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TESIS DOCTORAL

"Evaluación de los factores que determinan la efectividad del plasma atmosférico no térmico en la inactivación de microorganismos patógenos de transmisión alimentaria. Impacto del tratamiento sobre las membranas celulares"

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DOCTORANDO

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Índice

Resumen

Las actuales exigencias del consumidor hacia alimentos de elevada calidad nutritiva y sensorial, con características similares a las del producto fresco y libres de aditivos han llevado al desarrollo de nuevas tecnologías de procesado y conservación, como alternativa a otros métodos tradicionales más agresivos sobre sus propiedades organolépticas, nutritivas y funcionales, que han abierto nuevas expectativas para asegurar la calidad microbiológica de los alimentos. Recientemente, se ha conseguido la obtención, a temperatura ambiente y presión atmosférica, de plasmas de gases. Esta nueva técnica, el Plasma Atmosférico No térmico (PANT), por su capacidad para inactivar tanto células vegetativas como esporos bacterianos y fúngicos, abre un amplio abanico de posibilidades para su uso en la industria alimentaria para mejorar la calidad microbiológica de los alimentos o para la esterilización de material de envasado o diversas superficies de contacto. Además de su efectividad antimicrobiana, destaca el hecho de que presenta, en comparación con otras estrategias de conservación de alimentos, otras ventajas muy importantes, como son el bajo coste de aplicación, el empleo de tiempos cortos de tratamiento, la posibilidad de tratar una amplia variedad de alimentos, incluso previamente envasados, y el ser una técnica medioambientalmente sostenible, por lo que se considera como una tecnología sumamente prometedora para su aplicación en la industria alimentaria.

Sin embargo, para el diseño de tratamientos eficaces que garanticen el grado de inactivación deseado, resulta necesario un conocimiento extenso de aquellos factores que determinan su efectividad antimicrobiana, así como de los mecanismos responsables de la misma. El objetivo general de esta Tesis Doctoral fue evaluar la potencialidad del PANT como sistema alternativo de higienización de los alimentos, abordando su efectividad en la inactivación de algunos de los microorganismos patógenos de transmisión alimentaria más importantes (*Salmonella enterica* serovariedad Enteritidis, *Salmonella enterica* serovariedad Typhimurium, *Listeria monocytogenes* y *Escherichia coli* O157:H7), así como las bases moleculares de su efecto letal.

Se evaluó la influencia que las condiciones de generación del plasma, en relación a la composición (aire o nitrógeno) y velocidad de flujo (5, 10, 15 l/min) del gas precursor, ejercen sobre la efectividad antimicrobiana del PANT, comprobando que, en todos los casos, con los plasmas de aire se conseguían mayores tasas de inactivación.

Al determinar la resistencia al PANT que exhibían los patógenos tras su crecimiento en presencia de diferentes ácidos hasta un valor de pH de 4,5 y en un amplio rango de temperaturas, entre 10 y 45ºC, así como tras su exposición, durante diferentes períodos de tiempo (5 y 120 min), a diversas condiciones subletales, como un tratamiento térmico moderado (45ºC), o exposición a la acidez (pH 4,5) o frío (0ºC), se observó que ésta apenas se veía afectada, lo que supone una ventaja adicional para esta tecnología a la hora de su implementación a nivel industrial.

La aplicación de idénticos tratamientos por PANT sobre diferentes superficies puso de manifiesto que esta tecnología resulta más eficaz para la descontaminación de superficies abióticas que para la de alimentos, detectándose, asimismo, diferencias en el efecto letal conseguido entre los distintos alimentos estudiados (chorizo, salami, bacón, salmón ahumado, tofu y manzana), lo que indica que algunos factores asociados a su superficie, como rugosidad, adsorción de especies reactivas del plasma o humedad, podrían afectar a la supervivencia microbiana. Sin embargo, para cada alimento evaluado, las tasas de inactivación conseguidas para los diferentes patógenos, tras un determinado tiempo de tratamiento, mostraron una escasa variabilidad.

Finalmente, se evaluaron los cambios morfológicos y físico-químicos inducidos en los microorganismos tras su exposición al PANT. Concretamente, se determinaron los cambios en la composición química mediante espectroscopía de infrarrojos con transformada de Fourier (FTIR) y las modificaciones en la morfología y estructura celular por microscopía electrónica, evaluándose, asimismo, la integridad de la membrana citoplasmática aplicando técnicas de espectrofotometría UV y de citometría de flujo. El conjunto de información proporcionada sugiere que el mecanismo letal del PANT podría variar entre las especies bacterianas Gram positivas y Gram negativas. Los daños originados en las envolturas celulares serían la principal causa de la pérdida de viabilidad

de las bacterias Gram negativas, lo que podría facilitar la acción de algunos agentes antimicrobianos que, en condiciones normales, resultan ineficaces frente a este grupo bacteriano por su incapacidad de atravesar su membrana externa, abriendo la posibilidad de diseñar nuevos procesos combinados. Sin embargo, las lesiones infringidas en otras dianas celulares, como el ADN y/o sistemas enzimáticos, serían las que conducirían a la muerte celular en las especies Gram positivas.

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Introducción

En la actualidad los consumidores esperan y tienen derecho a disponer de alimentos sanitariamente seguros, aunque cada vez son más los que demandan también otros atributos como comodidad, alta calidad nutritiva y sensorial, larga vida útil, frescura, naturalidad, ausencia de aditivos, mínimo impacto sobre el medio ambiente y, todo ello, a un precio razonable (Fernández y col., 2012). Los métodos tradicionales utilizados por la industria para el procesado de los alimentos no permiten satisfacer todas estas exigencias. Aunque los tratamientos térmicos han sido ampliamente utilizados durante muchos años y resultan, en líneas generales, bien aceptados por los consumidores, presentan ciertos inconvenientes, como la pérdida de nutrientes y una considerable reducción de la calidad organoléptica de algunos alimentos (Jayasena y col., 2015). Por ello, en estos últimos años se ha hecho un gran esfuerzo investigador en el desarrollo de nuevas tecnologías de conservación, basadas en nuevos principios, diferentes al calor, que genéricamente se engloban bajo el nombre de "Tecnologías emergentes de conservación de los alimentos", cuyo objetivo es inactivar los microorganismos y enzimas presentes en los mismos sin alterar sensiblemente sus características nutritivas, organolépticas y funcionales (Kim y col., 2014). Entre ellas, han surgido las altas presiones hidrostáticas, los pulsos eléctricos de alto voltaje, los ultrasonidos, los pulsos de luz, los campos magnéticos oscilantes y, más recientemente, el plasma atmosférico no térmico (PANT).

El término plasma, en Física y Química, se utiliza para designar al estado de un gas ionizado. Según la energía de sus partículas, el plasma es considerado el cuarto estado de la materia. Aunque en la Tierra, por las condiciones de temperatura y presión, resultan más comunes los estados sólido, líquido y gaseoso, éstos son en términos globales exóticos, mientras que el plasma constituye el estado predominante en el universo, estimándose que hasta el 99% de la materia se halla en este estado, encontrándose, por ejemplo, en las auroras, la ionosfera, el viento solar, los canales de los rayos que se producen durante una tormenta, el Sol y el resto de las estrellas (Lackmann y Bandow, 2014). Además de los plasmas naturales, también hay plasmas producidos artificialmente y, muchos de ellos, forman parte de nuestra vida cotidiana (los televisores o monitores con pantalla de plasma, los tubos fluorescentes o

las lámparas de neón utilizados en la iluminación y como indicadores en equipos electrónicos, etc.) o diversas industrias los utilizan con distintos fines, como conferir ciertas propiedades funcionales a determinados materiales (papel, plásticos, tejidos o elementos electrónicos), conseguir la precipitación electrostática de partículas finas, generar ozono o como medios activos para procesos de síntesis química.

