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Effect of Atmospheric Pressure Plasma (APP) on Listeria monocytogenes attached to abiotic surfaces

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Running title: Plasma on *Listeria monocytogenes* attached to abiotic surfaces

Effect of Atmospheric Pressure Plasma (APP) on *Listeria monocytogenes* attached to abiotic surfaces

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Keywords: *Listeria monocytogenes*, Plasma treatment, VBNC, injured cells, RNA analysis

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ABSTRACT

Listeria monocytogenes is introduced into food processing plants via raw material of animal or plant origin and it can establish endemic populations through formation of biofilm. Biofilms represent a continuous source of contamination for food products and it has been repeatedly demonstrated that in this form *L. monocytogenes* is more resistant to stress and sanitizing agents than planktonic cells. The use of gas-discharge plasmas potentially offers a good alternative to conventional sanitization methods. Plasmas represent a mixture of charged particles, chemically reactive species and UV radiation, which are effective in the destruction of microorganisms. The purpose of this study was to measure the effectiveness of cold atmospheric pressure plasma (APP) treatments against bacteria attached to a solid surface, evaluating also the individual susceptibility of different *L. monocytogenes* strains. The attention was focused on the state of the cells after the treatment, combining detection by viable count and quantitative PCR (qPCR). Results showed how most of the culturable cells are inactivated after the Plasma exposure but the RNA analysis by qPCR targeting the 16S rRNA highlighted the presence of injured cells or their entrance in the viable-but non culturable state (VBNC). These results were at least partly confirmed by a resuscitation experiment. Incubation of cell suspensions, after APP treatment, in BHI broth showed that part of the *L. monocytogenes* population grew in the medium and therefore survived the treatment. The understanding of the effects of APP on the *L. monocytogenes* cells can support the development of sanitation programs with the use of APP for effective pathogen removal. It is important to consider that methods independent of the culturability of the cells have to be used to monitor pathogens in food processing plants since cultivation may underestimate microbial load.

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INTRODUCTION

The transmission of *Listeria monocytogenes* by contaminated food was first demonstrated by epidemiologic and laboratory investigation in 1983, and has since been shown to cause sporadic cases and outbreaks of listeriosis *(22).*

L. monocytogenes has been isolated from many different environments and especially from soil, water, vegetation, sewage, animal feeds, farm and food-processing environments *(21).* Transient populations of the microorganism are likely introduced in processing plants by raw materials naturally contaminated, and may be able to establish themselves on food contact (e.g. slicers, conveyers) and non-contact (drains, walls and cold store condensers) surfaces. Once *L. monocytogenes* populations become established, they can persist for many years *(15)*. Among other factors, differential aptitude to persist in a specific environment plays a role in the ability of a strain to contaminate foods and consequently to cause food-borne outbreaks.

Many bacteria are able to attach to and colonize environmental surfaces by producing biofilm *(8).* Biofilm formation allows microorganisms to persist in the environment and resist desiccation, UV light and treatment with antimicrobial and sanitizing agents. Established biofilms can tolerate antimicrobial agents at concentration of 10-1000 times higher than those needed to kill genetically equivalent planktonic bacteria, and are also extraordinarily resistant to phagocytosis, making biofilm extremely difficult to eradicate from living hosts (*15*). Due to these reasons, new sanitization techniques for the food plant environment and the development of antimicrobial measures that are not subject to evolving microbial resistance represent a new challenge to foodborne pathogen control. In this context, the cold atmospheric pressure plasma (APP) is a relatively new decontamination technique that has been widely tested at experimental level for applications in the food industry. The most attractive features of plasma is the possibility to apply it without altering the temperature characteristics of the product/element being treated and the high efficiency of microbial inactivation of both vegetative cells and spores (Lee et al. 2006), as well as viruses *(23)*. Plasma treatments can be useful in food processing including the dry disinfection of food surfaces. The reactive free radicals and H_2O_2 produced during APP generation play the main role in bacterial inactivation *(24)* together with the oxidation of amino acids and nucleic acids, which determine microbial death or injury *(25)*. Different studies *(5, 24)* showed the higher potential of APP against Gram negative bacteria if compared to Gram positive bacteria, that can be explained by the higher resistance offered by the peptidoglycan structure.

