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ATMOSPHERIC PLASMA PROCESSES FOR MICROBIAL INACTIVATION: FOOD APPLICATIONS AND STRESS RESPONSE IN *LISTERIA MONOCYTOGENES*

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Table of contents

Chapter 1. Emerging non-thermal technologies.

One of the main problems of the food industry is the microbial contamination of raw materials and finished products as the spoilage microflora reduces the product shelf-life, while the pathogenic species make foods unsafe. Nowadays, the industry is highly interested in replacing traditional food preservation techniques, which are mostly based on heat treatments and chemical preservatives, with new ones in order to satisfy the increasing consumer demand for “natural” and “fresh” food (Ross *et al.,* 2003). In this context numerous studies have been carried out in order to identify and develop new technological processes able to produce safe foods characterized at the same time by as-fresh-like properties. Indeed, these non-thermal technologies have the capability to inactivate microorganisms at room or near-room temperatures, therefore avoiding negative side effects including loss of sensory properties, such as aroma and colour, and nutritional value of products. On the other hand, depending to process conditions used, these technologies may positively affect some qualitative parameters. The mostly studied innovative technologies include:

- Pulsed Electric Fields (PEF)
- High Hydrostatic Pressure (HHP)
- High Pressure Homogenization (HPH)
- New packaging systems
- Biopreservation
- Natural antimicrobials
- Gas plasma (GP)

These technologies are fully placed within the "minimal food processing " and the concept of "hurdle technologies" which cause minimal damages to foods. The primary purpose of these technologies is to inactivate microorganisms with a limited use of heat, by keeping the organoleptic and nutritional quality of the food at high values.

1.1. Pulsed Electric Fields

Applying pulsed electric fields (PEF) consists in placing the food in an electrodes array and exposing to short electrical pulses of high field intensity (15–70 kV/cm) and very short duration (1–10 μs), which result in a really modest increase of temperature (below 60°C). The inactivation of microorganisms is related to changes in the cell membrane and its

electromechanical instability. Indeed the electric field can modify membrane permeability, due to its compression and pore formation, with subsequent loss of its functionality.

The number of pulses, pulse duration and electric field intensity play an important role in the microbial inactivation (Table 1), but also microbial species, cell sizes, cell wall construction and growth state affect the efficacy of the treatment (Aronsson *et al.,* 2001). Also the conductibility of the system strongly influences the inactivation effect, and for this reason PEF is usually applied to homogeneous liquids (such as fruit juices, milk, yogurt, beer and egg products) with a high protein content.

Table 1. Process parameters used for the inactivation of several pathogens in liquid foods by PEF treatments.

Microorganism	Fluid Food	E (kV/cm)	n[a]	τ^b (μs)	t_t^c (μs)	f (Hz)	T (°C)	Log_{10} reductions
L. innocua	Orange juice	30	6	2.0	12	—	54	6.0*
L. monocytogenes	Whole milk	30	400	1.5	600	1700	50	4.0
L. innocua	Skim milk	41	63	2.5	157.5	3	37	3.9
L. innocua	Liquid egg	50	32	2.0	64	3.5	36	3.4
L. innocua	Whole milk	29	312	0.8	250	100	36	2.0
L. innocua	Dairy cream	37.5	250	1.0	250	100	36	2.0
L. monocytogenes	Skim milk	20	10	3.25	32.5	—	35	1.0
E. coli	Liquid egg	26	100	4.0	400	2.5	37	6.0*
E. coli	Orange juice	30	6	2.0	12	-	54	6.0*
E. coli O157:H7	Apple cider	90	10	2.0	20	—	42	5.91*
E. coli 8739	Apple juice	29	43	4.0	172	1,000	42	5.4*
E. coli O157:H7	Apple juice	29	43	4.0	172	1,000	42	5.0*
E. coli	Liquid egg	32.89	180	0.17	30	—	20	4.7
E. coli O157:H7	Skim milk	41	63	2.5	157.5	3	37	4.0
E. coli O157:H7	Liquid egg	11	40	2.0	80	1	60	4.0
E. coli	Milk (1,5% fat)	23	20	—	—	—	45	4.0
Bacillus cereus	Skim milk	31	20		6.0		25	0.7
S. aureus	Raw milk	40	40	—	—	3.5	—	4.0
S. aureus	Skim milk	35	124	3.7	459	250	40	3.7
S. aureus	Skim milk	31	35		6.0		25	3.0
S. aureus	Skim milk	35	600	4.0	2,400	100	25	1.0
S. Typhimurium	Orange juice	90	50	2.0	100	—	55	5.9*
S. Dublin	Skim milk	35	164	1.0	164	2,000	50	4.0
S. Enteriditis	Eggs white	35	8	—	—	900	—	3.5

a: number of pulses, b: pulse width, c: treatment time (μs), −: No reported, *: log10 reductions at pasteurization levels (Mosqueda-Melgar *et al.,* 2008).

Some studies reported that PEF treatments combined with organic acids such as benzoic and sorbic acid (Liu *et al.,*1997) or natural antimicrobial agents such as nisin (Terebiznik *et al.*,

2000) had a synergistic effect on inactivating several bacteria. Such a synergism might be due to the fact that microbial cell membrane is the target of both treatments (Fernandez-Molina *et al.,* 2001). On the other hand, it is well known that the use of reduced pH as a sublethal hurdle may compromise the efficacy of other processes. Indeed, Evrendilek and Zhang (2001) observed that exposing *E. coli* O157:H7 to acid pH values before PEF treatments resulted in a lower inactivation than exposure to a neutral pH. These Authors concluded that the adaptation of *E. coli* to the acid stress determined an higher survival during PEF treatment. Moreover, some researches have shown that yeasts and Gram-negative bacteria are more sensitive to PEF than Gram-positive bacteria, while spores are difficult to be inactivated. This technology has the advantage of keeping the characteristics of the fresh product, while the main disadvantage is still the high cost that currently limits its development at the industrial level (Devlieghere *et al.,* 2004).

1.2. High Hydrostatic Pressure

The use of pressure in food processing is not new. Its application was proposed for the first time in 1899 by Hite as a way to preserve milk, but only in recent years the research has continued in this area. High hydrostatic pressure (HHP) is a discontinuous process that can be applied to liquid and solid matrices. The choice of process parameters depends on specific products, temperature and pressure-transmitting fluid, which can be a gas or, more frequently, water). Generally foods are subjected to pressures between 100 and 700 MPa. In this range it is possible to obtain the inactivation of many vegetative cells which is caused by the breakup of cellular membranes and the inactivation of enzymes. On the other hand, spores have shown to be very pressure resistant, being capable to survive pressures up to 1200 MPa. It has been also demonstrated that low-pressure treatments (*i.e.* 60 to 100 MPa) can induce spore germination. Therefore, a current trend is to combine HHP with some other treatments that allow the production of safe foods in which spore germination could be a problem (San Martìn *et al.,* 2002).

A HHP processing device basically consists of a bin containing a fluid (generally water) and a pressurization system that applies isostatically the compression on the fluid which acts as the pressure transmitting medium. The pressure is held for the desired treatment time and then released. In particular, the underlying principles of HHP are:

- Principle of the Chatelier: “When a system at equilibrium is subjected to change in pressure, then the system readjusts itself to (partially) counteract the effect of the

applied change and a new equilibrium is established". Consequently, all the reactions which lead to a decrease in volume, such as the rupture of complex molecule, are favoured. The breaking of chemical bonds does not concern the covalent ones, but only the hydrogen, disulfide bridges or ionic ones which are particularly sensitive to HHP treatments.

- Principle of Pascal: "A change in pressure at any point in an enclosed fluid at rest is transmitted undiminished to all points in the fluid regardless of the shape and volume".

Actually in Europe, despite the high cost of process, many industries commercialize pressurized products such as orange juice (by Ultifruit, Pernod Richard Company, France), acidified avocado puree (imported from Avomek Company, USA) and sliced ham (by Espona Company, Spain).

1.3. High Pressure Homogenization

Compared to HHP, during a High Pressure Homogenization (HPH) treatment a fluid is forced under high pressure to pass through a narrow gap in which undergoes an acceleration higher than the sound speed. Afterwards the fluid undergoes an extreme drop in pressure that leads to some effects such as high–speed friction, cavitation collapse, strong impacts, turbulence and heating. These last factors are responsible for cell wall rupture and cellular death (Pedras *et al.,* 2012). Also other indirect factors such as the composition and the viscosity of treated foods or the enhancement of the antimicrobial activity of naturally occurring antimicrobials *e.g.* lysozyme (Vannini *et al.,* 2004) seem to have an effect on the microbial inactivation.

Some Authors have reported that this innovative treatment can reduce several foodborne pathogens (e.g. *Salmonella enteritidis, Listeria monocytogenes, Bacillus cereus*) and spoilage microorganisms in model system and real food matrices (Table 2), such as milk, orange juices and mayonnaise-type products (Patrignani *et al.,* 2013, Guerzoni *et al.,* 2002). The sensitivity of various microorganisms to the homogenization pressures depends on several factors and in general spores (both bacterial or fungal) are more resistant than vegetative forms. Moreover, Gram-positive bacteria seem to be more resistant than Gram-negative ones, and cells in the exponential phase are more sensitive to HPH treatments compared to those in the stationary phase (Smelt, 1998). Some studies reported that some resistant spores were sensitized by HPH treatments followed by moderate thermal treatments. Adversely no sensitization for

these microorganisms was observed when thermal treatment was used before the HPH, indicating the importance of the process sequence.

Moreover, HPH is very attractive for its ability to modulate and preserve some functional and structural properties of foods, such as the retention of antioxidant capacity and polyphenolic compounds in HPH-treated apple juice (Suarez-Jacobo *et al.*, 2011), the increase of viscosity in HPH-treated apricot juice (Patrignani *et al.,* 2010) and the improvement of the structure in HPH dairy products including yogurt and soft cheeses (Patrignani *et al.,* 2007).

Table 2. Microbial inactivation in milk following HPH treatments at different pressure values.

Microorganism	Pressure	Inlet Temperature	Decimal Reduction
Bacillus subtilis A2 (vegetative cell)	130MPa	NI	2.90
Coliformes	150MPa	45ºC	> 1.12
Coliformes	200MPa	55ºC	>4.10
Escherichia coli 555	130MPa	NI	0.30
E. coli MG1655	300MPa	25ºC	3.50
Lactobacilli	200MPa	55ºC	> 3.80
Lactobacillus arizonensis 21	150 MPa	10ºC	1.50
Lactobacillus casei 28	150 MPa	10ºC	0.60
Lactobacillus pentosus 57	150 MPa	10ºC	1.00
Lactobacillus plantarum 58	150 MPa	10ºC	0.60
Listeria innocua ATCC 33090	300MPa	24ºC	1.80
Listeria monocytogenes CCUG 15526	400MPa	NI	7.95*
Listeria monocytogenes Scott A	130MPa	NI	1.03
Listeria monocytogenes Scott A	100MPa	2-4ºC	1.20
Micrococcus luteus ATCC 4698	300MPa	24ºC	2.50
Proteus vulgaris PV1	130MPa	NI	1.80
Pseudomonas	200MPa	55ºC	> 4.90
Pseudomonas fluorescens ATCC 13525	300MPa	24ºC	3.70
Pseudomonas fluorescens AFT36	200MPa	45ºC	6.00
Pseudomonas putida 754	130MPa	NI	2.42
Psychrotrophs	200MPa	55ºC	> 4.60
Salmonella enteritidis E4	130MPa	NI	1.40
Staphylococcus aureus	150MPa	45ºC	> 2.71
Staphylococcus aureus	200MPa	55ºC	>2.90
Staphylococcus aureus ATCC 13565	300MPa	20ºC	4.00
Staphylococcus aureus ATCC 13565	300MPa	6ºC	3.35
Staphylococcus aureus ST1	130MPa	NI	1.94
Staphylococcus carnosus CECT 4491	300MPa	20ºC	3.34
Staphylococcus carnosus CECT 4491	300MPa	6ºC	-0,01

(Pedras *et al.,* 2012)

1.4. New packaging systems

In the last decade the improvement of polymers manufacturing has greatly contributed to the production of new packaging systems able to extend the shelf life of minimally processed foods. Actually several systems are employed such as modified atmosphere packaging (MAP), active packaging and intelligent packaging.

MAP is largely applied by the food industry and some Authors defined it as 'the enclosure of food products in gas-barrier materials, in which the gaseous environment has been changed' (Young *et al.,*1988). The principal issue in MAP is defining the optimal gas atmosphere which depends on some intrinsic parameters of the food product (pH, aw, fat content and type) and the gas/product volume ratio in the chosen package type. For example food products whose spoilage is due to the development of Gram-negative bacteria and yeasts could be packaged in a CO_2 enriched atmosphere (including also a low percentage of O_2) as the growth of those microorganisms is significantly retarded by CO_2. On the contrary, in order to extend the shelf life of products which are spoiled due to mould growth or oxidation, it is essential to package in oxygen free atmospheres. On the other hand, the use of high concentration of CO_2 can cause some problem such as the collapse of package due to its solubility in water and fat (Devlieghere *et al.,* 2004).

Recently the food industry is highly interested in active and intelligent packaging. Active packaging is defined as "packaging in which subsidiary constituents have been deliberately included in or on either the packaging material or the package headspace to enhance the performance of the package system" (Robertson, 2006). This kind of packaging is able to change the condition of the packaged food product in order to extend its shelf-life and/or improve microbial food safety and/or improve sensorial properties. The most common active packaging system is represented by the use of oxygen scavengers which can remove the residual oxygen from the headspace and/or absorb oxygen diffusing through the packaging material during storage (Devlieghere *et al.,* 2004). Various studies showed the possibility to include some natural or synthetic antimicrobials which can extend the shelf-life and reduce the risk of foodborne diseases (Table 3).

Intelligent packaging can be defined as "packaging that contains an external or internal indicator to provide information about aspects of the history of the package and/or the quality of the food" (Robertson, 2006). Hence, the purpose of this system is to improve the quality or value of a product, to provide more convenience or tamper resistance. In particular, it can report the conditions of the environment outside the package, after the direct measurement of

the quality of the food product inside the package and then support the consumer in the decision making process to extend shelf life, enhance safety, improve quality, provide information, and warn of possible problems. The intelligent system mostly applied in the food industry is the "time/temperature indicator", which can tell the consumer when foods have been temperature abused.

Table 3: Antimicrobials directly incorporated into polymers used for food packaging,

Antimicrobials	Polymer/carrier	Main target microorganisms
Organic acids / anhydrides: Propionic, benzoic, sorbic, acetic, lactic, malic	Edible films, EVA, LLDPE	Molds
Inorganic gases: Sulfur dioxide, chlorine dioxide	Various polyolefins	Molds, Bacteria, Yeasts
Metals: Silver	Various polyolefins	Bacteria
Fungicide: Benomyl, imazalil	LDPE	Molds
Bacteriocins: Nisin, pediocins, lacticin	Edible films, cellulose, LDPE	Gram-positive bacteria
Enzymes: Lysozyme, glucose oxidase	Cellulose acetate, PS	Gram-positive bacteria
Chelating agents: EDTA	Edible films	Gram-negative bacteria
Spices: Cinnamic, caffeic, *p*-coumaic acids Horseradish (allylisothiocyanate)	Nylon/PE, cellulose	Molds, yeast, bacteria
Essential oils (plant extracts): Grapefruit seed extract, hinokitiol, bamboo powder, Rheum palmatum, Coptis chinesis extracts	LDPE, cellulose	Molds, yeast and bacteria
Parabens: Propylparaben, ethylparaben	Clay-coated cellulose LDPE	Molds
Miscellaneous: Hexamethyl-enetetramine	LDPE	Yeasts, anaerobes and aerobes

(Appendini *et al.,* 2002)

1.5. Biopreservation

Among the novel approaches for minimal processing also biopreservation is included. This technique refers to the extension of the shelf life and improvement of the safety of foods by using microorganisms and in particular their metabolites (Ross *et al.,*2002). The most studied and used bacteria are the lactic acid bacteria (LAB) which are considered GRAS (Generally Recognized As Safe) and have been studied since unmemorable time. In fact it is well known that LAB fermentation is an effective way of extending the shelf-life of several foods. Traditionally, foods have been preserved through naturally occurring fermentations, but nowadays at large scale production defined starter(s) are exploited in order to ensure

consistency and quality in the final products. LAB are able to produce several antagonistic primary and secondary metabolites including:

- Organic acids (*e.g.* lactic, acetic and propionic acids), which interfere with the maintenance of cell membrane potential, inhibit active transport and reduce cytoplasmic pH. They are active against Gram-positive and Gram-negative bacteria, yeasts and moulds (Caplice and Fitzgerald, 1999).
- Ethanol produced through the heterofermentative pathway.
- H_2O_2 produced during aerobic growth and having an oxidizing effect on cellular membranes (Condon, 1987).
- Diacetyl generated by the pyruvate pathway (Ray and Daeschel, 1992).

Moreover, some strains can produce bacteriocins, which are antimicrobial peptides, generally heat stable and apparently hypoallergenic as quickly degraded by proteolytic enzymes in the human intestine. Although several bacteriocins have been analyzed, actually only nisin is recognized as a preservative (E234) in various foods including beer, processed cheese products and tomato paste (Corbo *et al.,* 2009). Nisin display several functions against bacteria. Some studies have demonstrated that nisin inhibits peptidoglycan biosynthesis, causes pore formation in the membranes of Gram-positive bacteria and interacts with a docking molecule, lipid II, which is a membrane-bound precursor for cell wall biosynthesis (Ross *et al.,* 2002).

1.6. Natural antimicrobials

At the end of the 90's the consumer demand for "friendly" preservative, i.e. molecules of natural origin, not toxic for humans and environmentally safe, has highly increased. The main compounds actually studied are briefly described below.

- Essential oils (EOs): they are aromatic oily liquids obtained from plant materials. They exhibit various properties such as antiviral, antibacterial, antimycotic, antitoxigenic, antiparasitic and insecticidal properties. Their use is allowed in the food field as flavourings. EOs are composed by many components and, although their antimicrobial activity is not attributable to a specific compound, phenols seem to be the main ones responsible for the antibacterial properties of several EOs (Burt, 2004). EOs have been proved to be effective against several spoiling and pathogenic microorganisms, and a wide literature shows their great potential as antimicrobials in model and food systems. The most studied EOs derive from citrus fruit. For example

the action of the single constituents of these EOs, *i.e.* citral (3,7-dimethyl-2-7-octadienal) was studied in order to identify the cell targets and the most active molecules (Siroli *et al.,* 2014). The main targets of EOs and their components are the membrane and the cytoplasm, but in certain situations, they can completely alter the morphology of the cells. In most of the studies EOs have been used in direct contact with foods, but also their microencapsulation has been recently proposed (Ayala-Zavala *et al.,* 2008).

- Lactoferrin: is an iron-binding protein (transferring) that plays a protective role in various biological fluids such as milk and saliva. The main action site of lactoferrin is represented by the lipopolysaccharides of the outer membrane of Gram-negative bacteria. In literature it is reported that lactoferrin can act as a chelator or can cut the binding sites on the lipopolysaccharides, thus causing the formation of pores (Chapple *et al.,* 2004). Several studies report that lactoferrin is active against some pathogenic microorganisms including *Escherichia coli, Listeria monocytogenes, Streptococcus mutans* and *Vibrio cholerae* (Corbo *et al.,* 2009). Its antimicrobial action has been studied in several food- and drink-stored products *e.g.* soy powder and meat (Taylor *et al.,* 2004). Moreover, lactoferrin can provide antioxidant/iron chelating activity in products rich in fat such as milk and mayonnaise (Nielsen *et al.,* 2004).
- Lysozyme: is an enzyme naturally found in various foods and biological fluids, including white egg, saliva, tears and milk. It has been widely used in the pharmaceutical and food fields, for example to extend meat shelf life and to modulate cheese ageing, through the reduction of butyric fermentation bacteria which adversely affect the quality of final product (Corbo *et al.,* 2009). Lysozyme catalyses the hydrolysis of the β1–4 linkages between N-acetyl muramic acid and N-acetyl glucosamine in the peptidoglycan layers of the bacterial cell wall, and it is active against Gram-positive microorganisms (Vannini *et al.,* 2004), whereas it has not any effect against Gram-negative bacteria because of the presence of a lipopolysaccharide layer in the outer membrane. On the other hand, some Authors have shown that its effectiveness can be increased through the combined use of lysozyme with some chelating agents which are able to weaken the lipopolysaccharide layer allowing lysozyme to penetrate into microbial cells (Stevens *et al.,* 1991).
- Lactoperoxidase system: can be found in various biological fluids such us milk, colostrums and saliva, and it consists of three primary components: the lactoperoxidase enzyme, thiocyanate and hydrogen peroxide. In details, it is an

oxidoreductase and catalyses the oxidation of thiocyanate at the expense of hydrogen peroxide, to generate intermediate products, such as cyanosulphurous acid and cyanosulphuric acid, which have antibacterial properties (Corbo *et al.,* 2009). Its mechanisms of action include: inhibition of growth, oxygen uptake, production of lactic acid and some bacterial enzymes, leakage of potassium ions, amino acids and polypeptides causing damages to the cytoplasmic membrane. Lactoperoxidase system is active against Gram-positive bacteria as well as Gram-negative catalase positive organisms (Vannini *et al.,* 2004). It has been used to preserve raw/ pasteurized milk, cream, cheese, liquid whole eggs, ice cream and infant formula (Seifu *et al.,* 2004).

- Fatty acids (FFAs): display their antimicrobial action in several multicellular organisms including mammals, plants, molluscs and amphibians (Desbois and Smith, 2010). FFAs, and particularly C12:0, C14:0, C16:0, C16:1n-10, C18:1n-10, are also the most important antimicrobial agents on human skin (Takigawa *et al.,* 2005). Their antibacterial activity seems to be influenced by the length of the carbon chain and the presence, number, position and orientation of double bonds. In general, unsaturated FFAs, which have *cis* orientation, have a greater antibacterial effect than saturated FFAs with the same length of the carbon chain (Desbois *et al.,* 2008). Moreover, some Authors suggest that medium- and long-chain unsaturated FFAs tend to be less active against Gram-negative bacteria than Gram-positive ones because of their cell wall lipopolysaccharide and outer membrane (Branen *et al.,*1980). Even if their action mechanism is not clear yet, the main target seems to be the cellular membranes. In fact their detergent properties allow the solubilisation of some components and the creation of transient or permanent pores. Other processes that may kill bacteria or inhibit their growth are the inhibition of enzymatic activities, impairment of nutrient uptake and the generation of toxic oxidation products (Desbois and Smith, 2010). In the last years, some Authors suggested the use of fatty acids against foodborne and plant pathogenic moulds (Corbo *et al*., 2009). FFAs are widely used in food animal industry as fungistats (Dixon and Hamilton, 1981) or during meat processing as sanitizer (Dorsa, 1997).
- Chitosan: is a modified, natural carbohydrate polymer, derived by deacetylation of chitin, which is obtained by shellfish by-products. Nowadays chitosan is used in various foods e.g bread, egg, fruits and vegetables, juices, mayonnaise, milk, sausages, seafoods and soybean based products as an edible coating in order to increase their shelf life (No *et al.,* 2007). Changes in cell membrane permeability and the interaction

between its hydrolysis products and microbial DNA seem to be the main antimicrobial mechanisms of chitosan (Corbo *et al*., 2009).

1.7. Gas plasma

In the last years the attention to the decontaminating effect of gas plasma treatments has grown up and currently this technique is used for the sterilization of medical heat-sensitive materials (Laroussi, 2005). On the contrary applications in food area are still at research level. Gas plasma is generated by ionization of gases at environmental pressure condition, and the high amount of energy associated with gas plasma results in the production of UV radiations, electrons, ions and free radicalic species, which can damage biological systems. As gas plasma is the object of this thesis, it will described in more details in chapter n°2.

Chapter 2. Gas plasma

2.1. Definition

Gas plasma (GP) is the fourth state of the matter, following by order of increasing energy, the solid state, the liquid state and the gaseous state. In nature GP can be found in stars, thunders and aurora borealis, and it is composed only by ions and free electrons. The man-made GP is produced by subjecting a gas or a mixture of gases to an electric field thus causing their ionization. The electric field accelerates electrons which transmit their energy to the heavy species by collisions. Indeed, besides ions and electrons, ionized gases also consist of uncharged particles including atoms, molecules and free radicals which are in both fundamental and excited states (Moisan *et al.,* 2001). Moreover the excited species can emit UV photons when they lose their internal energy and return to the fundamental state (Moreau *et al.,* 2008).

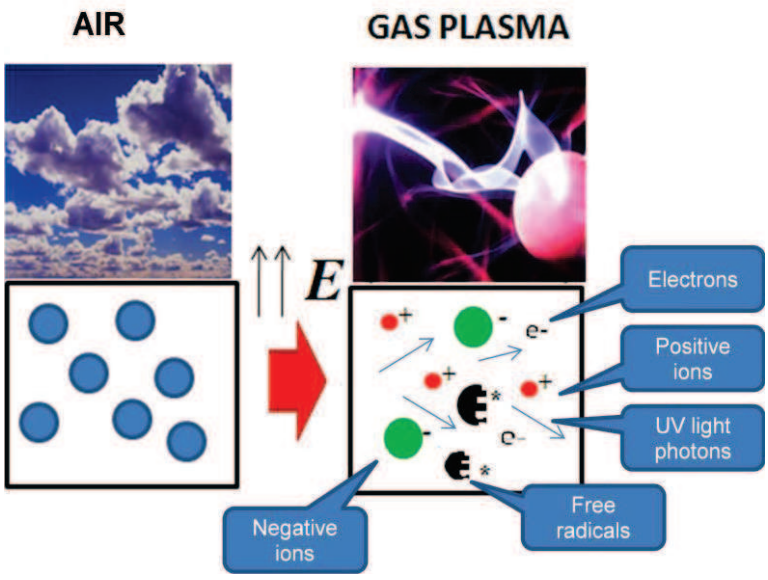

Figure 1. Gas plasma composition.

2.2. Thermal and non-thermal gas plasmas

The man-made GP can be distinguished into two main groups: high (thermal) temperature gas plasma, and low (non-thermal) temperature gas plasma. In the thermal GP, the temperature, typically ranging from 4000 K to 20 000 K, is the same for all the species and the pressure is high (about 10^5 Pa). Moreover, electrons energy is consumed through inelastic and elastic collisions with the heavy particles which consequently increase in temperature. On the contrary, in non-thermal GP electrons are characterized by much higher temperatures than

heavy particles (ions, atoms, molecules) and they consume their energy in inelastic collisions (Bogaerts *et al.,* 2002, Moisan *et al.,* 2001). As a rule, the temperature of the GP increases with pressure because of the increasing number of elastic collisions between electrons and heavy particles. In non-thermal GP the pressure is low, *i.e.* close to the atmospheric one. As temperature and pressure are close to room and atmospheric ones, respectively non thermal gas plasma is also named "cold atmospheric gas plasma".

2.3. Techniques able to generate cold atmospheric gas plasma

In the last decade, the field of GP has rapidly expanded and a large variety of non thermal GP employed in a wide range of applications has raised. This is due to the possibility to easily modify several processing parameters such as (Bogaerts *et al.,* 2002):

- The working gas: the nature of the gas (*e.g.* O_2, N_2, air, H_2, halogens, N_2O, H_2O, H_2O_2, CO_2, SO_2, SF_6, aldehydes, organic acids,…) or mixture of gases defines the different chemical species which are generated in the plasma such as electrons, atoms, molecules, ions and radicals;
- The pressure: ranging from 0.1 Pa to atmospheric pressure;
- The electromagnetic field structure and its frequency;
- The discharge configuration, *e.g.* with or without electrodes; the discharge vessel configuration and dimensions, discharge volume;
- The temporal behaviour, *e.g.* pulsing the plasma.

On the basis of the modulation of these multidimensional parameters, it is possible to classify cold gas plasmas in three categories: 1) running direct current discharges (DC), 2) radio-frequency (RF, 1–100 MHz), and 3) microwave (MW, < 300 MHz) discharges under low current and low power conditions (Moisan *et al.,* 2001).

2.3.1 Running direct current (DC) discharge and low frequency

As above reported , when a gas is subjected to an high potential difference applied between two electrodes, it breaks down into positive ions and electrons, giving rise to a gas discharge. In particular only if a potential difference is applied, the electrons are accelerated by the electric field in front of the cathode and consume their energy through inelastic collisions with the gas atoms, thus leading to excitation and ionization. The passage from the exited state to the de-excited one with the emission of an UV radiation, is responsible for the characteristic name of 'glow' discharge. The continual collisions give rise new electrons and ions which in

turn generate ionization collisions, creating new ions and electrons. Moreover, ions and fast atoms can release also atoms of the cathode material (sputtering phenomenon). These processes, which include the electron emission at the cathode, the ionization and the sputtering, make the glow discharge a self-sustaining plasma (Bogaerts *et al.,* 2002). It is possible to talk about *DC glow discharge* when the potential difference between the two electrodes is constant and a continuous current flows through the discharge. In the DC configuration the frequency is lower than RF and MW. With a DC glow discharge configuration is possible to choose various conditions: the pressure can vary from 1 Pa to atmospheric pressure, the voltage can be in the range between 300 and 1500 V (for specific condition it can increase to kV), the current is generally in the mA range and it is possible to work with various gases including Ar, He, N_2, O_2, H_2 etc.

One of the most investigated low frequency plasma sources is the dielectric barrier discharge (DBD). DBD operates at approximately atmospheric pressure (typically 0.1–1 atm) and an alternating current (AC) with an amplitude of 1–100 kV and a frequency between 500 Hz - 500 kHz is applied to the discharge. A DBD device (Figure 2) consists of two electrodes separated by a small inter-electrode distance variable from 0.1 mm (in plasma display) to several cm (in CO_2 lasers), and one or more dielectric layers (made of glass, quartz, ceramic material or polymers). The role of these dielectric layers is to ensure a uniform distribution of the discharge over the electrode area and prevent the arc transition.

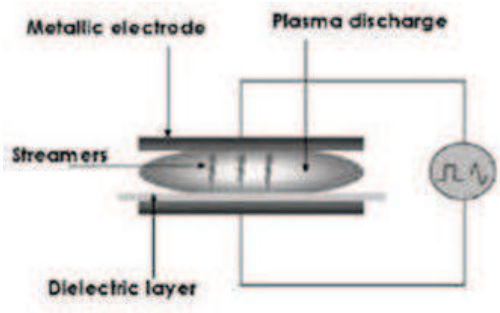

Figure 2. Schematic DBD configuration.

On the basis of the operating parameters two type of discharge can be obtained: micro-discharges and atmospheric pressure glow discharges (APGDs). The basic difference between micro-discharges and APGDs is that the latter are generally homogeneous across the electrodes and are characterized by only one current pulse per half cycle, whereas the micro-discharge consists in independent filaments of nanosecond duration (hence, with many current pulses per half cycle). These micro-discharges determine an accumulation of electrons on the dielectric layer and the production of high-energy electrons (Kogelschatz *et al.,* 1999).

Despite this distinction, the same electrode configuration can generate an APGD or a microdischarge, depending on the discharge conditions and the discharge gas.

Two basic configurations of DBD can be distinguished (Gibalov *et al.,* 2000) in:

- The v*olume discharge* (VD) composed by two parallel plates in which microdischarge takes place in thin channels which cross the discharge gap and are generally randomly distributed over the electrode surface.
- The *surface discharge* (SD) consists of a number of surface electrodes on a dielectric layer and a counter electrode on its reverse side.

The combination of VD and SD can generate other configurations (e.g. a co-planar arrangement, a packed bed reactor).

When one or both electrodes are covered by a high resistivity material, the DBD configuration is named resistive barrier discharge (RBD) and it can be driven by DC or AC power supplies (Laroussi *et al.,* 2002). The resistive layer is a kind of distributed ballast resistor that enhances the uniformity of the discharge.

Nowadays other interesting DBD configurations have been developed. One of them is the *microplasma* (Figure 3) which refers to discharges with dimensions that range from a few micrometers up to a few millimeters. This kind of configuration can combine the potential of low temperature plasmas with the advantages of being "micro" (Iza *et al.,* 2008). In literature a wide spectrum of applications of microplasma is reported including environmental application, radiation sources, micro-chemical analysis systems, gas analyzers, photodetectors, microlasers, material processing, bio-medical applications (Iza *et al.,* 2008), biofilm-forming bacteria (Abramzon *et al.,* 2006), and for food decontamination (Perni *et al.,* 2008).

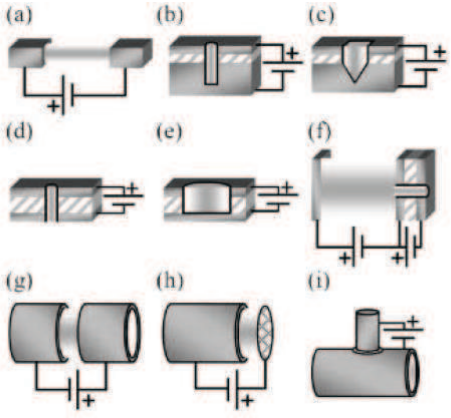

Figure 3. Examples of various DC microplasma sources: a) parallel electrode, b) cylindrical microhollow cathode, c) inverted pyramidal microhollow cathode, d) metal-insulator-metal microhollow cathode, e) cathode boundary layer, f) three electrode sources, g) microtubes, h) microtube with grid anode, and i) microtube with inserted anode (Iza *et al.,* 2008).

Another device that let to work at low frequency is the atmospheric gliding arc cold plasma. It is characterized by two diverging metallic electrodes and the gas is injected into the gap formed between the electrodes. The system offers the advantage to operate on an open-air bench top, and does not require a closed-batch process of placing the samples into an enclosed treatment chamber like the other systems (Niemira *et al.,* 2008).

2.3.2 Radio-frequency (RF)

When an alternative voltage is applied, the frequencies generally used are in the radiofrequency range (1 kHz–103 MHz; with a most common value of 13.56 MHz). In this configuration, the electrons and ions have a totally different behaviour, which can be explained by their different masses. The light electrons can follow the instantaneous electric fields produced by the applied RF voltage, while the ions can follow the field only if the RF is less than the ion plasma frequency.

The main plasma sources working in RF are: capacitively coupled plasma (CCP) and Inductively coupled plasma (ICP). These kind of sources can also be used in microplasma configuration. CCP sources, widely used in the semiconductor industry, operate at 1–100 MHz and the electrodes can be covered by low sputtering yield materials. CCP sources typically produce higher density plasmas than DBD sources even if the ion density achievable is limited, while ICP sources typically work at 0.5-28 MHz and can produce ion densities in excess of 10^{12} cm^{-3} even at submillitorr pressures (Hopwood 1999). These sources are known for their high efficiency, relative simplicity, and controllability of the density and energy of ion flux (Iza *et al.,*2008).

The Atmospheric Pressure Plasma Jet (APPJ) and the One Atmosphere Uniform Glow Discharge Plasma (OAUGDP) are the most recent RF plasma sources (Figure 4).

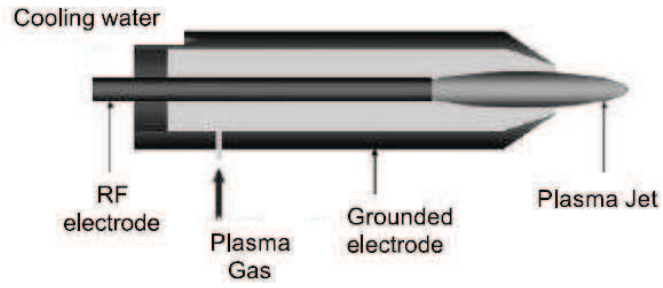

Figure 4. Atmospheric Pressure Plasma Jet (APPJ, Tendero *et al.,* 2005).

The APPJ is a type of CCP configuration which works at 13 or 27 MHz. APPJ is useful to decontaminate small areas or to penetrate into small structures having high aspect ratios, such as slits and tubes (Weltmann *et al.,* 2008).
The device is characterized by a nozzle equipped with one or two concentric electrodes, in which the discharge is operated, and the working gas, generally argon, flows from nozzle to outside. In this configuration, while the free electrons follow the oscillating field, the reactive species, formed after inelastic collisions with the electrons, are transported outside by the gas and contact the surface. In this way the surface is not in direct contact with the plasma sources. In order to assure stability and non-thermal properties, the APPJ devices necessarily need argon as a carrier gas. On the contrary OAUGDP can work with air. OAUGDP is a novel atmospheric plasma, which can be generated over large areas and in large volumes (Critzer *et al.,* 2007). This plasma source has the advantage to operate at ambient temperature and one atmosphere with electric fields of about 8.5 kV/cm, well below the electric field required to produce a DC glow discharge (Montie *et al.,* 2000).

2.3.3 Microwave (MW)

The microwave plasma sources are created by electromagnetic irradiation with a frequency higher than 300 MHz, and can operate in a large range of pressure and power values. The devices are mainly characterized by a microwave power source, a waveguide (or a tuning system) and a (noble and/or molecular) gas injector.
Nowadays various microwave plasma configurations have been developed such as Resonant Cavity Plasmas, Free Expanding Atmospheric Plasma Torches, Capacitive Microwave Plasmas (CMPs), Microstrip Plasmas (MSPs), Surface Wave Discharges (SWD) and they show an high efficiency (Bogaerts *et al.,* 2002).
The plasma generated by a microwave sources can be also directed into a jet (microwave plasma jet devices, Pau *et al.,* 2001).

2.4. Chemistry of electric discharges

According to the techniques and to the gas mixture used to drive the discharge, cold atmospheric gas plasma produces a mixture of reactive molecules that continually and rapidly react with other molecules and particles present in the system (Table 4).

Table 4. Collision processes of electrons and heavy particles in non-thermal plasmas.

Collisions of electrons	
$e^- + A \rightarrow A^{\bullet/m} + e^-$	excitation of atoms
$A^{\bullet/m} \rightarrow A + h\nu$	spontaneous de-excitation
$e^- + A^{\bullet/m} \rightarrow A + h\nu + e^-$	collisional-induced de-excitation
$e^- + A \rightarrow A^+ + 2e^-$	ionization of atoms
$e^- + AB \rightarrow AB^{\bullet/m} + e^-$	excitation of molecules
$AB^{\bullet} \rightarrow AB + h\nu$	spontaneous de-excitation
$e^- + AB^{\bullet} \rightarrow AB + h\nu + e^-$	collisional-induced de-excitation
$e^- + AB \rightarrow A^{(\bullet)} + B + e^-$	dissociation of molecules
$e^- + AB \rightarrow A + B^+ + 2e^-$	dissociative ionization
$e^- + A^{\bullet/m} \rightarrow A + e^- +$	*E*kin super-elastic collisions
$e^- + A^{\bullet/m} \rightarrow A^{\bullet\bullet} + e^-$	stepwise excitation
$e^- + A^{\bullet/m} \rightarrow A^+ + e^-$	stepwise ionization
$e^- + A \rightarrow A^-$	attachment
$e^- + A^- \rightarrow A + 2e^-$	detachment
$e^- + A^+ \rightarrow A$	recombination
$e^- + A^+ + M \rightarrow A + M$	three-body collision recombination
Collisions of heavy particles	
$A^+ + B \rightarrow A + B^+$	charge transfer
$A^m + B \rightarrow A + B^+ + e$	Penning ionization
$A^m + A^m \rightarrow A + A^+ + e$	pair collision
$A^{\bullet} + A \rightarrow A_2^+ + e$	Hornbeck–Molnar ionization
$A^+ + BC \rightarrow AC^+ + B$	ion–molecule reaction
$A + BC \rightarrow AC + B$	chemical reaction
$R + BC \rightarrow RC + B$	chemical reactions with radical R produced in the plasma
$A^{\bullet} + BC \rightarrow AC + B$	chemical reactions with excited atom or molecule

The black point ● denotes short-lived excited particles, the superscript m denotes long-lived metastable excited atoms or molecules (Schmidt and Becker 2001).

The particles generated in gas plasma include reactive oxygen species (ROS), reactive nitrogen species (RNS), energetic ions, and charged particles.

In particular, according to Gaunt *et al.* (2006) the ROS reported in air are: ozone (O_3), atomic oxygen (O or O●−), superoxide (O_2^-), peroxide (O_2^{-2} or H_2O_2), and hydroxyl radicals (OH●), while the RNS are N_2, nitric oxide radical (NO●), excited atomic N and ionic fragments such as N^+ and N^{2+} (Camacho *et al.,* 2007). ROS can be generated by the following reactions (Laroussi and Leipold, 2004):

$$e + O_2 \rightarrow e + O_2\left(A^3\sum_u^+\right) \rightarrow e + O(^3P) + O(^3P)$$

$$e + O_2 \rightarrow e + O_2\left(B^3\sum_u^-\right) \rightarrow e + O(^1D) + O(^3P)$$

$$O + O_2 + M \rightarrow O_3 + M \quad (M = O_2 or N_2)$$

$$e + H_2O \rightarrow OH + H + e$$

$$O + H_2O \rightarrow 2OH$$

$$H_2O + O_3 \leftrightarrow O_2 + 2OH$$

As emerged from these reactions, hydroxyl radicals OH•, which are highly reactive species, are caused by the presence of water vapour in a discharge feed gas. This radicals can be generated by electron impact dissociation of H_2O, but also by reactions of electronically excited oxygen atoms and nitrogen molecules or by the reactions between hydrogen peroxide and superoxide.

$$O_2\bullet^- + H_2O_2 \rightarrow O_2 + OH\bullet + HO^-$$

Some studies reported that the presence of water vapour promotes OH• production, but simultaneously reduces ozone concentration (Falkenstein and Coogan, 1997). Moreover, the water vapour increases the dielectric capacity, but reduces the surface resistance and the total charge transfer. For this reason dry air is used in the majority of plasma devices as water vapour reduces the number of microdischarges and thus the plasma volume. Some Authors choose to operate below 14% relative humidity (RH) in order to obtain a uniform discharge across the electrode gap (Montie *et al.,* 2000). On the contrary other Authors have published some data in which an increase of RH, ranging from 35% to 65%, has determined the best lethal effect of their DBD device against *Salmonella* (Ragni *et al.,* 2011).

Concerning superoxide (O2•−), which is difficult to detect because it is short-lived and does not accumulate, Gaunt *et al.* (2006) associated its presence with hydrogen peroxide (H_2O_2) because superoxide is a common precursor for this species.

As well as OH•, hydrogen peroxide requires the presence of water or water vapour to be generated, and generally its concentration increases by increasing RH.

In Table 5 the typical densities of some ROS and quantity of charge species in various plasma devices are reported.

Table 5. Typical densities of oxygen ions, oxygen atoms, ozone, and charged species in plasma discharges.

Source	**Typical Density (cm^{-3})**			
	$O^{\bullet-}$ **$O_2^{\bullet-}$** **O^-**	**O**	**O_3**	**Charged species in plasma**
Low pressure discharge	10^{10}	10^{14}	$<10^{10}$	10^{8}-10^{13}
Arc or plasma jet	10^{15}	10^{18}	$<10^{10}$	10^{16}-10^{19}
DBD	10^{10}	10^{12}	10^{18}	10^{12}-10^{15}
Plasma jet	10^{12}	10^{16}	10^{16}	10^{11}-10^{12}

(Gaunt *et al.,* 2006).

Concerning RNS, NO radicals can be formed from various reactions:

$$N + O + N_2 \rightarrow NO + N_2$$

$$NO_2 + O_2 + h\nu \rightarrow O_3 + NO$$

$$N + O_2 \rightarrow NO + O$$

The concentration of reactive species can be detected by various ways such as time-resolved UV absorbance spectroscopy, optical emission spectroscopy in UV-vis region and Fourier-transform infrared (FTIR) spectroscopy.

2.5. Mechanisms of gas plasma inactivation

The use of plasma as a sterilization process was first introduced towards the end of the 1960's. After that various researches have been performed in order to understand which chemical species and how can damage microbial cells. The decontaminating effect of gas plasma has been shown towards various microorganisms including Gram-negative pathogens (Misra *et al.,* 2001) such as *Salmonella* Enteritidis and *Salmonella typhimurium* (Ragni *et al*., 2010; Fernández *et al.,* 2013), Gram-positive ones as *Listeria monocytogenes* and *Bacillus cereus* (Vannini *et al.*, 2009), yeasts, moulds, spores (Roth *et al.,* 2010) and biofilms (Abramzon *et al*., 2006; Joaquin *et al*., 2009).

The mechanism of microbial inactivation by the gas plasma is complex and heterogeneous. Moisan *et al.* (2001) observed a different trend in the bacterial survival curves between classical sterilization and gas plasma. In particular, the sterilization by wet or dry heat determines a classical survival curve (Figure 5) with its unique straight line (B) which represents the survival curve of homogeneous microbial cultures exposed to a heat stress.

However, in most cases this curve has other common forms: indeed during the initial heating time period, the death rate can be lower (Figure 5, curves A and C) or higher (Figure 5, curve D) than the major straight-line portion. These trends have been attributed to the different heat-resistance of the population (Moisan *et al.,* 2001). Concerning the survival curves of microorganism subjected to a gas plasma exposure, in some cases, the kinetics of cell death demonstrate single-slope survivor curves (Laroussi *et al.,* 2000), but generally in others studies multi-slope curves are observed (Figure 6; Gaunt *et al.,* 2006).

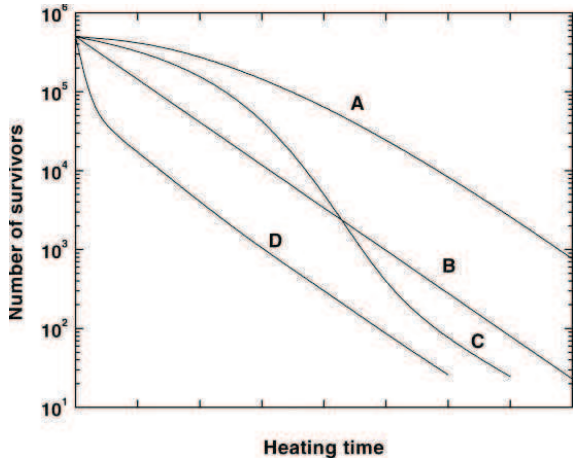

Figure 5. Survival curves of microorganisms subjected to heat treatment:) Classical survival curve(B); three commonly observed non-exponential survival curves (A, C, D) (Moisan *et al.,* 2001).

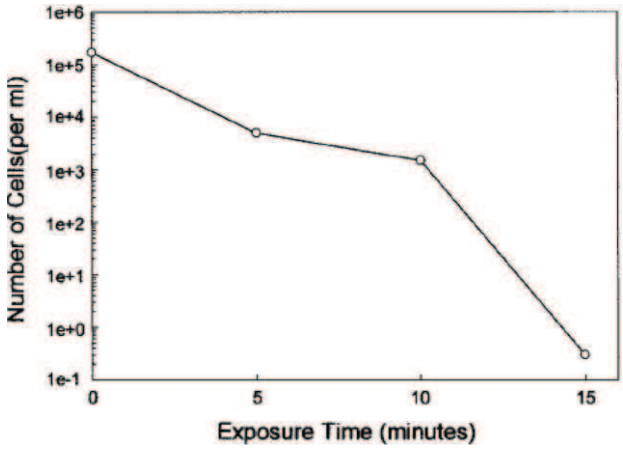

Figure 6. Example of a multi-slope survivor curve of a microorganism subjected to gas plasma exposure (Gaunt *et al*., 2006).

The reactive species briefly discussed before and UV radiation generated by gas plasma have been considered responsible for microbial cell damages. In particular, Moisan *et al.* (2001) hypothesized three main mechanisms involved in the microbial inactivation by gas plasma which can cause multi-slope curves:

a) the direct destruction of the genetic material by UV irradiation.

b) The erosion, atom by atom, of the microorganism through intrinsic photo-desorption by UV irradiation: the breaking of chemical bonds of microbial material by UV photons determines the formation of volatile compounds and generates the photodesorption.
c) The erosion, atom by atom, of the microorganism through etching: microorganism are exposed to an intense bombardment by the radical species such as ROS and RSN that most likely provoke surface lesions that the living cell cannot repair fast. Etching mechanism is also enhanced by UV photons.

UV photons of different wavelengths generated by gas plasma seem to be involved in dimerization of thymine bases of DNA (Misra *et al.,* 2011). In particular UV-C is the most effective radiation in the plasma (Roth *et al.,* 2010). Gaunt *et al.* (2006) reported that UV radiation plays the most important role in sterilization achieved through low-pressure plasma. On the contrary in atmospheric pressure plasma most of the UV radiation is reabsorbed in the plasma volume and does not reach the sample surface. Moreover, at the beginning of gas plasma studies, Laroussi (1996) observed that UV radiation is not sufficient to achieve complete sterilization, but charged particles and active free radicals play a significant role. More recent studies confirm that UV radiation play a minor role in the inactivation process (Lu *et al.,* 2008; Perni *et al.,* 2007). Lu *et al.* (2008) observed also that the charged particles generated by the working gas He/N_2 (3%) such as He^+, He^{2+}, or N_2^+ ions do not play an important role in the inactivation process. On the contrary when the working gas include O_2 its charged particles e.g. O_2^+ and O_2^- play a significant role in the inactivation of bacteria. Despite their short life, the oxygen radicals (e.g. **$O\bullet-$,$O_2\bullet^-$)** are the most efficient species because can cause lipid peroxidation, proteins and DNA oxidation (Montie *et al.,* 2000). Among these, the OH radicals seem to have the most important role in the etching effect (Figure 7).

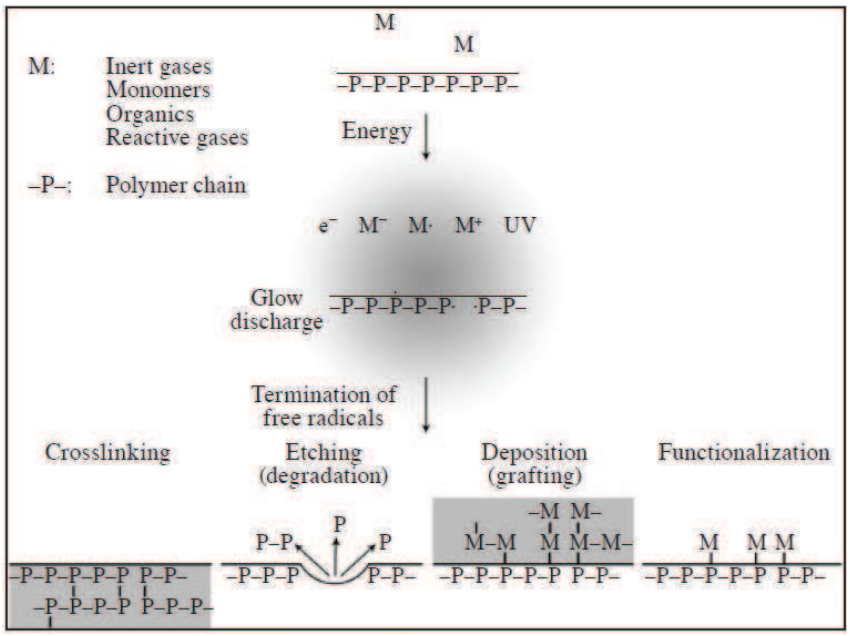

Figure 7. Speculated model of chemical reactions exposed by gas plasma (Shintani *et al.,* 2010)

Oxygen radicals can affect biological macromolecules, but the specific damages that lead to cell death are not clear yet.

Several studies reporting scanning electron microscopy (SEM) images of gas plasma treated microbial cells show that the cell membrane of Gram-negative bacteria, such as *E. coli* (Figure 8), is perforated after gas plasma exposure. This is related to the chemical reactive species that affect the cell membrane, which is essentially characterized by lipid bilayers and protein molecules, and generate pores. These ruptures are probably due to the fatty acids oxidation and, consequently, to the loss by the membrane of its role thus allowing the entrance of radical species into the cytoplasm. Moreover Ulbin-Figlewicz *et al.* (2014) observed that cells of *E. coli* appeared aggregated after gas plasma exposures. The same Authors have hypothesized a possible modification of the surface properties of cells exposed to plasma treatments. On the other hand, the capability of bacterial cells to aggregate each other in order to counteract stress is well known. Concerning Gram-positive species, which in general are more resistant to plasma than the Gram-negative ones, a direct contact between cytoplasmic material and radical species has been hypothesized after a chemical or physical adsorption of the latter by cell membranes (Weltmann *et al.,* 2012).

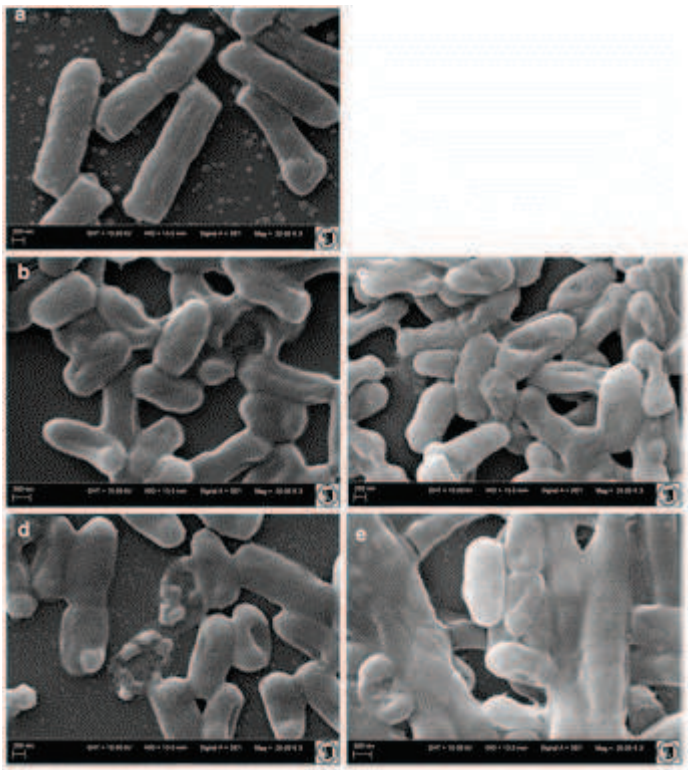

Figure 8. Scanning electron microscopy (SEM) images of *Escherichia coli*. a) Untreated control. b) After helium plasma exposure for 2 min. c) After argon plasma exposure for 2 min. d) After helium plasma exposure for 10 min. e) After argon plasma exposure for 10 min (Ulbin-Figlewicz *et al.,* 2014).

As reported before, when radical species generated by plasma react with cell membrane fatty acids, and in particular the polyunsaturated ones, lipid peroxidation can occur: a hydrogen atom is removed from fatty acids to form a lipid radical, which in turn can react with oxygen to form a lipid peroxyl radical. This radical in turn can affect other unsaturated fatty acids, thus perpetuating the chain reaction. During lipid peroxidation the fatty acids become shorter molecules and the integrity of membrane and the cell osmotic balance are compromised. Moreover, among the final products of peroxidation aldehydes are produced. Unlike radicals, aldehydes are longed lived and can damage structural proteins and enzymes, and consequently they can alter the metabolism of microorganism. DNA is another target of radicalic species generated by gas plasma or by lipids peroxidation and aldehydes.

In gas plasma others important molecules can inactivate bacteria *e.g.* ozone. It is a well-recognized disinfectant which interferes with cell respiration and attacks the double bonds of unsaturated lipids, and similarly to hydrogen peroxide acts as a bacteriostatic agent at concentrations of 25–50 μm (Gaunt *et al.,* 2006).

The concentration of UV, charged particles and radicals, and thus the efficiency of plasma, are strictly dependent on the device, the operating conditions (e.g. frequency and power of plasma), the gas composition (e.g. gas mixture, pressure, flow), time and type of exposure

(direct or remote). Concerning the latter condition, if the sample is exposed remotely to gas plasma, hardly the short-lived species, which play the most important role in the inactivation process, can reach the sample surface. In order to obtain an effective treatment, a good combination of all the parameters is necessary (Misra *et al.,* 2011).

Besides the operating conditions, also the medium supporting the microorganism (liquid or solid) and the characteristics of microorganism (e.g. taxonomy, cell load, phase of microbial growth) can also influence the decontamination efficacy (Laroussi *et al.,* 2000).

As previously reported, radical species attack in different way Gram-positive and Gram-negative bacteria. In general, the latter are less resistant to gas plasma exposure than the Gram–positive ones. Ponniah *et al.* (2003) also observed a major resistance in bacteria in stationary phase than those in the exponential one.

Concerning yeasts and moulds, a great deal of studies report the ability of gas plasma to reduce their loads on liquid media or food surfaces. Ulbin-Figlewicz *et al.* (2014) observed significant reductions of yeasts and moulds inoculated on pork and beef meat in ranges of 1.14-1.48 and 0.98-2.09 log cycles, respectively. Lee *at al.* (2006) calculated a D value of 1.55 min for *Saccharomyces cerevisiae* deposited on a nitrocellulose filter membrane. The cells of yeast have been exposed to atmospheric-pressure cold plasma (APCP) using as working gas helium/oxygen. In the same work some images of *Saccharomyces cerevisiae* cells acquired by SEM showed a sort of peeling on the treated cells.

Morgan (2009) obtained D-values of 7 and 4.7 min for *Saccharomyces cerevisiae* and *Candida* spp., respectively, by using a DBD discharge operated by oxygen at discharge current of 0.4, 0.8 and 1.0 mA. SEM pictures show some ruptures in the cell surface after treatments which assure the inactivation of the yeasts (Figure 9). The Authors suggest that ozone formed in oxygen plasma plays a major role in the inactivation of the yeasts.

Among various working gases used for gas plasma jet treatment, argon seems to have the best efficacy to prevent the growth of *Aspergillus niger*, *Aspergillus flavus*, *Penicillium* sp. and *Rhizopus* sp. spores on Malt Extract Agar medium and on brown rice cereal (Suem *at al.,* 2013).

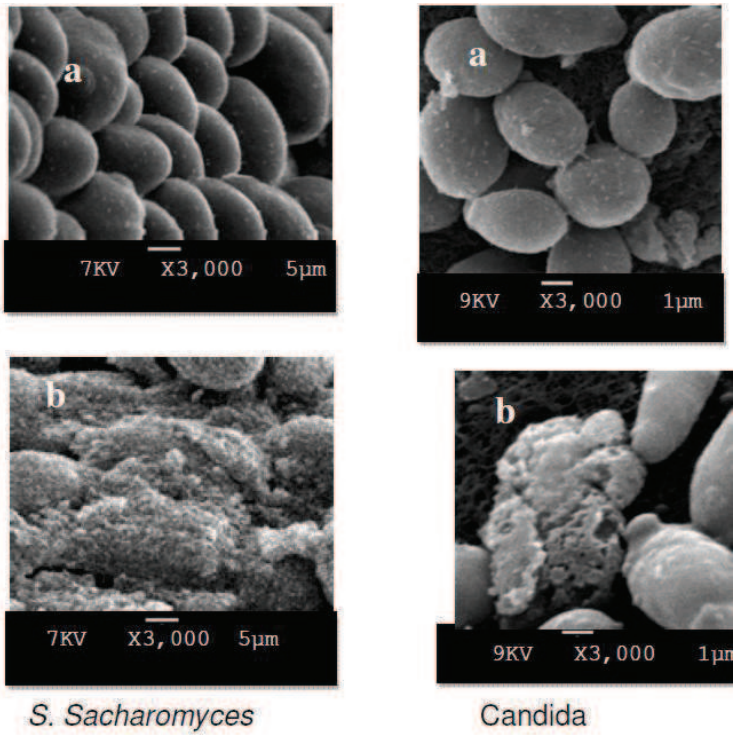

Figure 9. Cells of *Saccharomyces* and *Candida* spp. (a) before and (b) after a gas plasma treatment (Morgan, 2009).

Endospores have been one of the first issues in gas plasma studies. Indeed the first report on gas plasma as a sterilizing agent showed the possibility to sterilize vials containing 10^6 spores following treatments lower than one second (Menashi, 1968). The device used in the study was a pulsed RF field to achieve an argon plasma at atmospheric pressure and sterilize the inner surface of vials containing the spores. Since this publication, the capability of various plasma devices to inactivate spores has been widely studied. Almost all the studies investigating the inactivation kinetics of spores following plasmas show the same tailing phenomenon in the survivor curves. In particular, an initial linear relationship between log reduction of spore populations and treatment time is followed by a non-linear relationship in the quantal region of the curve (Shintani *et al.,* 2011). In a study conducted with a cold atmospheric gas plasma using several working gases, O_2/CF_4 (88%/12%) resulted to be the best mixture for killing *Bacillus subtilis* spores (Lerouge *et al.,* 1999). The Authors suggest that plasma etching could be a key contributor to spore mortality; moreover they hypothesized that plasma could be effective in destroying prions and endotoxins, which most sterilization processes fail to inactivate.

Unlike spores, the use of gas plasma to inactivate biofilm is quite recent. Several studies report that plasma is an effective technique in the treatment of biofilms on foods or medical devices (e.g. Abramzon *et al.,* 2006; Joaquin *et al.,*2009; Brelles-Mariño, 2012; Traba *et al.,* 2013). In these studies two main and simultaneously mechanisms of action are suggested:

- Killing both adhered and embedded bacteria in biofilms;
- Bacteria removal (both live and dead) from the contaminated surface through etching.

However, surface etching can cause a negative effect: if bacteria can be released from the surface before they are completely killed, they can contaminate new areas and remake biofilm (Traba *et al.,* 2013).

Also the lethal effect of non thermal gas plasma on viruses is demonstrated by various studies. Yasuda *et al.* (2010) describes activity of atmospheric pressure DBD on DNA viruses. The Authors suggest that damages in coat proteins precedes damage in DNA, and is the main responsible of λ phage inactivation. On the other hand DNA damages gradually increase by increasing the dose of the plasma. Each damage was introduced independently and a synergetic effect for the inactivation was not observed. Alshraiedeh *et al.* (2013) demonstrated that cold atmospheric gas plasma is a rapid and effective method for disinfection of surface contaminated by MS2 bacteriophages. Terrier *et al.* (2009) decontaminated the nebulized suspensions of viruses related to respiratory deseases.

In Table 6 some recent findings concerning the inactivation of several microorganism by gas plasma are reported.

Table 6. Some finding related to bacteria and spores inactivation by non thermal gas plasma.

Organism	Plasma conditions	Treatment surface/ medium	Salient result
Escherichia coli, Staphylococcus aureus	Atmospheric plasma corona discharge, with high-voltage (20 kV) DC power supply	On agar plates	Changes of pH levels from alkaline to acid, upon plasma application to bacteria in water, does not play a predominant role in cell death.
Staphylococcus aureus	DC cold-atmospheric pressure plasma microjet with compressed air as the working gas	Aqueous suspensions of the organism	First 10-min treatment led to insignificant inactivation. After 16 min, *S. aureus* was completely inactivated. Effective inactivation of *S. aureus* was found to start after the pH values decreased to about 4.5.
Bacillus atrophaeus, Geobacillus sterathermophilus, Clostridium sporogenes, Kocuria rhizophila, Staphylococcus aureus, Aspergillus niger	Low-pressure inductively coupled plasma (ICP) with different mixtures of gases	Glass substrates and silicon wafers coated with the organism by spraying.	All the organisms were found to be reduced by at least 4 orders of magnitude under optimized low-pressure argon plasma. Efficiency of inactivation was variable for different strains of a given species.
Bacillus subtilis	Oxygen and nitrogen treated using 8 Duo-Plasmalines driven by microwaves power	Microscopic slides stacked with spores	Plasma treatment of the spores caused release of DPA, generation of auxotrophic mutants, reduction in Kat X activity and damage to DNA. A biphasic model for the inactivation kinetics was proposed.
Escherichia coli, Bacillus subtilis, Candida albicans, and Staphylococcus aureus	High-frequency capacitive discharge (0.4 torr) and barrier discharge (0.4–0.5 torr) in air excited at commercial frequency of 5.28 MHz	Glass plate and Petri dish	The most probable sterilization agents of the plasma generated were established to be "hot" and "cold" OH radicals, the excited electrically neutral N_2 and O_2 molecules, and the UV plasma radiation.
Influenza viruses (RSV, hPIV-3 and A (H5N2))	Air subjected to high-energy deep-UV light using Biozone scientific COP generator	Air	More than 99.8% reduction of influenza virus A (H5N2).
Escherichia coli KCTC1039 *Bacillus subtilis*	Helium- and oxygen-based electric discharge plasma produced at a radio frequency (RF) of 13.56 MHz	Dried cells and endospore suspension on a cover-glass	Treated cells had severe cytoplasmic deformations and leakage of bacterial chromosome. UV from the plasma only slightly affected the viability of the spores.
Deinococcus radiodurans	Dielectric barrier discharge (DBD)	Cells dried in laminar flow hood and cells suspended in distilled water	4 log reduction of CFU count in 15 s of the extremophile organism suspended in distilled water. This was attributed to the fact that plasma compromises the integrity of the cell membrane of the organism.

Escherichia coli type 1 *Saccharomyces cerevisiae* *Gluconobacter liquefaciens* *Listeria monocytogenes*	Cold atmospheric plasma plume generated by an AC voltage of 8 kV at 30 kHz	Inoculated membrane filters and inoculated fruit surfaces	Efficacy of inactivation was markedly reduced for microorganisms on the cut surfaces than on filters due to the migration of microorganisms from the exterior of the fruit tissue to its interior and not quenching of reactive plasma species.
Escherichia coli O157:H7 *Salmonella* Stanley	Gliding Arc plasma	On agar plates and inoculated onto surfaces of Golden Delicious apples	Bacterial inactivation was shown to be a function of flow rate and duration of exposure.
Aspergillus parasiticus and Aflatoxins	Air gases and SF_6 plasma using total applied power of approximately 300 W	Hazelnuts, Peanuts and Pistachio nuts	SF_6 plasma application was more effective with a 5 log decrease in fungal population for the same duration as air gases plasma. 20-min air gases plasma treatment resulted in a 50% reduction in total aflatoxins (AFB1, AFB2, AFG1 and AFG2), while only a 20% with SF_6 plasma treatment. No significant organoleptic changes were observed.
Escherichia coli *Saccharomyces cerevisiae* *Pantoea agglomerans* *Gluconacetobacter liquefaciens*	Cold atmospheric plasma generated by an AC voltage (variable 12 kV and 16 kV)	Pericarps of mangoes and melons	*S. cerevisiae* was the most resistant among all test organisms. An increase in the applied voltage led to more efficient production of reactive plasma species (oxygen atoms), which was attributed for better inactivation.
Escherichia coli O157:H7 *Salmonella sp.* *Listeria monocytogenes*	One atmosphere uniform glow discharge plasma (OAUGDP) operated at 9-kV power and 6-kHz frequency	Apples, cantaloupe and lettuce	Inactivation was observed in all the cases. Extent of log reduction varied with the organisms.
Escherichia coli	Air in a dielectric discharge chamber	Raw almonds	Up to 5 log reduction observed
Escherichia coli NCTC 9001, *Campylobacter jejuni* ATCC 33560, *Campylobacter coli* ATCC 33559, *Listeria monocytogenes* NCTC 9863, *Salmonella enterica* serovar Enteritidis ATCC 4931, *Salmonella enterica* serovar Typhimurium ATCC 14028, and *Bacillus cereus* NCTC 11145 endospores.	Pulsed plasma gas discharge (PPGD) using high-voltage pulses (with 40-kV DC capacitor) in a coaxial treatment chamber. Different treatment gases viz. nitrogen, carbon dioxide, oxygen and air as sparging	Poultry wash water at 4 °C	Rapid reductions in microbial numbers (by $\leq$ 8 log CFU/ml). Use of oxygen alone produced the greatest reductions. In general, gram-negative test bacteria were more susceptible.
Aspergillus niger, *Bacillus atrophaeus*, *Bacillus pumilus*, *Clostridium botulinum* type A, *Clostridium sporogenes*, *Deinococcus radiodurans*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella mons*	Cascaded dielectric barrier discharge with air as the plasma gas	PET foils	Highest count reduction was observed for the vegetative cells with at least 6.6 $\log_{10}$ with in 1 s. *Aspergillus niger* was the most resistant test strain with an inactivation rate of about 5 $\log_{10}$ in 5 s.

(Misra *et al.,* 2011).

2.6. Use of gas plasma in the food field

As reported before, cold plasma is an emerging non-thermal technology that potentially could be exploited to decontaminate surfaces of food products or devices used for food processes.

Gas plasma has been already employed for bio-decontamination and sterilization of surfaces, medical instruments, water, air, and living tissues without causing damages.

By using non-equilibrium discharges, the decontamination can be achieved at room temperature and atmospheric pressure. Moreover, atmospheric gas plasma offers the advantage of being chemical and water-free, hence it has a positive consequence for the consumers and environment. It is also able to operate openly and continuously (Lacombe *et al.,* 2015). The increasing consumer demand for minimally processed food has created a challenge for the research and food industry to provide safe and high quality products. Gas plasma could satisfy this demand because it potentially offers a treatment step for fresh produce to reduce the microbial load without adversely affecting the nutritional and other key characteristics. On the other hand, this technique could have some limitations related to the characteristics of foods to be treated. Indeed the generation of radical species, especially those of oxygen, could compromise several food components, such as lipids and antioxidants, that can become a "perfect" substrate for oxidation.

In order to clarify the advantages and the disadvantages of the application of this promising technique in food field, a huge deal of studies have been carried out. In the following sections some results concerning food, processing surfaces, biofilm, packaging, and waste waters are reported.

2.6.1. Treatment of foods

One of the main problems of the food industry is the microbiological contamination of raw materials and finished products as the spoilage microflora reduces the product shelf-life and the pathogenic species make foods unsafe. Gas plasma could be useful to decontaminate several food surfaces because its reactive species are able to penetrate the cracks and crevices of even complex-shaped bodies unlike other potential surface treatments such as UV light. Therefore it may act more effectively and efficiently over undulated or cracked surfaces such as those found on many foods like seeds, species and meat (Misra *et al.,* 2011).

Critzer *et al.* (2007) published one of the first applications of atmospheric plasma on contaminated apples, cantaloupe and lettuce. By using one atmosphere uniform glow discharge plasma configuration (OAUGDP), a maximum inactivation of 3 log of *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* (7 log CFU per sample) inoculated on cut samples of apples, cantaloupes and lettuce, were obtained in afterglow after 5 min of treatment (about 11.4 cm form the air plasma source). Also Perni *et al.* (2008) analyzed the efficacy of non thermal gas plasma on cut sections of mangoes and honeydew melons

inoculated with a non-pathogenic species of *E. coli* and spoilage organisms such as *S. cerevisiae*, *P. agglomerans*, *Gluconobacter liquefaciens*. The glow was generated from a mixture of helium and oxygen gases. The Authors have observed that *S. cerevisiae* was more resistant than *P. agglomerans* and *G. Liquefaciens,* which were totally inactivated after only 2.5 seconds of samples exposure. On the contrary, *E. coli* was killed after 5 seconds of treatment.

Various researches on the entire surface of fruits and vegetables have been also conducted. Niemira and Sites (2008) treated Golden delicious apples inoculated with *E. coli* O157:H7 and *Salmonella* serovar Stanley by an open-air gliding arc cold plasma system. They obtained a reduction of about 3 Log CFU after 3 min of treatment. Deng *et al.* (2005) managed to reach maximum reductions of 5 Log CFU after 30 seconds of air plasma on almonds inoculated with *E. coli*. Selcuk *et al.* (2008) successfully treated grains (barley, oat, rye, corn and wheat) and legumes (bean, chickpea, soybean, lentil) infected with *Aspergillus* spp. and *Penicillium* spp. without or only marginally affecting products. They have observed maximum reductions of 3 Log CFU after 15 min of treatment with a low-pressure cold plasma (LPCP) prototype using air and SF_6 gases. Other recent studies have shown interesting results inactivating several microorganisms, including degradative and pathogens, inoculated on lettuce, carrots and tomatoes (Bermúdez-Aguirre *et al.,* 2013), leaves of corn salad (Baier *et al.,* 2013), apple juice (Surowsky *et al.,* 2014a). Other recent results have shown the capability of atmospheric gas plasma to inactivate the natural microflora occurring on the surface of Abate Fetèl pears (Berardinelli *et al.,* 2012), blueberries (Lacombe *et al.,* 2015) and strawberries (Misra *et al.,* 2014).

A huge amount of successful studies were carried out on food matrices of animal origin, inoculated with several foodborne pathogens and treated with different non thermal gas plasma devices, e.g. sliced cheese and ham (Song *et al.,* 2009), egg shells (Ragni *et al.,* 2010), chicken meat (Noriega *et al.,* 2011), bresaola (Rød *et al.,* 2012), pork butt and beef loin (Jayasena *et al.,* 2015) and milk (Kim *et al.,* 2015a). Some of these Authors have evaluated the effects of gas plasma exposure on food quality: the oxidation of the fat component seems to be the main issue (Korachi *et al.,* 2015). Jayasena *et al.* (2015) suggested the combination between gas plasma and some hurdles technologies as a possible solution to counteract this quality reduction.

2.6.2. Treatment of biofilms and processing surfaces

Several food components such as fats, if not correctly removed by processing surface, can promote biofilms formation, in which microorganisms proliferate and are largely protected from external stresses including washing and sterilization processes. Biofilms are a serious issue for many food industry sectors including brewing, dairy processing, fresh produce and meat processing. The study of the capability of gas plasma to counteract biofilm is quite recent. Denes *et al.* (2001) claimed in a patent (US 6096564) that their gas plasma generator can sterilize food processing surfaces contaminated by a mixed biofilm composed by *P. fluorescens*, *Salmonella typhimurium* and *S. epidermidis*. Abramzon *et al.* (2006) achieved almost total killing of *Chromobacterium violaceum* fixed in a 4-day old biofilm using a high-pressure cold plasma jet. Vleugels *et al.* (2005) were able to inactivate biofilm-forming bacterium *Pantoea agglomerans* on bell peppers with an atmospheric pressure glow discharges (APGD) device.

Moreover, some studies have reported the capability of gas plasma to denature proteins and remove allergens from food processing surfaces (Shama *et al.,* 2009).

Finally, some results have showed significant reductions of food-borne pathogens in planktonic form inoculated in some tools such as rotating knife (Leipold *et al.,* 2010).

2.6.3. Treatment of packaging

Non thermal gas plasma is also suitable to treat food packaging materials, especially those thermolabiles (Pankaj *et al*., 2014). This current application of plasma is limited and only at research level. Some data have shown that low-temperature gas plasma can quickly sterilize plastic bottles, lids and films without adversely affecting the properties of the material or leaving any residues (Misra *et al.,* 2011).

For instance, Muranyi *et al.* (2010) evaluated the influence of plasma treatment (with a DBD device) on viability of *Bacillus atrophaeus* spores and vegetative cells inoculated on PET / PE, PET, PS films. Moreover the possible changes of the films characteristics have been investigated. The results showed that the DBD device is able to reduce the cell load and do not significantly influence the functionality of the materials, even though some little changes have been observed. A study conducted by Yun *et al,* (2010) was focused on *L. monocytogenes* inoculated onto disposable food containers including paper cups, aluminum foil and disposable plastic trays. In aluminum foil and paper cups, three decimal reductions of viable cells were achieved after gas plasma treatment, while a completely cell inactivation was obtained on plastic tray.

Finally, in a recent work the corona discharges have been able to reduce over 2 Log of *S.* enteritidis, *P. aeruginosa*, and *Penicillium chrysogenum* deposited onto a polylactide (PLA) packaging film (Stepczyńska *et al.,* 2014). On the contrary, no significant results for *E. coli, B. subtilis and S. aureus* have been obtained.

2.6.4. Treatment of waste waters

Food industry, and namely especially poultry and meat industries, produces a large quantity of waste waters which must be properly sterilized and disposed. Beyond the active particles, electrical field and radiation, the mechanism of gas plasma generation in liquids can include the acoustic and shock waves. In particular, the application of high-voltage pulses favours and complete the breakdown of the gas in the liquid (Misra *et al.,* 2011). Nowadays in literature few works describe the possible use of gas plasma in waste waters treatment. In a study conducted with a pulsed plasma gas discharge device, significant reductions of *E. coli* and the completely inactivation of *S.* Enterica, *Listeria monocytogenes*, *Campylobacter jejuni* and *Campylobacter coli* were obtained after 30 second of treatment (Rowan *et al.,* 2007). When a working gas containing oxygen was used, a significant reduction of *Bacillus cereus* endospores were observed. The Authors have proposed this technology as an alternative approach for treating raw poultry wash waters and for preventing cross-contamination in processing environments.

Chapter 3. *Listeria monocytogenes*

3.1. Brief history

Murray *et al.* (1926) described for the first time *Listeria monocytogenes* as *Bacterium monocytogenes* because it caused monocytosis in infected laboratory rabbits and guinea pigs. Subsequently, Pirie renamed it *Listerella hepatolytica* in 1927 and finally in 1940 he call it with its present name. Following its initial description, Gille and Nyfeldt isolated it from sheep and humans, respectively (Farber *et al.,* 1991). After its identification, various works associated some severe diseases, such as meningitis, septicemia, infections of the central nervous system and spontaneous abortions, to these bacteria and merge them in one called "Listeriosis". Listeriosis can be lethal particularly for immune-compromised individuals. The interest in *Listeria* grew rapidly in the 90' when it was involved in various foodborne outbreaks associated with the consumption of minimally processed products in which the bacteria just exceeded 100 CFU/g (Chen *et al.,* 2003). *L. monocytogenes* can be found in a wide variety of raw and processed foods including milk, dairy products, beef and pork meats, fermented sausages, fish and vegetables. It can contaminate a great deal of foods because of its high ability to adapt, survive and grow in a wide range of environmental conditions.

The main factors that have influenced the incidence of listeriosis are the:

- increase of the average lifespan of people and survival of immune-compromised and elderly individuals.
- Development of new food production and food processing, *e.g.* minimally processed, ready-to-eat convenience foods and refrigerated or frozen foods.
- Globalization of the food industry.
- Growing demand for imported and ethnic foods.

Although listerosis is relatively rare, it is still one of the most deadly foodborne pathogens, with about one third of all clinical manifestations resulting in morbidity (Schuppler and Loessner, 2010). These rates overcome those from *Salmonella* and *Clostridium* and make listerosis the main cause of morbidity due to food related infections (Mead *et al.,* 2000).

In Italy listeriosis has been included among the "Notificable infectious and contagious diseases" at the end of 1990 (DM 15.12.1990). The Food and Drug Administration (FDA) has published a quantitative assessment of relative risks to public health from the consumption of selected categories of ready-to-eat foods that may be contaminated with the foodborne

pathogen *L. monocytogenes* and established a zero tolerance policy in place for this pathogen (Gandhi *et al.,* 2006).

3.2. Taxonomy, morphology and main characteristics

Listeria monocytogenes is a Gram-positive, non-spore forming, facultative anaerobic, catalase positive and oxidase negative rod shaped bacterium. It belongs to the Firmicutes and because of its characteristic low percentage of guanine/cytosine bases in its genome, it is closely related to *Bacillus, Staphylococcus, Streptococcus* and *Clostridium* species. Indeed it belongs to Bacilli class and Bacillales order. The genus *Listeria* includes six species: *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii, Listeria grayi, Listeria welshimeri* and *Listeria seeligeri.* Within these six species, only *Listeria monocytogenes* and *L. ivanovii* are pathogens, but only the former is fatal for humans, whereas the latter affects ungulates (Santagada *et al.*, 2004).

