treatment. In case of the SBD, there is no electrode gap and both electrodes are located on the top of the enclosure. Here, the sample was placed below the two electrodes while ensuring a similar distance between the sample and the electrodes as for the DBD configuration. Around both electrodes, an enclosure was provided to increase the residence time of the plasma species and to obtain a more controlled environment. Both enclosures (22.5 cm x 13.5 cm x 10 cm (DBD) and 10 cm x 10 cm x 4 cm (SBD)) were not airtight, which resulted in the presence of low amounts of oxygen and nitrogen from the environment. The plasma power supply connected to both electrodes transforms a low voltage DC input (0 - 60 V) into a high voltage AC signal (0 - 20 kV), at a frequency up to 30 kHz.

For all experiments, the plasma was generated in a gas mixture of helium (purity 99.996%, at a flow rate of 4 l/min) and oxygen (purity ≥ 99.995%). Three different oxygen levels were tested, i.e., 0.0, 0.5, and 1.0 (v/v) %, resulting in oxygen flow rates of 0, 20, and 40 ml/min. The helium and oxygen flow rates were mixed before entering the plasma enclosures. In this research, three different low input voltages were examined, i.e., 13.88, 17.88, and 21.88 V, resulting in peak-to-peak voltages between approximately 2 and 8 kV and a dissipated plasma power between approximately 2 and 13 W, depending on the applied conditions. For both the oxygen level and the intensity/input voltage, three levels were selected in order to examine if the CAP efficacy keeps on increasing as the value of the plasma characteristic increases or if there is an optimal (intermediate) value within the selected range. For all experiments, the frequency was set at 15 kHz.

After flushing the reactor chamber to ensure a homogeneous gas mixture, the high-voltage power source was energized, and the plasma was generated. Samples, which were rinsed three times with sterile Phosphate Buffered Saline (PBS) solution (to remove the remaining planktonic cells) and allowed to dry prior to treatment, were CAP treated up to 30 min.

## 2.4. Microbiological analysis

Following CAP treatment of the model biofilm, biofilm-associated cells were removed from the surface using the cell scraping method explained in Govaert et al. (2018). Serial decimal dilutions (in 0.85 (m/v) % NaCl solution) of the obtained cell suspension were prepared and plated on agar plates. BHI and PALCAM agar (VWR Chemicals, Leuven, Belgium) were used for *L. monocytogenes*, while Tryptic Soy Agar (TSA, TSB supplemented with 14 g/l biological agar, VWR Chemicals, Belgium) and Xylose Lysine Deoxycholate Agar (XLDA, Merck & Co, USA) were used for *S.* Typhimurium. For each of the serial dilutions, three drops of 20 µl were plated. Before counting the colonies, BHI/PALCAM and TSA/XLD agar plates were incubated for 24 h at 30 or 37°C, respectively. With the applied drop method, the detection limit is 1.7 log10 (CFU/cm²). Lower numbers should be interpreted with caution due to the high uncertainty.

## 2.5. Modelling, parameter estimation, and estimation of sub-lethal injury

The model of Geeraerd et al. (2000), describing a microbial inactivation curve consisting of a log-linear inactivation phase and a tail (Equation 1), was used to fit the experimental data.

$N\left(t\right)=(N\_{0}-N\_{res})\*e^{-k\_{max}\*t}+N\_{res}$ (1)

Where *N*(*t*) [CFU/cm²] is the cell density at time *t* [min], *N*0 [CFU/cm²] the initial cell density, *Nres* [CFU/cm²] is a more resistant subpopulation, and *kmax* [1/min] the maximum specific inactivation rate. Based on the difference between log10 *N0* and log10*Nres*, the final log10-reduction (following 30 min of CAP treatment) has been calculated for each combination of plasma characteristics.

The parameters of the Geeraerd et al. (2000) model were estimated via the minimization of the sum of squared errors (SSE), using the lsqnonlin routine of the Optimization Toolbox of Matlab version R2016a (The Mathworks, Inc.). At the same time, the parameter estimations were determined based on the Jacobian matrix. The Root Mean Squared Error (RMSE) served as an absolute measure of the goodness of the model to fit the actual obtained data.