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In meat-processing environments, all surfaces and tools (e.g. pipelines, knifes, hooks, gaskets, conveyor belts, etc) are likely to be colonized by microorganisms if sanitation procedures are inadequate and/or insufficiently frequent. In addition, most of the surfaces present potential harborage niches (hollow parts, crevices, cracks, unpolished or worn materials) that are difficult to clean and disinfect: different bacteria can colonize and occupy these surfaces, becoming more tolerant to sanitation procedures *(9).*

In evaluating any sanitization processes, it is important to understand the state of the cells after the treatments: cells can be stressed and non-culturable, but may not be necessarily dead. The most commonly used technique to assess bacterial inactivation is to grow cells in a suitable medium and then count the bacterial population using the classic colony counting methodology. However variation in the surrounding conditions can influence bacterial counts and because of the environmental instability, the bacteria can enter in a viable but non-cultivable state (VBNC) *(16).* The VBNC state induced in bacteria refers to a failure of bacterial growth using the conventional cultivation techniques in which they would normally grow and develop colonies *(6).* Normally, VBNC bacteria possess negligible metabolic activity and can be resuscitated in the proper conditions; becoming again cultivable *(16).* Also molecular methods, by targeting the ribosomal RNA and DNA by PMA-qPCR *(17)*, are described as a useful tool in the detection of viable microbiota *(4).*

The purpose of this study was to measure the effectiveness of APP treatments against different strains of *L. monocytogenes*. For this purpose, biofilm formation *in vitro* was promoted, cells were subjected to APP treatment and then viability was evaluated by both viable count and qPCR, targeting the 16S rRNA.

MATERIALS AND METHODS

Preparation of cultures. All strains of *L. monocytogenes* belonged to the DISAFA culture collection. More specifically, two International collection strains (EGDe, NCTC 10527), six strains isolated from food matrices (3, 19 and 36 from meat products, 70 and 162 from dairy products) and one of human origin (V7), were employed in the study.

Each strain, conserved at -80 °C in cryovials, was inoculated in BHI broth (Oxoid, Milan) at 37 °C for 24 h reaching a count of 9 Log CFU/ml. Cell density was determined by serial dilution and plating on Aloa selective medium (Biogenetics, Padova, Italy).

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Bacterial enumeration of attached cells. *L. monocytogenes* strains under analysis were evaluated for their ability to attach to a solid surface. Stainless steel (SS) coupons (7 x 2 cm) were the abiotic surface used for biofilm formation, since this material is frequently used for the manufacture of food processing equipment. Prior to use, coupons were cleaned and sterilized (121°C for 15 minutes) and then individually placed in sterile 50-ml conical centrifuge tubes containing 45 ml of BHI. The culture of *L. monocytogenes* prepared as described above was inoculated in the conical tubes containing a coupon and BHI broth at a ratio of 1%. The attachment ability of different *L. monocytogenes* strains on the stainless steel surface was determined after incubation at a temperature of 37 °C for 6, 24, 48 and 144 hours. The experiment was performed three times. Detachment of attached cells from the SS coupons was performed by using the bead vortexing method *(2 ,9)* with some modifications. SS coupons were aseptically removed, rinsed twice with sterile Ringer's solution (Oxoid, Milan, Italy) and left 5 min in Ringer solution between the two rinses to remove any loosely attached cells. SS coupons were then transferred into new sterile conical tubes containing 15 ml of Ringer's solution and 10 glass beads (Sigma aldrich, diameter: 3 mm, weight: 0.0034 g) and shaken with a benchtop vortex mixer set at maximum speed for 2 minutes. Immediately after vortexing, cell suspensions were tenfold serially diluted in Ringer's solution (Oxoid) and 0.1 ml of undiluted cell suspension or dilutions were spread-plated on Aloa selective agar to enumerate *L. monocytogenes* cells attached to the SS coupons. The plates were incubated at 37 °C for 48 hours. After incubation, colonies were counted.

Plasma treatment. Three *L. monocytogenes* strains (EDGe, 3 and 36) were selected on the basis of their provenience (a collection strain and two strains isolated from meat products) and their ability to form biofilm on SS coupons.

For this study, a prototype of dielectric barrier discharge (DBD) cold atmospheric plasma system was designed. The experiments were conducted using nitrogen gas: different powers and exposure times were tested to evaluate the efficacy of cold atmospheric plasma on the biofilm produced on SS coupons by the three strains after 144 hours. The plasma was generated at an input power of 1154, 760 or 430W with respective frequency of 142, 151 an 159 KHz for a time period of 10 min or 2 min for each side of the SS coupon. The APP conditions are reported in Table 1. The characterization of plasma was performed by the Optical Emission Spectrometry (OES) that highlighted the antimicrobial effects attributable to the generation of reactive oxygen and nitrogen species (RONS) and UV photons.