Cells are found as a single units, short chains or arrangend in V and Y forms (Rocourt and Buchirieser, 2007). They have peritrichous flagella, which give them a characteristic tumbling, motility, occurring only between 20 and 25°C. In Brain Heart Infusion agar, the colonies are 0.2-0.8 mm in diameter, smooth, punctiform, gray and translucid.

L. monocytogenes is ubiquitous and is widely present in plant, soil, silage, sewage, water and faeces of human and animals. Even if its primary environment is considered to be soil, where it lives as a saprophyte feeding off dead and decaying plant matter (Freitag *et al.*, 2009), *L. monocytogenes* can adapt to live in the cytosol of eukaryotic host cells. Indeed, following its ingestion by a susceptible individual, *L. monocytogenes* is capable of making the transition to a physiological state that promotes bacterial survival and replication in the host cells.

As above reported, *L. monocytogenes* contaminates foods because of its ability to survive in food processing plants, where it can resist to several adverse conditions including also environments specifically planned to inhibit bacterial growth. Although its optimum temperature is 37 °C, it is able to grow between -0.4 and 50 °C. Also the pH range is wide (5.6 – 9.0) and it grows in the presence of NaCl concentrations up to 10%, and at water activity (Aw) values below 0.93 (Rocourt and Buchirieser, 2007).

3.3. Mechanism of virulence

L. monocytogenes can affect the host because of its ability to cross the intestinal, the blood brain and fetal-placental barriers (Lecuit *et al.,* 2004). The first mentioned passing is the most

important one in listeriosis infections by foods. Inside the host cell *L. monocytogenes* follows a specific intracellular life cycle (Vàzquez-Boland *et al.,* 2001):

1) *Internalization:* it is the first step through which *Listeria* adheres to the surface of an eukaryotic cell and then penetrates into the host cell. During the invasion, a zipper-type mechanism is involved, in which the bacterium gradually sinks into dip-like structures of the host cell surface until it is finally engulfed. Hence, the membrane of the target cell closely surrounds the bacterial cell. The structures, mechanisms, and signal transduction cascades involved in the interaction between *Listeria* and the host cell during phagocytosis are not totally elucidated yet. In literature, some surface proteins such as the internalin A (InlA) and internalin B (InlB), Ami protein, the actin-polymerizing protein ActA, and p60 are recognized as bacterial ligands responsible for adhesion and phagocytosis. About 25 internalins are identified in *Listeria*, and the InlA InlB are the best characterized ones. InlA plays a fundamental role in the invasion of *L. monocytogenes* and in particular allows to enter the human intestinal epithelial cell line Caco-2 by binding the host cell adhesion transmembrane glycoprotein named Ecadherin (Gaillard *et al.,* 1991). The binding between *Listeria* and the E-cadehin activates a complex sequence of events which lead to the depolymerisation of the actin and subsequent envelopment of the bacterium with the membrane of the host cell (Cossart *et al.,* 2003). Hence *L. monocytogenes* enters the host cell within the phagosomal compartment.

   InlB allows the bacterium to invade hepatocytes cells by binding to Receptor Tyrosine Kinase (RTK) Met (Shen *et al.,* 2000). The RTK Met receptor consists of a single hydrophobic transmembrane-spanning domain, an extracellular N-terminal region, and an intracellular C-terminal region. The link between InlB and the extracellular part of the RTK Met causes the rapid tyrosine phosphorylation via the classical phosphatidylinositol 3 kinase pathway (PI3K) and triggers signaling pathways leading to actin cytoskeleton integration required for internalization of *L. monocytogenes*. Other proteins including Gab1 and CrkII can promote actin polymerisation.

2) *Escape from primary phagosome:* during the invasion, *L. monocytogenes* is internalized in a primary phagosome, but in order to survive and proliferate it needs to escape from this confinement. Little is known about the characteristics of the *Listeria*-containing vacuolar compartment, but the vacuoles become acidified soon after uptake. About 30 min after its entry, *L. monocytogenes* starts to destroy the phagosome membrane and exits in the cytoplasm. This membrane disruption is

mediated by the hemolysin in combination with phospholipases. Hemolysin, or Listeriosin O (LLO), is a 58 kDa protein belonging to a family of cholesterol dependent cytolysins which is encoded by the *hly* gene and regulated by *PrfA*, a central temperature sensitive regulator of virulence genes (Scortti *et al.*, 2007). LLO is activated by thiol reducing agents and is inactivated by the binding of cholesterol (Cossart *et al.,* 1989), and its function is to form pores into the membrane. It plays an important role also in the internalization and host cell interaction. Stavru and Cossart (2011) showed that LLO can interfere with host cellular mitochondria in order to preserve *L. monocytogenes* replication by inhibiting the death of host cells or killing agents which are inhibitory to bacterial dissemination. The phospholipases involved in the membrane disruption are: PI-PLC encoded by *plcA* gene and PC-PLC encoded by *plcB* (Freitag *et al.*, 2009). The first one is highly specific for phosphoinositol and glycosyl-PI-anchored proteins, while the second one hydrolyses a great deal of phospholipids (Geoffroy *et al.*, 1991). These proteins work synergistically with LLO causing the dissolution of the plasma membrane (Schnupf and Portnoy, 2007).

3) *Intracellular growth*: After escaping from the primary phagosome, *L.monocytogenes* actively multiplies in the host cytoplasm with a doubling time of approximately 1 h. Since the environment is permissive, *L. monocytogenes* does not use any stress response mechanism and three metabolic genes (*purH, purD*, and *pyrE*, involved in purine and pyrimidine biosynthesis) and an arginine ABC transporter (*arpJ*) ares induced within host cells. The mutation of these genes can be involved in metabolic pathway in order to improve the growth within cells. Indeed, a study indicates that pathogenic *Listeria* spp. may exploit hexose phosphates from the host cell cytoplasm for an efficient intracellular growth (Ripio *et al.,* 1997).

4) *Movement and spreading to adjacent cells:* intracytoplasmic *L. monocytogenes* is surrounded by a dense cloud, formed by host cell actin filaments, which polymerises to form an actin tail on one bacterium pole. This tail is composed by two cross-linked actin filaments and let bacterium to move quickly (0.3 mm/s) inside the host cell to infect the new cytoplasm. When bacterium comes into contact with the membrane, push it as a rocket and a sort of finger-like protrusion with a bacterium at the tip is generated. Later this protrusion penetrates in the neighboring cell and is "swallowed".

5) *Escape from secondary phagosome:* Inside the new cell, *L. monocytogenes* is in turn engulfed by a second phagosome delimited by a double membrane with the inner membrane originating from the donor cell. *L. monocytogenes* rapidly escapes from the

new formed vacuole by dissolving the double membrane, thus reaching the cytoplasm and initiating a new round of intracellular proliferation and direct intercellular spread. The actin-based intracytoplasmic movement and cell-to-cell spread are mediated by the surface protein ActA. ActA id encoded by the *ActA* gene and is a 639 amino acid, dimerised protein which is formed by three distinct parts (Smith *et al.*, 1996). The N terminus is associated with actin assembly and bacterial motility; the central part is responsible for the connection between protein and the bacterial cell wall, while the VCA region interacts with the Arp2/3 complex. Arp2/3 is another protein complex which facilitates the polymerisation of actin (Boujemaa-Paterski *et al.*, 2001). The polymerization involves other proteins such as VAPS and CapZ. These proteins mediate also the evasion of *L. monocytogenes* by the host cell.

A correct evolution of these steps is fundamental for a full *L. monocytogenes* virulence and defects at any point can lead to high attenuation. In Figure 10 the intracellular cell cycle is reported. Almost all genes reported before, and involved in the invasion, primary phagosomal escape and direct cell to cell transmission, are regulated by the PrfA protein. In particular *prfA, plcA, plcB, hly, mpl, actAB* and *hpt* are under the control of this protein.

PrfA is a 233 amino acid long, which up-regulates these gene when *Listeria* is in a host cell and down-regulates them when it lives in the environment.

The expression of the PrfA protein is temperature dependent: It is silent at 30°C and maximally expressed at 37°C (Sheehan *et al.,* 1995). In this way PrfA controls the virulence genes at the homeostatic temperature of the host cell.

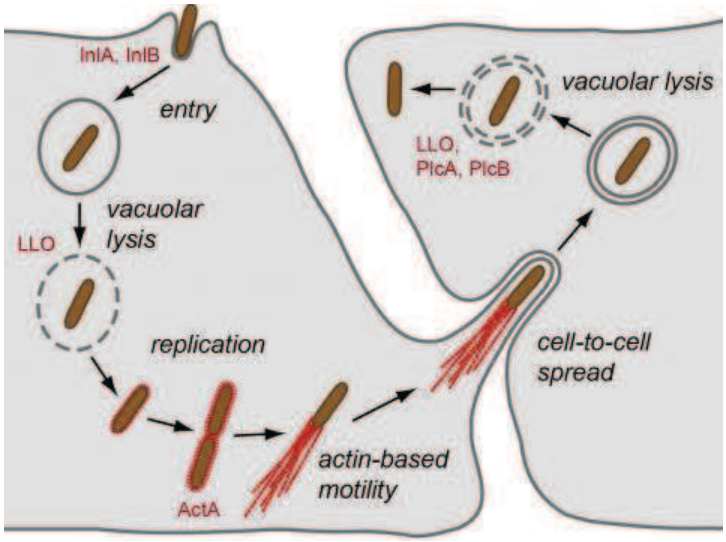

Figure 10. Intracellular cell cycle of *L. monocytogenes* (Pizarro *et al.*, 2012).

3.4. *Listeria monocytogenes* metabolism

L. monocytogenes can live and grow under both aerobic and anaerobic conditions. Most of its metabolic pathways are similar to those of *Bacillus subtilis*, which belongs to the group of low G+C Gram-positive bacteria similarly to *L. monocytogenes*. Neverthenless, there are various significant differences which may be essential for understanding the interference of listerial metabolism with that of the host cells (Joseph and Goebel, 2007).

In aerobic conditions, the respiration takes place and the respiration chains contains (as quinones only) menaquinone, but not coenzyme Q10, also called ubiquinone (Joseph and Goebel, 2007). Menaquinone derives from a branch of the aromatic amino acids pathway and it functions as a cofactor in the electron transport chain.

In aerobic conditions *Listeria* spp. uses hexoses and pentoses to grow, including maltose, glucose, rhamnose and lactose, but not sucrose (Farber *et al.,* 1991). Daneshvar *et al.* (1989) observed that the main metabolic end products in aerobic conditions are lactate (28%), acetate (23%) and acetoin (26%).

Under anaerobic conditions, only hexoses and pentoses support growth. In particular lactate is the major fermentation product (about 79%) thus indicating that the mixed acid fermentation is the major mode of fermentation in *L. monocytogenes* (Farber *et al.,* 1991). Romick *et al.* (1996) found other anaerobic end products which include formate (5.4%), ethanol (7.8%), carbon dioxide (2.3%) and acetate (2%). These results demonstrate that acetoin and lactate are good indicators of aerobic or anaerobic growth.

Concerning carbohydrates, glucose and other sugars are preferentially taken up by the bacterium via the phosphotransferase system (PTS).

Glucose and other PTS-sugars like fructose, mannose and cellobiose are the preferred carbon sources for *L. monocytogenes* when it grows in minimal liquid media. The study of its genome has revealed an unusually large number of genes (>40) encoding PTS. Unlike the other low G+C Gram-positive bacteria, which have *ptsG* gene encoding PTS-dependent glucose transporter, the genome of *L. monocytogenes* is incomplete. Despite this deletion, the growth of *L. monocytogenes* is unaffected in minimal media with glucose as the carbon source suggesting that this gene is not involved in the glucose uptake (Joseph and Goebel, 2007).

Mertins *et al.* (2007) investigated the possibility of a not PTS-dependent glucose uptake, but the *ptsH* mutant, which did not use the PTS-dependent systems, could not grow in minimal medium using glucose as a carbon source. This finding suggests that the PTS transport is the mainly one responsible for glucose transport.

L. monocytogenes catabolises glucose via the glycolytic and the pentose phosphate pathways, but not via the EntnereDoudoroff pathway (Joseph and Goebel, 2007).

The principal glycolysis genes, *i.e. gap, pgk, tpi, pgm* and *eno*, used by *L. monocytogenes* are the same as those found in most low G+C Gram-positive bacteria. These genes are down-regulated in minimal medium in favor of an up-regulation of the enzymes involved in the pentose phosphate pathway. This up-regulation indicates the need for an oxidative decarboxylation of glucose by glucose-6-phosphate and the production of CO_2 for the biosynthesis of aromatic amino acids, which are not present in the minimal medium. Joseph *et al.* (2006) observed a similar down-regulation of glycolysis genes and up-regulation of pentose phosphate pathway when *L. monocytogenes* grows in host cells, perhaps due to a limited availability of PTS sugars. Chico-Calero *et al.* (2002) observed the capability of *L. monocytogenes* to use phosphorylated hexoses (PHs), such as glucose-1-6-phosphate, fructose-6-phosphate, as carbon sources. The bacterium takes PHs by the host cytosol and transports them into the cell through the hpt transporter. This transporter is under the control of the PrfA virulence regulator, and is highly up-regulated during the internalization of bacterium onto the host cell (Camejo *et al.*, 2009). *L. monocytogenes* can use also glycerol as a carbon source (Figure 11). Glycerol is taken up via facilitated transport, phosphorylated by glycerol kinases (encoded by *lmo 1034)* and then oxidized by glycerol-3-phosphate dehydrogenase (encoded by *lmo 1538*) to glyceraldehyde-3-phosphate which is finally metabolized by the glycolytic pathway enzymes (Joseph and Goebel, 2007). The same Authors, instead, excluded amino acids and Acetyl-CoA as carbon fonts. The latter is not used by *L. monocytogenes* due to the lack of the glyoxlyate shunt genes and this also rule out the utilization of fatty acids as a carbon font.

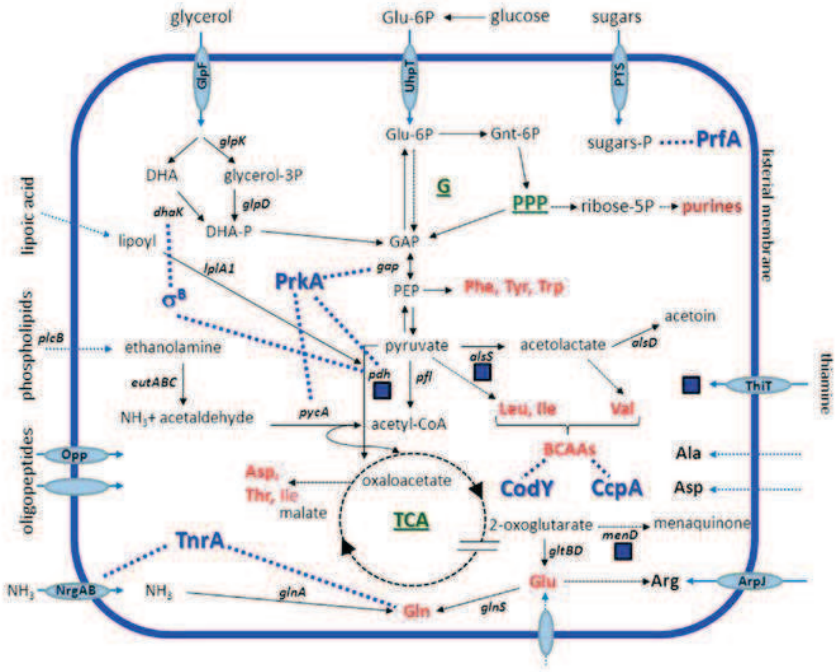

Figure 11. Simplified view of listerial metabolic enzymes, transporters, and pathways (Fuchs *et al.,* 2012).

L. monocytogenes has a regulatory mechanism, called carbon catabolite repression (CCR) which rules *prfA* virulence regulator and in general the expression of genes associated with secondary carbon sources when the primary carbon sources are available (Gorke and Stulke, 2008). This regulatory effect allows *L.monocytogenes* to grow optimally in the presence of various carbon sources using those preferential when they are available.

Joseph and Goebel (2007) reported that the listerial metabolism is relieved of CCR control when the bacteria replicate in the host cell cytosol. At the same time the Authors observed an up-regulation of genes encoding an uptake mechanism for phosphorylated hexoses (*hpt*), oligopeptides and amino acids (*lmo 2251*) and glycerol (*lmo1034, lmo1538*). On the basis of these observations it was hypothesized that glucose is not a predominant carbon source inside host cells. Moreover, when glucose or phosphorylated glucose are not available in the environment, an up-regulation of the genes involved in the pentose phosphate cycle and a down-regulation of those involved in glycolysis was observed. These results suggest that the pentose phosphate cycle is the favorite pathway in the absence of glucose.

Concerning nitrogen metabolism, glutamine is the preferential nitrogen source for *L. monocytogenes.* In the absence of this amino acid, especially when the bacterium is inside the host cell cytosol, it is capable to use alternative fonts, such as such ammonium, which is the favorite substitute, arginine and ethanolamine (Joseph and Goebel, 2007).

Inside the listerial cell, glutamine is converted to glutamic acid by glutamate synthetase (GOGAT) with 2-oxoglutarate (OG) as additional substrate.

On the other hand when ammonium is used as an alternative nitrogen source, it is transported in *L. monocytogenes* by the transporter NrgA which is encoded by the ngrAB operon. The transcription of the nrgAB promoter is activated during nitrogen-limited growth by the global regulator TnrA. Ammonium is then incorporated into glutamine, and further to glutamic acid, as above described. This pathway is also observed for *Bacillus subtilis*.

As previously reported, also arginine is a potential nitrogen sources. It is transported inside the cell by a specific arginine ABC transporter (encoded by *arpj)* and then degraded into citrulline and ammonia by arginine deaminase (encoded by *lmo0043-arcA*). Citrulline in turn is degraded into a further ammonia molecule and ornithine via the enzymes ornithine carbamoyl transferase (OCT) and carbamoyl carboxy kinase (CCK) encoded by the *L. monocytogenes*-specific arcBCD operon (*lmo 0036* and *lmo 0039*, respectively).

Also adenine (to a limited extent) and ethanolamine are two other possible nitrogen sources. The latter is generated through the degradation of phosphatidylethanolamine (PEA), which is an excellent substrate for PlcB, a listerial phospholipase C encoded by the PrfA-dependent gene *plcB*. Ethanolamine is hydrolyzed into ammonia and acetaldehyde by the vitamin B12-dependent ethanolamineammonia lyase encoded by the *eutBC* genes (Joseph and Goebel, 2007).

Concerning amino acids biosynthesis, Tsai and Hodgson (2003) observed the absence of the genes required for cysteine and methionine biosynthesis. Therefore these amino acids are essential for *L. monocytogenes* which have to absorb them from the environment. Moreover, *L .monocytogenes* lacks also sulphate and nitrate reductases, thus there is a dependency for reduced nitrogen and sulphate sources, which can be gained from cysteine and methionine. However, *L. monocytogenes* is capable of *de novo* synthesising branched chain amino acids (BCAA), *i.e.* valine, isoleucine and leucine, via the conventional pathways. Some studies have shown that *L. monocytogenes* has some requirement for them. In particular, the essential precursors of BCAA are pyruvate and threonine (deriving from aspartic acid via oxaloacetate), and their availability is directly or indirectly connected with the citrate cycle that is interrupted in *L. monocytogenes* due to the lack of 2-oxoglutarate dehydrogenase, which converts alpha-ketoglutarate into succinylCoA. As a result of this incomplete cycle (Figure 12), *L.monocytogenes* is incapable of regenerating oxaloacetate through the Krebs cycle from citrate. Therefore, oxaloacetate is produced by the carboxilation of pyruvate by pyruvate carboxylase, which is encoded by *pycA*. This step is fundamental for the entrance of Acetyl-CoA into the Krebs cycle and for the synthesis of asparagine, threonine, cysteine and

methionine (Figure 13). Because of the interruption of Krebs cycle, oxaloacetate is also the precursor of malate and succinate (Joseph and Goebel, 2007).

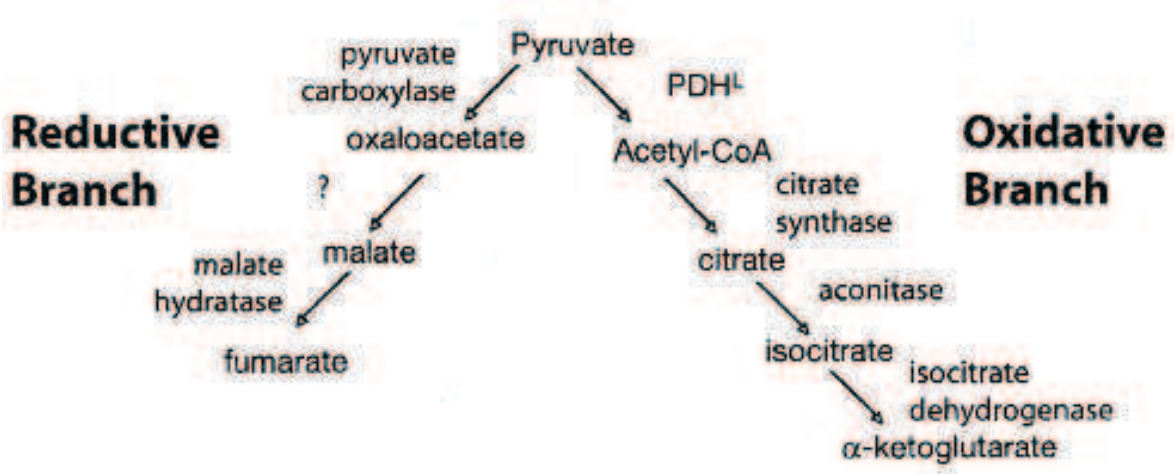

Figure 12. Krebs cycle of *Listeria monocytogenes* (Kenney, 2011). This cycle is split without the conversion of a-ketoglutarate to succinate. *L. monocytogenes* may possess a split non-cyclic citrate pathway with an oxidative portion (citrate synthase, aconitate hydratase, isocitrate dehydrogenase) and a reductive portion (malate dehydrogenase, fumarate hydratase, fumarate reductase).

Buzolyova and Somov (1999) observed that pyruvate carboxylase needs CO_2 to produce oxaloacetate. When glucose is the unique carbon source, the oxidative decarboxylation of glucose-6-phosphate, which is the first reaction in the pentose-phosphate pathway, seems to be necessary as suggested by the high induction of the gene for pyruvate carboxylase in *L. monocytogenes.* Some studies have reported that the major source of nitrogen inside the host cell, excluding alanine, asparagine and glutamate which are synthesized *de novo*, is provided by the host cell, as suggested by the up-regulation of the oligopeptide transporters (Chatterjee *et al.,* 2006). The Authors have also observed a down-regulation of the aminoacyl tRNA synthase genes *glyS, serS, cysS, alaS, hisS, valS, thrS, ileS, leuS, tyrS*, and *trpS*, as suggested availability of the respective amino acids within the cytosol.

L. monocytogenes cannot synthesize several vitamin and cofactor such as biotin, lipoic acid, riboflavin and thiamine which are fundamental for its growth. For instance, lipoic acid is an important co-factor of the pyruvate dehydrogenase enzyme (Pdh) complex, which is involved in acetyl CoA formation from pyruvate in the aerobic metabolism (Ramaswamy *et al.*, 2007). *L. monocytogenes* uses two lipoate ligases in order to absorb lipoic acid from the environment.

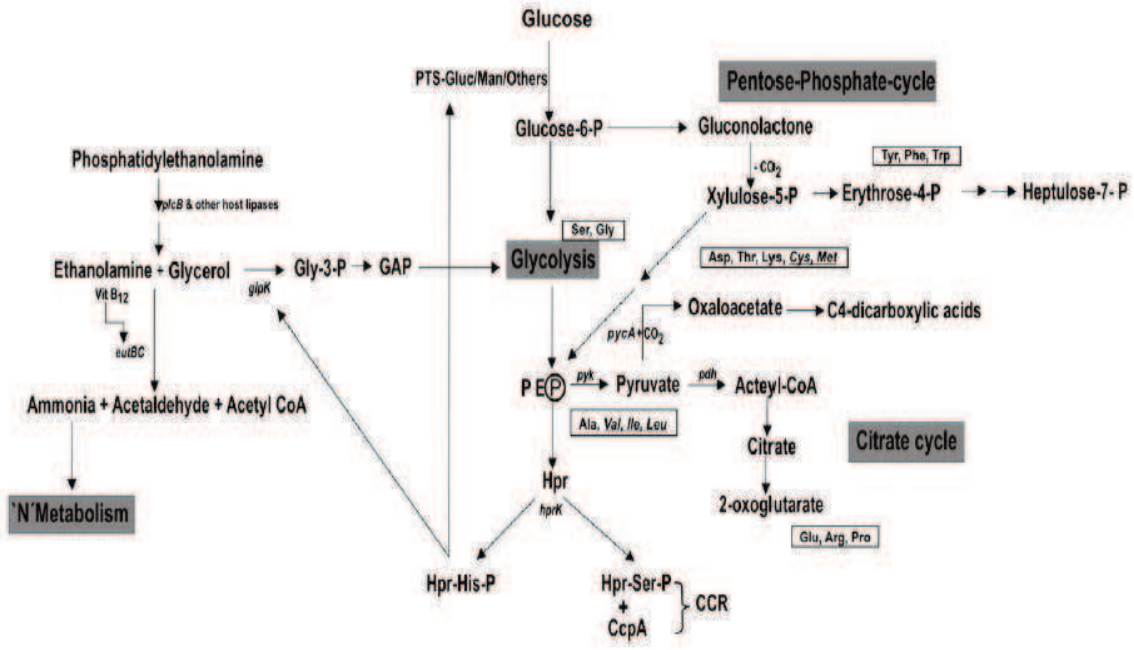

Figure 13. Carbon and Nitrogen metabolism in *L. monocytogenes* (Joseph and Goebel, 2007).

3.5. *Listeria monocytogenes* stress response mechanism

L. monocytogenes can survive and grow over various stress conditions. A microbial stress occurs when microorganisms are affected by harmful physical, chemical and biological causes (Yousef and Courtney, 2003). Various factors can be defined as stressor, including traditional (*e.g.* heat, low temperatures, high salt content, low or alkaline pH, chemical additives) and novel (*e.g.* HHP, HPH, ionizing radiation, PEF, MAP…) food preservation techniques, but also competition and metabolites produced by other microorganisms (microbial antagonism). These stressors can affect the growth, physiology and the activity of microorganism thus causing different degrees of damages. Indeed, on the basis of the extent, the stresses can be differentiated in "lethal" or "sub-lethal" stresses. The former cause irreversible damages to the microbial cells determining a complete death of the microbial population. On the other hand, a sub-lethal stress reversibly modifies the metabolic activity resulting in a delay in microbial growth (Donnelly, 2002). In order to counteract sub-lethal stresses, some microorganisms have evolved strategies resulting in modifications of their metabolism, thus becoming more resistant to subsequent similar or different stresses. This mechanism is named "stress adaptation" (Lou and Yousef, 1997). In the following sections, the survival mechanisms adopted by *L. monocytogenes* under adverse environmental conditions, *e.g.* low temperature, acid stress and osmotic stress, are described.

3.5.1. Survival mechanism at low temperatures

Refrigeration is one of the principal methods to increase the shelf-life of foods, but *L. monocytogenes* has the ability to survive and grow at 2–4 °C. This ability becomes a real issue for the food industry, and the understanding of the survival mechanisms adopted by *L. monocytogenes* can provide information to control and then avoid food contamination by this pathogen.

In order to counteract low temperature, *L. monocytogenes* has the capability to change its cell membrane fluidity. The fluidity, and the functionality of the cell membrane, are due to the presence of lipids in a fluid and crystalline state. When a change in temperature occurs, the bacterium has to modify the membrane lipid composition to maintain the membrane fluidity required for a proper solutes transport (Gandhi and Chikindas, 2007). The cell membrane of *Listeria* is characterized by an high amount (90%) of *iso* and *anteiso* odd-numbered branched-chain fatty acids (BCFAs), such as eptadecanoic (C17:0) and pentadecanoic (*i*C15:0, *a*C15:0) acids. The precursors of the BCFAs are alpha-keto acids which derive from BCAAs, such as isoleucine, valine, leucine (Figure 14). Alpha-keto acids are subsequently catalyzed by an alpha-keto acid dehydrogenase (BKD) complex in BCFA. Mutants deprived by gene encoding BKD were unable to grow at low temperature, *i.e.* 10 °C (Zhu *et al.,* 2005). On the other hand, the bacterium could recover the growth ability in a medium supplemented with 2-methylbutyric acid (2MB), which is a precursor for odd-numbered *anteiso*-fatty acids. The use of 2MB as a substrate bypassed the reaction driven by BKD (Figure 15). The Authors did not obtain the same results (at 10 °C) by using two other short chain fatty acids, *i.e.* isobutyric acid, precursor for even-numbered iso-fatty acids, and isovaleric acid, precursor for odd-numbered iso-fatty acids.

Beales (2004) observed an increase in the proportion of unsaturated fatty acids and C15:0 at the expense of C17:0 when *Listeria* grows at 7°C. The "cutting" of the chain length from C17:0 to C15:0 results in the reduction of carbon-carbon interaction between neighboring chains taking back the membrane fluidity to the optimum degree. Moreover, a change from *i*C15:0 to *a*C15:0 was observed when *Listeria* was grown at 5°C (Annous *et al.,* 1997).

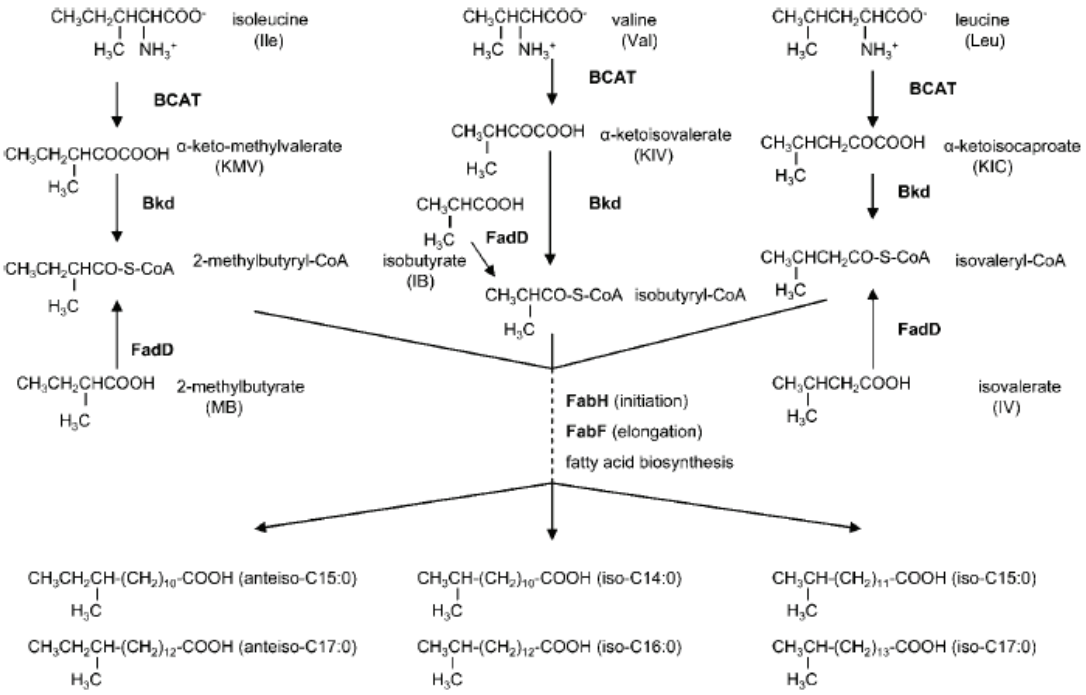

Figure 14. Pathway for the biosynthesis of branched-chain fatty acids (Zhu *et al.,* 2005).

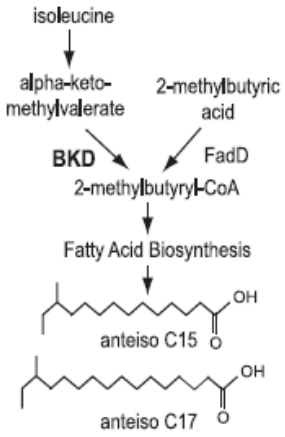

Figure 15. Use of 2-methylbutyric acid as a branched-chain fatty acids precursor (Sun and O'Riordan, 2010).

In order to response to a temperature down shock *L. monocytogenes* can also produce cold shock proteins (Csps). Bayles *et al.* (1996) observed an induction of 12 proteins in cold-shocked cells and a production of 4 cold acclimation proteins (Caps) during the growth at 5°C. This change in proteins production is accompanied by changes in the gene expression. Liu *et al.* (2002) reported some results concerning the ability of *L. monocytogenes* to increase the expression of mRNA for chaperone proteases GroEL, ClpP and ClpB. The Authors have hypothesized an involvement of these proteases in the degradation of anomalous polypeptides that are formed in the bacterium during the growth at cold temperatures.

Another way used by *Listeria* to counteract low temperature stresses is the accumulation of the cryoprotectants glycine betaine and carnitine, which are transported by three compatible

solute systems, *i.e.* glycine betaine porter I (BetL), glycine betaine porter II (Gbu) and the carnitine transporter (OpuC). In particular the uptake of glycine betaine into the cell is mediated mainly by Gbu and at lower extent by BetL and OpuC. The transport of glycine betaine at 7°C is 15-fold faster than at 30°C (Ko *et al.,* 1994). On the contrary, the transport of carnitine is conducted mainly by OpuC and in second way by Gbu and BetL (Angelidis and Smith, 2003). Moreover *Listeria* accumulates these solutes to thwart environmental osmotic changes.

The survival of *Listeria* under environmental stress conditions is modulated by sigmaB factor (σ^B). σ^B stimulates the accumulation of cryoprotectants during growth at low temperature (Becker *et al.,* 2000).

3.5.2. Survival under acid stress

Acidification is another method widely used to preserve foods, and it is achieved by the addition of some preservatives, *e.g.* citric acid, ascorbic acid, lactic acid or via fermentation.

The preservatives and the fermentation products are usually weak acids which cross the microbial membrane in a non-dissociated form and then dissociated them-selves into the cytoplasm and decrease the intracellular pH which compromise the cellular metabolism.

In the review by Farber *et al.* (1991) it is reported that various strains of *Listeria* spp. can grow in a nutrient broth with pH values from 4.5 to 7.0. Moreover, amongthe various acids used to lower the pH (e.g. acetic, lactic, citric, and hydrochloric acids), the acetic acid was the most effective growth inhibitor.

In real systems, *L. monocytogenes* can encounter low pH in foods, but also during gastric passage and in the phagosome of the macrophage (Gandhi and Chikindas, 2007) and can use a number of stress adaptation mechanisms in order to respond to and survive this environmental conditions.

Phan-Thanh and Mahouin (1999) observed the induction of several proteins during the growth under both lethal and sub-lethal low pH values. They found that most of the induced proteins, such as GroEL, ATP synthase, thioredoxin reductase, are common in both conditions. Also various transcriptional regulators and ferric uptake regulator were observed.

It is well known that acid adapted cells of *Listeria* spp. can subsequently counteract other stressor as heat (52 °C), salt (25–30% NaCl) and alcohols (Phan-Thanh et *al.,* 2000). This important ability must be considered by food industries when hurdles and processes applicable to their products are chosen and optimized.

Another method used by microorganisms including *L. monocytogenes* to survive in acid stress conditions includes the maintenance of their intra-cytoplasmic pH by homeostasis.

The homeostasis is achieved by proton transport across the cell membrane which works in a different way in aerobic and anaerobic organisms. In the former, the active transport of H^+ is coupled with the electron transport in respiratory chains, while in the latter H^+ transport is carried out via H^+- ATPase molecules using energy from ATP hydrolysis (Gandhi and Chikindas, 2007). *L. monocytogenes* can use both processes because is a facultative anaerobic bacterium (Shabala *et al.,* 2002). The F_0F_1-ATPase transports protons across the cell membrane by utilizing ATP (Figure 16). It is a multi-subunit enzyme highly conserved and it is composed by F_1 and F_0 portions. The proton gradient induces the rotation of F_0 permitting the F_1 subunit to lead ATP synthesis. In the reverse reaction, ATP hydrolysis determines the rotation of F_0 in the opposite direction (Yoshida *et al.,* 2001). In a study in which an inhibitor of this enzyme was used, a three-log reduction of *L. monocytogenes* was observed before and during acid stress (Cotter *et al*., 2000). This result highlights the contribution of F_0F_1-ATPase in *Listeria* acid adaptation.

Moreover, *L. monocytogenes* is one of the few microorganisms which can use glutamate decarboxylase (GAD) system to survive acid stress conditions. This system is composed of three genes: *gadA, gadB* and *gadC*. The first two encode two glutamate decarboxylase, while *gadC* encodes a glutamate-γ-aminobutyrate (GABA) antiporter (Cotter *et al.,* 2001). A specific transporter carries inside the cell glutamate which is decarboxilated in the cytoplasm. By this reaction γ-aminobutyrate is produced with intracellular proton utilization and it is taken out from the cell via GABA located in the cell membrane. During this last step the proton loss occurs and determines an increase in the pH in the cytoplasm. On the other hand, the release of γ-aminobutyrate into the external environment slightly raises the external pH (Small and Waterman, 1998). Cotter *et al.* (2001) observed an increase of listerial cell loads in a synthetic gastric fluid after the addition of glutamate. Moreover, mutants without *gadA*, *gadB* and *gadC* genes resulted less resistant to low pH values. Their results confirm the involvement of GAD into acid-tolerance of *L. monocytogenes*.

A study conducted by Wiedmann *et al.* (1998) showed the important role of σ^B–dependent proteins in acid resistance of *L. monocytogenes*. The alternative σ^B factor regulates the expression of the *gadB* gene, involved in acid stresssurvival, and OpuC, which is a chill-activated transporter for carnitine and virulence (Kazmierczak *et al.,* 2003).

Finally, *L. monocytogenes* has two-component signal transductions systems, consisting in *lisR* and *lisK* genes, which encode the response regulator and membrane-associated histidine

kinase, respectively. They can recognize environmental changes including low pH, oxidative and ethanol stresses via histidine kinase, and then they allow the cell to respond by altering gene expression (Cotter *et al.,* 1999). In the same study it is reported that *lisR* and *lisK* are involved also in the regulation of virulence.

3.5.3. Survival under osmotic stress

In food industry, salt is widely employed as a preservative to regulate water activity (Aw) of several products. As previously reported, *L. monocytogenes* can survive in the presence of high salt concentrations, and its salt tolerance makes the control of this pathogen in foods quite difficult. The response of bacteria as *Listeria monocytogenes* to osmotic stresses is called "osmoadaptation" and includes physiological changes and variations of gene expression (Hill *et al.,* 2002). As far as the latter, *Listeria monocytogenes* can modulate gene expression in order to increase or decrease the synthesis of various proteins. In a paper of Duche *et al.* (2002) the identification of twelve proteins highly induced in *Listeria monocytogtenes* after osmotic stress is reported. These proteins identified as salt shock proteins (Ssp; *e.g.* DnaK and Ctc) are rapidly over-expressed, while the proteins identified as stress acclimation proteins (Sap; *e.g.* GbuA) continue to be produced also after conditions return to normal levels. Gardan *et al.* (2003) observed that the expression of the *ctc* gene is dependent on σ^B in *L. monocytogenes.*

In order to counteract salt stresses, *Listeria monocytogenes* can adsorb osmoprotectans from the external environment, thus accelerating the recovery of an osmotic balance. These compounds, *e.g.* glycine betaine, proline betaine, acetyl carnitine, carnitine, γ-butyrobetaine and 3-dimethylsulphoniopropionate, are highly soluble, without charge and can be accumulated at high concentrations (Gandhi and Chikindas, 2007).The use of betaine and carnitine as osmoprotectans is regulated by the general stress sigma factor σ^B (Figure 16).

Kallipolitis and Ingmer (2001) identified two-component signal transduction system, i.e. *KdpE* and *orfX*, which are involved in the osmotic stress response. *KdpE* is involved in the transport of potassium (K^+) and encodes the response regulator and the downstream gene (*orfX*) in adaptation to salt stress. The effect of these systems is strictly dependent on the potassium level in the culture medium and the uptake of this element via the Kdp system has a protective effect on *L. monocytogenes* against salt stresses. Moreover, the *orfX* gene is responsible for triggering the activation of σ^B factor (Brøndsted et al., 2003).

3.5.4. Heat stress response

Thermal treatments are the earliest techniques employed to control microorganism in foods. Various microorganism, as well as *Listeria monocytogenes*, become thermo-tolerant when they are previously exposed to some environmental stresses, such as sub-lethal heat shock (Farber and Brown, 1990), osmotic and acid stress, ethanol and hydrogen peroxide (Lou and Yousef, 1997).

The thermo-tolerance of *L. monocytogenes* is highly variable because it depends by various factors such as the age of the culture, growth conditions, recovery media, and the characteristics of foods including its salt content, Aw, acidity, presence of inhibitors… (Doyle *et al.,* 2001). The bacterium adopts a heat shock response resulting in a transitory induction of heat shock proteins (HSPs) which defend the cells against heat and other damages. Most of the HSPs belong to the family of chaperones and their function is to stabilize new proteins to ensure correct folding or to re-fold proteins that were damaged during heat stress. All organisms, including humans, are able to produce these proteins which are encoded by *hsp60*, *hsp70*, *hsp80* and *hsp90* genes.

Under sub-lethal heat stress, *L. monocytogenes* induces synthesis of the conserved heat-shock proteins, DnaK, GroEL and GroES (Figure 16). These proteins are induced also following exposure to other environmental stresses such as low pH, high salt and ethanol indicating a protective role in the general stress response (Hill *et al.,* 2002).

Pang *et al.* (2007) shown that the expression of groEL can be used as an indicator of thermal stress response: listerial cells grown at 4 °C do not express this gene, while samples stored under stressful conditions (also swing of temperature between 4-30°C) express groEL. Some papers have reported a role of DnaK, GroEL and GroES in listeriosis (Hanawa *et al.,* 1999; Gahan *et al.,* 2001) and by a comparison with the results obtained for *E. faecalis*, an involvement of these proteins also in the protection against bile salts seems clear (Flahaut *et al.,* 1996).

Other proteins involved in *L. monocytogenes* thermo-tolerance are the family of Clp protease (*e.g.* clpC, clpE, clpP) which ensure stress tolerance and degradation of heat damaged proteins both Gram-positive and negative bacteria (Krüger *et al.,* 2001). In a review of Hill *et al.* (2001) it is highlighted the vital importance of these proteases in governing resistance to stress conditions. Mutants without the gene *clpC* encoding some of these proteases result not only in a reduced thermo-tolerance, but also reduce their virulence against mice.

Moreover, in the same review the same involvement in both thermo-tolerance and virulence is reported also for *clpE* and *clpP* genes.

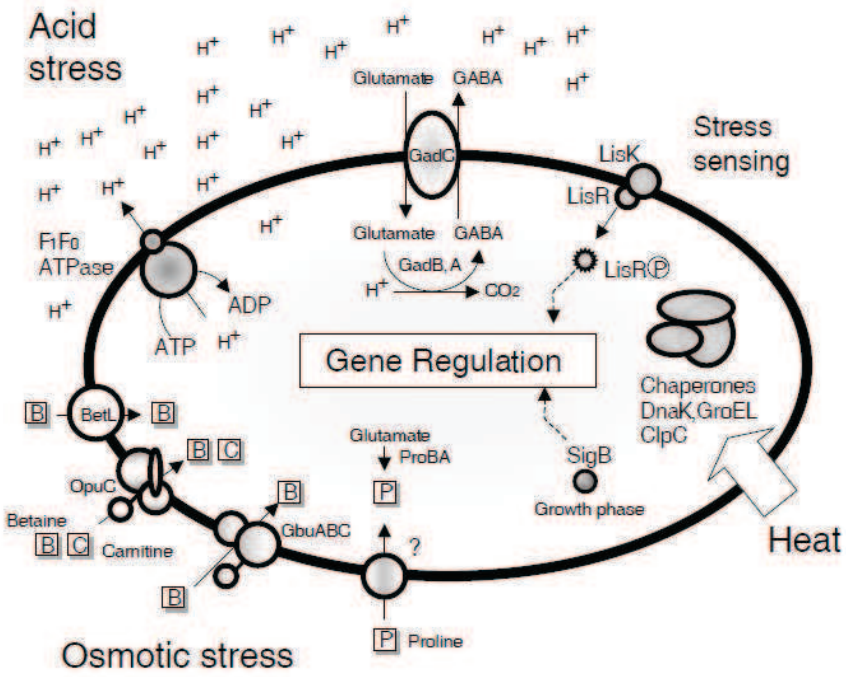

Figure 16. Schematic representation of gene regulation in *Listeria monocytogenes* during osmotic, acid and thermal stresses (Hill *et al.,* 2002).

3.5.5.oxidative stress

Oxidative stress is defined as interference in the equilibrium between the production/adsorption of reactive oxygen species (ROS) and the ability of bacteria to readily detect their presence and detoxify ROS or repair the resulting damage (Groves *et al.,* 2010). ROS can cause oxidative damage to macromolecules, *e.g.* proteins, DNA, and lipids, leading to an increased rate of mutagenesis, and cell death. In order to prevent damage to these essential macromolecules bacteria generally maintain a reducing environment within their cells. To preserve this state, they have developed highly complex nonenzymatic and enzymatic protection, repair and detoxification mechanisms. Most of the ROS are naturally generated endogenously by the same cells as a product of aerobic metabolism, or of enzymatic reactions (*e.g.* NO synthase). On the other hand, exogenous sources of ROS include irradiation (γ-ray, X-ray, UV), air pollutants, antibiotics and food.

Most of Gram-positive bacteria, as *Listeria* spp., have developed multiple strategies to counteract oxidative stress, including the production and excretion of a catalase-peroxidase

(KatG), superoxide dismutases (SodA and SodC), and alkylhydroperoxide reductase (Ahp) (Groves *et al.,* 2010).

In *B. subtilis* the expression of KatE is regulated by σ^B (Emgelmann *et al.,* 2005). In *Listeria monocytogenes* the σ^B regulon modulates the expression of *lmo0669,* which encodes an oxidoreductase and *lmo1433,* which is involved in a glutathione reductase synthesis (Chaturongakul *et al.,* 2008). Pleitner *et al.* (2014) observed in *Listeria monocytogenes* 10403S an up-regulation of 113 and 16 genes belonging to regulatory networks of σ^B and CtsR involved with protein fate activity upon ClO2 exposure. In particular some genes involved in the heat shock response seem to have a role also in oxidative one. The authors observed an over expression of genes such as *clpC*, *clpB*, and *clpP* which suggests a need for counteract the protein degradation and promote protein recycling, while the increased activity of genes encoding chaperone proteins (*e.g., dnaK*, *groEL*, and *groES*) indicated a need for maintenance or repair of protein structure damaged by oxidation.

Ferreira *et al.* (2001) reported that in stationary phase mutants of *Listeria monocytogenes* 10403S (serotype 1/2a) lacking of σ^B were 100-fold more sensitive to oxidative stress provided by 13.8 mM cumene hydroperoxide when compared to the wild type strain. However, σ^B contributions to oxidative-stress survival seem to vary among *L. monocytogenes* strains. Moorhead *et al.* (2003) observed no statistical difference in survival cells after cumene hydroperoxide exposure between *L. monocytogenes* L61 (serotype 1/2a) and its isogenic $\Delta\sigma^B$ mutant. Moreover, the same Authors observed that another strain (L99, serotype 4c) was significantly less resistant than its corresponding $\Delta\sigma^B$ mutant upon exposure to cumene hydroperoxide for 15 min. These experiments show a great strain-to-strain variability in σ^B contributions to oxidative-stress resistance in *L. monocytogenes*.

Gomes *et al.* (2011) identified an important role of three universal stress proteins (lmo0515, lmo1580 and lmo2673) in resistance and survival of *L. monocytogenes* in response to low pH conditions and oxidative stress.

Wonderling *et al.* (2004) observed the essential role of *htrA*, a gene coding for a serine protease identified as a stress response protein in several gram-positive and gram-negative bacteria, in the survival of *L. monocytogenes* in oxidative stress caused by hydrogen peroxide and acid sensitivity.

Chapter 4. *Salmonella enterica*

4.1. Characteristics and salmonellosis diseases

Salmonella, which was first identified in 1886 by Daniel Elmer Salmon during a case of pork plague, belongs to Proteobacteria phylum and *Enterobacteriaceae* family. The genus consists of two species: *S. enterica* and *S. bongori*.

Salmonella enterica is a rod-shaped, flagellated, facultative anerobic, non spore forming, Gram-negative bacteria which has 6 subspecies, *i.e arizonae, diarizonae, enterica, houtenae, indica* and *salamae.* Each of these subspecies has associated serovars that differ by antigenic specificity. Actually over 2500 serovars are known for *S. enterica*. The most common *Salmonella* serovars belong to *Salmonella enterica* subspecies *enterica*, which includes the serogroups *S.* Thyphi, *S.* Enteriditis, *S.* Paratyphi, *S*. Typhimurium and *S.* Choleraesuis. Most of the *Salmonella* subspecies are glucose and lactose fermenters, hydrogen sulfite producers, oxidase negative, and catalase positive. Other biochemical properties that allow identification of *Salmonella* include the ability to grow on citrate as a sole carbon source, decarboxylate lysine, and hydrolyze urea (Andino and Hanning, 2015).

Salmonella enterica is ubiquitous and is capable of colonizing and causing diseases in both animals, *e.g.*, poultry, cattle, swine, rodents, and humans intestinal tract. *Salmonella* can contaminate also feedstuff, soil, bedding, litter, and fecal matter.

When *Salmonella* colonizes the gastrointestinal system, the bacterial cells are evacuated by feces from which they may be transmitted by other animals (e.g. bugs or mice) to the waters. Although *Salmonella* do not originate in waters, its presence denotes fecal contamination (Andino and Hanning, 2015).

Both animals and humans are typically infected with *Salmonella* following ingestion of contaminated foods or water. Concerning humans, the main sources of *Salmonella* serovars include: contaminated or infected beef, pork, eggs, poultry, spices, fruits (*e.g.* mangoes, cantaloupe melons), vegetables (*e.g.* cucumber, sprouts) or derivatives/by-products of these foods (Centers for Disease Control and Prevention (CDC), 2010).

The foods contamination can occur during their production, preparation, or after cooking because of incorrect handling. Despite well-established instructions and measures for preventing salmonellosis, its incidence and severity have significantly increased. Indeed, nowadays *Salmonella* is the most common and primary cause of food-borne in many

countries, at least over the last 100 years. Usually, most of salmonellosis cases are self-limiting and the large outbreaks caused in schools, hospitals, and restaurants are not very common. Among the serotypes previously reported, those associated with human poisonings in the United States and European countries are *Salmonella enterica* serovar typhi (*S.* Typhi), *Salmonella enterica* serovar paratyphi (*S.* Parathyphi), *Salmonella enterica* serovar typhimurium (*S.* Typhimurium) and *S. enterica* serovar enteritidis (S. Enteritidis) (Lee *et al.,* 2015).

The salmonellosis by *S.* Typhi and *S.* Parathyphi clinically manifested as gastroenteritis, septicemia, or enteric fever. Infection severity varies on the basis of the individual resistance and the immune system. Even if *Salmonella* is not considered to be fatal to healthy people, enteric fevers cause 200,000 deaths and 22 million illnesses per year, with the highest incidence happening in Southeast and Central Asia where it is endemic (Crump *et al.,* 2004). Much more frequent are nontyphoidal salmonellosis which are spread via the fecal-oral route as enteric fevers. The main pathogens determining this food-borne poisoning are *S.* Typhimurium and *S.* Enteritidis. Clinically the nontyphoidal salmonellosis are characterized by gastroenteritis or bacteraemia; symptoms may involve nausea, vomiting, and diarrhea and are typically self-limiting lasting approximately 7 days (Andino and Hanning, 2015).

4.2. Stress response

Salmonella enterica is skilled at adapting to, growing and/or surviving in a diverse range of stressful environments. Although the optimum temperature is 37°C, it is able to grow between 2°C and 54°C. On the other hand, temperatures lower than 5°C prevent the bacterium to multiple and partially is inactivated, while the cooking temperatures completely inactive *Salmonella*. It can grow under low pH values down to 3.99 and up to 9.5, NaCl concentrations up to 4%, and Aw values between 0.999 and 0.945. Thus, these stresses can have a significant effect on the survival of *Salmonella* during food processing, preparation and storage as well as its passage through the host organism (Spector and Kenyon, 2012). In the following sections, the survival mechanisms under adverse environmental conditions, *e.g.* starvation, acid stress, oxidative stress, thermal stress, desiccation and osmotic stress, adopted by *Salmonella enterica* are reported.

4.2.1 Starvation

During the contamination cycle and the passage through various environment (*e.g.* polluted water), *Salmonella* may suffer a period of starvation. As well as *L. monocytogenes,*

salmonellae are not spore forming, but they can implement several physiological stress response mechanisms in order to survive.

S. Thyphimurium uses a starvation-stress response (SSR) when it is in an environment lacking of carbon sources (*e.g.* glucose). Firstly, the bacterium up-regulates genes involved in alternative carbon sources pathway and transport system. Subsequently, if carbon starvation continues, the bacterium "reprograms" its cellular metabolism in several ways including the production of: (i) new or higher affinity substrate transport and utilization systems (in the absence of substrates) for the scavenging of nutrients from the environment if they become available; (ii) enzymes for the "cannibalization" or turnover of unnecessary cellular apparatuses; (iii) new enzymes for an alternative and efficient metabolism of unusual C-sources (e.g. fuculose, xylulose, glucitol, sorbitol, xylitol, N-acetylglucosamine, ethanolamine, propanediol and aldehydes including glycolaldehyde and lactaldehyde); (iv) proteins that cause chromosome condensation thus protecting it from damages; (v) enzymes that modulate the cell membrane, and (vi) enzymes to prevent or repair cellular damages (Spector and Kenyon, 2012).

In *S.* Typhimurium, the SSR is regulated by two signal molecules, *i.e.* the cyclic 3′,5′-adenosine monophosphate (cAMP) with its receptor protein (CRP), and guanosine 5′-diphosphate-3′- diphosphate, which increases early during glucose-starvation. Moreover, two sigma factors, *i.e.* σ^S and σ^E, are involved in SSR (Spector and Kenyon, 2012).

4.2.2. Acid stress

Acid resistance is very important for *Salmonella* serovars and in general for all the foodborne pathogens because they have to survive the acidic pH (around the value of 2) of the stomach before colonizing the intestine. Some studies report that if *Salmonella* can adapt in chicken meat to a pH value around 4-5 due to bacterial lactic acid fermentation, subsequently the bacterium may survive to a more acidic pH such as that of the stomach (Andino and Hanning, 2015).

Moreover, *Salmonella* serovars can encounter acid stresses in many foods because of the food pH and the presence of preservatives (*e.g.* acetic and citric acids).

In order to resist to acid stresses, *Salmonella* involves an acid tolerance response (ATR) and an acid resistance (AR) mechanism. The ATR mechanism requires acid shock proteins including RpoS sigma factor and PhoP/PhoQ system which protect bacterial cells against inorganic acids. Moreover, PhoP/PhoQ is a virulence factor which act on the bacterial cell envelope by increasing the resistance to low pH and enhancing survival within the

macrophage (Spector and Kenyon, 2012). Salmonellae are also able to use an iron regulatory protein (Fur) and an adaptive response protein (Ada) in order to tolerate organic acids (Andino and Hanning, 2015).
Moreover, Gonzàlez-Gil *et al.* (2012) showed that virulence can be activated by an acetic acid stress through the *hil*A gene which is up-regulated in several strains of *S.* Typhiumurion during acid stress conditions.

4.2.3 Oxidative stress

When *Salmonella* serovars colonize the host, they may be exposed to diverse oxidizing agents such as superoxide anion ($O^{2\bullet-}$) and hydrogen peroxide (H_2O_2) produced through the endogenus aerobic metabolism or by the host immune system. These agents can strongly damage nucleic acids, proteins and lipids leading as a final result to the cell death.
To counteract oxidative stresses, *Salmonella* uses two major stress response pathways: the OxyR regulon, which responds to the presence of H_2O_2, and the SoxR/SoxS regulon, which detects changes in the cellular redox state generated by redox-cycling agents. The OxyR encodes various genes including *katG*, responsible of H_2O_2breakdown, *dps,* involved in DNA protection*, ahpCF*, which reduces oxided lipids, and *gorA, grxA, andtrxC* which reconstruct disulfide bonds. On the contrary the SoxR/SoxS encodes other genes such as *sodA* (detoxification of superoxide), *nfsA* (prevention of superxide production), *nfo* (DNA reparation), *zwf* (incrementation of reducing power) and others (Storz and Zheng, 2000).
In addition to OxyR and SoxR/SoxS, *Salmonella* uses other regulatory factors during acid stress including σS and *katE, xthA, sodC* genes. Also other factors, including σ^H and σ^E , involved also in other stress (*e.g.* starvation and thermal stress) may counteract the acid stress (Spector and Kenyon, 2012).

4.2.4. Thermal stress

As well as *Listeria,* salmonellae are able to survive at extremely low or high temperatures through the regulation of Sigma factors (σ^H and σ^E) and cold shock proteins (CSP). When a sigma factor senses an increase in temperature, it actives *rpoH* gene. The moisture and the food matrix can influence the thermo-tollerance of *Salmonella*. For example, low Aw values generally increase its resistance. Moreover, a simultaneous increment of thermo-tolerance and virulence (in particular an increase of the *hilA* gene) in *S.* Enteritidis strains exposed to heat stress has been observed. This result suggests that heat resistance confers a sort of pre-adaptation to subsequent stresses (Andino and Hanning, 2015).

Concerning cold shock proteins, they are quickly produced during the acclimation phase from 30 to 10°C. Rhodes and Kator (1988) observed that *S.* Enteritidis was able to survive in chicken parts at 2°C, and in shell eggs at 4°C, while *S.* Typhimurium can live in minced chicken at 2°C and in estuarine environments below 10°C.

4.2.5. Desiccation

Salmonella serovars can survive long time periods in dry products although they require Aw values higher than 0.93 for their growth. Recently, an increasing number of *Salmonella* outbreaks associated with dry foods, such as black or red pepper (Aw 0.409) and peanut butter (Aw 0.700), have occurred (Maki, 2009; CDC, 2010). Some studies have reported that *S.* Enteritidis, *S.* Typhimurium, and *S.* Mbandaka strains have great persistence (over one year) in dry products (Davies and Wray, 1996).

The survival mechanism of *Salmonella* is related to the *proP* (Proline permease II) gene and The Sigma factor RpoS. The latter regulates the *otsBA* operon, which is responsible for trehalose biosynthesis. Trehalose is a disaccharide that not only acts as a compatible solute, but also helps to maintain the structure and function of proteins and membrane lipids, replacing water during desiccation stress (Spector and Kenyon, 2012).

Moreover, salmonellae use glycocalyx surface layers, composed by exopolysaccharides and associated proteins, which form a gel-like extracellular matrix able to hold significant amounts of bound water (Spector and Kenyon, 2012).

Another way used by *Salmonella* to counteract low Aw values consists in the formation of multicellular filamentous cells by *rdar* (red, dry, and rough colony) morphology. *Rdar* morphology, monitored by CsgD protein, promotes the formation of aggregative fimbriae (curli) and cellulose which increase desiccation resistance in *Salmonella* cells (White *et al.,* 2006). Finally, Garmiri *et al.* (2008) observed the important role of the O-antigen polysaccharide chain of LPS in the desiccation resistance of *S.* Typhimurium.

4.2.6. *Osmotic stress*

Salmonella serovars can survive and grow in presence of NaCl concentrations up to 4% w/v. An high salt concentration determines the spillage of the intracellular water molecules which can cross directly the inner membrane or employ specific protein channels such as the AqpZ aquaporin (Calamita *et al.,* 1995). Similarly to *Listeria monocytogenes*, *Salmonella* counteracts osmotic stress through the increase in intracellular K^+ and the *de novo* synthesis or uptake from environment of osmoprotectants such as proline, glycine betaine, ectoine, or

trehalose (Spector and Kenyon, 2012). On the other hand, during the life cycle, *Salmonella* serovars can encounter environments with low osmolarity (*e.g.* water). An osmotic downshift determines an opposite movement of water resulting in its adsorption from the environment into cytoplasm with a subsequent increase in turgor pressure. Gram-negative bacteria not only have the peptidoglycan cell wall which can prevent the inner membrane ruptures, but they posses also some mechano-sensitive channels (MscL, MscM, and YggB) located in the inner membrane that are able to sense membrane tension and then mediate the release of compatible solutes, restoring osmotic balance (Spector and Kenyon, 2012).

It has been also observed an accumulation of osmoregulated periplasmic glucans (OPGs) in *S.* Typhimurium stressed cells (Bhagwat *et al.,* 2009). The Authors have suggested an involvement of OPGs towards virulence as well as growth and motility under low osmolarity growth conditions.

Chapter 5. *Escherichia coli*

5.1. Characteristics and pathogenesis

In 1885 the German-Austrian pediatrician Theodor Escherich discovered *Escherichia coli* in the colon of healthy individuals and he called it *Bacterium coli commune*. Subsequently, in 1919, Castellani renamed the bacterium with the current name in honour of its first discoverer. *Escherichia coli* is a Gram-negative, non spore forming, facultative anaerobic, rod-shaped bacterium belonging to the Protobacteria phylum and *Enterobacteriaceae* family. On the basis of genomic information, this species can be divided into six different phylogenetic groups: A (saprophyte), B1, B2 (pathogen), C, D (pathogen) and E (Touchon *et al.,* 2009).

Hundreds of *Escherichia coli* strains are commensal and can be commonly found in lower intestines of humans and mammals. In the intestines, *E. coli* can help digestion processes, food breakdown and absorption, and vitamin K production. *E. coli* can also be found in environments, and generally is used as an indicator of water microbiological quality. Indeed its presence is considered as an index of the level of human or mammal feces in waters. Most strains are not harmful to their hosts, but some of them can cause severe diseases. Pathogenic *Escherichia coli* isolates are classified into specific groups including the verotoxigenic (VTEC), enterohaemorrhagic (EHEC, a subclass of the VTEC class), enteroinvasive (EIEC), enterotoxigenic (ETEC), uropathogenic/extraintestinal pathogenic (UPEC/ExPEC) and diffusely adherent (DAEC) ones. The well-known *E. coli* O157:H7 is an example of a dangerous VTEC, which has caused several mortal cases all around the world. VTEC strains are capable of producing verotoxins causing mild to bloody diarrhea, which may culminates in the hemolytic uremic syndrome (van Elsas *et al.,* 2011).

Concerning the general metabolism, if oxygen is present the bacterium produces ATP by aerobic respiration; otherwise, it can use a mixed-acid fermentation in anaerobic conditions with the production of lactate, succinate, ethanol, acetate, and carbon dioxide. Some strains have flagella and are mobile. *E. coli* can transfer DNA via bacterial conjugation, transduction or transformation, which allows it to spread the genetic material an existing population. *Escherichia coli* is a mesophilic bacterium with optimal temperatures between 20- 45 °C; it can grow in a range of pH between 5.5-8.0 and is extremely sensitive to high salt concentrations. In particular, *E. coli* seems to be osmotollerant when cultured in nutrient rich

media (NaCl concentrations up to 5%), but in nutrient-depleted media it did not actively multiply at concentration of NaCl higher than 0.4% (Hrenovic *et al.,* 2009).

Most of the foods contaminated by *E. coli* are ground beef, un-pasteurizated milk and soft cheeses. The main natural reservoir of this bacterium is represented by ruminants, especially cattles. During slaughtering and processing, the intestinal *Escherichia coli* can get on the meat and then proliferate. Generally the combination of meat of different species increase the risk of contamination. Concerning milk and cheeses, *Escherichia coli* can colonize the udder of cows or the milking equipments. Also cross-contaminations resulting from environments contaminated by feces, manual milking (*E. coli* can come also from operators) and insufficient basic hygiene practices can occur (Espiè *et al.,* 2006). Lately, several *Escherichia coli* outbreaks due to vegetable consumption have occurred. The bacterium can contaminate vegetables during their production, harvesting, processing, distribution and preparation for consumption. Before harvesting, the pathogen can contaminate vegetables *via* fertilization with animal manure, fecally contaminated irrigation waters, feces of wild and domestic animals, poor hygiene of the operators, fecally contaminated farm equipments and insects (Ingham *et al.,* 2010). Also some processed crop products have been involved in several *Escherichia coli* outbreaks such as unpasteurized apple juice and cider (Cody *et al.,* 1999).

5.2. Stress response mechanisms

The survival and growth of both pathogenic and nonpathogenic *E. coli* in foods depend on the interactions of intrinsic (food related) and extrinsic (environmental) factors such as temperature, pH, and Aw (Buchanan and Doyle, 1997). This bacterium may encounter several stress conditions in foods and can implement several stress responses which let its survival under more severe conditions (*e.g.* subsequent food processes) and can enhance virulence. Therefore, understanding the effects of stresses on *E. coli* is important in order to assess and minimize the risk of food-borne diseases.

As well as the other pathogens previously described, *Escherichia coli* uses sigma factors. Sigma factors consist of small proteins able to bind RNA polymerase in order to improve/reduce the affinity of this enzyme with certain RNA regions. In particular, sigma factor manages the transcription of specific genes in response to unstressed/stressed conditions (Abee and Wouters, 1999). When *E. coli* lives and grows in normal unstressed conditions, the sigma factor σ^{70}, is responsible for transcription of many gene promoters. On the other hand under stress conditions, an alternative σ factor, σ^{S} (RpoS), with different promoter specificities is induced in order to start the expression of specific regulons to the

experienced stress. Under unstressed conditions, RpoS amount is very low because both its expression is down-regulated, and a protease (ClpXP) repeatedly degrades it in favour of σ^S expression (Schweder *et al.,* 1996). RpoS controls the transcription of more than 35 genes and plays a key role in the stationary phase stress response and other stress responses such as weak acids, starvation, high osmolarity, and high or low temperature (Lange and Hengge-Aronis, 1994). Under stress conditions *E. coli* can use other sigma factors such as σ^{32} and $\sigma^{24}(\sigma^E)$.

5.2.1 Thermal stress

In *Escherichia coli*, the heat shock response is mainly mediated by the sigma factor σ^{32} which directs transcription of RNA polymerase (RNAP) from the heat shock promoters and, thus, results in the induction of specific proteins called heat shock proteins (HSPs). Most HSPs, *e.g.* DnaK, DnaJ, GrpE, GroEL and GroES, act as molecular chaperones that stabilize non-native polypeptides generated by heat proteins denaturation, prevent misfolding or aggregation of proteins and promote a properly protein refolding (Georgopoulos and Welch, 1993). Some HSPs are involved in various fundamental cellular processes including proteolysis, cell wall synthesis, cell division and plasmid DNA replication. Moreover, some HSPs are also ATP-dependent proteases that digest heat-damaged polypeptides and facilitate some cellular functions such as nucleic acid synthesis, cell division and motility (Morris, 1993). In addition to σ^{32},also σ^E (σ^{24}) and σ^{54} (σ^N), are used by this bacterium under thermal stress (Chung *et al.,* 2006). As well as other bacteria, *E. coli* O157:H7 may become more resistant to subsequent heat treatments, which would otherwise be lethal, after a sub-lethal stress.

E. coli can develop also several mechanisms in order to survive and grow under a low temperature stress even if any specific sigma factor has not been identified. As well as *Listeria monocytogenes, E. coli* can change its membrane lipid composition. Some studies summarized by Chung *et al.* (2006) showed an increase of short and/or unsaturated fatty acids. In particular *E. coli* increases the amount of oleic acid (C18:1) at the expense of palmitic acid (C16:0) in favour of a greater fluidity when exposed to low temperatures (~12°C, Carty *et al.,* 1999).

Moreover, this bacterium expresses at least 15 different cold shock proteins (CSPs) involved in a variety of essential functions such as transcription, translation, mRNA degradation, protein synthesis, and recombination. CspA is the major cold shock protein of pathogenic and nonpathogenic *E. coli*, and it has the task of facilitating RNA translation at low temperature as

an RNA chaperone. Finally, some HSP proteins, which are protein chaperone at high temperature, have also an RNA chaperones function at low temperature.

5.2.2 Acid stress

The adaptation of pathogenic and nonpathogenic *E. coli* to the gastrointestinal environment of cattle may induce an acid tolerance response (ATR) which can make bacteria acid resistant in foods. Moreover, after the consumption of the contaminated food, the acid-adapted bacteria are able to counteract the gastric acid defense of human hosts and colonize the intestine or induce the disease in the case of pathogenic strains.

Gorden and Small (1993) observed a major acid tolerance in enteroinvasive, enteropathogenic, and enterohaemorrhagic *E. coli* than nonpathogenic strains such as *E. coli* K12. The acid stress response can be pH-dependent, pH-independent or a combination of both types (Lin *et al.,* 1995). Concerning the pH-dependent system, *E. coli* can employ different ways to counteract acid stress on the basis of the growth phase (log or stationary phase). During the log phase, an acid habituation induced by several compounds, *e.g.* glucose, glutamate, aspartate, $FeCl_3$, KCl, L-proline, phosphate and cAMP, can occur (Chung *et al.,* 2006). On the other hand, three acid resistance (AR) systems σ^S dependent, which include an oxidative system (AR1) and two fermentative acid resistance systems (glutamate decarboxylase, GAD-AR2 and arginine decarboxylase, AR3), have been identified in the stationary phase.

AR1 is induced into the stationary phase regardless the pH, while GAD-AR2 and AR3 are induced after the accumulation of glutamate and arginine in the external environment and they act as pH homeostasis systems (already discussed in the chapter 3). Other protective acid stress defense systems include changes in cell membrane composition (with the increase of the amount of membrane cyclopropane fatty acids, an homeostatic systems for internal pH, and pathways involved in the protection of essential cellular components (Chung *et al.,* 2006).

5.2.3. Starvation

When *E. coli* encounters a poor nutrient environment, firstly it stops the growth and induces the expression of both degradative enzymes (*e.g.* protease, lipase), in order to recover nutrients from useless cellular molecules, and enzymes responsible for the accumulation of storage compounds (*e.g.* glycogen and polyphosphate). Since the starvation response is regulated by RpoS, this bacterium may increase resistance to other stresses regulated by the same sigma factor, such as low pH, heat and oxidative stress. Moreover, *E. coli* expresses two

intracellular sensors, i.e. *cts* and *pex* genes, which are involved in C-starvation and C/N/P-starvation, respectively (Matin, 1991).

5.2.4. Osmotic stress

When pathogenic and nonpathogenic *E. coli* strains encounter an environment with low water content (e.g dried foods) or with an high concentration of salts, they respond with several osmoregulation systems to prevent shrinkage and eventual plasmolysis. Firstly, the increased osmolarity in bacterial cells determines the inhibition of DNA replication, cell growth, and nutrient uptake. After this, starvation Pex proteins, HSPs and osmoprotectants have been used in cells under an osmotic stress.

5.2.5. oxidative stress.

As well as previously described for *Listeria monocytogenes*, *Escherichia coli* uses various heat shock protein to manage oxidative stress. The roles of chaperone repair proteins DnaK, Hsp33, GroEL, and GroES are cross-functional for oxidative and heat shock response activities (Winter *et al.,* 2008). A partnership between Hsp33 and DnaK occurs where the oxidized dimers of Hsp33 bind to damaged proteins and, once a redox reaction occurs, transfer the protected substrate protein to DnaK for refolding (Winter *et al.,* 2005).
Wang *et al.* (2009) which analyzed the global gene expression profiles of two strains of *E. coli* O157:H7 (TW14359 and Sakai) under sodium hypochlorite or hydrogen peroxide treatments, observed increased transcript levels of *dnaK*, *groES*, *groEL*, and *clpP* following exposure to hydrogen peroxide and chlorine. Moreover, among 380 genes differentially expressed after exposure to low levels of chlorine or hydrogen peroxide, several regulatory genes responsive to oxidative stress (*e.g. katE* and *KatG)*, genes encoding putative oxidoreductases (*e.g. soxR)*, and genes associated with cysteine biosynthesis (*e.g cbl* and *fliY*), iron-sulfur cluster assembly (*e.g. iscRSUA-hscBA-fdx* and *sufABCDSE)*, and antibiotic resistance (*e.g. marRAB*) were found upregulated. Abram *et al.* (2008) observed also an important role of *marA* (an oxidoreductase) in *E. coli* oxidative and antibiotic resistance.
Moreover, UspA and UspD which belong to the universal stress protein superfamily are required by *E. coli* in the defense against superoxide-generating agents, and in the control of intracellular iron levels (Nachin *et al.,* 2005).

Chapter 6. Objectives

During the last decade, consumer expectations for safe food which are also characterized by fresh-like properties and high nutritional and qualitative values have strongly increased. In order to meet these demands, food manufactures have been increasingly interested in looking for new technologies which could be employed in place of the traditional ones which overall are based on the use of heat, chemical solutions and gases (*e.g.* ethylene oxide, hydrogen peroxide). Although all these technologies have disadvantages, *e.g.* being expensive and intrinsically toxic thus leaving residues on surfaces, causing damages to food matrices or being poorly sustainable, their use is well-consolidated at industrial level and most importantly they assure the production of foods meeting the safety criteria.

On the other hand several non-thermal technologies, *i.e*. preservation treatments that are effective at ambient or sub-lethal temperatures thereby minimizing negative thermal effects, are actually available. These include the application of gamma or beta (electron beam) irradiation, power ultrasound, ozonation, pulsed light, UV treatment, pulsed electric field (PEF), high hydrostatic pressure (HHP) and high pressure homogenization (HPH), and several others. However, for most of them practical applications are still limited due to adverse perceptions by the consumers, high initial investment or high energy costs.

During the last few years interest for cold atmospheric plasma has increasingly spread among researchers and this technology has been included among the emerging ones as a promising food preservation technology. Plasma is a neutral ionized gas which is composed of particles including free electrons, radicals, positive and negative ions, quanta of electromagnetic radiation, excited and nonexcited molecules (Misra *et al*., 2011). Several Authors reported evidences that it promotes an efficient inactivation of different types of microorganisms including spores and viruses, and some yeasts and fungi (Fernández and Thompson, 2012; Surowsky *et al.,* 2014b). Moreover, an increasingly number of studies in real food systems proved that it can be used for the inactivation of both natural contaminating microflora and deliberately inoculated pathogens in sliced cheese and ham (Song *et al*., 2009), beef (Kim *et al*., 2014), different fresh fruits and vegetables including apples (Niemira and Sites, 2008), cantaloupe, lettuce, mangoes and melon (Critzer *et al*., 2007; Perni *et al.,* 2008; Fernández *et al*., 2013; Baier *et al*., 2014), blueberries (Lacombe *et al*., 2015), cherry tomatoes and

strawberries (Ziuzina *et al*., 2014; Misra *et al*., 2014), lettuce, carrots and tomatoes (Bermúdez-Aguirre *et al*., 2013), apple juice (Surowsky *et al*., 2014a) herbs and spices (Hertwig *et al*., 2015) including also red pepper (Kim *et al*., 2014) and many others.

Although several studies have demonstrated the effectiveness of cold plasma for killing microorganisms, little is known about the effects of plasma on food matrices. In fact plasma can interact with food components such as water, lipids, proteins, carbohydrates and phenolic compounds. Therefore, studies related to the nutritional, chemical and enzymatic changes in plasma-treated foods are required to accurately assess the effects of the treatments also in relation to the intrinsic characteristics of the food matrices (*e.g.* composition, pH, Aw, …), the type of plasma generator used for the treatment and processing the conditions (*e.g.* exposure time, gas composition, gas humidity…).

Moreover, few studies are available on the effective mechanism(s) of action of plasmas against microbial cells which, otherwise, would be necessary in order to optimize processes also in relation to the target spoilage and/or pathogenic species that most frequently contaminate foods. In this context, the identification of the response mechanisms activated by the microbial cells to adapt and survive environmental challenges during food processing is of primary importance, also taking into consideration that adaptation can provide cell robustness to harsher stress conditions (den Besten *et al.,* 2010).

In this context, two main expects have been investigated in this thesis:

1) the effects of cold atmospheric plasma treatments on the inactivation of natural microflora and/or deliberately inoculated pathogens, *i.e. Listeria monocytogenes*, *Escherichia coli* and *Salmonella* Enteritidis, in three foods: soybean sprouts, Fuji apples and black pepper. Also the main qualitative parameters of the treated foods have been assessed immediately after the treatments and during storage

2) possible cell targets of plasma in two strains of *L. monocytogenes*, *i.e.* strains 56Ly and ScottA, exposed to different gas plasma treatments and processing conditions. In particular modifications in cell membrane fatty acids composition, volatile molecule profiles as well as relative expression of selected genes and proteome profiles have been studied trying to identify metabolic changes due to the plasma treatments.

Chapter 7. Effect of cold atmospheric gas plasma on soybean sprouts

7.1 Introduction

During last decades the consumption of raw or minimally treated fruit and vegetables has significantly increased due changes in dietary habits and/or to higher attention of consumers to healthier lifestyles. In fact the choice of vegetarian, vegan or Mediterranean diets, which are rich in fruit and vegetables, is increasing worldwide due to the well recognized role of these components in decreasing risk of cardiovascular diseases, certain cancers and type 2 diabetes. On the other hand, fruit and vegetable consumption among children and adults has been included among the non medical determinants of health by OECD (The Organization for Economic Co-operation and Development) as reported in the "2013 edition of Health at a Glance – OECD Indicators" which presents recent comparable data on key indicators of health and health systems across the 34 OECD member countries and the BRIICS. According to the report, average daily fruit consumption in 2011 was 57% for men and 69% for women, while those for vegetable ranged between 64% and 73% for men and women, respectively.

Despite beneficial effects, raw fruit and vegetables may harbor microbiological risks due to contamination with pathogens. Outbreaks of foodborne illnesses associated with the consumption of fresh produce have increased, being *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp, and viruses, and particularly *Norovirus* and hepatitis A, the agents more frequently implicated in causing illness outbreaks. *Salmonella enterica* was responsible for 76%, 60% and 30% of outbreaks caused by fruits, seed sprouts and leafy vegetables, respectively; *E. coli* O157:H7 were responsible for 19%, 40% and 48%, respectively. In May 2011, a large outbreak of illness caused by *E. coli* O104:H4-contaminated fenugreek seed sprouts occurred in northern Germany. A month later, a parallel outbreak developed in the Bordeaux region of France where 16 illnesses were reported. Both outbreaks were caused by the same *E. coli* O104:H4-contaminated fenugreek sprouts germinated locally from seeds believed imported from Egypt two years earlier (EFSA, 2011). In this context the European Commission asked the Panel on Biological Hazards to issue a scientific Opinion on the public health risk of Shiga toxin-producing *E. coli* (STEC) and other pathogenic bacteria that may contaminate seeds and sprouted seeds. According to the BIOHAZ assessment, sprouted seeds are ready-to-eat foods with microbial food safety

concern due to the potential for certain pathogenic bacteria to contaminate the raw materials (seeds) and to grow during germination and sprouting, and to their consumption patterns (raw or minimally processed).