Finally, to calculate the percentage of sub-lethal injury (% SI), theoretical concentrations obtained from the model of Geeraerd et al. (2000) were used for both the general and selective counts. The equation of Busch and Donnelly (1992) (Equation 2) was used to determine the percentage of injured cells at each treatment time. As a result, the percentage of sub-lethal injury was plotted as function of the treatment time.

$\%SI=\frac{CFU general medium-CFU selective medium}{CFU general medium}∙100$ (2)

## 2.8. Statistical analysis

​Analysis of variance (ANOVA) tests were conducted to determine whether there are any significant differences amongst means of the estimated model parameters, at a 95.0% confidence level (α = 0.05). Fisher´s Least Significant Difference (LSD) test was used to distinguish which means were significantly different from others. The analyses were conducted using the Statgraphics 18 software (Statistical Graphics, Washington, USA). For each parameter of the Geeraerd et al. (2000) model, different ANOVA tests (for each microorganism separately) were conducted. To determine the influence of one plasma characteristic (e.g., electrode), a separate ANOVA test was conducted for each level of the other characteristics (e.g., oxygen level and input voltage) and for each medium.

# 3. RESULTS and DISCUSSION

In Figure 2A (*L. monocytogenes*) and 2B (*S.*Typhimurium), (i) the biofilm cell density based on general and selective medium and (ii) the percentage of sub-lethally injured cells are expressed as function of the treatment time when applying the DBD and SBD configuration at different oxygen concentrations (i.e., 0.0, 0.5, and 1.0 (v/v) %) and at different intensities/input voltages (i.e., 13.88, 17.88, and 21.88V). In Table 1 and 2, all estimated parameters of the Geeraerd et al. (2000) model, including the statistical analyses, are represented for all possible combinations of plasma characteristics for *L. monocytogenes* and *S.* Typhimurium, respectively. However, in the following paragraphs, mainly the model parameters for the general medium will be discussed. While discussing the percentage of sub-lethal injury (as function of the treatment time), model parameters obtained for the selective media will only be discussed in an indirect way.

## 3.1. General observations

In general, several observations can be made independently from the electrode configuration, the oxygen level, and the intensity/input voltage. First of all, it can be observed that the shape of the inactivation curves (Figure 2A and 2B) is similar for both microorganisms and at all possible combinations of plasma characteristics, i.e., all curves contain a log-linear inactivation phase followed by a tail. However, since the duration time of the log-linear inactivation phase is in most of the cases relatively short (less than 10 min), a small variability of the cell densities obtained within this region can already result in significantly different *kmax* values. Therefore, results concerning the influence of the different plasma characteristics on the inactivation rate should be interpreted with caution.

Secondly, the initial cell density (log10 *N0*) is different for both microbial species, i.e., the cell density for the *L. monocytogenes* model biofilm (Table 1) is higher (on both the general and the selective medium) compared to the *S.*Typhimurium model biofilm (Table 2). This difference in initial cell density should be considered when assessing (possible) differences between the CAP efficacy for inactivation of both pathogenic biofilms.

Thirdly, a data set of 36 samples (for both the general and the selective medium) was used at each intensity/input voltage (for each species) to estimate the initial cell density (log10 *N0*) of the untreated biofilms. Consequently, only minor (non-significant) differences between the initial cell densitiesat the different treatment conditionscan be observed (for each microorganism separately). These minor differences are the result of the model fit and/or experimental/biological variability.