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After the treatment, in order to count the viable cells, the protocol described above for the bacterial enumeration was adopted. The cell suspension obtained was also conserved at -80°C in the presence of RNA*later* (Ambion, Italy) for subsequent use in RNA extraction.

RNA extraction and RT-qPCR. Master- Pure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA) following the manufacturer's instructions was adopted for total RNA extraction from the samples immediately after the APP treatment *(19).* Re-suspended RNA was treated with Turbo DNase (Ambion, Italy), in order to eliminate the DNA. Complete DNA digestion was confirmed using 1 μL in PCR and when a PCR product was obtained, the DNase treatment was repeated.

Reverse transcription (RT) reactions were performed using the M-MLV reverse transcriptase (Promega, Milan, Italy). Nine μL of RNA were mixed with 1 μL of 100 μM of Random primers (Promega) and the reverse transcription was carried out at 42 °C for 1 h. One μL of the obtained complementary DNA was used as template for the qPCR amplification of the V3 region of the 16S rRNA, using primers 338f and 518r *(3).* Amplifications were performed in a final volume of 25 μl in the Chromo 4 Real-Time PCR Detection System (Biorad, Milan, Italy) with the use of SSo Advanced Sybr Green Supermix (Biorad, Italy). Samples were amplified in triplicate using the following conditions: initial denaturation at 95 °C for 5 min and 40 cycles of 95 °C for 15 s and 60°C for 30 s, according to *(1).*

Construction of standard curve for enumeration of *L. monocytogenes*. In order to obtain a culture-independent enumeration of *L. monocytogenes* cells, a standard curve was constructed. Ten-fold serial dilutions of an overnight culture of *L. monocytogenes* strains were performed in Ringer's solution (Oxoid, Milan, Italy). One ml of each dilution was subjected to RNA extraction as described above. RT was performed using 9 μL of RNA and the resulting cDNA sample was submitted to qPCR. Standard curves were constructed by plotting the threshold cycle (Ct) values obtained against CFU/ml, as determined on BHI agar from each dilution. Correlation coefficient (R^2) and efficiency of amplification were calculated as previously described *(11).*

Resuscitation of the VBNC cells. After the APP exposure for 10 min at three APP intensities (conditions A, B, C; Table 1), coupons were aseptically inserted in BHI broth in order to evaluate the vitality of the cells. This resuscitation step was performed leaving the coupons in the medium at 37°C for 24 hours and visually evaluating increase in broth turbidity due to bacterial growth.

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Statistical analysis. Data were subjected to one-way ANOVA, and Duncan test was used to determine differences at $p < 0.05$, using the statistical software, STATISTICA 7.0 for Windows (Statsoft, Tulsa, USA).

RESULTS AND DISCUSSION

Several studies have demonstrated that bacteria in biofilm are more resistant to the action of sanitizers than planktonic cells *(18)*. In order to obtain the inactivation of biofilm cells, high concentration of sanitizers for long time are needed. In this context the use of alternative, non-chemical methods in the plant sanitization represents an interesting aspect warrant investigation. The purpose of this study was to investigate the effect of different combinations of time/intensity APP treatments on *L. monocytogenes* cells attached on a stainless steel surface. In this study nitrogen was adopted for plasma generation as substitute of other more expensive noble gases as helium and argon: nitrogen has been widely evaluated for plasma generation and several publications have reported the efficacy of this type of plasma treatments (*26*). The different levels of power tested were chosen in order to cover the operative range of the Plasma generator while the treatment time intervals in order to simulate applicative time in an industrial setting (2 min) and the sanitation at the end of the production day (10 min).