On the identification of risk factors, the BIOHAZ Panel concluded that pathogenic bacteria can be carried and transmitted by animals, humans and the environment, and they may contaminate seeds in the field and throughout the sprouted seed production chain. The most relevant risk factors are associated with the effect of agricultural practices on seed production, storage and distribution: contaminated irrigation water and/or manure, presence of birds and rodents in storage facilities, dust and soil particles are potential sources of contamination. Moreover, processing conditions (*e.g.* temperature, humidity) prevailing during germination and sprouting of contaminated seeds favour the growth and dissemination of pathogenic bacteria and should be considered as major risk factors. As mitigation options, the BIOHAZ Panel indicated that food safety management based on HACCP principles should be the objective of operators producing sprouted seeds including GMP along the whole chain from seed production to the final sprouted product. On the other hand, decontamination of seeds prior to sprouting, is currently practiced in some EU Member States as an additional risk mitigation measure as part of a combined intervention strategy. To date, no method of decontamination is available to ensure elimination of pathogens in all types of seeds without affecting seed germination or sprout yield. The safety and efficacy of different seed decontamination treatments (*e.g.* chemical, heat treatment, irradiation alone or in combination) should be evaluated in a harmonized way at EU level. The consequence of any decontamination treatment on the background microflora and its potential impact on the pathogenic bacteria during sprouting should be taken into account.

Based on the majority of published data, seeds should be rinsed in large volumes of potable water as many times as necessary to remove dirt and increase the efficiency of the chemical decontamination treatment. There have been extensive investigations into the efficacy of various chemical sanitizing agents and other disinfection treatments in reducing levels of pathogenic micro-organisms in contaminated seeds (Bang *et al.,* 2011; Bari *et al.,* 2011; Beuchat, 1997; Fett, 2002; Gandhi and Matthews, 2003; Jianxiong *et al.,* 2010; Saroj *et al.,* 2006). However, most of the scientific literature indicates that sanitizing reduces, but does not necessarily eliminate, pathogens from contaminated seed. Although chlorine washing is commonly used for seed decontamination, its efficacy seems to be very variable. Chlorine

washing of dry seeds at 200 and 20,000 ppm was shown to result in a reduction of pathogens by 3 Log CFU/g or less suggesting that other alternative treatments such as gaseous acetic acid could be more effective than chlorine washing in controlling pathogenic bacteria on seeds (Nei *et al.,* 2011).

In this perspective the principal aim of this work was to evaluate the efficacy of gas plasma treatments for the superficial decontamination of ready-to eat sprouts. In particular, the inactivation levels for both natural spoilage microflora and pathogenic species, *i.e. Salmonella* Enteritidis, *Escherichia coli* and *Listeria monocytogenes*, deliberately inoculated onto sprouts has been assessed immediately after the treatments and over 9 days of refrigerated storage. In order to better evaluate possible differences among strains in susceptibility to gas plasma, two strains for each target pathogen were tested. In addition, also the effects of GP treatments on some chemico-physical and quality parameters, including water activity, pH, water loss, antioxidant activity, oxidation degree and polyphenols content were monitored during the storage at 4°C.

7.2 Materials and Methods

7.2.1. Gas plasma device

Gas plasma treatments have been performed in the DBD device described by Berardinelli *et al.* (2012; Figure 17). In an hermetic chamber (70 dm^3) the atmospheric discharge was generated between three pairs of parallel plate electrodes made of brass. In order to ensure an uniform distribution of the discharge over the electrode area and prevent arc transition, one electrode of each pair was covered by a glass layer (5mm). The voltage at the electrodes was produced by three high voltage transformers and power switching transistors. Over each pair of electrodes three fans for driving the gas plasma towards the fruit samples were mounted.

The discharge originated by this device has been previously characterized by Ragni *et al.* (2010). Main results showed that gas plasma emission spectra are composed of several reactive species such as N_2^+ and NO and OH radicals. Moreover, the emission of OH radicals increased by increasing the humidity level of the air (RH).

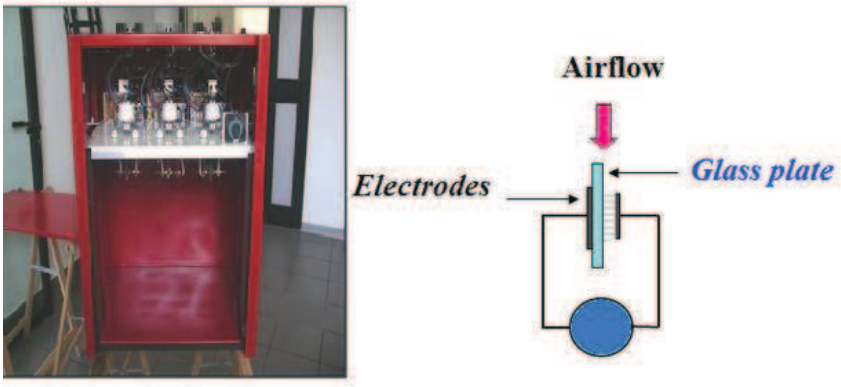

Figure 17. DBD device used for the experiment.

7.2.2. Soybean sprouts and sample preparation for challenge tests

Soybean sprouts were bought from a local supermarket (Bologna, Italy) the day before the experiments.

In order to evaluate the effects of gas plasma on some foodborne pathogens in addition to the indigenous microflora, a challenge tests were carried out by using strains of *Listeria monocytogenes*,*Salmonella* Enteritidis and *Escherichia coli*. The strains of the pathogens used in this work belong to the Department of Agricultural and Food Sciences (DISTAL) of Alma Mater Studiorum, University of Bologna. In particular the following strains have been selected:

- *Listeria monocytogenes* 56Ly (a wild type isolated from pork wastewater and characterized by high resistance to both high and low temperatures) and ScottA (sierotype 4b, clinical isolate);
- *Salmonella* Enteriditis 155 (isolated from poultry meat) and 86 (isolated from cabbage involved in an outbreak of salmonellosis which occurred in RS State, in Brazil, in 1999);
- *Escherichia coli* NCFB 555 (isolated from raw milk) and ATCC 25922 (clinical isolate).

The strains were cultured in Brain Heart Infusion (Oxoid, UK: 12.5 g/l brain infusion solids, 5.0 g/l beef heart infusion solids, 10.0 g/l proteoso peptone, 2.0 g/l glucose, 5.0 g/l sodium chloride, 2.5 g/l di-sodium phosphate) at 37°C for 24 h. Then, 7 ml of the overnight cultures (~9 Log CFU/ml) were separately transferred into 3.0l of BHI which were incubated at 37°C

for 12 h. The day after, the microbial cultures were transferred into sterile bags within a basket containing 350 g of soybean sprouts. Inoculum of soybean sprouts was performed by dipping for 2 min under agitation; subsequently sprouts were picked up and dried at room temperature under laminar flow hood for about 1 hour before gas plasma treatments were performed.

7.2.3. Treatment conditions

15.0 g of soybean sprouts, both uninoculated and inoculated with the target pathogens, were placed into sterile Petri dishes, which were placed into the DBD device at about 35 mm from the electrodes and then exposed to gas plasma for 20 and 40 min at RH of 60% (22°C). In order to have homogeneous treatments of the whole surface of the samples, after 20 and 40 min of treatment soybean sprouts were turned upside down into the Petri dishes and treated again for the same times.

Following GP treatments, samples were transferred into polypropylene cups which were closed with a polyethylene film, and stored at 4°C for 9 days. After 0, 2, 4, 6, 9 days sprout samples were analyzed in order to evaluate the effects of the exposure to gas plasma on: i) the surviving indigenous microflora and target inoculated pathogens, ii) chemico-physical and quality traits of soybean sprouts, and namely pH, Aw, water content, oxidation degree (Thiobarbituric Acid Reactive Substances - TBARS test), total polyphenols content (Folin-Chocolteau) and antioxidant activity (DPPH test).

7.2.4. Microbial analysis

10.0 g of soybean sprouts were transferred into a sterile sampling bag (International PBI S.p.A., Milan, Italy) containing 90.0 ml of sterile saline solution (NaCl 0.9% p/v, Merck KGaA, Germany) and homogenized for 2 min using a Stomacher mixer (Lab Blender Seward, PBI International, UK). Subsequently, a 1 ml aliquot was used to prepare decimal serial dilutions. Concerning uninoculated soybean sprouts, enumeration of total mesophilic bacteria and *Enterobacteriaceae* was done by surface plating, in triplicate, 100 µl of the appropriate dilutions onto Plate Count Agar (Oxoid, UK: 5.0 g/l peptone, 2.5 g/l yeast extract, 1.0 g/l glucose, 18.0 g/l agar) and Violet Red Bile Glucose Agar (Oxoid, UK: 3.0 g/l yeast extract, 7.0 g/l peptone, 5.0 g/l sodium chloride, 1.5 g/l bile salts No.3, 10.0 g/l glucose, 0.03 g/l neutral red, 0.002 g/l crystal violet, 12.0 g/l agar), respectively. Finally, plates were incubated

at 30 °C for 48 h and at 37°C for 24 h for mesophilic bacteria and *Enterobacteriaceae*, respectively.

Concerning soybean sprouts inoculated with the various pathogens, viable counts of *Listeria monocytogenes*, *Salmonella* Enteritidis and *Escherichia coli* were enumerated by surface plating, in triplicate, 100 µl of the appropriate dilutions onto the following media, respectively: Listeria Selective Agar Base - Oxoford formulation (Oxoid, UK: 39.0 g/l columbia blood agar base, 1.0 g/l aesculin, 0.5 g/l ferric ammonium citrate, 15.0 g/l lithium chloride, 400 mg/l cycloheximide, 20 mg/l colistin sulphate, 5 mg/l acriflavine, 2 mg/l cefotetan, 10 mg/l fosfomycin), Brilliant Green Agar (Modified) (Oxoid, UK: 5.0 g/l lab-lemco powder, 10.0 g/l peptone, 3.0 g/l yeast extract, 1.0 g/l disodium hydrogen phosphate, 0.6 g/l sodium dihydrogen phosphate, 10.0 g/l lactose, 10.0 g/l sucrose, 0.09 g/l phenol red, 0.0047 g/l brilliant green, 12.0 g/l agar) and ChromoCult® Coliform Agar (Merck KGaA, Germany: 3.0 g/l peptone, 5.0 g/l sodium chloride, 2.2 g/l sodium dihydrogen phosphate, 2.7 g/l disodium hydrogen phosphate, 1.0 g/l sodium pyruvate, 1.0 g/l tryptophan, 10.0 g/l agar, 1.0 g/l sorbitol, 0.15 g/l Tergitol®7, 0.4 g/l chromogenic mixture). Plates of all the 3 media were incubated at 37°C for 24 h.

7.2.5. Physico-chemical and quality assessment

7.2.5.1. pH measurement

5.0 g of sample were diluted into 5.0 ml of distilled water, homogenized for 2 min into a stomacher mixer and the pH was measured by using a pH meter (pH meter BasiC 20, Crison, Italy). The device was calibrated with calibration buffers at pH 7.0 and 4.00. The mean of three independent repetitions was calculated for each sample.

7.2.5.2. Aw measurement

About 3.0 g of soybean sprouts were placed into a disposable sample cup and the water activity was measured by using a water activity meter (Aqualab 4TE, USA). The device was previously calibrated with distilled water (aw=1.0000). Data are the mean of three independent repetitions.

7.2.5.3. Water content measurement

The moisture content was determined by measuring the mass of water in a known mass of sample. Exactly 5.0 g of soybean sprouts were place into weighed aluminium cups (previously dehydrated at 104°C and cooled before their use) and dried overnight in an oven (MOD 2100 Hight Performance Oven, Italy) set at 104°C to constant weight. Dried samples were then cooled into drier for about 1 hour and then their weights were recorded with an analytical balance (BL 120S, Sartorius, USA). The results were expressed as the percentage values by using the following formula:

$$\% \text{ Water} = \frac{(\text{Wi} - \text{Wf})}{\text{Wi}} \text{ x } 100$$

where: Wi refers to the weight of the fresh soybean sprouts; Wf refers to the weight of dried soybean sprouts. .Duplicate measurements were made for each sprout sample.

7.2.5.4. Thiobarbituric Acid Reactive Substances (TBARS) test

The assay of Thiobarbituric Acid Reactive Substances (TBARS) is a method for determining lipid peroxidation through the detection of Malondialdehyde present in the sample (MDA). Indeed MDA is a naturally occurring carbonyl compound generated through lipid peroxidation during cellular injuries of plants and animals.

1.0 g of soybean sprouts was accurately grinded and placed into test tubes containing 2.0 ml of thiobarbituric Acid (0.75% w/v in 0.25N HCl, Sigma-Aldrich, Germany), 2.0 ml of trichloroacetic Acid (30% w/v in 0.25N HCl, Carlo Erba reagents, Italy), and 40 µl of butylated hydroxytoluene (1% w/v in 0.25N HCl, Sigma-Aldrich, Germany). The control sample was prepared by mixing 1.0 g of grinded soybean sprouts with 4.0 ml of trichloroacetic acid, while the sample blank was made of 1.0 ml of distilled water mixed with all the reagents. All the test tubes were mixed and placed into a thermostatic bath (Lauda-Brinkmann, Germany) at 98°C for 10 min, then cooled with ice and centrifuged at 7000 r.p.m. for 5 min (Rotofix 32A, Hettich Lab Technology, Germany). 1.5 ml of supernatant was transferred into a cuvette and its absorbance at 530 nm measured by using a spectrophotometer (6705 UV/VIS spectrophotometer, Jenway, UK). Absorbance data were fitted with a calibration curve prepared with 1,1,3,3-tetraethoxypropane (TEP, Sigma-Aldrich,

United States) standards in the range 5.0-0.05µmol/l (y= 0.107x +0.053 R^2=0.992). MDA concentration (mg/kg sprout) was calculated according to the following formula:

$$TBARS\left(\frac{mgMDA}{kg\ sprouts}\right) = \frac{[\text{ABSsample} - (\text{ABScontrol})] - \text{b}}{\text{m}} \text{x } 72.06 \text{ x} \frac{1}{1000} \text{ x} \frac{1}{\text{w}}$$

where: *ABScontrol* is the absorbance of the control solution containing only the sample and trichloroacetic acid ; *ABS sample* is the absorption of the reaction mixture with the sample; *w* is weight (grams) of sample used. Two replicates were run per sample.

7.2.5.5. Antioxidant activity measurement (DPPH test)

2,2-Diphenyl-1-picryl-hydrazyl (DPPH) is a stable free radical which has an unpaired valence electron at one atom of nitrogen bridge and its scavenging activity is the basis of the well known DPPH antioxidant assay. DPPH radical scavenging activity was assessed according to the method of Hsu (2010) and modified as follow. Exactly 0.2 g of grinded soybean sprouts were incubated into a test tube containing 400 µl of 0.5 mM DPPH (Sigma-Aldrich, Germany) and 1.4 ml of 99.5 % methanol. 200 µl of L-ascorbic acid (0.1 mg/ml) and water were used to replace samples and referred to as positive and negative control, respectively. The mixture was mixed and incubated for 30 min in the dark at room temperature. Then the absorbance at 517 nm was measured by using a UV spectrophotometer (6705 UV/VIS spectrophotometer, Jenway, UK). Methanol was used as a blank. For each sample, the percentage of radical scavenging activity was calculated according to the following formula:

$$\%DPPH = \frac{(1 - (\text{ABS control} - \text{ABS sample}))}{\text{ABS control}} \text{x}100$$

where: *ABScontrol* is the absorbance of the negative control containing only DPPH and water, while *ABSsample* is the absorbance of the solution with samples. Two replicates were made per sample.

7.2.5.6. Determination of total phenolic compounds content

Total phenolic compounds content (TPC) was determined using the Folin-Ciocalteau's method according to Singelton *et al.* (1999). Phenolic compounds were extracted from 1.0 g of grinded soybean sprouts by continuous stirring with 10.0 ml of 80% methanol at 25 °C for

1 h. The suspension was filtered by Whatman® filter papers and the liquid phase was collected. 100 µl of extract was added 500 µl of Folin-Ciocalteau's reagent and 6.0 ml of distilled water. After 2.5 min of incubation, 1.5 ml of 15% (w/v) sodium carbonate solution and distilled water were added to the mixture to have a final volume of 10.0 ml. Blanks were prepared by replacing samples with 100 µl of water. After 2.5 h (in the dark), the absorbance was measured at 750 nm using a UV spectrophotometer (6705 UV/VIS spectrophotometer, Jenway, UK). A standard calibration curve was prepared by using gallic acid with a concentration range of 0.5-0.01 mg/ml ($y= 1.020x + 0.013$ $R^2=0.985$), and the content of total phenolics in each extract was calculated and expressed as milligrams of gallic acid equivalent (GAE) per gram of soybean sprouts (w/w). For each sample, TPC was measured in duplicate.

7.2.6. Statistical analysis

Overall differences between means were tested according to Tukey's test, performed at 95% confidence level and considered to be significant when $p < 0.05$. Analysis was carried out using Statistica 8.0 (StatSoft Italy srl, Italy).

7.3 Results

7.3.1. Efficacy of gas plasma treatments as a decontamination technology

In order to evaluate the effects of gas plasma treatments on the microbial traits of sprout samples, cell viability immediately after treatments and over refrigerated storage was measured for total mesophilic bacteria, *Enterobacteriaceae* and lactose/sucrose fermenting bacteria, which were chosen as representatives of spoilage contaminating microflora.

Initial contamination levels were quite high as ranged between 6-7 Log CFU/g and 7-8 Log CFU/g for mesophiles and enterobacteria, respectively. On the other hand such values are in agreement with data reported in literature (Olaimat and Holley, 2012). According to a survey of fresh and minimally-processed fruit and vegetables, and sprouts conducted in several retail establishments in Spain during 2005-2006, sprouts were highly contaminated with mesophilic (7.9 Log CFU/ g), psychrotrophic microorganisms (7.3 Log CFU/g) and *Enterobacteriaceae* (7.2 Log CFU/g), and also showed a high incidence of *E. coli* (40% of samples; Abadias *et al.,* 2008).

Overall, gas plasma treatments resulted in significant ($p< 0.05$) immediate reductions in cell viability of the indigenous bacteria by increasing the treatment time. The highest inactivation

levels were observed for *Enterobacteriaceae* being reduced by 1.9 ± 0.2 and 2.6 ± 0.1 Log units after 20 and 40 min of treatment, respectively. On the other hand, cell load reductions changed from 1.3 ± 0.3 Log CFU/g to 2.3 ± 0.2 Log CFU/g for the mesophilic bacteria by increasing the exposure to gas plasma, while lactose/sucrose fermenting bacteria were not affected by GP treatments regardless the exposure time (Table 7).

During refrigerated storage different trends in the evolution of the surviving cells were observed for the mesophiles and enterobacteria also in relation to the treatment time. Concerning mesophilic bacteria (Figure 18), no change was observed in untreated samples, while a 1 log unit increase was detected after 1 day for samples exposed to the shortest treatment (20 min) which attained final values similar to those of the control ones (7 ± 0.1 Log CFU/g). By contrast, a slower recovery ability was found for 40 min-treated samples. In fact a maximum cell increase of 1 log unit was observed only after 3 days of storage, and the final cell load attained did not exceed 6 Log CFU/g, being significantly ($p< 0.05$) lower than those of control and 20 min treated sprouts.

Unlike mesophyles, enterobacteria did not present any growth ability as their cell loads remained unchanged over storage regardless GP treatment time, similarly to the control products (Figure 19). On the contrary, the fate of lactose/ sucrose fermenting bacteria was significantly delayed in GP treated samples compared to the control ones over refrigerated storage (Figure 20). In fact while loads higher than 8 Log CFU/g were reached after 6 days in the latter, no cell increase was observed for almost one week in the former, being the 40 min treated ones the most stable ones.

Regarding challenge tests with the target pathogens, sprouts were deliberately contaminated with an average of 6.5-7.5 Log CFU/g for *Salmonella* Enteritidis (strains 86 and 155), 6.7-7.5 Log CFU/g for *Escherichia coli* (strains 555 and ATCC 25922) and 8.0-8.2 Log CFU/g for *Listeria monocytogenes* (strains 56Ly and Scott A). GP treatments displayed different effects in relation to the microbial species and the strains. As far as *Salmonella* Enteritidis, immediate cell reductions of 0.5 and 1 Log CFU/g were observed for the strain 86 following 20 and 40 min treatments, respectively (Figure 21). Such differences in cell counts between control and GP-treated sprouts were maintained also during the whole refrigerated storage. On the contrary, the strain 155 presented a higher resistance and no change in cell viability was observed immediately after treatments (Figure 22). However, during refrigerated storage *S.* Enteritidis strain 155 presented an initial increased up to 6 Log CFU/g after 6 days (following

an initial lag phase of ~ 4 days) in untreated sprouts, while it showed a 1 log viability loss in both the treated products. At the end of storage, 1 and 1.5 log units lower values were found for *S.* Enteritidis strain 155 in 20 min and 40 min treated products, respectively compared to the untreated ones.

Also *L. monocytogenes* was sensitive to GP treatments as both the strains were reduced by 1 Log unit regardless the exposure time to gas plasma (Figures 23 and 24). Moreover, hardly cells were able to recover damages during refrigerated storage as cell increases were limited (*i.e.* < 1 Log CFU/g) and were observed only at the end of storage in samples exposed to the shortest treatment.

In general *E. coli* was the most resistant species. In fact GP exposure resulted in no or limited cell inactivation. Moreover, no significant differences in cell counts were found during storage among control and GP treated products regardless the treatment time and the strain used (Figures 25 and 26).

7.3.2. Effect of gas plasma treatments on compositive and quality parameters

7.3.2.1. Weight loss

Water loss is one of the main causes of deterioration in raw vegetables because it results not only in direct quantitative losses, but also in decreases of qualitative parameters *e.g.* appearance, textural quality, and nutritional value. As a direct effect of gas plasma treatments reductions in water content of 3.4% and 5% were recorded after 20 and 40 min treatments (Figure 27). Such changes may be related to the temperature increase up to 22 °C occurring during GP treatments. However, no further dehydration was observed during storage in both GP treated samples similarly to the control ones.

7.3.2.2. Aw and pH

As expected, Aw values of GP treated products were significantly reduced compared to the control ones immediately after the treatments, with final values of 0.994 and 0.987 for sprouts exposed to GP for 20 and 40 min, respectively (Figure 28). On the other hand, differences among untreated and treated samples were limited at the end of storage as a consequence of water absorption from atmosphere as products were stored at 4°C and RH of 37±2 %.

pH values of the sprouts were found to slightly decrease due to GP treatments moving from 6 down to 5.1 ± 0.02 (Figure 29). Also this reduction is likely to be related to the water loss that

led to concentration of acid compounds in the cells. However, no further variation was observed during storage regardless the GP treatment.

7.3.2.3.Total phenolic compounds and oxidation degree

The levels of total phenolics (TP), which were expressed as mg of gallic acid equivalents/g of sprouts, are shown in Figure 30. Interestingly, GP treatments did not negatively affect TP regardless the treatment time. In fact higher values were recorded for the treated products compared to the raw ones due to their higher concentration resulting from water loss. During chilled storage, such levels were retained, although quite a high variability was observed in the treated samples.

Although exposure to GP did not impair the antioxidant potentials of sprouts, TBARS values were significantly higher in treated samples. In fact even the shortest treatment resulted in a 3 fold increase in TBARS although no clear differences were found in relation to the treatment time. Moreover, a tendency to slightly increase was recorded for TBARS during storage indicating that the oxidation phenomena induced by GP treatments continued also over storage (Figure 31).

Table 7. Inactivation levels (Δ Log_{10} CFU/g) of the indigenous bacteria after GP treatments.

GP treatment time (min)	Mesophilic bacteria	*Enterobacteriaceae*	Lactose/sucrose fermenting bacteria
20	1.3±0.3	1.9±0.2	0.23±0.07
40	2.3±0.2	2.6±0.1	0.031±0.01

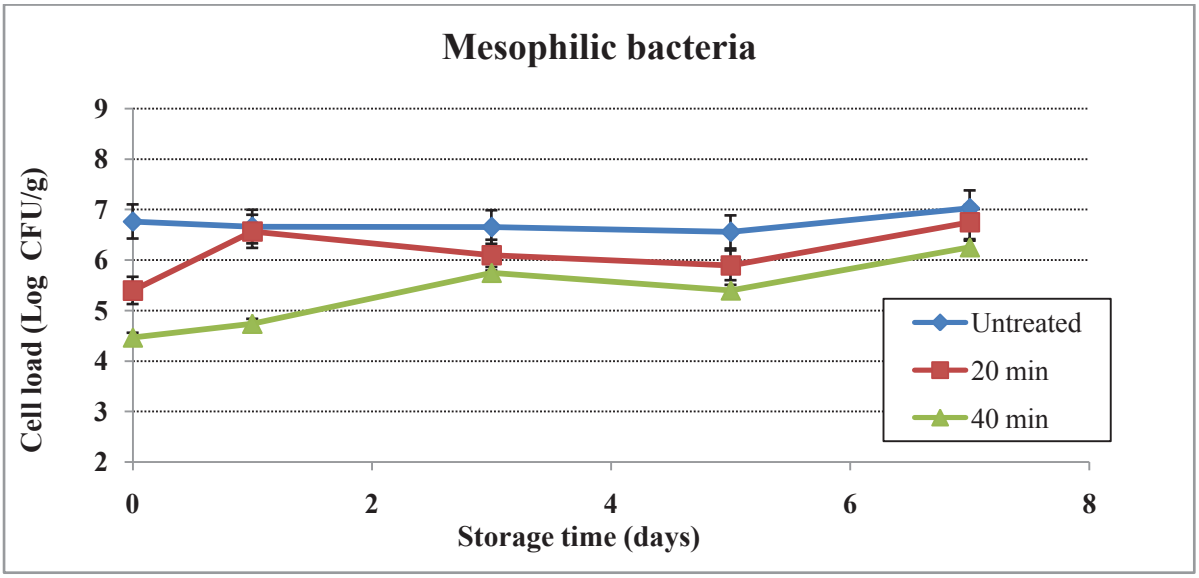

Figure 28. Counts during storage (4°C) of the surviving cells of mesophilic bacteria in sprouts exposed to GP.

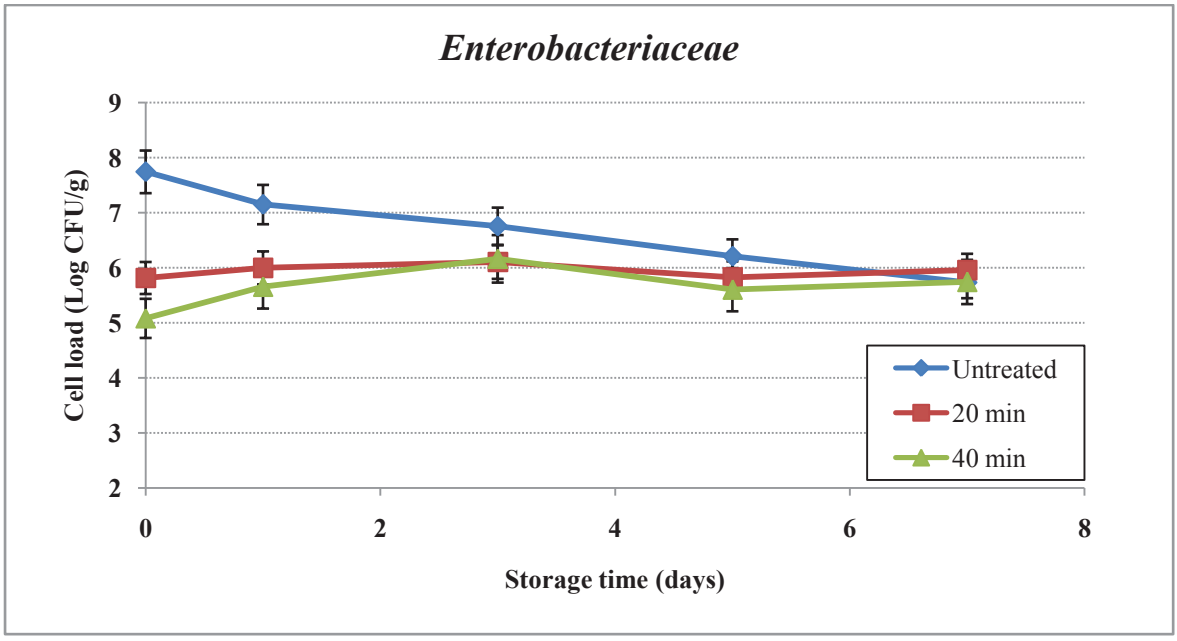

Figure 19. Counts during storage (4°C) of the surviving cells of *Enterobacteriaceae* in sprouts exposed to GP.

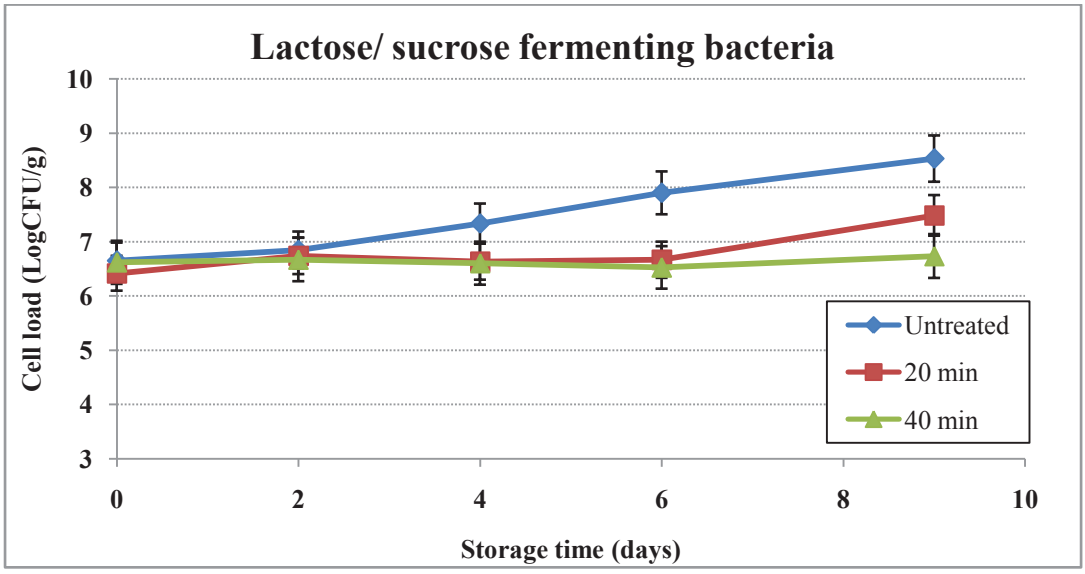

Figure 20. Counts during storage (4°C) of the surviving cells of lactose/ sucrose fermenting bacteria in sprouts exposed to GP.

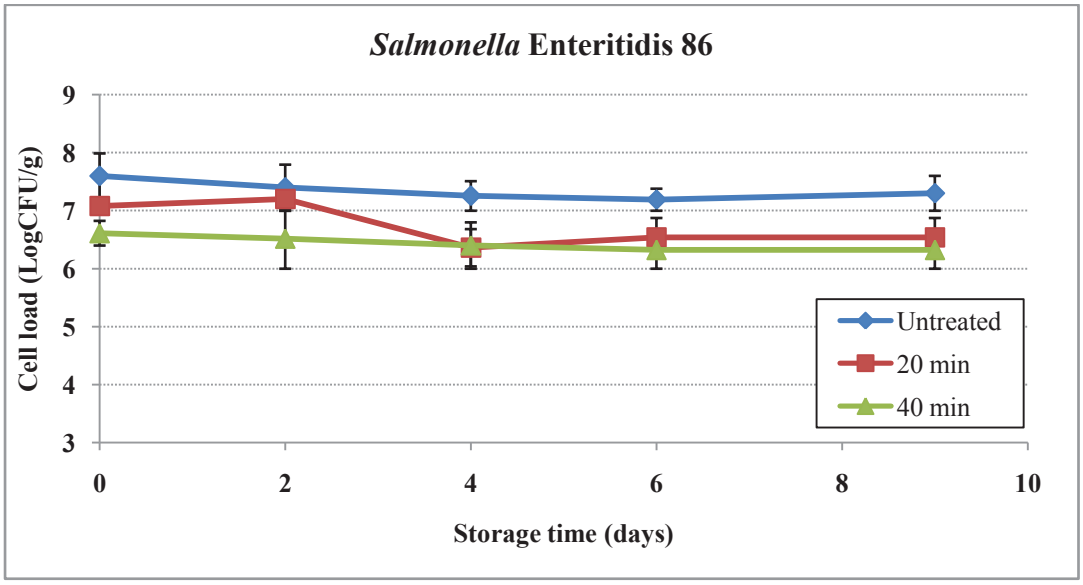

Figure 21. Counts during storage (4°C) of the surviving cells of *Salmonella* Enteritidis 86 artificially inoculated on sprouts exposed to GP.

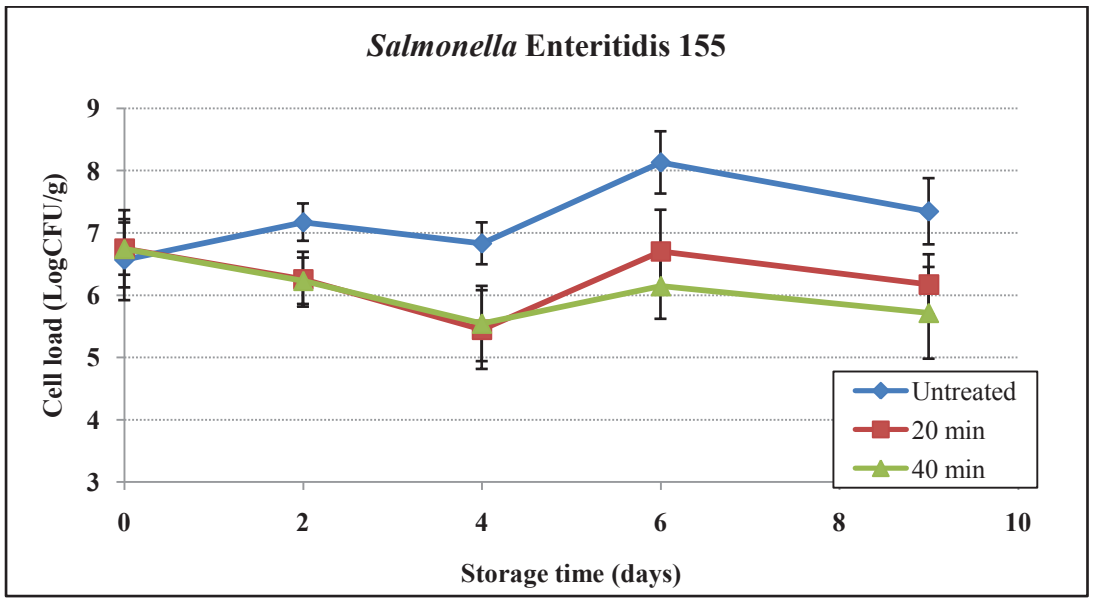

Figure 22. Counts during storage (4°C) of the surviving cells of *Salmonella* Enteritidis 155 artificially inoculated on sprouts exposed to GP.

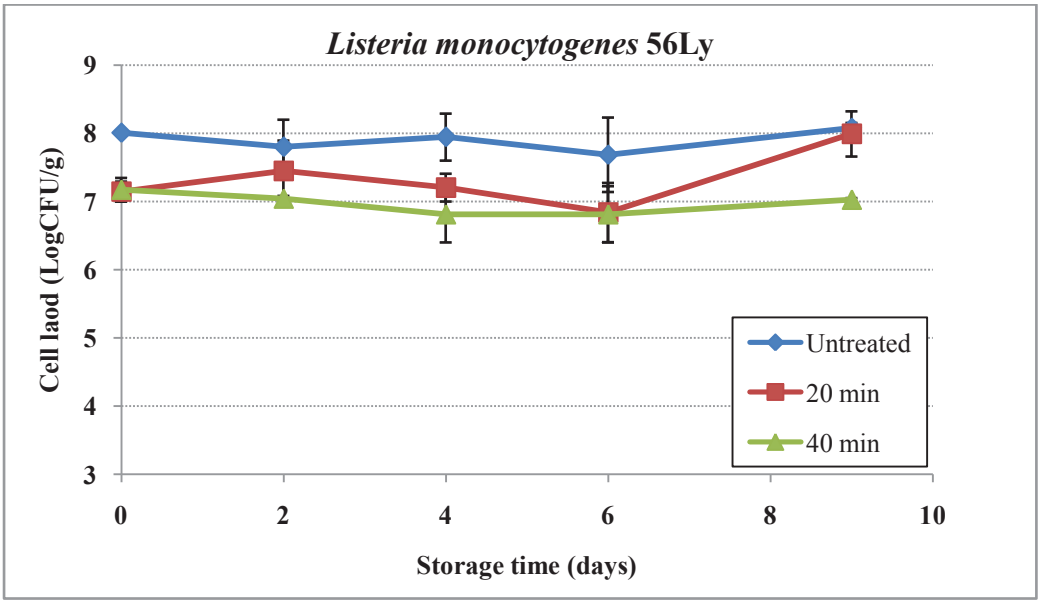

Figure 23. Counts during storage (4°C) of the surviving cells of *Listeria monocytogenes* 56Ly artificially inoculated on sprouts exposed to GP.

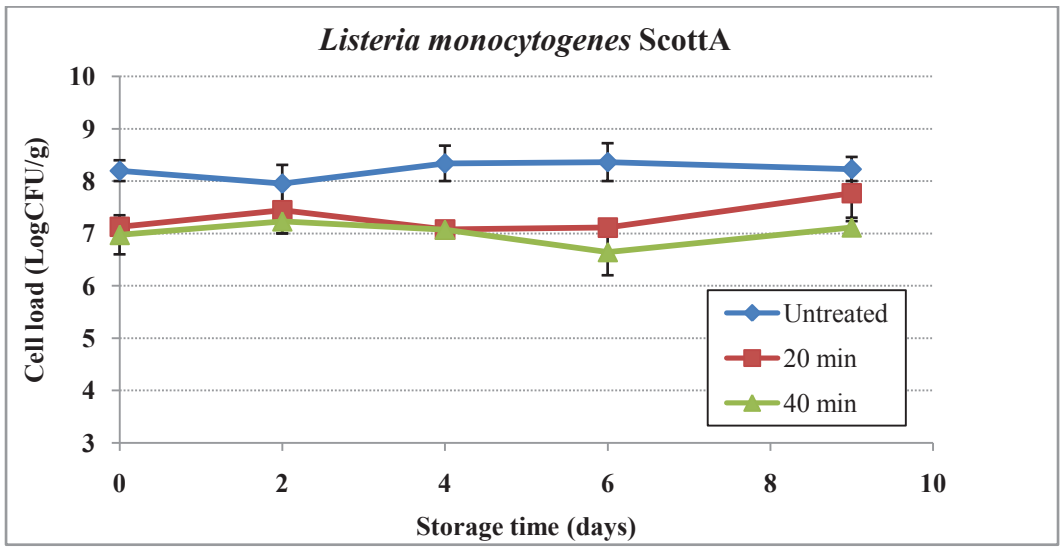

Figure 24. Counts during storage (4°C) of the surviving cells of *Listeria monocytogenes* ScottA artificially inoculated on sprouts exposed to GP.

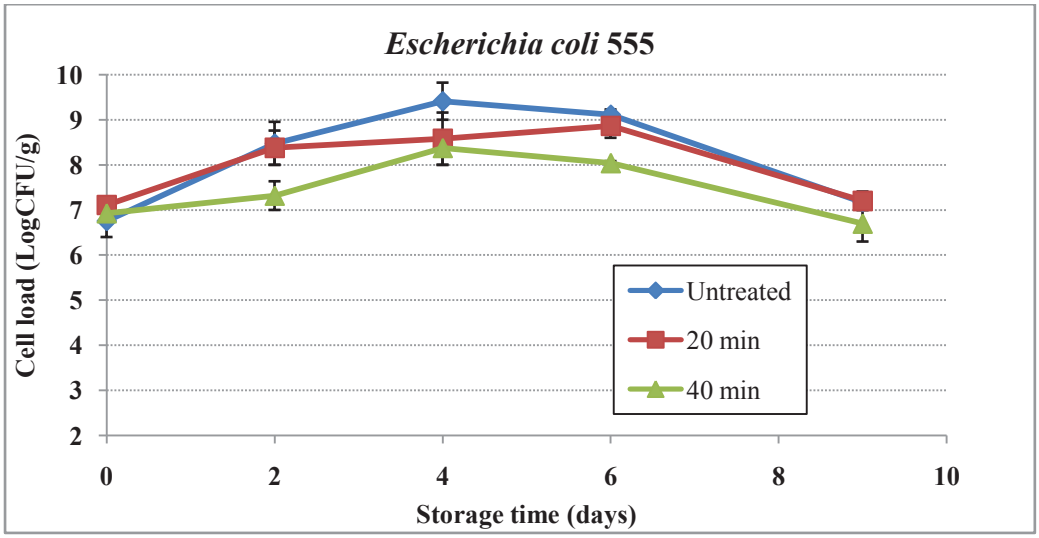

Figure 25. Counts during storage (4°C) of the surviving cells of *Escherichia coli* 555 artificially inoculated on sprouts exposed to GP.

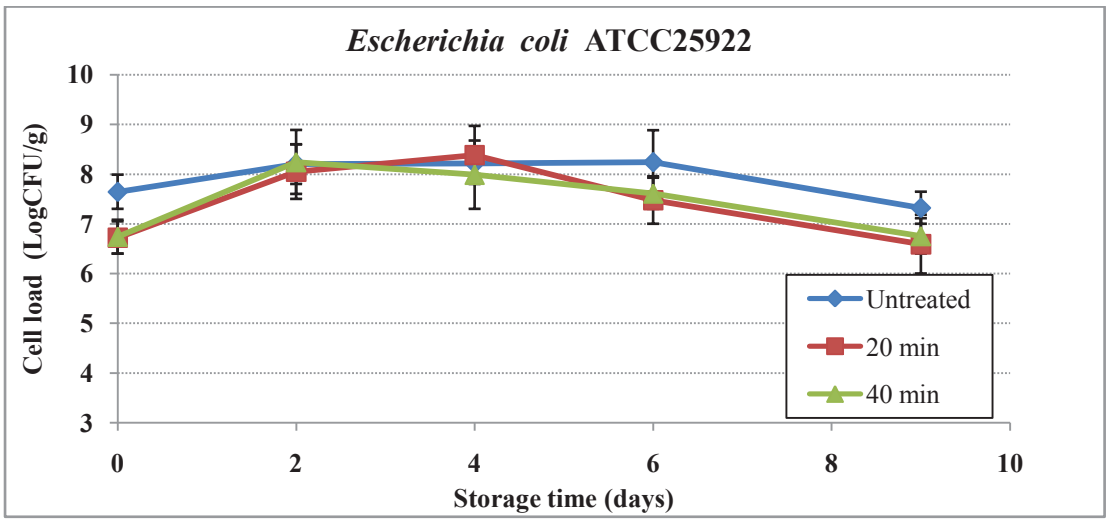

Figure 26. Counts during storage (4°C) of the surviving cells of *Escherichia coli* ATCC25922 artificially inoculated on sprouts exposed to GP.

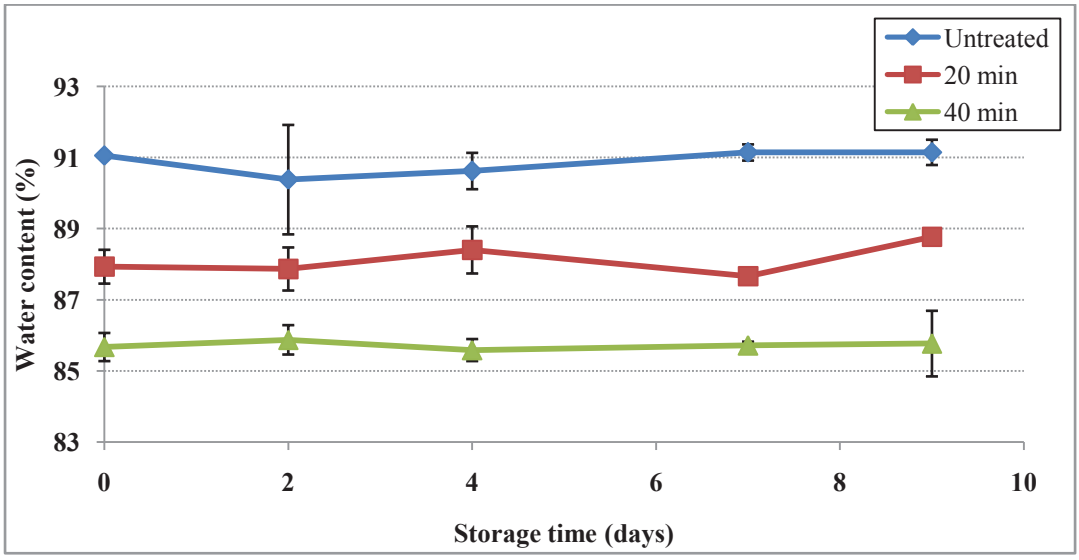

Figure 27. Evolution of water content of sprouts treated with GP during storage time.

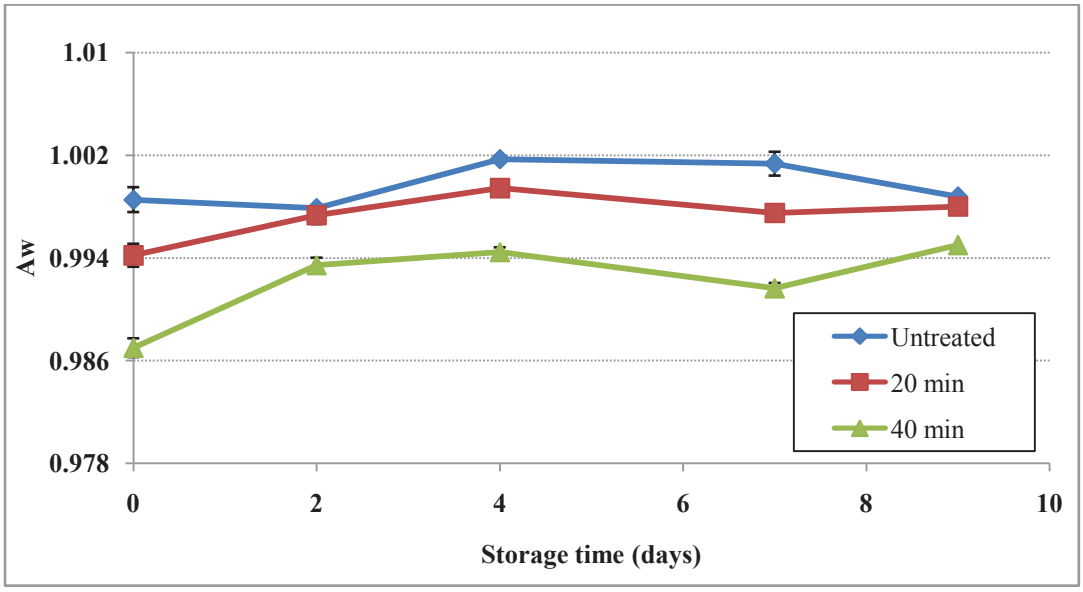

Figure 28. Evolution of water activity of sprouts treated with GP during storage time.

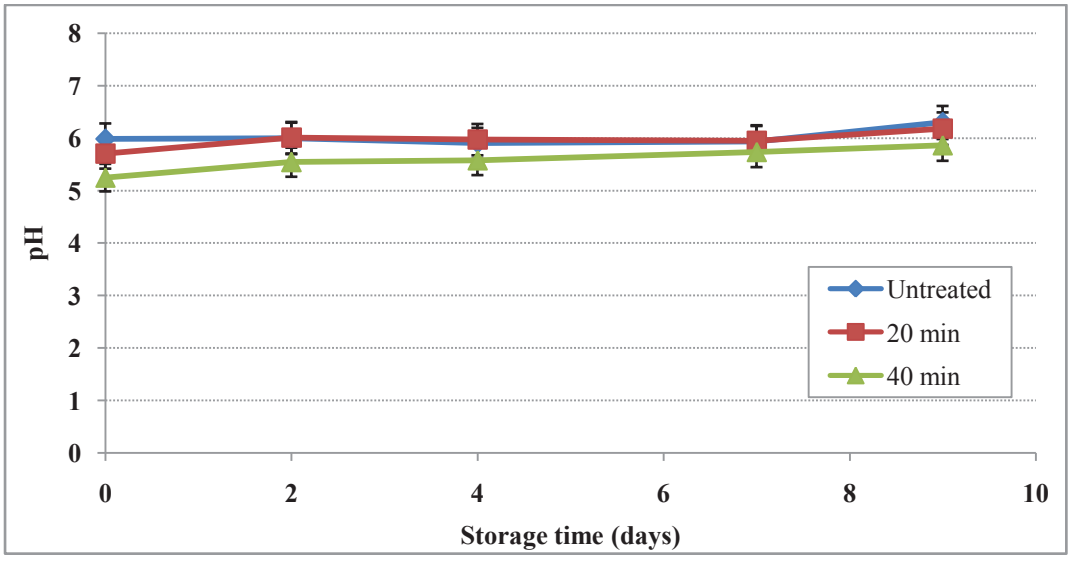

Figure 29. Evolution of pH value of sprouts treated with GP during storage time.

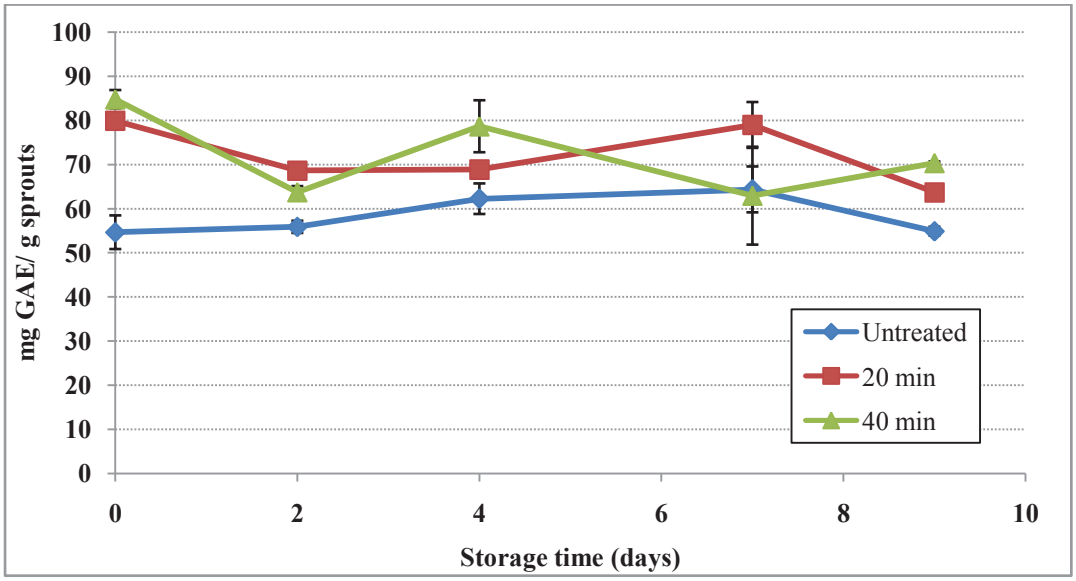

Figure 30. Evolution of total phenolic compounds of sprouts treated with GP during storage time.

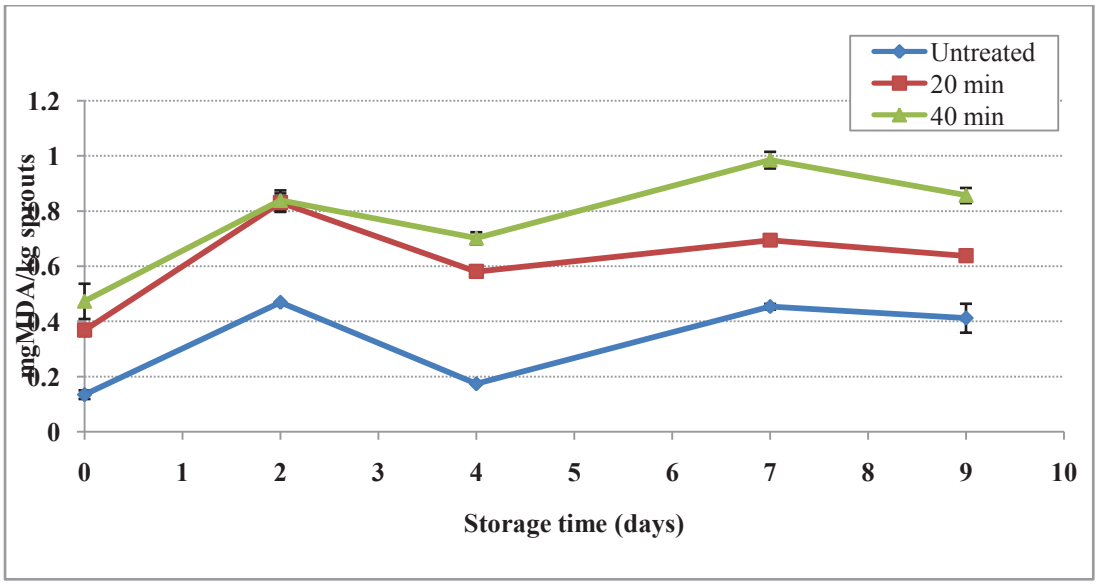

Figure 31. Evolution of oxidation degree (TBARS assay) of sprouts treated with GP during storage time.

7.4. Discussion

In this study the efficacy of gas plasma treatments has been evaluated against inoculated target pathogens and background microflora present on the surface of a ready-to-eat vegetable. Sprouts have been chosen as they have been associated with recent foodborne

illness outbreaks and represent a common raw food ingredient of salads which is not subjected to any sanitization treatment during processing.

In general, GP treatments were effective in reducing the contamination level of both inoculated pathogens or natural microflora and/or inhibiting their growth during refrigerated storage. Inactivation rates obtained in this work ranged between 1 and 2.6 Log CFU/g depending on the exposure time and microbial species. These data are in accordance with those reported in literature for traditional technologies to reduce/eliminate the microorganisms present in food products. Among the different methods commonly used to reduce microorganism's population on whole and fresh-cut fruit and vegetable products, washing with sanitizing agents (*e.g*. chlorine) represents the most widely diffused one. Several studies have shown that chlorine rinses can decrease the bacterial load by values ranging from <1 Log CFU/g to 3.1 Log CFU/g, depending on inoculation method, chlorine concentration, contact time, and the target bacteria (Ramos *et al*., 2013; Gil *et al*., 2009; Hua and Reckhow, 2007; Baur *et al*., 2005). On the other hand, it is quite ineffective in reducing pathogens on vegetables (Oliveira *et al.,* 2012; Gil *et al.,* 2009), and has several side effects as chlorine-based compounds are corrosive, cause skin and respiratory tract irritation and is inactivated by organic material and can also lead to the liberation of chlorine vapours and formation of chlorinated by-products, with potential adverse health effects (Selma *et al.,* 2008; Sao Josè and Vanetti, 2012; Lopez-Galvez *et al.,* 2010).

Although several studies on the use of GP treatments for the decontamination of different raw fruit and vegetables have already been published, to the best of our knowledge this is the first experimental work made on sprouts. On the other hand, a critical assessment of the performances of gas plasma treatments as an emerging non thermal technology for the decontamination of fresh produce based on literature data is difficult due to wide differences in the equipments and operating conditions used (sources, processing , conditions to generate plasma…). In this work a Dielectric Barrier Discharge (DBD) generator was used for the treatments and all samples were treated in the plasma after-glow chamber.

Selcuk *et al.* (2008) used DBD system to inactivate species of *Aspergillus* and *Penicillum* inoculated onto the surface of various seeds. The GP treatment reduced the fungal attachment to seeds within 20 min of exposure by 3 log below 1% of the initial concentration and the germination quality of the seeds was not affected. Klockow and Keener (2009) reduce of 3–5 log10 CFU/leaf the presence of *E. coli* artificially inoculated in fresh spinach after 5 min of

treatment with a DBD ozone generation system (PK-1). Critzer *et al.* (2007) reported reductions of strains of *Escherichia coli* O157:H7, *Salmonella* sp. and *Listeria monocytogenes*, artificially inoculated on apples, cantaloupe and iceberg lettuce, by at least 2 log units within a few minutes of treatments with an atmosphere uniform glow discharge plasma (OUAGDP). Recently, a commercially available nitrogen plasma-jet was employed to inactivate *Salmonella* Typhimurium on fresh produce by Fernández *et al.* (2013) who recorded bacterial reductions of 2.72, 1.76, and 0.94 log units on lettuce, strawberry, and potato, respectively after 15 min. Similar results were obtained after indirect treatment of romaine lettuce and cocktail tomatoes in the afterglow of a needle array at high voltage, resulting into a 1.6 log unit reduction after 10 min (Bermúdez-Aguirre *et al.,* 2013). Corn salad treated with an atmospheric pressure plasma-jet up to 30 sec allowed the inactivation of *E. coli* by 2.1 up to 3.6 log units from initial cell loads of 7 and 4 Log CFU/cm^2, respectively (Baier *et al.,* 2013). Tests performed on corn salad, cucumber, apple, and tomato treated with an atmospheric pressure plasma-jet allowed an inactivation of artificially inoculated *Escherichia coli* DSM 1116 of 4.1 ± 1.2, 4.7 ± 0.4, 4.7 ± 0, and 3.3 ± 0.9 log units, respectively, after 60 s treatment time (Baier *et al.,* 2014). Additional tests with a dielectric barrier discharge plasma and indirect plasma treatment within a remote exposure reactor, fed by a microwave induced plasma torch, did not result in equivalent levels of quality retention as observed using the plasma-jet.

While immediate inactivation rates obtained in this thesis for natural microflora and target inoculated pathogens are comparable with data reported in literature, no comparison on the effects of GP on microbial quality of treated products during storage is possible. In fact most of the published papers do not consider the fate of the survivors over subsequent storage. Among the three bacteria studied, *Salmonella* Enteritidis seems to be the most sensitive to GP, while *E. coli* the most resistant one.

It has been reported that Gram positive bacteria are more resistant to cold atmospheric plasma than Gram negative ones (Montie *et al.,* 2000; Lee *et al.,* 2006; Ermolaeva *et al.,* 2011; Frohling *et al.,* 2012; Ziuzina *et al.,* 2014). Such effects have been attributed to cell envelope differences as the thicker membrane of the Gram positive bacteria may present a barrier to the diffusion of plasma reactive species through the bacterial cell wall, thus impacting antimicrobial efficacy. However, some Authors reported a greater sensitivity of Gram positive *Listeria innocua* than Gram negative *Salmonella* and *E. coli* inoculated on tomato surface

(Fan *et al.,* 2012). In contrast, other studies indicated no significant differences in the effect of plasma treatment between Gram-positive and Gram-negative bacteria (Kostov *et al.,* 2010; Olmez and Temur, 2010; Klampfl *et al.,* 2012).

According to this study, no clear relationship between sensitivity to gas plasma and microbial outer cell structures can be envisaged, also considering that effectiveness of the treatment was strain dependent and differences in the fate of the surviving cells were found during subsequent storage. In agreement with our results, Lu *et al.* (2013) reported that the effects of atmospheric cold plasma (ACP) inactivation are also dependent on bacterial strains studied. These Authors hypothesized that the greater resistance to ACP stress of *E. coli* NCTC 12900 compared to *E. coli* ATCC 25922 can be related to the intrinsic characteristic of the former. In particular the stronger resistance to acid stress, multidrug resistance and higher rate of mutations may have a cross-protective effect against a wide range of environmental stresses including oxidative stress produced during GP treatments.

Despite a huge amount of data on efficiency of gas plasma treatments, information about physicochemical changes that might occur in the product due to the interaction of charged species from plasma with the food components is still scarce. On the basis of the experimental results reported in this thesis, the processing conditions adopted did not negatively affect the quality parameters of sprouts. In fact no visual differences were recorded colour and appearance of treated sprouts immediately at the end of treatments and during storage. Slight differences were evidenced for pH, TP and Aw as a consequence of the initial water loss due to the treatments. It is important to note, however, that total loss in weight due to dehydration did not exceed 5% immediately after treatment. This value is similar to that reported by Javanmardi and Kubota (2006), who observed up to 5% weight loss after 7 days storage at room temperature (25-27 °C) for cluster tomatoes (cv. *Clermon*). Also the increase of TBARS values was overall limited for both GP treatments also considering that air was used as working gas and ROS species are produced during treatments, being one of the main causes of bacterial inactivation. On the other hand, Kim *et al.* (2010) reported that the TBARS values of plasma-treated bacon fluctuated, and after 7 days of storage, plasma treatment for 60 or 90s produced higher TBARS values than untreated control.

Chapter 8. Effects of cold atmospheric gas plasma treatments on "Fuji" apples

8.1. Introduction

The consumption of fresh fruit and vegetables has increased over the last 2 decades also as a consequence of advices and campaigns encouraging consumers to eat at least 5 servings of fruit and vegetables each day (WHO, 2003; FSA, 2006). It is well known that fresh products are an important source of nutrients, vitamins and fibre for humans. Diets rich in fruit and vegetables have been shown to be protective against cancers and chronic illnesses such as coronary heart disease due to biologically active components in plant-based foods, particularly phytochemicals, which have important potential to modulate many processes in the development of diseases, including cancer, cardiovascular disease, diabetes, pulmonary disorders, Alzheimer's disease, and other degenerative disease states (CAC, 2010). Among fruit, apples and derived products, including juices and extracts, have been included in health-related studies around the world due to their rich content of various phytochemicals. In fact apples are a good source of antioxidants and have a rather high concentration of total phenolic compounds ranging from 110 to 357 mg/100 g of fresh apple (Wolfe *et al.,* 2003).

According to a recent study on consumption frequency within different European countries in relation to age and gender, Italian consumers most often indicated eating 3–5 apples per week (39.3%) (Konopacka *et al.,* 2010). Furthermore, older people (61–70 years) consume apples more often than the adults (36–60), while within the youngest group of consumers (16–35) eating apples is not at all popular. Although the major part of fruits and vegetables are consumed fresh or as industrially processed (canned, dried, juice, paste, pulp, sauce and soup preparations), the consumer trend is currently oriented also to ready-to-eat salads and ready-to-drink beverages (Endrizzi *et al.,* 2006). Moreover, also distribution of fresh fruit through vending machines is becoming quite popular following interventions aimed at reducing risks associated with unhealthy dietary intakes, particularly among the youngest students. In response to concerns about child health and obesity, a 2007 Institute of Medicine report recommended eliminating all sugar-sweetened beverages and restricting snack foods and beverages sold in school venues outside of the federal breakfast and lunch programs (Hartstein *et al.,* 2013; Committee on Nutrition Standards for Foods in Schools, 2007). As a

result, nutrition standards for vending machine items have been implemented, and high-fat and high-sugar items offered in vending machines have been significantly reduced or replaced with fruit and vegetable products (Blum *et al.,* 2007; Samuels *et al.,* 2009).

In the framework of the "National Prevention Plan 2005-2007" issued by the Italian Ministry of Health, which identified obesity as a priority health issue, one of the interventions was related to the promotion of the pilot national project called "Fruit Snacks". Such a project started with the beginning of the school year 2007/2008, with the involvement of 80 schools in the areas of Bologna, Rome, Bari and 60.000 students, and aimed at educating and encouraging the consumption of fruit and vegetables in school and in the family also throughout fruit snacks distributors in schools. Products often offered by vending machines include fruit salads, sliced fresh fruit (mainly apples), vegetables with vinaigrette, fruit juices… However, this new tendency poses several questions related to the microbial quality and safety of the products in relation to the new storage and vending conditions, *e.g.* non homogeneity of the temperature and relative humidity in the vending machines, unpredictable storage time of the fruits, impossibility to peel fresh fruit by the consumers, unnecessity of washing vegetables and the fruit that can be eaten also without peeling. In this scenario, a critical evaluation of the microbiological quality standards of fresh fruit and vegetables also in relation to processing operations is necessary.

Fresh fruits and vegetables, including tree components (*e.g.* leaves, roots, bulbs and tubers) are usually contaminated by spoilage microorganisms which are introduced to the crop on itself, during growing in the field, harvesting and postharvest handling, or during storage and distribution. The number and type of microorganisms found on fresh produce are highly variable. Mesophilic bacteria are around 10^3–10^9 CFU/g in raw vegetables after harvest, depending on the produce and the growing conditions. Gram-negative bacteria dominate the microflora associated with most vegetables, whereas yeasts and moulds are often the majority microflora of raw fruits (Burnett and Beuchat, 2000; Tournas, 2005). The microflora of vegetables and fruits is made up largely of *Pseudomonas* spp., *Erwinia herbicola*, *Flavobacterium*, *Xanthomonas*, and *Enterobacter agglomerans* as well as various moulds, *Alternaria*, *Penicillium*, *Fusarium* and *Aspergillus*. Lactic acid bacteria, such as *Leuconostoc mesenteroides* and *Lactobacillus* spp., are also commonly found, while yeasts such as *Torulopsis*, *Saccharomyces* and *Candida* are part of dominant microorganisms mostly on fruits because of their high sugar content (Caponigro *et al.,* 2010; de Azeredo *et al.,* 2011;

Pianetti *et al.,* 2008). Although natural microflora of raw fruits and vegetables is usually nonpathogenic for humans, the produce can be contaminated with pathogens (mainly *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli*, and *Shigella* spp.) from human, animal, or environmental sources during growth, harvest, transportation and further processing (Berger *et al.,* 2010).

Among the various chemical and physical treatments available to reduce/eliminate the microorganisms present in food products (Ramos *et al.,* 2013), chlorine solutions still remain the most widely used one due to its efficacy, cost-effectiveness ratio and simple use. However, the association of chlorine with the possible formation of carcinogenic chlorinated compounds in water has called into question the use of chlorine in food processing. Moreover, regulatory restrictions on the use of chlorine constrain the food industry to find alternatives for preservation of whole and fresh-cut fruit and vegetables.

As a consequence, several innovative approaches have been proposed and explored such as antioxidants, irradiation, ozone, organics acids, modified atmosphere packaging, natural preservatives, electrolyzed water, whey permeate, etc…(Ramos *et al.,* 2013; Rico *et al.,* 2007). However, none have yet gained widespread acceptance by the industry. For this reason the development of alternatives and markers in order to measure the efficacy of these alternatives are needed.

In this context, cold gas plasma can be considered an interesting emerging technology for decontaminating surfaces also taking into consideration that it can be used to treat the food at low temperatures.

The principal aim of this work was to evaluate the potentialities of cold atmospheric gas plasma as an emerging technology for the decontamination of whole "Fuji" apples. In particular the effectiveness in reducing the natural spoilage microflora was assessed following direct exposure to gas plasma. Moreover, also the use of gas plasma treated water as an alternative to sanitization (washing) with hypochlorite was investigated. Moreover also the impact of the various treatments on quality attributes, and antioxidant and enzymatic activities was examined.

8.2. Materials and methods

8.2.1. Fruit samples

"Fuji" apples were bought from local producer (APOFRUIT Italia soc.coop. Agricola) immediately after harvest (Emilia Romagna region, Italy). Fruits were then stored at about 0°C without any washing process until the gas plasma treatments were performed.

8.2.2. Gas plasma device and treatment conditions

Gas plasma treatments were conducted in the DBD device reported by Berardinelli *et al.,* (2012) and previously describe in paragraph *7.2.1.* (Figure 32).

Figure 32. DBD device used for "Fuji" apples.

The decontamination efficacy of the gas plasma device on the superficial indigenous microflora was evaluated by directly exposure of "Fuji" apples to gas plasma for 45 and 90 min at RH of 60% (22°C). For each treatment time five fruits were considered.
Gas plasma treated water was prepared by using distilled water which was put into glass bowl (with a maximum height of 1.5 cm) and exposed to gas plasma for 50 min (65% RTH, 22°C). Following treatments apples were transferred into plastic boxed which were stored at 4°C for 1 month.

8.2.3. Washing treatments

Washing treatments were performed with tap water, GP-treated water and hypochlorite (20 ppm) aqueous solution. Five fruits were dipped into the various solutions (3 l) for 10 min, air dried and then transferred into plastic boxes which were stored at 4°C for 1 month.

8.2.4. Microbiological analysis

The decontamination efficacy of the various treatments on the superficial indigenous microflora was evaluated for apples exposed to direct gas plasma (45 and 90 min), and fruits washed with GP-treated water, hypochlorite solution and tap water immediately after treatments and after 1 month of chilled storage.

Each apple was transferred into a sterile sampling bag (International PBI S.p.A., Milan, Italy) containing 100 ml of sterile saline solution (NaCl 0.9%, Merck KGaA, Germany) and hand-rubbed through the bag for 3 min in order to detach bacteria from the fruit surface. Following, serial dilutions in sterile saline solutions were carried out for each sample, and 100µl of the appropriate dilutions were inoculated onto Plate Count Agar (Oxoid, UK: 5.0 g/l peptone, 2.5 g/l yeast extract, 1.0 g/l glucose, 18.0 g/l agar) or Sabouraud Agar (Oxoid, UK: 10.0 g/l peptone, 20.0 g/l glucose, 18.0 g/l agar) added with chloramphenicol (100 ppm) in order to enumerate the surviving cells of total mesophilic bacteria and moulds, respectively. Then Petri dishes were incubated at 30°C for 48 h. For each treatment condition five fruits were analysed.

8.2.5. Physicol-chemicals analyses

The possible effects of the gas plasma on "Fuji" apple quality traits were assessed after each gas plasma treatment and compared with the traditional washing procedures with/without sodium hypochlorite (20 ppm). In particular, the influence of the treatments on several properties of the peel and pulp such as lipid peroxidation (Thiobarbituric Acid Reactive Substances - TBARS test), polyphenol-oxidase (PPO) activity, antioxidant activity (DPPH test) were investigated.

8.2.5.1. Thiobarbituric Acid Reactive Substances (TBARS) test

The assay of Thiobarbituric Acid Reactive Substances (TBARS) is a method for monitoring peroxidation through the detection of Malondialdehyde (MDA) level in a sample. Indeed MDA is a naturally occurring carbonyl compound produced through lipid peroxidation during cellular injury of plants and animals.

0.2 g of peel or pulp were placed into test tubes containing 1.5 ml of thiobarbituric acid (0.75% w/v in 0.25N HCl, Sigma-Aldrich, Germany), 1.5 ml of trichloroacetic acid (30% w/v in 0.25N HCl, Carlo Erba reagents, Italy), and 30 µl of butylated hydroxytoluene (1% w/v in

0.25N HCl, Sigma-Aldrich, Germany). The control sample was prepared by mixing 0.2 g of peel (or pulp) with 3.0 ml of trichloroacetic acid, while the sample blank was made of 0.2 ml of distilled water mixed with all the reagents (1.5 ml of thiobarbituric acid, 1.5 ml of trichloroacetic acid and 30 µl of butylated hydroxytoluene). Subsequently, all the test tubes were mixed and placed into a thermostatic bath (Lauda-Brinkmann, Germany) at 98°C for 10 min. After the heating, samples were cooled in ice and centrifuged at 7000 r.p.m. for 5 min (Rotofix 32A, Hettich Lab Technology, Germany). 1.5 ml of each supernatant were transferred into cuvettes and the absorbance was measured at 530 nm using an UV spectrophotometer (6705 UV/VIS spectrophotometer, Jenway, UK). Absorbance data was fitted with a calibration curve prepared with 1,1,3,3-tetraethoxypropane (TEP, Sigma-Aldrich, United States) in the concentration range 6.0-0.05µmol/l (y= 0.099x-0.015 R^2=0.989). MDA content (mg/kg apples) was calculated according to the following formula:

$$TBARS\left(\frac{mgMDA}{kg\ apple}\right) = \frac{[\mathrm{ABSsample} - (\mathrm{ABScontrol})] - \mathrm{b}}{\mathrm{m}} \mathrm{x}\ 16\ \mathrm{x}\ 72.06\ \mathrm{x}\frac{1}{1000}\ \mathrm{x}\frac{1}{\mathrm{w}}$$

where: *ABScontrol* is the absorbance of reference solution containing only TCA and peel/pulp; *ABSsample* is the absorption of the TBARS solution with sample; *w* is the weight (grams) of sample used. Two replicates were run per sample.

8.2.5.2. Antioxidant activity (DPPH test)

2,2-Diphenyl-1-picryl-hydrazyl (DPPH) is a stable free radical which has an unpaired valence electron at one atom of nitrogen bridge and its scavenging activity is the basis of the well known DPPH antioxidant assay. DPPH radical scavenging activity was assessed according to the method of Hsu (2010), previously described in the paragraph *7.2.5.5.* and modified as follow. Briefly, 0.1 g of grinded peel (or pulp) were incubated into a test tube containing 2.5 ml of methanol (Carlo Erba reagents, Italy) for 1 h. Then samples were filtered and mixed with DPPH and methanol. 0.1 mg/ml of L-ascorbic acid and water were used to replace samples and referred to as positive and negative control, respectively. After incubation in the dark at room temperature, the absorbance at 517 nm was spectrophotometrically measured. For each sample, data were repeated in twice and the percentage of radical scavenging activity was calculated according to the formula:

$$\%DPPH = \frac{(1 - (\text{ABS control} - \text{ABS sample}))}{\text{ABS control}} \text{x}100$$

where: ABS control is the absorbance of negative control containing only DPPH and water, and ABS sample is the absorption of the DPPH solution with samples.

8.2.5.3. PPO activity

The enzyme polyphenol oxidase (PPO) catalyzes the hydroxylation of monophenols into ortho-diphenols and the oxidation of o-diphenols into quinones. The quinones polymerize to form dark-colored phytomelanins, most often responsible for browning of damaged plant tissues.

The enzyme was extracted from samples according to the following protocol: 20 g of pulp or 5 g of peel were mixed with 20 ml or 5 mL of phosphate buffered saline pH 6.5 (PBS Dulbecco A, Oxoid, UK) solution, respectively. Samples were homogenized in ice for 2 min using a Stomacher mixer (Lab Blender Seward, PBI International, UK) and then centrifuged at 4000 r.p.m for 5 min at 4°C. PPO activity was determined by measuring the increase in absorbance at 420 nm over 30 min with an UV spectrophotometer (T80 + UV/VIS spectrometer, PG instrument Ltd, UK). The sample cuvette contained 100 µl of the extracted enzyme and 1.4 ml of L-DOPA as reaction substrate (Sigma-Aldrich, Germany), which was used at various concentrations (*i.e.* 20mM, 10mM, 7mM, 5mM and 2.5mM in PBS buffer, pH 6.5). The sample blank contained only 1.5 ml of the substrate solution. Each reaction was carried out at 20°C.PPO activity was measured in duplicate for each sample.

For each sample, V_{max} and K_m values were calculated through the Michaelis-Menten equation. By comparing these parameters, which describe the kinetics of the PPO enzyme, it is possible evaluate the effects of reactive oxygen species generated by gas plasma treatment on the PPO enzyme activity.

$$\text{Michaelis} - \text{Menten equations} \qquad V = \frac{\text{Vmax x [S]}}{\text{Km} + \text{[S]}}$$

8.2.6. Statistical analysis

Significant differences ($p<0.05$) between control and treated mean values were found by using Student's t-test and the Analysis of Variance (ANOVA) according to Tukey's HSD. Analysis was carried out using Statistica 8.0 (StatSoft Italy srl, Italy).

8.3. Results

8.3.1. Microbial analysis

The efficacy of gas plasma treatments for the superficial decontamination of apples was evaluated by detecting counts of total viable mesophilic bacteria and moulds following 45 and 90 min of direct exposure to plasma. Such treatment times were chosen on the basis of previous experiments on pears showing reductions about 1.0 and 3.0 log units after 45 and 90 min, respectively (Berardinelli *et al.*, 2012). A comparison with washing treatments with tap water, chlorine and with gas plasma-treated water was also made.

Data reported in Figure 33 clearly show that mesophilic microflora was sensitive to gas plasma as significant ($p<0.05$) reductions were obtained for both direct treatments. In particular a 0.9 and a 1.5 log reduction were achieved following 45 and 90 min of direct exposure to plasma, respectively. Washing fruits with GP-treated water resulted in a 1 log inactivation similarly to that achieved by using chlorine. After 1 month of refrigerated storage, no significant differences in cell counts were detected compared to time zero, regardless the sanitizing treatment adopted. These results indicate that all the tested treatments were effective in inactivating spoilage microflora as no ability to recover damages induced by physical process and chemical agents was observed during subsequent storage.

Unlike mesophilic bacteria, moulds were poorly susceptible to the direct exposure to gas plasma (Figure 34). In fact mean viability losses not exceeding 0.8 Log CFU/fruit were achieved regardless the treatment time, while no effect was observed following washing with GP pre-treated water. On the other hand, also chlorine proved to have no activity as no significant differences were detected immediately after the treatments in moulds counts in comparison to both raw whole apples and fruits washed with tap water. After 1 month of refrigerated storage, the fungal contamination level was stable as indicated by data reported in Figure 34, which were unchanged compared to time zero except for the fruits exposed to direct gas plasma for 90 min which showed a significantly ($p<0.05$) lower value.

8.3.2. Quality assessment

In order to evaluate the effects of gas plasma treatments on quality parameters of apples, colour, antioxidant activity (DPPH test) and oxidation level (TBARs test) were assessed immediately after the various treatments and following 1 month of refrigerated storage.

As far as colour, no significant differences were detected for the CIE *L**, *a** and *b** parameters regardless the decontamination procedure and treatment condition used (data not shown).

According to TBARS data (Figure 35), none of the tested treatments gave rise to oxidation phenomenon of the flesh. On the contrary, the peel was affected by either direct and indirect exposure to gas plasma. In fact immediate 2-fold and 3-fold increases were observed after direct washing with GP-treated water and the shortest GP treatment (direct exposure), respectively. However, such increases were not found after 1 month of storage thus suggesting that sub-lethal oxidative stresses occurred. On the contrary, when the longest GP treatment was used, the increase in TBARS values occurred later as it was detected after a 1-month storage.

Results of the DPPH test (Figure 36) revealed that the scavenging activity of apple peel was unaffected by both washing into GP-treated water and direct GP treatments for 45 min, and ranged between 55 and 60% similarly to the untreated samples. On the contrary, increasing the direct exposure up to 90 min resulted in a reduction down to 37% ± 3.6. A similar behavior was observed also for the flesh although DPPH values were lower as expected. During storage all the samples underwent a decrease in DPPH values similarly to the untreated fruits, with the exception of those washed with GP-treated water. In fact no significant ($p<0.05$) change in their scavenging activity was detected both for peel and the flesh after 1 month of storage compared to time zero.

8.3.3. Enzymatic activity

The impact of gas plasma on PPO activity was different in relation to the processing conditions adopted as evidenced by the Vmax and Km values shown in Figure 37. In fact a 45 min direct exposure to GP caused a significant ($p<0.05$) change in the activity in the flesh and in the peel which showed a 4-fold and 20-fold increase, respectively. On the contrary, prolonging the exposure up to 90 min resulted in a half reduction. When apples were washed

with the GP-treated water no effect on the enzyme activity was observed similarly to the washing with chlorine or tap water.