Finally, sub-lethal injury can be observed for the untreated (time = 0 min) *L. monocytogenes* (Figure 2A) and *S.* Typhimurium (Figure 2B) model biofilms. For *L. monocytogenes*, the initial percentage of sub-lethal injury ranges between 0 and approximately 35%. For *S.* Typhimurium, this percentage ranges approximately between 40 and 50%. The (small) difference in sub-lethal injury prior to treatment between both bacterial species has been observed before in Govaert et al. (2018), i.e., 3-dimensional Confocal Laser Scanning Microscopy (CLSM) images taken following live/dead staining of the (24 h old) model biofilms indicated that the bottom layers of the *S.* Typhimurium biofilm contained more dead or damaged cells compared to the *L. monocytogenes* biofilm. This could possibly be the result of the heterogeneous microenvironment present in the biofilm. In the bottom layers, cells can encounter problems with waste accumulation, nutrient availability, and oxygen limitations, which might result in sub-lethal injury of the cells (Anwar 1992). The lower amount of sub-lethally injured *L. monocytogenes* biofilm-associated cells might be a consequence of the higher (general) resistance of *L. monocytogenes* to environmental stress (Marriott & Gravani 2006; Food and Drug Administration 2012). However, since there is quite some variation in the percentage of sub-lethal injury of the untreated *L. monocytogenes* biofilms (due to experimental/biological variability and/or the use of different stock cultures for each data set of 36 samples to estimate log10 (*N0*) at each intensity/input voltage), conclusions concerning the difference in initial sub-lethal injury between both species should be drawn with caution. Following CAP treatment of the biofilm, in general, all curves for the percentage of sub-lethal injury as function of the CAP treatment time have a similar shape (Figure 2A and 2B). There is an initial increase in sub-lethal injury, which decreases again until a residual amount of sub-lethal injury is obtained. In some cases (i.e., 17.88 V – 0.0 (v/v) % oxygen - SBD, 21.88 V - 0.5 (v/v) % oxygen – DBD, and 21.88 V – 1.0 (v/v) % oxygen – SBD), however, there is only an increase in sub-lethal injury, which is immediately followed by a residual percentage. Nevertheless, for each possible condition, the increase and subsequent decrease (if applicable) of sub-lethal injury coincides with the log-linear inactivation phase, suggesting a mechanism of injury accumulation eventually resulting in cell lysis (Noriega et al. 2013).

## 3.2. Influence electrode configuration

Although the shape of the inactivation curve is not influenced by the applied electrode configuration (Figure 2A), the electrode does have an influence on the inactivation rate and the residual *L. monocytogenes* cell density (Table 1).

For the inactivation rate, the influence of the electrode configuration is only minor. In two cases (i.e., 13.88 V – 0.0 (v/v) % oxygen and 17.88 V – 0.5 (v/v) % oxygen), significantly higher inactivation rates are obtained with the DBD configuration. For all other cases, this model parameter is independent of the applied electrode configuration. As mentioned before, one should interpret these observations concerning the influence of the electrode on the inactivation rate with caution. Therefore, apart from focusing on the *kmax* value, it is important to stress out that the final residual cell densities are already obtained following a (very) short CAP treatment time. While using the DBD, final log10-reductions are already obtained following less than 5 min of CAP treatment. Only in three cases (i.e., 17.88 V – 0.0 (v/v) % oxygen, 21.88 V – 0.0 (v/v) % oxygen, and 21.88 V – 0.5 (v/v) % oxygen), treatment up to 10 min is required to reach the final cell density. While using the SBD, a similar conclusion can be drawn, i.e., in most of the cases, less than 5 min of CAP treatment is sufficient to obtain the final cell density. Here, also three exceptions can be observed, i.e., 13.88 V – 0.0 (v/v) % oxygen, 17.88 V – 0.0 (v/v) % oxygen, and 17.88 V – 0.5 (v/v) % oxygen.

Despite a minor influence of the electrode configuration on the inactivation rate (and the duration of this log-linear inactivation phase), a significant influence on the residual *L. monocytogenes* population (log10 *Nres*) can be observed. While using the DBD configuration, significantly lower log10 *Nres* values are obtained for almost any possible combination of oxygen level and input voltage. Only at 13.88 V exceptions can be observed. At 0.5 (v/v) % oxygen, significantly lower values are obtained with the SBD configuration and at 0.0 and 1.0 (v/v) % oxygen, the electrode configuration does not significantly influence the residual cell density. Related to this, the obtained log10-reductions are also significantly higher while applying the DBD configuration, with the same exceptions as mentioned before. Depending on the intensity and the oxygen level, overall *L. monocytogenes* log10-reductions (for the general medium) obtained with the DBD and SBD electrode configuration range between 1.1 - 3.6 and 1.3 – 2.4 log10 (CFU/cm²), respectively.

As for *L. monocytogenes*, the shape of the (general medium) *S.* Typhimurium inactivation curves is independent of the electrode configuration (Figure 2B), however, the configuration does have an influence on the inactivation rate and the residual cell density (Table 2).