El fundamento de la producción de plasma se basa en aportar energía a un gas para provocar su ionización, pudiendo ser ésta de diferente naturaleza. Ahora bien, dependiendo del tipo y cantidad de energía transferida al gas se obtienen plasmas que presentan diferentes características y que pueden ser clasificados, en función de su temperatura, en 2 grandes grupos: plasmas térmicos y plasmas fríos (Lieberman y Lichtenberg, 2005). Los plasmas térmicos pueden alcanzar temperaturas de hasta varios miles de grados centígrados y se emplean en aplicaciones donde se requieren altas temperaturas como, por ejemplo, en procesos de fundición en la industria metalúrgica, en la incineración de residuos o como medio activo para procesos de síntesis química (producción de acetileno a partir de gas natural). Los plasmas fríos, si bien este término no se refiere a temperaturas de refrigeración sino a temperaturas próximas a la ambiental, resultan adecuados para el tratamiento de materiales sensibles al calor.

El PANT se genera mediante la aplicación, a presión atmosférica, de un campo eléctrico o electromagnético a un gas, en el que los electrones libres toman la energía del campo lo que produce su aceleración hasta que sus energías se elevan lo suficiente para ionizar los átomos o las moléculas del gas con las que colisionan, liberando más electrones que provocan, a su vez, nuevas ionizaciones. Además, los electrones con energías adecuadas producen disociación molecular, formándose átomos y radicales libres, siendo, asimismo, capaces de excitar átomos y moléculas a niveles superiores de energía que, al retornar al estado más estable, emiten el exceso de energía en forma de radiaciones electromagnéticas de amplio espectro, incluyendo radiaciones en el rango ultravioleta. En consecuencia, el PANT está constituido básicamente por moléculas y átomos en estado o no de excitación, iones positivos y negativos, radicales libres, electrones y radiación ultravioleta, estando presentes especies reactivas del oxígeno y

del nitrógeno, tales como ozono, superóxido, radicales hidroxilo, oxígeno singlete, oxígeno atómico, óxido nítrico o dióxido de nitrógeno, con capacidad de inactivar una amplia gama de microorganismos, incluyendo bacterias, mohos, levaduras, esporos e incluso virus, priones y parásitos (Klämpfl y col., 2012; Tseng y col., 2012; Hayashi y col., 2013; Alkawareek y col., 2014; Mai-Prochnowa y col., 2014; Takamatsu y col., 2015; Dasan y col., 2016).

A continuación, se presenta un artículo de revisión que se ha publicado en el contexto de esta Tesis Doctoral, donde se recoge una visión general de los principios de funcionamiento y las aplicaciones del PANT en la industria alimentaria. En particular, se discuten los numerosos estudios llevados a cabo en la última década destinados a descifrar la influencia de los diferentes factores ambientales y de los parámetros de procesado en la inactivación microbiana mediante esta tecnología. Además, esta revisión también considera algunos de los estudios más importantes destinados a elucidar los complejos mecanismos de inactivación microbiana por PANT. Por último, se describe brevemente otras de sus potenciales aplicaciones en la industria alimentaria, así como algunas limitaciones existentes para la implementación inmediata de esta tecnología, como son su impacto nutricional y sensorial sobre los atributos de calidad de los alimentos tratados, el conocimiento de los componentes del plasma y las especies químicas reactivas responsables de la efectividad antimicrobiana, la posible toxicidad de alguna de ellas y el diseño de equipos a escala industrial que se adecúen a su propósito.

A review on Non-Thermal Atmospheric Plasma for Food Preservation: mode of action, determinants of effectiveness and applications

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Summary

Non-Thermal Atmospheric Plasma (NTAP) is a cutting-edge technology which has gained much attention during the last decade in the food-processing sector as a promising technology for food preservation and maintenance of food safety, with minimal impact on the quality attributes of foods, thanks to its effectiveness in microbial inactivation, including of pathogens, spoilage fungi and bacterial spores, simple design, ease of use, cost-effective operation, short treatment times, lack of toxic effects and significant reduction of water consumption. This review article provides a general overview of the principles of operation and applications of NTAP in the agri-food sector. In particular, the numerous studies carried out in the last decade aimed at deciphering the influence of different environmental factors and processing parameters on the microbial inactivation attained are discussed. In addition, this review also considers some important studies aimed at elucidating the complex mechanism of microbial inactivation by NTAP. Finally, other potential applications of NTAP in the agri-food sector, apart from food decontamination, are briefly described, and some limitations for the immediate industrial implementation of NTAP are discussed (e.g., impact on the nutritional and sensory quality of treated foods; knowledge on the plasma components and reactive species responsible for the antimicrobial activity; possible toxicity of some of the chemical species generated; scale-up by designing fit-for-purpose equipment).

1.1 Potential of NTAP as a preservation technology in the food industry

The possibility of using plasma as a surface decontamination technology was first pointed out in the late 60´s in an American patent (Menashi, 1968). However, NTAP was not implemented yet in the food industry at that point, since cold plasmas could only be obtained under vacuum and at small-scale conditions, which was expensive and not applicable in industrial settings (Lackmann and Bandow, 2014). Nonetheless, the technological advances experienced at the late 90´s in relation to the sources of plasma generation allowed the development of equipment capable of generating plasmas at atmospheric pressure, thus avoiding the need for vacuum cameras and pumps and facilitating continuous treatments with relatively simple and inexpensive equipment.

NTAP offers very important advantages for food industries, which makes it a very promising novel food preservation technology. Firstly, it allows short processing times. Indeed, it has been described that very short treatment times (between a few seconds and 2 minutes) can cause more than 5 log reductions for different microorganisms, including pathogens such as *Salmonella* Typhimurium, *S.* Enteritidis, *Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, Campylobacter jejuni, Campylobacter coli*, *Aeromonas hydrophila*, and even sporulated microorganisms such as *Bacillus cereus* and *Clostridium botulinum* (Deng et al., 2007; Muranyi et al., 2007; Rowan et al., 2007; Song et al., 2009; Lee et al., 2012b; Shi et al., 2011; Jahid et al., 2014; Ziuzina et al., 2014). In addition, it is effective at room temperature, which makes it particularly interesting for heat-sensitive products and can be used to treat pre-packaged foods (Fröhling et al., 2012a; Rød et al., 2012; Ziuzina et al., 2014; Jayasena et al., 2015), which prevents their subsequent recontamination. Finally, its non-toxic nature and the reduced consumption of water and chemical agents result in a significant reduction of effluents, which is beneficial not only from an economic but also from an environmental point of view.

This set of advantages has led in recent years to explore the use of NTAP for food preservation, and there are already numerous studies, focused on characterizing its antimicrobial effectiveness and on deciphering the inactivation mechanisms involved.

Nevertheless, a great research effort is still necessary to accomplish its successful implementation at industrial level as a safe and effective alternative to traditional preservation methods, with the main challenges arising from the difficulty in interpreting the data obtained by different research groups which use very diverse equipment and operating conditions, resulting in very different plasmas in terms of properties and, consequently, with very different antimicrobial effectiveness. However, some general conclusions can be drawn on various aspects related to the mechanisms of microbial inactivation by NTAP and the factors that determine its lethal efficacy, which will be discussed in the following sections of this review article.

1.2 Mechanisms of microbial inactivation by NTAP

Although several studies have tried to elucidate the mode of microbial inactivation by various plasmas obtained under atmospheric conditions, the specific mechanisms leading to microbial death are not precisely known yet.