Following refrigerated storage, Vmax values recorded for both control samples and fruits treated with tap water or hypochlorite solution were higher than those at the beginning of storage untreated ones (Figure 38).

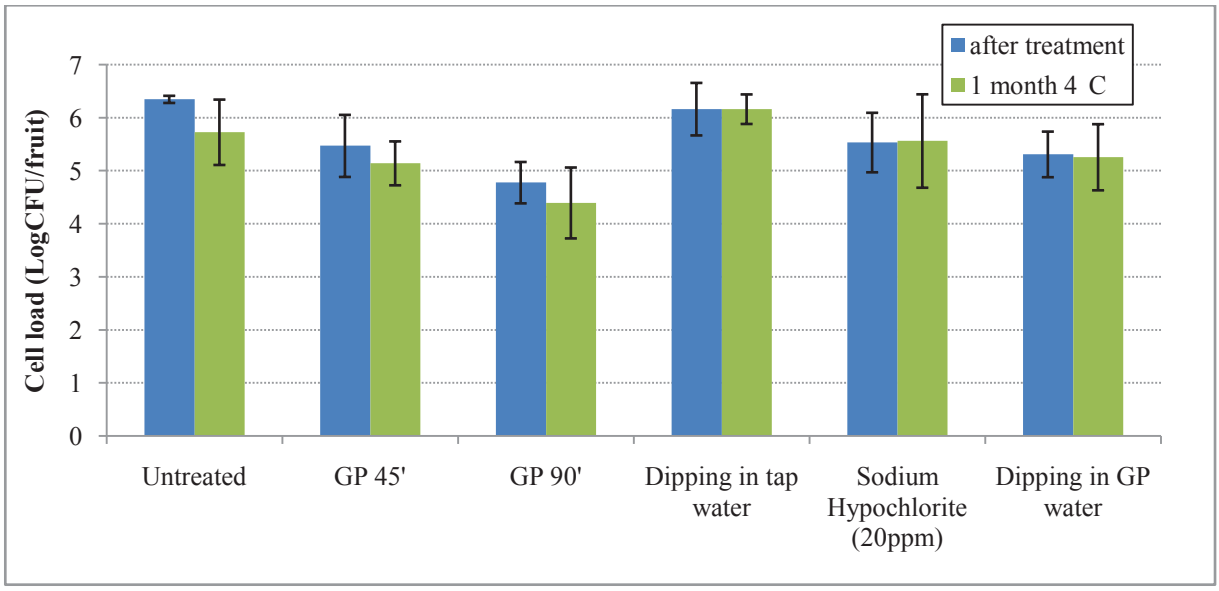

Figure 33. Fate of total aerobic mesophilic bacteria (LogCFU/fruit) in apples' surface in relation to the treatment conditions (GP and different washing procedures).

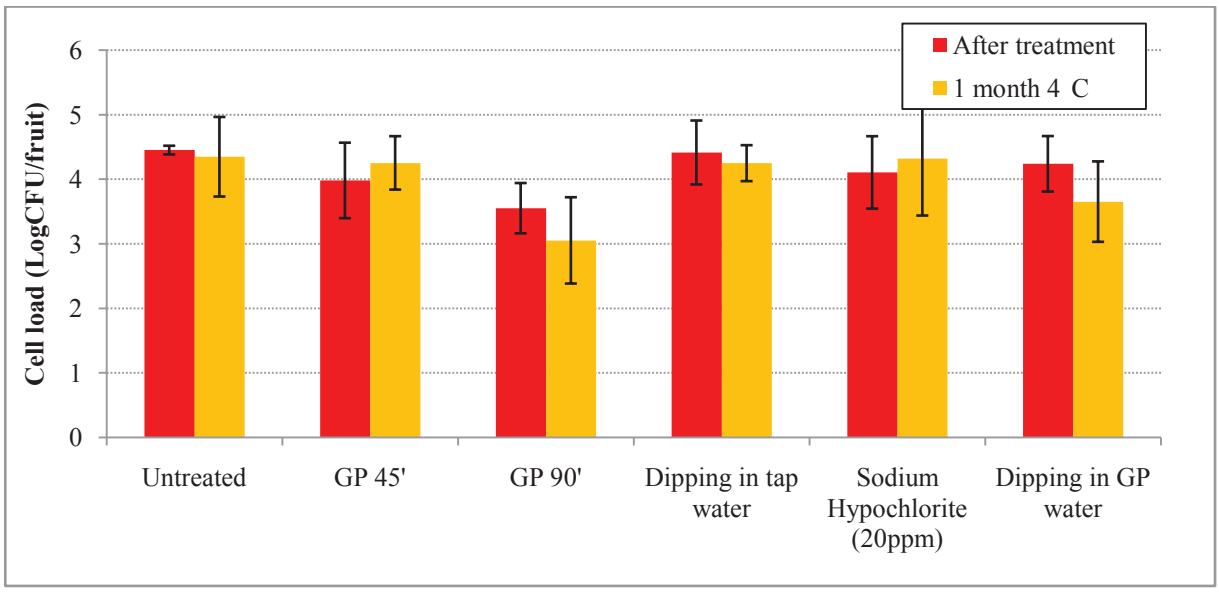

Figure 34. Fate of moulds (LogCFU/fruit) in apples' surface in relation to the treatment conditions (GP and different washing procedures).

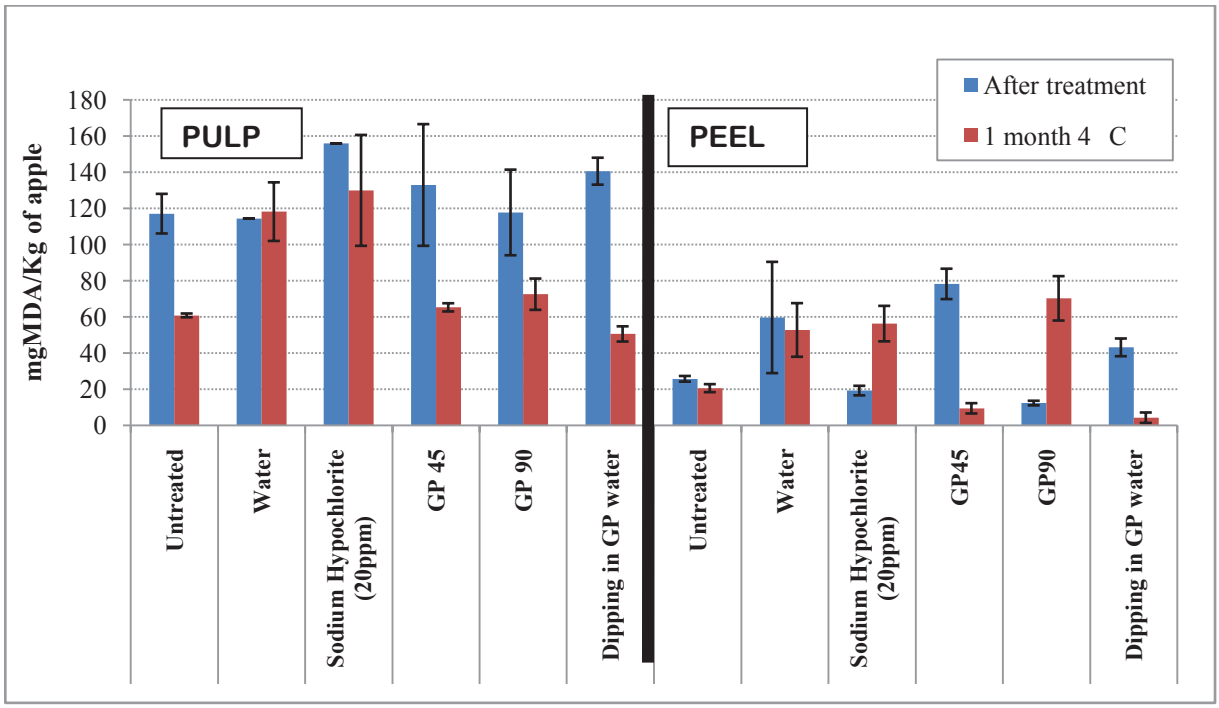

Figure 35. Evolution of oxidation degree (TBARS assay) of peel and pulp of apples in relation to the treatment conditions (GP and different washing procedures).

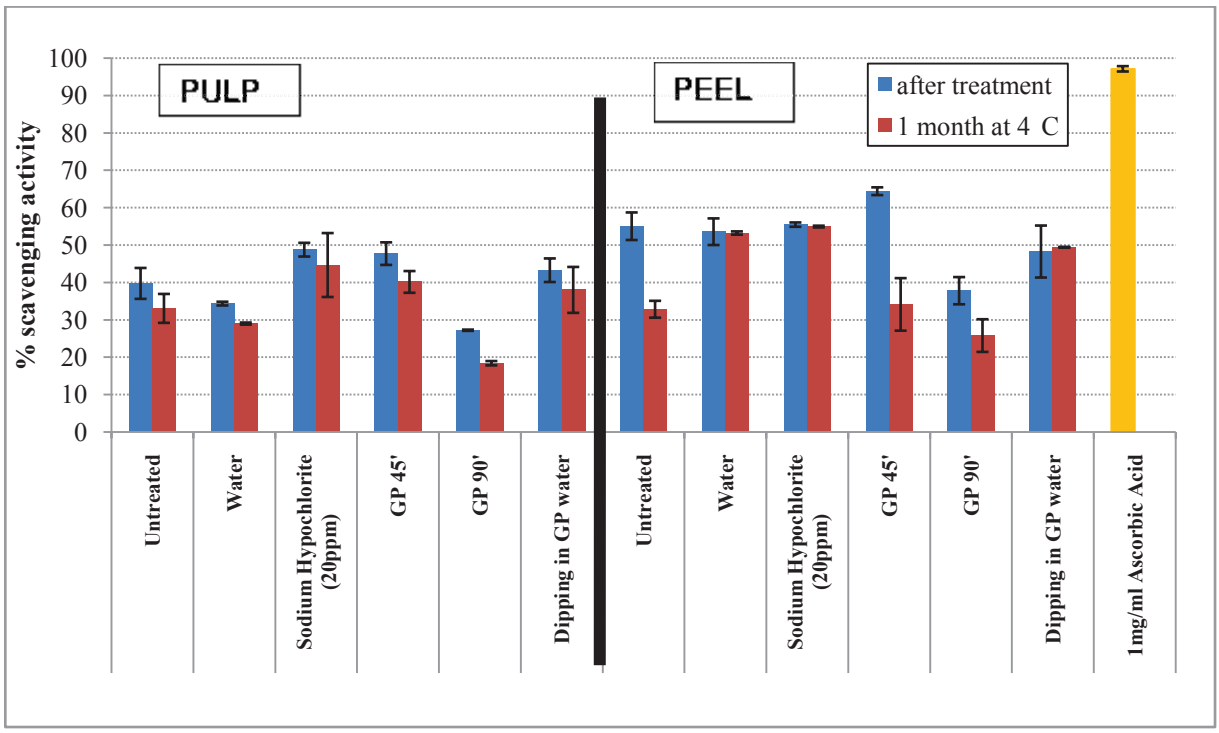

Figure 36. Evolution of antioxidant activity (DPPH assay) of peel and pulp of apples in relation to the treatment conditions (GP and different washing procedures).

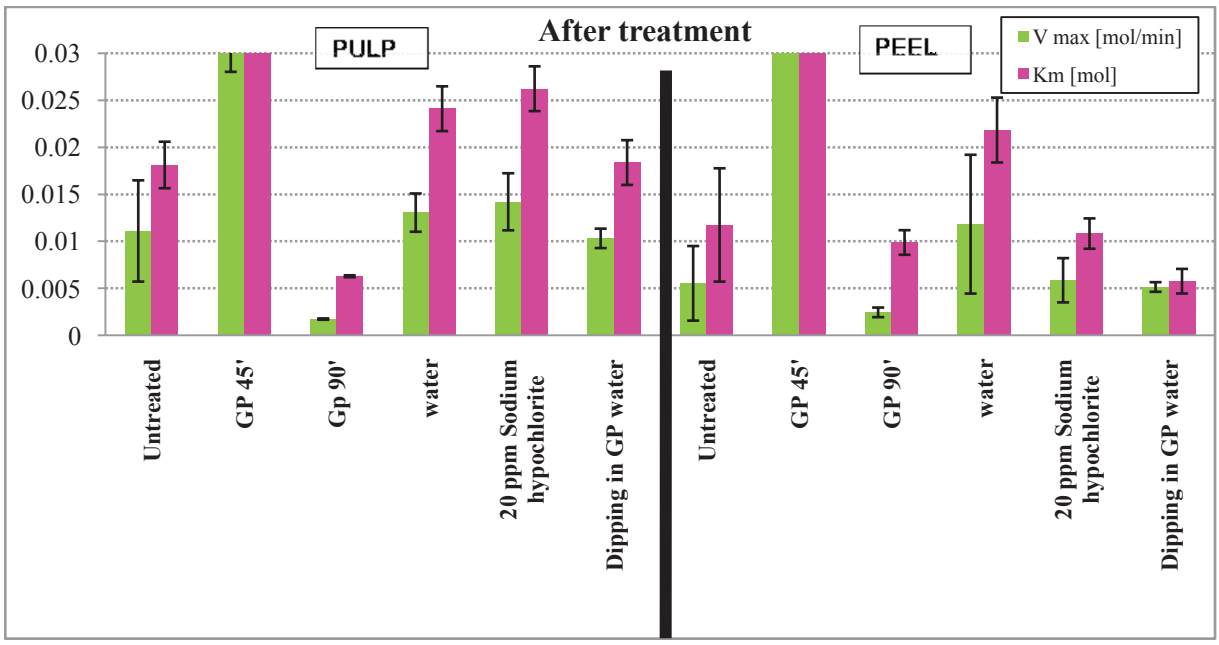

Figure 37. Vmax (mol/min) and Km (mol) of polyphenol oxidase enzyme extracted from pell and pulp of apples immediately after treatment (GP and different washing procedures).

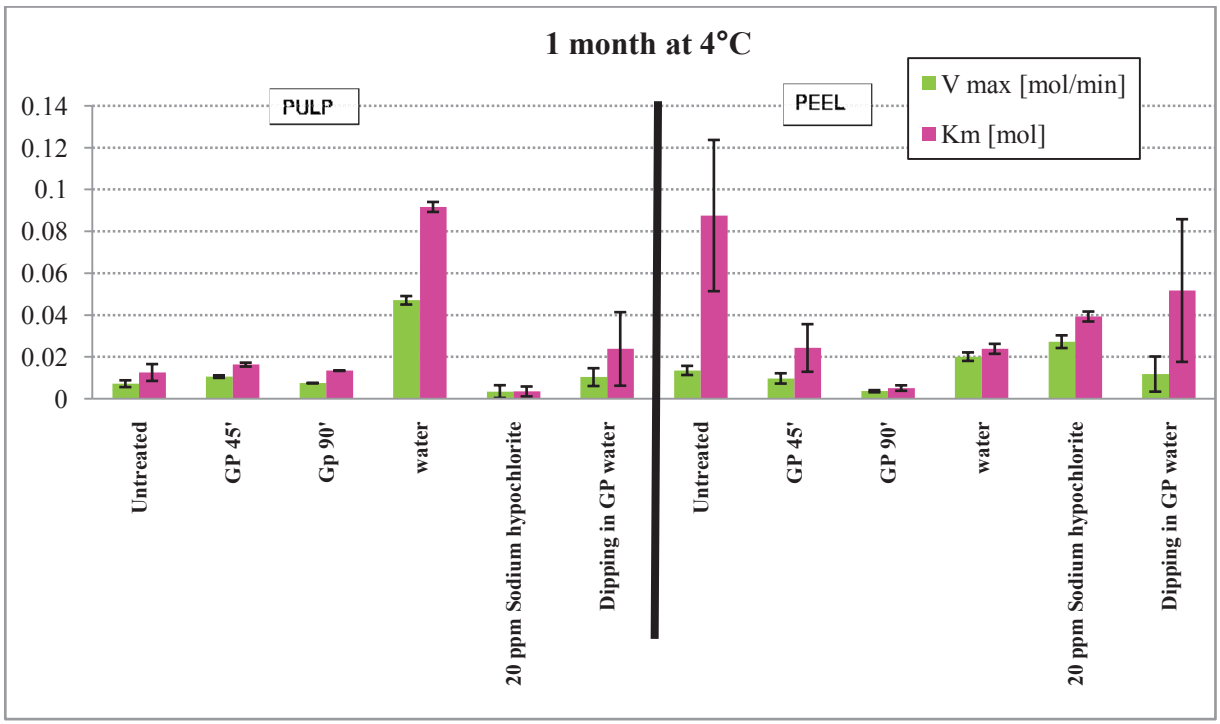

Figure 38. Evolution during the storage of Vmax (mol/min) and Km (mol) of polyphenol oxidase enzyme extracted from pell and pulp of apples in relation to the treatment conditions (GP and different washing procedures).

8.4. Discussion

In this study the efficacy of atmospheric plasma based treatments for the superficial decontamination of "Fuji" apples was investigated. In particular washing with plasma treated water was compared to the conventional treatment currently used in the food industry also for RTE fruit and vegetables, which is based on chlorine sanitizers. Also direct exposure to gas plasma was investigated as an alternative to washing.

The experimental results obtained showed that GP effectively reduced mesophilic microbiota on fresh apples, and the immediate reductions persisted over 1 month of refrigerated storage. In particular a 1 Log inactivation was achieved by using GP-treated water similarly to cleaning with the hypochlorite solution. When direct GP treatments were used, the same efficacy as washing was shown for the shortest treatment, while it was enhanced up to 1.5 log cycles by increasing treatment time. Such inactivation levels are in agreement with literature data reporting that conventional postharvest washing and sanitizing treatments are not highly effective for fresh produce, often resulting in less than 2 log unit reductions of pathogens (Niemira, 2012). Atmospheric cold plasma technology has recently attracted quite a lot of research as a non-thermal antimicrobial treatment of several foods including also fruits and

vegetables (Ramos *et al.,* 2013). To the best of our knowledge this is the first work investigating the use of GP-treated water as an alternative to chlorine sanitizers as almost all the published papers only refer to the use of gas plasma for a direct samples exposure. Nevertheless, differences in the equipments and processing conditions employed make a comparison rather difficult. Moreover, most of the papers are focused on the evaluation of treatment effectiveness towards pathogens deliberately inoculated onto the tested foods, while literature data on the response to GP by background microbiota are scarce. Critzer *et al*. (2007) reported the ability of one atmosphere uniform glow discharge plasma (OAUGDP) for reduction of inoculated microbial populations on fresh produce surfaces, and namely *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* on Red delicious apples, cantaloupe, and lettuce, respectively. *E. coli* O157:H7 was generally more resistant to plasma treatment than *Salmonella*, and it was reduced by >1 log after 30-s and 1-min exposures and >2 log after a 2-min exposure. Niemera and Sites (2008) investigated a gliding arc plasma system for the treatment of *Escherichia coli* O157:H7 and *Salmonella* Stanley both on agar plates and inoculated onto the surfaces of Golden Delicious apples. According to the study, inactivation of both the pathogens on apples followed a time-dependent reduction, and higher flow rates resulted in greater inactivation at shorter times. At the maximum flow rate (40 ml/min) a 3 log CFU reduction was observed after 1 and 3 min for *E. coli* and *Salmonella*, respectively. Recently, Misra *et al.* (2014) found that the background microflora (aerobic mesophilic bacteria, yeast and mould) of strawberries treated for 5 min with dielectric barrier discharge (DBD) system was reduced by 2 log units within 24 h of post-Atmospheric cold plasma treatment.

Despite a wide literature on the impact of cold gas plasma treatments on several matrices inoculated with pathogenic bacteria, studies on moulds are few and mainly limited to pure liquid cultures. In our study, mould population contaminating the surface of apples was resistant to both direct gas plasma exposure, irrespective of treatment time, and washing with the GP- treated aqueous solution. On the other hand, also hypochlorite did not lead to any significant fungal reduction compared to untreated products. Similarly to our results, Lacombe *et al.* (2015) found that cold plasma was not effective in significantly reducing the numbers of yeast and molds on blueberries. On the contrary, Herceg *et al.* (2014) achieved the greatest inactivation of *Aspergillus ochraceus* 318 (~ 3.5 Log reduction) and *Penicillium expansum* 565 (2.5-3 log reduction) by treating aqueous cell suspensions with a single

electrode atmospheric jet for 5 min and by using lowest sample volume (2 ml). Furthermore, Suhem *et al.* (2013) indicated that Ar plasma by a plasma jet system provided good protection against mold (*Aspergillus flavus*, *Aspergillus niger*, *Rhizopus* sp. and *Penicillium* sp.) on brown rice cereal for at least 19 days under storage conditions at 25°C and 100% RH, while air and water vapor gas plasma were ineffective in reducing mold spore spoilage.

Few studies have been conducted on the physicochemical properties or nutritional components of food after plasma processing. Since this technology uses charged particles (*e.g.* O_3, O●−, O_2^-, O_2^{-2}, H_2O_2,OH●, NO●, etc.), interaction with some food components is possible thus leading to losses in nutritional and quality parameters. Despite data reported in literature and processing condition adopted in this work (*i.e.* quite long treatment time for the direct GP exposure), the tested GP treatments did not result in any significant change in colour which is notably important as product appearance is one of the primary criterion taken into consideration by consumers. Similarly to our results, Niemira and Sites (2008) reported that no changes in color, texture, aroma or other sensory properties occurred when testing cold plasma in apples to inactivate *Salmonella* Stanley and *E. coli* O157:H7.

According to Critzer *et al.* (2007), the main limitation of plasma in the food industry is the treatment of those food products with high lipid content and antioxidants, because of the possible oxidation generated by the plasma species. In the present work, the scavenging activity of both the peel and flesh did not change following washing with GP-treated water or direct exposure to GP for 45 min. These results suggest that nor direct exposure to GP, nor treatment with GP-treated water affect phenolic and /or flavonoid contents of both the peel and the pulp. Phenolic compounds, which are secondary plant metabolites, not only are important determinants in the sensory and nutritional quality of fruits, vegetables and other plants, but also are one of the most widely occurring groups of phytochemicals. They are considered of considerable physiological and morphological importance in plants since may act as phytoalexins, antifeedants, attractants for pollinators, contributors to plant pigmentation, antioxidants and protective agents against UV light, amongst others (Naczk and Shahidi, 2006). Due to their high redox potential, which allows them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers, flavonoids help to protect the plant against UV light, fungal parasites, herbivores, pathogens and oxidative cell injury. Apples are a good source of phenolic compounds (Eberhardt *et al.,* 2000), and their concentration is much greater in the peel of apples than in the pulp (Burda *et al.,* 1990; Wolfe *et al.,* 2003).

The result of this work are in disagreement with the work of GrzeGorzewSkI *et al.* (2009) aimed at investigating the stability of selected flavonoids with different antioxidative potential upon exposure to an atmospheric pressure Ar plasma jet. The Authors reported that the flavonoids 1–4 degrade upon plasmo-chemical reactions probably due to existing ROS and radicals in the plasma effluent (GrzeGorzewSkI *et al.,* 2009). On the contrary Kim *et al.* (2015b) reported that the biotransformation of naringin using DBD plasma resulted in the formation of two new flavanone derivatives, one of which showed significantly enhanced antioxidant effects relative to the parent naringin.

Concerning TBARS, data indicated that none of the tested treatments gave rise to oxidation phenomenon of the pulp. Moreover, during storage a reduction in MDA content was observed for all the GP samples unlike apples cleaned with tap and chlorinated water. On the contrary, the peel resulted to be much more susceptible to oxidation when washing treatments (both with hypochlorite and GP-treated water) and direct exposure to plasma for 45 min were performed. However, increasing GP treatment time up to 90 min led to a significant reduction of MDA content compared to untreated apples. Such an opposite result evidenced for direct GP exposure by increasing treatment time can be explained by considering that the shortest treatment caused a sub-lethal stress, while the longest one could represent a lethal dose. The formation of fatty acid hydroperoxides may occur either by chemical oxidation or by the action of enzymes such as lipoxygenase (LOX) (Mosblech *et al.,* 2009). LOXs occur ubiquitously in plants and mammals, and they have been detected in coral, moss, fungi and a number of bacteria as well (Andreou *et al.,* 2009;, Oliw, 2002). LOX-derived fatty acid hydroperoxides can be further metabolized into volatile aldehydes and jasmonates (JA) in plants (Mosblech *et al.,* 2009), in diols and lactones in fungi (Tsitsigiannis and Keller, 2007) and in lipoxins and leukotrienes in mammals (Samuelsson *et al.,* 1987; Sigal *et al.,* 1994). The array of molecules derived from LOX pathway, known as oxylipins, play an important role as signals in wound healing and defense processes in plants, while in mammals they are involved in inflammation, asthma and heart diseases. A steadily increasing number of studies support a role of these compounds as a ‘‘master switch’’ in plant development and stress adaptation. Among oxylipins, salicylic acid and jasmonic acid-induced defense responses could be mediated by an increase of reactive Oxygen species (ROS) (Torres, 2010). By investigating the scald development in Fuji apples, which is believed to be associated with adverse effects of oxidative stress induced by prolonged chilled storage, Lu *et al.* (2014)

evidenced that MDA content in "Fuji" apples increased similarly to H_2O_2 levels during cold storage. On the other hand Surowsky *et al.* (2013) hypothesized that chemical reactive species generated during GP treatments (OH, O^{2-}, HOO and NO radicals) induced chemical modifications of chemically reactive side-chain of the amino acids, such as cysteine, aromatic rings of phenylalanine, tyrosine, and tryptophan in enzymes such as polyphenoloxidase (PPO) and peroxidase (POD). As a consequence a loss of enzyme activity was observed in a model food system. In particular, the activity of PPO was reduced by about 90% after a treatment time of 180 s, while POD was more stable and was reduced by about 85%after 240 s. Cullen *et al.* (2013) indicated that treatment voltage and time were both found to have a significant effect on POD inactivation, which was not linear. By using the same DBD prototype employed in this study, Tappi *et al.* (2013) found that PPO residual activity in fresh-cut Pink Lady® apples linearly decreased by increasing the treatment time from 5+5, 10+10 and 15+15min. In these conditions, residual activities were about 88, 68 and 42%, respectively. These findings are in accordance with our results showing a significant inactivation of PPO due to GP direct exposure particularly for the longest treatment. On the contrary, following the 45 min exposure a significant increase of PPO activity was observed similarly to the peroxidation phenomen thus strengthening the idea that such a treatment time causes a sub-lethal stress to apple fruits. In fact the reduced PPO values observed after 1 month of storage confirm that the 45 min GP treatment led to reversible modification in the enzyme macromolecules.

Chapter 9. Effects of cold atmospheric gas plasma treatments on black pepper

9.1. Introduction

Similarly to several spices, pepper is cultivated in many countries and consumed fresh or in dried form as a food ingredient. Given their provenance, spices are contaminated by various microorganisms coming mainly from soil and in particular aerobic and anaerobic spore-forming bacteria. Moreover, most of the spices are produced in tropical and subtropical countries where hygienic conditions are overall poor. Therefore, if not properly handled during harvest, drying and storage, they are an important source of contamination for foods to which they are added. Pathogenic microorganisms, including *Aspergillus flavus*, *Bacillus cereus*, *Clostridium perfringens*, *Salmonella*, *Escherihcia coli* are often present in spices (Aydin *et al.,* 2007; Buckenhuskes and Rendlen, 2004).

Several salmonellosis outbreaks attributed to contaminated spices have been documented, including two large-scale illness outbreaks in the United States attributed to consumption of contaminated white, black or red pepper (Centers for Disease Control and Prevention, 2010; Higa, 2011). Spices have been found to be contaminated with *Salmonella* at several points along the food supply chain including at the point of import into the United States, in spice processing/packing and food manufacturing facilities and at retail (Keller *et al.,* 2013). In 1993, a single foodborne salmonellosis outbreak in Germany which caused an estimated 1000 illnesses (Lehmacher *et al.,* 1995), highlighted that even minor ingredients such as spices (and namely paprika) can cause large-scale foodborne illness outbreaks.

In order to reduce contamination level of spices, several decontamination techniques are used worldwide and/or are available and include fumigation with ethylene oxide, irradiation, steam heat sterilization, and ultraviolet (UV) treatments (Schweiggert *et al.,* 2007). Although fumigation with ethylene oxide is the technique used for the longest period as it effectively inhibits several microbial species, its employment is actually forbidden in several countries due to its carcinogenicity (Fowles *et al.,* 2001). Also gamma irradiation at 2-7 kGy has been shown to effectively decontaminate various spices (Farkas, 1998), but its use is generally unpopular among consumers and is allowed only in few countries. On the other hand, thermal treatments using superheated steam causes sensory and nutritional losses and is quite

expensive. UV treatments present several limitations, mainly due to the poor penetration ability of UV radiations, the strong dependence of inactivation on the distance from the UV source, which reduces their practical applications.

In the view of the increasingly interest and request by the consumers for fresh-like products, characterized by low or no content of chemical preservatives and produced by using technologies with limited impact on nutritional and qualitative properties of foods, several studies have investigated and promoted the use of gas plasma-based technology as an emerging decontamination technique. In fact an increasing number of papers have been published in the last 5 years reporting the effectiveness of different gas plasma devices on several pathogenic and spoilage organisms inoculated in food matrices (Surowsky *et al.,* 2014b). By considering that cold plasma is a non-thermal technology in which the surface temperature of the treated sample is kept at temperatures below thermal treatment temperatures, thus limiting alteration of aroma, odour and nutritional properties, its use has been mainly investigated for perishable foods, such as fresh fruits and vegetables and to a latter extent for meat products (Baier *et al.,* 2014). On the contrary, investigation for the decontamination of dried products, like herbs and spices, are still limited. Two studies (Basaran *et al.*, 2008; Selcuk *et al.,* 2008) reported the application of low-pressure cold plasma on grain, legumes and nuts that were infected with *Aspergillus* spp., *Aspergillus parasiticus* and *Penicillium* spp., while Pignata *et al.* (2014) investigated the effect of plasma-enhanced chemical vapour deposition (PECVD) treatment on naturally contaminated pistachios. Kim *et al.* (2014) used a microwave-powered CPT system to study the microbial inhibition effects of cold plasma treatments on the inhibition of naturally occurring aerobic microorganisms in red pepper powder including *A. flavus* and *B. cereus* spores.

The aim of this study was to test the effectiveness of cold pressure plasma generated by a DBD device on the inactivation of selected pathogens which frequently contaminate pepper. In particular the sensitiveness to gas plasma treatments of strain of *Salmonella* Enteritidis, *Escherichia coli* and *Listeria monocytogenes*, deliberately inoculated onto black pepper, and their survival during typical spices storage conditions was evaluated. Also the effects of the treatments on colour, lipid peroxydation, antioxidant activity and volatile compounds immediately after the treatments and over a 3-months storage were assessed.

9.2. Materials and methods

9.2.1. Black pepper

Organic black pepper grains were bought in a Brazilian market (Porto Alegre) and were stored at room temperature until the gas plasma treatments were performed.

9.2.2. Bacterial strains and inocula preparation

In order to evaluate the effect of gas plasma exposures on some foodborne pathogens, which can contaminate black pepper, a Challenge test was carried out. The strains of *Listeria monocytogenes, Salmonella* Enteritidis and *Escherichia coli* used in this Ph.D. thesis belong to the Department of Agricultural and Food Sciences (DISTAL), of Alma Mater Studiorum, University of Bologna. In particular the following strains have been selected:

- *Listeria monocytogenes* 56Ly (a wild type isolated from pork wastewater and characterized by high resistance to both high and low temperatures) and ScottA (sierotype 4b, clinical isolate);
- *Salmonella* Enteriditis 155 (isolated from poultry meat) and 86 (isolated from cabbage involved in an outbreak of salmonellosis which occurred in RS State, in Brazil, in 1999);
- *Escherichia coli* NCFB 555 (isolated from raw milk) and ATCC 25922 (clinical isolate).

All the strains were cultivated in Brain Heart Infusion (BHI, Oxoid: 12.5 g/l brain infusion solids, 5.0 g/l beef heart infusion solids, 10.0 g/l proteoso peptone, 2.0 g/l glucose, 5.0 g/l sodium chloride, 2.5 g/l di-sodium phosphate) at 37°C for 24 h. 1.0 ml of overnight cultures (~9 Log CFU/ml) were transferred into 50.0 ml of BHI and incubated at 37°C for 12 h. The day after, microbial cultures of each strain were harvested by centrifugation and re-suspended in 150 ml of saline solution (NaCl 0.9% w/v, Merck KGaA, Germany). Final concentration of cells was around 9 Log CFU/ml and was quantified by pour-plating.

9.2.3. Produce preparation

About 15.0 g of whole black pepper were placed into a sterile Petri dish and sprayed (about 10 sprays) with the *Listeria monocytogenes*, *Salmonella* Enteritidis and *Escherichia coli*

saline suspension. Samples were then air-dried at room temperature under a laminar flow hood for approximately 1 h before being used for gas plasma treatments.

9.2.4. Gas plasma device and treatment conditions

Gas plasma treatments were conducted in the DBD device described by Berardinelli *et al.,* (2012) and reported in the paragraph *7.2.1.* (Figure 39).

Figure 39. Black pepper grains treated with DBD device.

Petri dishes containing uninoculated or inoculated samples were transferred into the chamber of the DBD device and exposed to gas plasma discharge. The distance between the samples and the electrodes was within the range from 9 to 12 cm. The inoculated and uninoculated samples were treated for gas plasma for 10, 20, 30, 45, 60 and 90 min at RH of 60% (22°C). For each experimental condition three Petri plates containing black pepper were placed under the three electrodes.

After treatments samples were transferred into sterile glass vials and stored at room temperature for three months. Immediately after treatments and after 1 and 3 months they were analysed in order to evaluate the effects of gas plasma on the surviving cells of inoculated pathogens, and main quality traits including pH, Aw, colour, thiobarbituric acid reactive substances (TBARS test) and antioxidant activity (DPPH test). Moreover also volatile compounds were analyzed by GC/MS-SPME.

9.2.5. Microbial analysis

10.0 g of black pepper were transferred into a sterile sampling bag (International PBI S.p.A., Milan, Italy) containing 90.0 ml of sterile saline solution and homogenized for 2 min using a Stomacher mixer (Lab Blender Seward, PBI International, UK). Subsequently, 1-ml aliquot was used to prepare decimal serial dilutions.

In order to evaluate viable cells of the target pathogens, 100 µl of the appropriate dilutions were inoculated onto different selective agar medium including Listeria Selective Agar Base (Oxoford formulation, Oxoid, UK: 39.0 g/l columbia blood agar base, 1.0 g/l aesculin, 0.5 g/l ferric ammonium citrate, 15.0 g/l lithium chloride, 400 mg/l cycloheximide, 20 mg/l colistin sulphate, 5 mg/l acriflavine, 2 mg/l cefotetan, 10 mg/l fosfomycin), Brilliant Green Agar (Modified) (Oxoid, UK: 5.0 g/l lab-lemco powder, 10.0 g/l peptone, 3.0 g/l yeast extract, 1.0 g/l disodium hydrogen phosphate, 0.6 g/l sodium dihydrogen phosphate, 10.0 g/l lactose, 10.0 g/l sucrose, 0.09 g/l phenol red, 0.0047 g/l brilliant green, 12.0 g/l agar) and ChromoCult® Coliform Agar (Merck KGaA, Germany: 3.0 g/l peptones, 5.0 g/l sodium chloride, 2.2 g/l sodium dihydrogen phosphate, 2.7 g/l disodium hydrogen phosphate, 1.0 g/l sodium pyruvate, 1.0 g/l tryptophan, 10.0 g/l agar, 1.0 g/l sorbitol, 0.15 g/l Tergitol®7, 0.4 g/l chromogenic mixture). All the media were incubated at 37°C for 24 h.

9.2.6. Black pepper quality traits

9.2.6.1. pH measurement

2.0 g of black pepper were grinded and suspended into 5.0 ml of distilled water. Samples were homogenized for 2 min using a Stomacher mixer, and the pH was measured by using a pH meter (pH meter BasiC 20, Crison, Italy). The device were calibrated with pH 7 and pH 4 calibration buffers. The mean of three independent repetitions was calculated for each sample.

9.2.6.2. Aw measurement

About 3.0 g of whole black pepper were placed into a disposable sample cup and the water activity was measured by using water activity meter (Aqualab 4TE, USA). The device was previously calibrated with distilled water (aw=1.0000). The mean of three independent repetitions was calculated for each sample.

9.2.6.3. Colour measurement

About 3.0 g of black pepper grains were placed in a disposable sample cup and the colour profile of lightness (L*), redness (a*) and yellowness (b*) (CIE, Commission Internationale de l'Eclairage, 1978) was measured for each sample in triplicate with a reflectance colorimeter (Minolta Chroma Meter CR-400, Minolta Italia S.p.A., Italy). The CR-400 colorimeter measures the red, green, blue and total amount of light reflected from an object

using an 8-mm-diameter measuring area, a d/0° illuminating and viewing geometry, and illuminant C.

Chroma (C*), which is a measure of saturation, was calculated with the formula:

$$C^* = \sqrt{a^{*2} + b^{*2}}$$

Hue-angle, in degrees, is a measure of an object's color in the a*–b* plane and was calculated from the following formula:

$$h = \tan^{-1}\left(\frac{b^*}{a^*}\right)$$

9.2.6.4. Lipid peroxidation and antioxidant activity of black pepper

a. Sample preparation

The assessment of lipid peroxidation and antioxidant activity was made through the TBARs and DPHH assays, respectively by using black pepper extracts. Samples extracts were prepared from 2.0 g of black pepper accurately ground and extracted for 1 h with 50 ml of aqueous methanol (80%). TBARS and DPPH tests were performed according to the methods of Suhaj *et al.* (2006), previously described in the paragraphs 7.2.5.4. and 7.2.5.5. respectively, and modified as follow.

b. Thiobarbituric Acid Reactive Substances (TBARS) test

1 ml of methanolic extract was placed into a test tube containing 2.0 ml of thiobarbituric acid (0.67% w/v in 0.25N HCl, Sigma-Aldrich, Germany), 2.0 ml of trichloroacetic acid (20% w/v in 0.25N HCl, Carlo Erba reagents, Italy), and 40 µl of butylated hdroxytoluene (1% w/v in 0.25N HCl, Sigma-Aldrich, Germany). All the test tubes were mixed and placed into a thermostatic bath (Lauda-Brinkmann, Germany) at 98°C for 10 min. After heating, samples were cooled in ice and centrifuged at 3000 r.p.m. for 20 min (Rotofix 32A, Hettich Lab Technology, Germany). The absorbance of 1.5 ml of supernatant was measured at 530 nm using a spectrophotometer (6705 UV/VIS spectrophotometer, Jenway, UK). 1 ml of water was used to replace samples and referred to as the blank. For each sample, data were repeated in twice. The data was fitted with a calibration curve made with serial dilution of 1,1,3,3-tetraethoxypropane (TEP, Sigma-Aldrich, United States) in the concentration range 6.0-0.025

µmol/l (y= 0.180x-0.024 R^2=0.997). MDA content (mg/kg apples) was calculated according to the following formula:

$$TBARS\left(\frac{mgMDA}{kg\ pepper}\right) = \frac{[\text{ABSsample}] - \text{b}}{\text{m}} \text{x } 26 \text{ x } 72.06 \text{ x} \frac{1}{1000} \text{ x} \frac{1}{\text{w}}$$

where: *ABScontrol* is the absorbance of reference solution containing only TCA and peel/pulp; *ABSsample* is the absorption of the TBARS solution with sample; *w* is the weight (grams) of sample used. Two replicates were run per sample.

c. DPPH test

Briefly, 52.0 µl of the methanolic extract were incubated into a test tube containing 2.0 ml of 60 µM DPPH (Sigma-Aldrich, Germany) for 15 min in the dark at room temperature. 0.1 mg/ml of L-ascorbic acid and water were used to replace samples and referred to as positive and negative control, respectively. Then the absorbance at 515 nm was measured using a spectrophotometer (6705 UV/VIS spectrophotometer, Jenway, UK). For each sample, data were repeated in twice and the percentage of radical scavenging activity was calculated according to the formula:

$$\%DPPH = \frac{(1 - (\text{ABS control} - \text{ABS sample}))}{\text{ABS control}} \text{x}100$$

where: ABS control is the absorbance of negative control containing only DPPH and water, and ABS sample is the absorption of the DPPH solution with samples.

9.2.7. Gas-chromatography mass spectrometry-solid-phase microextraction (GC-MS/SPME) analysis of black pepper volatile compounds

After preconditioning according to the manufacturer's instructions, a SPME fiber covered by 50/30 µm Carboxen Polydimethyl Siloxane (DVB/CAR/PDMS StableFlex) (Supelco Inc., Germany) and a manual solid phase micro-extraction (SPME) holder (Supelco Inc., Germany) were used. Before headspace sampling, the fiber was exposed to GC inlet for 1 h for thermal desorption at 250°C. 1.0 g of black pepper, placed into 10 ml glass vials, were equilibrated for 10 min at 50°C. SPME fiber was exposed to each sample for 40 min. The fiber was then inserted into the injection port of the gas chromatograph for 10 min for sample desorption. GC-MS analyses were carried out with an Agilent 7890A gas chromatograph (Agilent

Technologies, Palo Alto, CA) coupled to an Agilent 5975C mass selective detector operating in an electron impact mode (ionization voltage, 70 eV). A Supelcowax 10 capillary column (length, 60 m; inside diameter, 0.32 mm; Supelco, Bellefonte, PA) was used. The temperature program was 50°C for 0 min, followed by an increase, at a rate of 5°C/min, to 230°C, and then 230°C for 10 min. The injector, interface and ion source temperatures were 250, 250, and 230°C, respectively. The mass charge ratio interval was 30 to 350 Da at a rate of 2.9 scans per s. Injection was carried out in splitless mode, and helium (flow rate, 1 ml/min) was used as the carrier gas. Compounds were identified by computer matching of mass spectral data with those of compounds contained in the Agilent Hewlett–Packard NIST 98 and Wiley vers. 6 mass spectral database. When it was possible, molecules were also identified by comparison of their retention times with those of pure compounds (Sigma-Aldrich, Milan, Italy). Quantitative data were expressed as relative percentages, *i.e.* ratio of each individual peak area and the total peak area. GC-MS/SPME data were organized into a matrix for subsequent statistical analysis.

9.2.8. Statistical analysis

Significant differences ($p<0.05$) between control and treated mean values were found by using Student's t-test and the Analysis of Variance (ANOVA) according to Tukey's HSD. Concerning GC-MS/SPME data, a Principal Component Analysis (PCA) was performed to obtain a visual overview of the differences in aroma compounds.
All statistical analysis were carried out using Statistica 8.0 (StatSoft Italy srl, Italy).

9.3. Results

9.3.1. Efficacy of gas plasma treatments for the decontamination of black pepper

In order to evaluate the efficacy of gas plasma as a superficial decontamination technology, black pepper were deliberately inoculated with different strains of foodborne pathogens, namely *Listeria monocytogenes*, *Salmonella* Enteritidis and *Escherichia coli,* and were exposed to gas plasma treatments for a various times ranging from 10 and 90 min.
Microbial data highlighted different behaviours among species and weak differences between strains. Concerning *L. monocytogenes*, both strains (56Ly and Scott A) were sensitive to GP treatments and were reduced by 1 Log unit already after 10 min. After 30 min, cell viability

decreased linearly and reached maximum inactivation levels up to 4 Log unit after the longest treatment (Figure 40).

Also *Salmonella* Enteritidis (strains 155 and 86) was sensitive to GP treatments and final inactivation degrees similar to those of *L. monocytogenes* were achieved (Figure 41) although the dynamics were slightly different. The strain 155 presented a considerable viability loss after 45 min and then cell viability was maintained rather constant thus suggesting the presence of a sub-population resistant to GP. The strain 86 seems to be slightly more sensitive than the 155 one. Indeed, after the longest treatment a reduction of about 3 Log unit was observed for the strain 86, while a 2 Log unit inactivation for the strain 155.

In general, *E. coli* (strains 555 and ATCC25922) was the most resistant species even if GP treatments determined different effects in relation to the strain. In particular the strain ATCC25922 showed a low sensitivity to GP treatments with a maximum inactivation of only 2 Log units even after longest process (Figure 42). As far as the strain 555, a cell reduction of 1.7 Log CFU/g was observed following the 30 min treatment and a maximum viability loss of about 4 Log CFU/g was recorded by increasing treatment time up to 90 min.

Moreover, data relative to the storage at room temperature highlighted that all the three species and strain tested were not able to recover the damages caused by GP treatments. Indeed their cell loads remained unchanged over storage (compared to time zero) regardless GP treatment time. In Figure 43 for example the behaviour of *Escherichia coli* 555 during the storage time is shown.

9.3.2. Effect of gas plasma treatments on compositive and quality parameters of black pepper

In order to evaluate the effects of GP treatments on some chemico-physical parameters and quality traits of black pepper, pH, Aw, the antioxidant activity (DPPH test) and the oxidation degree (TBARS test) immediately after treatments and during storage for 1 and 3 months were assessed.

9.3.2.1. Aw and pH

Aw values seem to be strongly influenced by the GP treatments. In fact the exposure of black pepper grains to GP determined a gradual increase in Aw values which was more evident for treatments longer than 30 min, thus resulting in final values of 0.65± 0.02 (Figure 44). This

increase is attributable to the water absorption by the black pepper from the hermetic chamber during GP exposure since all the treatments were carried out at RH of 60± 5%. On the other hand, this phenomenon seems to be temporary because the Aw values of the treated samples recorded during storage were similar to those of the control ones (*i.e.* about 0.55± 0.03) regardless the GP exposure time.

pH values of the black pepper rapidly decreased by 0.3-0.5 units due to GP treatments already after 10 min (Figure 45). On the other hand, by increasing the treatment time no further variations were observed, and the pH values remained close to 6.0± 0.1. Moreover, this difference between the untreated sample and those exposed to GP was unchanged within 3 months of storage.

9.3.2.2. Antioxidant activity and oxidation degree

Among the quality parameters considered, the antioxidant activity, assessed by the DPPH test, seems to be positively affected by the GP treatments (Figure 46). Indeed, an increase of this parameter from 20% to 30% already after exposures of 10-20 min was observed. These differences between the control sample and those GP-treated did not change during the whole storage time.

On the other hand, also the TBARS values significantly increased after GP treatments, particularly following the longest one. Furthermore, as expected, the oxidation process continued during storage reaching the highest levels after 3 months. However, the extent of the oxidation process during storage was overall limited and involved all samples, also including the untreated one (Figure 47).

9.3.2.3. Colour parameters

As far as colorimetric parameters, the brightness (L*) and the yellow-blue index (b*) underwent the largest modifications. The tendency to increase by increasing GP exposure time was observed for both parameters. Consequently also a significant increase in the chroma parameter to the progress of the treatment time was observed (data not shown).

9.3.3. Effect of gas plasma on volatile compounds in black pepper by (GC-MS/SPME).

In order to evaluate possible changes in the aroma of black pepper following the GP treatments, GC-MS/SPME analyses were carried out. About 70 molecules mostly belonging to the following chemical classes, monoterpene alcohols, aldehydes, monoterpenes, sesquiterpenes, which are typical of pepper and other spices, were detected (Table 8). Among these compounds, β-pinene, γ-terpinene, β-bisabolene, terpineol, α-selinene, caryophylla-4(12),8(13)-dien-5β-ol were found to be discriminant by one-way ANOVA ($p< 0.05$). PCA results showed that the control sample, particularly at the beginning of storage, was well separated by the treated ones (Figure 48). Moreover, also the samples exposed to GP for the longest time generated a separate cluster from the all the other GP treated samples. The multivariate analysis shows that the small differences found seem unable to produce a real differentiation between sample treated for time lower than 90 min. Control sample was characterized by an higher amount of α-zingiberene, terpineol, α-selinene than the treated pepper thus indicating that the GP treatments may degrade the aroma compounds. In fact some molecules, such as terpinene and terpineol, were not detectable in the samples exposed to the longest treatment.

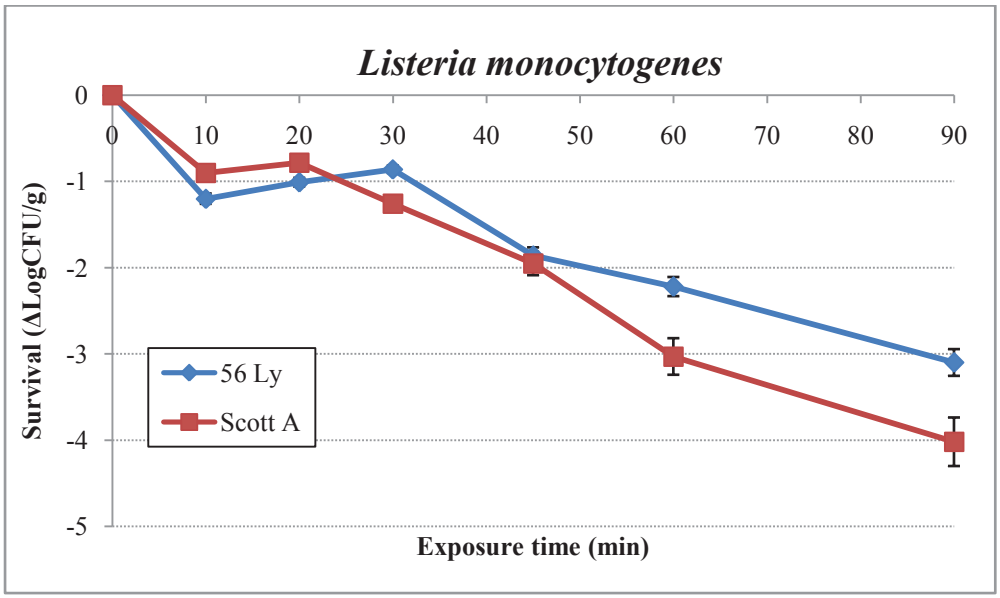

Figure 40. Survival (ΔLog CFU/g) curves of *Listeria monocytogenes* (strains 56Ly and ScottA) deliberately inoculated on black pepper exposed to GP for different treatment times.

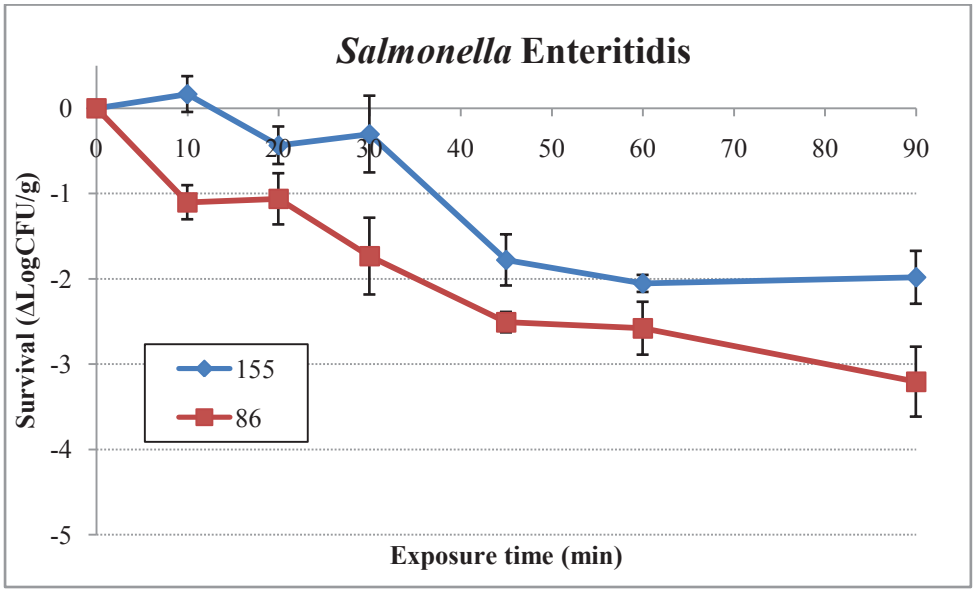

Figure 41. Survival (ΔLog CFU/g) curves of *Salmonella* Enteritidis (strains 155 and 86) deliberately inoculated on black pepper exposed to GP for different treatment times.

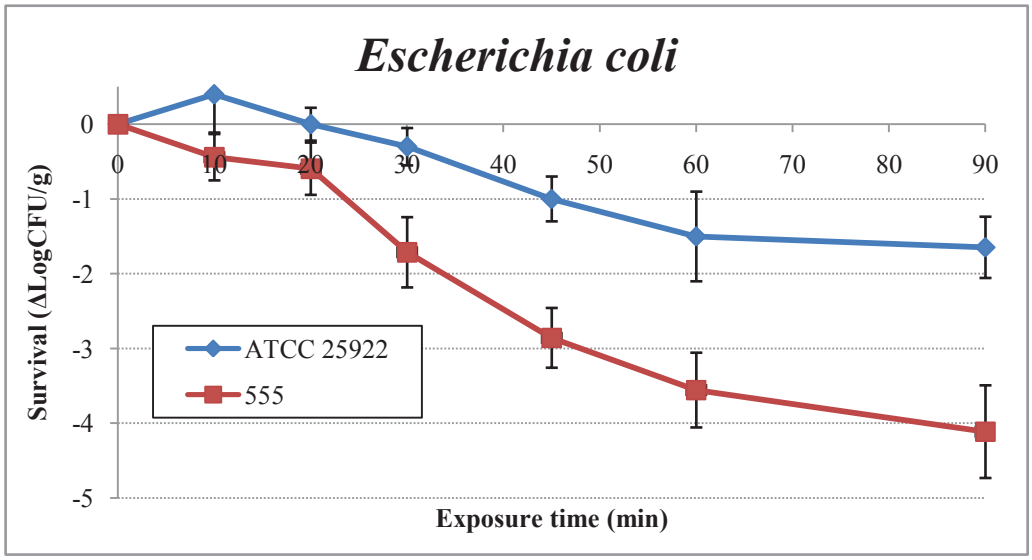

Figure 42. Survival (ΔLog CFU/g) curves of *Escherichia coli* (strains 555 and ATCC 25922) deliberately inoculated on black pepper exposed to GP for different treatment times.

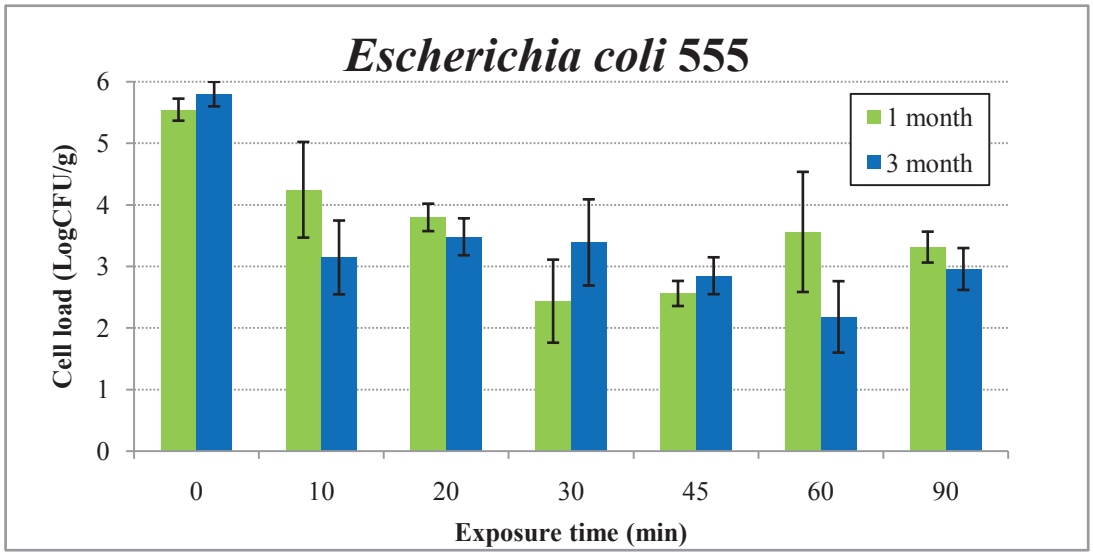

Figure 13. Counts (Log CFU/g) during storage at room temperature of the surviving cells of *Escherichia coli* 555 deliberatelly inoculated on black pepper exposed to GP treatments.

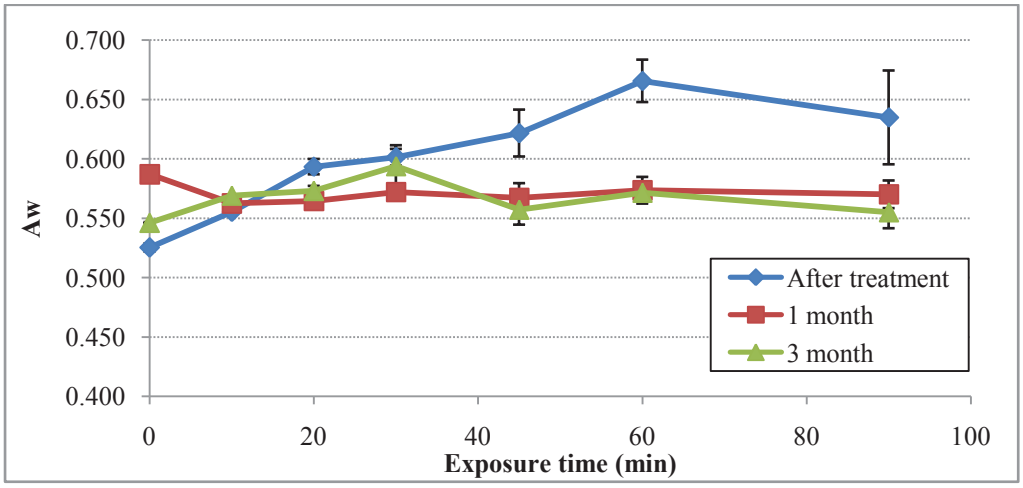

Figure 44. Evolution during storage at room temperature of water activity of black pepper exposed to GP treatments.

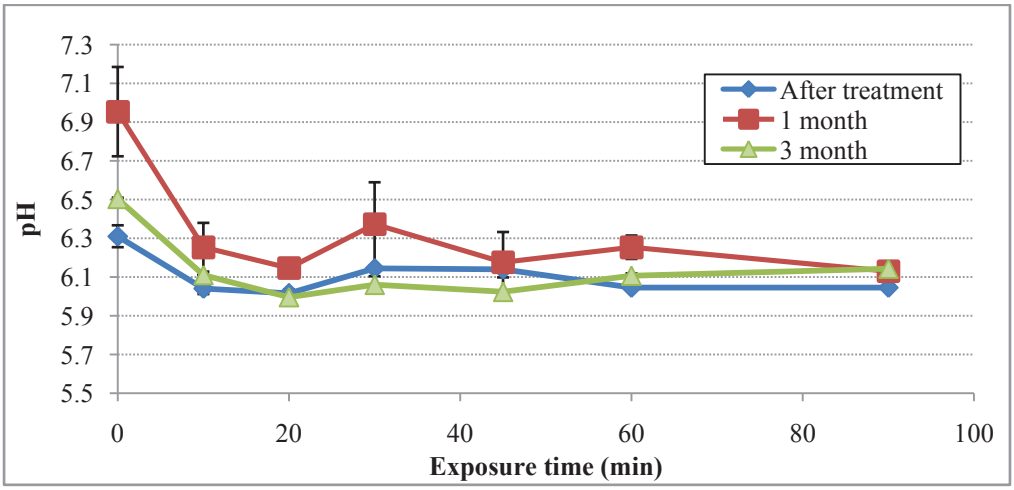

Figure 45. Evolution during storage at room temperature of pH values of black pepper exposed to GP treatments.

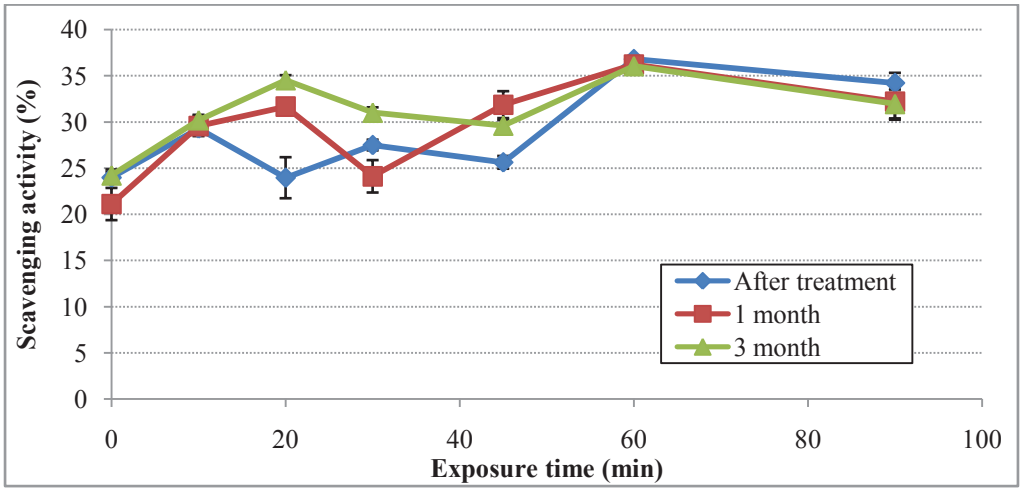

Figure 46. Evolution during storage at room temperature of antioxidant activity (DPPH assay) of black pepper exposed to GP treatments.

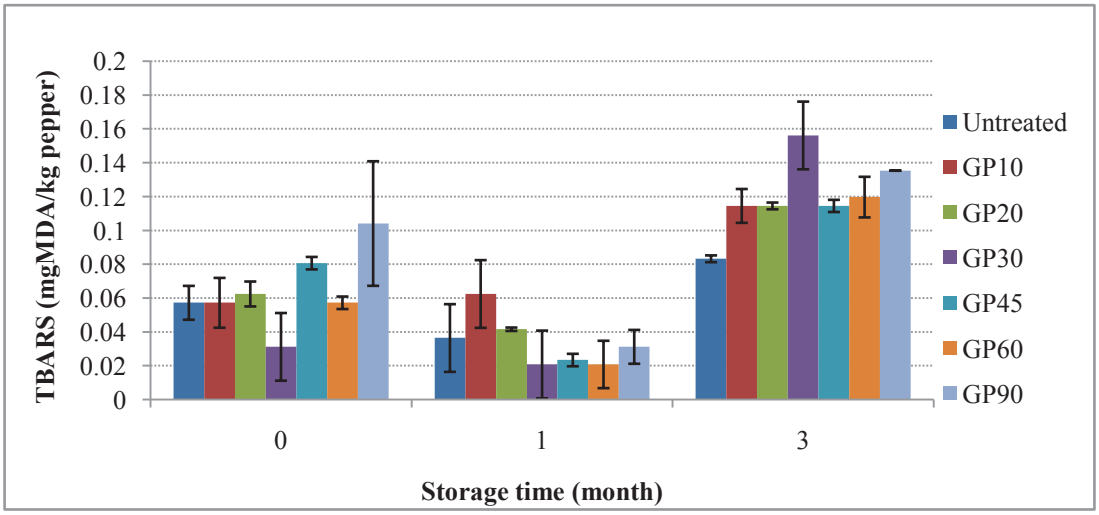

Figure 47. Evolution during storage at room temperature of oxidation degree (TBARS assay) of black pepper exposed to GP treatments.

Table 8. Volatile aroma compounds (expressed as relative %) detected in black pepper exposed to GP during and then stored at room temperature (0, 2, 3 months).

		Untreated			GP10		GP20		GP30		GP45		GP60		GP90	
	RT	0	2	3	2	3	2	3	2	3	2	3	2	3	2	3
Hexamethyl-cyclotrisiloxane	**3.301**	0.028	0.082	0.079	0.093	n.d.	0.109	0.035	0.069	0.074	0.064	0.097	0.065	0.036	0.056	0.102
Acetone	**3.467**	0.059	n.d.	0.090	0.094	n.d.	0.198	0.038	0.149	n.d.	0.087	0.099	0.086	0.103	0.084	0.173
cyclotetrasiloxane	**4.194**	0.014	n.d.	0.030	0.052	n.d.	0.043	0.016	0.048	0.025	0.041	0.052	0.042	0.018	0.054	0.053
Ethyl alcohol	**4.795**	0.050	0.215	0.088	0.075	n.d.	0.138	0.064	0.174	0.116	0.088	0.136	0.060	0.105	0.107	0.120
1R-α-Pinene	**6.523**	3.210	4.964	2.690	3.746	3.776	3.411	4.382	4.456	2.825	2.743	3.925	4.116	5.722	3.741	3.968
Camphene	**7.457**	0.077	0.042	0.037	n.d.	n.d.	0.027	0.053	n.d.	n.d.	n.d.	n.d.	0.079	n.d.	0.032	0.032
β-Pinene	**8.384**	3.007	5.185	2.862	3.929	3.957	3.853	4.162	2.504	1.567	3.364	4.373	4.847	4.348	1.307	2.581
β-Phellandrene	**8.693**	0.431	0.208	1.093	5.455	5.448	4.729	0.674	1.417	2.188	3.672	5.970	5.445	1.267	2.495	1.348
β-Thujene	**8.812**	4.062	5.933	3.591	n.d.	n.d.	n.d.	8.125	3.302	3.522	n.d.	5.485	n.d.	3.797	0.220	2.174
Sabinene	**9.028**	1.382	0.678	0.959	0.175	0.300	0.160	n.d.	3.363	n.d.	0.090	0.422	0.431	4.178	5.590	4.506
Δ3-Carene	**9.450**	1.596	2.143	0.491	1.423	1.713	0.949	0.979	0.335	0.119	0.812	1.611	0.589	n.d.	n.d.	0.257
β-myrcene	**9.660**	1.580	0.787	1.135	1.838	1.890	1.808	1.265	0.428	0.660	1.500	2.061	1.775	0.982	1.195	1.411
α-phellandrene	**9.896**	0.398	0.069	0.741	0.488	0.767	0.475	1.635	1.752	1.535	0.282	0.563	0.353	1.946	2.106	2.175
α-Terpinene	**10.283**	0.161	0.370	0.557	0.417	0.547	0.405	1.246	0.343	n.d.	0.326	0.472	0.276	0.612	0.674	0.647
D-limonene	**10.858**	3.993	2.423	5.077	8.206	8.217	8.200	12.272	0.519	n.d.	7.755	10.488	7.580	14.302	0.101	12.500
τ-Terpinene	**11.150**	1.178	0.461	5.819	2.512	2.170	2.185	3.477	9.813	12.772	2.054	2.461	0.577	2.629	11.508	3.111
γ -Terpinene	**12.086**	0.226	0.272	0.135	0.815	0.814	0.690	n.d.	n.d.	n.d.	0.659	0.884	0.494	n.d.	n.d.	n.d.
1S-α-Pinene	**12.273**	0.413	n.d.	0.326	0.034	0.151	0.047	1.092	0.907	0.484	n.d.	0.109	0.092	1.302	1.055	1.175
o-Cymene	**12.814**	0.311	0.266	0.111	0.866	0.882	0.925	1.285	n.d.	0.650	0.842	1.092	0.686	1.768	n.d.	1.561
Terpinolene	**13.133**	0.115	0.224	0.048	0.291	0.287	0.242	0.437	0.883	0.596	0.234	0.254	0.173	0.370	1.187	0.399
Nonanal	**16.258**	n.d.	n.d.	0.162	0.205	n.d.	0.280	n.d.	0.448	0.584	0.312	0.202	0.381	0.318	0.322	0.032
cis-β-Terpineol	**17.921**	0.898	1.038	0.780	0.866	0.896	1.178	1.129	0.554	1.224	1.224	0.876	1.081	0.980	1.020	1.006
δ-Elemene	**18.210**	3.091	0.882	0.791	1.047	1.304	0.782	1.829	0.764	0.641	1.034	0.475	0.847	0.972	1.054	0.998
Copaene	**18.997**	0.163	1.738	0.382	1.047	2.498	n.d.	2.715	1.745	0.396	n.d.	n.d.	0.178	n.d.	0.076	0.064
α-Cubebene	**19.026**	2.567	n.d.	1.530	2.127	n.d.	2.314	n.d.	1.694	1.305	2.344	1.724	1.687	1.664	2.070	1.736
Linalool	**19.797**	0.394	0.706	0.727	0.520	0.506	0.755	0.525	0.584	1.087	0.867	0.640	1.082	0.767	0.727	0.772
trans-β-Terpineol	**20.102**	0.935	0.832	0.679	0.647	0.795	1.005	0.927	0.555	1.037	1.072	0.701	0.780	0.893	0.994	1.048
cis-p-Menth-2-en-1-ol	**20.511**	0.138	0.318	0.169	0.190	0.404	0.255	0.225	0.739	n.d.	0.217	0.254	0.209	0.794	0.473	0.735
α-Bergamotene	**20.775**	1.853	2.704	1.896	1.356	0.935	1.494	0.942	0.888	2.836	1.845	0.951	2.267	0.897	0.857	0.625
Tricyclene	**20.938**	0.307	0.948	4.617	0.694	0.394	0.540	n.d.	1.315	3.391	0.976	n.d.	1.313	n.d.	n.d.	n.d.
2-Undecyl-pyridine	**21.229**	0.543	3.862	2.977	2.159	1.728	2.026	1.226	0.242	2.582	2.819	1.291	3.409	0.945	1.386	0.860
β-Elemene	**21.420**	2.987	1.204	1.104	1.259	1.262	1.257	1.407	1.224	0.908	1.270	0.871	0.990	0.944	1.016	0.718
p-Menth-1-en-4-ol	**21.605**	2.302	1.742	1.214	1.603	2.273	2.015	1.825	1.977	1.070	2.696	2.353	1.895	2.849	2.604	3.387
Caryophillene	**21.845**	23.712	18.028	16.552	19.877	23.050	20.781	24.063	22.883	22.623	18.589	18.497	15.836	18.209	23.450	23.213
γ-Elemene	**22.582**	1.778	1.324	1.723	1.703	2.431	1.708	0.978	1.068	0.749	1.874	1.027	1.585	1.131	2.164	1.772
trans-β-Farnesene	**22.941**	2.760	3.231	2.116	1.828	1.483	1.579	0.907	1.124	1.928	2.147	1.197	2.488	1.090	1.137	0.951
α-Caryophyllene	**23.577**	4.175	2.310	0.109	2.617	2.907	2.417	3.016	0.188	n.d.	2.617	n.d.	1.675	0.239	n.d.	n.d.
Z,Z,Z-1,5,9,9-Tetramethyl-1,4,7-cycloundecatriene	**23.698**	1.074	1.679	1.891	2.617	0.812	0.808	0.708	1.894	1.806	n.d.	1.748	0.928	2.048	2.179	1.996
cis-β-Farnesene	**23.788**	0.287	0.536	1.483	1.033	0.672	0.485	n.d.	1.663	1.642	0.611	1.267	0.218	1.442	1.224	1.286
β-Bisabolene	**24.069**	0.463	0.270	0.156	n.d.	n.d.	n.d.	n.d.	0.385	n.d.	n.d.	n.d.	n.d.	n.d.	0.451	0.519
Terpineol	**24.449**	3.360	3.414	2.141	1.171	0.733	1.851	0.998	2.223	1.321	1.800	2.138	1.885	0.604	n.d.	n.d.
α-Zingiberene	**24.622**	5.151	6.402	5.880	3.422	2.757	4.618	2.396	3.732	4.462	4.730	3.540	4.191	1.930	2.263	2.028
α-Selinene	**24.907**	2.363	1.871	1.583	1.423	1.415	0.827	1.253	1.211	0.529	1.129	0.953	0.788	0.922	2.316	2.074
Cadina-1,4-diene	**25.415**	1.897	3.991	2.143	1.226	1.570	2.328	1.683	1.649	1.295	1.466	1.686	3.349	1.651	1.887	1.426
α-Curcumene	**25.704**	2.942	3.702	3.061	2.541	2.270	2.573	1.300	1.786	3.052	3.007	1.904	3.309	1.669	1.858	1.678
β-Selinene	**26.205**	0.520	0.441	0.375	0.356	n.d.	0.485	0.171	0.393	0.283	0.495	0.216	0.304	0.391	0.453	0.580
p-Anethole	**27.176**	n.d.	0.030	0.129	0.228	n.d.	0.432	0.116	0.268	0.148	0.583	0.184	0.480	n.d.	0.212	0.254
Elixene	**27.355**	1.546	1.036	0.662	1.021	1.003	0.939	0.571	0.580	0.316	1.185	0.560	0.562	0.644	0.766	0.744
Benzyl Alcohol	**27.883**	0.713	0.028	0.141	n.d.	n.d.	n.d.	n.d.	0.211	n.d.	n.d.	n.d.	n.d.	n.d.	0.114	n.d.
Curzerene	**28.004**	0.115	0.422	0.832	0.849	0.898	0.862	0.262	0.696	0.190	0.866	0.494	0.622	0.580	0.796	0.597
Octadecanal	**29.113**	n.d.	n.d.	n.d.	0.204	n.d.	0.201	0.096	0.414	n.d.	0.502	0.217	0.815	0.234	0.198	0.137
β-Patchoulene	**29.474**	0.454	n.d.	0.404	0.125	0.352	0.462	0.225	n.d.	0.123	0.552	0.394	0.398	n.d.	n.d.	0.290
γ-Cadinene	**29.511**	0.046	0.340	0.068	0.351	n.d.	0.330	0.209	0.393	0.371	n.d.	0.273	0.117	0.228	0.417	0.063
Caryophyllene oxide	**30.796**	3.380	3.440	3.661	3.795	4.204	4.257	2.354	3.934	4.497	4.796	3.360	4.135	3.138	3.745	2.618
Nerolidol 2	**31.102**	0.315	0.371	0.331	0.305	0.170	0.279	n.d.	0.382	0.183	n.d.	n.d.	0.426	n.d.	0.220	0.127
2-Decyl-thiophene	**31.384**	0.153	0.364	0.352	0.308	0.336	0.364	0.212	0.822	0.495	0.368	0.439	0.297	0.514	0.675	0.440
α-Humulene epoxide	**31.960**	0.179	0.231	0.259	0.290	0.235	0.277	0.197	0.285	0.350	0.363	0.225	0.255	0.219	0.227	0.163
Elemol	**32.186**	0.717	1.712	3.116	1.457	1.417	1.608	0.816	2.118	3.518	1.696	1.482	2.023	1.411	1.466	1.099
y-Gurjunene 0.12 1413	**32.542**	0.161	0.274	0.245	0.210	0.266	0.294	n.d.	0.262	0.226	0.369	n.d.	0.257	n.d.	0.360	0.269
Spatulenol	**33.235**	0.078	0.196	0.188	0.147	0.176	0.225	0.103	0.301	0.131	0.278	0.136	0.152	n.d.	0.342	0.155
α-Himachalene	**33.705**	0.172	0.410	0.530	0.397	0.515	0.457	0.143	0.729	0.328	0.488	0.307	0.562	0.219	0.614	0.307
3-allyl-6-methoxyphenol	**33.868**	0.026	0.121	0.115	0.131	n.d.	0.203	n.d.	0.163	0.387	0.233	n.d.	0.294	0.362	0.189	0.122
β-Maaliene	**34.045**	0.111	0.348	0.289	0.295	0.363	0.322	0.176	0.305	0.261	0.405	0.375	0.398	0.277	0.382	0.260
α-Copaene	**34.536**	0.288	0.428	0.434	0.286	0.470	0.271	0.203	0.443	0.307	0.491	0.373	0.336	0.293	0.674	0.438
α-Bisabolol	**34.878**	0.866	0.209	2.673	1.738	2.011	1.988	0.964	0.265	2.228	2.032	1.983	2.329	1.666	0.446	0.361
β-Eudesmol	**35.359**	0.148	0.526	0.432	0.290	0.378	0.296	0.233	0.414	0.232	0.366	0.280	0.401	0.239	0.623	0.440
γ-Eudesmol	**35.179**	0.126	0.370	0.361	0.247	0.317	0.287	0.223	0.364	n.d.	0.403	0.342	0.303	0.149	0.449	0.337
Piperonal	**35.613**	0.696	0.597	1.202	2.104	1.384	1.416	0.695	2.080	1.030	2.934	2.302	3.552	1.780	1.545	1.346
N,N,2,6-Tetramethyl-4-pyridinamine	**35.804**	0.257	n.d.	0.679	0.648	0.665	0.718	0.311	0.391	n.d.	0.614	0.513	0.295	0.655	0.880	0.756
Caryophylla-4(12),8(13)-dien-5β-ol	**36.495**	0.281	0.724	0.274	0.167	0.423	0.259	0.221	0.285	0.293	0.289	0.188	0.219	0.149	0.692	0.485
Alloaromadendrene	**38.040**	0.224	0.325	0.425	0.361	0.403	0.295	0.204	0.505	0.502	0.439	0.504	0.330	0.409	0.478	0.393

RT: retention time (min); n.d.: under the detection level.

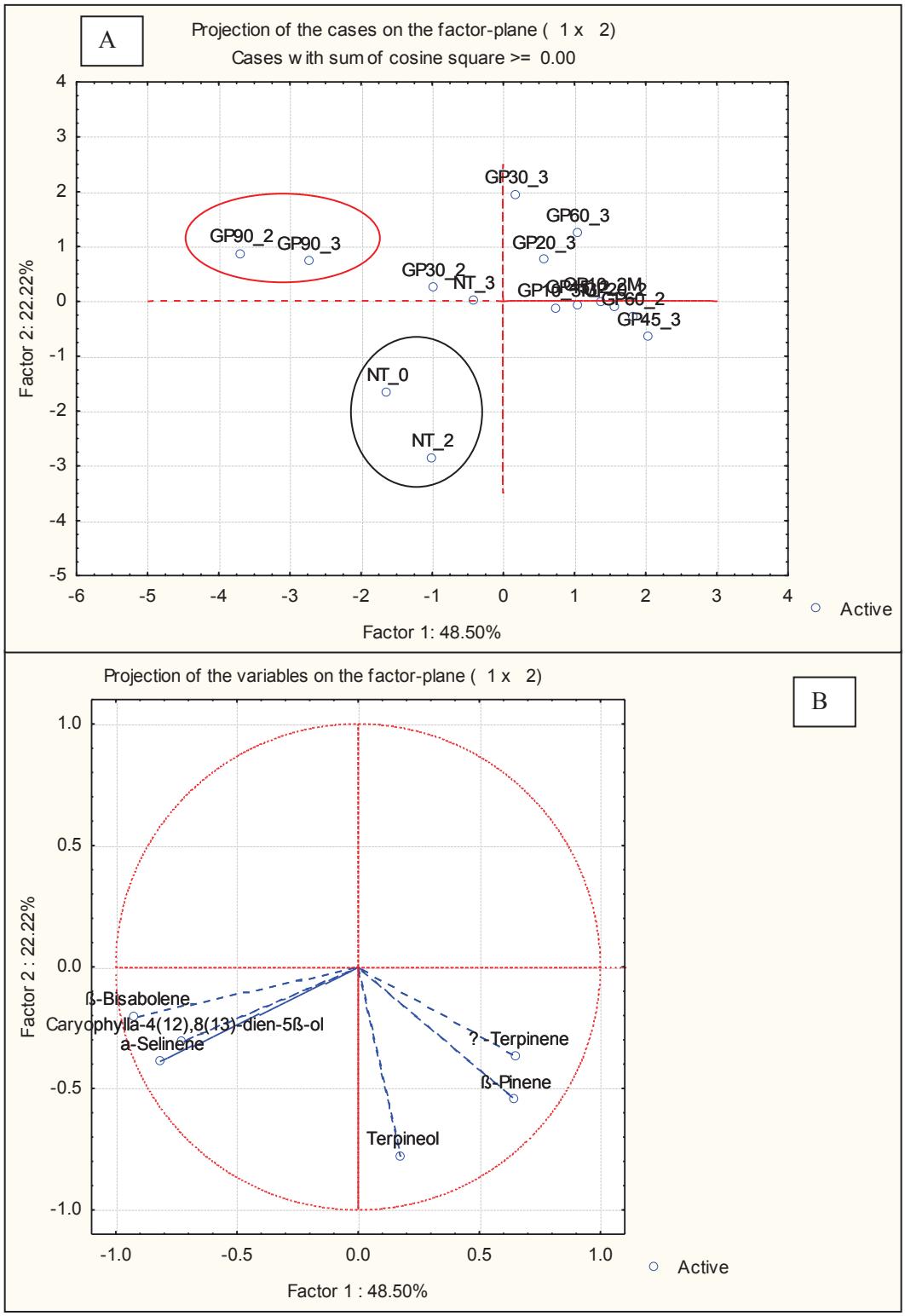

Figure 48. Projection of the cases A): pepper exposed to GP for various treatment times) and loadings B): molecules resulting discriminating by One way Anova, ($p < 0.05$) on the factor-plane (1x2). PC1 and PC2 explained 48.50% and 22.22% of the total variance, respectively.

