

Microbial consortia in meat processing environments

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Abstract. Microbial contamination in food processing plants can play a fundamental role in food quality and safety. The description of the microbial consortia in the meat processing environment is important since it is a first step in understanding possible routes of product contamination. Furthermore, it may contribute in the development of sanitation programs for effective pathogen removal. The purpose of this study was to characterize the type of microbiota in the environment of meat processing plants: the microbiota of three different meat plants was studied by both traditional and molecular methods (PCR-DGGE) in two different periods. Different levels of contamination emerged between the three plants as well as between the two sampling periods. Conventional methods of killing free-living bacteria through antimicrobial agents and disinfection are often ineffective against bacteria within a biofilm. The use of gas-discharge plasmas potentially can offer a good alternative to conventional sterilization methods. The purpose of this study was to measure the effectiveness of Atmospheric Pressure Plasma (APP) surface treatments against bacteria in biofilms. Biofilms produced by three different *L. monocytogenes* strains on stainless steel surface were subjected to three different conditions (power, exposure time) of APP. Our results showed how most of the culturable cells are inactivated after the Plasma exposure but the RNA analysis by qPCR highlighted the entrance of the cells in the viable-but non culturable (VBNC) state, confirming the hypothesis that cells are damaged after plasma treatment, but in a first step, still remain alive. The understanding of the effects of APP on the *L. monocytogenes* biofilm can improve the development of sanitation programs with the use of APP for effective pathogen removal.

1. Introduction

Spoilage organisms can be transferred from the environment to intermediates of production and may negatively affect the production process and the quality of the final product. The first purpose of this study was to characterize the type of microbiota in the environment of meat processing plants situated in north west of Italy and gain insights regarding potential microbial contamination risks for the final products.

The second step of the work focused on the evaluation of an alternative sanitization technique that has become an important aspect in the food environment. The development of antimicrobial measures that are not subject to evolving microbial resistance represent a new challenge in the food control. In this context, the cold atmospheric pressure plasma (APP) is a relatively new antimicrobial technique that has been recently adopted also for applications in the food industry. The reactive free radicals and H₂O₂ produced during APP generation play the main role in bacterial inactivation [1] together with the oxidation of amino acids and nucleic acids that are involved in microbial death or injury [2]. Evaluating any sanitization process, particular attention has to be focused in the understanding of the state of the cells after the treatments: cells can be stressed and non culturable but may not necessarily be dead. Variation in the surrounding conditions can influence bacterial counts and because of the environmental instability, the bacteria can enter in a viable but non-culturable state (VBNC) [3].



The purpose of this second part of the study was to measure the effectiveness of APP treatments against bacteria organized in biofilm on surfaces, evaluating also the individual susceptibility of different *L. monocytogenes* strains. The attention focused in particular on the state of the cells after the treatment and on their possible entry in the VBNC state by traditional and molecular methods.

2. Material and methods

2.1. Traditional and molecular methods in the analysis of food processing plants

The microbiota of three different meat plants was studied by both traditional and molecular methods (PCR-DGGE) in two different periods (winter and summer). Environmental samples from surfaces and tools (swabs from knives, blades, walls, conveyor belts, hoppers, bagging, saw bones, table surfaces, cutter, meat mixers) were analyzed and the occurrence of pathogens (*Listeria monocytogenes* and *Salmonella* spp.) was also investigated.

Plate Count Agar was used for total mesophilic aerobic count. *L. monocytogenes* and *Salmonella* spp. were investigated following the ISO methods.

DNA was extracted directly from swabs and culture-independent analyses were carried out by PCR-DGGE of V3 region of 16S rRNA gene [4].

2.2. Plasma treatment

Three *L. monocytogenes* strains and in particular a collection strain (EDGE) and two from the Unito culture collection (3 and 36) were selected for the evaluation of their ability to attach to abiotic surface. Different conditions of APP were tested on the biofilm produced on stainless steel (SS) coupons by these three strains after 144 hours. The plasma was generated at an input power of 1154, 760 or 430W for a time period of 10 min or 2 min each side of the SS coupon (Table 1). Detachment of attached cells from the SS coupons and plate counts were performed by using the bead vortexing method [5]. with some modifications. The cell suspension obtained was also conserved at -80°C in the presence of RNAlater (Ambion, Italy) for future use in RNA extraction.