For the initial inactivation rate (*kmax*), the influence of the electrode configuration is again only minor. For two combinations (i.e., 13.88 V – 0.0 (v/v) % oxygen and 21.88 V – 1.0 (v/v) % oxygen), using the DBD results in a significantly higher inactivation rate. For all other combinations, the electrode configuration has no influence on this model parameter, although the average values obtained with the SBD are in many of these cases higher than the ones obtained with the DBD. As for *L. monocytogenes*, it is difficult to draw (significant) conclusions concerning the influence of the electrode configuration on the inactivation rate since in most of the cases, the tail phase is already observed following less than 10 min of CAP treatment (Figure 2B). Only for one case (13.88 V - DBD - 0.5 (v/v) % oxygen), 15 min of treatment is required to obtain the residual cell density. Nevertheless, it is again important to stress out that the final residual *S.* Typhimurium cell density (log10 *Nres*) is already obtained following a (very) short CAP treatment time.

As it was mentioned before, the log10 *Nres* value for *S.* Typhimurium is influenced by the applied electrode configuration. In general, significantly higher log10 *Nres* values are obtained while using the SBD configuration. However, in some cases (i.e., (i) 13.88 V - 0.5 (v/v) % oxygen, (ii) 13.88 V - 1.0 (v/v) % oxygen, (iii) 17.88V - 1.0 (v/v) % oxygen, and (iv) 21.88 V - 1.0 (v/v)% oxygen), the electrode configuration has no influence on this model parameter, although the average log10 *Nres* values are still slightly lower while using the DBD compared to the SBD. Related to the results obtained for the residual *S.* Typhimurium population, significantly higher log10-reductions are (in general) obtained while applying the DBD configuration (1.4-3.5 log10 (CFU/cm²)) compared to the ones obtained with the SBD configuration (1.3-2.2 log10 (CFU/cm²)). Exceptions are the same as the ones previously mentioned, i.e., for these cases, the electrode configuration has no influence on the log10-reduction values. Consequently, it can be concluded that (in general) the highest efficacy for *S.* Typhimurium biofilm inactivation can be obtained with the DBD configuration. Depending on the oxygen level and the intensity, using the SBD configuration might result in a similar efficacy.

Based on the higher log10-reductions obtained with the DBD configuration (in most cases), it can be concluded that this electrode configuration is more effective for inactivation of both pathogenic biofilms. The lower efficacy applying the SBD can be explained based on the research of Lerouge et al. (2001) and Olszewski et al. (2014). It has been reported that the type of reactor design has a strong influence on the concentrations of active species. If the sample is placed in direct contact with the plasma (DBD), it gets into contact with all created species (short and long living). If the sample is further located from the sample (SBD), only the long living species can interact with the sample (Lerouge et al. 2001). In the latter case, the most reactive species, which have the shortest lifetime, recombine before they reach the sample, resulting in a less effective treatment (Olszewski et al. 2014). However, depending on the targeted application, it could still be advised to use the SBD configuration since its more practical scale-up aspect for industrial applications (Xu 2001; Olszewski et al. 2014) and the lower (experimental or biological) variability observed in the presented research (Figure 2A and 2B). Nevertheless, in this case, a compromise should be find between applicability and efficacy.

Despite the higher CAP efficacy obtained with the DBD configuration, it is important to mention that the residual percentage of sub-lethally injured cells is in general higher while applying this configuration than while applying the SBD. Since the total kill with the DBD electrode is still higher than with the SBD electrode, it would still be advised to use the former electrode configuration. Nevertheless, when samples are taken to assess the degree of contamination of (food) contact surfaces following CAP treatment, it should be taken into account that there might be an underestimation of the cell density since up to 85% of the cells are sub-lethally injured.

## 3.3. Influence oxygen level

As for the influence of the electrode configuration, the oxygen level does have an influence on the *kmax* value and the residual cell density, but without altering the shape of the *L. monocytogenes* inactivation curves (Figure 2A and Table 1).