9.4. Discussion

In this study the efficiency of gas plasma for the decontamination of black pepper deliberately contaminated with pathogens has been evaluated. The decontamination of dried products, like spices is difficult because the resistance of microorganisms, especially sporulated ones, is higher in media with low Aw compared to the behaviour of the same microorganism in a water-rich or liquid medium. Moreover, processing dry products in the presence of medium-high humidity atmospheres may negatively affect the product shelf-life due to the absorbance of water which would favour the growth of spoilage and pathogenic species.

Concerning relative gas humidity, Muranyi *et al.* (2008) reported that water molecules in the process gas significantly affect the inactivation efficiency of a cascade dielectric barrier discharge (CDBD) in air against *Aspergillus niger* and *Bacillus subtilis* spores on PET foils. In particular, an improvement of *A. niger* inactivation with increasing relative gas humidity was observed with a maximum viability loss of 3.3 Log units for 70% relative gas humidity and 7 seconds as treatment time. In contrast, the kinetics for *B. subtilis* endospores were found to slightly worsen with increasing gas humidity. By using the same device as that of the present study, Ragni *et al*. (2010) evidenced a higher sensitiveness of *Salmonella* when exposed to the gas plasma in the presence of humid atmosphere (35 vs 65% RH).

In general this technology proved to be effective for the inactivation of all pathogens examined (*i.e. Salmonella* Enteritidis, *Escherichia coli* and *Listeria monocytogenes*) when deliberately inoculated onto the product. Indeed reduction levels ranging from 2 to 4 logarithmic cycles were achieved following treatments comprised between 20 and 90 min. Among the three microbial species, *Salmonella* was the most resistant one, while *L. monocytogenes* and *E. coli* showed a similar behavior. It should be noted, however, that slight differences were observed between strains which were much more evident in the case of *E. coli*. Nevertheless, none of the tested strain was able to recover damages caused by the exposure to plasma as no proliferation over 3 months of storage was observed.

Overall, microbial results obtained in this work are in accordance with those of Hertwig *et al.* (2015) although a critical and accurate comparison cannot be made as the Authors evaluated the effects of remote gas plasma on the natural flora of black pepper, while a challenge test with pathogens was not taken into consideration. Nevertheless, in their study Hertwig *et al.* (2015) achieved complete inactivation of molds and yeasts on the surface of the black pepper seeds after 5 min of remote plasma treatment. On the other hand, within the first 30 min of

treatment a reduction of about 3 Log was observed for the total mesophilic aerobic count and for the total spore count. No further significant reduction after 60 min and 90 min treatment was measured for the total spore count. In case of the total mesophilic aerobic count the highest inactivation of about 4 Log was achieved after 60 min of remote plasma treatment. Kim *et al.* (2014) reported that the number of *A. flavus* in red pepper powder was reduced by 2.5 ± 0.3 log spores/g by a plasma treatment with nitrogen at 900 W and 667Pa for 20min. The same processing conditions resulted in the inhibition of the naturally occurring total aerobic bacteria by approximately 1 log CFU/g, while *B. cereus* spores were inhibited (3.4 ± 0.7 log spores/g reduction) only when a heat treatment at 90°C for 30 min was integrated with the plasma treatment using a helium-oxygen gas mixture at 900 W. According to Pignata *et al.* (2014), treating pistachios with pure oxygen plasma give rise to a low reduction (0.6-1.0 Log reduction/g) in the fungal population that naturally contaminated the product, while the reduction reached 2 log cycles on the pistachios that were treated for 1 min with argon/oxygen (10 : 1 v/v) plasma.

Studies regarding also the effects of cold plasma on the quality parameters of the treated products are scarce. Similarly to our result, Hertwig *et al.* (2015) did not find any significant effect on the surface color of the black pepper seeds exposed to a remote plasma up to 90 min. Furthermore, no significant change in the L*, a* and b* values were reported by Kim *et al.* (2014) for red pepper powder following cold plasma treatments both with nitrogen gas (900 W, 20 min) and a helium-oxygen mixture (900 W, 20 min).

As expected, gas plasma caused an increase in the TBARs values resulting from the generation of reactive compounds. However, immediately after treatments, only samples exposed to plasma for the longest time (90min) presented significantly higher TBARS values. On the other hand, after 3 months of storage both untreated and treated samples (regardless the treatment time) showed increased values thus suggesting that not only gas plasma, but also storage time favoured lipid peroxidation. On the other hand significant changes in TBARS values were observed by Suhaj *et al.* (2006) in ground black pepper following γ-irradiation. The results showed that the increase of reactive substances in black pepper caused by ionizing radiation was proportional to the dose of irradiation, while no effect due to storage time was detected up to 5 months following irradiation. On the contrary, the same Authors reported that irradiation resulted in a significant tendency to decreasing of DPPH radical-scavenging activity of black pepper methanolic extracts, mainly immediately after irradiation

and after the first month of irradiation. However, the differences gradually disappeared during the storage of irradiated black pepper. These results are not in accordance with those of this thesis showing that DPPH the antioxidant activity of treated pepper significantly increased following plasma treatments. The longer the exposure, the higher the DPPH values were recorded. Furthermore, these differences among untreated and treated samples were retained over 3 months of storage. The increase of the DPPH activity was unexpected as during plasma treatments reactive oxygen and reactive nitrogen species are formed, as previously evidenced by Ragni *et al*. (2010) by using the same plasma generator as the one employed for this work. It could be hypothesized that the increase in the antioxidant activity in treated samples is related to the generation of new compounds having a higher scavenging activity than those in untreated pepper. According to literature, one of the main components of black pepper oleoresins, *i.e.* piperine, has been demonstrated to protect against oxidative damages in *in vitro* studies by inhibiting or quenching free radicals and reactive oxygen species. Black pepper or piperine treatment has also been evidenced to lower lipid peroxidation *in vivo* (Srinivasan, 2007). GC-MS/SPME analysis showed that GP-treated pepper have a higher content of piperonal, compared to untreated samples, which has been reported to be one of the metabolites deriving from the biotransformation of piperine *in vivo* (Srinivasan, 2007). On the other hand, PCA analysis indicated that control and GP-treated pepper were significantly discriminated by several terpenes including β-pinene, γ-terpinene, β-bisabolene, terpineol, α-selinene, caryophylla-4(12),8(13)-dien-5β-ol, which have been widely reported to be responsible for antioxidant activity in several plants and spices and to protection against several stresses (Graßmann, 2005).

Chapter 10. Gas plasma treatments of pathogenic species in model system

10.1. Introduction

The increasingly higher consumers demand for high-quality products that are safe and inexpensive has been one of the main reasons driving researches to the development of technologies alternative to the traditional food processing. In this context several non-thermal food preservation processes have been studied and implemented with the aim of improving the level of food safety, enhancing shelf life while maintaining food quality attributes.

Among the various non-thermal technologies, atmospheric pressure plasma has attracted interest of researchers and food industry since the mid-1990s when generation of non-thermal plasma under atmospheric pressure became possible. Consequently, in the past 20 years, the total number of publications dealing with cold plasma strongly increased, proving the importance of this emerging technology (Surowsky *et al*., 2014b). In addition to the medical area, where plasma was tested to cancer cells and in the initiation of apoptosis, prion and other biomolecule inactivation, prevention of nosocomial infections or the therapy of infected wounds and sterilization of medical devices, most of the recently published papers refers to the food area. In fact several papers have been published on the effects of cold gas plasma treatments for the inactivation of yeasts, moulds and bacteria in different foods. Both natural contaminating microflora and deliberately inoculated pathogens have been studied in several foods including sliced cheese and ham (Song *et al*., 2009), beef (Kim *et al*., 2014), different fresh fruits and vegetables including apples (Niemira and Sites, 2008), cantaloupe, lettuce, mangoes and melon (Critzer *et al*., 2007; Perni *et al.,* 2008; Fernández *et al*., 2013; Baier *et al*., 2014), blueberries (Lacombe *et al*., 2015), cherry tomatoes and strawberries (Ziuzina *et al*., 2014; Misra *et al*., 2014), lettuce, carrots and tomatoes (Bermúdez-Aguirre *et al*., 2013), apple juice (Surowsky *et al*., 2014a) herbs and spices (Hertwig *et al*., 2015) including also red pepper (Kim *et al*., 2014) and many others. On the other side, several Author evaluated the efficacy of this technology by studying the inactivation curves in model systems (*i.e.* solid or liquid culture media, buffered aqueous solutions, paper disc..) as influenced by different processing parameters such as plasma exposure time, gas composition, gas flow rate, input power and relative humidity or the type of plasma application, *i.e*. direct *vs* indirect treatment (Lu *et al.,* 2014).

In principle, plasma inactivation may be induced by the heat, charged particles, electric fields, UV photons, and reactive species (*e.g.,* atomic oxygen, metastable oxygen molecules, ozone, and OH) that are commonly present in a gas discharge. Generally the properties of gas plasmas can be generally modified by varying the main parameters such as pressure, power, process gas…In fact each alteration of one of these parameters changes the whole plasma chemistry and influences, for example, the electron density, concentrations of charged or reactive particles and the amount of emitted UV radiation. These physical quantities have long been linked to the microbial inactivation. In particular, several studies have been published on plasma chemistry describing mainly the variety of reactive species, which are produced in non-thermal plasma due to collisions between electrons, atoms, and molecules (Surowsky *et al*., 2014b). It has been reported the majority of reactive species include: i) electronically and vibrationally excited oxygen O_2 and nitrogen N_2, ii) active form of oxygen molecules and atoms (reactive oxygen species, ROS) such as atomic oxygen O, singlet oxygen $^1O_2$, superoxide anion O_2^- and ozone O_3, iii) reactive nitrogen species (RNS) such as atomic nitrogen N, excited nitrogen N_2 (A), nitric oxide NO•; moreover, if humidity is present, H_2O^+, OH^- anion, OH• radical or hydrogen peroxide (H_2O_2) are also generated (Scholtz *et al*., 2015).

In particular ROS and RNS have attracted much attention due to their role in biology and consequently some implications for plasma applications to medicine and biology. Recent research suggests that ROS/RNS are significant and perhaps even central actors in the actions of antimicrobial and anti-parasite drugs, cancer therapies, wound healing therapies and therapies involving the cardiovascular system (Graves, 2012).

It has been proposed by Vatansever *et al*. (2013) that bacterial killing may occur via three different mechanisms: (i) direct permeabilization of the cell membrane or wall, leading to leakage of cellular components, including potassium, nucleic acid and proteins; (ii) critical damage of intracellular proteins from oxidative or nitrosative species; and (iii) direct chemical DNA damage. However, there are very few studies in the literature that have established their effective bactericidal capabilities and evaluated the stress response mechanisms activated by bacteria following non-thermal plasma exposure.

Therefore, in this part of the thesis some experiments have been performed in model systems, *i.e.* aqueous saline solutions and liquid culture media, in order to better understand how bacteria respond to gas plasma treatments. Specific objectives have been to:

1) evaluate whether the use of electrodes made of different materials, *i.e.* silver, brass, steel or glass, may affect the efficacy of a DBD plasma generator towards two target bacteria, *i.e. Listeria monocytogenes* (strain 56 Ly) and *Escherichia coli* (strain 555)
2) assess the effects of almost lethal gas plasma treatments, performed with the DBD device by using different electrodes, on volatile metabolites released by the treated cells of *L. monocytogenes* and *E. coli*
3) investigate modifications in cell membrane fatty acids composition and volatile molecule profiles of two strains of *L. monocytogenes*, *i.e.* strains 56Ly and ScottA, following sub-lethal gas plasma treatments
4) identify possible metabolic changes due to gas plasma treatments by analysing the proteome profiles of the strains *L. monocytogenes* 56Ly and ScottA through Multidimensional Protein Identification Technology (MudPIT)

10.2. Materials and methods

10.2.1. Bacterial strains

In order to evaluate the efficacy of two DBD equipments, one of which working with electrodes of different materials, and the response to lethal or sub-lethal gas plasma treatments, two strains of *Listeria monocytogenes* and one strain of *Escherichia coli* belonging o the Department of Agricultural and Food Sciences (DISTAL) of Alma Mater Studiorum, University of Bologna were used. In particular the following strains have been selected:

- *Listeria monocytogenes* 56Ly (a wild type isolated from pork wastewater and characterized by high resistance to both high and low temperatures) and ScottA (sierotype 4b, clinical isolate);
- *Escherichia coli* NCFB 555 (isolated from raw milk).

All the strains were cultivated in Brain Heart Infusion (BHI, Oxoid: 12.5 g/l brain infusion solids, 5.0 g/l beef heart infusion solids, 10.0 g/l proteoso peptone, 2.0 g/l glucose, 5.0 g/l sodium chloride, 2.5 g/l di-sodium phosphate) at 37°C for 24 h.

10.2.2. Inocula preparation

Samples for the experiments made with the DBD generator equipped with electrodes of different materials (which resulted in lethal treatments) were prepared as follow. 1.0 ml of overnight cultures (~9 Log CFU/ml) were transferred into 400 ml of BHI and incubated at 37°C for 12 h. Subsequently, cells were harvested by centrifugation (7000 r.p.m. for 10 min) and re-suspended into 400 ml of sterile saline solution (NaCl 0.9% w/v, Merck KGaA, Germany). 25.0 ml of the cell suspension were transferred into Petri dishes and subjected to gas plasma treatments by using the prototype shown in Figure 49.

Sample used for the experiments with the DBD equipment having only brass electrodes (which led to sub-lethal treatment conditions) were prepared as follow. 7 ml of overnight cultures (~9 Log CFU/ml) were transferred into 250 ml of BHI and incubated at 37°C for 12 h. Subsequently, 25.0 ml of the culture broth were poured into Petri dishes and exposed to gas plasma by using the prototype shown in Figure 50.

10.2.3. Gas plasma devices and treatment conditions

The prototype used to test the effects of different electrodes was built by Dr. Luigi Ragni and is shown in Figure 49. This device worked with air at atmospheric pressure and each sample was placed at about 24 mm from the discharge. The glow discharge was generated between one pair of parallel plate electrodes one of which was covered by a glass layer (5mm). In order to evaluate the effect of different materials, the electrode not covered by glass could be removed and electrodes made of different material*s, i.e.* brass, steel, silver and glass, were tested. Over the electrode a fan for driving the gas plasma towards the samples was mounted. The voltage (19.5 V) at the electrodes was produced by an high voltage transformer and power switching transistors. The electrode was inserted in an hermetic chamber with a volume of about 5.29 dm^3 and during treatments the atmosphere became saturated of water.

Petri dishes containing 25 ml of the cell suspensions (into saline solution) of *Listeria monocytogenes* 56Ly or *Escherichia coli* 555 were placed into the DBD generator and subjected to gas plasma treatments for 20, 40 and 60 min with each different pair of electrodes. At the end of each treatment microbiological analysis and the measurement of pH were carried out. Furthermore, the influence of gas plasma on volatile metabolites profile was evaluated by GC-MS/SPME analysis.

In order to better evaluate the possible role of the short-life radicalic species and the other compounds which are generated by plasma discharges by using different electrodes, cells of *Listeria monocytogenes* 56Ly were put in contact with saline solutions previously exposed to gas plasma discharges generated by the two most efficient electrodes. 25.0 ml aliquots of sterile saline solution were poured into Petri dishes and treated with the DBD device for 20, 40 and 60 min by using the brass and silver as electrodes. After each gas plasma treatment, the pellet of 25.0 ml overnight cultures (~9 Log CFU/ml) were re-suspended in the plasma-treated saline solutions. After 0, 20, 40 and 60 min from the re-suspension, the number of viable cells was evaluated by plate counting. Also pH was measured and the volatile compounds were evaluated by GC-MS/SPME analysis.

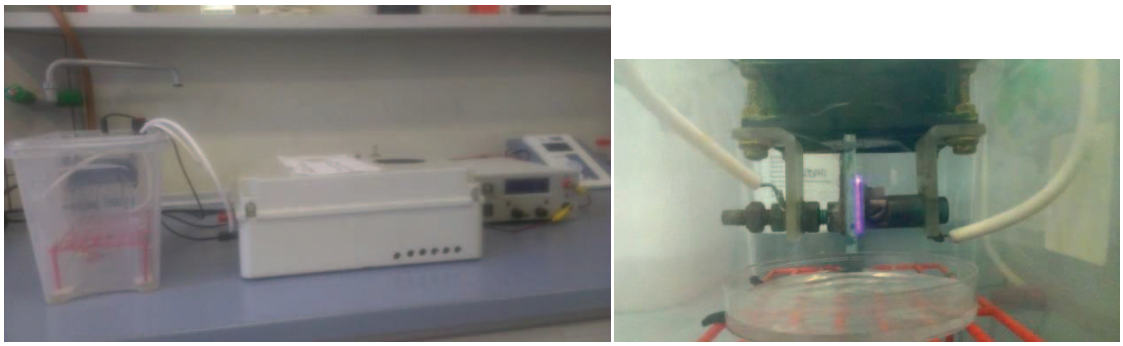

Figure 49: DBD prototype and discharge between the electrodes.

Sub-lethal GP treatments were conducted in the DBD device described by Berardinelli *et al.* (2012) and in the paragraph *7.2.1*. (Figure 50).

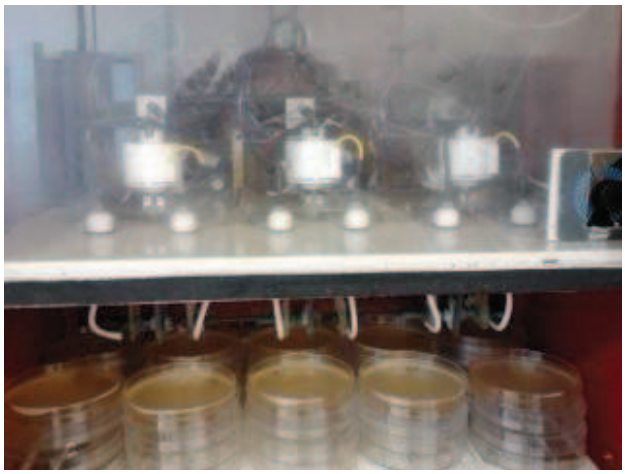

Figure 50. Petri dishes containing *L. monocytogenes* exposed to sub lethal treatments.

Petri dishes containing 25 ml of overnight cultures of *Listeria monocytogenes* strains 56Ly and ScottA were placed into the chamber of the DBD device and exposed to the gas plasma discharge for 10, 20, 30 and 60 min. After each treatment cell suspensions were recovered into sterile flasks and incubated at 37 °C. Immediately after each treatment (time 0) and after 15, 30, 60, 180, 360 min and 24 h from gas plasma exposure samples were collected in order to verify by plate countings the effects of the treatments on cell viability and the possible recovery over time. Furthermore, the influence of gas plasma on cellular fatty acids composition (by GC-MS analysis), metabolites profile (by GC-MS/SPME and ^{1}H-NMR analyses) and gene expression (by Real Time RT-PCR) were evaluated.

10.2.4. Microbial analysis

For each experiment enumeration of the surviving cells was done by surface plating, in triplicate, 100 µl of the appropriate dilutions onto non-selective BHI agar plates which were incubated at 37°C for 24 h.

10.2.5. pH measurement

1 ml of each sample was used to measure the pH with a pH meter (pH meter BasiC 20, Crison, Italy). The device were calibrated with pH 7 and pH 4 calibration buffers. The mean of three independent repetitions was calculated for each sample.

10.2.6. GC-MS/SPME analysis of volatile compounds

In order to evaluate possible changes in the volatile profile of *Listeria monocytogenes* and *Escherichia coli* cell suspensions following lethal or sub-lethal gas plasma treatments, 5 ml of each sample were collected into 10 ml glass vials immediately after each treatment and subjected to GC-MS/SPME analysis.

After preconditioning, according to the manufacturer's instructions, a SPME fiber covered by 50/30 µm Carboxen Polydimethyl Siloxane (DVB/CAR/PDMS StableFlex) (Supelco Inc., Germany) and a manual solid phase micro-extraction (SPME) holder (Supelco Inc., Germany) were used. Before headspace sampling, the fiber was exposed to GC inlet for 1h for thermal desorption at 250°C. 15 µl of 4-methyl-2-pentanol (final concentration of 30 mg/l) were added to each sample as the internal standard. Subsequently, samples were equilibrated for 10 min at 45°C. SPME fiber was exposed to each sample for 40 min. The fiber was then inserted

into the injection port of the gas chromatograph for 10 min of sample desorption. GC-MS analyses were carried out with an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA) coupled to an Agilent 5975C mass selective detector operating in an electron impact mode (ionization voltage, 70 eV). A Supelcowax 10 capillary column (length, 60 m; inside diameter, 0.32 mm; Supelco, Bellefonte, PA) was used. The temperature program was 40° C for 1 min, followed by an increase, at a rate of 4.5°C/min, to 65°C, an increase, at a rate of 10°C/min, to 230°C, and then 230°C for 17 min. Injection was carried out in splitless mode, and helium (flow rate, 1 ml/min) was used as the carrier gas. Compounds were identified by computer matching of mass spectral data with those of compounds contained in the Agilent Hewlett–Packard NIST 98 and Wiley vers. 6 mass spectral database. Quantitative data for the identified compounds were obtained by the interpolation of the relative areas versus the internal standard area and were expressed as ppmEq. All the GC-MS raw files were also converted to netCDF format via Chemstation (Agilent Technologies, USA) and subsequently processed by the XCMS toolbox (http://metlin.scripps.edu/download/). XCMS software allows automatic and simultaneous retention time alignment, matched filtration, peak detection, and peak matching. GC-MS/SPME data were organized into matrix for subsequent statistical analysis.

10.2.7. Analysis of cellular fatty acid composition

10.2.7.1. Cell lipid extraction

Cellular fatty acids (FAs) extraction and methylation were performed with the Microbial Identification System (MIS) protocol produced by Microbial ID (MIDI, Newark, DE) and described by Welch (1991).

For the extraction of cellular FAs the following reagents were prepared:

Reagent 1: 45g NaOH, 150 ml methanol, 150 ml distilled water.

Reagent 2: 54.17 ml HCl (6N), 45.83 ml methanol.

Reagent 3: 200 ml hexane, 200 ml diethyl ether.

Reagent 4: 10.8 g NaOH, 900 ml distilled water.

5.0 ml of untreated or plasma-treated culture suspensions were transferred into test tubes and centrifuged at 5000 r.p.m for 10 min (Rotofix 32A, Hettich Lab Technology, Germany). The pellets were re-suspended in 2ml of the reagent 1 and then transferred into souvirel tubes. Samples were mixed and placed into a thermostatic bath (Lauda-Brinkmann, Germany) set at 100°C for 5 min. Then they were mixed again and re-placed into the thermostatic bath for 30 min. After this treatment, samples were cooled in ice, added with 4ml of the reagent 2 and placed at 80°C for 10 min in order to favour the FAs methylation. Subsequently, samples were cooled and added with 1.5 ml of the reagent 3. The upper organic phase was collected in truncated cone-shaped tubes and added with 3ml of the reagent 4. 490 µl of the upper phase were transferred into a test tube and added with 10 µl of methyl undecanoate (final concentration of 20 mg/l, Sigma Aldrich, Germany) as an internal standard. Samples were stored to -20°C until GC/MS analysis.

10.2.7.2. Gas chromatographic analysis of cellular fatty acids

Hexane extracts of cellular FAs methyl esters were analyzed for the identification and detection of the cellular FAs according to the method previously described by Montanari *et al.,* (2013). Briefly, an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA) coupled to an Agilent 5975C mass selective detector operating in an electron impact mode (ionization voltage, 70 eV) and a capillary column (Supelco SPB-5-24036; 60m x 250µm x 0.25 µm) were used for the analysis. The injector and the detector were both held at 240°C. The temperature program was 120° C for 5 min, followed by an increase, at a rate of 4.5°C/min, to 215°C, an increase, at a rate of 0.5°C/min, to 225°C, and then 225°C for 12 min. The carrier gas was helium, with a rate of 1 ml/min, and the split was 1:10. FAs were quantified with the internal standard and identified by comparing their retention times with those of the standards the BAME mix (Bacterial Acid Methyl Ester, Supelco ,Sigma-Aldrich, Germany). Data were expressed as a relative percentage of each fatty acid and were organized into matrix for subsequent statistical analysis.

10.2.8. Gene expression

10.2.8.1. RNA extraction

500 µl of control (untreated) or plasma-treated cells were added with 1ml of RNA protect™ Bacteria Reagent (Qiagen) and centrifuged at 5000 r.p.m. for 10 min at 4°C (Himac CT15RE,

Hitachi Koki, Japan). The pellets were stored at -20°C until RNA extraction. For RNA extraction, 100 µl of lysozyme buffered solution (100 mg of lysozyme/1 ml of TE buffer) weres added to each pellet which was then incubated at 37°C for 25 min. 1.5ml of TRIzol® reagent (Invitrogen, Thermo Fisher Scientific, UK) were subsequently added to each sample and after a short stirring they were centrifuged at 12000 r.p.m for 10 min at 4°C. The supernatants were added with 200 µl of chloroform (Carlo Erba, Italy), stirred for 15 sec and placed in ice for 5 min. After this time, samples were centrifuged at 12000 r.p.m. for 15 min at 4°C and the upper phases were collected into new test tubes. An equal volume of frozen isopropanol (Carlo Erba, Italy) was added and samples were energetically shacked and placed in ice for 15 min. Then they were centrifuged at 12000 r.p.m. for 15 min at 4°C and the supernatants were removed. The pellets were washed twice with 750 µl of 70% (v/v) frozen ethanol diluted into DEPC water (Carlo Erba, Italy) and then centrifuged at 12000 r.p.m. for 10 min at 4°C. The pellets were dried on air, re-suspended in 60 µl of DEPC water and finally incubated at 55°C for 10 min. DEPC water was prepared by adding 1ml of DEPC (Diethyl pyrocarbonate, Sigma-Aldrich, Germany) to bi-distilled water, placed overnight at 37°C under stirring and then autoclaved at 121°C for 20 min.

10.2.8.2. RNA purification

Once RNA integrity had been verified, 20 µl RNA samples were treated with 5 µL DNase (7 Units, Promega, Madison WI) in 4 µl DNase buffer (Promega, WI) and incubated at 37°C for 1 h before the reaction was stopped by the addition of 4 µl of stop solution (Promega, WI) and incubation at 65°C for 10 min.

10.2.8.3. Reverse transcription

10 µl of RNA were used to synthesized cDNA from the mRNA using 2 µl of random primers (Promega, WI). Samples were incubated at 70°C for 5 min for RNA denaturation, placed in ice for 5 min to let the primer anneal to the RNA and then added with 2 µl of deoxynucleoside triphosphates 2.5 mM (dNTPs, Takara, WI), 4 µl reverse transcription (RT) buffer (Promega, WI), 0.5 µl Moloney murine leukemia virus (MMLV, 0.5 Units) reverse transcriptase (Promega, WI). Reverse transcriptase reaction was performed according to the following conditions: 15 min at 25°C, 1 h at 42°C and finally 70°C for 15 min.

10.2.8.4. Real Time-PCR

The evaluation of the expression of some selected genes was performed fluorometrically by using Syber ® Premix Ex TaqTM (Takara, WI) and a Rotor Gene 6000 (Corbett, Germany) equipment. A set of genes was selected as identified to have an important role in general stress response and virulence (Table 9).

Table 9. List of genes used in the experimental plan

Gene	Response	Gene function	Annealing temperature (°C)
16S rRNA	Housekeeping gene	Higly conserved region in bacterial genome	55
rpoB	Housekeeping gene	DNA-directed RNA polymerase, beta subunit/140 kD subunit	60
sigB	General stress	σB network	56.5
hrcA	General stress	Heat inducible transcription repressor	58.5
dnaK	General stress	Molecular chaperone	60
cspB	General stress	Cold shock proteins	56.5
cspD	General stress	Cold shock proteins	51.6
hly	Virulence	Listeriosin O	58.5
fri	Oxidative stress	DNA-binding ferritin-like protein (oxidative damage protectant)	56.3
kat	Oxidative stress	Catalase	58.5
pdhD	Metabolism	Pyruvate dehydrogenase complex	58.4
pgm	Metabolism	Phosphoglyceromutase	54

2.0 µl of cDNA were mixed with 12.5 µl of Syber ® Premix Ex TaqTM (Takara, WI), 1.5 µl of each forward and reverse primers (100 µM) and 7.5 µl of sterile water. The temperature program was 50° C for 15 min, 95°C for 15min, followed by the amplification cycles: 94°C for 15 sec, annealing temperature (Table 9) for 25 sec and then 72°C for 30 sec. Once concluded all cycles, a temperature of 72°C was held for 5 min followed by a melt step in which temperature increased from 60°C to 95°C, at a rate of 0.2°C/sec. Serial dilutions of *Listeria monocytogenes* (56 Ly and ScottA) DNA extracted with the ISTAGENE Matrix Kit (Bio-Rad, USA) from 1 ml of pure cultures were used to build calibration curves of the selected primers and calculate their efficiency with the following formula:

$$Efficiency\ E = 10^{-1/slope}$$

Samples were examined for differences in gene expression by relative quantification according to Pfaffl (2001) by using the following formula:

$$Relative\ gene\ expression\ RGE = \frac{\mathrm{E}^{(\mathrm{Control-Sample})\text{gene of interest}}}{\mathrm{E}^{(\mathrm{Control-Sample})\text{housekeeping gene}}}$$

where “control” refers to *Listeria monocytogenes* untreated cells, “sample” are *Listeria monocytogenes* cells exposed to gas plasma for different times, *i.e.* 10, 20, 30 or 60 min to gas plasma. E is the efficiency calculated with the formula above reported. Both *16S rRNA* and *rpoB* were used as the housekeeping genes.

10.2.9. ^{1}H nuclear magnetic resonance (NMR) spectroscopy

10.2.9.1. Sample preparation

1ml of control (untreated) or plasma-treated cell culture was placed into test tubes and centrifuged at 5000 r.p.m. for 10 min. 800 µl of each supernatant was thoroughly mixed with 100 µl of NMR buffer (1M Na_2HPO_4, 1M NaH_2PO_4, 2mM NaN_3, 10mM sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4, D_2O, pH 7.0) and stored overnight at -20°C. Before transferring 700 µl of each sample into 5 mm NMR tubes, they were re-centrifuged at 15000 r.p.m. for 10 min to remove any solid debris.

10.2.9.2. ^{1}H-NMR data acquisition

All ^{1}H-NMR spectra were recorded at 298 K using a Bruker US+ Avance III spectrometer operating at 600.13 MHz (Bruker BioSpin, Karlsruhe, Germany). To avoid the presence of broad signals arising from slowly tumbling molecules, a T2 filter of 400 echoes, separated by an echo time of 400 ms, was applied. The signals were assigned by comparing their chemical shift and multiplicity with Chenomx software data bank (Chenomx Inc., Canada, ver 8.02).

10.2.10. Proteomic profile analysis

10.2.10.1. Protein extraction

20 ml the cell suspensions of *Listeria monocytogenes* strains 56Ly and ScottA were transferred into Petri dishes and exposed for 60 min to gas plasma generated by the DBD device described in the paragraph *7.2.1.*.

Immediately after GP exposure, 20 ml of treated cells were centrifuged at 6000 r.p.m. for 5 min (Rotofix 32A, Hettich Lab Technology, Germany) and the pellets were washed three times with 2ml of PBS pH 7.4 (Oxoid, UK). Cells were re-suspended in 1 ml of buffer (50 mM tris-HCl pH 7.5,) with 10 µl of Halt™ Protease Inhibitor Cockatail (Thermo Scientific, USA) and then sonicated (Vibra cell CV188, Sonics and Materials, USA) six times for 1 min

each at intervals of 1 min on ice. Samples were centrifuged at 13000 r.p.m. for 15 min and supernatants were frozen at −80 °C, and finally lyophilized.

10.2.10.2. MudPIT and Mass Spectrometry

The proteomic profile of the lyophilized protein extracts of the two strains of *Listeria monocytogenes* 56 Ly and ScottA before and after exposure to gas plasma for 60 min was analyzed in collaboration with Dr. Lucélia Santi of the Scripps Research Institute of San Diego (USA) through Multidimensional Protein Identification Technology (MudPIT). Samples were prepared and analyzed according to Santi *et al.* (2014).

10.2.11. Statistical analysis

Significant differences ($p<0.05$) between control and treated mean values were found by using Student's t-test and the Analysis of Variance (ANOVA) according to Tukey's HSD. All the analyses were carried out using Statistica 8.0 (StatSoft Italy srl, Italy).

In order to identify differences between samples, GC/MS/SPME and ^{1}H-NMR data were analyzed using a multivariate technique of Canonical Analysis of Principal Coordinates (CAP) using the CAP program (Anderson, 2004). CAP provides a constrained ordination that finds the axis that best discriminates among *a priori* groups (treatments). Binary data were subjected to Principal Coordinate Analysis (PCA) using an Euclidean distance matrix. Canonical Analysis of the Principal Coordinates was subsequently conducted (Ndagijimana *et al.,* 2009). The number of PCO dimensions used for canonical analysis was automatically selected by the program to optimize the overall variability used. The power of classification was tested through a leave-one-out procedure and a traditional canonical analysis on the first PCO was carried out. The total variance obtained in PCO used to perform the CAP was higher than 50% for all the samples. Significance testing was carried out using 999 permutations (Anderson, 2004). The correctly performed permutation test assigns ca. 90% of the samples.

10.3. Results

10.3.1. Effects of different electrodes on E. coli and L. monocytogenes by direct exposure to gas plasma

In order to assess the potential of gas plasma two pathogens such as *E. coli* 555 (Gram-negative bacterium) and *L. monocytogenes* 56Ly (Gram- positive) were examined.
Cell suspensions of both microorganisms were exposed to GP treatments for different times ranging from 20 and 60 min, using a DBD prototype. In particular treatments were performed by using electrodes made of different materials, and namely of brass, steel, glass or silver.
The effects of the treatments on *L. monocytogenes* and *E. coli* are shown in the Figures 51 and 52, respectively. The results evidenced that the material had a significant effect giving rise to different inactivation dynamics. In particular, as far as *L. monocytogenes* Ly56, glass was found to be less efficient than the other materials determining a maximum decrease of about 5 Log units after the longest treatment (60 min). On the contrary, in the presence of the silver electrode, an almost complete inactivation (final cell load 0.93±0.80) was obtained after 1 h of treatment. By analyzing the inactivation curve of the cells treated with the brass electrode, it seems that a straight line is followed. On the other hand, when *Listeria monocytogenes* was exposed to plasma generated with the silver and steel electrodes, during the initial period, both the death curves are linear and death rates are lower than the final step, where cell viability related to the silver electrode considerably decreased reaching an inactivation level lower than the brass one (7 *vs* 8 Log reductions).
As previously observed in real systems examined in this work (*e.g.* soybean sprouts and to a lesser extent black pepper), *E. coli* appeared to be more resistant to GP treatments than *L. monocytogenes.* Moreover, the effects played by the type of electrode was evident only after the longest treatment where it is possible to detect differences in terms of the microbial inactivation. More precisely, cell viabilities were reduced approximately of about 3-4 Log units by using the electrodes made of brass, steel and silver, whereas in the presence of the glass electrode the effectiveness of the plasma treatment increased up to 6 Log units.
After all the treatments, the pH of cell suspensions was measured. Results showed in Figures 53 and 54, relative to GP exposure of *L. monocytogenes* and *E. coli*, respectively, suggested that the differences between microorganisms in their inactivation curves in relation to the electrodes material were not attributable to the reduction of pH. In fact the dynamics of this

parameters were quite similar regardless the various electrodes considered, and decreased down to 3-3.5 and 2.5 following 20 and 60 min of treatment, respectively. Therefore, the effect of the various treatments could be due to the generation of different reactive species.

10.3.2. Effects of different electrodes on L. monocytogenes by indirect exposure to gas plasma

In order to better evaluate the role of the pH reduction on the cell viability, cells of *Listeria monocytogenes* were re-suspended and stored at 37°C until 60 min into saline solutions previously treated with GP for various times ranging from 20 and 60 min. For this experiment only silver and brass electrodes were considered. Data relative to the silver electrodes showed that *Listeria monocytogenes* was not affected by the re-suspension in saline solution previously treated for the shortest time as no change in cell viability was observed during all the storage time (Figure 55). On the other hand when cells were transferred into the saline solutions treated for 40 min and 60 min, a gradual decrease in cell viability was observed, especially for that exposed to plasma for longest time. Moreover, the re-suspension into the solution treated for 60 min determined an immediate reduction of about 2 log units which was not observed for the other samples. Data relative to pH showed that this parameter decreased by increasing the treatment time (as observed in the previous experiment and reported in the paragraph *10.3.1*), but did not change during the storage (Table 10). A comparison between results relative to the changes in viability of *L. monocytogenes* and pH could suggest that an accumulation of some reactive species, which did not influence the pH, but remain stable into the plasma-treated solution thus affecting the microorganism over time, have occurred.
Concerning brass, a similar behaviour was observed, but with a lower extent in the final inactivation level (Figure 56). In particular comparing the results obtained during 60 min of storage with the saline solutions treated for the longest time, the final cell loads of *Listeria monocytogenes* were about 4 Log CFU/ml and 2 Log CFU/ml for the solutions treated with the brass and silver electrodes, respectively. The pH values recorded with solutions relative to the brass electrode were similar to those obtained with the silver one (Table 10).

10.3.3. Volatile metabolites released by L. monocytogenes and E. coli following exposure to gas plasma with different electrodes

Concerning the release of volatile metabolites analyzed by GC-MS/SPME, about 50 molecules belonging to different chemical groups including aldehydes, ketones, alcohols, short chain fatty acids and phenols were detected for both microorganisms.

As far as *L. monocytogenes* 56Ly, the direct exposure to gas plasma determined some qualitative differences between the control and the treated cells regardless the exposure time and the material used for the electrodes. Indeed the untreated samples were found lacking some aldheydes (*e.g.* octanal and octadecanal) and short chain fatty acids (*e.g.* eptanoic, octanoic, n-decanoic and dodecanoic acids) which increased by increasing the exposure time to gas plasma (Table 11). Moreover, other molecules, including nonanal, acetophenone and 2,4-bis(1-methylethyl)-phenol, which were present in the control samples at low levels, rapidly increased already after the shortest treatments. CAP analysis showed that the control samples can be completely separated from the treated ones (Figure 57). The molecules discriminating the untreated samples were some ketones (*e.g.* 5-methyl- 3-hexanone, 4-methyl-3-penten-2-one) and 4-ethyl-benzaldehyde. Among the treated samples, those exposed to plasma in the presence of the glass electrode generated an isolated cluster more distant to all the other samples for which it was not possible to recognized distinct groups. The molecules discriminating the samples exposed to glass electrode plasma treatment were 2-pentyl-furan, 2-methyl-3-decen-5-one, (e)-2-decenal, heptanoic acid, octanoic acid, m-tert-butyl- phenol, which showed higher amounts than in the samples treated with the other electrodes.

E. coli 555 presented a different profile in volatile molecules than *L. monocytogenes* (Table 12). The control samples were characterized by a huge amount of indole, which was totally absent in the treated samples. On the contrary, ethyl alcohol, 2-ethyl-1-hexanol, ethyl acetate, diethyl ester of butanedioic acid, were significantly ($p < 0.05$) associated to the treated cells.

As observed for *Listeria monocytogenes*, CAP analysis completely discriminated the control samples from the treated ones (Figure 58). Among the GP treated samples, those exposed to the discharge generated by the brass electrode were well discriminated from the other ones due to high amounts of some benzaldehydes, *e.g.* 4-ethyl-benzaldehyde and 4-methyl-benzaldehyde, and an ester of thiophene.

Also volatile metabolites of *Listeria monocytogenes* re-suspended in saline solutions previously treated with GP (indirect exposure to gas plasma) were analyzed by GC-MS/SPME (Tables 13 and 14). CAP analysis showed that all the samples, *i.e.* the untreated and the treated ones relative to the brass and silver electrodes, generated well separated clusters thus suggesting that the material of the electrodes could determine different response mechanisms resulting in different volatile profiles (Figure 59). 4-methyl-3-penten-2-one, 4-(1,1,3,3-tetramethylbutyl)-phenol and 2-heptanol were the molecules significantly associated to the control sample. Short chain fatty acids, *e.g.* nonanoic and octanoic acids, acetophenone, benzaldehyde and phenols characterized the samples treated with silver, while those exposed to brass-related treatments were discriminated due to diethyl ester of butanedioic acid, 2-chloro-4-(1,1-dimethylpropyl)- phenol, benzaldehyde and octadecanal.

10.3.4. Effect of sub-lethal gas plasma treatments on the stress response in Listeria monocytogenes

Although the inactivation of several pathogens including *Listeria monocytogenes* by atmospheric gas plasma has been reported for different food systems, few data considering the possible cellular targets and the response mechanisms as a result of sub-lethal gas plasma treatments have been published. In this work the effects of sub-lethal GP treatments on the viability of two strains of *Listeria monocytogenes* (56Ly, ScottA) were evaluated. Also changes in cellular fatty acids and volatile metabolites, analysed by gaschromatographic and ^{1}H-NMR techniques, were detected for gas plasma treated cells and compared to control ones. Finally an investigation on the expression of some selected genes by using Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) was performed.

10.3.4.1. Viability of Listeria monocytogenes after sub-lethal gas plasma treatments

In order to produce sub-lethal treatments by reduce the efficacy of GP treatments, cell suspensions in BHI broth were used. In fact nutrients of the medium are targets of the reactive species, which are generated during gas plasma treatments, thus protecting bacterial cells from strong damages and direct interactions.

Data relative to cell counts immediately after GP treatments and during the following 24 h showed that sub-lethal processing conditions had been effectively chosen for both strains

(Figures 60 and 61). Indeed no significant changes in cell viability were observed for all the exposure times thus suggesting that only reversible damages may have occurred.

10.3.4.2. Effect of gas plasma treatments on cellular fatty acids composition

To assess whether sub-lethal GP treatments affected cellular membranes, cellular fatty acids composition under the different processing conditions was analysed. Eleven fatty acids were detected and quantified for both strains of *L. monocytogenes*. However, only six FAs were found discriminating by one-way ANOVA ($p<0.05$). Concerning *L. monocytogenes* 56Ly, in the control cells the predominant FAs were C16:0 (33%), *a*C15:0 (22%), *i*C15:0 (10.5%), C18:0 (15.8%) and *i*C17:0 (9.2%). Following the exposure to gas plasma the amounts of C14:0, C16:0 and *i*C17:0 were significantly reduced compared to the untreated cells. Moreover C18:0 tended to disappear (<2%) by increasing gas plasma treatment time. On the contrary, *i*C15:0 and *a*C15:0, which accounted for 15 and 30% of the total FAs in the control samples respectively, increased by 3 and 10% already after 10 min of treatment. In Figure 62 the main changes in fatty acids composition of *L. monocytogenes* 56Ly are shown. A similar behaviour was observed for the strain ScottA (Figure 63). This finding is in agreement with scientific literature reporting that *Listeria monocytogenes* increases the amount of branched FAs as a response to different environmental stresses (Giotis *et al.*, 2007; Gianotti *et al*., 2008). This result suggests that although there were no reductions in cell viability, gas plasma treatments resulted in a stress for *Listeria monocytogenes*. Some studies demonstrated that the modulation of such branched-chain FAs also regulates bacterial virulence (Sun *et al.,*2010*).*

10.3.4.3. Effect of gas plasma treatments on metabolites released by L. monocytogenes

Concerning the results of volatile metabolites analyzed by GC-MS/SPME, about 70 molecules belonging to different chemical groups including aldehydes, ketones, alcohols, short chain fatty acids and pyrazine were detected for both strains (Tables 15-20). In contrast to what observed after the exposure of *L. monocytogenes* to lethal GP treatments (paragraph *10.3.3.*), a sub-lethal stress did not determine any qualitative differences regardless the treatment time (Tables 15-17). However, CAP analysis showed that the control samples can be completely separated from the treated ones for both strains (Figures 64 and 65) especially

from the samples treated for the longest time (GP60). In particular, as far as *L. monocytogenes* 56Ly, the molecules discriminating the GP60 samples were short chain fatty acids (*e.g.* 3-methyl-butanoic acid, dodecanoic acid, nonanoic acid, octanoic acid, heptanoic acid, acetic acid etc.) and ketones (*e.g.* acetophenone, 2,3-butanedione, 3-hydroxy-2-butanone, 2-hexadecanone etc.), while higher amounts of aldehydes (*e.g.* 3-methyl-butanal, pentanal, 3-furaldehyde), alcohols *(e.g.* ethanol) and pyrazine (*e.g.* pyrazine, ethyl-pyrazine, 2,6-dimethyl-pyrazine etc.) characterized the untreated samples (Figure 66). The molecules discriminating the GP60 samples were found also in the sample exposed to lowest treatment time, although their content was lower compared to the longest treatment. In particular acetophenone and 3-methylbutanoic acid were significantly ($p < 0.05$) associated to the treated cells. The content of the former strongly increased by increasing the gas plasma exposure time. Moreover, its accumulation was higher until half an hour following the treatment.

Significantly ($p < 0.05$) higher concentrations of ethyl alcohol, furaldehyde and butanoic acid characterized the untreated cells. In particular the release of ethanol, which attained mean levels of 3.5-4.0 ppm Eq in the controls, decreased down to 1.5-1.8 ppm Eq after the longest treatments. This results was also confirmed by ^{1}H-NMR analysis. Also a strong reduction in the treated cells of acetic acid, which presented the highest levels in the controls, could suggest a metabolic slowdown induced by gas plasma. Moreover several pyrazines were detected in control samples compared to the gas plasma treated ones. In general pyrazines are reported to be produced by several microorganisms although knowledge about their biosynthesis is limited.

As far as the strain ScottA, a behaviour similar to that of *L. monocytogenes* 56Ly was observed (Tables 18-20). In fact the same chemical groups characterized and discriminated the untreated samples (*i.e.* pyrazines, adheydes and alcohols) and treated ones (*i.e* ketones, short fatty acids). However, some specific molecules were slightly different (Figure 67). As example, cells exposed to the longest treatment were found rich in 4-methyl-3-penten-2-one, 1-hydroxy-2-propanone, 2,6-dimethyl-4-heptanone. Moreover, some esters (*i.e.* esters of the butanoic, hexadecanoic and sulfurous acids) characterized the treated cells of *Listeria monocytogenes* ScottA, while a detectable level of these molecules was not found for the strain 56Ly thus indicating a strain variability in the stress response mechanism to sub-lethal gas plasma conditions.

Among the molecules detected with ^{1}H-NMR analysis, the amino acids resulted very interesting (Tables 21-26). As far as *L. monocytogenes* 56Ly, methionine and tryptophan decreased by increasing the exposure times, while phenylalanine and 4-aminobutyrate increased (Tables 21-23). Different results were obtained for the strain ScottA (Tables 24-26). In fact no differences between untreated and treated samples were observed for phenylalanine, while tyrosine seemed to increase after GP exposure. In both strains riboflavin and sarcosine increased. Other molecules detected in treated cells of *L. monocytogenes* 56Ly were hypoxanthine and inosine. Lactate seemed to increase only following the shortest treatments (10 and 20 min), while orotate increased immediately after all the treatments although its content returned to similar values as those of the untreated samples after the first hour of storage. As far as *L. monocytogenes* ScottA an interesting increase in acetate and orotate levels was observed. The latter did not follow the same trend detected for the strain 56Ly. The amount of pyruvate was lower in the treated cells compared to the control ones, and during the storage it decreased more rapidly than in the control samples. CAP analysis of the ^{1}H-NMR data confirmed the outcomes observed for the GC-MS/SPME analysis, *i.e.* that the control samples were completely separated from the treated ones especially from the samples treated for the longest time (Figures 68 and 69).

10.3.4.4. Gene expression in L. monocytogenes

In order to assess the impact of gas plasma treatments on the expression of some genes related to general stress response and metabolism, their relative gene expression (RGE) was calculated, considering as a control samples the untreated ones (Tables 27 and 28). The results obtained for both strains showed that in general the exposure to gas plasma gave rise to slight changes in RGE values for all the selected genes. As far as *L. monocytogenes* 56 Ly, in general a small reduction was detected for all the genes following the shortest treatments, while a modest up-regulation was observed when cells were exposed to gas plasma for 30 and 60 min. The gene *fri* encoding a protein involved in oxidative stress resulted down-regulated in samples GP20 and GP30, while it was slightly up-regulated after the longest treatment. The regulation of *pdhD* coding pyruvate dehydrogenase enzyme (Pdh) complex, which is involved in acetyl CoA formation from pyruvate in the aerobic metabolism, diminished by the increasing the GP exposure time.

10.3.5. Proteomic profile of Listeria monocytogenes following gas plasma treatments

In order to evaluate whether exposure to gas plasma affect proteomic profile of *L. monocytogenes*, a comparative analysis of protein expression of untreated and gas plasma treated (for 60 min) cells of both the strains 56 Ly and ScottA was performed. In Figure 70 and in Tables 29 and 32 the distribution of the proteins and the list of those that were found differentially expressed for both strains and processing conditions are reported.

As far as *L. monocytogenes* strain 56 Ly, a total of 1.008 proteins were identified from the control cells and 975 from the treated ones (Figure 70). The majority of these proteins were common to both conditions (871), while 137 (12.32%) were identified only in the control cells, and 104 (9.35%) were exclusive to cells exposed to gas plasma. Among the proteins uniquely identified in treated cells (Table 31), proteins related to glutamate decarboxylase, propanediol dehydratase, succinate-semialdehyde dehdyrogenase and oxidoreductase were found, while several proteins related to the phosphotransferase (PTS) system for sugar transport were uniquely related to the control cells (Table 30). Concerning those that were differentially regulated, a total of 92 proteins were identified up- and 17 down-regulated in control cells of the strain 56 Ly compared to the treated ones. In particular, those that were up-regulated more than 1.5 fold included several proteins involved in transport mechanisms, energetic metabolism, proteolysis (peptidases) and flagella in addition to ribosomial proteins. On the other hand, those down-regulated in control cells (*i.e.* expressed at higher levels following the treatment) were mainly related to related to oxidation−reduction (oxidoreductase), response to stress (cold shock proteins) and proteolysis (peptidases).

A comparison between the two strains of *L. monocytogenes* clearly evidenced that they are characterized by different responses. In fact, for the strain ScottA a significantly higher number of proteins were detected only in the untreated cells (259= 23.60%) compared to the strain 56 Ly (137), while a limited number of proteins (35=3.19% *vs* 137) were found following the gas plasma treatment (Tables 33 and 34). Unique proteins of the control cells were mainly related to the energy metabolism and aminoacid biosynthesis, while several (11 out of 35) hypothetical proteins were unique for the treated cells. Most of the proteins that were differentially expressed in the control cells were involved in the energetic metabolism, while only 3 proteins were significantly associated to the treated cells and namely proteins related to PTS galactitol transporter subunit IIB, glycosyl hydrolase and ATP-dependent Clp protease (ATP-binding protein).

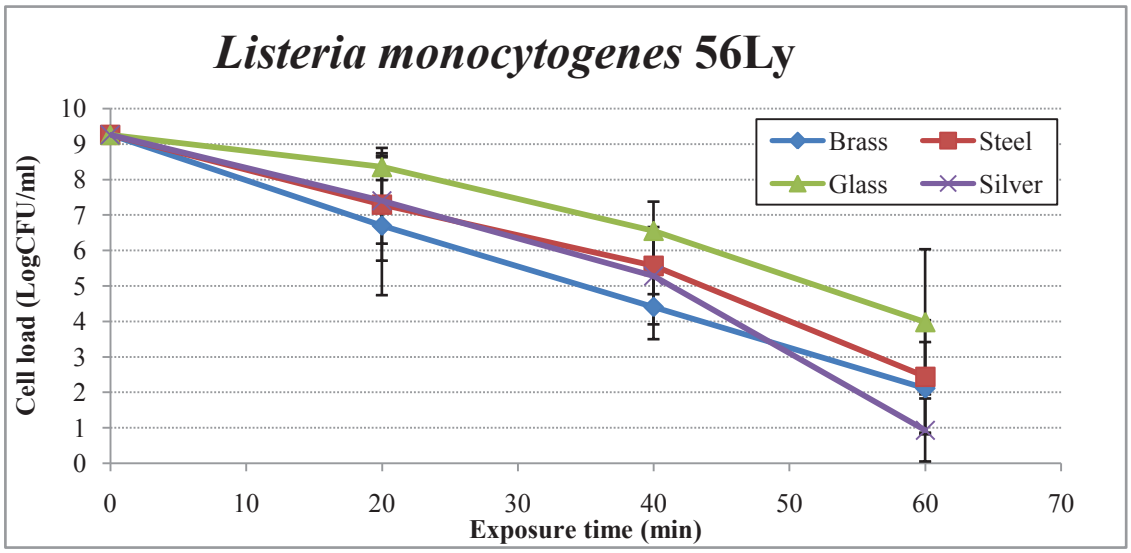

Figure 51. Cell counts (Log CFU/ml) of the surviving cells of *Listeria monocytogenes* 56Ly (into saline solutions) in relation to the gas plasma treatment conditions: time - 20, 40, 60 min - and electrode material- steel, silver, glass and brass.

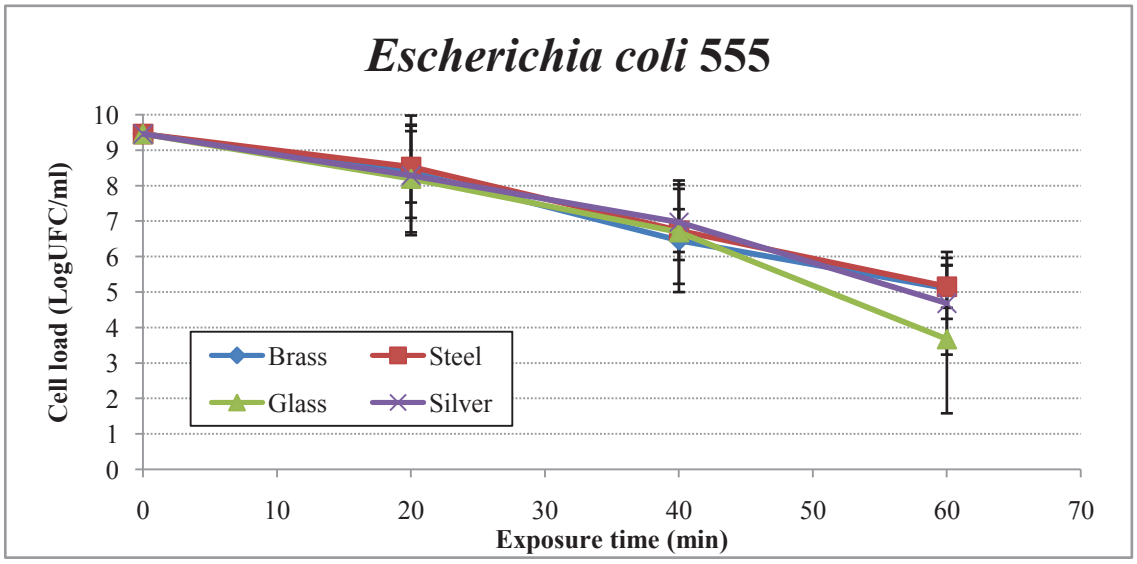

Figure 52. Cell counts (Log CFU/ml) of the surviving cells of *Escherichia coli* 555 (into saline solutions) in relation to the gas plasma treatment conditions: time - 20, 40, 60 min - and electrode material - steel, silver, glass and brass.

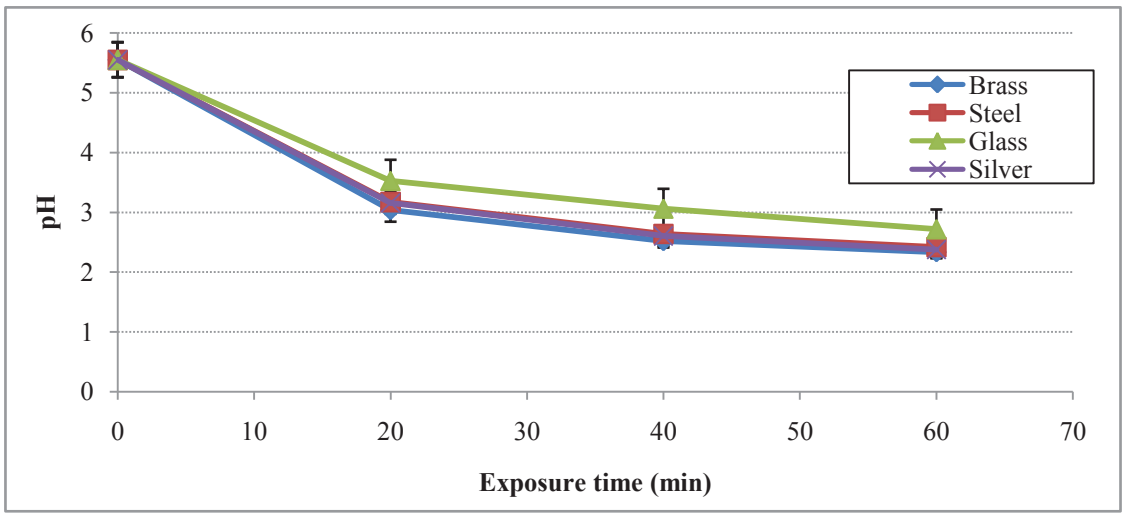

Figure 53. pH values of cell suspensions (into saline solutions) of *Listeria monocytogenes* in relation to the gas plasma treatment conditions: time- 20, 40, 60 min- and electrode material - steel, silver, glass and brass.

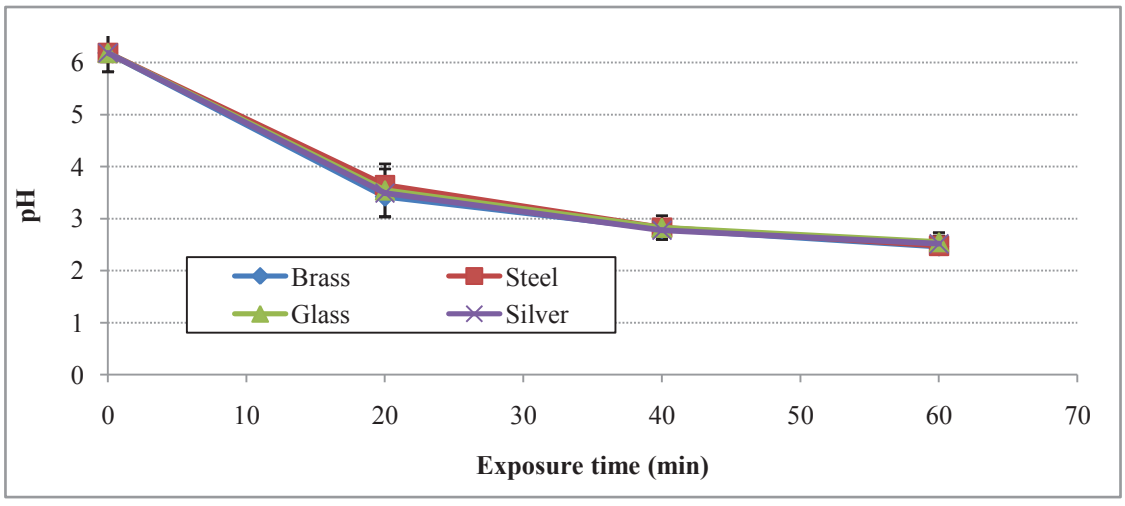

Figure 54. pH values of cell suspensions (into saline solutions) of *Escherichia coli* in relation to the gas plasma treatment conditions: time - 20, 40, 60 min - and electrode material, steel, silver, glass and brass.

Table 10. pH values of cell suspensions of *Listeria monocytogenes* into saline solutions previously exposed to gas plasma treatments with the silver or brass electrode for 20, 40 or 60 min.

		GP treatment time (min)					
		Silver			Brass		
re-suspension time (min)	Untreated	20	40	60	20	40	60
Pre-treatment	5.7±0.156	6.1±0.16	5.99±0.16	5.98±0.16	5.84±0.16	5.84±0.16	6.14±0.16
After treatment	5.71±0.45	2.83±0.17	2.54±0.01	2.29±0.05	2.64±0.15	2.35±0.15	2.18±0.01
0	5.69±0.36	3.99±0.08	3.09±0.25	2.69±0.10	3.53±0.25	2.98±0.44	2.56±0.07
20	5.67±0.34	4.11±0.12	3.18±0.23	2.78±0.11	3.58±0.32	3.04±0.45	2.7±0.11
40	5.96±0.57	4.17±0.17	3.24±0.25	2.76±0.14	3.63±0.25	3.07±0.49	2.7±0.15
60	5.93±0.71	4.21±0.13	3.32±0.35	2.74±0.22	3.7±0.26	3.14±0.48	2.72±0.13

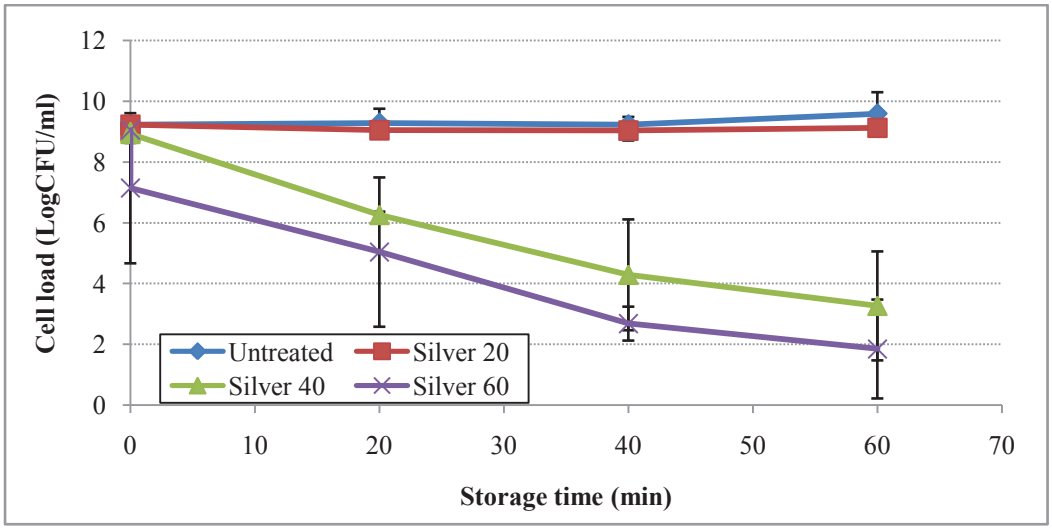

Figure 55. Cell counts (Log CFU/ml) of the surviving cells of *Listeria monocytogenes* suspended into saline solutions previously exposed to gas plasma treatments with the silver electrode for 20, 40 or 60 min.

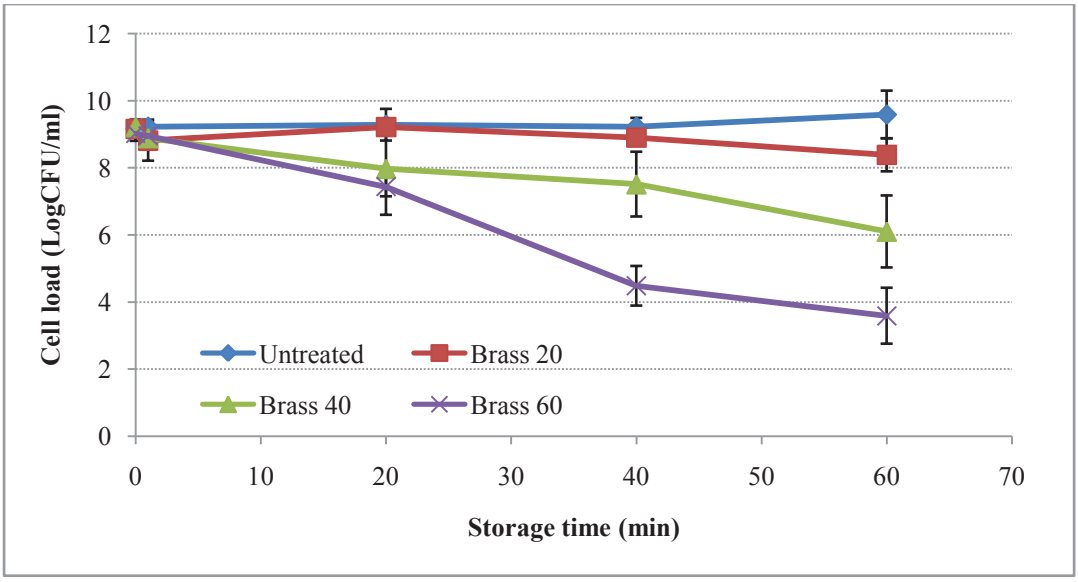

Figure 16. Cell counts (Log CFU/ml) of the surviving cells of *Listeria monocytogenes* suspended into saline solutions previously exposed to gas plasma treatments with the brass electrode for 20, 40 or 60 min.

Table 11. Volatile compounds (expressed as ppmEq) detected for *Listeria monocytogenes* 56Ly cells (into saline solutions) in relation to gas plasma treatment conditions: time - 20, 40, 60 min - and electrode material - steel (ST), silver (SI), glass (DV), brass (BR). The results are means of two independent experiments.

		ST			SI			DV			BR		
molecules	**Untreated**	**20**	**40**	**60**	**20**	**40**	**60**	**20**	**40**	**60**	**20**	**40**	**60**
Pentadecanal	1.495	0.212	0.217	1.168	0.939	1.021	0.956	0.914	1.496	0.912	0.188	0.184	1.279
Hexanal	0.472	0.100	0.076	0.075	0.065	0.079	0.106	0.149	0.115	0.083	1.015	0.101	0.092
Octanal	n.d.	0.979	0.350	0.848	0.722	0.733	0.822	1.150	1.213	5.836	1.426	0.305	5.648
Nonanal	0.593	1.440	0.850	0.825	1.060	0.833	1.158	1.849	1.839	1.350	1.309	0.769	1.020
2-Dodecenal	0.072	0.287	0.260	0.220	0.215	0.094	0.119	0.353	0.224	0.407	0.197	0.140	0.147
(E)-2-Decenal	0.338	0.634	n.d.	0.647	0.345	n.d.	0.576	0.511	0.937	0.415	0.513	0.441	0.537
Benzaldehyde	0.381	0.546	0.854	1.974	0.265	1.531	1.448	1.968	1.547	1.102	1.175	0.780	1.299
4-Ethyl-Benaldehyde	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.564	n.d.	n.d.	n.d.	n.d.	n.d.
Octadecanal	n.d.	0.168	0.564	0.719	0.133	1.152	1.051	0.418	1.068	0.704	0.153	0.427	0.539
Total Aldehydes	**3.351**	**4.367**	**3.172**	**6.476**	**3.744**	**5.443**	**6.236**	**7.876**	**8.439**	**10.809**	**5.977**	**3.147**	**10.562**
5-Methyl-3-hexanone	0.057	0.068	0.052	0.082	0.051	0.045	0.080	0.078	0.082	0.049	0.423	0.052	0.041
4-Methyl-2-Hexanone	0.604	1.096	n.d.	0.232	n.d.	n.d.	1.224	1.103	0.702	0.684	0.663	0.605	0.221
4-Methyl-3-Penten-2-one	1.480	1.305	n.d.	1.714	n.d.	0.327	1.504	1.061	0.983	0.960	n.d.	0.962	1.118
2,6-Dimethyl-4-Heptanone	8.785	11.191	0.847	8.390	2.429	n.d.	10.709	12.819	8.953	n.d.	9.215	n.d.	n.d.
3-Chloro-5,5-dimethyl-2-cyclohexen-1-one	2.290	1.155	n.d.	1.297	0.861	n.d.	0.951	1.083	1.593	0.447	3.385	n.d.	n.d.
5-Amino-2,4(1H,3H)-Pyrimidinedione	1.215	1.194	0.848	1.333	0.913	0.830	1.233	1.608	1.380	0.911	1.422	1.038	0.565
2-Methyl-3-Decen-5-one	4.402	6.571	0.133	6.111	n.d.	n.d.	6.495	7.507	8.029	5.963	3.843	2.311	6.600
Acetophenone	0.144	0.367	0.297	0.294	n.d.	0.197	0.226	0.294	0.392	0.323	0.255	0.211	0.334
Total Ketones	**18.977**	**22.946**	**2.177**	**19.454**	**4.253**	**1.398**	**22.422**	**25.553**	**22.115**	**9.335**	**19.206**	**5.179**	**8.879**
Ethyl alcohol	0.233	0.206	0.142	0.205	0.125	0.136	0.231	0.444	0.547	0.303	0.282	0.169	0.191
2-Hexyl-1-Decanol	0.260	0.676	0.156	0.485	0.075	0.142	0.607	0.502	0.491	n.d.	0.567	0.181	0.244
2-Ethyl-1-Hexanol	0.164	0.698	0.193	0.433	0.907	0.209	0.531	0.174	0.448	0.293	0.278	0.241	0.244
(S)- 2,5-Dimethyl-2-Hexanol	0.901	1.678	0.761	1.000	4.097	0.762	n.d.	1.950	n.d.	1.252	0.801	0.820	1.265
2-Hepatanol	n.d.	0.791	0.220	0.600	n.d.	0.357	0.590	0.693	0.292	0.499	n.d.	0.105	0.297
5-Methyl-3-Hexanol	0.611	n.d.	n.d.	0.348	n.d.	n.d.	n.d.	0.429	n.d.	0.455	0.554	0.046	0.420
1-Tridecanol	n.d.	n.d.	n.d.	n.d.	0.213	0.172	0.200	n.d.	0.242	0.204	0.472	0.154	n.d.
2,6-Bis(1,1-Dimethylethyl)-1,4-Benzenediol	n.d.	2.469	0.903	1.420	n.d.	2.094	1.135	0.705	2.660	2.014	n.d.	3.045	1.898
Total Alcohols	**2.169**	**6.518**	**2.374**	**4.492**	**5.417**	**3.873**	**3.294**	**4.897**	**4.680**	**5.021**	**2.954**	**4.761**	**4.559**
Heptanoic acid	n.d.	n.d.	0.456	n.d.	n.d.	n.d.	n.d.	0.160	n.d.	0.563	n.d.	0.585	n.d.
Dodecanoic acid	n.d.	0.398	0.859	1.784	0.096	0.668	2.726	2.117	2.664	2.182	0.831	1.856	2.256
Octanoic acid	n.d.	n.d.	1.147	0.313	0.803	1.103	2.095	1.913	2.235	2.839	n.d.	3.816	n.d.
n-Decanoic acid	n.d.	n.d.	0.930	0.515	0.103	0.611	0.898	n.d.	n.d.	1.164	n.d.	1.509	0.486
Total Carboxylic acids	**0.000**	**0.398**	**3.392**	**2.612**	**1.002**	**2.383**	**5.719**	**4.190**	**4.898**	**6.748**	**0.831**	**7.766**	**2.741**
Thiophene-2-acetic acid, dodec-9-ynyl ester	8.210	14.388	13.032	13.852	1.088	11.744	17.260	20.370	19.233	16.393	6.226	7.067	17.356
Butanoic, butyl ester	n.d.	0.242	n.d.	n.d.	n.d.	0.437	0.966	0.380	0.163	0.401	n.d.	n.d.	4.537
Total Esters	**8.210**	**14.630**	**13.032**	**13.852**	**1.088**	**12.181**	**18.226**	**20.750**	**19.396**	**16.794**	**6.226**	**7.067**	**21.893**
2,4-Bis(1-Methylethyl)-Phenol	0.790	1.760	1.140	2.004	1.015	1.454	1.938	1.836	2.281	1.904	1.713	1.683	1.956
m-tert-Butyl-Phenol	0.783	0.564	0.377	0.444	0.629	0.402	0.536	0.669	1.051	0.554	0.449	0.342	0.552
4-(1,1,3,3-Tetramethylbutyl)-Phenol	1.959	1.061	0.840	0.959	1.401	0.814	1.113	1.207	2.513	1.079	0.864	0.813	1.043
Total Phenols	**3.532**	**3.385**	**2.357**	**3.407**	**3.045**	**2.670**	**3.587**	**3.711**	**5.845**	**3.537**	**3.026**	**2.838**	**3.551**
2,4,4-Trimethyl-1-Pentene	0.832	0.679	0.553	1.672	n.d.	0.400	1.140	1.347	0.188	0.529	2.009	0.675	0.922
6-Azathymine	3.774	4.063	2.672	3.872	4.709	2.562	4.121	5.079	4.394	2.928	4.708	3.388	1.750
1-Chloro-Hexane	0.129	0.151	0.080	0.144	0.215	0.079	0.131	0.133	0.094	0.094	0.143	0.088	0.067
1-Chloro-Octane	0.460	0.378	0.299	n.d.	0.856	0.694	0.410	n.d.	n.d.	0.250	1.614	n.d.	n.d.
Ethylbenzene	0.403	1.105	n.d.	n.d.	n.d.	n.d.	1.033	0.946	0.502	0.173	0.317	0.288	0.587
2-Pentyl-Furan	0.693	1.946	1.417	1.260	0.858	0.726	1.185	1.533	1.055	1.107	0.322	0.197	0.464
1-Chloro-Decane	1.706	2.505	2.984	2.012	3.244	n.d.	2.254	2.417	n.d.	2.612	0.195	0.875	2.262
2,2,4,6,6-Pentamethyl-3-Heptene	4.609	6.113	n.d.	5.533	1.153	n.d.	6.214	6.562	6.714	0.956	4.606	2.688	0.753
2,4,4,6,6,8,8-Heptamethyl-1-nonene	0.985	n.d.	n.d.	n.d.	n.d.	n.d.	1.243	n.d.	0.512	0.497	n.d.	n.d.	n.d.
trans-1,1,3,5-Tetramethyl-Cyclohexane	0.358	0.167	n.d.	n.d.	n.d.	n.d.	0.043	n.d.	n.d.	n.d.	0.503	n.d.	n.d.
n.d.102	0.582	0.847	0.545	0.749	4.984	0.484	0.813	0.993	1.129	0.712	0.525	0.204	0.779
2-Pentyl-Thiophene	0.698	0.667	0.522	0.829	0.619	0.514	0.687	0.835	1.068	0.558	2.613	0.621	0.822
Total others molecules	**15.230**	**18.622**	**9.071**	**16.071**	**16.639**	**5.458**	**19.272**	**19.845**	**15.657**	**10.416**	**17.554**	**9.024**	**8.406**

n.d. under the detection level

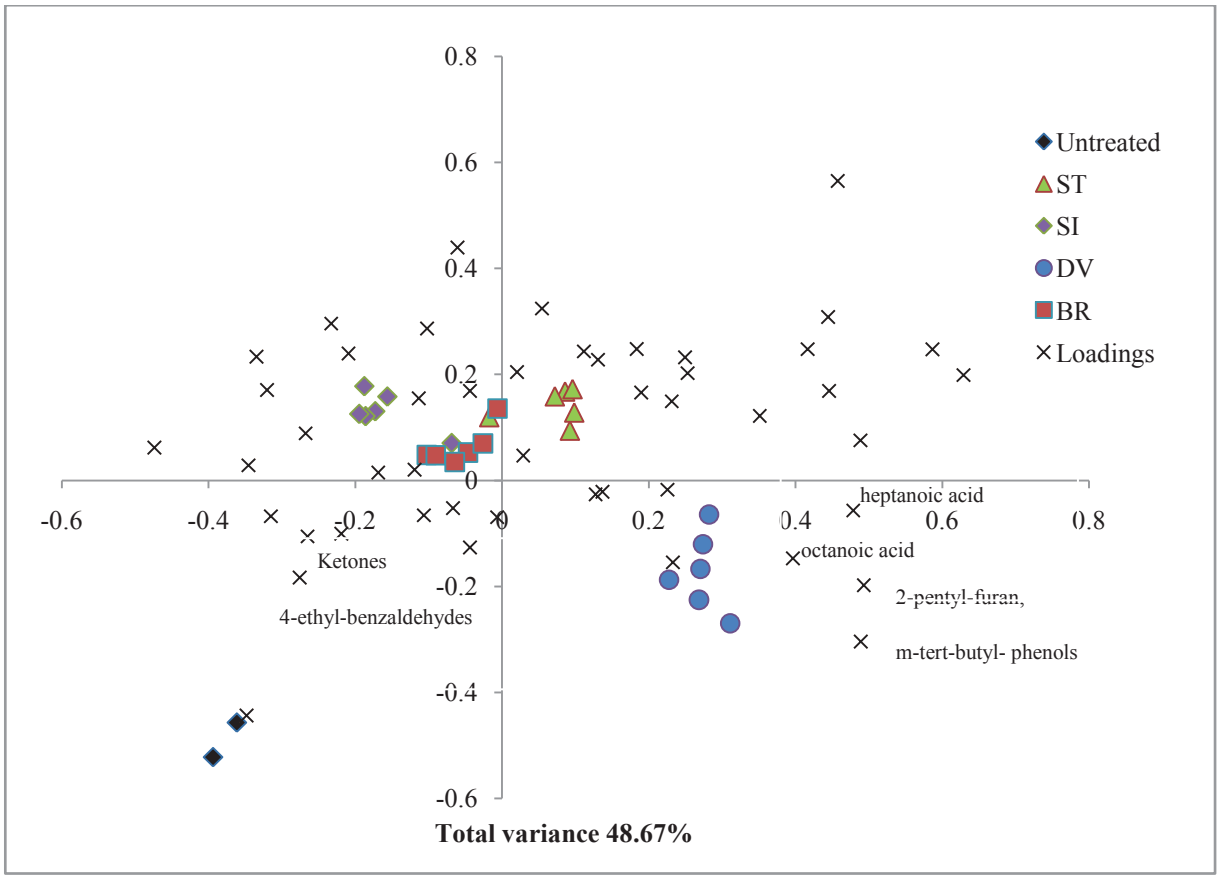

Figure 57. Plots of the first two canonical axes produced by Canonical Analysis of Principal Coordinates (CAP) of GC-MS/SPME analysis for *Listeria monocytogenes* 56Ly cells (into saline solutions) exposed to different gas plasma treatment conditions: time - 20, 40, 60 min - and electrode material - steel (ST), silver (SI), glass (DV), brass (BR).