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-151KHz
C	2 min each side- 1154W-142KHz
D	2min each side-741W-151KHz
E	2min each side-431W-159KHz

2.3 Resuscitation of the VBNC cells and enumeration of *L. monocytogenes*

After an APP exposure for 10 min, 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. In order to count the viable cells, the same protocol described above with bead vortexing was used.

RNA Purification Kit (Epicentre, Madison, WI, USA) following the manufacturer's instructions was adopted for the RNA extraction. Reverse transcription (RT) reactions were performed and one μ L of the obtained complementary DNA was used as template for the qPCR amplification of the bacterial V3 region of the 16S rRNA, using primers 338f and 518r [4]. Amplifications were performed 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, 60 °C for 30 s, according to [6].

In order to obtain a culture-independent enumeration of the active *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 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 coefficients (R^2) and efficiency of amplification were calculated as previously described [7].

3. Results and Discussion

3.1. Microbiota in food processing plants

Differences in the microbial populations (composition, load) between the three plants among the two periods were detected by traditional methods. More than 50% of samples analyzed in Plant 1 showed the presence of *L. monocytogenes* during the first sampling. Also *Salmonella* spp. was found with a high occurrence in Plant 1.

Plant 1 and 2 showed differences in the mesophilic aerobic counts between the two seasons, characterized by a high incidence of samples (more than 50%) with values $> 10^4$ cfu/cm² in the summer. Plant 3 did not report relevant differences among the two seasons.

By PCR-DGGE, tools and environmental samples were found to be characterized by the presence of several contaminants such as *Pseudomonas*, *Acinetobacter*, *Brochotrix*. Genera *Staphylococcus* and *Lactobacillus* were detected mainly in Plant 1. Swabs analyzed in winter and summer, for the same plant, showed a degree of microbial diversity (Table 2).

Table 2. Results of the identification of selected PCR-DGGE band sequencing

closest relative	Plant 1		Plant 2		Plant 3	
	winter	summer	winter	summer	winter	summer
<i>Pseudomonas</i> sp.	black	green	black	black	black	blue
<i>Pseudomonas migulae</i>	green	green	black	red	black	blue
<i>Pseudomonas graminis</i>	green	green	black	red	black	blue
<i>Acinetobacter xiamenensis</i>	black	green	red	red	black	blue
<i>Acinetobacter johnsonii</i>	black	green	black	black	black	blue
<i>Acinetobacter haemolyticus</i>	green	green	red	black	black	blue
<i>Acinetobacter calcoaceticus</i>	green	green	red	red	black	black
<i>Acinetobacter</i> sp.	green	black	red	red	black	black
<i>Staphylococcus saprophyticus</i>	black	green	red	red	black	blue
<i>Staphylococcus xylosum</i>	black	green	red	red	black	blue
<i>Staphylococcus lentus</i>	green	black	red	red	black	blue
<i>Staphylococcus sciuri</i>	green	black	red	red	black	blue
<i>Staphylococcus</i> sp.	green	green	red	red	black	black
<i>Leuconostoc citreum</i>	green	black	red	red	black	blue
<i>Lactobacillus plantarum</i>	green	black	red	red	black	blue
<i>Lactococcus piscium</i>	green	green	black	red	black	blue
<i>Micrococcus</i> sp.	green	green	red	black	black	blue
<i>Bacillus</i> sp.	black	green	red	red	black	blue
<i>Psychrobacter</i> sp.	black	green	red	red	black	blue
<i>Klebsiella</i> sp.	green	green	red	black	black	blue
<i>Moraxella</i> sp.	black	green	red	red	black	blue
<i>Corynebacterium</i> sp.	black	black	red	red	black	blue
<i>Blastocloris</i> sp.	black	green	red	red	black	blue
<i>Flavobacterium</i> sp.	green	green	black	red	black	blue
<i>Micrococcus</i> sp.	green	green	red	black	black	blue
Uncultured <i>Brochotrix</i> sp.	green	green	red	black	black	blue
<i>Exiguobacterium</i> sp.	green	green	red	black	black	blue
<i>Arthobacter</i> sp.	green	green	red	red	black	blue
<i>Spingomonas</i> sp.	green	green	red	red	black	blue
<i>Pantoea vagans</i>	green	green	red	black	black	blue

The presence of the black box indicates the presence of the bands in the DGGE profiles. The sequences obtained were aligned with those in GenBank with Blast program.