It is well known that UV radiation with wavelengths in the 220-280 nm range is capable of inhibiting microbial growth by inducing the formation of DNA thymine dimers. Indeed, UV light has been used for years for the decontamination of water, air and surfaces. However, the contribution of UV radiation to the antimicrobial effect of plasmas obtained at atmospheric pressure is controversial. Thus, although some researchers hypothesize that UV-C radiation present in plasma plays an important inactivating role (Boudan et al., 2006; Eto et al., 2008; Muranyi et al., 2010), most authors (Laroussi and Leipold, 2004; Deng et al., 2006; Lee et al., 2006; Dobrynin et al., 2009, 2011; Joshi et al., 2011; Miao and Yun, 2011; Reineke et al., 2015) believe that UV radiations are not generated at the most effective wavelengths or are absorbed by the gas molecules themselves (Reineke et al., 2015) and, therefore, are not involved in microbial inactivation (Patil et al., 2014; Surowsky et al., 2014). Indeed, Reineke et al. (2015) compared the effectiveness of different plasmas for the inactivation of *Bacillus atrophaeus* and *Bacillus subtilis* spores and found that, although plasmas containing oxygen and nitrogen emitted four times more UV radiation than pure argon plasmas,

the greatest lethal effect was achieved when pure argon was used as the working gas. These authors suggested that the antimicrobial effect was determined by reactive species of oxygen and nitrogen generated in the pure gas, and especially by hydroxyl radicals. Other authors have also tested the contribution of UV light by exposing *E. coli* cells (Liu et al., 2008; Gweon et al., 2009) and *Bacillus cereus* (van Bokhorst-van de Veen at al., 2015) and *B. subtilis* spores (Hong et al., 2009) to the action of plasma by intercalating between the plasma generation point and the treatment medium a lithium fluoride filter or a fused silica quartz plate transparent to UV light, which allows cell/spore exposure to the radiation potentially emitted by the plasma source but avoids contact with chemical reactive species. These studies demonstrated that the contribution of UV light to microbial inactivation was negligible, compared to that observed for direct exposition to plasma. In addition, exposure of *B. cereus* vegetative cells to nitrogen plasma has been shown to result in a transcriptional response in which the expression of genes involved in UV damage repair (*uvrA*, *uvrB*) was unaffected, while various genes involved in the response to oxidative stress, such as those encoding nitric oxide dioxygenase, as well as membrane-associated enzymes that catalyze oxidation-reduction reactions, were overexpressed (Mols et al., 2013). Finally, it has also been shown that microorganisms exhibiting variability in resistance to UV light exhibit a similar tolerance against NTAP. For instance, although *B. cereus* spores were more sensitive to UV light than spores of *Geobacillus stearothermophilus* and *B. atrophaeus*, a given NTAP treatment produced a similar degree of inactivation for the three species (van Bokhorst-van de Veen et al., 2015), suggesting that the mechanism of inactivation through both technologies was different.

There exists on the contrary a general agreement in that reactive chemical species generated through gas ionization exert an antimicrobial effect through a direct and non-specific attack on various microbial structures and components, including cellular envelopes, DNA and proteins (Song et al., 2009; Colagar et al., 2010; Surowsky et al., 2014; Yost and Joshi, 2015).

1.2.1 Lesions at the cellular envelopes

Various authors (Dobrynin et al., 2009; Muranyi et al., 2010; Miao and Yun, 2011; Tseng et al., 2012; Tian et al., 2015) have identified the mechanical or oxidative damage caused to cellular envelopes as the main cause of death after NTAP treatments. The mechanical erosion of cellular envelopes could be due either to the impact of plasma energy particles, such as electrons and excited atoms (Butscher et al., 2016), or to the accumulation of charges in certain parts of the cell surface, which would lead to its permeabilization through the formation of pores, in a phenomenon known as electroporation (Laroussi et al., 2003). Moreover, damage at this level would facilitate the release of important intracellular components and the invasion of reactive species which would attack other cellular components, such as DNA and proteins, thus accelerating the inactivation process. In addition to this mechanical effect, neutral reactive species generated in plasma, such as atomic oxygen, hydroxyl and peroxyl radicals, metastable oxygen or ozone, are capable of causing oxidative damage to various cellular structures and macromolecules. If this damage is very intense and exceeds the physiological repair capacity, it will lead to microbial death (Leipold et al., 2010). In fact, the use of substances that chelate reactive species has been shown to reduce the antimicrobial efficacy of NTAP. Thus, several sequestering agents of various reactive oxygen species, such as catalase, superoxide dismutase, dimethyl sulfoxide, thiourea, sodium azide, and various antioxidants (glutathione, ascorbic acid, sodium pyruvate, mannitol, histidine or tocopherol) have been reported to protect microorganisms, such as *E. coli* (Joshi et al., 2011; Yost and Joshi, 2015) or *P. aeruginosa* (Takamatsu et al., 2015), from the action of NTAP. Yost and Joshi (2015) showed that previous incubation of *E. coli* with catalase provided a greater protective effect than vitamin E and thiourea, concluding that peroxides such as H_2O_2 could be the major chemical species responsible for microbial inactivation by NTAP. On the other hand, Takamatsu et al. (2015) observed that the presence of dimethyl sulfoxide or sodium azide resulted in lower *P. aeruginosa* inactivation rates than those observed when catalase or superoxide dismutase were used, suggesting that hydroxyl radicals and singlet oxygen were the main agents involved in microbial inactivation.

Reactive species exert their oxidative effect especially on the polyunsaturated fatty acids of the cytoplasmic membrane (Laroussi and Leipold, 2004), which are very susceptible to lipid peroxidation phenomena. Lipid peroxidation can compromise cell viability by modifying membrane properties, decreasing its permeability and even its integrity (Joshi et al., 2011; Colagar et al., 2013). Lipid peroxidation is a chain reaction that begins with the attack of unsaturated fatty acids by reactive oxygen species (or any reactive species), which extract a hydrogen atom from a methylene group (-CH2), giving rise to the formation of a lipid radical (L*) which can react rapidly with an oxygen molecule to give a peroxyl radical (LOO*). These radicals can extract new hydrogen atoms from other lipids and become hydroperoxides (LOOH), which undergo chemical degradative phenomena to produce very toxic degradation compounds, such as alkoxy (LO*), peroxyl and hydroxyl radicals and reactive aldehydes, including malondialdehyde and 4-hydroxynonenal. In fact, malondialdehyde is commonly used as a biological marker of oxidative stress (Liu et al., 2008), and a linear correlation between the content of this compound in *E. coli*, the time of exposure to NTAP and microbial viability has been described (Joshi et al., 2011; Colagar et al., 2013; Yost and Joshi, 2015). These intermediate reactive compounds also behave as secondary toxic messengers of the reactive species generated in the plasma, amplifying their lethal damage to the cell (Joshi et al., 2011). Furthermore, many have greater stability than the plasma reactive species and, consequently, a much longer cytotoxic action, and can disseminate from the membrane towards more distant molecules, like DNA (Yost and Joshi, 2015), where they exert their action by interacting with nucleotides, inducing important modifications and cross-links and making cell growth and DNA repair more difficult (del Río et al., 2005). In *S*. Typhimurium, malondialdehyde has been shown to be capable of inducing base insertions, deletions and substitutions, thus potentially contributing to significant DNA alterations (Dobrynin et al., 2009). In addition, some of the aldehydes generated in the peroxidation process, including malondialdehyde, are capable of forming cross-links in the polypeptide chains of proteins, affecting the activity of enzymes and membrane-associated proteins. To sum up, NTAP causes an uncontrolled process of lipid peroxidation which results in (i) changes in the membrane chemical composition,

ultrastructural organization and permeability, (ii) a decrease in membrane fluidity, and (iii) the inactivation of membrane-associated enzymes, thereby compromising cellular viability.