Table 12. Volatile compounds (expressed as ppmEq) detected for *Escherichia coli* 555 cells (into saline solutions) in relation to different gas plasma treatment conditions: time - 20, 40, 60 min - and electrode material - steel (ST), silver (SI), glass (DV), brass (BR). The results are means of two independent experiments.

		ST			SI			DV			BR		
molecules	**Untreated**	**20**	**40**	**60**	**20**	**40**	**60**	**20**	**40**	**60**	**20**	**40**	**60**
Octanal	0.136	0.110	0.342	0.535	0.606	n.d.	0.444	0.459	1.169	0.621	n.d.	n.d.	0.074
Nonanal	0.221	0.740	0.679	1.128	0.626	0.463	0.566	1.138	0.929	0.714	0.140	n.d.	0.150
2-Hydroxy-Benzaldehyde	n.d.	n.d.	n.d.	2.120	n.d.	2.082	0.234	n.d.	0.944	2.793	n.d.	2.732	2.794
2-Chloro-Benzaldehyde	n.d.	0.407	n.d.	0.123	0.181	0.148	0.104	0.492	0.248	0.293	0.174	n.d.	n.d.
4-Ethyl-Benzaldehyde	0.262	1.983	4.183	2.080	1.041	2.030	1.079	1.183	1.105	1.516	0.754	1.042	1.835
Benzaldehyde	0.158	3.960	5.629	7.735	3.279	6.425	2.969	2.847	4.092	3.515	2.207	2.906	3.601
4-Methyl-Benzaldehyde	n.d.	n.d.	n.d.	2.861	n.d.	0.871	0.338	n.d.	0.545	0.956	n.d.	0.814	2.089
Total Aldehydes	**0.778**	**7.200**	**10.834**	**16.584**	**5.733**	**12.019**	**5.733**	**6.118**	**9.033**	**10.408**	**3.274**	**7.494**	**10.544**
2-Butanone	0.670	0.650	1.213	1.760	0.889	1.261	1.033	1.266	1.450	1.981	0.972	1.123	1.315
5-Methyl-3-Hexanone	0.060	n.d.	0.050	0.134	0.270	0.201	0.329	0.063	0.142	0.237	n.d.	0.231	0.062
3-Methyl-2,4-Pentanedione	0.225	n.d.	0.055	0.286	0.528	0.408	0.552	0.065	0.513	0.356	n.d.	0.141	0.225
4-Methyl-2-Hexanone	0.442	0.481	0.282	n.d.	0.672	0.489	n.d.	0.308	n.d.	0.142	0.334	0.569	0.614
4-Methyl-3-Penten-2-one,	0.832	0.431	1.259	0.724	1.409	0.773	1.318	1.050	1.513	1.097	0.992	n.d.	0.845
2,6-Dimethyl-4-Heptanone	4.421	3.853	4.330	3.400	4.575	4.510	4.369	3.619	5.135	4.647	4.275	4.398	5.001
5-Amino-2,4(1H,3H)-Pyrimidinedione	1.336	1.029	0.911	1.039	1.139	0.993	1.213	0.932	1.115	1.190	0.882	1.690	1.123
3-Chloro-5,5-dimethyl-2-cyclohexen-1-one	0.198	n.d.	1.667	n.d.	2.536	1.683	n.d.	1.343	2.706	2.589	0.558	n.d.	0.324
Acetophenone	n.d.	0.102	0.050	n.d.	n.d.	0.103	0.089	0.100	0.259	0.261	n.d.	n.d.	n.d.
Total Ketones	**8.183**	**6.546**	**9.816**	**7.343**	**12.018**	**10.421**	**8.904**	**8.747**	**12.833**	**12.500**	**8.012**	**8.152**	**9.508**
Ethyl alcohol	0.102	0.436	0.420	0.917	0.402	1.032	0.815	1.177	1.184	6.348	0.514	1.004	1.084
5-Methyl-3-Hexanol	0.176	0.046	0.316	0.481	0.218	0.303	0.042	0.360	0.326	0.863	0.164	n.d.	n.d.
6,6-Dimethyl-1,3-Heptadien-5-ol	0.509	n.d.	0.017	0.176	0.483	0.413	0.296	0.138	0.488	0.335	n.d.	n.d.	n.d.
2-Ethyl-1-Hexanol	n.d.	0.116	0.280	0.596	0.167	0.316	0.151	0.377	1.057	2.635	0.143	0.291	0.365
2-Hepatanol	0.109	0.116	0.203	0.134	0.233	0.127	0.239	0.105	0.157	0.155	0.091	0.167	0.162
1-Dodecanol	1.609	0.496	0.438	0.518	0.575	0.530	0.239	0.704	0.609	0.513	0.936	0.727	0.554
2,6-bis(1,1-Dimethylethyl)-1,4-Benzenediol	1.442	n.d.	n.d.	1.506	1.580	2.161	n.d.	1.373	1.617	n.d.	1.147	1.769	n.d.
2,5-bis(1,1-Dimethylethyl)-1,4-Benzenediol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.448	n.d.	n.d.
Total Alcohols	**3.948**	**1.211**	**1.674**	**4.328**	**3.657**	**4.882**	**1.782**	**4.234**	**5.439**	**10.848**	**4.443**	**3.959**	**2.165**
Ethyl Acetate	n.d.	0.066	0.159	0.306	0.103	0.176	0.157	0.136	0.265	1.019	n.d.	0.142	0.311
Nitric acid, ethyl ester	n.d.	0.049	0.180	0.170	n.d.	0.063	0.055	n.d.	0.038	0.178	n.d.	0.057	0.061
Butanedioic acid, diethyl ester	n.d.	0.259	0.266	0.398	0.360	0.406	0.319	0.400	1.039	2.037	0.251	0.717	0.569
Thiophene-2-acetic acid, dodec-9-ynyl ester	0.246	0.088	4.518	1.662	4.602	4.707	2.184	3.017	5.670	5.508	0.234	n.d.	n.d.
Isobutyl isothiocyanate	n.d.	0.211	0.181	0.093	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.070	n.d.	n.d.
Total Esters	**0.246**	**0.674**	**5.303**	**2.630**	**5.066**	**5.352**	**2.715**	**3.553**	**7.011**	**8.743**	**0.555**	**0.917**	**0.941**
2,5-bis(1-Methylpropyl)-Phenol	n.d.	0.180	n.d.	0.208	0.156	0.184	n.d.	n.d.	0.165	n.d.	n.d.	n.d.	n.d.
2,5-bis(1,1-Dimethylethyl)-Phenol	0.822	0.134	n.d.	n.d.	0.119	0.103	n.d.	0.337	0.166	0.284	n.d.	n.d.	0.231
2,4-bis(1-Methylethyl)-Phenol	n.d.	0.617	0.515	0.503	0.670	0.646	0.518	0.692	0.588	0.676	n.d.	n.d.	n.d.
m-tert-Butyl-Phenol	0.330	0.277	0.365	0.212	0.519	0.416	0.210	0.393	0.502	0.610	n.d.	n.d.	n.d.
4-(1,1,3,3-Tetramethylbutyl)-Phenol	0.832	0.865	0.814	0.686	1.256	0.972	0.474	1.358	0.989	1.415	0.184	n.d.	n.d.
Total Phenols	**1.984**	**2.073**	**1.693**	**1.609**	**2.720**	**2.320**	**1.202**	**2.781**	**2.410**	**2.985**	**0.184**	**0.000**	**0.231**
2-Isocyanato-Butane	n.d.	0.063	0.249	0.113	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Indole	2.404	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6-Azathymine	3.605	2.434	2.262	2.289	3.013	2.728	3.095	2.652	2.832	2.732	2.293	3.724	2.888
Hexane, 1-chloro	0.066	0.045	0.046	0.050	0.115	0.091	0.119	n.d.	0.112	0.102	n.d.	0.085	0.073
Ethylbenzene	0.097	n.d.	0.191	0.171	0.413	0.242	0.652	0.085	0.609	0.570	n.d.	0.094	0.261
3,9-dimethyl-Undecane	n.d.	n.d.	0.104	0.124	n.d.	n.d.	0.154	0.127	n.d.	0.887	0.071	0.103	0.105
2-Pentyl-Furan	n.d.	0.174	0.266	0.195	0.127	n.d.	0.114	n.d.	0.174	0.279	n.d.	0.158	0.357
1-Chloro-Octane	0.150	0.059	0.526	0.449	0.766	n.d.	0.650	0.502	1.431	1.062	0.125	0.083	0.127
2,2,4,6,6-Pentamethyl-3-Heptene	0.402	0.812	1.738	1.427	3.113	3.093	2.098	1.558	3.915	3.448	0.171	n.d.	0.118
2,2,4-Trimethyl-4-nitro-Pentane	n.d.	n.d.	0.253	0.196	0.405	0.909	0.260	0.230	0.741	0.468	n.d.	n.d.	n.d.
1,1'Propyldenebis-Cyclohexane	0.210	0.473	0.508	0.154	0.375	0.343	0.442	0.497	0.928	0.510	n.d.	n.d.	n.d.
Benzonitrile	n.d.	3.115	6.515	6.972	1.862	6.407	3.843	1.575	3.341	3.047	2.882	1.855	2.016
N.d.97	1.779	4.794	2.121	3.830	8.529	7.941	6.382	2.826	8.172	7.551	0.173	n.d.	n.d.
2-Pentyl-Thiophene	0.351	0.459	0.565	0.601	0.444	0.374	0.269	0.491	0.453	0.573	0.676	1.408	0.251
Methyl-Cycloheptane	5.910	1.238	1.247	1.221	1.312	1.430	0.252	2.096	1.651	1.424	3.627	1.354	1.343
Total others molecules	**14.974**	**13.666**	**16.591**	**17.792**	**20.474**	**23.557**	**18.332**	**12.640**	**24.360**	**22.654**	**10.018**	**8.863**	**7.540**

n.d. under the detection level.

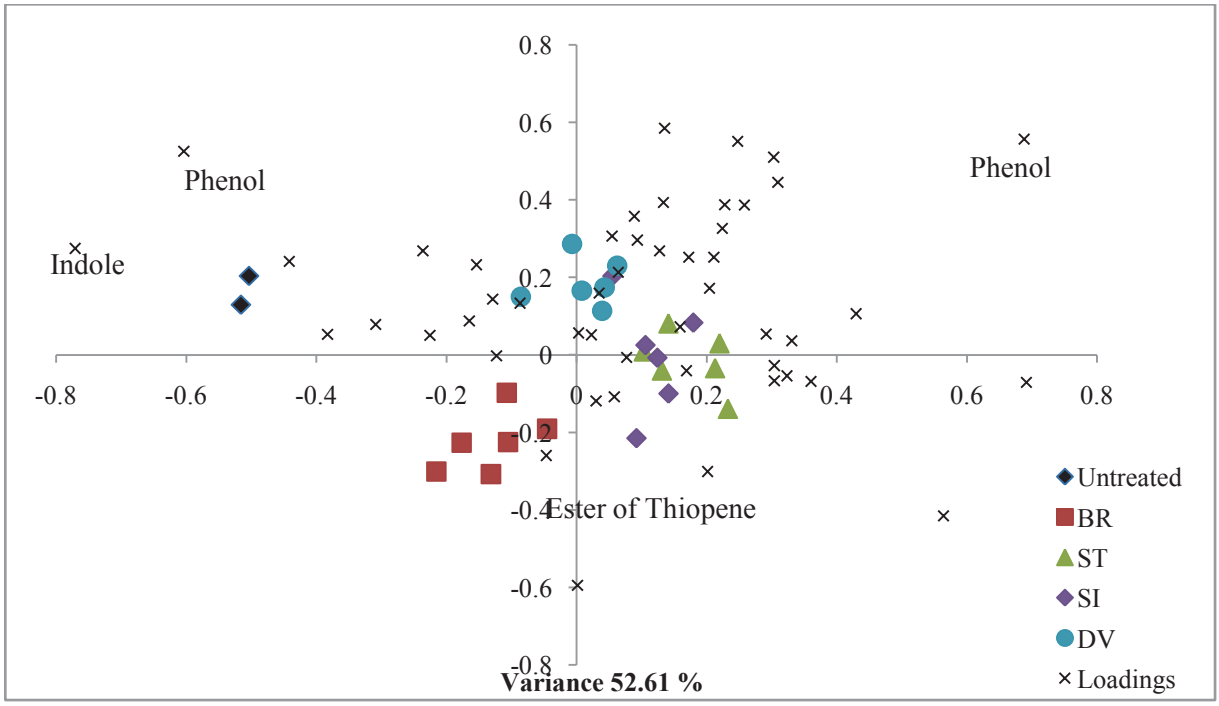

Figure 58. Plots of the first two canonical axes produced by Canonical Analysis of Principal Coordinates (CAP) of GC-MS/SPME analysis for *Escherichia coli* 555 cells (into saline solutions) exposed to different gas plasma treatment conditions: time - 20, 40, 60 min - and electrode material, steel (ST), silver (SI), glass (DV), brass (BR).

Table 13. Volatile compounds (expressed as ppmEq) detected for *Listeria monocytogenes* 56Ly cells re-suspended into saline solutions previously exposed to gas plasma treatments with the silver (SI) electrode for 20, 40, 60 min. The results are means of two independent experiments.

	Untreated				SI20				SI40				SI60			
Sampling after treatment (min)	**0**	**20**	**40**	**60**	**0**	**20**	**40**	**60**	**0**	**20**	**40**	**60**	**0**	**20**	**40**	**60**
Hexanal	0.836	0.537	0.624	0.679	0.917	0.481	0.123	0.797	0.896	0.650	n.d.	0.978	n.d.	n.d.	0.104	0.726
Octanal	1.239	0.743	1.060	1.244	0.917	0.967	1.072	0.885	1.292	0.577	1.032	1.685	1.017	1.273	0.941	1.038
Nonanal	0.637	0.328	0.608	0.717	0.689	0.650	0.308	0.402	0.991	0.785	1.042	1.064	0.795	1.078	n.d.	0.844
Octadecanal	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.092	0.197	n.d.	n.d.	n.d.	n.d.	n.d.	0.613	0.458
Tetradecanal	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Benzaldehyde	0.922	0.194	0.667	0.601	2.719	2.396	3.554	2.122	1.977	1.423	2.702	2.339	2.218	3.161	2.285	2.530
4-Ethyl-Benzaldehyde	n.d.	2.057	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.749	n.d.	n.d.	n.d.	1.115	0.525	0.879	0.853
Total Aldehydes	**3.634**	**3.859**	**2.958**	**3.241**	**5.242**	**4.494**	**5.058**	**4.298**	**6.103**	**3.436**	**4.777**	**6.066**	**5.145**	**6.037**	**4.822**	**6.448**
5-Methyl-3-hexanone	0.218	0.247	n.d.	0.130	0.341	n.d.	n.d.	0.247	0.896	0.204	n.d.	n.d.	n.d.	n.d.	0.076	n.d.
4-Methyl-2-Hexanone	0.692	0.612	0.610	0.671	0.436	0.680	0.620	0.430	0.738	0.328	0.425	0.776	0.283	0.729	0.364	0.623
4-Methyl-3-Penten-2-one	1.784	1.422	1.388	1.785	0.455	1.304	0.574	0.425	1.581	0.487	0.939	2.150	1.171	0.853	1.089	1.150
2,6-Dimethyl-4-Heptanone	5.470	4.052	5.312	5.456	4.011	5.225	4.178	3.372	4.775	2.845	4.886	4.872	4.741	4.251	4.757	4.374
3-Chloro-5,5-dimethyl-2-cyclohexen-1-one	2.530	2.222	2.194	2.678	4.777	n.d.	5.574	4.345	2.613	5.859	0.351	2.772	1.861	1.636	1.661	2.367
5-Amino-2,4(1H,3H)-Pyrimidinedione	1.065	1.011	1.020	1.105	0.919	1.038	0.779	0.844	1.067	0.696	0.913	0.979	1.050	0.799	1.350	1.333
2-Methyl-3-Decen-5-one	3.911	2.392	4.931	4.138	n.d.	3.885	n.d.	1.273	3.858	0.658	4.691	4.623	4.625	5.977	3.557	4.020
Acetophenone	0.153	n.d.	0.134	0.167	0.298	0.296	0.232	0.235	0.388	0.255	0.269	0.272	0.350	0.302	0.211	0.286
Total Ketones	**15.823**	**11.959**	**15.588**	**16.130**	**11.236**	**12.427**	**11.957**	**11.170**	**15.916**	**11.331**	**12.474**	**16.444**	**14.082**	**14.548**	**13.066**	**14.152**
Ethyl alcohol	0.361	0.284	0.231	0.257	0.421	0.318	0.294	0.345	0.510	0.402	0.465	0.517	0.654	0.535	0.798	0.688
2-Ethyl-1-Hexanol	0.120	n.d.	0.251	0.126	n.d.	n.d.	0.261	n.d.	0.236	n.d.	0.270	n.d.	0.320	0.343	0.268	0.260
(S)-2,5-Dimethyl-, 2-Hexanol	0.957	0.572	0.872	0.863	0.756	0.364	0.179	0.733	1.126	1.139	0.439	1.395	0.815	1.063	0.830	0.979
2-Hepatanol	0.414	0.100	0.443	0.498	0.397	n.d.	n.d.	0.202	0.149	0.293	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5-Methyl-3-Hexanol	n.d.	n.d.	0.927	n.d.	n.d.	n.d.	1.098	n.d.	0.408	n.d.	0.975	n.d.	n.d.	n.d.	n.d.	n.d.
2-Methylene cyclopentanol	0.465	n.d.	0.350	0.429	0.423	0.407	0.531	0.403	6.906	0.518	0.717	0.746	0.510	0.886	0.380	0.586
2,6-bis(1,1-Dimethylethyl)-1,4-Benzenediol	n.d.	n.d.	n.d.	n.d.	0.812	1.081	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.064	0.359	0.487
2,5-bis(1,1-Dimethylethyl)-1,4-Benzenediol	n.d.	n.d.	2.609	1.970	1.761	0.576	3.370	n.d.	n.d.	n.d.	2.796	2.100	1.548	0.883	n.d.	n.d.
1-Tridecanol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total Alcohols	**2.317**	**0.956**	**5.682**	**4.143**	**4.571**	**2.746**	**5.732**	**1.683**	**9.334**	**2.351**	**5.661**	**4.757**	**3.848**	**4.775**	**2.635**	**2.999**
Nonanoic acid	0.120	n.d.	0.187	n.d.	n.d.	n.d.	n.d.	n.d.	1.224	n.d.	n.d.	n.d.	n.d.	1.663	n.d.	n.d.
Octanoic acid	n.d.	0.345	0.547	n.d.	n.d.	n.d.	n.d.	n.d.	1.043	0.462	0.805	0.769	0.656	2.026	0.521	0.434
Total Carboxylic Acid	**0.120**	**0.345**	**0.734**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**2.267**	**0.462**	**0.805**	**0.769**	**0.656**	**3.688**	**0.521**	**0.434**
Oxalic acid, isobutyl heptadecyl ester	0.627	n.d.	0.470	0.537	n.d.	0.460	0.452	0.735	0.349	0.277	0.470	0.382	n.d.	0.225	0.240	n.d.
Propanedioic acid, diethyl ester	1.128	0.646	n.d.	n.d.	n.d.	n.d.	1.165	n.d.	1.148	n.d.	n.d.	1.378	0.863	n.d.	0.778	1.073
Thiophene-2-acetic acid, dodec-9-ynyl ester	8.732	7.909	9.876	9.412	n.d.	8.127	6.685	12.211	7.949	14.085	4.786	8.838	9.757	13.217	5.333	7.888
Butanedioic acid, diethyl ester	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.630	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total Esters	**10.487**	**8.554**	**10.345**	**9.949**	**0.000**	**8.587**	**8.301**	**12.946**	**10.076**	**14.361**	**5.256**	**10.598**	**10.621**	**13.442**	**6.351**	**8.961**
2-chloro-4-(1,1-dimethylpropyl)-Phenol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.263	0.360
2,4-bis(1-methylethyl)-Phenol	0.209	n.d.	0.329	1.087	0.713	0.548	2.814	0.920	1.597	1.041	0.654	0.710	1.014	3.479	2.038	1.969
m-tert-butyl-Phenol	0.711	0.420	0.857	0.770	0.517	0.507	0.689	0.389	n.d.	0.346	n.d.	0.188	n.d.	1.592	0.641	n.d.
4-(1,1,3,3-tetramethylbutyl)-Phenol	2.142	1.001	1.924	1.741	1.583	1.349	1.922	0.905	0.507	0.965	0.783	0.842	0.334	0.627	n.d.	0.204
Total Phenols	**3.063**	**1.421**	**3.109**	**3.597**	**2.813**	**2.404**	**5.425**	**2.213**	**2.104**	**2.352**	**1.437**	**1.739**	**1.348**	**5.698**	**2.941**	**2.533**
6-Azathymine	3.105	2.796	2.978	3.073	2.443	2.790	2.090	2.095	2.797	1.625	2.481	2.568	2.648	2.263	3.093	3.046
2,4,4-Trimethyl-1-Pentene	2.346	1.052	0.362	0.632	1.595	0.566	0.420	1.675	1.483	1.663	0.948	1.966	0.821	1.550	1.556	2.260
1-Chloro-Hexane	0.237	0.162	0.101	0.153	0.092	n.d.	0.072	0.124	0.110	0.121	0.149	0.256	0.095	0.151	0.109	0.092
1-Chloro-Octane	0.142	n.d.	0.331	n.d.	0.610	n.d.	0.989	0.545	n.d.	0.634	1.068	n.d.	0.516	n.d.	3.178	1.105
2,6-Dimethyl-Undecane	0.274	0.239	n.d.	0.378	0.189	1.068	1.098	0.236	0.327	0.277	0.975	0.395	n.d.	n.d.	0.830	n.d.
2-Pentyl-Thiophene	0.644	0.345	0.547	0.737	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.805	n.d.	n.d.	0.768	n.d.	0.572
2-Pentyl-Furan	0.603	0.241	0.692	0.578	0.387	n.d.	n.d.	0.365	0.408	0.403	n.d.	1.124	0.897	1.081	n.d.	0.578
1-Chloro-Decane	1.742	1.135	1.536	1.727	1.152	1.449	1.615	1.040	1.556	1.336	1.922	2.197	1.907	1.830	1.199	1.121
3,5,5,-Trimethyl-Cyclohexene	2.279	1.961	2.853	2.577	1.609	2.435	4.575	1.521	2.329	1.538	2.263	2.671	n.d.	2.131	1.835	1.714
Total Others molecules	**11.372**	**7.930**	**9.401**	**9.855**	**8.076**	**8.309**	**10.860**	**7.602**	**9.010**	**7.595**	**10.610**	**11.178**	**6.884**	**9.773**	**11.800**	**10.488**

n.d. under the detection level

Table 14. Volatile compounds (expressed as ppmEq) detected for *Listeria monocytogenes* 56Ly cells re-suspended into saline solutions previously exposed to gas plasma treatments with the brass (BR) electrode for 20, 40, 60 min. The results are means of two independent experiments.

	BR20				BR40				BR60			
Sampling after treatment (min)	**0**	**20**	**40**	**60**	**0**	**20**	**40**	**60**	**0**	**20**	**40**	**60**
Hexanal	n.d.	0.837	0.325	0.047	n.d.	0.755	n.d.	0.047	0.265	0.210	0.128	0.051
Octanal	0.439	0.522	0.242	0.076	n.d.	0.700	0.115	0.530	0.115	n.d.	n.d.	0.263
Nonanal	0.878	0.582	0.562	0.262	0.175	1.405	0.271	1.003	0.721	0.655	0.304	1.237
Octadecanal	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.240	0.093	0.153	0.525
Tetradecanal	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.128	n.d.	n.d.
Benzaldehyde	1.082	0.521	0.873	1.033	0.761	0.972	0.502	1.247	1.446	1.186	1.732	2.343
4-Ethyl-Benzaldehyde	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.993	n.d.	n.d.	n.d.	1.506	n.d.
Total Aldehydes	**2.399**	**2.463**	**2.002**	**1.418**	**0.936**	**3.832**	**1.880**	**2.827**	**2.787**	**2.272**	**3.824**	**4.418**
5-Methyl-3-hexanone	0.306	n.d.	0.282	0.068	0.262	0.151	0.043	0.056	0.242	0.241	0.242	0.073
4-Methyl-2-Hexanone	0.523	0.418	0.442	0.624	0.404	0.607	0.255	0.320	0.414	0.429	0.496	0.638
4-Methyl-3-Penten-2-one	0.955	0.969	0.708	0.980	1.130	1.076	0.502	1.047	0.572	0.617	0.522	0.936
2,6-Dimethyl-4-Heptanone	4.221	3.517	3.259	5.320	4.632	4.961	3.528	4.832	2.996	2.925	2.836	5.789
3-Chloro-5,5-dimethyl-2-cyclohexen-1-one	n.d.	1.079	1.181	n.d.	n.d.	1.053	n.d.	n.d.	1.668	1.280	1.746	n.d.
5-Amino-2,4(1H,3H)-Pyrimidinedione	1.221	0.942	0.992	1.163	1.256	1.171	0.835	0.978	0.932	0.983	0.911	1.167
2-Methyl-3-Decen-5-one	1.129	1.158	0.532	0.769	1.491	0.887	0.901	1.540	0.295	n.d.	0.165	0.154
Acetophenone	0.100	0.100	0.081	0.089	n.d.	0.176	n.d.	n.d.	0.156	0.120	0.112	0.161
Total Ketones	**8.455**	**8.183**	**7.478**	**9.013**	**9.175**	**10.084**	**6.066**	**8.772**	**7.275**	**6.595**	**7.029**	**8.917**
Ethyl alcohol	0.607	n.d.	0.161	0.169	0.167	0.283	0.216	0.192	0.096	0.100	0.109	0.144
2-Ethyl-1-Hexanol	0.289	n.d.	n.d.	0.348	n.d.	n.d.	0.078	n.d.	n.d.	n.d.	n.d.	0.215
(S)-2,5-Dimethyl-, 2-Hexanol	n.d.	0.261	n.d.	0.082	n.d.	0.522	n.d.	0.128	0.181	0.145	n.d.	n.d.
2-Hepatanol	n.d.	n.d.	n.d.	0.174	0.123	0.328	0.097	0.086	0.163	0.158	0.155	0.166
5-Methyl-3-Hexanol	n.d.	n.d.	n.d.	0.238	0.118	n.d.	0.173	0.242	n.d.	n.d.	n.d.	n.d.
2-Methylene cyclopentanol	n.d.	n.d.	0.507	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2,6-bis(1,1-Dimethylethyl)-1,4-Benzenediol	n.d.	n.d.	1.107	n.d.	1.709	n.d.	2.162	1.787	1.815	n.d.	0.479	0.434
2,5-bis(1,1-Dimethylethyl)-1,4-Benzenediol	1.376	n.d.	1.060	n.d.	1.741	n.d.	1.870	1.334	n.d.	n.d.	1.020	n.d.
1-Tridecanol	n.d.	n.d.	n.d.	0.481	n.d.	n.d.	0.146	0.177	0.132	0.095	0.090	0.181
Total Alcohols	**2.272**	**0.261**	**2.836**	**1.492**	**3.857**	**1.133**	**4.742**	**3.947**	**2.386**	**0.498**	**1.853**	**1.140**
Nonanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Octanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total Carboxylic Acid	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Oxalic acid, isobutyl heptadecyl ester	n.d.	n.d.	n.d.	n.d.	n.d.	0.355	n.d.	n.d.	n.d.	n.d.	n.d.	0.131
Propanedioic acid, diethyl ester	0.727	0.381	0.460	0.856	0.453	0.597	n.d.	0.606	n.d.	n.d.	n.d.	0.091
Thiophene-2-acetic acid, dodec-9-ynyl ester	2.514	3.808	3.496	n.d.	n.d.	1.233	n.d.	3.110	4.364	3.279	0.404	0.308
Butanedioic acid, diethyl ester	n.d.	0.447	n.d.	0.674	0.701	0.543	0.616	0.653	0.332	0.263	0.655	0.344
Total Esters	**3.242**	**4.636**	**3.955**	**1.530**	**1.155**	**2.729**	**0.616**	**4.368**	**4.696**	**3.542**	**1.060**	**0.873**
2-chloro-4-(1,1-dimethylpropyl)-Phenol	n.d.	n.d.	n.d.	0.843	1.021	0.411	1.400	0.735	0.371	n.d.	0.600	0.436
2,4-bis(1-methylethyl)-Phenol	0.821	0.277	0.851	n.d.	0.373	1.614	1.770	0.906	0.518	0.570	0.053	0.413
m-tert-butyl-Phenol	n.d.	n.d.	0.186	n.d.	n.d.	n.d.	n.d.	0.289	0.184	0.148	0.173	0.073
4-(1,1,3,3-tetramethylbutyl)-Phenol	0.817	0.412	0.599	n.d.	0.352	1.082	0.592	0.635	0.641	0.369	n.d.	0.230
Total Phenols	**1.638**	**0.690**	**1.636**	**0.843**	**1.745**	**3.107**	**3.763**	**2.565**	**1.714**	**1.088**	**0.826**	**1.151**
6-Azathymine	3.082	2.378	2.329	3.177	3.325	2.949	2.130	2.633	2.066	2.184	2.002	3.448
2,4,4-Trimethyl-1-Pentene	n.d.	0.609	0.229	n.d.	n.d.	1.002	n.d.	0.139	0.344	0.618	0.237	n.d.
1-Chloro-Hexane	0.361	n.d.	n.d.	0.097	0.156	0.084	0.051	0.055	n.d.	0.048	0.128	0.060
1-Chloro-Octane	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2,6-Dimethyl-Undecane	0.447	0.480	0.192	n.d.	n.d.	0.300	n.d.	n.d.	0.160	0.096	0.144	0.183
2-Pentyl-Thiophene	0.463	0.359	0.441	1.096	1.496	0.599	n.d.	0.673	n.d.	n.d.	0.851	0.166
2-Pentyl-Furan	n.d.	n.d.	n.d.	n.d.	n.d.	0.193	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1-Chloro-Decane	0.410	0.410	0.150	0.251	n.d.	0.492	0.290	0.454	0.131	0.083	0.065	0.244
3,5,5,-Trimethyl-Cyclohexene	0.584	0.620	0.225	n.d.	0.361	0.562	n.d.	0.657	0.227	0.229	0.110	n.d.
Total Others molecules	**5.347**	**4.856**	**3.567**	**4.620**	**5.337**	**6.181**	**2.471**	**4.612**	**2.929**	**3.259**	**3.537**	**4.103**

n.d. under the detection level

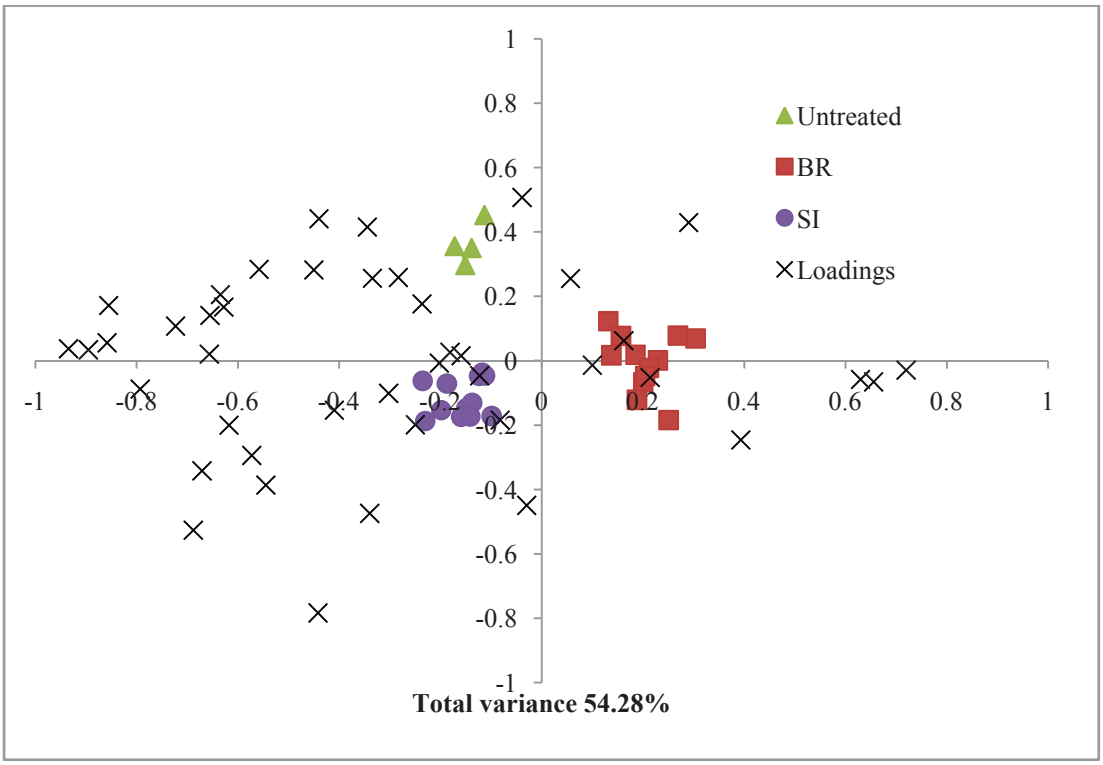

Figure 59. Plots of the first two canonical axes produced by Canonical Analysis of Principal Coordinates (CAP) of volatile compounds detected for *Listeria monocytogenes* 56Ly cells re-suspended into saline solutions previously exposed to gas plasma treatments with the brass (BR) or silver (SI) electrode for 20, 40, 60 min.

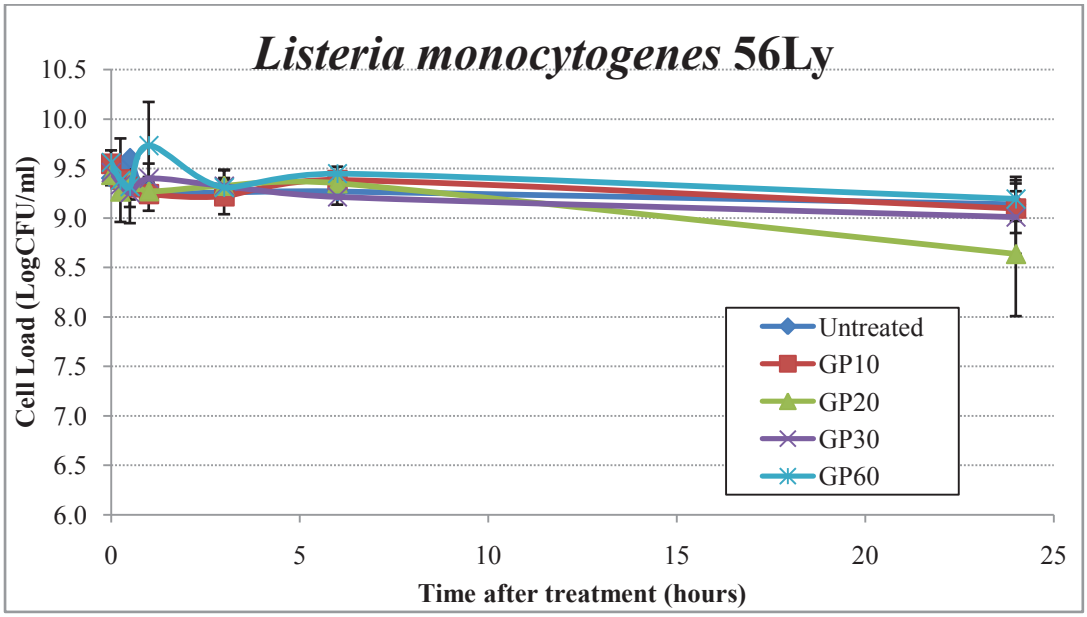

Figure 60. Cell counts (Log CFU/ml) of the surviving cells of *Listeria monocytogenes* 56Ly (into BHI broth) after exposure to gas plasma for 0, 10, 20, 30 and 60 min.

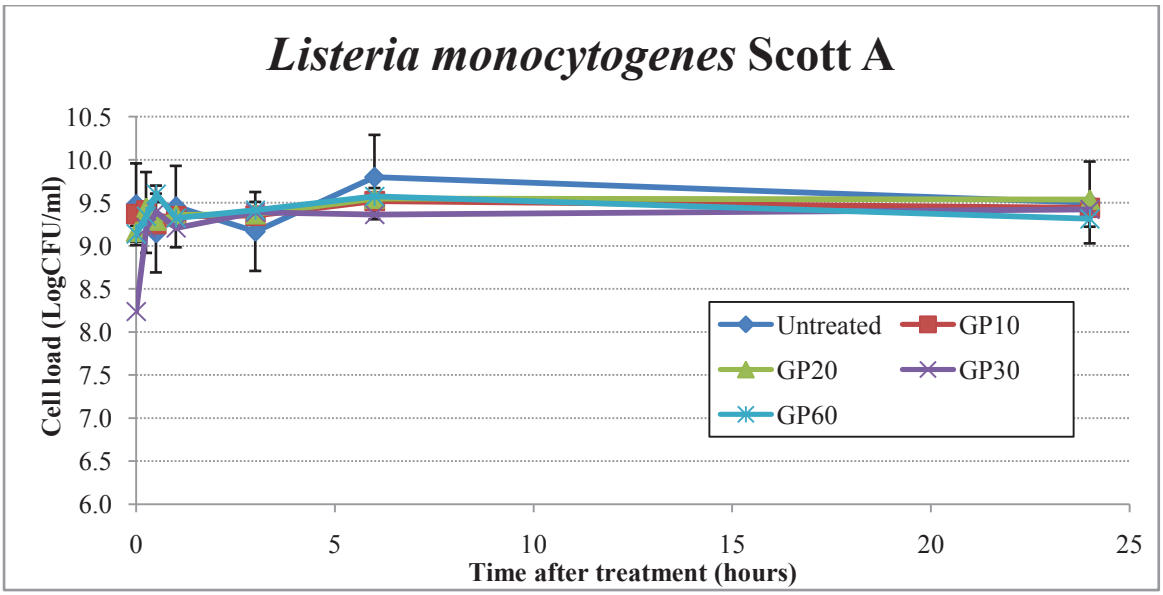

Figure 61. Cell counts (Log CFU/ml) of the surviving cells of *Listeria monocytogenes* ScottA (into BHI broth) after exposure to gas plasma for 0, 10, 20, 30 and 60 min.

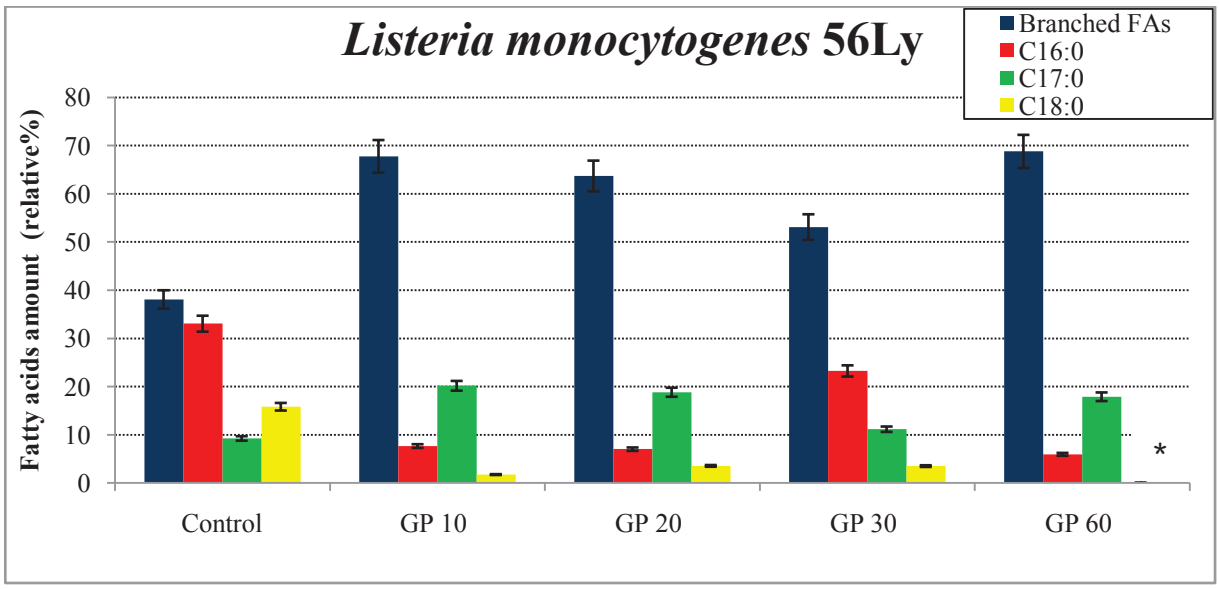

Figure 62. Main cellular fatty acids extracted from *Listeria monocytogenes* 56Ly cells after exposure to gas plasma treatments. Fatty acid relative percentages were calculated with respect to the total fatty acid methyl esters. The results are means of two independent experiments. * under the detection level.

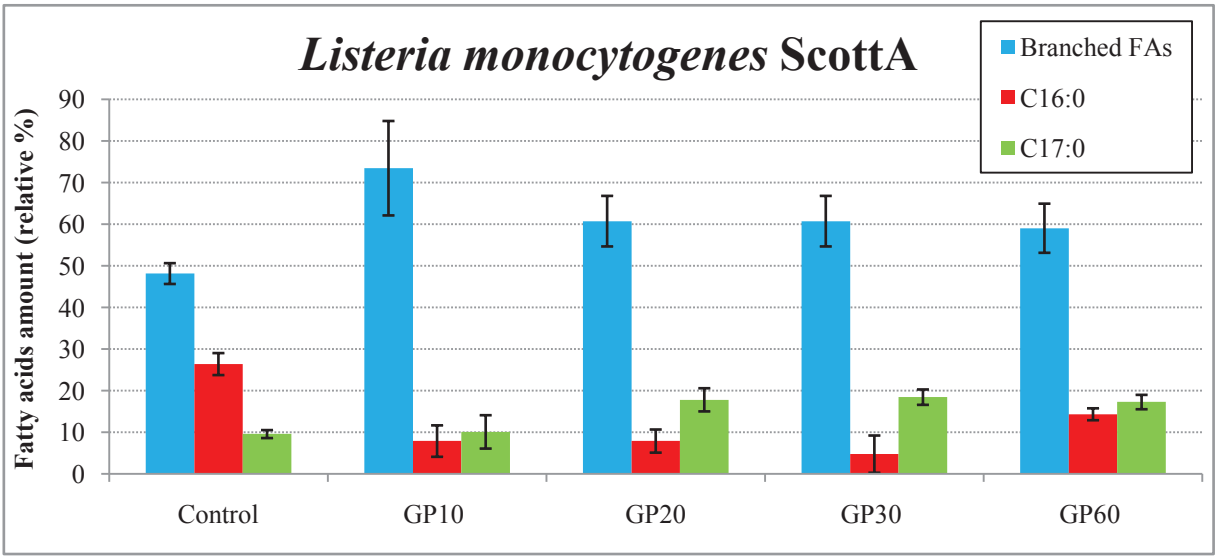

Figure 63. Main cellular fatty acids extracted from *Listeria monocytogenes* 56Ly cells after exposure to GP treatments. Fatty acid relative percentages were calculated with respect to the total fatty acid methyl esters. The results are means of two independent experiments.

Table 15. Volatile compounds (expressed as ppmEq) detected for *Listeria monocytogenes* 56Ly cells (into BHI broth) in untreated samples. The results are means of two independent experiments.

	Untreated						
Sampling after treatment (min)	**0**	**15**	**30**	**60**	**180**	**360**	**1440**
Pentanal	0.276	1.764	0.271	0.240	1.576	0.393	0.205
3-Methyl-butanal	0.367	2.326	0.363	0.311	2.109	0.544	0.273
3-Furaldehyde	0.223	0.205	0.254	0.230	0.219	0.269	0.269
Benzaldehyde	2.538	2.619	2.879	3.032	2.454	2.956	2.287
4-Methyl-benzaldehyde	0.756	0.329	0.744	1.060	0.256	0.429	0.767
Total Aldehydes	**4.159**	**7.243**	**4.512**	**4.873**	**6.614**	**4.590**	**3.800**
Acetone	0.678	1.089	0.633	0.667	1.258	1.017	1.098
2-Butanone	0.777	2.069	0.613	0.781	1.884	1.052	1.093
2,3-Butanedione	1.388	1.340	1.446	1.056	1.187	1.218	0.796
5-Amino-2,4(1H,3H)-Pyrimidinedione	0.481	0.388	0.338	0.439	0.449	0.476	0.384
2-Heptanone	0.279	0.227	0.207	0.253	0.249	0.266	0.229
4-Methyl-3-penten-2-one	1.040	0.839	0.889	0.867	0.890	0.982	0.813
2,6-Dimethyl-4-heptanone	3.812	3.727	3.709	3.129	3.484	4.100	3.731
5-Nonanone	1.312	1.293	1.252	1.244	1.193	1.416	1.217
3-Hydroxy-2-butanone	0.188	0.271	0.215	0.005	0.200	0.184	0.223
1-Hydroxy-2-propanone	0.034	0.065	0.036	0.019	0.064	0.034	0.041
Acetophenone	0.102	0.175	0.146	0.123	0.132	0.130	0.150
2,6-Dimethyl-4-hepten-3-one	2.261	3.213	3.333	2.281	2.662	2.667	3.277
4,5-Dimethyl-1,3-benzenediol	0.510	0.643	0.716	0.394	0.501	0.572	0.716
2-Pyrrolidinone	0.167	1.074	0.442	0.203	0.716	0.358	0.252
2-Hexadecanone	0.010	0.007	0.014	0.011	0.008	0.013	0.012
Total Ketones	**13.039**	**16.420**	**13.989**	**11.471**	**14.877**	**14.485**	**14.034**
Ethyl alcohol	3.560	3.242	3.428	3.401	3.236	3.369	3.035
1-Butanol	0.078	0.084	0.093	0.093	0.077	0.116	0.098
3-Methyl-2-heptanol	0.070	0.058	0.057	0.063	0.076	0.060	0.070
2,2-Dimethyl-4-octen-3-ol	0.183	0.226	0.242	0.112	0.178	0.199	0.176
2-Ethyl-1-hexanol	0.033	0.053	0.072	0.032	0.069	0.075	0.068
2-Furanmethanol	0.432	0.645	0.593	0.358	0.479	0.513	0.494
Phenylethyl alcohol	0.084	0.162	0.130	0.086	0.128	0.145	0.181
1-Dodecanol	0.022	0.022	0.083	0.019	0.029	0.086	0.012
2-Chloro-4-(1,1-dimethylpropyl)-phenol	0.866	1.299	1.529	1.005	1.298	1.647	2.191
Total Alcohols	**5.328**	**5.791**	**6.228**	**5.169**	**5.571**	**6.211**	**6.326**
Acetic acid	0.788	5.014	4.324	0.906	12.225	2.957	3.080
Butanoic acid	n.d	0.049	n.d	n.d	0.131	n.d	n.d
Hexanoic acid	0.015	0.108	0.054	0.016	0.164	0.036	0.030
3-Methyl-butanoic acid	0.102	0.622	0.556	0.110	0.527	0.487	0.326
Hepatanoic acid	n.d	0.002	n.d	n.d	n.d	n.d	n.d
Octanoic acid	0.020	0.144	0.110	0.018	0.168	0.057	0.040
Nonanoic acid	n.d	0.010	n.d	0.003	0.007	n.d	n.d
n-Decanoic acid	0.016	0.216	0.063	0.018	0.183	0.047	0.034
Dodecanoic acid	n.d	0.033	0.007	n.d	0.020	0.001	0.002
Tetradecanoic acid	0.000	0.143	0.070	0.002	0.057	0.004	0.002
Total Carboxylic acids	**0.968**	**6.433**	**5.227**	**1.074**	**13.659**	**3.650**	**3.572**
Cyclohexylmethyl hexyl ester of sulfurous acid	0.545	0.555	0.625	0.155	0.353	0.453	0.374
Bis(1-methylethyl) ester of hexanedioic acid	0.083	0.099	0.103	0.066	0.193	0.218	0.174
Total Esters	**0.628**	**0.655**	**0.728**	**0.222**	**0.546**	**0.671**	**0.548**
2-Methyl-furan	0.096	0.084	0.139	0.030	0.213	0.088	0.092
Guanidina	0.385	0.321	0.277	0.356	0.360	0.387	0.313
1R-alpha-Pinene	0.213	0.191	0.183	0.038	0.126	0.253	0.243
1-Chloro-5-methyl-hexane/1chloro-heptane	0.449	0.373	0.399	0.147	0.335	0.576	0.504
1,1'-Oxybis-Heptane	0.797	0.762	0.809	0.160	0.315	0.683	0.559
(1S)-3,7,7-trimethyl-bicyclo[4.1.0]hept-3-ene	0.469	0.275	0.436	0.067	0.235	0.427	0.257
3,5,5-Trimethyl-cyclohexene	0.151	0.152	0.155	0.042	0.100	0.132	0.108
Styrene	0.094	0.121	0.115	0.082	0.087	0.102	0.060
4-Pyridinamine	0.557	0.607	0.604	0.611	0.583	0.600	0.599
1,2,3-Trichloro-2-methyl-propane	0.198	0.229	0.245	0.058	0.207	0.303	0.330
1,2,4,5-Tetramethyl-benzene	0.428	0.560	0.590	0.213	0.405	0.591	0.509
3-Acetamidofuran	0.440	0.586	0.557	0.305	0.568	0.839	0.811
n.d.102/97	1.811	2.278	2.446	1.023	1.680	1.933	1.679
n.d.102/97	1.825	2.270	2.433	1.075	1.717	1.943	1.725
(E)-3-(2-butenyl)-thiophene	0.757	0.989	1.083	0.641	0.792	0.868	1.084
3-Phenyl-furan	0.214	0.373	0.362	0.286	0.250	0.230	0.150
4-Methyl-5-(2methyl-2-propenyl)-2(5H)-furanone	0.613	0.938	1.015	0.749	0.759	0.905	1.218
n.d.57	0.489	0.854	1.680	0.576	0.690	0.776	0.924
1-Dodecene	n.d	n.d	0.011	n.d	0.001	0.005	0.002
Total other molecules	**9.988**	**11.962**	**13.540**	**6.460**	**9.426**	**11.643**	**11.165**
Pyrazine	1.220	1.159	1.209	1.290	1.202	1.267	1.191
2,6-dimethyl-pyrazine	5.443	6.155	6.275	6.184	5.549	5.934	6.024
Ethyl-pyrazine	0.712	0.750	0.766	0.785	0.708	0.745	0.736
2-Ethyl-6-methyl-pyrazine	0.502	0.566	0.556	0.571	0.513	0.532	0.554
Trimethyl-pyrazine	0.657	0.787	0.752	0.741	0.689	0.706	0.742
2,3-Dimethyl-5-ethylpyrazine	0.646	0.760	0.732	0.736	0.654	0.692	0.727
4,5-Dihydro-3,5,5-trimethyl-1H-pyrazole	0.046	0.040	0.061	0.025	0.056	0.046	0.055
Total Pyrazine	**9.227**	**10.217**	**10.350**	**10.332**	**9.372**	**9.921**	**10.029**

n.d. under the detection level

Table 16. Volatile compounds (expressed as ppmEq) detected for *Listeria monocytogenes* 56Ly cells (into BHI broth) in relation to gas plasma treatment times (10 and 20 min). The results are means of two independent experiments.

	GP10							GP20						
Sampling after treatment (min)	**0**	**15**	**30**	**60**	**180**	**360**	**1440**	**0**	**15**	**30**	**60**	**180**	**360**	**1440**
Pentanal	0.200	0.354	0.282	0.187	0.264	0.612	0.786	0.161	0.347	0.273	0.177	0.246	0.377	1.920
3-Methyl-butanal	0.276	0.484	0.377	0.254	0.351	0.813	1.008	0.223	0.455	0.367	0.245	0.335	0.501	2.382
3-Furaldehyde	0.230	0.237	0.262	0.296	0.270	0.320	0.210	0.216	0.235	0.251	0.202	0.213	0.219	0.159
Benzaldehyde	2.140	2.408	2.627	2.916	2.439	3.203	2.148	2.323	2.556	2.622	2.211	2.404	2.465	2.098
4-Methyl-benzaldehyde	0.827	0.397	0.904	1.243	0.726	1.069	0.678	1.077	1.180	1.295	1.132	0.752	0.902	0.724
Total Aldehydes	**3.673**	**3.880**	**4.452**	**4.895**	**4.050**	**6.017**	**4.830**	**4.000**	**4.773**	**4.809**	**3.968**	**3.949**	**4.465**	**7.282**
Acetone	0.543	0.558	0.489	0.521	0.600	0.913	1.130	0.462	0.489	0.438	0.525	0.641	0.760	1.321
2-Butanone	0.608	0.468	0.444	0.513	0.622	0.814	2.097	0.491	0.378	0.360	0.527	0.612	0.712	2.680
2,3-Butanedione	1.449	1.263	1.233	1.335	1.065	1.076	0.728	1.664	0.957	1.067	1.348	1.087	0.951	0.919
5-Amino-2,4(1H,3H)-Pyrimidinedione	0.462	0.357	0.321	0.288	0.300	0.391	0.426	0.421	0.295	0.187	0.416	0.488	0.456	0.407
2-Heptanone	0.268	0.224	0.194	0.180	0.186	0.227	0.237	0.268	0.176	0.131	0.266	0.262	0.243	0.031
4-Methyl-3-penten-2-one	1.047	0.853	0.718	0.926	0.831	0.891	0.669	1.183	0.689	0.577	1.072	0.923	0.821	0.586
2,6-Dimethyl-4-heptanone	3.665	3.520	3.439	3.799	3.776	3.726	3.953	3.751	3.293	3.440	3.705	3.663	3.738	4.219
5-Nonanone	1.438	1.189	1.186	1.160	1.168	1.330	1.246	1.298	1.130	0.917	1.293	1.385	1.366	1.065
3-Hydroxy-2-butanone	0.163	0.179	0.199	0.182	0.162	0.174	0.140	0.158	0.162	0.116	0.184	0.184	0.203	0.193
1-Hydroxy-2-propanone	0.031	0.030	0.038	0.079	0.042	0.042	0.115	0.028	0.039	0.037	0.025	0.059	0.050	0.048
Acetophenone	0.270	0.363	0.376	0.360	0.303	0.406	0.365	0.534	0.678	0.657	0.509	0.514	0.523	0.552
2,6-Dimethyl-4-hepten-3-one	2.697	3.248	3.518	3.794	3.184	3.987	1.864	3.034	3.379	4.308	2.689	2.981	2.426	1.690
4,5-Dimethyl-1,3-benzenediol	0.538	0.590	0.642	0.720	0.572	0.739	0.437	0.632	0.611	0.747	0.595	0.538	0.506	0.351
2-Pyrrolidinone	0.182	0.408	0.412	0.312	0.273	0.578	0.632	0.241	0.513	0.419	0.204	0.278	0.350	2.285
2-Hexadecanone	0.004	0.017	0.022	0.017	0.009	0.008	0.008	0.005	0.018	0.016	0.010	0.009	0.011	0.003
Total Ketones	**13.366**	**13.267**	**13.231**	**14.188**	**13.093**	**15.302**	**14.048**	**14.170**	**12.808**	**13.418**	**13.369**	**13.622**	**13.114**	**16.351**
Ethyl alcohol	3.027	3.261	2.910	2.905	2.901	2.877	2.723	2.643	2.591	2.499	2.759	2.581	2.408	2.574
1-Butanol	0.135	0.072	0.061	0.073	0.072	0.084	0.074	0.077	0.055	0.044	0.067	0.060	0.076	0.044
3-Methyl-2-heptanol	0.073	0.055	0.036	0.052	0.041	0.057	0.056	0.056	0.035	0.031	0.072	0.058	0.060	0.050
2,2-Dimethyl-4-octen-3-ol	0.205	0.218	0.223	0.249	0.206	0.255	0.079	0.231	0.205	0.239	0.216	0.167	0.118	0.118
2-Ethyl-1-hexanol	0.050	0.046	0.072	0.082	0.062	0.061	0.052	0.055	0.056	0.076	0.050	0.053	0.082	0.068
2-Furanmethanol	0.442	0.537	0.574	0.562	0.486	0.608	0.331	0.454	0.486	0.515	0.424	0.399	0.363	0.441
Phenylethyl alcohol	0.084	0.137	0.148	0.112	0.114	0.187	0.180	0.094	0.139	0.124	0.094	0.118	0.134	0.165
1-Dodecanol	0.012	0.006	0.013	0.015	0.009	0.009	0.010	0.026	0.036	0.024	0.011	0.010	0.009	0.009
2-Chloro-4-(1,1-dimethylpropyl)-phenol	0.888	1.255	1.372	1.175	1.022	1.804	1.802	1.047	1.437	1.436	1.197	0.961	1.277	1.529
Total Alcohols	**4.916**	**5.588**	**5.408**	**5.225**	**4.914**	**5.943**	**5.307**	**4.681**	**5.041**	**4.989**	**4.888**	**4.408**	**4.528**	**4.999**
Acetic acid	1.138	3.149	3.155	1.425	2.227	3.767	4.859	1.438	2.801	2.299	1.421	2.755	3.700	9.302
Butanoic acid	0.010	n.d	0.007	n.d	n.d	0.018	0.022	0.000	n.d	n.d	n.d	n.d	n.d	0.067
Hexanoic acid	0.025	0.053	0.059	n.d	0.024	0.045	0.051	0.000	0.057	0.057	0.019	0.030	0.032	0.094
3-Methyl-butanoic acid	0.104	0.603	0.621	0.233	0.353	0.656	0.449	0.430	0.579	0.459	0.135	0.482	0.483	0.452
Hepatanoic acid	0.018	n.d	n.d	0.003	n.d	n.d	n.d	0.021	n.d	0.041	n.d	n.d	0.004	0.005
Octanoic acid	0.018	0.071	0.077	0.041	0.028	0.068	0.047	0.030	0.011	n.d	0.020	0.030	0.043	0.115
Nonanoic acid	0.003	0.008	0.009	0.006	n.d	0.007	0.008	0.027	0.016	0.014	0.003	0.011	0.014	0.009
n-Decanoic acid	0.018	0.067	0.085	0.041	0.043	0.092	0.071	0.022	0.118	0.083	0.018	0.034	0.044	0.162
Dodecanoic acid	0.005	0.016	0.024	n.d	0.011	0.015	0.015	n.d	0.028	0.022	n.d	n.d	0.006	0.025
Tetradecanoic acid	n.d	0.055	0.086	0.001	0.004	0.042	0.052	0.001	0.065	0.008	0.001	0.001	0.041	0.070
Total Carboxylic acids	**1.338**	**4.023**	**4.172**	**1.751**	**2.731**	**4.786**	**5.651**	**2.002**	**3.675**	**3.015**	**1.643**	**3.395**	**4.419**	**10.511**
Cyclohexylmethyl hexyl ester of sulfurous acid	0.514	0.538	0.493	0.596	0.483	0.509	0.115	0.681	0.439	0.524	0.581	0.359	0.211	0.199
Bis(1-methylethyl) ester of hexanedioic acid	0.082	0.088	0.113	0.106	0.083	0.129	0.143	0.091	0.083	0.097	0.085	0.097	0.111	0.121
Total Esters	**0.596**	**0.627**	**0.607**	**0.701**	**0.566**	**0.638**	**0.258**	**0.772**	**0.522**	**0.621**	**0.666**	**0.456**	**0.323**	**0.320**
2-Methyl-furan	0.052	0.052	0.074	0.122	0.078	0.042	0.060	0.057	0.016	0.099	0.063	0.035	0.052	0.047
Guanidina	0.377	0.288	0.266	0.246	0.256	0.323	0.344	0.345	0.248	0.165	0.339	0.396	0.367	0.332
1R-alpha-Pinene	0.134	0.194	0.121	0.117	0.115	0.148	0.172	0.204	0.043	0.110	0.212	0.148	0.126	0.168
1-Chloro-5-methyl-hexane/1chloro-heptane	0.331	0.348	0.285	0.334	0.294	0.327	0.333	0.424	0.146	0.262	0.408	0.326	0.309	0.295
1,1'-Oxybis-Heptane	0.668	0.732	0.603	0.679	0.197	0.638	0.195	0.975	0.317	0.607	0.797	0.490	0.337	0.111
(1S)-3,7,7-trimethyl-bicyclo[4.1.0]hept-3-ene	0.341	0.271	0.243	0.287	0.245	0.357	0.125	0.450	0.143	0.262	0.444	0.297	0.189	0.145
3,5,5-Trimethyl-cyclohexene	0.136	0.139	0.120	0.153	0.127	0.129	0.045	0.186	0.101	0.125	0.154	0.099	0.066	0.026
Styrene	0.125	0.141	0.137	0.124	0.090	0.084	0.053	0.140	0.156	0.137	0.110	0.093	0.080	0.058
4-Pyridinamine	0.570	0.583	0.600	0.681	0.561	0.674	0.488	0.573	0.551	0.540	0.511	0.486	0.498	0.419
1,2,3-Trichloro-2-methyl-propane	0.208	0.220	0.220	0.243	0.204	0.264	0.261	0.242	0.076	0.233	0.223	0.192	0.193	0.270
1,2,4,5-Tetramethyl-benzene	0.415	0.532	0.483	0.531	0.446	0.512	0.313	0.579	0.431	0.562	0.495	0.401	0.371	0.280
3-Acetamidofuran	0.460	0.494	0.470	0.499	0.435	0.679	0.652	0.542	0.395	0.485	0.469	0.488	0.586	0.549
n.d.102/97	2.037	2.162	2.266	2.661	2.164	2.419	0.668	2.386	2.225	2.661	2.178	1.604	1.081	0.884
n.d.102/97	2.014	2.161	2.274	2.590	2.104	2.430	0.750	2.354	2.168	2.575	2.138	1.637	1.121	0.874
(E)-3-(2-butenyl)-thiophene	0.834	0.939	1.020	1.129	0.908	1.169	0.653	0.962	0.975	1.210	0.888	0.863	0.779	0.541
3-Phenyl-furan	0.156	0.283	0.336	0.244	0.184	0.195	0.122	0.116	0.184	0.191	0.117	0.114	0.104	0.083
4-Methyl-5-(2methyl-2-propenyl)-2(5H)-furanone	0.628	0.906	0.925	0.932	0.664	1.115	0.929	0.592	0.964	1.108	0.613	0.664	0.753	0.803
n.d.57	0.491	0.761	0.781	0.727	0.544	0.942	0.781	0.472	0.849	0.909	0.489	0.543	0.646	0.731
1-Dodecene	n.d	n.d	0.002	n.d	0.003	0.005	0.006	n.d	n.d	0.003	n.d	0.001	0.005	0.003
Total other molecules	**9.977**	**11.206**	**11.228**	**12.297**	**9.619**	**12.451**	**6.951**	**11.599**	**9.989**	**12.243**	**10.648**	**8.879**	**7.663**	**6.619**
Pyrazine	1.238	1.119	1.126	1.389	1.162	1.287	0.919	1.178	1.007	0.967	1.043	0.974	0.980	0.687
2,6-dimethyl-pyrazine	5.397	6.224	6.352	6.654	5.307	6.979	4.820	5.665	5.803	5.593	5.160	4.861	4.907	4.103
Ethyl-pyrazine	0.716	0.767	0.761	0.836	0.660	0.851	0.588	0.706	0.665	0.617	0.640	0.593	0.581	0.469
2-Ethyl-6-methyl-pyrazine	0.480	0.566	0.566	0.547	0.426	0.617	0.433	0.481	0.480	0.420	0.448	0.411	0.402	0.364
Trimethyl-pyrazine	0.653	0.797	0.787	0.749	0.583	0.894	0.598	0.676	0.685	0.596	0.627	0.585	0.592	0.532
2,3-Dimethyl-5-ethylpyrazine	0.712	0.887	0.858	0.793	0.597	0.995	0.626	0.681	0.689	0.580	0.631	0.583	0.577	0.500
4,5-Dihydro-3,5,5-trimethyl-1H-pyrazole	0.039	0.041	0.046	0.061	0.048	0.049	0.034	0.049	0.046	0.049	0.045	0.043	0.035	0.036
Total Pyrazine	**9.235**	**10.401**	**10.496**	**11.028**	**8.783**	**11.672**	**8.018**	**9.436**	**9.374**	**8.822**	**8.594**	**8.050**	**8.073**	**6.692**

n.d. under the detection level

Table 17. Volatile compounds (expressed as ppmEq) detected for *Listeria monocytogenes* 56Ly cells (into BHI broth) in relation to gas plasma treatment times (30 and 60 min). The results are means of two independent experiments.

	GP30							GP60						
Sampling after treatment (min)	**0**	**15**	**30**	**60**	**180**	**360**	**1440**	**0**	**15**	**30**	**60**	**180**	**360**	**1440**
Pentanal	0.218	2.114	0.442	0.162	0.248	0.315	0.708	0.137	0.328	0.225	0.861	1.410	0.308	0.466
3-Methyl-butanal	0.297	2.739	0.593	0.220	0.340	0.426	0.902	0.193	0.437	0.310	1.131	1.824	0.407	0.604
3-Furaldehyde	0.191	0.179	0.196	0.214	0.242	0.281	0.205	0.122	0.154	0.168	0.126	0.141	0.194	0.168
Benzaldehyde	2.430	2.482	2.481	2.528	3.046	3.496	2.555	2.244	2.781	3.090	2.463	2.987	3.384	2.867
4-Methyl-benzaldehyde	1.156	0.854	0.763	1.158	0.982	0.763	0.495	1.182	1.258	1.464	1.115	0.981	0.980	0.488
Total Aldehydes	**4.292**	**8.369**	**4.475**	**4.282**	**4.858**	**5.280**	**4.865**	**3.879**	**4.958**	**5.257**	**5.695**	**7.343**	**5.273**	**4.593**
Acetone	0.441	1.034	0.478	0.426	0.620	0.754	0.909	0.343	0.413	0.381	0.733	0.966	0.599	0.650
2-Butanone	0.445	2.243	0.350	0.479	0.614	0.621	0.559	0.302	0.263	0.277	0.368	1.379	0.459	0.482
2,3-Butanedione	3.071	1.905	1.880	2.407	1.738	1.342	0.889	3.494	2.230	2.271	3.135	2.461	2.085	1.859
5-Amino-2,4(1H,3H)-Pyrimidinedione	0.448	0.260	0.285	0.363	0.395	0.349	0.398	0.401	0.189	0.221	0.462	0.450	0.386	0.457
2-Heptanone	0.042	0.168	0.188	0.227	0.234	0.222	0.226	0.249	0.133	0.150	0.267	0.230	0.222	0.238
4-Methyl-3-penten-2-one	1.015	0.652	0.723	0.977	0.877	0.935	0.683	1.029	0.506	0.698	0.950	0.787	0.841	0.792
2,6-Dimethyl-4-heptanone	3.538	3.397	3.766	3.553	3.643	3.797	4.470	3.561	3.384	3.698	3.645	3.750	3.649	3.983
5-Nonanone	1.312	1.041	1.155	1.232	1.298	1.263	1.231	1.314	0.924	1.044	1.338	1.361	1.316	1.365
3-Hydroxy-2-butanone	1.939	0.177	0.206	0.220	0.223	0.236	0.214	0.204	0.212	0.255	0.307	0.391	0.404	0.397
1-Hydroxy-2-propanone	0.010	0.043	0.045	0.036	0.044	0.053	0.039	0.016	0.029	0.135	0.056	0.055	0.036	0.047
Acetophenone	0.669	0.873	0.822	0.682	0.721	0.827	0.754	1.194	1.639	1.793	1.296	1.370	1.493	1.562
2,6-Dimethyl-4-hepten-3-one	2.935	2.419	3.235	2.975	3.500	4.988	2.908	2.373	3.404	3.941	2.578	3.275	4.214	2.552
4,5-Dimethyl-1,3-benzenediol	0.618	0.434	0.584	0.564	0.654	0.804	0.538	0.543	0.562	0.726	0.423	0.521	0.737	0.479
2-Pyrrolidinone	0.257	0.942	0.535	0.302	0.337	0.452	0.682	0.337	0.665	0.590	0.603	1.058	0.510	0.470
2-Hexadecanone	0.007	0.008	0.014	0.017	0.009	0.014	0.011	0.008	0.016	0.019	0.014	0.009	0.015	0.010
Total Ketones	**16.747**	**15.595**	**14.266**	**14.458**	**14.907**	**16.656**	**14.512**	**15.368**	**14.568**	**16.196**	**16.177**	**18.064**	**16.966**	**15.342**
Ethyl alcohol	2.547	2.481	2.437	2.196	2.318	2.305	2.010	1.295	1.517	1.441	1.275	1.135	1.161	1.142
1-Butanol	0.071	0.037	0.039	0.080	0.057	0.061	0.064	0.043	0.026	0.027	0.040	0.030	0.040	0.034
3-Methyl-2-heptanol	0.081	0.026	0.041	0.049	0.050	0.048	0.043	0.072	0.037	0.037	0.062	0.046	0.048	0.040
2,2-Dimethyl-4-octen-3-ol	0.202	0.154	0.224	0.213	0.228	0.321	0.142	0.170	0.180	0.261	0.136	0.128	0.245	0.171
2-Ethyl-1-hexanol	0.054	0.064	0.100	0.071	0.076	0.098	0.103	0.086	0.095	0.094	0.054	0.077	0.079	0.175
2-Furanmethanol	0.395	0.560	0.493	0.453	0.490	0.616	0.372	0.298	0.340	0.456	0.297	0.327	0.424	0.338
Phenylethyl alcohol	0.077	0.159	0.056	0.093	0.120	0.183	0.184	0.076	0.113	0.132	0.102	0.141	0.138	0.145
1-Dodecanol	0.022	0.023	0.031	0.017	0.017	0.021	0.016	0.015	0.018	0.013	0.013	0.013	0.011	0.011
2-Chloro-4-(1,1-dimethylpropyl)-phenol	1.117	1.375	1.242	0.847	1.227	1.817	2.135	0.997	1.460	1.660	0.791	1.209	1.756	1.648
Total Alcohols	**4.566**	**4.879**	**4.663**	**4.019**	**4.584**	**5.471**	**5.067**	**3.050**	**3.788**	**4.121**	**2.771**	**3.105**	**3.904**	**3.704**
Acetic acid	1.206	7.111	3.352	1.539	3.308	3.579	6.080	1.608	3.596	3.200	3.497	6.896	3.446	7.197
Butanoic acid	n.d.	0.098	0.032	n.d.	n.d.	0.013	0.037	n.d.	n.d.	n.d.	0.056	0.086	n.d.	0.059
Hexanoic acid	0.023	0.160	0.064	n.d.	0.033	n.d.	0.067	0.028	0.073	n.d.	0.121	0.137	0.065	0.098
3-Methyl-butanoic acid	0.425	0.634	0.581	0.209	0.575	0.646	0.483	0.500	0.719	0.726	0.734	0.822	0.726	0.652
Hepatanoic acid	n.d.	0.009	n.d.	0.024	n.d.	0.049	n.d.	0.009	n.d.	0.054	0.029	0.033	0.013	0.012
Octanoic acid	0.028	0.160	0.079	0.026	0.046	0.058	0.103	0.034	0.125	0.079	0.146	0.189	0.058	0.081
Nonanoic acid	0.006	0.033	0.014	0.007	0.010	0.014	0.019	0.026	0.045	0.028	0.083	0.112	0.023	0.027
n-Decanoic acid	0.026	0.310	0.144	0.032	0.043	0.050	0.108	0.025	0.121	0.058	0.145	0.208	0.046	0.060
Dodecanoic acid	n.d.	0.041	0.027	n.d.	0.009	0.009	0.014	n.d.	0.031	n.d.	0.069	0.031	n.d.	0.008
Tetradecanoic acid	n.d.	0.124	0.124	n.d.	0.064	n.d.	0.042	n.d.	0.118	0.005	0.104	0.118	n.d.	0.054
Total Carboxylic acids	**1.714**	**8.680**	**4.416**	**1.837**	**4.087**	**4.417**	**6.952**	**2.230**	**4.828**	**4.151**	**4.983**	**8.633**	**4.377**	**8.248**
Cyclohexylmethyl hexyl ester of sulfurous acid	0.475	0.313	0.529	0.538	0.547	0.739	0.227	0.456	0.261	0.626	0.322	0.220	0.497	0.394
Bis(1-methylethyl) ester of hexanedioic acid	0.095	0.092	0.108	0.082	0.119	0.174	0.160	0.093	0.092	0.110	0.084	0.120	0.148	0.140
Total Esters	**0.570**	**0.404**	**0.637**	**0.619**	**0.666**	**0.913**	**0.388**	**0.548**	**0.353**	**0.735**	**0.406**	**0.340**	**0.646**	**0.534**
2-Methyl-furan	0.080	0.092	0.070	0.072	0.033	0.039	0.049	0.115	0.109	0.078	0.069	0.048	0.066	0.088
Guanidina	0.365	0.238	0.233	0.301	0.321	0.286	0.321	0.330	0.168	0.189	0.378	0.369	0.314	0.363
1R-alpha-Pinene	0.168	0.058	0.140	0.133	0.196	0.190	0.166	0.171	0.091	0.106	0.171	0.131	0.129	0.172
1-Chloro-5-methyl-hexane/1chloro-heptane	0.321	0.186	0.273	0.332	0.356	0.410	0.360	0.383	0.217	0.234	0.330	0.317	0.275	0.350
1,1'-Oxybis-Heptane	0.602	0.082	0.220	0.691	0.752	0.980	0.158	0.659	0.118	0.342	0.491	0.345	0.588	0.541
(1S)-3,7,7-trimethyl-bicyclo[4.1.0]hept-3-ene	0.249	0.136	0.214	0.272	0.280	0.413	0.253	0.317	0.130	0.279	0.249	0.217	0.304	0.323
3,5,5-Trimethyl-cyclohexene	0.123	0.086	0.128	0.142	0.145	0.184	0.077	0.124	0.075	0.148	0.098	0.076	0.122	0.112
Styrene	0.150	0.154	0.186	0.147	0.119	0.123	0.079	0.174	0.177	0.211	0.138	0.186	0.139	0.161
4-Pyridinamine	0.481	0.534	0.482	0.487	0.480	0.502	0.390	0.294	0.308	0.321	0.301	0.323	0.305	0.290
1,2,3-Trichloro-2-methyl-propane	0.204	0.155	0.222	0.212	0.234	0.340	0.289	0.188	0.097	0.111	0.168	0.182	0.244	0.282
1,2,4,5-Tetramethyl-benzene	0.464	0.349	0.498	0.447	0.522	0.713	0.406	0.458	0.363	0.561	0.314	0.357	0.524	0.416
3-Acetamidofuran	0.474	0.368	0.491	0.468	0.574	0.918	0.767	0.472	0.434	0.572	0.447	0.619	0.712	0.715
n.d.102/97	2.014	1.485	2.368	2.226	2.224	3.066	1.379	1.767	1.761	2.800	1.342	1.151	2.351	1.682
n.d.102/97	2.001	1.456	2.281	2.193	2.225	3.056	1.374	1.726	1.709	2.714	1.356	1.176	2.326	1.661
(E)-3-(2-butenyl)-thiophene	0.931	0.701	0.938	0.887	1.027	1.342	0.858	0.799	0.933	1.151	0.700	0.878	1.198	0.763
3-Phenyl-furan	0.109	0.167	0.167	0.110	0.113	0.142	0.118	0.056	0.103	0.109	0.076	0.102	0.104	0.104
4-Methyl-5-(2methyl-2-propenyl)-2(5H)-furanone	0.713	0.850	0.210	0.779	0.926	1.130	1.152	0.793	1.034	1.061	0.555	0.897	1.120	1.011
n.d.57	0.560	0.814	0.789	0.615	0.746	0.926	0.942	0.656	0.927	0.930	0.567	0.848	0.922	0.852
1-Dodecene	n.d.	n.d.	0.009	n.d.	n.d.	0.005	0.011	n.d.	0.020	0.014	0.026	0.044	n.d.	0.011
Total other molecules	**10.009**	**7.913**	**9.919**	**10.514**	**11.271**	**14.767**	**9.149**	**9.482**	**8.774**	**11.931**	**7.777**	**8.267**	**11.743**	**9.897**
Pyrazine	1.026	0.974	0.887	1.029	0.993	0.978	0.712	0.668	0.611	0.645	0.647	0.632	0.607	0.572
2,6-dimethyl-pyrazine	4.800	5.437	5.040	4.818	4.888	5.337	4.126	2.762	3.090	3.428	2.836	2.961	3.108	2.709
Ethyl-pyrazine	0.591	0.612	0.568	0.587	0.582	0.619	0.461	0.325	0.320	0.363	0.325	0.323	0.337	0.296
2-Ethyl-6-methyl-pyrazine	0.414	0.461	0.427	0.400	0.416	0.459	0.354	0.210	0.219	0.253	0.226	0.240	0.244	0.217
Trimethyl-pyrazine	0.573	0.667	0.627	0.551	0.588	0.673	0.509	0.323	0.345	0.402	0.354	0.383	0.390	0.328
2,3-Dimethyl-5-ethylpyrazine	0.572	0.644	0.623	0.538	0.580	0.681	0.506	0.274	0.290	0.343	0.294	0.317	0.341	0.282
4,5-Dihydro-3,5,5-trimethyl-1H-pyrazole	0.043	0.047	0.051	0.041	0.052	0.073	0.062	0.037	0.064	0.086	0.038	0.052	0.071	0.051
Total Pyrazine	**8.019**	**8.843**	**8.223**	**7.962**	**8.099**	**8.820**	**6.730**	**4.600**	**4.939**	**5.521**	**4.719**	**4.909**	**5.099**	**4.455**

n.d. under detectable level

Table 18. Volatile compounds (expressed as ppmEq) detected for *Listeria monocytogenes* ScottA cells (into BHI broth) in relation to gas plasma treatment times (30 and 60 min). The results are means of two independent experiments.