In general, it can be observed that significantly higher *kmax* values are obtained with oxygen, either at 0.5 or 1.0 (v/v) % of oxygen. Only for two conditions (i.e., 13.88 V – DBD and 21.88 V - SBD), the oxygen level has no significant influence on this model parameter. In other words, addition of oxygen to the feed gas results in a significantly faster inactivation of the biofilm-associated *L. monocytogenes* cells. In addition, the duration of the log-linear inactivation phase increases when no oxygen is added to the feed gas, i.e., a longer treatment time is required to obtain the final log10-reduction. With oxygen, the residual *L. monocytogenes* cell density (log10 *Nres*) is in most cases already obtained following less than 5 min of treatment. For two exceptions (i.e., 17.88 V – 0.5 (v/v) % oxygen – SBD and 21.88 V – 0.5 (v/v) % oxygen – DBD), CAP treatment up to 10 min is required. For most combinations without oxygen, CAP treatment times up to 10 min are needed to reach this tail phase. Consequently, if (very) short CAP treatment times are required, addition of oxygen favors the efficacy of the treatment for inactivation of the *L. monocytogenes* biofilms. Depending on the intensity and/or the electrode configuration, this should be 0.5 or 1.0 (v/v) % oxygen.

Despite the lower inactivation rate without oxygen, it should be stressed out that, in general, the residual *L. monocytogenes* cell densities (log10 *Nres*) are significantly lower without oxygen than the ones obtained with oxygen (either 0.5 or 1.0 (v/v) % oxygen). Only for one condition, i.e., 21.88 V – SBD, the oxygen level has no influence on the residual cell density. Consequently, the log10-reductions obtained without oxygen (1.8 – 3.6 log10 (CFU/cm²)) are in general also significantly higher compared to the ones obtained with addition of oxygen to the feed gas (0.5 (v/v) %: 1.1 – 3.5 log10 (CFU/cm²)); 1.0 (v/v) %: 1.3 – 2.7 log10 (CFU/cm²)). Based on this, it can be concluded that addition of oxygen results in a lower CAP inactivation efficacy for the *L. monocytogenes* biofilms. Nevertheless, it requires a shorter treatment time.

As for *L. monocytogenes*, the oxygen level has an influence on the inactivation rate and the residual cell density for inactivation of *S.* Typhimurium biofilms (Table 2), but it does not influence the shape of the inactivation curves (Figure 2B).

The influence on the *kmax* value is similar as for *L. monocytogenes*, i.e., the absence of oxygen in the feed gas results in a lower inactivation rate for most combinations of electrode configuration and intensity/input voltage. Two exceptions can be observed, i.e., 13.88 V – DBD and 17.88 V – SBD. For the former case, the lack of oxygen results in a (significantly) higher inactivation rate compared to the results obtained with addition of oxygen. For the latter case, the oxygen level does not have a significant influence on the obtained *kmax* values. In all other cases, inactivation rates without oxygen are lower compared to the ones obtained with oxygen, although not always significantly. In addition, a longer treatment time (up to 15 min) is required to reach the residual *S.* Typhimurium cell density when no oxygen is added to the feed gas (Figure 2B). Exceptions are observed at (i) 13.88 V – DBD, (ii) 21.88 V – SBD, and (iii) 17.88 V – SBD. Based on these observations, similar conclusions can be drawn as for *L. monocytogenes*, i.e., addition of oxygen results (in most of the cases) in a faster inactivation of the *S.* Typhimurium biofilm.

When assessing the effect of the oxygen level on the log10 *Nres* values, again similar conclusions can be drawn as for *L. monocytogenes*, i.e., in most of the cases, the lack of oxygen in the feed gas results in a (significantly) lower residual cell density compared to the results obtained with oxygen (either 0.5 or 1.0 (v/v) %). Only at 13.88 V – SBD, the oxygen level has no influence on this model parameter and at 21.88 V – DBD, (significantly) lower log10 *Nres* values are obtained at 1.0 (v/v) % oxygen. Consequently, log10-reductions obtained without oxygen (with two exceptions, i.e., 13.88 V – SBD and 17.88 V - SBD) are also (significantly) higher compared to those obtained with oxygen. Final log10-reductions range between 1.7 - 3.5, 1.3 - 2.5, and 1.3 – 2.1 log10 (CFU/cm²)) for 0.0, 0.5 and 1.0 (v/v) % oxygen, respectively. So, despite the slower inactivation without oxygen, it can be concluded that the lack of oxygen results in a higher CAP efficacy for inactivation of the *S.*Typhimurium model biofilms.