The existence of damages in the cellular envelopes upon exposure to NTAP has been repeatedly demonstrated by using direct and indirect methods. Micrographs obtained through scanning electron microscopy for both vegetative cells (*Staphylococcus aureus, E. coli, B. subtilis, Campylobacter jejuni, Citrobacter freundii, Pseudomonas aeruginosa, Enterococcus faecalis, Saccharomyces cerevisiae, Candida albicans*) and bacterial (*B. cereus, B. subtilis* and *G. stearothermophilus*) and fungal (*Aspergillus parasiticus, Aspergillus flavus*) spores have allowed the identification of morphological alterations, such as size reductions, changes of shape, surface structure modifications, presence of pores in membranes and envelope disruptions (Yu et al., 2006; Hong et al., 2009; Miao and Jierong, 2009; Joshi et al., 2011; Miao and Yun., 2011; Bermúdez-Aguirre et al., 2013; Ryu et al., 2013; Zhang et al., 2013; Kim et al., 2014; Surowsky et al., 2014; Ma et al., 2015; van Bokhorst-van de Veen et al., 2015; Butscher et al., 2016; Cui et al., 2016a; Dasan et al., 2016; Gabriel et al., 2016b; Lai et al., 2016; Nishime et al., 2017). For bacterial spores, a loss of refringence, not accompanied by the onset of germination, has been observed for *B. cereus* by phase contrast microscopy (van Bokhorst-van de Veen et al., 2015). Damages in the cell envelopes have also been evidenced indirectly by determining the release of different intracellular components, including small ions such as K^+ (Zhang et al., 2013), ATP (Cui et al., 2016a), nucleic acids (Zhang et al., 2013; Tian et al., 2015; Cui et al., 2016a), proteins (Liu et al., 2008; Miao and Jierong, 2009; Miao and Yun., 2011; Zhang et al., 2013; Tian et al., 2015; Cui et al., 2016a) or dipicolinic acid in the case of bacterial spores (Tseng et al., 2012), or by monitoring the entry into the cell of fluorescent dyes which cannot cross intact membranes, such as propidium iodide (Joshi et al., 2011; Surowsky et al., 2014; Ma et al., 2015; Tian et al., 2015), the decrease in membrane potential or its depolarization (Joshi et al., 2011; Surowsky et al., 2014; Tian et al., 2015; Yost and Joshi, 2015), as well as the inability of cells to retain the Gram staining (Muranyi et al., 2010; Joshi et al., 2011).

1.2.2 Damages at intracellular level

However, it appears that microbial cell envelopes are not the only damaged cell structure. Indeed, reactive species, such as singlet oxygen, hydrogen peroxide, nitric oxide and excited atoms and molecules, can rapidly and easily diffuse into the cells, even when the membrane is intact, and oxidize many macromolecules (Dobrynin et al., 2011; Joshi et al., 2011; Yost and Joshi, 2015). Recently, Ziuzina et al. (2015b) detected and quantified, in *L. monocytogenes* and *E. coli,* the intracellular pool of reactive oxygen species after exposure to NTAP, using fluorescence techniques. They observed an increase in their concentration after NTAP treatment, which was more pronounced in *L. monocytogenes*. Similarly, Joshi et al. (2011), using highly selective fluorescent compounds for singlet oxygen and hydrogen peroxide quantification, found that the singlet oxygen concentration inside *E. coli* cells increased steadily within a 60 second NTAP treatment, whereas hydrogen peroxide content reached a maximum value after 12 seconds of treatment. In *S. cerevisiae*, an increase in the intracellular concentration of reactive species of oxygen and, especially, of nitric oxide has also been reported (Ryu et al., 2013). Once inside the cell, reactive substances provoke damage not only on lipids, but also on proteins, nucleic acids and carbohydrates. In order to neutralize the deleterious effects of such oxidant species or to repair the damage they cause, microorganisms have a number of mechanisms of elimination and transformation of these compounds, such as catalases, superoxide dismutases and glutathione and thioredoxin systems, among others. Nevertheless, when the accumulation of reactive oxygen species exceeds the cellular detoxifying capacity, and their concentration surpasses a critical level, the microbial cell suffers from oxidative stress, which induces the expression of certain genes and the activation of different defense responses (Pomposiello and Demple, 2002). For instance, in *E. coli*, the regulators *oxyRS* and *soxRS* are involved in the cellular response to oxidative stress induced by NTAP. Indeed, the exposure of *E. coli* to NTAP results in the overexpression of the *oxyS*, *soxS* and *soxR* genes, as well as of those genes encoding for the catalase (KatG) and superoxide dismutase (SodA) enzymes (Sharma et al., 2009; Yost and Joshi, 2015). In addition, *E. coli* mutants lacking the *oxyR* or *soxS* genes have been shown to be more susceptible than

wild-type strains to a plasma treatment using as working gases helium or a mixture of helium and oxygen (Perni et al., 2007). Similarly, Yost and Joshi (2015) reported that strains deficient in superoxide dismutase (SodA) were more rapidly inactivated by a NTAP treatment than wild-type strains. Overall, these results demonstrate the occurrence of reactive oxygen species within the cells during plasma treatments and suggest that the membrane is not the unique cellular component targeted by NTAP.

At the intracellular level, reactive species of oxygen and nitrogen, such as atomic oxygen, hydroxyl radicals (OH*), hydroperoxyls (HOO*), superoxides (O_2-*) and nitric oxide (NO), are capable of interacting with various macromolecules and, according to some authors (Yost and Joshi, 2015), DNA and proteins are especially susceptible.

1.2.3 DNA damage

Damage to DNA is caused through the oxidation of bases. For example, oxidation of guanine generates 8-hydroxy-2'deoxyguanosine (OHdG), a compound which is used as a marker of oxidative DNA damage (Joshi et al., 2011; Yost and Joshi, 2015). The oxidation of DNA bases leads to a transversion (substitution of a purine for a pyrimidine or *vice versa*), which alters the bonds between DNA bases, causing changes in DNA conformation, and frequently leading to errors in the reading of the strand, mutagenesis and cell death. Some of the chemical species of plasma also react with deoxyribose carbons, breaking the N-glucosidic bond, thus generating apurinic or apyrimidine sites (abasic sites). All these changes may eventually lead to DNA strand ruptures (Colagar et al., 2013; Ryu et al., 2013; Yost and Joshi, 2015). Microorganisms can nevertheless respond to DNA damage inflicted by NTAP. Indeed, transcriptional studies using microarrays have demonstrated that up to 18 genes involved in the SOS response are overexpressed upon exposure to NTAP (Sharma et al., 2009). Such overexpression is triggered when the level of accumulated damage is so high that microbial replication mechanisms are blocked. Similarly, Sharma et al. (2009), studying the transcriptomic response of *E. coli* cells exposed for 2 minutes to an argon plasma, observed an intense overexpression of several genes encoding for polymerase enzymes involved in the repair

of extremely damaged DNA (*polB*, *dinB*, *umuC* and *umuD*). In addition, these authors also detected an incomplete induction of some more specific DNA repair mechanisms, such as the nucleotide cleavage repair system, and concluded that plasma exerted massive damage at DNA level. The existence of significant damage to microbial DNA has also been corroborated using agarose gel electrophoresis techniques (Joshi et al., 2011; Colagar et al., 2013; Ryu et al., 2013; Lu et al., 2013). For instance, Joshi et al. (2011) observed fragmentation of *E. coli* DNA after NTAP treatment, which increased with increasing treatment times, and Colagar et al. (2013) found that treatment times longer than 3 minutes resulted in a complete degradation of *E. coli* genomic DNA. Similar results have been obtained for *Saccharomyces cerevisiae* by Ryu et al. (2013), who also found complete DNA fragmentation after exposure to plasma for up to 3 minutes, and by Muranyi et al. (2010), who detected massive DNA damage in *B. subtilis* vegetative cells and, to a lesser extent, in their sporulated forms.