	Untreated						
Sampling after treatment (min)	**0**	**15**	**30**	**60**	**180**	**360**	**1440**
Pentanal	0.198	0.233	0.341	0.367	0.258	0.271	0.269
3-Methyl-butanal	0.263	0.322	0.412	0.465	0.324	0.350	0.343
3-(Methylthio)-propanal	0.356	0.620	0.599	0.549	0.704	0.984	0.752
3-Furaldehyde	0.432	0.478	0.396	0.394	0.435	0.559	0.316
Benzaldehyde	3.583	4.732	4.122	3.741	5.674	7.953	8.751
4-Methyl-benzaldehyde	0.994	1.646	1.650	1.595	2.068	3.394	3.092
Total Aldehydes	**5.826**	**8.031**	**7.519**	**7.111**	**9.463**	**13.511**	**13.523**
Acetone	0.518	0.701	0.640	0.619	0.726	0.893	1.643
2-Butanone	0.330	0.435	0.480	0.414	0.648	0.633	1.195
2,3-Butanedione	1.689	1.751	2.209	1.868	2.024	1.895	2.503
5-Amino-2,4(1H,3H)-Pyrimidinedione	1.604	1.239	2.453	1.915	1.872	1.295	1.525
3,4-Dimethyl-2-eptanone	0.004	0.001	0.009	0.003	0.006	0.005	0.030
2-Heptanone	0.292	0.026	0.407	0.016	0.321	0.236	0.357
4-Methyl-3-penten-2-one	0.521	0.460	0.609	0.404	0.565	0.408	0.765
2,6-Dimethyl-4-heptanone	4.309	4.969	5.802	5.460	6.036	4.664	6.190
5-Nonanone	2.150	3.219	3.907	3.892	3.942	2.519	3.947
3-Hydroxy-2-butanone	0.894	0.128	0.864	1.109	0.871	1.387	1.131
1-Hydroxy-2-propanone	0.662	0.502	0.599	0.795	0.624	0.933	0.863
Acetophenone	0.132	0.149	0.141	0.129	0.175	0.232	0.330
2,6-Dimethyl-4-hepten-3-one	0.020	0.087	0.005	0.027	n.d.	0.091	n.d.
2-Pyrrolidinone	n.d.	0.000	0.000	n.d.	0.001	n.d.	0.002
Total Ketones	**13.124**	**13.669**	**18.127**	**16.652**	**17.811**	**15.191**	**20.481**
Ethyl alcohol	3.714	4.746	2.953	3.996	3.213	3.961	3.051
1-Butanol	0.044	0.043	0.047	0.040	0.058	0.046	0.056
3-Methyl-2-heptanol	n.d.	0.012	0.039	0.032	0.002	0.033	0.044
2,2-Dimethyl-4-octen-3-ol	0.169	0.150	0.113	0.084	0.156	0.103	0.214
2-Ethyl-1-hexanol	0.016	0.023	0.020	0.023	0.030	0.063	0.065
2-Furanmethanol	0.424	0.420	0.346	0.244	0.426	0.413	0.527
Phenylethyl alcohol	0.083	0.118	0.101	0.099	0.116	0.210	0.196
1-Dodecanol	n.d.	n.d.	n.d.	0.002	n.d.	0.004	0.001
Total Alcohols	**4.451**	**5.511**	**3.620**	**4.520**	**4.001**	**4.834**	**4.154**
Acetic acid	0.565	0.924	1.343	1.443	0.834	1.135	1.146
Butanoic acid	n.d.	n.d.	n.d.	0.006	n.d.	0.018	n.d.
3-Methyl-butanoic acid	n.d.	0.005	0.011	0.006	0.005	0.011	0.005
Hepatanoic acid	0.004	0.010	0.017	0.012	0.012	0.019	0.014
Octanoic acid	0.007	0.015	0.024	0.020	0.013	0.025	0.016
Nonanoic acid	0.005	0.012	0.020	0.005	0.012	0.024	0.014
n-Decanoic acid	0.000	0.008	0.015	0.022	0.012	0.013	0.015
Dodecanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total Carboxylic acids	**0.581**	**0.975**	**1.431**	**1.538**	**0.890**	**1.245**	**1.231**
Cyclohexylmethyl hexyl ester of sulfurous acid	0.364	0.212	0.202	0.093	0.330	0.104	0.493
Cyclohexylmethyl heptyl ester of sulfurous acid	2.865	2.338	2.563	1.848	3.341	3.337	4.076
Bis(1-methylethyl) ester of hexanedioic acid	0.071	0.078	0.070	0.053	0.096	0.147	0.165
Butyl ester of butanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total Esters	**4.461**	**4.577**	**5.697**	**5.072**	**5.546**	**6.077**	**7.195**
Guanidina	1.151	0.920	1.776	1.395	1.369	0.969	1.124
6-Azathymine	0.780	0.612	1.169	0.926	0.932	0.644	0.890
1R-alpha-Pinene	0.102	0.065	0.111	0.053	0.128	0.063	0.220
1-chloro-5-methyl-hexane/1chloro-heptane	0.207	0.200	0.267	0.158	0.319	0.254	0.673
Dimethyl disulfide	0.006	0.006	0.006	0.004	0.010	0.011	0.031
1,1'-Oxybis-Heptane	0.011	0.010	0.003	0.005	0.010	0.003	0.110
(1S)-3,7,7-Trimethyl-bicyclo[4.1.0]hept-3-ene	0.027	0.017	0.019	n.d.	0.020	0.010	0.062
3,5,5-Trimethyl-cyclohexene	0.082	n.d.	0.051	0.004	0.078	0.032	0.141
1,2,3-Trimethyl-benzene	0.384	0.002	0.399	0.001	0.402	0.454	0.590
Styrene	0.055	n.d.	0.054	n.d.	0.056	0.064	0.073
4-Pyridinamine	0.599	0.017	0.598	0.014	0.609	0.792	0.658
1,2,3-Trichloro-2-methyl-propane	0.115	0.103	0.146	0.093	0.148	0.157	0.353
1,2,4,5-Tetramethyl-benzene	0.288	0.283	0.229	0.138	0.361	0.276	0.645
3-Acetamidofuran	0.328	0.356	0.280	0.204	0.399	0.582	0.800
n.d.102/97	1.726	1.349	1.072	0.641	1.626	0.908	2.604
4,5-Dimethyl-1,3-benzenediol	0.495	0.473	0.382	0.265	0.559	0.554	0.800
(E)-3-(2-Butenyl)-thiophene	0.792	0.822	0.649	0.449	0.914	0.922	1.243
4,5-Dihydro-3,5,5-trimethyl-1H-pyrazole	0.140	0.137	0.136	0.107	0.146	0.219	0.095
3-Phenyl-furan	0.304	0.356	0.310	0.264	0.275	0.322	0.102
4-Methyl-5-(2methyl-2-propenyl)-2(5H)-furanone	0.707	0.854	0.711	0.535	0.889	1.573	1.330
n.d.57	0.504	0.619	0.525	0.407	0.636	1.123	0.955
2-Chloro-4-(1,1-dimethylpropyl)-phenol	1.031	1.283	1.073	0.818	1.945	2.678	2.301
2-Methyl-furan	0.066	0.049	0.056	0.035	0.038	0.093	0.177
Total other molecules	**9.898**	**8.532**	**10.022**	**6.515**	**11.868**	**12.704**	**15.977**
Pyrazine	1.026	0.001	0.982	0.003	0.985	1.222	1.044
2,6-Dimethyl-pyrazine	5.743	6.980	6.222	5.646	6.229	8.495	6.797
Ethyl-pyrazine	0.712	0.828	0.784	0.687	0.748	0.929	0.850
2-Ethyl-6-methyl-pyrazine	0.521	0.620	0.640	0.556	0.608	0.791	0.737
Trimethyl-pyrazine	0.702	0.849	0.875	0.775	0.838	1.129	1.020
2,3-Dimethyl-5-ethylpyrazine	0.841	1.044	1.114	0.981	1.078	1.463	1.273
Total Pyrazine	**9.545**	**10.321**	**10.617**	**8.648**	**10.487**	**14.029**	**11.720**

n.d. under the detection level

Table 19. Volatile compounds (expressed as ppmEq) detected for *Listeria monocytogenes* ScottA cells (into BHI broth) in relation to gas plasma treatment times (10 and 20 min). The results are means of two independent experiments.

	GP10							GP20						
Sampling after treatment (min)	**0**	**15**	**30**	**60**	**180**	**360**	**1440**	**0**	**15**	**30**	**60**	**180**	**360**	**1440**
Pentanal	0.125	0.217	0.237	0.258	0.220	1.758	0.314	0.655	0.129	0.160	0.154	0.171	0.221	0.368
3-Methyl-butanal	0.158	0.274	0.303	0.332	0.279	2.185	0.386	0.835	0.168	0.202	0.191	0.221	0.285	0.444
3-(Methylthio)-propanal	0.426	0.595	0.954	0.920	0.706	0.953	0.487	0.269	0.178	0.224	0.343	0.413	0.411	0.330
3-Furaldehyde	0.260	0.596	0.560	0.479	0.376	0.252	0.205	0.302	0.206	0.215	0.234	0.290	0.312	0.251
Benzaldehyde	1.969	4.962	6.269	5.467	5.011	4.742	5.621	2.883	1.750	1.763	2.503	3.592	4.686	4.763
4-Methyl-benzaldehyde	1.425	2.655	3.754	3.784	2.713	2.495	2.157	1.140	0.776	0.907	1.209	1.632	1.884	2.027
Total Aldehydes	**4.364**	**9.298**	**12.078**	**11.242**	**9.305**	**12.386**	**9.170**	**6.083**	**3.208**	**3.471**	**4.633**	**6.318**	**7.798**	**8.183**
Acetone	0.424	0.662	0.857	0.760	0.672	1.381	1.326	0.703	0.395	0.452	0.559	0.700	0.912	1.034
2-Butanone	0.262	0.510	0.521	0.558	0.511	0.106	0.907	0.383	0.254	0.310	0.423	0.509	0.549	0.805
2,3-Butanedione	0.468	2.095	1.744	2.849	2.198	2.447	2.416	2.560	1.945	1.954	0.640	1.542	2.002	1.723
5-Amino-2,4(1H,3H)-Pyrimidinedione	1.229	1.308	1.556	2.303	1.817	2.270	1.521	2.485	1.753	1.396	1.114	1.249	1.678	1.317
3,4-Dimethyl-2-eptanone	0.031	n.d.	0.006	0.010	0.003	0.008	0.039	0.011	0.001	0.007	0.005	0.005	0.006	0.008
2-Heptanone	0.334	0.273	0.305	0.411	0.339	0.017	0.381	0.456	0.348	0.331	0.313	0.336	0.356	0.311
4-Methyl-3-penten-2-one	0.985	0.633	0.647	0.887	0.669	0.131	0.989	0.804	0.829	0.710	0.737	0.899	0.887	0.646
2,6-Dimethyl-4-heptanone	5.216	5.405	5.070	5.696	6.531	5.980	5.842	7.016	6.161	5.223	4.482	5.661	6.974	5.475
5-Nonanone	3.184	3.047	2.764	2.791	4.071	4.429	3.862	4.956	4.181	3.141	2.590	3.309	4.517	2.962
3-Hydroxy-2-butanone	0.227	1.344	1.487	1.324	1.103	2.094	1.015	1.280	0.740	0.819	1.113	0.901	1.573	1.690
1-Hydroxy-2-propanone	0.438	1.003	1.092	1.037	0.825	1.882	0.741	0.951	0.556	0.588	0.766	0.737	1.173	1.191
Acetophenone	0.103	0.299	0.327	0.304	0.264	0.264	0.330	0.312	0.159	0.167	0.226	0.362	0.514	0.713
2,6-Dimethyl-4-hepten-3-one	n.d.	0.164	0.098	0.195	0.050	0.050	n.d.	0.023	n.d.	n.d.	n.d.	n.d.	n.d.	0.059
2-Pyrrolidinone	n.d.	0.001	0.001	0.002	0.000	n.d.	0.003	0.005	0.001	0.002	n.d.	n.d.	0.001	0.006
Total Ketones	**12.901**	**16.745**	**16.478**	**19.128**	**19.052**	**21.059**	**19.372**	**21.943**	**17.323**	**15.100**	**12.971**	**16.209**	**21.142**	**17.940**
Ethyl alcohol	1.871	3.582	4.089	3.655	2.843	3.438	2.509	2.648	1.654	1.702	2.439	2.127	2.602	2.363
1-Butanol	0.048	0.048	0.043	0.050	0.059	0.033	0.043	0.038	0.032	0.028	0.031	0.042	0.034	0.028
3-Methyl-2-heptanol	0.055	n.d.	0.030	0.039	0.039	0.042	0.058	0.044	0.032	0.043	0.031	0.024	0.043	0.023
2,2-Dimethyl-4-octen-3-ol	0.185	0.318	0.260	0.329	0.261	0.072	0.179	0.170	0.123	0.112	0.118	0.250	0.246	0.229
2-Ethyl-1-hexanol	0.015	0.037	0.052	0.043	0.041	0.041	0.023	0.039	0.016	0.041	0.032	0.030	0.068	0.039
2-Furanmethanol	0.452	0.730	0.684	0.667	0.551	0.368	0.430	0.427	0.293	0.286	0.303	0.500	0.542	0.439
Phenylethyl alcohol	0.091	0.183	0.234	0.188	0.152	0.219	0.151	0.145	0.073	0.079	0.092	0.121	0.177	0.146
1-Dodecanol	n.d.	n.d.	n.d.	n.d.	n.d.	0.003	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total Alcohols	**2.717**	**4.899**	**5.391**	**4.971**	**3.945**	**4.216**	**3.392**	**3.511**	**2.224**	**2.292**	**3.045**	**3.094**	**3.711**	**3.267**
Acetic acid	1.104	1.850	1.797	1.797	1.024	4.934	0.924	3.347	1.166	1.171	0.850	0.661	1.692	0.891
Butanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.013	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-Methyl-butanoic acid	0.005	0.011	0.018	0.021	0.009	0.017	n.d.	0.013	n.d.	0.002	n.d.	0.009	n.d.	0.005
Hepatanoic acid	n.d.	0.023	0.030	0.035	0.014	0.039	0.013	0.020	0.004	0.005	0.007	0.011	0.018	0.010
Octanoic acid	0.004	0.024	0.044	0.039	0.014	0.076	0.014	0.042	0.004	0.007	0.006	0.012	0.018	0.015
Nonanoic acid	0.005	n.d.	0.007	0.006	0.003	0.020	0.015	0.003	0.004	0.007	0.007	0.014	0.018	0.004
n-Decanoic acid	0.005	0.015	0.018	0.025	0.014	0.084	0.011	0.059	n.d.	0.009	n.d.	0.009	0.023	0.016
Dodecanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.034	n.d.	0.009	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total Carboxylic acids	**1.123**	**1.922**	**1.914**	**1.923**	**1.077**	**5.288**	**0.977**	**3.537**	**1.178**	**1.213**	**0.870**	**0.716**	**1.791**	**0.940**
Cyclohexylmethyl hexyl ester of sulfurous acid	0.528	0.508	0.376	0.732	0.601	0.038	0.445	0.386	0.426	0.324	0.247	0.749	0.644	0.500
Cyclohexylmethyl heptyl ester of sulfurous acid	3.969	5.991	4.817	4.639	4.571	1.420	3.476	2.918	1.980	2.341	2.360	3.953	3.883	4.194
Bis(1-methylethyl) ester of hexanedioic acid	0.114	0.163	0.179	0.167	0.143	0.108	0.131	0.125	0.085	0.090	0.095	0.154	0.186	0.172
Butyl ester of butanoic acid	0.002	0.006	0.006	0.006	0.006	0.003	0.002	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total Esters	**6.859**	**10.512**	**9.206**	**9.390**	**7.475**	**12.145**	**6.010**	**10.504**	**4.847**	**5.181**	**4.443**	**6.287**	**8.294**	**6.747**
Guanidina	0.897	0.975	1.148	1.657	1.325	1.626	1.110	1.773	1.269	1.020	0.836	0.924	1.236	0.983
6-Azathymine	0.660	0.672	0.782	1.137	0.968	1.061	0.785	1.185	0.832	0.681	0.534	0.692	0.889	0.753
1R-alpha-Pinene	0.139	0.097	0.098	0.210	0.174	0.079	0.171	0.172	0.138	0.108	0.033	0.174	0.150	0.164
1-chloro-5-methyl-hexane/1chloro-heptane	0.288	0.273	0.332	0.452	0.398	0.325	0.424	0.303	0.205	0.188	0.127	0.424	0.419	0.375
Dimethyl disulfide	0.001	0.004	0.005	0.011	0.010	0.015	0.031	0.003	0.002	n.d.	0.003	0.005	0.009	0.018
1,1'-Oxybis-Heptane	0.103	0.017	0.017	0.031	0.015	n.d.	0.086	0.016	0.070	0.060	0.007	0.134	0.047	0.019
(1S)-3,7,7-Trimethyl-bicyclo[4.1.0]hept-3-ene	0.043	n.d.	0.029	0.063	n.d.	n.d.	0.041	n.d.	0.022	0.017	n.d.	n.d.	0.035	n.d.
3,5,5-Trimethyl-cyclohexene	0.128	0.120	0.115	0.182	0.141	0.012	0.126	0.096	0.109	0.076	0.054	0.185	0.167	0.118
1,2,3-Trimethyl-benzene	0.490	0.432	0.555	0.554	0.447	0.011	0.536	0.458	0.505	0.483	0.467	0.452	0.613	0.447
Styrene	0.031	0.090	0.110	0.100	0.065	0.002	0.054	0.073	0.055	0.041	0.047	0.057	0.070	0.054
4-Pyridinamine	0.361	0.770	0.808	0.691	0.571	0.577	0.451	0.517	0.294	0.324	0.407	0.481	0.583	0.429
1,2,3-Trichloro-2-methyl-propane	0.224	0.153	0.264	0.324	0.284	0.181	0.237	0.204	0.098	0.097	0.087	0.277	0.298	0.270
1,2,4,5-Tetramethyl-benzene	0.476	0.541	0.560	0.610	0.514	0.180	0.520	0.310	0.295	0.229	0.200	0.686	0.669	0.505
3-Acetamidofuran	0.525	0.619	0.617	0.653	0.569	0.343	0.579	0.390	0.301	0.290	0.275	0.610	0.679	0.576
n.d.102/97	2.410	3.235	2.551	3.084	2.634	0.547	2.272	1.707	1.578	1.376	1.212	3.046	2.727	2.333
4,5-Dimethyl-1,3-benzenediol	0.679	0.904	0.850	0.861	0.799	0.250	0.655	0.415	0.380	0.388	0.381	0.789	0.767	0.808
(E)-3-(2-Butenyl)-thiophene	1.093	1.553	1.396	1.375	1.262	0.395	1.042	0.736	0.590	0.637	0.627	1.213	1.179	1.270
4,5-Dihydro-3,5,5-trimethyl-1H-pyrazole	0.180	0.354	0.347	0.249	0.274	0.013	n.d.	n.d.	0.105	0.104	0.127	0.199	0.245	0.225
3-Phenyl-furan	0.052	0.213	0.212	0.197	0.158	0.116	0.049	0.130	0.054	0.053	0.059	0.065	0.068	0.065
4-Methyl-5-(2methyl-2-propenyl)-2(5H)-furanone	0.920	1.504	1.531	1.209	1.071	0.688	0.946	0.689	0.459	0.600	0.677	0.894	0.998	1.439
n.d.57	0.645	1.049	1.115	0.888	0.746	0.599	0.690	0.533	0.348	0.458	0.506	0.658	0.738	1.028
2-Chloro-4-(1,1-dimethylpropyl)-phenol	1.417	2.429	2.270	1.954	1.640	1.270	1.633	1.322	0.633	0.738	0.815	1.480	1.652	2.169
2-Methyl-furan	0.040	0.038	0.070	0.050	0.044	0.041	0.044	0.051	0.026	0.034	0.006	0.038	0.062	0.069
Total other molecules	**11.803**	**16.042**	**15.784**	**16.540**	**14.107**	**8.331**	**12.482**	**11.086**	**8.370**	**8.004**	**7.488**	**13.483**	**14.300**	**14.118**
Pyrazine	0.666	1.274	1.296	1.066	0.898	0.876	0.765	0.859	0.557	0.597	0.755	0.822	0.966	0.697
2,6-Dimethyl-pyrazine	3.491	7.703	8.698	7.823	6.095	6.171	4.500	5.358	2.717	3.072	3.855	4.778	5.867	4.193
Ethyl-pyrazine	0.434	0.894	0.979	0.919	0.731	0.713	0.557	0.648	0.342	0.382	0.461	0.579	0.682	0.496
2-Ethyl-6-methyl-pyrazine	0.326	0.686	0.840	0.832	0.620	0.629	0.450	0.517	0.242	0.275	0.324	0.447	0.529	0.391
Trimethyl-pyrazine	0.450	0.970	1.257	1.237	0.878	0.959	0.651	0.754	0.311	0.357	0.490	0.633	0.801	0.553
2,3-Dimethyl-5-ethylpyrazine	0.553	1.196	1.625	1.625	1.126	1.129	0.781	0.861	0.381	0.458	0.525	0.757	0.892	0.638
Total Pyrazine	**5.921**	**12.723**	**14.694**	**13.503**	**10.350**	**10.479**	**7.705**	**8.997**	**4.549**	**5.141**	**6.410**	**8.017**	**9.737**	**6.969**

n.d. under the detection level

Table 20. Volatile compounds (expressed as ppmEq) detected for *Listeria monocytogenes* ScottA cells (into BHI broth) in relation to gas plasma treatment times (30 and 60 min). The results are means of two independent experiments.

	GP30							GP60						
Sampling after treatment (min)	**0**	**15**	**30**	**60**	**180**	**360**	**1440**	**0**	**15**	**30**	**60**	**180**	**360**	**1440**
Pentanal	0.171	0.164	0.179	0.579	0.197	0.249	0.536	0.167	0.345	0.202	0.102	0.190	0.289	0.314
3-Methyl-butanal	0.231	0.215	0.227	0.731	0.264	0.307	0.667	0.218	0.413	0.249	0.129	0.239	0.360	0.381
3-(Methylthio)-propanal	0.232	0.240	0.267	0.298	0.256	0.314	0.282	0.094	0.113	0.136	0.099	0.138	0.142	0.165
3-Furaldehyde	0.282	0.234	0.267	0.267	0.269	0.290	0.263	0.218	0.194	0.219	0.147	0.195	0.203	0.213
Benzaldehyde	3.064	2.779	3.309	3.697	3.406	4.873	5.808	2.451	3.037	3.306	2.081	3.248	3.640	4.294
4-Methyl-benzaldehyde	1.401	1.002	1.243	0.959	0.930	1.395	1.309	1.200	1.487	1.417	0.811	1.044	1.049	0.982
Total Aldehydes	**5.382**	**4.633**	**5.492**	**6.531**	**5.321**	**7.429**	**8.866**	**4.348**	**5.589**	**5.527**	**3.369**	**5.055**	**5.681**	**6.349**
Acetone	0.615	0.524	0.594	0.937	0.737	0.972	1.433	0.484	0.571	0.606	0.488	0.685	0.824	1.027
2-Butanone	0.449	0.289	0.393	0.391	0.392	0.430	0.652	0.361	0.390	0.422	0.367	0.449	0.570	0.562
2,3-Butanedione	1.935	2.932	3.035	3.312	2.020	3.137	2.434	2.139	2.888	2.256	1.503	3.082	2.359	2.501
5-Amino-2,4(1H,3H)-Pyrimidinedione	1.263	2.786	2.670	2.544	1.770	2.268	2.187	2.106	2.615	2.581	1.340	2.586	2.508	2.349
3,4-Dimethyl-2-eptanone	0.001	0.013	0.012	0.047	0.002	0.035	0.038	0.000	0.014	0.014	0.007	0.011	0.008	0.011
2-Heptanone	0.028	0.392	0.465	0.432	0.378	0.396	0.406	0.422	0.431	0.448	0.364	0.450	0.438	0.437
4-Methyl-3-penten-2-one	0.612	0.756	0.817	0.843	0.804	0.742	0.769	0.855	0.684	0.713	0.845	0.694	0.710	0.727
2,6-Dimethyl-4-heptanone	5.740	5.298	6.619	7.132	6.405	6.883	6.884	6.941	4.285	4.777	5.146	6.567	6.628	6.781
5-Nonanone	3.712	3.298	4.418	5.176	3.940	4.925	4.948	4.639	2.412	2.897	3.252	4.647	5.058	4.883
3-Hydroxy-2-butanone	0.145	1.650	1.604	1.416	1.581	1.406	1.737	1.574	1.468	1.574	0.922	1.461	1.375	1.641
1-Hydroxy-2-propanone	0.920	1.151	1.138	1.093	1.176	1.050	1.253	1.206	1.012	1.082	0.718	1.053	0.989	1.169
Acetophenone	0.415	0.471	0.547	0.582	0.440	0.659	0.960	0.530	0.525	0.707	0.419	0.668	0.780	1.145
2,6-Dimethyl-4-hepten-3-one	0.272	0.073	n.d.	n.d.	n.d.	n.d.	n.d.	0.036	n.d.	0.028	n.d.	n.d.	n.d.	n.d.
2-Pyrrolidinone	0.035	n.d.	0.001	n.d.	0.000	0.000	n.d.	0.004	0.005	n.d.	0.007	0.003	0.030	0.003
Total Ketones	**16.141**	**19.633**	**22.312**	**23.904**	**19.645**	**22.903**	**23.701**	**21.298**	**17.300**	**18.106**	**15.378**	**22.356**	**22.276**	**23.236**
Ethyl alcohol	3.496	2.657	2.613	2.387	2.281	2.252	2.344	1.693	1.659	1.783	1.095	1.564	1.526	1.688
1-Butanol	0.025	0.031	0.032	0.041	0.032	0.045	0.031	0.000	0.004	0.002	0.021	0.018	0.027	0.019
3-Methyl-2-heptanol	0.002	0.044	0.030	0.042	0.035	0.020	0.043	0.044	0.047	0.010	0.047	0.041	0.042	0.044
2,2-Dimethyl-4-octen-3-ol	0.240	0.163	0.164	0.186	0.242	0.169	0.173	0.227	0.130	0.122	0.170	0.109	0.083	0.133
2-Ethyl-1-hexanol	0.042	0.064	0.047	0.024	0.035	0.028	0.036	0.064	0.039	0.047	0.031	0.094	0.050	0.109
2-Furanmethanol	0.465	0.353	0.390	0.475	0.490	0.443	0.427	0.415	0.274	0.268	0.313	0.247	0.226	0.283
Phenylethyl alcohol	0.172	0.134	0.161	0.173	0.153	0.201	0.233	0.174	0.153	0.155	0.102	0.144	0.151	0.157
1-Dodecanol	n.d.	n.d.	n.d.	0.002	n.d.	0.004	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total Alcohols	**4.443**	**3.446**	**3.437**	**3.330**	**3.268**	**3.163**	**3.288**	**2.617**	**2.307**	**2.387**	**1.780**	**2.217**	**2.104**	**2.433**
Acetic acid	1.903	1.602	1.794	2.758	2.055	1.990	2.363	2.856	2.584	2.703	0.857	2.196	2.075	2.229
Butanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-Methyl-butanoic acid	0.016	n.d.	n.d.	0.016	n.d.	n.d.	n.d.	0.014	0.011	n.d.	n.d.	0.007	0.006	n.d.
Hepatanoic acid	0.019	0.009	0.014	0.027	0.014	0.019	0.025	0.014	0.022	0.012	0.010	0.010	0.014	0.012
Octanoic acid	0.028	0.008	0.014	0.043	0.010	0.023	0.033	0.014	0.028	0.014	0.008	0.011	0.015	0.012
Nonanoic acid	0.030	0.008	0.012	0.047	0.009	n.d.	0.031	0.014	0.023	0.011	0.009	0.010	0.016	0.011
n-Decanoic acid	0.025	n.d.	0.007	0.050	0.008	0.011	0.026	0.010	0.022	0.007	0.007	n.d.	0.016	n.d.
Dodecanoic acid	n.d.	n.d.	n.d.	0.004	n.d.	n.d.	0.002	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total Carboxylic acids	**2.020**	**1.627**	**1.841**	**2.945**	**2.096**	**2.043**	**2.480**	**2.921**	**2.691**	**2.747**	**0.891**	**2.257**	**2.163**	**2.285**
Cyclohexylmethyl hexyl ester of sulfurous acid	0.443	0.424	0.385	0.480	0.668	0.407	0.403	0.562	0.177	0.187	0.476	0.176	0.134	0.226
Cyclohexylmethyl heptyl ester of sulfurous acid	4.147	2.218	2.535	3.448	3.695	3.574	3.685	3.426	2.133	2.212	2.846	2.088	2.046	2.566
Bis(1-methylethyl) ester of hexanedioic acid	0.197	0.126	0.134	0.190	0.187	0.243	0.244	0.113	0.161	0.164	0.125	0.178	0.158	0.221
Butyl ester of butanoic acid	0.033	0.024	0.032	0.031	0.023	0.034	0.033	0.009	0.006	0.006	0.005	0.005	0.005	0.005
Total Esters	**8.860**	**6.046**	**6.767**	**10.040**	**8.766**	**8.344**	**9.325**	**9.953**	**7.859**	**8.064**	**5.235**	**6.961**	**6.668**	**7.588**
Guanidina	0.945	1.985	1.898	1.817	1.294	1.642	1.581	1.524	1.889	1.857	0.981	1.863	1.821	1.695
6-Azathymine	0.665	1.262	1.219	1.222	0.963	1.145	1.119	1.000	1.182	1.162	0.646	1.198	1.140	1.073
1R-alpha-Pinene	0.099	0.103	0.121	0.171	0.244	0.175	0.166	0.196	0.027	0.043	0.050	0.111	0.040	0.103
1-chloro-5-methyl-hexane/1chloro-heptane	0.259	0.273	0.311	0.472	0.559	0.472	0.451	0.264	0.201	0.266	0.183	0.334	0.256	0.272
Dimethyl disulfide	0.001	0.004	0.004	0.003	0.010	0.013	0.036	0.002	0.003	0.002	0.002	0.006	0.006	0.013
1,1'-Oxybis-Heptane	0.017	0.015	0.013	0.098	0.125	0.073	0.079	0.023	0.006	0.007	0.068	0.009	0.021	0.009
(1S)-3,7,7-Trimethyl-bicyclo[4.1.0]hept-3-ene	0.041	0.019	0.028	0.040	0.053	0.047	0.003	0.031	0.009	0.013	0.019	0.013	0.009	0.014
3,5,5-Trimethyl-cyclohexene	0.021	0.102	0.091	0.118	0.166	0.104	0.101	0.135	0.036	0.051	0.095	0.039	0.032	0.051
1,2,3-Trimethyl-benzene	0.012	0.554	0.518	0.592	0.562	0.520	0.602	0.636	0.540	0.540	0.290	0.472	0.475	0.502
Styrene	0.000	0.066	0.076	0.089	0.075	0.083	0.083	0.088	0.085	0.089	0.050	0.078	0.082	0.095
4-Pyridinamine	0.013	0.419	0.453	0.482	0.410	0.475	0.488	0.361	0.309	0.323	0.214	0.290	0.304	0.342
1,2,3-Trichloro-2-methyl-propane	0.243	0.105	0.193	0.250	0.308	0.258	0.332	0.132	0.107	0.116	0.140	0.157	0.105	0.195
1,2,4,5-Tetramethyl-benzene	0.469	0.308	0.265	0.430	0.617	0.485	0.534	0.357	0.176	0.182	0.309	0.182	0.151	0.268
3-Acetamidofuran	0.510	0.357	0.404	0.582	0.620	0.653	0.768	0.478	0.289	0.351	0.397	0.385	0.368	0.558
n.d.102/97	2.405	1.617	1.538	2.132	2.682	1.919	1.851	2.301	1.187	1.020	1.994	0.894	0.695	1.127
4,5-Dimethyl-1,3-benzenediol	0.666	0.450	0.429	0.535	0.717	0.636	0.696	0.563	0.346	0.347	0.512	0.343	0.294	0.465
(E)-3-(2-Butenyl)-thiophene	1.118	0.699	0.705	0.916	1.121	1.029	1.117	0.941	0.585	0.601	0.815	0.582	0.525	0.752
4,5-Dihydro-3,5,5-trimethyl-1H-pyrazole	0.143	0.116	0.155	0.102	0.062	0.053	0.073	0.161	0.135	0.125	0.060	0.120	0.029	0.039
3-Phenyl-furan	0.071	0.058	0.075	0.072	0.053	0.072	0.068	0.012	0.026	0.031	0.011	0.035	0.042	0.053
4-Methyl-5-(2methyl-2-propenyl)-2(5H)-furanone	1.007	0.663	0.698	0.852	0.811	1.025	1.268	0.837	0.787	0.793	0.778	0.841	0.810	1.154
n.d.57	0.734	0.494	0.507	0.627	0.585	0.735	0.924	0.618	0.597	0.600	0.580	0.633	0.623	0.866
2-Chloro-4-(1,1-dimethylpropyl)-phenol	1.459	0.897	0.879	1.160	1.477	1.960	2.423	1.017	0.805	0.753	0.938	1.060	1.017	1.775
2-Methyl-furan	0.066	0.118	0.050	0.072	0.055	0.045	0.088	0.035	0.008	0.008	0.005	0.032	0.015	0.030
Total other molecules	**10.963**	**10.682**	**10.629**	**12.836**	**13.566**	**13.620**	**14.849**	**11.712**	**9.333**	**9.279**	**9.140**	**9.678**	**8.859**	**11.450**
Pyrazine	0.005	0.712	0.768	0.837	0.721	0.789	0.791	0.578	0.501	0.533	0.371	0.472	0.497	0.535
2,6-Dimethyl-pyrazine	5.063	4.437	4.825	4.856	4.103	5.087	5.104	3.720	3.224	3.433	2.100	3.019	3.139	3.524
Ethyl-pyrazine	0.582	0.527	0.567	0.586	0.505	0.584	0.589	0.409	0.351	0.374	0.242	0.332	0.344	0.387
2-Ethyl-6-methyl-pyrazine	0.441	0.411	0.451	0.462	0.379	0.489	0.509	0.300	0.263	0.283	0.176	0.252	0.262	0.312
Trimethyl-pyrazine	0.644	0.600	0.670	0.669	0.549	0.713	0.749	0.491	0.416	0.447	0.248	0.393	0.412	0.485
2,3-Dimethyl-5-ethylpyrazine	0.701	0.647	0.729	0.748	0.596	0.823	0.857	0.396	0.359	0.384	0.233	0.336	0.364	0.442
Total Pyrazine	**7.435**	**7.334**	**8.009**	**8.158**	**6.852**	**8.484**	**8.599**	**5.895**	**5.115**	**5.453**	**3.371**	**4.804**	**5.019**	**5.684**

n.d. under the detection level

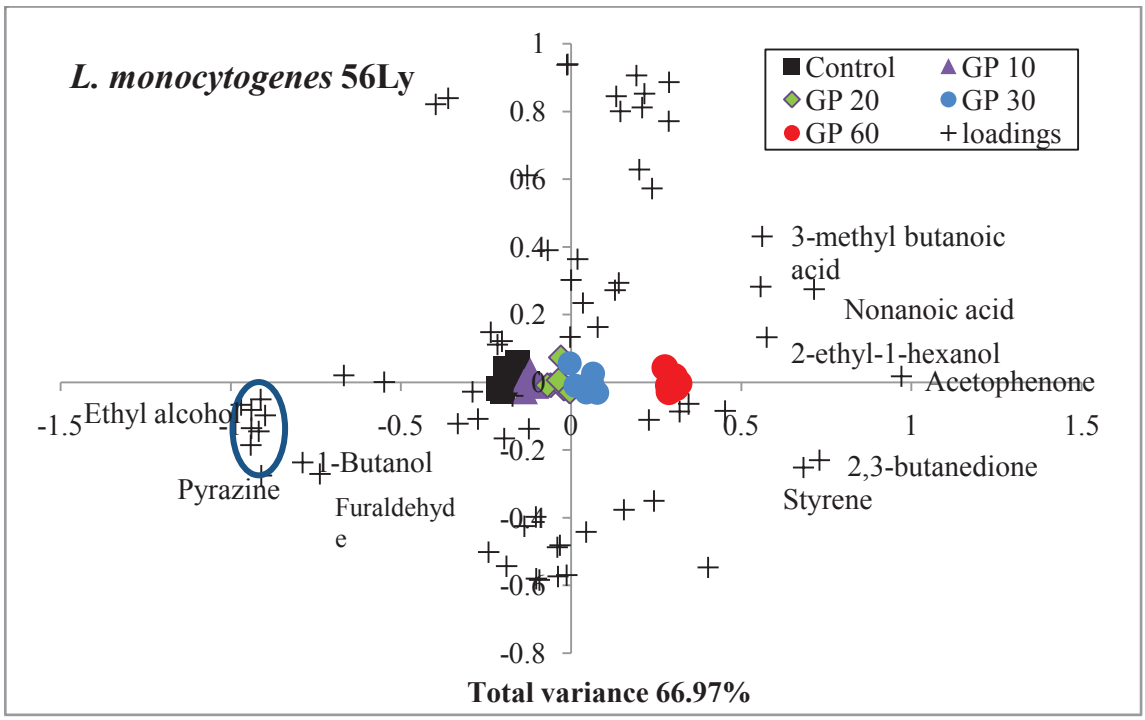

Figure 64. Plots of the first two canonical axes produced by Canonical Analysis of Principal Coordinates (CAP) of GC-MS/SPME analysis for *Listeria monocytogenes* 56Ly cells (into BHI broth) exposed to gas plasma for different times (10, 20, 30 and 60 min; control= untreated cells).

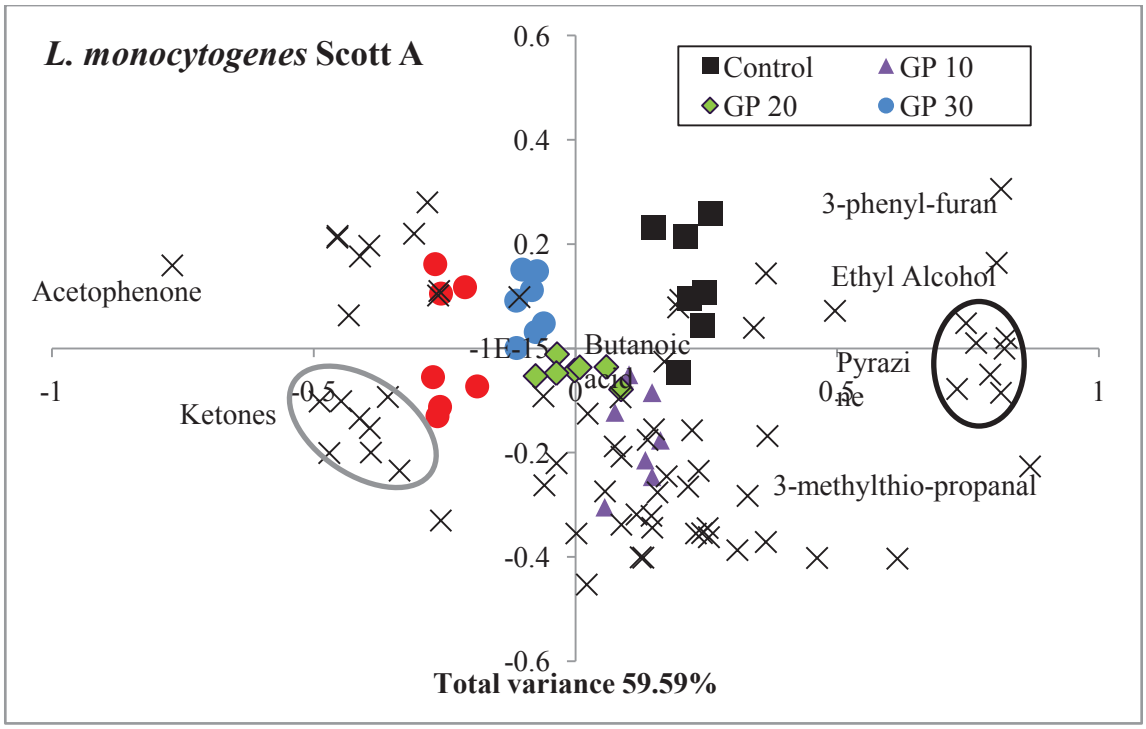

Figure 65. Plots of the first two canonical axes produced by Canonical Analysis of Principal Coordinates (CAP) of GC-MS/SPME analysis for *Listeria monocytogenes* ScottA cells (into BHI broth) exposed to gas plasma for different times (10, 20, 30 and 60 min; control= untreated cells).

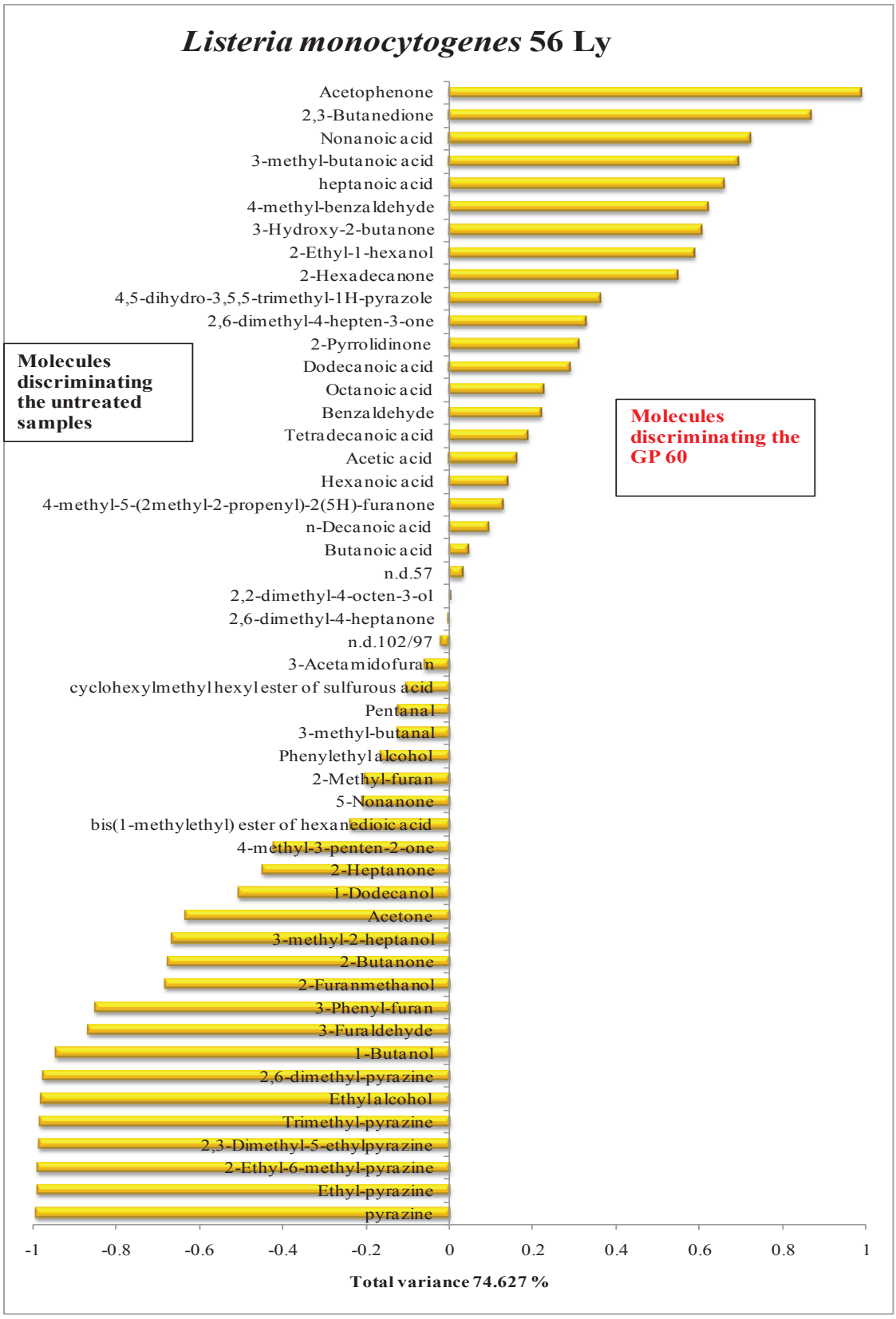

Figure 66. Canonical Discriminant Analysis of Principal Coordinates (CAP) loading coefficient plot of the volatile compounds of *Listeria monocytogenes* 56Ly cells (into BHI broth) exposed to gas plasma for 0 and 60 min**.**

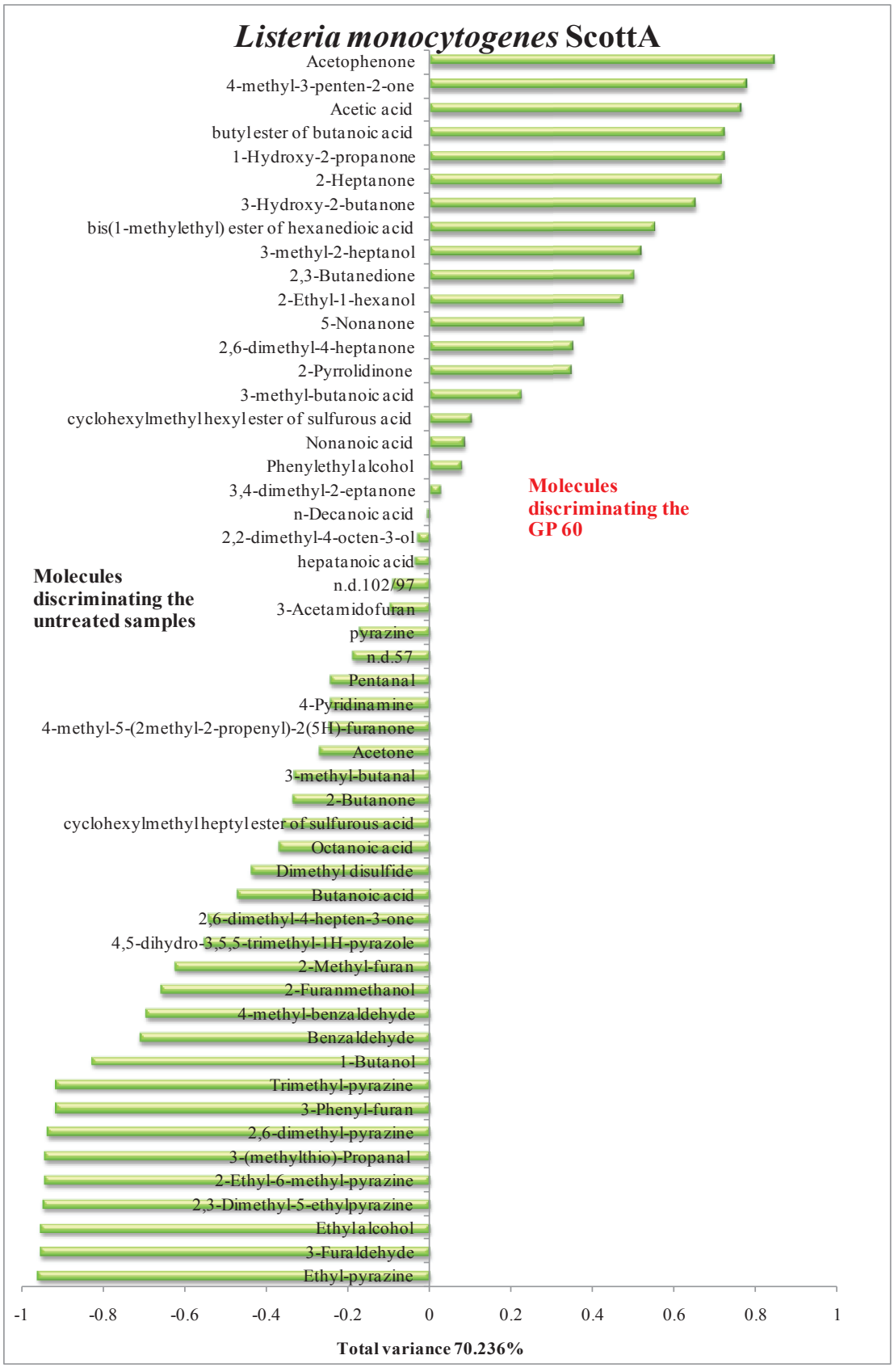

Figure 67. Canonical Discriminant Analysis of Principal Coordinates (CAP) loading coefficient plot of the volatile compounds of *Listeria monocytogenes* ScottA cells exposed to gas plasma for 0 and 60 min.

Table 21. ^{1}H-NMR spectral data (expressed as μM) of the compounds detected for *Listeria monocytogenes* 56Ly cells (into BHI broth) in relation to the different gas plasma treatment times (Untreated and 10 min=GP10) and storage times following plasma treatments (0, 15, 30, 60, 180, 360, 1440 min).

	Untreated							GP10						
	0	**15**	**30**	**60**	**180**	**360**	**1440**	**0**	**15**	**30**	**60**	**180**	**360**	**1440**
Leucine	28156.72	27777.86	28228.77	29210.90	27976.61	28838.65	29790.72	29627.18	28310.03	28249.47	28273.64	23603.06	28439.54	29486.24
Isoleucine	3665.55	3625.91	3686.09	3794.88	3708.53	3835.02	4032.64	3837.79	3693.33	3746.99	3664.84	2345.55	3795.79	3969.60
Valine	4609.46	4521.34	4646.62	4792.95	4559.89	4745.16	5001.80	4887.10	4584.97	4634.29	4595.67	4249.81	4682.96	4921.14
3-Methyl-2-oxovalerate	163.85	142.80	153.24	161.07	144.74	155.47	186.58	175.42	140.53	148.11	150.56	160.85	151.54	169.27
Ethanol	5041.56	3315.00	4886.78	5054.46	2883.02	4816.39	4916.02	4951.71	4632.12	3046.79	4499.20	4592.62	4512.38	4641.21
Alanine	6056.93	5052.98	5155.73	5314.82	5176.72	5394.79	5567.25	6327.86	6286.50	5229.97	5100.24	6609.45	6474.51	6566.86
Lysine	22195.69	21972.55	22522.24	23597.72	22613.18	23778.94	28339.24	23376.46	22905.91	22055.82	22841.30	24424.70	24408.15	30030.37
Acetate	15803.58	15527.39	15500.46	16690.84	17343.13	19771.38	22121.42	17856.50	17274.64	17756.15	17599.78	19393.39	20517.07	22913.86
Glutamate	4711.75	4450.03	9057.43	4607.82	4134.72	4315.31	4935.05	9135.96	4057.43	4069.39	4588.99	4583.15	3990.01	5117.19
Methionine	3490.91	3413.77	3532.09	3642.03	3450.12	3568.41	3701.25	3220.77	2987.94	3010.28	3026.29	3099.42	3024.36	3095.25
O-Acetylcholine	281.88	284.06	288.53	302.23	289.64	304.36	292.10	292.00	288.24	292.00	283.54	294.96	298.15	282.99
Acetone	166.51	173.59	172.40	184.96	187.52	198.50	206.15	168.55	167.40	176.12	183.41	185.32	191.66	197.12
4-aminobutyrate	1466.84	1981.20	1942.49	1752.74	2209.81	2346.85	3771.53	2249.44	2276.38	2319.92	2257.64	2333.57	2499.14	4558.72
Pyruvate	3404.69	3627.24	3539.18	3397.17	2714.60	1838.94	245.29	3227.18	3230.36	3262.03	2815.08	2338.72	1504.71	227.04
Pyroglutamate	2083.24	2465.26	2513.26	2057.89	2532.23	2467.05	2232.00	2015.81	2397.67	2452.22	1905.97	2101.75	2123.56	2251.14
Succinate	603.45	665.43	662.56	585.13	661.37	635.82	652.48	579.56	624.78	636.16	576.88	595.66	582.62	621.53
Riboflavin	7.15	8.40	7.59	8.54	5.13	9.85	11.67	12.64	6.18	4.35	7.99	7.30	5.66	9.99
Sarcosine	137.98	144.25	143.38	148.86	137.32	146.83	163.73	542.89	504.63	506.22	520.63	523.14	513.77	563.28
Aspartate	1263.78	1306.66	1389.02	1416.82	1271.40	1485.98	1489.93	1353.51	1274.34	1282.23	1460.00	1496.80	1348.24	1488.57
Asparagine	1188.43	1200.22	1281.05	1324.31	1199.84	1314.07	1327.57	1329.94	1181.31	1278.06	1332.61	1335.30	1254.64	1343.64
Creatinine3.03	2300.54	2283.50	2353.65	2431.90	2421.64	2447.05	2590.28	2337.24	2387.94	2338.04	2364.64	2413.05	2476.59	2670.54
Creatine	1218.14	1210.60	1250.15	1292.39	1268.90	1291.04	1311.95	1256.23	1244.27	1222.91	1243.42	1269.33	1286.40	1306.01
Choline	213.61	211.20	224.37	230.40	222.30	220.85	199.84	211.02	218.93	239.57	224.05	227.83	217.66	206.05
Betaine	2930.84	3009.17	3094.73	3383.23	3087.88	3098.72	3000.36	3158.85	3072.25	3385.52	3261.97	3315.62	3111.84	3274.89
Methanol	159.20	297.07	552.58	372.78	464.92	333.73	125.12	186.28	81.67	209.23	175.48	365.87	217.59	198.26
Proline	514.71	573.55	515.54	524.97	477.70	517.47	559.18	540.11	522.70	460.76	544.06	543.58	551.70	528.56
Glycine	2253.10	2810.14	2383.41	2440.67	2500.55	2626.81	2808.67	2435.66	2379.03	2451.12	2390.07	2564.41	2613.53	2729.79
Glycerol	3390.45	8113.75	3629.54	3764.02	3282.09	3489.16	3782.67	3833.91	3337.85	3178.80	3491.39	3902.53	3443.88	3712.58
Threonine	2538.17	2471.60	2702.63	2823.23	2479.23	2697.36	3035.29	2863.53	2534.38	2478.58	2667.69	2716.03	2635.09	2922.46
Serine	6549.45	7029.19	7078.27	7192.56	7431.04	7683.27	6742.70	6098.02	7522.76	7451.80	6972.77	7362.16	7657.66	6686.83
Lactate	25296.86	25801.39	26547.35	27003.30	25815.83	27600.22	28491.10	27160.68	27030.74	27646.76	26912.52	27522.02	27727.00	28026.24
Uracil	203.58	182.08	194.91	188.21	199.76	189.12	233.30	203.72	182.85	191.10	193.66	205.09	193.07	226.21
Uridine	254.05	236.14	234.12	250.39	235.75	227.45	271.94	260.20	219.00	238.87	235.59	257.92	245.69	267.79
Adenosine	41.76	28.13	37.13	34.40	38.00	26.60	38.26	28.29	28.19	53.38	34.69	35.71	37.75	32.83
Inosine	14.89	7.05	17.46	12.62	2.21	6.78	12.88	9.45	-0.95	25.21	22.18	19.71	1.56	16.16
Orotate	5.99	5.71	12.33	6.90	10.74	15.22	10.83	11.02	7.89	9.10	11.37	9.01	2.07	5.16
Fumarate	5.64	4.50	5.98	4.33	7.51	6.59	8.61	5.30	6.33	7.73	7.04	3.46	7.33	5.90
Tyrosine	2275.79	2238.32	2225.90	2259.35	2339.91	2377.59	2413.72	2348.42	2307.94	2355.68	2236.59	2397.32	2523.67	2329.81
Phenylalanine	4782.20	4786.39	4867.31	5078.03	4796.87	5010.65	5086.45	5086.80	4922.93	4968.79	4855.37	5090.01	5000.93	5025.75
Tryptophan	1310.06	1333.35	1357.27	1417.56	1363.48	1449.17	1493.85	1141.63	1135.83	1168.86	1143.64	1188.78	1224.51	1237.28
1-Methylhistidine	605.48	689.23	255.47	654.08	649.75	791.76	144.45	176.43	827.20	795.17	727.46	700.62	875.25	491.42
Hypoxanthine	300.30	322.46	304.98	322.94	340.08	352.37	364.19	338.30	362.92	372.20	363.36	389.24	395.86	419.18
Formate	13195.76	12841.44	13172.01	13518.77	13022.14	13381.68	12523.85	14652.30	13907.65	14095.15	13798.21	14167.86	13868.17	14328.02

Table 22. ^{1}H-NMR spectral data (expressed as μM) of compounds detected for *Listeria monocytogenes* 56Ly cells (into BHI broth) in relation to the different gas plasma treatment times (GP20 and GP30 min) and storage times following plasma treatments (0, 15, 30, 60, 180, 360, 1440 min).

	GP20							GP30						
	0	**15**	**30**	**60**	**180**	**360**	**1440**	**0**	**15**	**30**	**60**	**180**	**360**	**1440**
Leucine	28622.21	28846.79	21746.43	28275.17	22679.15	29107.38	24512.42	29029.24	23633.93	29383.01	23697.17	28394.15	28920.96	24580.71
Isoleucine	3741.31	3793.10	2151.92	3721.12	2283.15	3902.16	2565.23	3763.80	2259.50	3817.69	2269.71	3705.88	3814.85	2542.45
Valine	4700.58	4700.06	4034.56	4653.49	4251.59	4790.56	4458.72	4800.64	4290.91	4856.35	4266.46	4650.77	4741.01	4497.40
3-Methyl-2-oxovalerate	158.54	150.61	161.27	158.69	167.73	155.13	159.41	174.86	177.01	176.86	174.93	153.23	158.69	167.02
Ethanol	4303.04	4280.50	4241.17	4218.82	4115.21	4159.58	4395.53	4106.25	4147.13	4093.06	4069.79	3816.58	3788.33	3974.50
Alanine	5217.01	6388.06	6337.27	5146.96	6592.63	5451.87	6749.54	5248.84	6394.09	5331.72	6421.40	6314.72	5280.47	6762.71
Lysine	24010.15	24420.17	24148.02	24000.60	25476.84	26449.83	34645.42	21567.77	22743.38	22057.18	23032.15	22773.41	23961.46	29430.59
Acetate	18443.56	18914.17	18980.66	18956.18	20444.43	21148.96	22879.96	16231.58	16459.00	16811.87	17084.16	17718.16	19192.52	22290.50
Glutamate	4263.30	4297.85	4218.54	4100.82	4124.75	4200.73	3623.72	7795.38	9136.71	4547.17	4539.08	4292.23	4409.41	3695.96
Methionine	2431.42	2435.68	2491.53	2410.66	2557.70	2446.23	2345.89	2562.64	2589.05	2548.83	2558.14	2419.83	2502.31	2485.53
O-Acetylcholine	280.99	272.68	281.39	278.53	295.41	277.08	255.87	262.34	277.44	271.07	278.55	270.03	274.94	273.95
Acetone	177.04	183.71	194.48	184.51	200.95	207.51	232.90	162.31	170.20	186.61	198.44	209.10	262.65	310.87
4-aminobutyrate	2366.64	2419.13	2751.30	2435.47	3141.37	3347.52	5799.21	1896.19	1958.37	2069.99	2129.31	1979.08	2319.98	4590.81
Pyruvate	2423.94	2242.53	2276.50	2263.37	1712.05	1241.62	475.73	3593.55	3804.69	3565.65	3528.83	2624.80	1701.01	318.10
Pyroglutamate	2050.93	2059.46	1846.84	2039.64	2103.53	2153.98	2501.93	1974.27	1911.69	2042.10	1828.03	1937.27	1997.02	2156.68
Succinate	579.87	626.74	637.77	641.17	642.77	629.45	713.03	578.55	573.63	596.73	625.81	596.04	599.65	668.53
Riboflavin	8.09	8.68	6.98	7.09	9.08	8.53	7.77	14.46	12.42	13.86	12.83	7.44	7.28	9.50
Sarcosine	992.01	999.31	1004.54	975.67	1045.47	1001.16	1044.21	1004.66	1027.87	1029.02	1040.77	963.08	987.20	1071.72
Aspartate	1491.55	1365.98	1376.79	1497.97	1504.48	1362.91	1621.90	1332.83	1385.38	1376.93	1413.82	1446.96	1494.17	1542.33
Asparagine	1355.53	1299.50	1275.29	1350.33	1320.23	1265.00	1409.63	1301.58	1338.88	1337.45	1327.05	1306.01	1313.87	1362.39
Creatinine3.03	2438.94	2505.09	2440.05	2444.40	2582.18	2611.55	2942.28	2363.72	2369.86	2386.69	2430.80	2425.05	2470.04	2792.74
Creatine	1297.36	1313.56	1305.97	1301.09	1362.34	1336.83	1372.70	1292.58	1302.67	1306.49	1335.12	1299.76	1322.90	1390.75
Choline	220.18	236.87	214.16	223.54	209.88	230.08	229.24	215.26	211.97	217.74	214.39	220.75	216.97	211.36
Betaine	3104.90	3364.20	3040.48	3169.66	3137.28	3371.94	3369.82	3204.71	2982.01	3274.34	3025.94	3174.12	3192.95	3103.26
Methanol	83.74	93.16	313.37	108.89	778.40	360.70	100.84	180.54	345.58	94.80	99.59	92.21	380.48	233.54
Proline	540.27	491.36	547.82	511.62	554.66	566.63	550.68	528.02	538.12	541.55	533.68	552.52	549.06	580.51
Glycine	2456.00	2506.32	2526.48	2459.61	2692.52	2662.71	2745.73	2402.98	2471.10	2460.75	2435.50	2469.56	2577.45	2787.97
Glycerol	3533.29	3715.18	3706.58	3566.42	3900.21	3529.69	3653.62	3762.82	3887.30	3902.63	3709.95	3593.17	3567.57	3831.66
Threonine	2702.66	2610.85	2728.85	2644.15	2936.73	2753.76	2906.14	2833.41	2818.91	2932.81	2743.41	2673.35	2692.65	3000.97
Serine	6956.52	7599.53	6974.75	7016.26	7266.85	7703.83	7988.40	6211.51	6279.38	6329.33	6430.02	7148.13	7277.11	7784.46
Lactate	27859.17	28437.21	28688.39	27452.85	29060.83	28930.70	29451.80	26388.22	26782.18	27045.83	26444.05	26823.10	27675.26	29527.60
Uracil	198.16	189.00	190.27	186.14	199.10	194.42	205.20	198.50	209.25	218.73	191.56	194.22	193.82	226.66
Uridine	247.35	231.91	239.48	246.15	249.00	233.69	264.37	254.49	258.06	277.85	253.84	230.19	224.30	268.98
Adenosine	36.45	43.73	31.21	39.56	47.11	31.11	36.80	28.36	26.82	42.70	40.56	17.93	29.40	45.55
Inosine	17.36	23.54	13.32	11.21	29.82	9.23	16.89	15.69	5.05	28.50	15.07	5.47	5.11	25.76
Orotate	11.28	14.19	12.13	6.38	8.15	10.04	13.18	16.05	10.47	10.61	8.89	5.91	10.66	10.26
Fumarate	6.15	2.53	5.15	4.06	3.99	5.62	6.31	3.31	2.99	3.76	4.18	3.78	5.02	6.76
Tyrosine	2315.48	2302.69	2367.72	2327.98	2407.70	2400.96	2590.45	2338.34	2377.85	2361.60	2379.65	2331.51	2320.62	2495.17
Phenylalanine	5031.89	5043.92	5067.89	4870.00	5254.47	5115.84	5240.07	5108.05	5022.70	5190.45	5192.01	4987.31	5060.11	5296.27
Tryptophan	917.40	939.32	933.82	898.94	959.49	991.98	997.34	881.87	865.98	893.21	919.45	894.63	921.87	1001.08
1-Methylhistidine	769.21	857.05	566.30	824.16	583.45	873.48	765.32	205.32	231.68	212.62	546.27	308.34	700.19	621.05
Hypoxanthine	420.91	413.78	416.37	436.41	473.13	446.69	465.31	387.51	348.07	394.12	490.78	423.98	437.58	490.31
Formate	14361.62	14730.95	14235.49	14158.76	14419.26	14473.72	14181.41	13259.81	13526.04	13072.81	13503.08	12916.48	13380.69	13301.81

Table 23. ^{1}H-NMR spectral data (expressed as μM) of compounds detected for *Listeria monocytogenes* 56Ly cells (into BHI broth) in relation to gas plasma treatment time (GP60) and storage times following the plasma treatment (0, 15, 30, 60, 180, 360, 1440 min).

	GP60						
	0	**15**	**30**	**60**	**180**	**360**	**1440**
Leucine	22158.30	29811.00	29215.90	21685.18	29087.05	29119.09	24615.39
Isoleucine	2229.24	3883.14	3820.93	2060.36	3802.88	3848.44	2529.56
Valine	4199.95	4921.51	4818.43	3850.46	4750.30	4788.76	4510.86
3-Methyl-2-oxovalerate	165.66	179.52	169.78	150.33	155.82	156.51	173.50
Ethanol	3060.49	3246.21	3065.48	2656.40	3002.39	3092.22	3120.30
Alanine	6486.28	5393.77	5287.14	6310.13	5282.45	5345.53	6568.89
Lysine	22722.11	22846.54	21328.13	19851.27	23174.24	25529.14	35622.66
Acetate	17148.78	17026.49	17460.91	17482.71	17774.63	19146.91	21378.26
Glutamate	5058.20	4936.66	4605.68	5126.37	4519.65	8348.92	7743.12
Methionine	1412.51	1430.40	1413.83	1396.51	1364.63	1356.93	1279.46
O-Acetylcholine	251.96	243.07	240.42	206.55	236.31	235.32	211.05
Acetone	185.20	196.62	216.82	260.59	300.32	424.25	435.47
4-aminobutyrate	2422.50	2185.93	2482.15	2981.36	2593.96	3107.25	5981.13
Pyruvate	2957.23	3149.81	2546.90	2311.27	1896.15	1583.68	337.35
Pyroglutamate	1975.99	2041.31	1923.90	2123.13	1987.72	1999.11	2464.78
Succinate	620.36	582.37	596.56	661.21	638.28	583.44	690.85
Riboflavin	11.38	13.64	14.44	9.58	9.30	9.07	13.29
Sarcosine	1956.75	1930.72	1894.21	1805.87	1871.79	1881.56	2002.98
Aspartate	1480.28	1420.56	1377.65	1356.19	1424.14	1497.92	1485.65
Asparagine	1284.45	1205.06	1242.05	1234.39	1291.10	1342.51	1289.32
Creatinine3.03	2453.65	2406.64	2401.74	2528.76	2467.80	2606.91	2981.74
Creatine	1358.62	1337.30	1322.26	1420.50	1342.89	1371.83	1410.79
Choline	212.48	215.38	215.48	221.33	215.18	232.47	213.97
Betaine	3116.77	3261.31	3233.15	3230.07	3185.48	3390.09	3353.48
Methanol	113.83	629.60	400.65	286.08	648.20	375.73	486.80
Proline	492.49	554.70	539.66	605.84	529.60	546.28	564.23
Glycine	2462.09	2572.50	2484.78	2646.51	2591.45	2582.46	2720.96
Glycerol	3832.88	3907.81	3841.25	3839.58	3710.82	3562.80	3960.17
Threonine	2809.72	2873.10	2851.89	2781.78	2741.26	2740.22	3041.03
Serine	6701.76	5898.33	6343.47	7401.64	7082.19	7464.06	6859.89
Lactate	26533.09	26360.73	26333.42	26734.07	27156.91	26933.14	27585.17
Uracil	218.09	222.64	204.39	227.71	217.18	216.42	222.18
Uridine	259.38	265.88	255.64	271.34	250.35	245.72	271.34
Adenosine	47.78	34.46	35.37	75.87	40.97	32.82	47.29
Inosine	28.32	20.18	21.44	57.01	21.73	12.79	29.43
Orotate	13.67	12.25	10.16	19.85	11.43	9.12	10.88
Fumarate	3.15	4.44	3.35	4.82	3.83	4.07	25.14
Tyrosine	2399.02	2368.95	2339.16	2248.28	2310.06	2466.43	2464.46
Phenylalanine	5299.96	5297.94	5208.39	5099.74	5113.35	5043.41	5398.58
Tryptophan	510.17	529.83	517.85	521.79	525.02	464.11	552.67
1-Methylhistidine	503.14	428.63	306.16	817.79	661.32	851.94	304.07
Hypoxanthine	585.77	661.88	521.75	426.44	523.34	499.86	565.83
Formate	13993.32	14461.82	13903.31	13402.42	13929.49	13860.80	14343.23

Table 24. ^{1}H-NMR spectral data (expressed as μM) of compounds detected for *Listeria monocytogenes* ScottA cells (into BHI broth) in relation to the different gas plasma treatment times (Untreated and GP10 min) and storage times following the plasma treatments (0, 15, 30, 60, 180, 360, 1440 min).

	Untreated							GP10						
	0	15	30	60	180	360	1440	0	15	30	60	180	360	1440
Leucine	29094.57	28287.68	28123.91	28493.72	28651.63	28811.36	28749.74	27546.55	23145.76	28480.87	23352.30	28853.00	28440.33	22501.20
Isoleucine	3822.04	3733.26	3672.55	3729.54	3778.12	3779.67	3819.65	3718.64	2285.37	3743.23	2205.27	3451.48	3741.48	2238.89
Valine	4760.03	4639.84	4591.69	4668.27	4683.59	4618.81	4739.35	4653.45	4254.46	4648.84	4185.67	4410.77	4641.06	4198.78
3-Methyl-2-oxovalerate	160.81	147.66	149.04	148.93	152.69	153.64	172.82	134.62	153.28	150.95	152.26	149.78	151.91	194.72
Ethanol	5518.23	4881.91	5351.49	4851.97	5036.13	3237.50	5363.40	2994.38	4583.49	4639.54	4360.51	4055.28	4797.19	5299.20
Alanine	4917.24	4862.23	5887.40	5950.71	5900.87	4727.58	5856.01	4973.99	6126.84	4893.57	6002.34	4936.53	5822.09	5831.39
Lysine	20061.16	20858.60	19202.52	19863.95	20206.41	20926.46	23576.48	20113.69	21330.05	20506.24	20921.65	21441.09	19522.92	24508.68
Acetate	17749.48	16755.33	17443.80	17530.40	18294.62	17765.26	21210.12	16495.41	17405.70	17880.48	18569.92	19410.24	19297.58	21327.12
Glutamate	4042.33	4781.91	3935.10	4017.50	4480.77	4086.50	4144.49	4341.11	4167.18	4678.05	4729.02	4649.68	3788.24	3126.24
Methionine	3629.20	3457.70	3473.38	3530.18	3509.50	3462.20	3489.29	2938.37	3005.14	2975.55	3043.64	3057.06	2979.08	2919.47
O-Acetylcholine	339.04	335.77	340.84	341.99	328.80	340.40	317.98	315.61	327.99	314.23	329.36	323.44	314.84	318.86
Acetone	250.28	237.37	245.32	240.70	250.36	245.14	286.21	231.98	247.61	257.43	265.51	274.74	263.30	286.63
4-aminobutyrate	1182.92	1648.62	1578.58	1232.12	1591.53	1668.51	2217.12	1798.45	1835.21	1676.77	1743.39	1884.95	1974.42	2908.76
Pyruvate	2319.23	2300.22	2256.47	1989.73	1592.28	1142.66	179.77	2227.48	2708.54	2506.60	2071.60	1519.27	858.21	211.25
Pyroglutamate	1919.69	2369.74	2333.64	2015.64	2320.57	2394.11	1941.14	2545.05	2525.01	2329.83	2383.60	2377.89	2091.57	2150.02
Succinate	610.43	680.78	630.08	584.51	676.70	650.59	679.45	676.28	673.91	631.43	616.85	717.23	648.09	616.75
Riboflavin	6.74	7.20	6.95	7.71	4.12	6.43	5.56	5.77	6.59	6.79	5.89	5.10	4.91	6.52
Sarcosine	164.68	205.20	151.53	149.35	151.95	177.93	197.93	583.60	578.89	559.43	585.46	598.84	570.73	627.46
Aspartate	1434.50	1414.49	1454.63	1461.45	1307.25	1302.35	1397.72	1211.31	1455.96	1328.10	1331.91	1307.84	1449.45	1246.26
Asparagine	1329.21	1294.14	1340.75	1335.59	1224.49	1205.85	1273.68	1209.51	1385.31	1215.66	1254.94	1227.56	1309.12	1154.41
Creatinine3.03	2353.13	2285.69	2226.86	2303.40	2314.04	2293.13	2455.59	2283.00	2348.28	2304.45	2332.09	2399.67	2287.25	2376.20
Creatine	1315.03	1276.50	1245.69	1283.69	1294.09	1274.91	1325.02	1273.14	1315.74	1284.72	1311.78	1339.00	1290.90	1288.42
Choline	223.30	218.44	221.20	219.78	220.86	216.33	207.74	220.96	236.29	224.98	209.78	229.65	214.65	196.05
Betaine	3312.76	3130.88	3225.02	3274.82	3132.81	2974.79	2998.12	3076.36	3345.09	3274.09	3068.70	3297.88	3234.69	2692.66
Methanol	269.10	527.19	304.20	553.61	441.85	267.21	81.00	297.14	977.52	649.65	919.72	614.30	312.06	187.01
Proline	514.35	508.92	492.07	506.66	512.06	468.50	502.31	547.31	506.46	522.92	511.08	528.17	520.58	480.09
Glycine	2200.80	2177.32	2104.10	2162.47	2167.33	2130.65	2145.67	2252.51	2349.92	2255.87	2292.56	2281.79	2180.26	2086.66
Glycerol	3018.19	2957.17	2836.58	2949.45	2777.20	2682.17	2559.07	3159.69	3114.25	2937.57	2965.95	2912.58	2824.34	2609.96
Threonine	2653.32	2646.11	2572.62	2638.61	2607.03	2479.72	2613.98	2556.67	2629.54	2593.28	2652.85	2633.04	2587.75	2515.98
Serine	7025.27	7157.14	7301.95	7033.39	7184.29	7382.09	6968.51	7432.51	7358.77	7185.77	6983.61	7554.03	7099.88	6069.01
Lactate	28711.86	28436.95	27996.82	28736.10	29237.17	28466.37	30360.73	28374.17	29484.48	28245.50	28286.22	29027.66	29411.11	28665.66
Uracil	262.93	240.83	237.58	250.00	238.98	229.54	233.67	235.28	230.45	238.30	212.59	240.58	195.90	222.38
Uridine	284.62	270.18	268.27	271.13	268.25	269.81	252.55	272.78	255.46	268.58	219.34	276.87	213.35	239.23
Adenosine	54.75	52.24	51.54	44.60	59.24	40.24	44.69	46.20	38.62	45.98	37.93	55.63	44.09	36.31
Inosine	39.65	36.91	26.89	27.42	37.13	31.78	37.02	29.59	17.54	25.84	21.17	30.65	23.20	23.08
Orotate	12.78	5.21	7.99	6.03	12.58	9.62	11.76	4.59	5.83	7.67	10.06	10.86	4.83	10.35
Fumarate	6.06	3.50	2.23	6.84	2.64	2.64	4.86	5.91	2.29	5.79	5.63	5.26	4.44	3.59
Tyrosine	2221.70	2141.13	2171.76	2211.35	2239.80	2194.43	2328.38	2214.37	2250.51	2220.81	2319.25	2314.15	2193.95	2270.90
Phenylalanine	4980.73	4812.08	4800.59	4927.52	4859.06	4879.01	4883.52	4975.49	5030.19	4923.95	5014.93	5024.08	4817.71	4888.60
Tryptophan	1501.15	1401.30	1395.94	1484.90	1467.64	1533.67	1495.31	1206.21	1263.11	1241.80	1217.27	1250.62	1224.24	1240.45
1-Methylhistidine	621.48	725.98	642.82	634.28	851.88	807.31	609.50	677.52	958.76	747.77	478.42	829.72	680.02	276.43
Hypoxanthine	386.05	386.34	372.21	379.61	389.97	403.99	397.80	412.53	451.95	429.19	421.48	449.13	431.15	438.16
Formate	12060.33	11493.97	11218.70	11415.41	11751.57	11345.31	11848.59	11722.59	12345.30	12048.86	12181.93	12183.57	11804.04	12103.50

Table 25. ^{1}H-NMR spectral data (expressed as μM) of compounds detected for *Listeria monocytogenes* ScottA cells (into BHI broth) in relation to the different gas plasma treatment times (GP20 and GP30 min) and storage times following the plasma treatments (0, 15, 30, 60, 180, 360, 1440 min).