For both microorganisms and for most combinations of electrode configuration and intensity/input voltage, (significantly) lower *kmax* values and (significantly) higher log10-reductions are obtained while using 0.0 (v/v) % oxygen. These observations are in contradiction with most studies previously reported in literature. According to Laroussi (2005) and Fernández & Thompson (2012), the presence of oxygen enhances the killing efficacy of CAP. Oxygen-based reactive species have been reported to have a strong oxidative effect on the outer surfaces of the cells, i.e., unsaturated fatty acids present in the membrane of the cells can react with oxygen-based reactive species such as hydroxyl radicals (OH.), resulting in a compromised function of the membrane lipids. Atomic oxygen on the other hand can oxidize proteins, resulting in structural changes and malfunctioning of proteins/enzymes (Laroussi 2005). Nevertheless, previously mentioned studies were not performed using bacterial cells growing as biofilms. A possible explanation for the contradictory results obtained for CAP inactivation of biofilms can be found in the research of Duan et al. (2017). In this research, the penetration depth of the reactive oxygen species (ROS) and reactive nitrogen species (RNS) through biological tissue was investigated. For a tissue thickness of 500 µm, only 5 % of the ROS could penetrate through the complete tissue, while for the RNS, up to 80 % of the species were able to do this. In addition, in the research of Hao et al. (2014), it was reported that NO is an important reactive species for bacterial inactivation and dispersal of biofilms. According to Patil et al. (2016), the highest amount of NO is formed if the ratio of N2 and O2 in the feed gas is equal to 2. If no oxygen is added to the feed gas, the ratio of N2 and O2 becomes closest to this optimal value. Assuming that the helium feed gas contains mainly impurities from air (due to the enclosure that is not completely airtight and due to the impurities of the used helium gas), this ratio would be 3.71 (78 % N2 and 21 % O2 in air). Consequently, the formation of NO (and other RNS) would be more optimal without addition of oxygen to the feed gas, resulting in the formation of RNS which are able to penetrate further into the biofilm. As a result, biofilm-associated cells might get more efficiently inactivated when no oxygen is added to the gas flow. However, in the research of Puligunda & Mok (2017), it was mentioned that ROS can easily penetrate biofilms and kill the biofilm-associated cells. Future research investigating the biofilm inactivation mechanism of CAP could possibly aid understanding the influence of the oxygen concentration on the CAP inactivation efficacy for biofilms in particular.

Finally, since no correlation can be observed between the oxygen level of the gas flow and the residual percentage of sub-lethal injury (Figure 2A and 2B), the percentage of sub-lethally injured cells is not a deciding factor to determine the most optimal oxygen level of the gas flow. Consequently, if a more effective biofilm inactivation is required, it would be advised not to add oxygen to the feed gas, while addition of oxygen would favor a faster (but less efficient) treatment.

## 3.4. Influence intensity

As for the influence of the electrode configuration and the oxygen level, the intensity/input voltage does not have an effect on the shape of the inactivation curves obtained for both microorganisms (Figure 2A and 2B). However, the applied intensity/input voltage does have an influence on the inactivation rate and the residual population (Table 1 and 2).

For *L. monocytogenes*, the highest *kmax* values are in most of the cases obtained at the highest input voltage of 21.88 V. It should be mentioned, however, that in two of these cases (i.e., DBD – 0.0 (v/v) % oxygen and SBD – 0.5 (v/v) % oxygen), no significant difference can be observed between the values obtained at 13.88 and 21.88 V. In addition, for three combinations of electrode configuration and oxygen level, the highest *kmax* value is obtained at 17.88 V (DBD – 0.5 (v/v) % oxygen) or the input voltage has no influence on this model parameter (DBD/SBD – 1.0 (v/v) % oxygen). For these combinations with 1 (v/v) % oxygen, the obtained average values are, however, still the highest at the highest intensity. For the DBD configuration, the log-linear inactivation phase tends to become longer as the intensity/input voltage increases. In other words, as the input voltage increases, a longer CAP treatment time will be required to obtain the final log10-reduction. For the SBD configuration, there seems to be no major influence of the intensity/input voltage on the duration of the log-linear inactivation phase.