This study underlined the high incidence of spoilage microorganisms in the environment of meat processing plants and tools.

Traditional methods showed the presence of pathogenic bacteria such as *L. monocytogenes* and *Salmonella* spp. Differences emerged between the two sampling periods. The corrective actions taken after the first sampling, allowed a % decrease of contaminated samples. The mesophilic count was higher in the samples analyzed in the summer.

DGGE analysis underlined the occurrence of members of *Enterobacteriaceae*, lactic acid bacteria, *Pseudomonas* spp. and *Brochothrix* spp. that are recognized as the principal players in meat spoilage.

3.2. Plasma treatment

Due to the microbiota contamination found in the cleaned environment, the second step of the work focused on the evaluation of new sanitization techniques. In this context, the APP is a relatively new antimicrobial technique that has been recently adopted also for applications in the food industry.

This study investigated the effect of different combinations of time/intensity APP treatments on *L. monocytogenes* cells attached on stainless steel surface. After 10 minutes of AAP treatment, for all 3 intensities tested, *L. monocytogenes* was not detected by plate counts. In the case of shorter APP exposure time (2 min), the lowest power (431W-159Hz) reduced the count more than 2 Log CFU/cm² (data not shown).

After the APP exposure for 10 min, coupons were inserted in BHI broth in order to evaluate if cells could be resuscitated. Growth in BHI was observed in the coupons treated with the lowest plasma power 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 entrance of these cells in the VBNC state after APP treatment since no *L. monocytogenes* growth was observed without incubation in BHI at 37 °C for 24 hours.

In order to enumerate *L. monocytogenes* in a culture-independent way, qPCR was applied on RNA extracted from cell suspensions recover from the SS coupons. The results obtained analysing the RNA by the amplification of the 16S showed the presence of viable cells also in the coupon treated with the highest power for 10 minutes. Non treated SS coupons showed the highest count (6 Log CFU/cm² for strain 36). Differences between strains were observed. Regarding strain 3, the conditions D and E (treatment for 2 minutes) were no different compared to the control. In the case of strains 36 and EGDe, all the Plasma conditions reduced the count significantly compared to the control (Table 3).

Table 3. Inactivation kinetics after the APP treatments as resulted by molecular methods expressed as CFU/cm²

		Plasma conditions						sig.
		Control	A	B	C	D	E	
strains	3	5,48 b	3,82 a	3,91 a	3,65 a	5,13 b	4,99 ab	p<0,05
	36	6,04 b	4,5 a	3,825 a	4,125 a	4,095 a	4,79 a	p<0,05
	EGDe	5,44 b	4,57 a	4,22 ab	4,34 a	4,88 a	4,68 a	p<0,05

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

Traditional methods showed how most of the culturable cells are inactivated after the Plasma exposure but the RNA analysis obtained by q(PCR) highlighted the entrance of the cells in the viable-but non culturable (VBNC) state, confirming the hypothesis that cells are damaged after plasma treatment, but still remain alive.

The results showed that bacterial biofilms can be reduced by using gas-discharge plasma thus confirming the potential of plasma as an alternative sterilization method. However, discrepancies were observed between the two microbial enumeration methods employed: the plate count highlighted the suitability of the APP in eliminating *L. monocytogenes* cells organized in biofilms while by targeting the 16S rRNA by qPCR, the presence of VBNC populations was revealed and no significant differences

emerged between the different conditions of the treatments. These results were at least partly confirmed by the resuscitation experiment: incubating the cell suspensions, after APP treatment (at low intensity) in BHI broth, plate count results showed that part of the *L. monocytogenes* population survived the treatment. 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 [8]. The resuscitation in medium was not obtained for coupons treated at high APP intensity but the results of qPCR counts with about 4 Log CFU/cm² showed the vitality of cells. 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 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.

4. Conclusion

The results of this study highlight how it is vital for food producing companies to have control of the contaminants in the plants. This can be achieved with the implementation of adequate cleaning and disinfection procedures and it may contribute in the development of programs for effective pathogen removal.

The description of the microbial consortia in the meat processing environment is important since it is a first step in understanding possible routes of product contamination. Sanitizing procedures and VBNC state of the cells cover a fundamental role in the food safety: considering that VBNC populations can subsequently recover and grow [9]. As also affirmed by [10], it is important to use methods independent of cell culturability to monitor pathogens in food processing plants since cultivation may underestimate the microbial load.

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