	GP20							GP30						
	0	**15**	**30**	**60**	**180**	**360**	**1440**	**0**	**15**	**30**	**60**	**180**	**360**	**1440**
Leucine	23701.47	23570.81	28103.48	27847.63	28869.31	28879.35	29030.04	22471.14	27796.94	28647.78	28139.51	27971.02	28796.62	28549.56
Isoleucine	2289.10	2275.31	3700.62	3706.80	3805.51	3795.33	3868.07	2261.43	3671.03	3712.36	3728.59	3696.60	3807.83	3783.94
Valine	4343.92	4329.03	4566.87	4665.55	4741.56	4754.98	4883.88	4278.10	4569.17	4687.68	4581.50	4574.49	4477.79	4700.38
3-Methyl-2-oxovalerate	159.45	154.39	143.50	146.45	165.27	163.62	175.12	174.03	140.82	163.79	144.90	145.68	145.38	163.87
Ethanol	4567.28	4170.55	4210.59	4011.94	3939.12	4225.42	4629.19	4484.76	3768.79	3715.85	2417.24	2348.12	3816.16	4212.22
Alanine	6173.56	6140.98	5830.27	4853.76	6326.62	5986.96	5086.11	6093.67	5889.86	5870.15	4758.43	4629.90	5886.50	5756.72
Lysine	21041.92	20523.50	19155.89	20941.93	23851.29	20929.97	25742.28	20992.37	20328.09	22242.30	20844.31	21754.25	21095.64	27340.95
Acetate	18156.82	17628.08	17948.25	16550.83	20059.33	21182.27	25193.49	20555.28	19037.30	19557.13	19238.61	19276.56	19401.54	21838.18
Glutamate	4822.48	4026.01	4551.89	4312.40	3445.61	3815.90	4478.48	4392.91	4507.23	4421.39	3905.64	4220.40	3643.90	3279.89
Methionine	2507.88	2457.72	2320.97	2308.67	2514.81	2416.89	2329.49	2133.63	1930.41	2016.58	1790.70	1774.96	1940.81	1834.97
O-Acetylcholine	330.96	323.78	300.67	287.78	271.38	336.27	336.68	314.93	285.56	293.67	250.33	245.14	281.61	269.30
Acetone	279.51	268.17	278.77	273.89	177.49	338.13	558.48	317.95	294.75	293.67	302.61	323.06	295.15	360.86
4-aminobutyrate	1741.05	1670.32	1877.37	2703.33	2730.56	2056.84	3257.42	1940.86	2681.96	2249.77	2074.91	2904.95	1839.74	4243.69
Pyruvate	2061.10	1900.24	1925.49	1899.24	1554.54	778.64	260.31	1501.21	1672.12	1511.59	1290.92	1148.51	552.50	151.81
Pyroglutamate	1933.35	1926.59	2266.57	2886.71	1885.37	2006.76	2579.06	2086.30	2874.06	1962.13	2374.94	2834.68	2090.04	2167.91
Succinate	607.17	599.47	656.85	757.32	633.84	618.01	711.37	649.80	726.17	616.37	660.50	689.93	660.12	672.95
Riboflavin	8.27	7.38	8.22	7.80	6.10	5.91	8.27	13.47	9.70	6.31	4.86	6.42	1.40	4.83
Sarcosine	1113.15	1124.26	1029.05	1060.40	994.50	1080.06	1142.70	1398.55	1293.65	1381.17	1281.74	1286.83	1280.27	1336.33
Aspartate	1381.97	1363.77	1273.36	1438.19	1379.18	1376.41	1496.45	1435.32	1250.51	1386.01	1180.69	1220.97	1102.12	1419.56
Asparagine	1260.95	1278.61	1197.14	1324.14	1265.88	1231.16	1329.13	1358.08	1289.99	1299.79	1245.06	1206.77	1165.95	1303.54
Creatinine3.03	2426.83	2391.79	2330.10	2361.59	2477.69	2395.85	2572.41	2441.67	2398.14	2391.55	2427.04	2396.30	2481.17	2617.65
Creatine	1380.18	1357.99	1300.76	1334.23	1318.70	1357.34	1380.51	1398.18	1343.48	1351.81	1342.50	1325.41	1383.49	1342.01
Choline	214.01	215.76	221.46	219.60	208.01	208.01	206.22	212.88	215.77	205.70	234.58	211.08	210.21	206.04
Betaine	3152.34	3126.98	3212.52	3272.15	3026.53	3067.25	3052.51	3249.80	3020.37	2998.42	3333.54	2973.73	3144.16	3135.87
Methanol	376.67	750.21	558.37	530.44	782.55	1219.18	116.43	119.34	623.86	791.66	604.63	432.11	619.29	281.71
Proline	542.30	530.66	503.28	462.60	534.07	526.09	525.77	556.99	459.30	533.25	523.90	508.63	522.29	574.66
Glycine	2268.59	2275.97	2201.20	2213.66	2611.42	2313.08	2204.08	2231.65	2240.74	2212.33	2251.90	2203.97	2184.40	2121.99
Glycerol	3163.11	3176.11	2923.84	3001.28	3676.03	2931.18	2004.54	3112.54	2970.24	2979.84	2737.07	2800.81	2316.00	2654.44
Threonine	2785.07	2739.91	2560.13	2580.71	2703.51	2708.51	2844.93	2766.08	2516.21	2624.14	2482.50	2540.84	2121.09	2562.87
Serine	7197.78	7149.28	7259.48	6952.51	7010.09	7110.43	7152.57	6936.79	7710.94	6846.94	7366.54	7208.04	7108.49	6960.64
Lactate	29870.15	29655.45	28078.91	28081.70	27697.11	29678.49	28178.40	30178.04	29290.88	27497.92	29590.64	28488.92	28635.73	29614.52
Uracil	263.80	244.07	226.22	205.17	196.02	237.97	257.69	257.62	221.50	236.80	236.05	232.88	152.03	227.60
Uridine	297.67	284.97	257.07	235.90	243.92	270.39	279.93	289.69	265.29	278.18	267.04	263.37	205.67	258.08
Adenosine	57.36	55.31	54.43	49.67	29.95	59.08	56.17	58.24	53.26	51.06	43.52	42.93	50.14	43.90
Inosine	41.66	42.15	27.83	32.16	8.36	45.92	44.65	37.03	34.82	35.70	13.86	30.99	-10.14	29.70
Orotate	11.78	13.94	15.01	9.50	8.04	9.49	12.12	13.84	4.69	7.44	12.67	12.38	6.42	8.09
Fumarate	4.64	4.99	3.20	4.73	4.73	5.27	4.07	5.09	4.26	4.17	6.65	4.29	5.09	4.08
Tyrosine	2243.65	2339.98	2175.76	2170.86	2396.11	2241.98	2370.19	2288.45	2328.91	2259.67	2184.24	2178.10	2227.30	2208.40
Phenylalanine	5208.18	5159.24	4867.42	4913.74	5032.28	5030.38	5084.99	5277.29	4923.98	5080.42	4880.44	4886.57	4753.48	4911.33
Tryptophan	962.83	958.10	920.45	918.87	893.57	975.61	960.43	846.17	770.86	812.99	730.26	810.60	775.91	800.37
1-Methylhistidine	451.38	545.60	851.32	702.79	560.70	467.79	475.52	486.83	835.12	464.28	816.61	812.41	210.42	448.26
Hypoxanthine	488.14	500.65	448.78	465.29	446.96	489.27	544.59	603.74	487.32	534.90	592.98	500.97	702.96	493.39
Formate	12250.90	12132.58	11554.71	11639.46	14541.01	11925.70	12257.63	12260.74	12288.50	12358.23	12494.86	12146.42	12132.01	11342.82

Table 26. ^{1}H-NMR spectral data (expressed as μM) of compounds detected forn *Listeria monocytogenes* ScottA cells (into BHI broth) in relation to gas plasma treatment time (GP60 min) and storage times following the plasma treatments (0, 15, 30, 60, 180, 360, 1440 min).

	GP60						
	0	**15**	**30**	**60**	**180**	**360**	**1440**
Leucine	22376.27	22765.35	29156.79	28279.84	28358.88	22168.98	28008.47
Isoleucine	2279.10	2205.04	3841.16	3696.52	3723.08	2152.05	3694.14
Valine	4279.43	4161.48	4798.88	4662.39	4659.98	4022.88	4587.09
3-Methyl-2-oxovalerate	182.63	157.47	165.38	166.88	163.70	156.50	150.85
Ethanol	3707.23	3545.42	3606.05	3545.99	3495.32	3583.53	3415.32
Alanine	6016.10	6083.08	4934.25	5769.54	5721.75	5903.62	5700.50
Lysine	23187.08	21497.09	20823.69	21703.05	22003.70	23795.21	31413.67
Acetate	21121.59	20441.39	21018.05	20592.89	20633.09	21654.35	21581.42
Glutamate	4783.70	4292.29	4310.57	4302.81	4414.66	5194.81	7150.07
Methionine	1374.35	1392.24	1343.78	1325.92	1329.90	1350.18	1108.45
O-Acetylcholine	277.53	281.33	270.75	271.10	265.79	267.02	211.34
Acetone	296.75	286.82	309.27	320.68	314.61	313.69	305.41
4-aminobutyrate	2342.85	2303.63	2252.48	2766.16	2667.66	2719.73	5272.62
Pyruvate	1282.44	1268.97	1150.72	1109.62	687.37	380.98	104.91
Pyroglutamate	2047.28	2082.30	2074.03	2271.10	1930.38	2133.96	2412.03
Succinate	608.75	664.41	647.88	699.27	674.56	714.00	710.24
Riboflavin	13.62	9.29	6.33	6.75	5.87	8.53	6.97
Sarcosine	1994.95	1914.61	1900.73	1841.67	1824.90	1887.76	1827.81
Aspartate	1405.29	1522.90	1373.56	1399.76	1340.40	1479.94	1300.95
Asparagine	1181.32	1409.01	1288.53	1300.32	1304.10	1339.86	1206.66
Creatinine3.03	2499.43	2546.08	2457.88	2384.60	2449.70	2554.52	2793.26
Creatine	1430.16	1439.41	1381.82	1362.76	1382.16	1426.32	1343.07
Choline	207.76	219.23	222.76	204.60	210.80	216.76	215.49
Betaine	3063.39	3277.40	3300.46	2956.35	2998.55	3215.56	3166.92
Methanol	246.62	625.48	1019.85	456.05	463.05	483.13	450.63
Proline	483.93	534.74	558.62	501.32	510.16	523.46	540.70
Glycine	2232.93	2329.37	2347.84	2163.22	2185.88	2269.48	2176.85
Glycerol	3232.86	3282.61	3175.59	2954.14	2942.74	3050.93	2812.43
Threonine	2837.02	2785.39	2687.71	2613.47	2618.42	2706.14	2620.51
Serine	6754.55	7525.38	7445.89	6595.42	7002.01	7401.35	7118.55
Lactate	29858.21	30242.89	29690.24	28123.38	28745.16	30150.68	28672.83
Uracil	264.07	240.08	241.42	206.01	242.60	233.44	236.42
Uridine	294.37	274.20	273.77	221.77	263.40	267.37	260.79
Adenosine	53.32	56.73	62.68	36.98	50.86	61.34	52.47
Inosine	45.19	37.04	41.64	21.09	34.82	49.66	30.63
Orotate	12.25	14.25	12.48	12.18	12.55	14.94	7.13
Fumarate	2.20	2.59	4.41	4.00	4.98	3.48	6.68
Tyrosine	2392.12	2397.11	2268.78	2260.06	2214.40	2303.04	2214.20
Phenylalanine	5121.70	5274.71	5162.21	4970.05	4974.30	5016.33	4918.03
Tryptophan	527.82	562.38	561.53	549.90	556.16	509.35	545.01
1-Methylhistidine	363.95	712.19	895.54	533.28	521.20	418.49	910.17
Hypoxanthine	504.07	589.34	565.69	613.97	557.69	525.22	550.02
Formate	13111.41	13528.54	13403.03	13277.72	13314.74	13675.41	13058.71

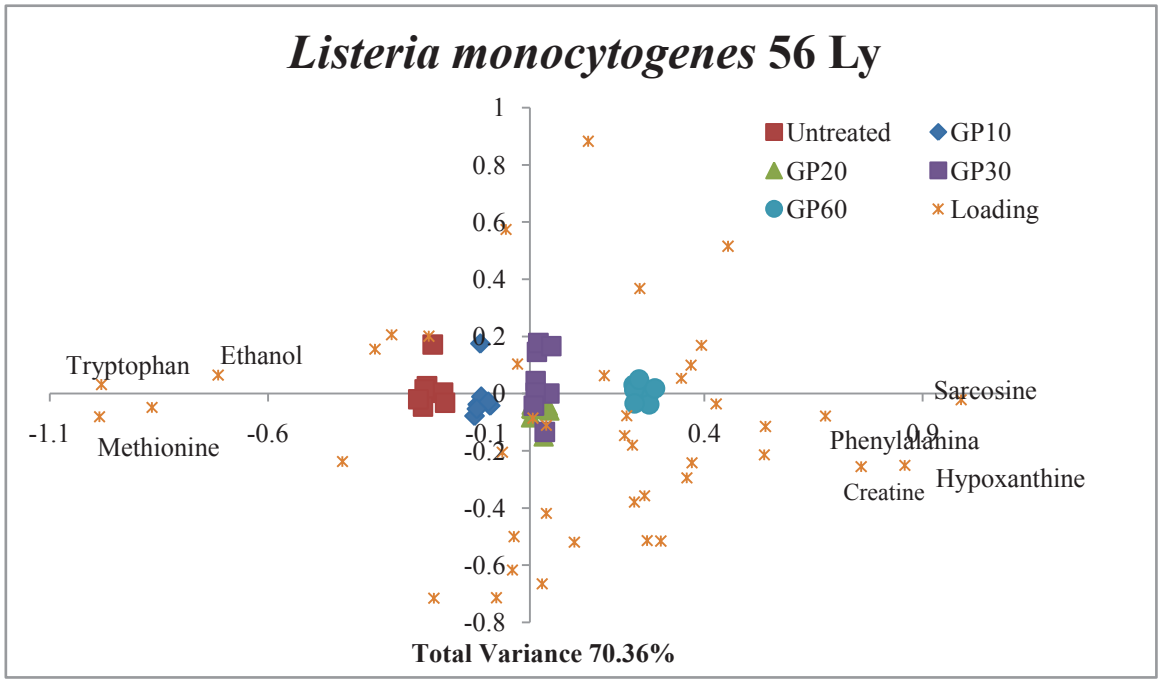

Figure 68. Plots of the first two canonical axes produced by Canonical Analysis of Principal Coordinates (CAP) of ^{1}H-NMR spectral data relative to *Listeria monocytogenes* 56Ly cells (into BHI broth) exposed to gas plasma for different times (0, 10, 20, 30, 60 min).

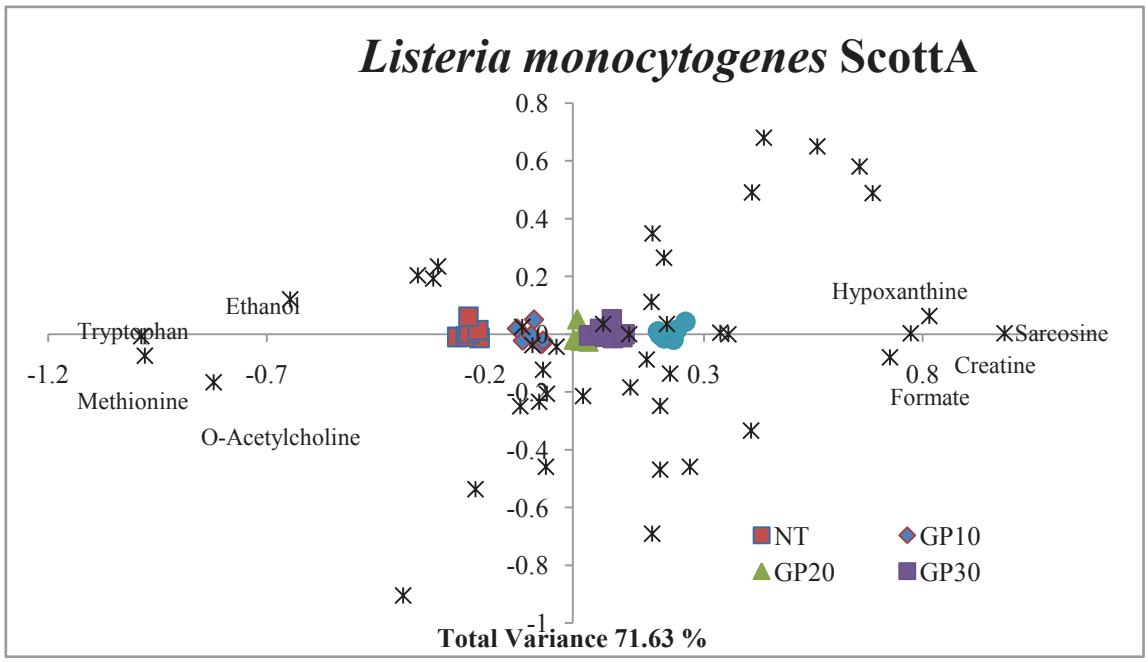

Figure 69. Plots of the first two canonical axes produced by Canonical Analysis of Principal Coordinates (CAP) of ^{1}H-NMR spectral data relative to *Listeria monocytogenes* ScottA cells (into BHI broth) exposed to gas plasma for different times (0, 10, 20, 30, 60 min).

Table 27. RGE values relative to different genes following exposure of *Listeria monocytogenes* 56Ly cells (into BHI broth) to gas plasma treatments for 10, 20, 30 and 60 min.

		RGE			
	genes	**GP 10**	**GP 20**	**GP 30**	**GP 60**
cspB	*General stress*	0.95 ± 0.01	0.92 ± 0.01	1.06 ± 0.01	1.07 ± 0.01
cspD	*General stress*	0.92 ± 0.01	0.84 ± 0.01	1.24 ± 0.01	1.15 ± 0.01
kat	*Oxidative stress*	0.89 ± 0.01	0.91 ± 0.01	0.97 ± 0.01	0.75 ± 0.01
sigB	*General stress*	0.89 ± 0.05	0.91 ± 0.01	1.21 ± 0.04	1.23 ± 0.05
hrcA	*General stress*	0.93 ± 0.01	0.96 ± 0.01	1.49 ± 0.01	1.33 ± 0.01
hly	*Virulence*	0.84 ± 0.07	0.79 ± 0.04	1.28 ± 0.01	1.24 ± 0.05
fri	*Oxidative stress*	1.02 ± 0.05	1.00 ± 0.04	1.08 ± 0.03	1.17 ± 0.05
dnaK	*General stress*	0.94 ± 0.02	0.93 ± 0.01	1.01 ± 0.01	0.98 ± 0.02
pgm	*Phosphoglyceromutase*	0.80 ± 0.04	0.84 ± 0.04	0.89 ± 0.03	0.85 ± 0.01
pdhD	*Pyruvate dehydrogenase enzyme (Pdh) complex*	0.97 ± 0.01	1.08 ± 0.02	0.65 ± 0.01	0.56 ± 0.01

Table 28. RGE values relative to different genes following exposure of *Listeria monocytogenes* ScottA cells (into BHI broth) to gas plasma treatments for 10, 20, 30 and 60 min.

		RGE			
	genes	**GP 10**	**GP 20**	**GP 30**	**GP 60**
cspB	*General stress*	0.93 ± 0.01	0.99 ± 0.02	0.97± 0.02	1.04 ± 0.02
hly	*Virulence*	1.03 ± 0.02	0.99 ± 0.02	0.97 ± 0.01	1.04 ± 0.05
fri	*Oxidative stress*	1.23 ± 0.01	0.67 ± 0.00	0.64 ± 0.00	1.47 ± 0.03
dnaK	*General stress*	1.07 ± 0.02	0.98 ± 0.01	0.98 ± 0.02	1.04 ± 0.02
pgm	*Phosphoglyceromutase*	1.16 ± 0.02	1.25 ± 0.02	1.36 ± 0.02	1.00 ± 0.01
pdhD	*Pyruvate dehydrogenase enzyme (Pdh) complex*	1.01 ± 0.03	1.02 ± 0.03	0.96 ± 0.01	0.87 ± 0.02

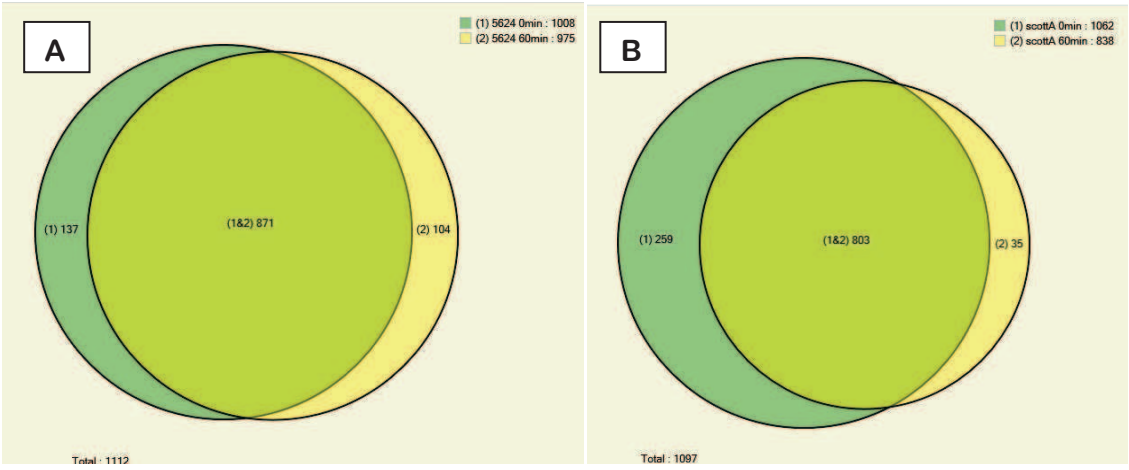

Figure 70. Distribution of *Listeria monocytogenes* strain 56Ly (A) and ScottA (B) proteins obtained in untreated and GP treated (60 min) cells. Venn diagram shows the dispersion of total proteins identified under both conditions, according PatternLab's AAPV module, using 0.01 probability.

Table 29. Proteins identified in *Listeria monocytogenes* 56Ly that are differentially expressed in untreated and treated (60 min) cells.

Accession Number	Fold Change	pValue	Protein name
AGR13932.1	34.75	0.0013	neopullulanase
AGR13931.1	22.75	0.0000	sugar ABC transporter substrate-binding protein
AGR14407.1	20.67	0.0439	PTS mannose transporter subunit IIA
AGR14405.1	16.67	0.0100	PTS beta-glucoside transporter subunit IIB
AGR14260.1	10.67	0.0085	F0F1 ATP synthase subunit gamma
AGR14384.1	10.50	0.0014	transketolase
AGR14441.1	10.00	0.0079	acyl--CoA ligase
AGR14202.1	8.00	0.0001	ATP-dependent Clp protease proteolytic subunit
AGR13189.1	8.00	0.0022	elongation factor P
AGR13055.1	8.00	0.0064	peptidase M28
AGR14709.1	7.41	0.0008	sugar ABC transporter substrate-binding protein
AGR13206.1	7.00	0.0008	2-oxoisovalerate dehydrogenase subunit alpha
AGR17326.1	6.25	0.0014	PTS-associated protein
AGR13553.1	6.25	0.0014	PTS-associated protein
AGR15137.1	6.00	0.0009	flagellar hook protein FlgE
AGR13554.1	6.00	0.0323	PTS mannose transporter subunit IIA
AGR13858.1	5.86	0.0404	50S ribosomal protein L32
AGR14004.1	5.85	0.0012	3-oxoacyl-ACP synthase
AGR13301.1	5.83	0.0128	heat shock protein GrpE
AGR14797.1	5.80	0.0001	sugar ABC transporter ATP-binding protein
AGR13207.1	5.68	0.0060	2-oxoisovalerate dehydrogenase subunit beta
AGR13287.1	5.50	0.0016	glycine-tRNA synthetase subunit beta
AGR15130.1	5.38	0.0006	flagellin
AGR14774.1	5.23	0.0133	50S ribosomal protein L1
AGR13442.1	5.22	0.0066	D-alanine aminotransferase
AGR13060.1	5.11	0.0002	phenylalanyl-tRNA synthase subunit beta
AGR14333.1	5.07	0.0355	30S ribosomal protein S11
AGR14288.1	4.67	0.0122	CTP synthetase
AGR14356.1	4.51	0.0174	50S ribosomal protein L4
AGR15129.1	4.33	0.0001	chemotaxis protein CheV
AGR14725.1	4.21	0.0397	regulatory protein
AGR14775.1	4.15	0.0023	50S ribosomal protein L10
AGR14378.1	4.15	0.0058	elongation factor P
|AGR14181.1	4.13	0.0001	glyceraldehyde-3-phosphate dehydrogenase
AGR14068.1	3.79	0.0005	PTS fructose transporter subunit IIC
AGR14323.1	3.69	0.0171	50S ribosomal protein L13
AGR13420.1	3.63	0.0061	30S ribosomal protein S4
AGR15276.1	3.59	0.0026	DEAD/DEAH box helicase
AGR14350.1	3.54	0.0072	50S ribosomal protein L16
AGR13092.1	3.53	0.0031	trehalose-6-phosphate hydrolase
AGR15463.1	3.45	0.0087	GTP-binding protein
AGR14334.1	3.28	0.0137	30S ribosomal protein S13
AGR13280.1	3.25	0.0016	DEAD/DEAH box helicase
AGR13813.1	3.25	0.0030	glucosamine-fructose-6-phosphate aminotransferase
AGR14003.1	3.22	0.0015	3-oxoacyl-ACP synthase
AGR13443.1	3.20	0.0024	diguanylate cyclase
AGR14744.1	3.15	0.0045	hypothetical protein M643_11320
AGR14332.1	3.06	0.0228	DNA-directed RNA polymerase subunit alpha
AGR15163.1	2.95	0.0118	chemotaxis protein
AGR14363.1	2.80	0.0180	NADH dehydrogenase
AGR14286.1	2.80	0.0401	fructose-bisphosphate aldolase
AGR13164.1	2.78	0.0319	30S ribosomal protein S15
AGR14344.1	2.69	0.0001	30S ribosomal protein S8
AGR14773.1	2.69	0.0195	50S ribosomal protein L11
AGR14259.1	2.64	0.0057	F0F1 ATP synthase subunit beta
AGR13423.1	2.63	0.0093	3-deoxy-7-phosphoheptulonate synthase
AGR14357.1	2.59	0.0022	50S ribosomal protein L3
AGR13329.1	2.58	0.0244	alanyl-tRNA synthase
AGR13198.1	2.50	0.0052	cold-shock protein
AGR14776.1	2.48	0.0282	50S ribosomal protein L7/L12
AGR13760.1	2.47	0.0095	glycerol-3-phosphate dehydrogenase
|AGR13489.1	2.45	0.0100	30S ribosomal protein S2
AGR13156.1	2.43	0.0063	transcription elongation factor NusA
AGR13344.1	2.35	0.0030	aspartyl-tRNA synthetase
AGR14337.1	2.33	0.0171	adenylate kinase

Table 29. continue

Accession Number	Fold Change	pValue	Protein name
AGR13373.1	2.31	0.0266	rod shape-determining protein Mbl
AGR13113.1	2.31	0.0015	DNA topoisomerase I
AGR13488.1	2.28	0.0245	elongation factor Ts
AGR15303.1	2.28	0.0074	serine-protein kinase
AGR13834.1	2.24	0.0345	isoleucyl-tRNA synthetase
AGR14343.1	2.23	0.0159	50S ribosomal protein L6
AGR13266.1	2.22	0.0257	ribonuclease J
AGR13395.1	2.22	0.0065	pyruvate kinase
AGR13586.1	2.21	0.0341	glutamyl-tRNA amidotransferase subunit C
AGR14322.1	2.14	0.0131	30S ribosomal protein S9
|AGR13777.1	2.10	0.0078	purine nucleoside phosphorylase
AGR14377.1	2.10	0.0042	elongation factor Tu
AGR13761.1	2.06	0.0121	GTP-binding protein Der
AGR14353.1	2.05	0.0001	30S ribosomal protein S19
AGR13741.1	2.02	0.0002	formate acetyltransferase
AGR17514.1	2.01	0.0002	formate acetyltransferase
AGR14753.1	2.00	0.0086	lysyl-tRNA synthetase
AGR14355.1	2.00	0.0192	50S ribosomal protein L23
AGR13159.1	2.00	0.0194	translation initiation factor IF-2
AGR13995.1	1.94	0.0217	peptide ABC transporter substrate-binding protein
AGR15455.1	1.92	0.0225	dihydrolipoamide dehydrogenase
AGR16016.1	1.90	0.0267	spore coat protein
AGR14331.1	1.86	0.0332	50S ribosomal protein L17
AGR13105.1	1.83	0.0332	trigger factor
AGR13615.1	1.80	0.0057	translation initiation factor IF-3
AGR13165.1	1.74	0.0078	polyribonucleotide nucleotidyltransferase
AGR13396.1	1.72	0.0219	6-phosphofructokinase
AGR15080.1	-1.80	0.0407	aldo/keto reductase
AGR13318.1	-1.92	0.0015	oligoendopeptidase
AGR18189.1	-2.15	0.0321	dihydroxyacetone kinase subunit K
AGR13831.1	-2.22	0.0194	cold-shock protein
AGR14269.1	-2.23	0.0237	serine hydroxymethyltransferase
AGR14422.1	-2.24	0.0153	glyoxal reductase
AGR13404.1	-2.27	0.0225	alanine dehydrogenase
AGR13071.1	-2.79	0.0044	thioredoxin
AGR15162.1	-3.27	0.0117	pyruvate oxidase
AGR18830.1	-3.63	0.0356	N-acetylmuramoyl-L-alanine amidase
AGR14987.1	-4.62	0.0113	tagatose-bisphosphate aldolase
AGR13363.1	-4.80	0.0091	glycerol kinase
AGR14588.1	-4.87	0.0028	PTS beta-glucoside transporter subunit IIABC
AGR13910.1	-5.76	0.0230	pyridoxal biosynthesis protein
AGR18185.1	-6.19	0.0033	amidase
AGR12993.1	-22.33	0.0007	propanediol utilization protein PduB
AGR13704.1	-32.43	0.0003	cold-shock protein

The proteins listed in this Table were found to be statistically differentially expressed using PatternLab's Tfold module with an absolute fold change greater than 2.0 (BH-FDR 0.05). Fold change positive values: up-regulated in untreated cells; negative values: up-regulated in cells treated 60min; in this case, protein must be at least 1.5 times differentially regulated.

Table 30. Unique proteins identified in untreated cells of *Listeria monocytogenes* 56 Ly.

accession number	spec count	protein name
AGR17703.1	154	neopullulanase
AGR14648.1	102	PTS mannose transporter subunit IID
AGR14712.1	98	oligo-1,6-glucosidase
AGR14386.1	75	ribose 5-phosphate isomerase
AGR18157.1	72	transketolase
AGR15216.1	53	hypothetical protein M643_14280
AGR15205.1	48	FMN-dependent NADH-azoreductase
AGR14431.1	34	hypothetical protein M643_09150
AGR14711.1	30	alpha-glucosidase
AGR17784.1	25	ferrochelatase
AGR17941.1	19	hypothetical protein M640_11455
AGR13772.1	17	PhoP family transcriptional regulator
AGR15204.1	17	PTS sugar transporter subunit IIA
AGR18170.1	15	universal stress protein UspA
AGR17127.1	15	hypothetical protein M640_06520
AGR13899.1	14	argininosuccinate synthase
AGR15202.1	12	PTS friuctose transporter subunit IIB
AGR14826.1	12	6-phospho-beta-glucosidase
AGR13817.1	11	PTS sugar transporter
AGR14389.1	11	PTS galactitol transporter subunit IIC
AGR13927.1	11	maltose phosphorylase
AGR13323.1	10	O-methyltransferase
AGR15780.1	10	oxidoreductase
AGR14391.1	9	PTS fructose transporter subunit IIA
AGR14091.1	9	COF family hydrolase
AGR15115.1	9	flagellar motor switch protein FliN
AGR13193.1	9	transcription antitermination protein NusB
AGR18099.1	8	cobalt ABC transporter ATP-binding protein
AGR14136.1	8	cysteine desulfurase
AGR15366.1	8	peptidase
AGR15472.1	8	N-acetylmuramoyl-L-alanine amidase
AGR13521.1	8	lysyl-tRNA synthetase
AGR15793.1	8	agmatine deiminase
AGR15907.1	7	hypothetical protein M643_11875
AGR15480.1	7	monooxygenase
AGR15142.1	7	hypothetical protein M643_13870
AGR13417.1	7	aminotransferase V
AGR27331.1	6	hypothetical protein M641_01805
AGR15133.1	6	flagellar motor switch protein FliN
AGR14214.1	6	pyrophosphatase
AGR14388.1	6	alcohol dehydrogenase
AGR13629.1	6	DNA-binding protein
AGR13540.1	6	RNA methyltransferase
AGR13361.1	6	prephenate dehydratase
AGR15364.1	6	GntR family transcriptional regulator
AGR13016.1	6	ethanolamine transporter
AGR14820.1	6	threonine aldolase
AGR14053.1	6	aspartate aminotransferase
AGR14011.1	6	GNAT family acetyltransferase
AGR14033.1	6	membrane protein
|AGR13385.1	6	primosomal protein DnaI
AGR13979.1	6	3-ketoacyl-ACP reductase
AGR13338.1	5	cysteine desulfurase
AGR13694.1	5	glyoxalase
AGR14052.1	5	methionine ABC transporter ATP-binding protein
AGR13722.1	5	peptidase
AGR14857.1	5	hypothetical protein M643_12145

Table 30. Continue

AGR13419.1	4	hypothetical protein M643_02465
AGR15148.1	4	flagellar biosynthesis protein FliS
AGR14750.1	4	dienelactone hydrolase
AGR14155.1	4	hypothetical protein M643_07300
AGR14385.1	4	ribulose-phosphate 3-epimerase
AGR15255.1	4	endoribonuclease L-PSP
AGR13511.1	4	dehydrogenase
AGR13605.1	4	N5-carboxyaminoimidazole ribonucleotide mutase
AGR13971.1	4	hypothetical protein M643_06215
AGR14383.1	4	ribulose-phosphate 3-epimerase
AGR13203.1	4	phosphate acetyltransferase
AGR13776.1	4	diaminopimelate decarboxylase
AGR13279.1	4	endonuclease IV
AGR13376.1	4	folylpolyglutamate synthase
AGR15412.1	4	aminotransferase A
AGR17622.1	4	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase
AGR17548.1	4	diaminopimelate decarboxylase
AGR15120.1	4	flagellar biosynthesis protein FlhA
AGR13467.1	4	helicase SNF2
AGR14671.1	4	hypothetical protein M643_10920
AGR14649.1	4	ManO-protein
AGR15259.1	4	methionine ABC transporter ATP-binding protein
AGR15295.1	4	alanine racemase
AGR14647.1	4	PTS alpha-glucoside transporter subunit IIBC
AGR15207.1	4	2-hydroxyglutaryl-CoA dehydratase
AGR14862.1	3	hypothetical protein M643_12170
AGR14562.1	3	ribonuclease P
AGR13157.1	3	hypothetical protein M643_01090
AGR13727.1	3	thioredoxin
AGR13958.1	3	flavodoxin
AGR14912.1	3	transcriptional regulator
AGR13843.1	3	cell division protein SepF
AGR13123.1	3	CoA-binding protein
AGR14755.1	3	excinuclease Uvr
AGR15021.1	3	GntR family transcriptional regulator
AGR13461.1	3	peptidase S66
AGR13491.1	3	hypothetical protein M643_03225
AGR16655.1	3	ribose 5-phosphate isomerase
AGR13332.1	3	transcriptional regulator
AGR15784.1	3	diphosphomevalonate decarboxylase
AGR14275.1	3	homoserine kinase
AGR13358.1	3	Holliday junction DNA helicase RuvA
AGR15415.1	3	hypothetical protein M643_15370
AGR17303.1	3	recombination protein RecX
AGR13438.1	3	tRNA (guanine-N(7)-)-methyltransferase
AGR18716.1	3	hypothetical protein M640_07680
AGR15161.1	3	ferrous iron transporter A
AGR14513.1	3	16S rRNA methyltransferase
AGR13679.1	3	ATPase P

Table 30. Continue

AGR14450.1	2	cupin
AGR15345.1	2	protein tyrosine phosphatase
AGR13347.1	2	D-tyrosyl-tRNA(Tyr) deacylase
AGR13336.1	2	alpha/beta hydrolase
AGR14662.1	2	diguanylate phosphodiesterase
AGR13608.1	2	ABC transporter ATPase
AGR14569.1	2	recombinase F
AGR14189.1	2	hypothetical protein M643_07590
AGR14715.1	2	ribonuclease M5
AGR13844.1	2	hypothetical protein M643_05505
AGR13754.1	2	heptaprenyl diphosphate synthase subunit II
AGR13805.1	2	isopropylmalate isomerase
AGR13798.1	2	dihydroxy-acid dehydratase
AGR15317.1	2	membrane protein
AGR16004.1	2	LacI family transcriptional regulator
AGR14529.1	2	amidohydrolase
AGR14144.1	2	histidine kinase
AGR14527.1	2	amidohydrolase
AGR15258.1	2	glutamine ABC transporter permease
AGR13742.1	2	hypothetical protein M643_04915
AGR14133.1	2	hypothetical protein M643_07190
AGR13076.1	2	ribonuclease PH
AGR14824.1	2	phosphomethylpyrimidine kinase
AGR13934.1	2	LacI family transcriptional regulator
AGR13072.1	2	excinuclease ABC subunit C
AGR13650.1	2	primosomal protein N'
AGR13295.1	2	hydrolase
AGR13577.1	2	ABC transporter permease

Table 31. Unique proteins identified in *Listeria monocytogenes* 56 Ly exposed to gas plasma for 60 min

accession number	spec count	protein name
AGR14966.1	32	phosphoglycerate mutase
AGR12995.1	23	propanediol dehydratase medium subunit
AGR16597.1	20	succinate-semialdehyde dehdyrogenase
AGR14162.1	19	hypothetical protein M643_07335
AGR15322.1	14	succinate-semialdehyde dehdyrogenase
AGR13693.1	14	pyruvate phosphate dikinase
AGR14314.1	14	oxidoreductase
AGR14920.1	14	glutamate decarboxylase
AGR13346.1	11	N-acetylmuramoyl-L-alanine amidase
AGR13306.1	10	30S ribosomal protein S20
AGR15101.1	10	carboxymuconolactone decarboxylase
AGR27499.1	8	hypothetical protein M641_07630
AGR14434.1	8	hypothetical protein M643_09170
AGR12996.1	7	propanediol dehydratase small subunit
AGR13126.1	7	S-ribosylhomocysteinase
AGR15047.1	7	GNAT family acetyltransferase
AGR18805.1	6	hypothetical protein M640_10805
AGR27697.1	6	peptidase M15
AGR13444.1	6	DNA mismatch repair protein MutT
AGR13064.1	6	membrane protein
AGR15696.1	6	peptidase M15
AGR14890.1	5	glyoxalase
AGR14313.1	5	hypothetical protein M643_08420
AGR27471.1	5	phage tail protein
AGR14838.1	5	transaldolase
AGR13004.1	5	ethanolamine utilization protein EutN
AGR14235.1	5	peptidase P60
AGR14885.1	5	alpha-mannosidase
AGR14479.1	5	aryl-phospho-beta-D-glucosidase
AGR14157.1	5	glutamate decarboxylase
AGR15898.1	5	internalin
AGR14183.1	5	RNA polymerase sigma54 factor
AGR13151.1	5	1-deoxy-D-xylulose 5-phosphate reductoisomerase
AGR13965.1	5	trehalose utilization protein
AGR13499.1	5	adhesin
AGR14742.1	4	hypothetical protein M643_11310
AGR15385.1	4	glycosyl transferase family 8
AGR14159.1	4	transcription antiterminator BglG
AGR13317.1	4	haloacid dehalogenase
AGR15109.1	4	oxidoreductase
AGR14223.1	4	hydrolase
AGR15383.1	4	hypothetical protein M643_15210
AGR13167.1	4	hypothetical protein M643_01140
AGR13918.1	4	FMN reductase
AGR13651.1	4	phosphopantothenoylcysteine decarboxylase
AGR13253.1	4	glycine/betaine ABC transporter ATP-binding protein
AGR15052.1	4	multidrug ABC transporter ATP-binding protein
AGR15309.1	3	hypothetical protein M643_14810
AGR15742.1	3	hypothetical protein M643_07790

Table 31. Continue

AGR18918.1	3	hypothetical protein M640_14510
AGR15105.1	3	hypothetical protein M643_13680
AGR14197.1	3	antirepressor
AGR14324.1	3	tRNA pseudouridine synthase A
AGR27494.1	3	hypothetical protein M641_07590
AGR14461.1	3	hypothetical protein M643_09320
AGR27731.1	3	hypothetical protein M641_15460
AGR14665.1	3	XRE family transcriptional regulator
AGR16165.1	3	glyoxalase
AGR15187.1	3	lipoate-protein ligase A
AGR14602.1	3	hypothetical protein M643_10150
AGR14622.1	3	antirepressor
AGR15825.1	3	hypothetical protein M643_10415
AGR13612.1	3	exodeoxyribonuclease III
AGR13759.1	3	protein-tyrosine phosphatase
AGR27462.1	3	hypothetical protein M641_07350
AGR13007.1	3	iron-containing alcohol dehydrogenase
AGR13597.1	3	phosphoribosylaminoimidazole synthetase
AGR14030.1	3	penicillin-binding protein
AGR13069.1	3	DNA polymerase
AGR15025.1	2	seryl-tRNA synthetase
AGR13090.1	2	hypothetical protein M643_00745
AGR14840.1	2	ribose 5-phosphate isomerase
AGR13142.1	2	hypothetical protein M643_01010
AGR13601.1	2	phosphoribosylformylglycinamidine synthase
AGR13172.1	2	hypothetical protein M643_01165
AGR15465.1	2	hypothetical protein M643_15660
AGR15294.1	2	4'-phosphopantetheinyl transferase
AGR14905.1	2	PTS mannose transporter subunit IIAB
AGR14937.1	2	heme-degrading monooxygenase IsdG
AGR14122.1	2	disulfide oxidoreductase
AGR14115.1	2	membrane protein
AGR13623.1	2	glutamine amidotransferase
AGR13755.1	2	ubiquinone/menaquinone biosynthesis methyltransferase
AGR14653.1	2	NAD(P)H nitroreductase
AGR14980.1	2	SAM-dependent methyltransferase
AGR14760.1	2	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase
AGR13637.1	2	DeoR family transcriptional regulator
AGR15357.1	2	hypothetical protein M643_15075
AGR13904.1	2	carbohydrate kinase
AGR13241.1	2	PadR family transcriptional regulator
AGR15447.1	2	molybdenum cofactor biosynthesis protein MoeA
AGR13581.1	2	RNA methyltransferase
AGR27732.1	2	cell division protein Fic
AGR14713.1	2	hydrolase TatD
AGR15442.1	2	molybdopterin biosynthesis protein MoeA
AGR14730.1	2	Ivanolysin
AGR13303.1	2	coproporphyrinogen III oxidase
AGR12983.1	2	propanediol utilization protein PduS
AGT06926.1	2	hypothetical protein M643_p00635
AGR15053.1	2	ABC transporter
AGR14598.1	2	arginine deiminase
AGR13224.1	2	branched-chain amino acid ABC transporter permease

Table 32. Proteins identified of *Listeria monocytogenes* ScottA that are differentially expressed in untreated and treated (60 min) cells.

Accession	Fold Change	pValue	Description
AGR13910.1	78.00	0.0165	pyridoxal biosynthesis protein
AGR14415.1	31.33	0.0163	dihydroxyacetone kinase subunit K
AGR12992.1	30.67	0.0478	carboxysome shell protein
AGR14416.1	23.33	0.0011	dihydroxyacetone kinase
AGR14987.1	23.06	0.0093	tagatose-bisphosphate aldolase
AGR14588.1	18.14	0.0269	PTS beta-glucoside transporter subunit IIABC
AGR13404.1	16.45	0.0034	alanine dehydrogenase
AGR13704.1	14.94	0.0362	cold-shock protein
AGR13254.1	11.67	0.0327	ABC transporter permease
AGR13716.1	10.25	0.0417	penicillin-binding protein
AGR14095.1	9.85	0.0320	glutamate decarboxylase
AGR12993.1	9.78	0.0031	propanediol utilization protein PduB
AGR17475.1	9.67	0.0003	formate--tetrahydrofolate ligase
AGR13702.1	9.67	0.0003	formate--tetrahydrofolate ligase
AGR13130.1	8.71	0.0012	glycerol-3-phosphate dehydrogenase
AGR15349.1	8.70	0.0042	general stress protein
AGR14843.1	8.17	0.0407	dihydroxyacetone kinase subunit K
AGR18185.1	7.63	0.0187	amidase
AGR13426.1	7.22	0.0203	aminopeptidase
AGR17219.1	7.22	0.0203	aminopeptidase
AGR14790.1	7.20	0.0352	aryl-phospho-beta-D-glucosidase
AGR13424.1	7.18	0.0283	general stress protein
AGR13141.1	7.09	0.0099	transketolase
AGR13443.1	7.06	0.0469	diguanylate cyclase
AGR13403.1	7.00	0.0011	dipeptidase
AGR13613.1	6.97	0.0368	50S ribosomal protein L20
AGR13221.1	6.38	0.0228	hypothetical protein M643_01420
AGR14090.1	6.25	0.0144	glucosamine-6-phosphate isomerase
AGR13352.1	6.17	0.0255	preprotein translocase subunit SecD
AGR16353.1	6.08	0.0362	aldo/keto reductase
AGR15080.1	6.08	0.0362	aldo/keto reductase
AGR15409.1	5.69	0.0476	phosphoenolpyruvate-protein phosphotransferase
AGR13422.1	5.48	0.0205	catabolite control protein A
AGR13845.1	5.44	0.0216	cell division protein FtsZ
AGR13610.1	5.40	0.0036	peptidase T
AGR13501.1	5.33	0.0171	dihydroxynaphthoic acid synthetase
AGR13271.1	4.87	0.0273	superoxide dismutase
AGR13173.1	4.83	0.0149	glucokinase
AGR14073.1	4.78	0.0149	pseudouridine-5'-phosphate glycosidase
AGR13821.1	4.76	0.0169	acetolactate synthase
AGR14748.1	4.58	0.0081	cysteine synthase
AGR16015.1	4.50	0.0019	dTDP-4-dehydrorhamnose 3,5-epimerase
AGR14346.1	4.48	0.0136	50S ribosomal protein L5
AGR13990.1	4.45	0.0074	oligopeptidase PepB
AGR14138.1	4.38	0.0020	iron ABC transporter ATP-binding protein
AGR15452.1	4.35	0.0011	pyruvate dehydrogenase E1 subunit alpha
AGR13912.1	3.35	0.0023	phosphotransacetylase
AGR15403.1	-6.67	0.0099	ATP-dependent Clp protease ATP-binding protein
AGR14710.1	-15.00	0.0269	glycosyl hydrolase family 31
AGR14390.1	-19.47	0.0083	PTS galactitol transporter subunit IIB

The proteins listed in this Table were found to be statistically differentially expressed using PatternLab's Tfold module with an absolute fold change greater than 2.0 (BH-FDR 0.05). Fold change positive values: up-regulated in untreated cells; negative values: up-regulated in cells treated 60min; in this case, protein must be at least 1.5 times differentially regulated.

Table 33. Unique proteins identified in untreated cells of *Listeria monocytogenes* ScottA.

accession number	spec count	protein name
AGR18189.1	144	dihydroxyacetone kinase subunit K
AGR17792.1	136	foldase PrsA
AGR15676.1	123	alkyl sulfatase
AGR12994.1	96	propanediol dehydratase large subunit
AGR17648.1	57	choloylglycine hydrolase
AGR14094.1	55	glutamate:gamma-aminobutyrate antiporter
AGR13877.1	50	choloylglycine hydrolase
AGR16597.1	50	succinate-semialdehyde dehdyrogenase
AGR14118.1	45	NADH dehydrogenase
AGR14120.1	44	NAD dependent epimerase/dehydratase
AGR15322.1	43	succinate-semialdehyde dehdyrogenase
AGR14157.1	41	glutamate decarboxylase
AGR14966.1	35	phosphoglycerate mutase
AGR13656.1	34	short-chain dehydrogenase
AGR12995.1	30	propanediol dehydratase medium subunit
AGR13693.1	30	pyruvate phosphate dikinase
AGR13911.1	28	glutamine amidotransferase
AGR13471.1	27	hypothetical protein M643_02735
AGR17978.1	27	NADPH dehydrogenase
AGR14202.1	27	ATP-dependent Clp protease proteolytic subunit
AGR14839.1	26	short-chain dehydrogenase
AGR17466.1	26	pyruvate phosphate dikinase
AGR13387.1	25	NrdR family transcriptional regulator
AGR14422.1	24	glyoxal reductase
AGR13346.1	24	N-acetylmuramoyl-L-alanine amidase
AGR17682.1	23	glutamine amidotransferase
AGR13006.1	22	aldehyde dehydrogenase
AGR13320.1	22	hypothetical protein M643_01960
AGR13004.1	18	ethanolamine utilization protein EutN
AGR13851.1	18	penicillin-binding protein 2B
AGR13107.1	17	signal peptidase
AGR14661.1	17	lipase
AGR14314.1	17	oxidoreductase
AGR18087.1	17	oxidoreductase
AGR17330.1	16	oxidoreductase
AGR18274.1	16	plasmid partitioning protein ParB
AGR13752.1	15	chorismate synthase
AGR16057.1	15	6-phospho-beta-glucosidase
AGR17982.1	15	phosphoglucomutase
AGR13114.1	14	tRNA (uracil-5-)-methyltransferase
AGR15269.1	14	sugar ABC transporter substrate-binding protein
AGR16904.1	14	tRNA (uracil-5-)-methyltransferase
AGR13336.1	14	alpha/beta hydrolase
AGR14608.1	13	50S ribosomal protein L9
AGR14579.1	13	6-phospho-beta-glucosidase
AGR13599.1	13	phosphoribosylformylglycinamidine synthase
AGR14300.1	12	amidase
AGR13009.1	12	acetate kinase
AGR14207.1	12	glmZ(sRNA)-inactivating NTPase
AGR15104.1	11	maltose O-acetyltransferase
AGR27501.1	11	hypothetical protein M641_07650
AGR14503.1	11	sporulation initiation inhibitor Soj
AGR13588.1	11	DNA ligase LigA
AGR15110.1	10	hypothetical protein M643_13710
AGR13962.1	10	hypothetical protein M643_06170
AGR14842.1	10	dihydroxyacetone kinase subunit DhaL
AGR14154.1	10	iron ABC transporter substrate-binding protein

Table 33. continue

AGR15196.1	10	ROK family transcriptional regulator
AGR13520.1	10	epimerase
AGR15364.1	9	GntR family transcriptional regulator
AGR13343.1	9	hypothetical protein M643_02075
AGR13389.1	9	5-hydroxymethyluracil DNA glycosylase
AGR13765.1	9	peptidoglycan-binding protein LysM
AGR14755.1	9	excinuclease Uvr
AGR14022.1	9	3'-5' exoribonuclease
AGR14162.1	9	hypothetical protein M643_07335
AGR13381.1	9	porphobilinogen deaminase
AGR15986.1	9	ABC transporter substrate-binding protein
AGR27471.1	9	phage tail protein
AGR14395.1	9	glyoxalase
AGR13601.1	8	phosphoribosylformylglycinamidine synthase
AGR14340.1	8	50S ribosomal protein L30
AGR13078.1	8	phosphodiesterase
AGR13168.1	8	hypothetical protein M643_01145
AGR15359.1	8	hypothetical protein M643_15085
AGR14742.1	8	hypothetical protein M643_11310
AGR13889.1	8	hypothetical protein M643_05740
AGR14327.1	8	cobalt ABC transporter ATP-binding protein
AGR15078.1	8	SAM-dependent methlyltransferase
AGR14182.1	8	central glycolytic genes regulator
AGR13580.1	8	Molybdate metabolism regulator
AGR13142.1	7	hypothetical protein M643_01010
AGR13110.1	7	GTPase
AGR16000.1	7	ABC transporter ATP-binding protein
AGR14214.1	7	pyrophosphatase
AGR14128.1	7	HAD family hydrolase
AGR15428.1	7	LuxR family transcriptional regulator
AGR13515.1	7	enoyl-ACP reductase
AGR13697.1	7	phosphoglucomutase
AGR14427.1	7	hypothetical protein M643_09130
AGR12997.1	7	glycerol dehydratase
AGR13905.1	7	PTS galactitol transporter subunit IIC
AGR16613.1	7	lipoate-protein ligase A
AGR13574.1	7	hypothetical protein M643_04020
AGR15447.1	7	molybdenum cofactor biosynthesis protein MoeA
AGR15898.1	7	internalin
AGR15418.1	7	N-acetyldiaminopimelate deacetylase
AGR14846.1	7	dihydroxyacetone kinase
AGR13925.1	7	hypothetical protein M643_05950
AGR13967.1	6	oxidoreductase
AGR14129.1	6	hypothetical protein M643_07170
AGR15456.1	6	hypothetical protein M643_15615
AGR13918.1	6	FMN reductase
AGR15649.1	6	serine dehydratase subunit alpha
AGR15441.1	6	molybdate ABC transporter substrate-binding protein
AGR15038.1	6	hypothetical protein M643_13280
AGR15462.1	6	inositol monophosphatase
AGR13793.1	6	oxidoreductase
AGR13080.1	6	hypothetical protein M643_00675
AGR13358.1	6	Holliday junction DNA helicase RuvA
AGR13595.1	6	purine biosynthesis protein purH
AGR14159.1	6	transcription antiterminator BglG
AGR13007.1	6	iron-containing alcohol dehydrogenase

Table 33. continue

AGR14786.1	6	glyoxalase
AGR13397.1	6	acetyl-CoA carboxylase subunit alpha
AGR14210.1	6	UDP-glucose 4-epimerase
AGR15436.1	6	PTS beta-glucoside transporter subunit IIABC
AGR13730.1	5	methylglyoxal synthase
AGR13306.1	5	30S ribosomal protein S20
AGR14176.1	5	hypothetical protein M643_07415
AGR14890.1	5	glyoxalase
AGR27697.1	5	peptidase M15
AGR14014.1	5	uroporphyrinogen decarboxylase
AGR14650.1	5	hypothetical protein M643_10770
AGR14620.1	5	2-hydroxyacid dehydrogenase
AGR14345.1	5	30S ribosomal protein S14
AGR13008.1	5	glycerol transporter
AGR13754.1	5	heptaprenyl diphosphate synthase subunit II
AGR27732.1	5	cell division protein Fic
AGR13108.1	5	signal peptidase
AGR14782.1	5	hydrolase
AGR15668.1	5	cell division protein FtsQ
AGR14575.1	5	quinol oxidase subunit 2
AGR14136.1	5	cysteine desulfurase
AGR27492.1	5	hypothetical protein M641_07580
AGR15182.1	5	glyoxalase
AGR13926.1	5	membrane protein
AGR14572.1	5	spermidine N1-acetyltransferase
AGR15338.1	5	lipoate-protein ligase A
AGR27494.1	5	hypothetical protein M641_07590
AGR14017.1	5	ABC transporter ATP-binding protein
AGR14103.1	5	aspartate kinase
AGR13963.1	5	dehydrogenase
AGR14808.1	5	transcriptional regulator
AGR15356.1	5	hypothetical protein M643_15070
AGR13867.1	5	hypothetical protein M643_05625
AGR14885.1	5	alpha-mannosidase
AGR14439.1	5	cytochrome D ubiquinol oxidase subunit I
AGR13820.1	5	NADP-dependent aryl-alcohol dehydrogenase
AGR13236.1	5	DNA mismatch repair protein MutS
AGR14183.1	5	RNA polymerase sigma54 factor
AGR13128.1	5	peptidoglycan O-acetyltransferase
AGR14965.1	5	CapA domain-containing protein
AGR14092.1	5	membrane protein
AGR13573.1	5	adenine deaminase
AGR13888.1	4	ATP/GTP hydrolase
AGR15151.1	4	flagellar basal body rod protein FlgC
AGR13722.1	4	peptidase
AGR14244.1	4	hypothetical protein M643_08055
AGR14851.1	4	NAD(P)-dependent oxidoreductase
AGR13706.1	4	5'-3' exonuclease
AGR15062.1	4	hypothetical protein M643_13405
AGR14694.1	4	sulfate transporter
AGR14763.1	4	serine acetyltransferase
AGR27731.1	4	hypothetical protein M641_15460
AGR15093.1	4	membrane protein
AGR13307.1	4	DNA polymerase III subunit delta
AGR15194.1	4	diacylglycerol kinase
AGR14934.1	4	hypothetical protein M643_12700
AGR13513.1	4	membrane protein
AGR13334.1	4	hypothetical protein M643_02030
AGR12999.1	4	propanediol utilization protein PduK
AGR13018.1	4	ethanolamine utilization protein EutL
AGR14632.1	3	major tail shaft protein

Table 33. continue

AGR15737.1	3	hypothetical protein M643_07760
AGR12984.1	3	propanediol utilization: polyhedral bodies pduT
AGR15309.1	3	hypothetical protein M643_14810
AGR13854.1	3	cell division protein MraZ
AGR13366.1	3	hypothetical protein M643_02190
AGR13773.1	3	ribosomal large subunit pseudouridine synthase B
AGR13158.1	3	hypothetical protein M643_01095
AGR13470.1	3	1-acyl-sn-glycerol-3-phosphate acyltransferase
AGR15424.1	3	copper homeostasis protein CutC
AGR15841.1	3	scaffold protein
AGR15109.1	3	oxidoreductase
AGR15183.1	3	phospholipase
AGR13303.1	3	coproporphyrinogen III oxidase
AGR15670.1	3	cell wall surface anchor protein
AGR13033.1	3	precorrin-8X methylmutase
AGR14233.1	3	phospholipase D
AGR13576.1	3	transcriptional regulator
AGR15223.1	3	spermidine/putrescine ABC transporter ATP-binding protein
AGR13361.1	3	prephenate dehydratase
AGR15114.1	3	Motility gene repressor mogR
AGR13819.1	3	GntR family transcriptional regulator
AGR13691.1	3	hypothetical protein M643_04650
AGR14062.1	3	hypothetical protein M643_06690
AGR15988.1	3	GntR family transcriptional regulator
AGR14265.1	3	ATP synthase subunit A
AGR15261.1	3	hypothetical protein M643_14540
AGR13364.1	3	glycerol transporter
AGR14096.1	3	DeoR faimly transcriptional regulator
AGR13654.1	3	hypothetical protein M643_04465
AGR16004.1	3	LacI family transcriptional regulator
AGR15442.1	3	molybdopterin biosynthesis protein MoeA
AGR15367.1	3	protease
AGR13265.1	3	pyridine nucleotide-disulfide oxidoreductase
AGR13212.1	3	ATPase
AGR15252.1	3	calcium-transporting ATPase
AGR13883.1	3	multidrug ABC transporter ATP-binding protein
AGR14739.1	3	peptidyl-tRNA hydrolase
AGR14050.1	3	inorganic phosphate transporter
AGR16005.1	3	fucose isomerase
AGR15334.1	3	glycerol phosphate lipoteichoic acid synthase
AGR15450.1	3	FMN-binding split barrel domain-containing protein
AGR13723.1	3	DNA polymerase III subunit epsilon
AGR13083.1	2	hypothetical protein M643_00690
AGR13090.1	2	hypothetical protein M643_00745
AGR18806.1	2	hypothetical protein M640_10810
AGR13172.1	2	hypothetical protein M643_01165
AGR15415.1	2	hypothetical protein M643_15370
AGR13555.1	2	PTS cellobiose transporter subunit IIB
AGR13100.1	2	transcriptional regulator
AGR13836.1	2	transcriptional regulator
AGR14440.1	2	deaminase
AGR15095.1	2	serine/threonine protein phosphatase
AGR14432.1	2	hypothetical protein M643_09155
AGR13893.1	2	transcriptional regulator
AGR13255.1	2	hypothetical protein M643_01600
AGR16253.1	2	SAM-dependent methyltransferase
AGR13844.1	2	hypothetical protein M643_05505
AGR14510.1	2	haloacid dehalogenase
AGR13751.1	2	3-dehydroquinate synthase
AGR14453.1	2	phosphosugar-binding protein

Table 33. continue

AGR13681.1	2	D-alanyl-D-alanine carboxypeptidase
AGR14653.1	2	NAD(P)H nitroreductase
AGR14125.1	2	low temperature requirement C protein
AGR14106.1	2	MFS transporter
AGR13272.1	2	membrane protein
AGR18205.1	2	gluconate kinase
AGR14045.1	2	ribosomal large subunit pseudouridine synthase D
AGR15295.1	2	alanine racemase
AGR13632.1	2	ribonuclease III
AGR16009.1	2	beta-glucosidase
AGR16259.1	2	peptidase M20
AGR13862.1	2	hypothetical protein M643_05600
AGR15499.1	2	bifunctional uroporphyrin-III C-methyltransferase/uroporphyrinogen-III synthase
AGR14976.1	2	hypothetical protein M643_12965
AGR13230.1	2	damage-inducible protein
AGR14074.1	2	carbohydrate kinase
AGR14036.1	2	NADH oxidase
AGR13594.1	2	phosphoribosylamine--glycine ligase
AGR15959.1	2	glycosyltransferase
AGR13099.1	2	membrane protein
AGR15357.1	2	hypothetical protein M643_15075
AGR15178.1	2	lipase/acylhydrolase
AGR13620.1	2	tRNA (guanine-N1)-methyltransferase
AGR15185.1	2	GTP-binding protein
AGR15870.1	2	aldehyde oxidase

Table 34. Unique proteins identified in *Listeria monocytogenes* ScottA cells exposed to gas plasma for 60 min.

accession number	spec count	protein name
AGR14672.1	37	hypothetical protein M643_10925
AGR15216.1	34	hypothetical protein M643_14280
AGR14386.1	33	ribose 5-phosphate isomerase
AGR15878.1	24	hypothetical protein M643_10905
AGR14669.1	20	hypothetical protein M643_10890
AGR14671.1	20	hypothetical protein M643_10920
AGR13772.1	10	PhoP family transcriptional regulator
AGR15907.1	9	hypothetical protein M643_11875
AGR14828.1	8	hypothetical protein M643_11940
AGR15204.1	7	PTS sugar transporter subunit IIA
AGR14862.1	6	hypothetical protein M643_12170
AGR15205.1	6	FMN-dependent NADH-azoreductase
AGR13096.1	6	alpha/beta hydrolase
AGR15029.1	6	preprotein translocase subunit SecA
AGR17941.1	5	hypothetical protein M640_11455
AGR14874.1	5	hypothetical protein M643_12255
AGR13190.1	5	acetyl-CoA carboxylase
AGR15780.1	4	oxidoreductase
AGR14388.1	4	alcohol dehydrogenase
AGR15371.1	4	adenylate cyclase
AGR15159.1	4	PadR family transcriptional regulator
AGR13521.1	4	lysyl-tRNA synthetase
AGR14509.1	3	PTS mannitol transporter subunit IIA
AGR14174.1	3	carboxylesterase
AGR14891.1	3	hypothetical protein M643_12390
AGR14147.1	2	thioredoxin
AGR12976.1	2	antibiotic biosynthesis monooxygenase
AGR14485.1	2	membrane protein
AGR14820.1	2	threonine aldolase
AGR15793.1	2	agmatine deiminase
AGR13429.1	2	cell division protein FtsK
AGR13843.1	2	cell division protein SepF
AGR13197.1	2	geranyltranstransferase
AGR15200.1	2	PTS mannose transporter subunit IID

10.4. Discussion

Although several studies showing promising results for atmospheric pressure plasma-mediated inactivation of bacteria, including biofilm removal, are available in literature, the mode of action of this technology and the resulting bacterial response are not fully understood. In fact the interpretation of the microbicidal results remains difficult due to the non-standardized methods used by various Authors and due to the fact that even small variations in the setup (*e.g.* GP generating device, processing conditions, working gas, …) can strongly influence the results. This effect was partly confirmed in this thesis by using electrodes of different materials in a DBD device. In fact, microbial results showed that both *E. coli* and *L. monocytogenes* presented different inactivation curves in relation to the material. This effect was less evident with the strain of *E. coli* which presented reductions ranging from 4 (with steel, bras and silver) to 6 (with glass) Log units, while *L. monocytogenes* was characterized by significantly different inactivation levels when the four

electrodes were employed. In particular, the least efficient was glass (5 Log reductions), followed by brass and steel (~7 Log units), while the silver one gave rise to an almost complete inactivation (8 Log units).

The differences between bacteria can be attributed to intrinsic differences between the two microbial species. However, the results of this experiment are partly in disagreement with several Authors reporting that Gram-negative organisms are generally more susceptible to atmospheric pressure plasma than Gram-positive ones due to damages induced to the cell membrane and cell wall, which are therefore critical factors in the mechanism of action of plasma (Ziuzina *et al*., 2014). By contrast, other Authors found no relationship between bacterial structures and sensitivity to cold plasma (Fan *et al*., 2012) similarly to this work which evidenced that *L. monocytogenes* is endowed with a significantly higher sensitivity than *E. coli*.

It should be considered however, that although the treatments with four electrodes resulting in strong efficacies thus allowing strong log reductions, they did not lead to complete bacterial killing. Moreover, it is likely that bacterial cells activated stress response mechanisms, particularly for the longest treatments, as outlined by changes in the volatile metabolite profile detected by GC/MS-SPME analysis in GP-treated cells compared to the untreated ones for both the microbial species. In this view, comparative analysis of the metabolite spectra evidenced that shifts in the metabolite profiles were “electrode-dependent”. As far as *L. monocytogenes* is concerned, short chain fatty acids (SCFA), and mainly octanoic and dodecanois acids, increased by increasing treatment time, regardless the electrode used. A similar tendency was observed for benzaldehyde, and a thiophenic compound. On the other hand, cells treated in the presence of the glass electrode, which resulted in the lowest inactivation, were characterized by the highest accumulation of the SCFA octanoic and dodecanoic acid immediately after 20 min, and heptanoic acid after 60 min. Also 2-methyl-3-decen-5-one and 2-pentyl-furan were detected at significantly higher levels immediately after treatments. On the contrary, when silver electrode was employed, which gave rise to the highest bacterial inactivation, all these volatiles were found at the lowest levels. In the case of *E. coli*, the compounds that were mainly affected by the exposure to gas plasma were the aldehydes and alcohols, in addition to some esters. In particular, cells treated with the glass electrode, which resulted in the best final inactivation level, presented a significantly higher level of ethanol, 2-ethyl-hexanol, ethyl-acetate, butanedioic acid diethyl ester and the

thiophene derivative. A marked increase in hexanol, aldehydes and thiophene following exposure to chemical stresses (*i.e.* hexanal and 2-E-hexanal) has been reported by Patrignani *et al*. (2008). On the other hand, aldehydes are generally regarded as end products of the breakdown of peroxidated unsaturated fatty acids (Deighton *et al,* 1999) or can be formed through alternative pathways involving for example chemical autoxidation of oleic and linoleic acids, or through the lipoxygenase pathway.

The conversion of phenylalanine to benzaldehyde has been reported for several bacteria according to different pathways. In *L. plantarum*, this conversion involves both an enzymatic step and a chemical reaction. In the cell extract of this strain, phenylalanine is initially converted to phenyl-pyruvic acid by the action of an aminotransferase. In the presence of oxygen, the keto acid is then oxidized to benzaldehyde in a nonenzymatic reaction (Nierop Groot and De Bont, 1999). Another pathway involves phenylalanine ammonia lyase (PAL), resulting in the production of cinnamic acid as an intermediate. However, the intermediates of this metabolic pathway are still not well known, and Nierop Groot and De Bont (1998) speculated that the last step is a chemical and not a biological one.

On the basis of these results and according to literature data, it seems that both microbial species responded to an oxidative stress rather than to the acidic conditions generated during plasma treatments. On the other hand, changes in pH values due to the 4 electrodes were similar despite different inactivation levels and volatile metabolite profiles were found for both the microbial species. Various Authors reported that gas plasma consists in several species that can damage microbial cells such as radical, ions and electrons. Among the radical species, those of oxygen (reactive oxygen species – ROS) are widely considered by several Authors the most effective in microbial inactivation, while ions such as N^+ ,N^{2+} and other species (*e.g.* H_2O_2) seem to play a minor role. On the other hand, radical species have a very short life (~1µs) and their action is closer to the electrodes (Yin *et al.,* 2015) than ions which can remain in the sample for a longer time. Oehmigen *et al.* (2011) detected nitrate (NO^-_3), nitrite (NO^-_2), and hydrogen peroxide (H_2O_2), respectively, as well as strong acidification in plasma treated liquids (NaCl solutions), and partially attributed to these compounds the bactericidal activity observed towards *E. coli* cells (≥ 7 log). It is important to note that this system (*i.e.* saline solutions) is very similar to the one employed in this thesis. Using FT-IR analysis, the Authors measured stable molecules like nitrous oxide (N_2O), ozone (O_3), carbon dioxide (CO_2), and traces of nitric acid (HNO_3) and/or peroxynitrous acid (ONOOH). Authors

hypothesized that reactions of these molecules from the plasma/gas phase with the aqueous liquid can result in acidification and generation of H_2O_2, NO_2^-, and NO_3^- or peroxynitrite ($ONOO^-$), respectively, via reactions which are associated with the occurrence of several more or less stable but biologically active chemical intermediates like $NO^\bullet$ or nitrogen dioxide ($NO_2^\bullet$). On the other hand, H_2O_2, NO_2^-, and NO_3^-/$ONOO^-$ could serve as starting reaction partners to generate $NO^\bullet$, $HO^\bullet$, $NO_2^\bullet$, or hydroxyl radicals ($HOO^\bullet$) in the liquid. Therefore it is likely that the acid/base and oxidation/reduction properties govern the rapid formation of nitrite which is later followed by its conversion into nitrate (resulting from the formation of strong nitric acid) and involves peroxynitrite as an intermediate.

On the other hand, the results of this thesis seem to indicate that not only ROS, but also other chemical species (*e.g.* RNS and H_2O_2) are responsible for microbial inactivation. This hypothesis is supported by data on metabolite volatiles released by cells of *L. monocyogenes* in saline solutions during almost lethal treatments. In fact the profile of volatiles detected following direct exposure to plasma generated by DBD equipment with different electrodes only partly coincided with that produced by indirect plasma exposure, *i.e.* by suspending the cells into NaCl solutions pre-treated with the same equipment and with the same treatment conditions (*i.e.* time and electrode). Common volatiles detected were mainly represented by aldehydes and short and medium chain fatty acids, whose accumulation has been widely related by several Authors to oxidative stress conditions. On the other hand, when sub-lethal treatments were applied instead of lethal ones, a wider range of volatiles was detected, particularly with the longest treatment, indicating that *L. monocytogenes* cells can better adapt to the changing environment challenges. In fact additional aldehydes, short chain fatty acids and ketones (including 3- methyl-butanal, 3-methyl-butanoic acid, acetophenone, 2,3-butanedione, 3-hydroxy-2-butanone) were significantly associated to the treated cells in addition to those detected after lethal treatments. On the other hand several pyrazines were associated to the untreated cells as well as higher levels of both ethanol and acetic acid, thus suggesting that a metabolic slowdown was induced by gas plasma. These responses were common to both the strains of *L. monocytogenes* used, *i.e.* strain 56Ly and ScottA. Pyrazines are generally reported to be produced by several microorganisms although knowledge about their biosynthesis is limited. Dickschat *et al.* (2010) reported a direct biosynthetic link between pyrazines and the amino acids valine, leucine and isoleucine, while other Authors suggested the pathway via acetoin. It can be hypothesized that the decrease of these

metabolites in *Listeria monocytogenes* after gas plasma treatments is related to a change in amino acids pathway. Concerning acetophenone, Lapadatescu *et al.* (2000) reported that could be generated via the phenylalanine degradation pathway which involves a beta-oxidation reaction and a phenylalanine ammonia lyase. This enzyme is over-produced by the Fungus *Bjerkandera adusta* following stress conditions. Moreover, Bandyopadhaya *et al.* (2012) observed that 2-amino-acetophenone, an ammine derivative of acetophenone, was involved in quorum sensing mechanisms in *Pseudomonas aeroginosa*. In gas plasma treated cells an increase of 3-methylbutanoic acid was also detected. Serrazanetti *et al*. (2011) reported that a metabolic shift toward an overproduction of 3-methylbutanoic acid by *L. sanfranciscensis* following exposure to acid stress could support its growth in restricted environmental conditions. Furthermore it is well known that isovaleric acid is involved in branched FAs pathway.

According to the results of this work, also cellular membrane resulted to be one of the cellular target for the chemical species generated during gas plasma exposure. In fact, following sub-lethal treatments the amounts of branched fatty acids, and namely *i*C15:0 and *a*C15:0, significantly increased already after 10 min of treatment for both the strains 56Ly and Scott A. This finding is in agreement with scientific literature reporting that *L. monocytogenes* increases the amount of branched FAs as a response to different environmental stress (Giotis *et al.*, 2007; Gianotti *et al*., 2008). Some studies also demonstrated that the modulation of such branched-chain FAs regulates bacterial virulence (Sun *et al.,*2010). In addition, also a reduction in the contents of the linear FA C16:0 and C18:0 was observed. It can be hypothesized that these FAs underwent degradation as evidenced by data of the volatiles indicating that shorter chain fatty acids and the corresponding aldehlydes and ketones were released following plasma exposure.

While common responses involving membrane fatty acids and volatile compounds were shared by the *L. monocytognes* strain 56Ly and strain ScottA, result on proteomic profile evidenced that the 2 strains of *L. monocytogenes* responded in a different way to a gas plasma treatment as evidenced by the significantly different number of proteins that were differentially expressed. This outcome is in agreement with the study of Melo *et al*. (2013) evidencing that a significant intra-strain variation in the protein arsenal used to respond to the adaptation in the cheese-based medium and to the gastric stress occurred in the intracellular proteome profiles of three isolates of *L. monocytogenes*.

As far as the strain 56Ly, gas plasma treatments seems to affect several proteins involved in carbohydrates transport into the cell and energetic metabolism. In particular a reduction in the abundance of F0F1 ATP synthase subunit gamma was observed following gas plasma which could result in a decrease in cellular energy production. In fact, gamma subunit of ATP synthase F1 complex forms the central shaft that connects the F0 rotary motor to the F1 catalytic core of ATP synthase which provides energy for the cell to use through the synthesis of ATP. Moreover, the reduced expression of the proteins involved in carbohydrates transport system via phosphotransferase system (PTS) result in limited carbohydrate intake, and consequently metabolism, which could deplete cellular energy. Importation of sugars via the PTS has been shown to be important also for buffering of the cell cytoplasm (Shabala *et al.,* 2002) while increasing substrates for glycolysis. Glycolysis, the pentose phosphate shunt and PTS system produce by-products that are associated with the electron transport chain. This multi-step energy generating system involves a number of protein components that transfer electrons from the initial NADH and succinate donors, culminating in energy production by an ATP synthase powered by a proton motive force (Alberts *et al.,* 2004).

On the other hand treated cells were characterized by up-regulation of several oxidoreductases, in addition to cold shock proteins and proteins involved in pyridoxal biosynthesis and thioredoxin. Pyridoxal 5′-phosphate (PLP) is an essential cofactor in all living systems and participates in catalysis by a diverse group of enzymes including oxidoreductases, transferases, hydrolases, lyases and isomerases (Percudani and Peracchi, 2003). In addition, PLP participates in radical mediated reactions (*e.g.* lysine 2,3-aminomutase; Frey, 2001), aminosugar deoxygenation (He *et al*., 2000) and catalysis involving the phosphate group of PLP in muscle glycogen phosphorylase b (Livanova *et al*., 2002). Moreover, PLP is the catalytically active form of vitamin B_6 whose role as a singlet oxygen quencher has been recently reported in fungi (Bilski *et al*., 2000; Ehrenshaft *et al.*, 1999; Jain and Lim, 2001).

Thioredoxin is a class of small redox proteins known to be present in all organisms. It plays a role in many important biological processes, including redox signaling. The thioredoxin (Trx) system, which is composed of NADPH, thioredoxin reductase (TrxR), and thioredoxin, is a key antioxidant system in defense against oxidative stress through its disulfide reductase activity regulating protein dithiol/disulfide balance. The Trx system provides the electrons to thiol-dependent peroxidases (peroxiredoxins) to remove reactive oxygen and nitrogen species

with a fast reaction rate. Trx antioxidant functions are also shown by involvement in DNA and protein repair by reducing ribonucleotide reductase, methionine sulfoxide reductases, and regulating the activity of many redox-sensitive transcription factors (Lu and Holmgren, 2014). Among the oxidoreductases, also an aldo/keto reductase was over expressed following gas plasma exposure. These enzymes reduce carbonyl substrates such as sugar aldehydes; keto-steroids, keto-prostaglandins, retinals, quinones, and lipid peroxidation by-products (Penning, 2014). This result is in line with GC-MS/SPME analysis of volatile metabolites which evidenced that gas plasma treated cells accumulated higher amounts of several aldehydes compared to the control ones, regardless the treatment type, *i.e.* a sub-lethal or lethal one.

As far as the strain ScottA, only 3 proteins were significantly associated to the treated cells and namely proteins related to PTS galactitol transporter subunit IIB, glycosyl hydrolase and ATP-dependent Clp protease (ATP-binding protein). Glycoside hydrolase family 31 is a widespread group of enzymes that assist the hydrolysis of glycosidic bonds between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety in complex sugars. In bacteria, they are found both as intracellular and extracellular enzymes that are largely involved in nutrient acquisition in addition to anti-bacterial defense strategies (*e.g.* lysozyme). The up-regulation in the plasma treated cells could favor energy production through the catabolism of substrates which are not usually employed by *Listeria monocytogenes*. ClpP, are energy-dependent proteases involved in protein degradation which plays a key role in cell physiology of all organisms by regulating the availability of certain short-lived regulatory proteins or preventing the accumulation of abnormal proteins. Although Clp proteins are highly conserved and ubiquitous in bacteria and higher organisms, only a few data are available about the importance of Clp-mediated proteolysis in organisms other than *E.coli*. In *B. subtilis* ClpP synthesis has been found to increase during heat shock, but also during salt and oxidative stress, glucose and oxygen deprivation (Völker *et al*.,1994). Furthermore, *B.subtilis* Clp proteins were found to be required for several cellular processes such as cell division, motility and degradative enzyme synthesis, and for developmental processes such as sporulation and genetic competence (Krüger *et al*., 2001). In *L. monocytogenes* transcriptional analyses have provided clear evidence that several virulence and stress-response genes are co-regulated by either multiple alternative σ factors, or alternatives factors and other transcriptional regulators (Chaturongakul *et al*., 2008). In particular σ^B co-regulates stress-response and virulence genes with negative regulators such

as CtsR and HrcA, which both contribute to the regulation of expression of heat shock genes and genes important for virulence (Hu *et al*., 2007a; 2007b). One example is the co-regulation by the σ^B and CtsR of genes encoding Clp proteins, which have endopeptidase and chaperone functions. Moreover, the gene *clpP* is co-regulated by σ^B, CtsR and σ^L. Networks between different transcriptional regulators, including alternative σ factors, thus seem to contribute to fine-tuning gene expression under various different stress conditions which may also include those generated during gas plasma treatments.

Chapter 11. Conclusions

The overall objective of this PhD thesis was to evaluate the potentials of cold atmospheric plasma, produced by a DBD generator, as an emerging decontamination technology. To achieved this goal, several experiments were made by using both some real foods and model systems.

Concerning the former, raw fruit and fresh vegetables, *i.e.* soybean sprouts and Fuji apples, as well as a dry matrix represented by black pepper were taken into consideration. With all the 3 foods, the efficacy of gas plasma was proved as the natural microflora and the pathogens deliberately inoculated onto the products were inactivated, although complete killing was not achieved. Nevertheless, mean reductions ranging between 1 and 2.6 Log CFU/g depending on the exposure time and microbial species were obtained for soybean sprouts and apples. These data are in accordance with those reported in literature for traditional technologies to reduce/eliminate the microorganisms present in food products. In fact washing with sanitizing agents, *e.g*. chlorine, which is the most widely diffused for fresh and ready-to-eat fruit and vegetables, is reported to decrease the bacterial load by values ranging from <1 Log CFU/g to 3.1 Log CFU/g, depending on inoculation method, chlorine concentration, contact time, and the target bacteria. Compared the fresh produce, treatments on black pepper resulted in higher inactivation levels (2 to 4 logarithmic cycles) regardless the target pathogen. This result is promising as actually no effective decontamination technologies are available or allowed in several European countries for spices. Nevertheless, it should be considered that low water activity of the matrix, which is not favorable for the growth of the target pathogens, may have contributed to their inactivation in synergistic action with chemical species generated during gas plasma treatments. On the other hand these species clearly interacted also with the chemical components of the food resulting in changes in some quality parameters. Nevertheless the extent of these modifications was limited (*e.g.* the increase in TBARs for all the 3 products) and quality parameters were considered acceptable for consumption. In interesting aspect that was evidenced while analyzing apple, was the strong inactivation of polyphenoloxidase activity following direct exposure to gas plasma or washing with plasma treated water. This outcome is really promising as it could be exploited also for other fresh or ready-to-eat products to limit browning phenomena while assuring microbial quality by

reducing the risk of contamination from pathogens. In this contest, knowledge on the actual mechanism of action of gas plasma and possible cellular target in different pathogenic species would be useful to optimize processing conditions. Although chemical species generated during gas plasma treatments were not measured, the results of the experiments related to the selected target pathogens, *i.e. E. coli* and mainly *L. monocytogenes*, in model systems provided information on the possible targets at cellular level. In fact the tested processing conditions induced noticeable modifications in the membrane fatty acids and volatile compound profiles in relation to the severity of the treatments. On the basis of the observed changes in the membrane FAs and accumulated volatiles, mainly represented by aldehydes, ketones and short- and medium chain fatty acids, it is likely that oxidative stress plays a key role against microbial cells. Moreover, the analysis of the proteomic profile indicated that other compounds, which are notably involved in redox signaling or antioxidant system in defense against oxidative stress, were up-regulated following exposure to gas plasma. Although this part of work is not exhaustive and deeper researches are necessary, it represents the first study evaluating how *L. monocytogenes* respond to gas plasma treatments and the provided information can contribute to the optimization of gas plasma processing conditions also in the view of preventing the induction of (cross-)protection towards other stresses that pathogens may encounter during food processing.

Chapter 12. References

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