With respect to the influence of the input voltage on the residual *L. monocytogenes* cell density, it can be observed that the log10 *Nres* value decreases as the intensity/input voltage increases. There is only one exception, i.e., the intensity/input voltage does not influence this model parameter while using the SBD at 0.0 (v/v) % oxygen. Combined with the log10 *N0* value, it can be concluded that the obtained log10-reductions increase as the intensity/input voltage increases, i.e., obtained log10-reductions (depending on the applied electrode configuration and oxygen level) range between 1.1 – 2.3, 1.4 – 2.8, and 1.7 – 3.6 log10 (CFU/cm²) at 13.88, 17.88, and 21.88 V, respectively. This means that the efficacy of the CAP treatment for inactivation of *L. monocytogenes* biofilms increases as the intensity/input voltage increases.

For inactivation of the *S.* Typhimurium biofilms, the influence of the input voltage on the inactivation rate is not straightforward. For two cases (i.e., DBD – 0.0 (v/v) % oxygen and SBD – 1.0 (v/v) % oxygen), the highest inactivation rate is obtained at the lowest input voltage of 13.88 V. On the other hand, using the SBD at 0.0 (v/v) % oxygen and the DBD at 0.5 (v/v) % oxygen results in significantly higher *kmax* values at an input voltage of 21.88 and 17.88 V, respectively. For the remaining two cases (i.e., SBD – 0.5 (v/v) % oxygen and DBD 1.0 (v/v) % oxygen), the input voltage does not influence the inactivation rate. With respect to the duration of the log-linear inactivation phase, it is not possible to observe any general trend concerning the influence of the intensity/input voltage on this parameter (Figure 2B).

For the influence of the intensity/input voltage on the log10 *Nres* value, no significant differences can be observed for most of the cases. However, for two cases (i.e., DBD – 0.0 (v/v) % oxygen and SBD 0.5 (v/v) % oxygen), the lowest residual cell densities are obtained at 13.88 V, although not always significantly different from the values obtained at 21.88 V. While comparing the log10-reductions, it can be concluded that the highest values are in most cases obtained while using the highest input voltage of 21.88 V. The obtained log10-reductions at 21.88 V range between 1.7 and 3.5 log10 (CFU/cm²), while at the other two input voltages log10-reductions between 1.4 – 2.6 log10 (CFU/cm²) (13.88 V) and 1.3 – 2.6 log10 (CFU/cm²) (17.88 V) are obtained. Only for two cases (i.e., SBD – 0.5 (v/v) % oxygen and SBD - 1.0 (v/v) % oxygen), the input voltage has no influence on the log10-reductions, indicating that CAP treatment with the SBD configuration is less affected by the intensity/input voltage. The significant influence of the intensity/input voltage on the log10-reduction might be in contradiction with the minor influence of this plasma characteristic on the residual cell density, however, observed differences between the log10-reductions can be explained based on the slightly different initial *S.* Typhimurium cell densities for each intensity/input voltage.

Based on the obtained results, it can be concluded that (in general) the efficacy of CAP for inactivation of both pathogenic biofilms increases with an increased intensity/input voltage. In the research of Deng et al. (2007) and Han et al. (2014), although these studies were performed with planktonic *Escherichia coli* cells, a similar conclusion was drawn, i.e., increased log10-reductions were obtained with an increasing voltage. In general, the higher inactivation efficacy at a higher intensity/input voltage can be explained based on the research of Lerouge et al. (2001) and Xu (2001). According to the former, increasing the plasma power (or intensity) leads to an increased electron density and a corresponding increased concentration of active plasma species (Lerouge et al. 2001). According to the latter, the discharge created while using a DBD or SBD configuration results in a large number of current filaments (micro-discharges). Since these are proportional to the voltage applied on the electrodes, the higher efficacy of CAP at an increased input voltage could be the result of an increased number of created micro-discharges (Xu 2001).