1.2.4 Damage to proteins

According to some authors, proteins and cytoplasmic enzymes would also suffer damage during plasma treatments. Indeed, reactive species are able to (i) break peptide bonds, (ii) oxidize amino acid side chains, especially of sulfur amino acids, such as methionine and cysteine, and amino acids harboring aromatic rings (tryptophan, phenylalanine and tyrosine), (iii) produce crosslinks within proteins and (iv) give rise to aggregation phenomena, which are favored by the formation of intra- and inter-molecular disulfide bonds. All these effects result in modifications in the conformation and three-dimensional structure of proteins and enzymes (Surowsky et al., 2013). In addition, in the case of enzymes, their activity could also be compromised by the oxidation of their cofactors (Colagar ettbla al., 2010). Actually, the oxidation of just one amino acid in a protein can affect its function (Lackmann et al., 2013). These latter authors showed that the loss of 3-phosphate glyceraldehyde activity after NTAP treatment was due to the oxidation of a cysteine residue (cys150) of its active site, which is known to be irreversible (Brandes et al., 2009). However, it is still unknown which enzymes and proteins are specific targets for the action of NTAP. In the particular case

of vegetative cells, Colagar et al. (2013) analyzed *E. coli* proteins after exposure to plasma using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) techniques and found that high molecular weight proteins, of between 50 and 90 KDa, were especially sensitive to NTAP, observing a decrease in their content as treatment time increased. In addition, these authors also observed that the concentration of free amino acids linearly increased with exposure time, which suggested that NTAP is not only able to break peptide bonds, but also to fragment proteins liberating free amino acids. Concerning bacterial spores, several authors (Klämpfl et al., 2012; Tseng et al., 2012) have pointed out that the effectiveness of NTAP against these resistant forms is due to the inactivation of various enzymes responsible for spore germination, such as GerA and GerB, as well as to the inactivation of channel proteins involved in the transport of ions and dipicolinic acid, which also results in the inability to initiate germination processes.

1.2.5 Indirect mechanism of action

Finally, apart from the direct effects caused by reactive species present in plasmas, an indirect mechanism of action, through the formation of other cytotoxic compounds, has been also described. Thus, Yost and Joshi (2015) showed that hydroxyl radicals (OH*) can be generated from H_2O_2 and superoxide radicals (O_{2^-}*) in the presence of transition metals such as iron or copper. Although OH* radicals can be produced by different routes, the Haber-Weiss reaction is the most habitual (Lemire et al., 2013). This reaction consists in the transformation of the ferric cation to ferrous cation ($Fe^{3+} + O_2^{-*} \rightarrow Fe^{2+} + O_2$) by the superoxide radical. Afterwards, the ferrous cation reacts with H_2O_2 to produce Fe^{3+}, hydroxyl anions and hydroxyl radicals, which also exert and antimicrobial activity. Nitric oxide (NO) can also react with oxygen or superoxide radicals to form nitrogen dioxide (NO_2), peroxynitrites ($ONOO^-$ or ONO_2^-) and nitrous anhydride (N_2O_3) (Lu et al., 2013), which all have a broad spectrum of antimicrobial activity, although they differ in reactivity, stability and biological activity (Fang et al., 2004).

1.3 Factors determining the antimicrobial effectiveness of NTAP

There are several factors known to influence the microbial inactivation achieved through NTAP treatments. Some of these factors are related to the conditions used for plasma generation and application, while others are associated with the particular properties of the microorganism targeted or with the characteristics of the treatment medium or food. The following sections of this review article will compile and discuss the information available in the literature in relation to these aspects.

1.3.1 Processing Parameters

NTAP may be obtained through electrical discharges, which may be accomplished by using very diverse systems, e.g., corona discharge, micro hollow cathode discharge, atmospheric pressure plasma jet, gliding arc discharge, dielectric barrier discharge (DBD), radiofrequency (rf) and microwaves. In any case, electrical discharges are initiated and sustained through electron collision processes under the action of specific electric or electromagnetic fields. Among all the existing plasma sources, DBD and plasma jets are the most widely explored configurations in food research, due to their easiness of construction and adoption. Indeed, there are some commercially available DBD and plasma jet configurations. Nevertheless, regardless of the equipment used, the molecular composition of plasma will depend on the energy supplied, which in turn is determined by voltage, power and excitation frequency, and even on the composition and flow rate of the working gas, with these parameters being very variable among different NTAP units, which makes it very difficult to carry out direct comparisons among research studies executed employing different equipment and NTAP processing conditions. However, it is possible to draw some general conclusions on how some processing parameters affect the level of microbial inactivation achieved through NTAP treatments.

With processing times described in the literature ranging from a few seconds up to several minutes, it is well known that microbial inactivation increases with processing time, although not in all cases exponentially. Thus, a wide variety of inactivation kinetics

are observed in the published data, where it is frequent to find both linear, concave-upward (tailing) and concave-downward (shoulders) kinetics (Lee et al., 2011; Calvo et al., 2016, 2017).

Voltage (difference of potential between two points per unit of charge, expressed in Volts), power (electrical energy consumed per unit of time, expressed in Watts) and frequency (number of cycles per second, expressed in Hertz) have a great effect on the antimicrobial effectiveness of NTAP, as they are known to determine the input energy and, consequently, the chemical reactive species generated and their concentration. It has been repeatedly demonstrated that an increase in voltage increases microbial inactivation of both vegetative cells and bacterial spores (Table 1). In fact, Liu et al. (2013) have found, after using different voltages to generate helium plasmas, that the content of reactive chemical species and, in particular, of N_2^+, OH, He and O, and the level of inactivation achieved for *S. aureus*, increased with the voltage applied. It has also been shown that, working under a constant voltage, an increase in excitation frequency or in power gives rise to greater lethal effects in a wide range of microbial species (Table 1). Taking these findings into account it would be advisable to use high voltages, power and frequencies for the generation of plasmas. However, some technological constraints, including high costs, and potential negative impacts on food quality, due to an excessive temperature rise, represent a limit towards the use of extreme processing conditions in industrial practice (Kim et al., 2011; Alkawareek et al., 2012; Daeschlein et al., 2012; Butscher et al., 2016).

Another variable to take into account when considering the antimicrobial effectiveness of NTAP treatments is the working gas used. A wide variety of gases have been used to generate plasma, with the most frequent ones being nitrogen, oxygen, carbon dioxide, argon, helium, air, or mixtures of some of these gases (Table 1). All of them give rise to plasmas capable of achieving certain level of microbial inactivation, and there is no general agreement on which one is the most effective gas. Lee et al. (2011) compared the effectiveness against *L. monocytogenes* of plasmas obtained with helium or nitrogen and observed a higher inactivation level for nitrogen-based plasmas.

Table 1. Summary of major research articles assessing the influence of processing parameters on the performance of non-thermal atmospheric plasma as a food decontamination technique.

Reference	Treatment Regime	Foods / medium	Microorganism	Main findings
Marsili et al. (2002)	23 kV; Pulse rate: 320 pps; N_2, CO_2, air (10 L/min); Up to 50 sec; batch	0.1% peptone solution	*E. coli* NCTC 9001, *S. aureus* NCTC 4135, *S.* Enteritidis NCTC PT4, *B. cereus* NCTC KD4	Air gas facilitated a greater inactivation after a 50 sec treatment. *B. cereus* was the most susceptible microorganism.
Deng et al. (2007)	16- 25 kV; 1000- 2500 Hz; Air; Up to 30 sec	Almonds	*E. coli* 12955 (Collection of the Dept of Food Science and Nutrition, University of Minnesota)	Effectiveness increased with the applied voltage and the frequency. Cells at log growth phase were more sensitive than those at stationary phase.
Rowan et al. (2007)	23,5 kV; Pulse rate: 124 pps; N_2, O_2, CO_2, air (10 L/min); Up to 30 sec; 4ºC; batch	Distilled water	*B. cereus* NCTC 11145 spores	The order of effectiveness was: $O_2 > CO_2$; air > N_2
Muranyi et al. (2008)	170 W; Air (0-80% RH); 20ºC; For up to 7 sec	PET foils	*Bacillus subtilis* DSM 4181 spores, *Aspergillus niger* DSM 1957 spores	For *A. niger*, increased effectiveness at relative gas humidity of 70%; in contrast, *B. subtilis* spores showed slightly poorer inactivation at high gas humidity.
Miao and Jierong (2009)	13.56 MHz; 20–120 W; O_2 (20–100 cm^3/min); Up to 120 sec	Poly (vinyl chloride) (PVC)	*E. coli**	The optimum conditions (highest efficiency) are 100 W and O_2 60 cm^3/min.
Song et al. (2009)	13.56 MHz; 75- 150 W; Helium (10 L/min); Up to 120 sec	Sliced cheese and ham	3-strain cocktail of *L. monocytogenes* (ATCC 19114, 19115, and 19111)	Increased effectiveness at high input power. Higher D values were obtained in sliced ham.