In the first step of the work the ability of different strains to attach to abiotic surfaces was evaluated. Table 2 shows numbers of Log CFU/cm^2 of attached *L. monocytogenes* on SS as determined on Aloa medium. Starting from the same inoculum concentration, after 6 hours a variation in the counts was already evident. Strain EGDe showed the lowest counts, with 1.43 Log CFU/cm^2 together with strain 3 (1.99 Log CFU/cm^2). The Duncan test showed significant differences between strains (as reported by the lower case letters, Table 2) but no correlations were detected with their origin. Strain V7 isolated from human, manifested a good capacity to attach to the surface as demonstrated by the higher count at 6 hours. Some strains, namely strains 70, 162 and V7, showed strong adhesion ability at the beginning of the followed period, but the concentration of attached cells reduced with time. On the contrary, other strains displayed a trend of increasing attachment with time and reached highest counts after 144 hours. Accordingly, it can be deduced that the attachment ability was strain dependent. Furthermore, it was evident that the time of contact with the abiotic surface played a role in the attachment. Under the conditions tested here, significant increase in the concentration of cells attached was observed between 6 and 24 hours, for

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all strains except one (Table 2, capital letters). In the evaluation of these results it has also to be considered that an amount of cells may remain attached to the coupons after the vortex treatment: in this case an underestimation can occur in the count of the cells.

In the second step of the work we focused on the effect of the APP treatment on biofilm removal, considering both cultivability and vitality of cells attached to the SS coupons. In order to investigate the effect of APP on the destabilization of these cells, 6 combinations of intensity and time were tested using 3 different strains of *L. monocytogenes*, namely strains EGDe, 3 and 36, which showed the highest values of adhesion among the strains tested in this study. After the period of adhesion (144 hours), strains on coupons were treated with APP and after 10 minutes of treatment for all 3 intensities tested, *L. monocytogenes* was not detected by plate counts. In the case of shorter APP exposure time the lowest power (condition F) was not suitable for the inactivation of the cells but contributed to reduce the count by more than 2 Log, while in the case of conditions D and E no colonies were detected on Aloa medium (data not shown).

In order to enumerate *L. monocytogenes* in a culture-independent way, qPCR was applied on RNA extracted from cell suspensions recovered from the SS coupons. Beforehand, it was necessary to construct standard curves that correlate the C_t value obtained from the qPCR analysis with the plate count. The linearity range of the standard curve was from 10^8 to 10^3 CFU/ml, covering 5 orders of magnitude (data not shown).

The standard curves were used to quantify *L. monocytogenes* cells attached to the SS coupons after APP treatment. The results obtained analysing the RNA (targeting the 16S rRNA) showed the presence of cells also in the coupon treated for 10 minutes. Non APP treated cells showed the highest count (6 Log CFU/cm^2 for strain 36), while differences between strains and treatments were observed. Regarding strain 3, in the conditions E and F (treatment for 2 minutes) RT-qPCR results were similar to those of non-treated SS coupon (control). In the case of strains 36 and EGDe all the APP conditions reduced the counts significantly compared to the control (Table 3).

As can be seen, differences were observed in the population load determined by viable count and qPCR, after the APP treatment. In order to assess if the APP treatment for 10 min induced a VBNC state, coupons were inserted in BHI broth. After 24 hours of incubation at 37 °C, growth in BHI was observed, through the increase in broth turbidity, highlighting that a viable population of *L. monocytogenes* was resuscitated. In the coupons treated with the lowest plasma power, the broth turbidity was observed for all the strains tested underlining the capability of cells to survive for as much as 10 minutes at the APP treatment (data not shown). This resuscitation step confirmed the presence of injured cells or their entrance in the VBNC state due to the APP treatment. This result

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suggests that cells of *L. monocytogenes*, that are not cultivable immediately after APP treatment, have the potential to recover from the injury during resuscitation and become culturable. The RNA analysis performed by RT-qPCR, also suggested a vitality of cells on the contrary of what traditional methods showed. The traditional methods showed that bacteria attached to surfaces can be reduced by using gas-discharge plasma, thus confirming the potential of plasma as an alternative sterilization method. However, the discrepancies observed between the methods employed have to be considered in the evaluation of the efficacy of APP treatment. The plate count highlighted the suitability of the APP in eliminating *L. monocytogenes* cells colonizing a SS surface while by targeting the 16S rRNA, the presence of viable populations was revealed. These results were at least partly confirmed by the resuscitation experiment: incubation of cell suspensions, after APP treatment (condition A) in BHI broth, showed that part of the *L. monocytogenes* population survived the treatment and grew in the BHI medium. Therefore, plasma treatment damaged, but did not eliminate the *L. monocytogenes* cells attached to SS coupons.