As for the influence of the electrode configuration, it is important to mention that the residual percentage of sub-lethal injury in general tends to increase as the input voltage increases. So, despite the higher log10-reductions obtained at the highest input voltage, this increased amount of sub-lethally injured cells following CAP treatment (up to 85%) should be taken into account as sub-lethally injured cells might recover once they re-encounter favorable environmental conditions (Noriega et al. 2013). Consequently, they can be a possible source of contamination, which might result in health risks and/or economic losses. Nevertheless, since the total kill of bacterial cells is still the highest at the highest input voltage, these treatment conditions are still advised to obtain an optimal biofilm inactivation efficacy.

When the log10-reductions obtained for both microorganisms are compared with each other, it can be concluded that CAP inactivation is as effective for both pathogenic biofilms, i.e., maximum average log10-reductions (on general medium) are 3.6 and 3.5 log10 (CFU/cm²) for *L. monocytogenes* (Gram positive) and *S.* Typhimurium (Gram negative), respectively. This means that the difference in cell wall composition between the selected Gram positive (thick peptidoglycan layer and no outer cell membrane) and Gram negative bacterium (thin peptidoglycan layer and outer cell membrane consisting of lipopolysaccharides) does not influence the CAP inactivation efficacy. However, due to a higher initial cell density of the *L. monocytogenes* model biofilms compared to the model biofilms for *S.*Typhimurium, the remaining residual cell density for the former is higher, which makes it more of a risk with respect to possible (cross) contamination of food products.

# 4. Conclusions

The goal of this research was to investigate the influence of three different plasma characteristics (i.e., the electrode configuration, the oxygen level of the gas flow, and the intensity/input voltage) on the CAP inactivation efficacy for *L. monocytogenes* and *S.* Typhimurium biofilms. The obtained datasets were fitted using the predictive model of Geeraerd et al. (2000), leading to several (general) conclusions based on the obtained model parameters for this specific model. These conclusions are: (i) the DBD configuration has the highest biofilm inactivation efficacy, (ii) the highest log10-reductions are obtained while using 0.0 (v/v) % oxygen, however, addition of oxygen results in a significantly faster inactivation, and (iii) increasing the input voltage results in an increased efficacy as well. At these specific conditions (i.e., 21.88 V – 0.0 (v/v) % oxygen – DBD), (general medium) log10-reductions of approximately 3.5 log10 (CFU/cm²) have been obtained for both pathogenic biofilms following less than 10 min of CAP treatment, which is promising for the application of CAP for inactivation of biofilms. Nevertheless, the CAP treatment alone is not sufficient for complete biofilm inactivation. Therefore, it should be incorporated within a complete cleaning protocol, combined with other treatments and/or control actions. In this case, despite the lower obtained log10-reductions, addition of oxygen to the feed gas might be considered to decrease the total equipment downtime.

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# DECLARATIONS OF INTEREST

None.

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# FIGURES

Figure 1 Dielectric Barrier Discharge (DBD) and Surface Barrier Discharge (SBD) electrode configuration used for this research – A: Schematic representation of the DBD configuration, B: Specific DBD used in this study, C: Schematic representation of the SBD configuration, and D: Specific SBD used in this research.

Figure 2 Cell density (log10 (CFU/cm²)) and percentage (%) of sub-lethally injured (SI) cells as function of the treatment time when applying two different electrode configurations (DBD/SBD), three different oxygen levels (0.0/0.5/1.0 (v/v) % oxygen), and three intensities (13.88/17.88/21.88 V input voltage). For the cell density as function of the treatment time, both the experimental data (symbols) and the global fit (line) of the Geeraerd et al. (2000) model are represented: total viable population on general medium (o, solid line) and uninjured viable population on selective medium (x, dashed line). For both the cell density and the percentage of SI, different oxygen levels are indicated in different colors, i.e., black, red, and blue are used to illustrate the results obtained with 0.0, 0.5, and 1.0 (v/v) % oxygen, respectively. A: results *L. monocytogenes* biofilms and B: results *S.* Typhimurium biofilms.

# TABLES

Table 1 Estimated model parameters Geeraerd et al. (2000) model for all examined combinations of plasma characteristics (electrode configurations/oxygen levels/intensities) for inactivation of the *L. monocytogenes* model biofilms.

Table 2 Estimated model parameters Geeraerd et al. (2000) model for all examined combinations of plasma characteristics (electrode configurations/oxygen levels/intensities) for inactivation of the *S.* Typhimurium model biofilms.