(Continued)

Table 1. Summary of major research articles assessing the influence of processing parameters on the performance of non-thermal atmospheric plasma as a food decontamination technique. Continued

Reference	Treatment Regime	Foods / medium	Microorganism	Main findings
Jung et al. (2010)	13.56 MHz; 75 and 100 W; Helium and argon (6 L/min); Up to 120 sec	Slide glass	*Bacillus subtilis* Spores*	Helium plasma was much less efficient than argon plasma; Increased effectiveness at high input power.
Leipold et al. (2010)	10.4 kV; 21.7 kHz; 1.8 W-0.36 kW; Air; Up to 340 sec	Knife	*Listeria innocua* (DMRI 0011)	Increased effectiveness at high discharge power.
Ragni et al. (2010)	12.7 kHz; 15 kV; Air (RH 35 and 65%); 25ºC; Up to 90 min	Shell eggs	*S.* Enteritidis MB2509, *S.* Typhimurium T5	Increased effectiveness at high air moisture content; *S.* Enteritidis resulted to be more plasma sensitive.
Yun et al. (2010)	13.56 MHz; 75-150 W; Helium (4 L/min); Up to 120 sec	Disposable plastic trays, aluminum foil, and paper cups	3-strain cocktail of *L. monocytogenes* (ATCC 19114, 19115, and 19111)	Increased effectiveness at high input power. The lowest D values were obtained on disposable plastic trays.
Kim et al. (2011)	13.56 MHz; 75-125 W; Helium (10 L/min) and He+O_2 (10 sccm); Up to 90 sec	Sliced bacon	*L. monocytogenes* (KCTC 3596), *E. coli* (KCTC 1682), *S.* Typhimurium (KCTC 1925)	Increased effectiveness at high input power and with the addition of oxygen to the working gas.
Lee et al. (2011)	2 kV; 50 kHz; He, N_2 (7 L/min), He+O_2, N_2+O_2 (0.07 L/min); Up to 2 min	Agar plates, slices of cooked chicken breast and ham	*L. monocytogenes* KCTC 3596	N_2 was more effective than He. The addition of O_2 to both gases improved their effectiveness, being N_2 + O_2 the most effective mixture. The highest and the lowest levels of inactivation were obtained on agar plates and sliced chicken breast, respectively.

(Continued)

Table 1. Summary of major research articles assessing the influence of processing parameters on the performance of non-thermal atmospheric plasma as a food decontamination technique. Continued

Reference	Treatment Regime	Foods / medium	Microorganism	Main findings
Alkawareek et al. (2012)	6 kV; 20-40 kHz; 0.5% O2+ 99.5% helium (2 L/min); Up to 240 sec	Agar plates	*Pseudomonas aeruginosa* PA01 (ATCC BAA-47)	Increased effectiveness at high frequencies
Fröhling et al. (2012b)	27.12 MHz; 10-40 W; Argon (20 L/min); Up to 4 min	Gelrite® - a polysaccharide gel	*L. innocua* DSM 20649; *E. coli* DSM 1116	Increased effectiveness at high discharge power
Niemira (2012)	47 kHz; 549 W; Air and N_2; Distance from the plasma jet: 2-6 cm; Up to 20 sec	Almonds	*S.* Anatum F4317, *S.* Stanley H0558, *S.* Enteritidis PT30, *E. coli* O157:H7 (C9490, ATCC 35150, ATCC 43894)	Air was more effective The greatest reductions were observed for *E. coli* O157:H7 C9490 at a 6 cm distance
Bermúdez – Aguirre et al. (2013)	3.95- 12.83 kV; 60 Hz; Argon (455.33 sccm); Up to 10 min	Lettuce, baby carrots, tomatoes	*E. coli* ATCC 11775	Effectiveness increased with voltage and decreased with increasing contamination level. Tomatoes, followed by lettuce, were easier to decontaminate than carrots.
Lu et al. (2013)	56 and 70 kV; 50 Hz; Air, 90% N_2 + 10% O_2, 65% O_2 + 30% CO_2 + 5% N_2; Direct and indirect exposure; Up to 120 sec; batch	Phosphate Buffered Saline (PBS)	*E. coli* ATCC 25922, *E. coli* NCTC 12900, *L. monocytogenes* NCTC11994	Greater reduction of viability using higher voltage and with working gas mixtures with higher oxygen content. Indirect mode of exposure more effective than direct exposure. *L. monocytogenes* resulted to be more sensitive
Ziuzina et al. (2013)	40 kV; Air; Direct and indirect exposure; Up to 300 sec; batch	Maximum recovery diluent (MRD) or PBS	*E. coli* ATCC 25922	Direct exposure resulted to be more effective than indirect exposure, especially at lower plasma treatment times.

(Continued)

Table 1. Summary of major research articles assessing the influence of processing parameters on the performance of non-thermal atmospheric plasma as a food decontamination technique. Continued

Reference	Treatment Regime	Foods / medium	Microorganism	Main findings
Patil et al. (2014)	50 Hz; 70 kV; Air (3-70% RH), 90% N_2 + 10% O_2, 65% O_2 + 30% CO_2 + 5% N_2; 20ºC; Direct and indirect exposure; Up to 120 sec	Sterile polystyrene Petri dish containing *B. atrophaeus* spore strips	*B.atrophaeus* standard spore strips (Sportrol®_ Namsa®)	The gas mixture 65% O_2 + 30% CO_2 + 5% N_2 was the most effective; Increased effectiveness at high air moisture content and after direct exposure to plasma
Edelblute et al. (2015)	24 kV; 500 Hz; Air (5, 10 L/min); Up to 3 min	Brain Heart Infusion (BHI) agar plates	*Ecoli* ATCC 25922 and *S. epidermidis* ATCC 12228.	Effectiveness decreased with the flow rate
Takamatsu et al. (2015)	16 kHz; 9 kV; 10 W; Ar, O_2, N_2, CO_2, air (1 L/min); 20ºC; Up to 120 sec; batch	PBS	*S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853	The greatest antimicrobial effect was obtained with N_2 and CO_2
Butscher et al. (2016)	6-10 kV; 5-15 kHz; Argon; Up to 60 min	Wheat grains; Polypropylene granules	*Geobacillus stearothermophilus* (ATCC 7953) spores	Higher efficiency by applying faster pulse frequency or higher pulse voltage. Less decontamination effect on wheat grains than on polypropylene
Calvo et al. (2016)	1 kHz; 1 W; O_2, N_2 (5-15 L/min); Up to 4 min	Polycarbonate membrane filters	*L. monocytogenes* CECT 4301, *L. innocua* CECT 910	A higher sensitivity to plasma was observed when the treatment was performed using air; increases in flow rate from 5 to 10 L/min caused an acceleration of bacterial inactivation when air was used; gas flow rate hardly affected NTAP efficiency when nitrogen was used.