Vitality of bacteria is an important aspect, especially in the food safety sector. Cells that appear unculturable in laboratory conditions may still possess several functions and activities typical of living cells *(13)*. It is widely accepted that exposure of foodborne pathogens to sublethal stresses as those applied in food industries may alter their subsequent survival and proliferation characteristics *(12)*. Unculturability may result from the lack of suitable growth or from survival mechanisms triggered by stress conditions *(13)*. The resuscitation in BHI was not obtained for coupons treated at high APP intensity, but the results of qPCR counts with about 4 Log CFU/cm^2 may suggest the vitality of cells. It should be underlined that confirmation of the VBNC state can only come from the resuscitation at least of part of the population that was previously non-culturable. In this study, this was confirmed in one out of the APP treatments applied. Whether the culturable cells which appear following removal of the inducing environmental stress are a result of true resuscitation or of regrowth of a few residual culturable cells is a matter of further discussion *(26)*. Similarly to *(7)* we can state that the conventional cultivation methods overestimate the decontamination efficiency of the APP, and must therefore be complemented by alternative techniques capable of detecting damaged and/or viable but non-culturable bacteria. Notwithstanding the discrepancies observed between culture dependent and independent approach, the APP resulted to be effective in decreasing the load of attached cells. The untreated sample has shown higher counts by both traditional and molecular methods, compared with those that were treated, confirming that APP activity may challenge the physiology of microorganisms.

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The effect of APP treatment on the bacterial populations was often determined by plate count only: on the base on the results obtained in this study, it could be interesting to evaluate the state of the microbiota on food after the APP by RNA analysis or resuscitation approaches. Considering that VBNC populations can subsequently recover, grow and/or contaminate the food *(20)*, as also affirmed by *(17)*, it is important that methods independent of the culturability of the cells are used to monitor pathogens in food processing plants.

In conclusion the results of this study are of relevance to food safety. Quantification of cells through the combined use of traditional and molecular methods, underlined the presence of populations after APP treatment. VBNC populations and the presence of injured cells of foodborne pathogens able to resuscitate represent potential risk to the health of consumers. The understanding of the effects of APP on the *L. monocytogenes* biofilm is a prerequisite for the development of sanitation programs, based on APP, for effective pathogen removal.

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TABLE 1. The APP conditions on SS coupons: time and power

	CONDITIONS
CONTROL	No treatment
A	10 min each side - 431W-159 KHz
B	10 min each side - 724W-151 KHz
C	10 min each side - 1154W-142 KHz
D	2 min each side - 1154W-142 KHz
E	2 min each side - 741W-151 KHz
F	2 min each side - 431W-159 KHz

TABLE 2. Results of variance analysis and Duncan test performed on *L. monocytogenes* adhesion on SS coupons expressed as Log CFU/cm^2 after 6, 24, 48 and 144 hours

		time			
		6 h	**24 h**	**48 h**	**144 h**
strains	**EGDE**	1.43 a A	3.17 a B	3.54 bc B	4.46 cd C
	3	1.99 b A	3.34 ab B	3.46 ab B	4.71 cd C
	36	2.89 c A	3.83 bc BC	3.7 d B	4.39 cd C
	70	3.69 d AB	3.78 c B	3.15 a A	3.12 b A
	162	3.85 d C	3.57 ab BC	3.33 ab B	1.87 a A
	NCTC	3.69 d NS	3.76 bc NS	4.02 e NS	4.09 c NS
	19	3.05 c A	4.92 d D	4.54 f C	3.42 b B
	V7	4.82 e B	5.6 e C	4.73 f B	4.02 c A

Values with different letters are significantly different, P < 0,05. Lowercase indicate differences between strains. Capital letters indicate differences among different time points. Values without letters are not significantly different. NS not significant.

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TABLE 3. Enumeration of *L. monocytogenes,* by RT-qPCR, after the APP treatments. Results are expressed as Log CFU/cm^2 and are based on the conversion of the C_t value obtained by qPCR into CFU/cm^2, using as a reference the standard curve previously constructed.

		Plasma conditions							
		Control	**A**	**B**	**C**	**D**	**E**	**F**	sig.
strains	**3**	5.48 b	3.82 a	3.91 a	3.5 a	3.65 a	5.13 b	4.99 ab	p<0.05
	36	6.04 b	4.5 a	3.825 a	3.48 a	4.125 a	4.095 a	4.79 a	p<0.05
	EGDe	5.44 b	4.57 a	4.22 a	4.01 a	4.34 a	4.88 a	4.68 a	p<0.05

The Plasma conditions (A-E) are reported in Table 1. Values with different letters are significantly different among treatments for each strain, P < 0,05.

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