(Continued)

Table 1. Summary of major research articles assessing the influence of processing parameters on the performance of non-thermal atmospheric plasma as a food decontamination technique. Continued

Reference	Treatment Regime	Foods / medium	Microorganism	Main findings
Cui et al. (2016a)	300 - 600 W; N_2; Up to 3 min	Lettuce; Stainless steel coupons	Biofilms of *Escherichia coli* O157:H7 (CICC 21530).	Significant lower inactivation was observed at 300W; The combination of plasma and clove oil exhibited a remarkable synergistic effect
Cui et al. (2016b)	300 - 600 W; N_2 (100 sccm); Up to 3 min	Eggshell	*Salmonella* Enteritidis (CICC 21482); *Salmonella* Typhimurium (CICC 22956)	Significant lower inactivation was observed at 300W; The combination of plasma and thyme oil exhibited a remarkable synergistic effect
Gabriel et al. (2016a)	2.45 GHz; 450 and 650 W; Air (5 L/min); Up to 25 min; batch	Young coconut liquid endosperm	Multi-strain cocktails of *E. coli* O157:H7 (7 strains), *S. enterica* (5 strains), *L. monocytogenes* (2 strains) and spoilage bacteria (*Klebsiella* spp., *Staphylococcus* spp., and *Kluyvera* spp.)	Significant lower inactivation was observed at 450W; *S. enterica* exhibited the greatest plasma resistance, *while L. monocytogenes* and *Staphylococcus* spp. exhibited the least.
Lai et al. (2016)	4 W; Air (2-7 m/s); 52-90% RH	Sterilized distilled water	*Micrococcus luteus* ATCC 4698, *Staphylococcus epidermidis* ATCC 12228, *E. coli* ATCC 10536, *Serratia marcescens* ATCC 6911, *Pseudomonas alcaligenes* ATCC 14909.	The inactivation efficacy increased with flow rate and decreased with relative humidity. The inactivation efficacy at 90% R.H. dropped to 10% of the value measured at 55% R.H.
Calvo et al. (2017)	1 kHz; 1 W; O_2, N_2 (5- 15 L/min); Up to 12 min	Polycarbonate membrane filters	*S.* Typhimurium CECT 443, *S.* Enteritidis CECT 4300	Microbial inactivation was higher when air was used and with increasing flow rates

®Non-specified strain. RH: relative humidity; pps: pulses per second; sccm: standard cubic centimeters per minute

Other studies have described that air-based plasmas are most effective in inactivating *E. coli* O157:H7, *B. cereus, S. aureus, L. monocytogenes, L. innocua* and various serovars of *Salmonella enterica*, such as *S*. Anatum, *S*. Stanley, *S*. Typhimurium and *S*. Enteritidis, than plasmas generated with nitrogen (Niemira, 2012; Calvo et al., 2016, 2017; Marsili et al., 2002). On the contrary, Takamatsu et al. (2015) obtained a greater lethal effect against *S. aureus* and *P. aeruginosa* with nitrogen and CO_2 based plasmas than with plasmas generated with air, O_2 or argon, and Rowan et al. (2007) concluded that oxygen is the gas of choice, over CO_2 or nitrogen, when generating plasmas for the inactivation of *E. coli, C. jejuni, C. coli, L. monocytogenes, S.* Typhimurium*, S.* Enteritidis and *B. cereus* spores. On the other hand, it seems to exist an agreement, although with some exceptions (Reineke et al., 2015), in that the addition of small amounts of oxygen to noble gases, such as helium (Gweon et al., 2009; Kim et al., 2011, 2013; Lee et al., 2011, 2012a; Galvin et al., 2013) and argon (Surowsky et al., 2014), or nitrogen (Lee et al., 2011, 2012b) improves the antimicrobial effectiveness of NTAP against vegetative cells and spore-forming bacteria (Table 1). This effect is mainly attributed to a higher formation of reactive oxygen species, such as hydroxyl and hydroperoxyl radicals, atomic oxygen, hydrogen peroxide, singlet oxygen and ozone, all of them with a high antibacterial activity.

Another processing parameter determining the antimicrobial effectiveness of NTAP is the gas moisture content. In fact, it has been occasionally observed (Dobrynin et al., 2011) that the use of completely dry gases is ineffective for *E. coli* inactivation, and there are several studies which show that an increase in the water content of the gas improves its effectiveness (Table 1). Thus, Ragni et al. (2010) reported that an increase in the air relative humidity from 35 to 65% increased the inactivation of *S.* Enteritidis and *S.* Typhimurium from 2.5 to 4.5 log cycles, and attributed this effect to a higher concentration of hydroxyl radicals in the plasma. Similar results were found by Patil et al. (2014) for *B. atrophaeus* spores. These authors used plasmas with different moisture content (3, 10, 30, 50 and 70%), and obtained a 5 to 6 log reduction at humidities of 3 and 10%, while a complete inactivation was observed at higher humidities. These authors related this higher antimicrobial activity to the increased

generation of numerous reactive species, such as N_2O_5, H_2O_2, HNO_4, or hydroxyl radicals, and, especially, to the decomposition of ozone in the presence of water, with the consequent formation of highly oxidizing species, such as hydroxyl and hydroperoxyl radicals, superoxide anion and H_2O_2. On the contrary, a recent study by Lai et al. (2016) has shown that when the relative air humidity increased from 52 to 81% or from 62 to 81%, the inactivation efficiency of NTAP against *E. coli* and *Staphylococcus epidermidis* decreased by 87 and 58%, respectively, which was linked to a decrease in the concentration of negative ions in plasma. These apparently contradictory results could be explained by the existence of an optimum moisture value for achieving a maximum antimicrobial activity. For instance, in the particular case of *Aspergillus niger*, a progressive increase in microbial inactivation was observed up to a moisture content of 70%, which was the optimum moisture content for NTAP generation (Muranyi et al., 2008).

Gas flow rate also seems to influence the effectiveness of NTAP treatments (Table 1). Lai et al. (2016) reported a steady increase in *E. coli, S. epidermidis* and *P. alcaligenes* inactivation when air flow rates increased from 2 to 7 meters/s, linked to a linear rise in the concentration of negative ions in the generated plasma. Results from our research group have demonstrated that the effect of gas flow rate on *L. monocytogenes* and *L. innocua* inactivation through NTAP depended on the type of gas used to generate plasma. Indeed, increases in flow rate from 5 to 10 L/min caused an acceleration of bacterial inactivation when air was used, while an additional increase of gas flow from 10 to 15 L/min had a minor impact on microbial inactivation. On the other hand, gas flow rate hardly affected NTAP treatment efficiency when nitrogen was used to generate plasma (Calvo et al., 2016). A similar behavior was also observed for *S.* Enteritidis and *S.* Typhimurium (Calvo et al., 2017). In contrast, Edelblute et al. (2015) found that an increase in air flow rate from 5 to 10 liters per minute was accompanied by a loss of efficacy of NTAP for the inactivation of *E. coli* and *S. epidermidis*, and attributed this effect to a reduction in ozone and NO_2 concentration. Moreover, Miao and Jierong (2009), using oxygen as working gas, suggested the existence of an optimal flow rate for the inactivation of *E. coli*, reporting that, once this optimal flow rate is

surpassed, the lethal effect achieved is reduced. According to these authors, at low flow rates, the number of reactive species, mainly constituted by oxygen radicals, is lower than at high flow rates, but they have a higher average energy and, therefore, the likelihood of each one colliding with microbial cells increases and, consequently, also their antimicrobial effectiveness. However, at higher flow rates the number of reactive species will be higher, but they will have a lower average energy, and their antimicrobial action would be comparatively lower.

NTAP effectiveness can also depend on whether treatments are carried out directly or indirectly. In direct treatments, the product is physically located in the field where plasma is generated, and, therefore, it is in intimate contact with all the photons and chemical species produced. In contrast, in indirect treatments plasma is produced at some distance from the product, and is usually displaced towards it, using a rapid flow of the feed gas. In principle, one would expect that indirect treatments would be less effective in microbial inactivation. Patil et al. (2014) demonstrated that the direct exposure of *B. atrophaeus* spores to plasmas generated with gases of different composition (air; a mixture of nitrogen [90%] and oxygen [10%]; a mixture of oxygen [65%], CO_2 [30%] and nitrogen [5%]) caused the inactivation of at least 6 log cycles, whereas for indirect exposures inactivation rates ranged from 2.1 to 6.3 log units, depending on the type of gas used. Nevertheless, other authors have obtained conflicting results. Thus, Lu et al. (2013) found that indirect treatments were more effective against *E. coli* and *L. monocytogenes* than direct treatments, and suggested that this fact could be due to the recombination of reactive radicals with a short life before reaching the microorganisms in indirect treatments, giving rise to new chemical species with strong bactericidal effects. However, there are also some authors (Ziuzina et al., 2015a) who did not detect differences in effectiveness between direct and indirect treatments when treating *L. monocytogenes*, *E. coli* and *S. aureus* biofilms.

Finally, it should be noted that, in the case of indirect treatments, another parameter that seems to determine NTAP lethality is the distance between the point of plasma generation and the sample. Thus, the results obtained by several authors show

that, in general, the antimicrobial effectiveness decreases as that distance increases (Gabriel et al., 2016b; Nishime et al., 2017).

1.3.2 Microbial-related factors

One of the key advantages of NTAP in comparison to other non-thermal food preservation technologies is its ability to inactivate not only bacteria, molds and yeasts, but also mold ascospores and bacterial spores (Muranyi et al., 2007; Rowan et al., 2007; Shi et al., 2011; Klämpfl et al., 2012; Dasan et al., 2016).

In general, molds and yeasts are more tolerant against the action of plasma than bacteria, and vegetative cells are more sensitive than bacterial spores (Lee et al., 2006; Muranyi et al., 2007; Rowan et al., 2007; Hong et al., 2009; Shi et al., 2011; Klämpfl et al., 2012; Tseng et al., 2012; Takamatsu et al., 2015). However, results obtained by numerous authors studying the effectiveness of NTAP under the same experimental conditions for a wide range of microbial groups have demonstrated that differences in NTAP resistance among these microbial groups are not as marked as those observed for thermal and other non-thermal processing technologies.

Some studies, in particular, provide interesting insights into the inter-kingdom and inter-species variability in NTAP resistance. Klämpfl et al. (2012) evaluated the effectiveness of an air plasma for the inactivation of vegetative cells of fifteen bacterial species, including *E. coli, P. aeruginosa, S. aureus, E. faecalis, B. cereus, B. pumilus,* and *C. difficile*, one yeast species (*Candida albicans*), and spores of four bacterial species (*B. subtilis, B. pumilus, B. atrophaeus* and *G. stearothermophilus*). They observed that after 30 seconds of NTAP treatment, between 4 and 6 log reductions were achieved for vegetative cells, while after a 1 minute exposure under the same working conditions, from 1 (*G. stearothermophilus*) to 4 (*B. subtilis*) log reductions were attained for bacterial spores. Lee et al. (2006), using a plasma obtained from a mixture of helium and oxygen, also reported a higher sensitivity to NTAP for vegetative cells than for yeasts and bacterial spores, with D values of 0.3 minutes for *E. coli* and *S. aureus*, 2 minutes for *S. cerevisae* and 14 minutes for *B subtilis* spores. Similarly, Takamatsu et al. (2015) found

that treatments of around 1 minute with a nitrogen-based plasma were required in order to achieve 6 log cycles of inactivation for *E. coli, P. aeruginosa, E. faecalis* and *S. aureus*, while 5 and 15 minutes were required to achieve the same inactivation levels for *Aspergillus niger* and *B. cereus* spores, respectively. Moreover, Tseng et al. (2012) described D values of 0.50 min for vegetative cells of *E. coli* and *B. subtilis*, while D values obtained for bacterial spores of species belonging to the genus *Bacillus, Geobacillus* and *Clostridium* (*B. subtilis, G. stearothermophilus*, *C. sporogenes, C. perfringens, C. difficile* and *C. botulinum* Type A and type B) ranged between 2.66 (*C. perfringens*) and 8.04 (*C. botulinum* type A) min. Interestingly, spores of *G. stearothermophilus*, generally used as biological indicators of heat sterilization treatments given their extreme thermoresistance, showed a similar NTAP resistance to that of spores of *B. subtilis*, a mesophilic microorganism, suggesting that mechanisms of inactivation by heat and plasma differ, at least to a certain extent. Similar observations were also made by van Bokhorst-van de Veen et al. (2015), who hardly found differences in NTAP resistance among spores of *B. cereus, G. stearothermophilus* and *B. atrophaeus*, obtaining between 3.7 and 4.9 log reductions after a 20 min treatment with a nitrogen-based plasma, while identified large inter-species variations in spore resistance to heat, UV light and chemical oxidants (hydrogen peroxide and sodium hypochlorite).

The higher NTAP resistance exhibited by bacterial spores may be due to the low water content and the high concentration of dipicolinic acid in the spore core, the low permeability of the spore inner membrane, the very robust spore coat, which represents a physical barrier to external agents, the special conformation of the spore DNA, which is saturated by a group of soluble acid proteins (SASP), the presence of detoxifying enzymes in the spore cortex, or the absence of metabolic activity within the spore, which limits the activity of plasma-generated components (Henriques and Moran, 2007). In a recent study by Reineke et al. (2015) analyzing two *B. subtilis* mutant strains, unable to synthesize dipicolinic acid during sporulation and two of the major SASPs, respectively, it was reported that only this latter strain was more sensitive to NTAP than the wild type strain, what suggested that DNA stabilization through SASPs may be responsible, at least in part, for the increased resistance of bacterial spores against NTAP.

The higher NTAP resistance exhibited by yeasts and molds in comparison to vegetative bacterial cells could be due to differences between prokaryotic and eukaryotic cells in cellular structure and molecular composition. On the one hand, the fungal genome is protected by the nuclear membrane, which constitutes an additional diffusion barrier that would contribute to increase the cell resistance to DNA damaging agents. On the other hand, the fungal cell wall is very thick and consists of rigid layers of polysaccharides, which would provide further protection to the cell (Klämpfl et al., 2012).

In relation to bacterial vegetative cells, Gram-positive bacteria are generally considered to be more resistant to NTAP than Gram-negative bacteria due to the fact that they have a thicker layer of peptidoglycan in their cell wall, which increases the rigidity of cellular envelopes, making them more resistant to mechanical damage and hindering the diffusion of reactive species through the envelopes (Lee et al., 2006; Ziuzina et al., 2014, 2015a; Edelblute et al., 2015; Jayasena et al., 2015; Yong et al., 2015a; Puligundla et al., 2017). However, other studies have found similar resistance to NTAP for particular species of both bacterial groups (Lee et al., 2006; Klämpfl y col., 2012; Tseng et al., 2012; Takamatsu et al., 2015), and, even, some authors have described strains of *L. innocua* (Baier et al., 2014), *L. monocytogenes* (Gabriel et al., 2016a), *B. cereus* (Marsili et al., 2002), *S. aureus* and *E. faecalis* (Nishime et al., 2017) as being more sensitive to NTAP than strains of *E. coli, S.* Typhimurium, *S*. Enteritidis, *Vibrio parahaemolyticus* and *Pseudomonas aeruginosa*, respectively. These results seem to indicate that it is not possible to draw general conclusions on whether Gram-positive and Gram-negative bacteria have differential sensitiveness against NTAP.

Intraspecific variability in NTAP resistance may also exist. Indeed, Galvin et al. (2013) and Burts et al. (2009) have reported differences of around 1 log cycle of inactivation among *S. aureus* strains exposed either to helium-based or air-based plasmas. Similar results have been described by Niemira (2012) for a group of *E. coli* O157:H7 strains exposed to air plasmas.

It is well known that the cellular physiological state markedly determines microbial resistance to different inactivation treatments. In fact, bacterial cells harvested during exponential growth phase are known to be more sensitive against heat, pulsed electric fields and high hydrostatic pressures than cells obtained in the stationary phase of growth (Álvarez et al., 2000; Martínez et al., 2003; Mañas and Mackey, 2004). However, based on the results available in the literature, the microbial physiological state exerts little or no influence at all on microbial resistance against NTAP. Thus, no significant differences in D-values were found among *E. coli* (Yu et al., 2006; Deng et al., 2007) and *S.* Typhimurium (Fernández et al., 2013) cultures obtained in logarithmic, early and late stationary phases of growth.

It is worth noting that NTAP treatments have been shown to be capable of inactivating different bacteria (e.g., *S. aureus*, *E. faecium*, *L. monocytogenes, E. coli* O157:H7, *A. hydrophila* or *P. aeruginosa*) in the form of biofilms (Jahid et al., 2014; Flynn et al., 2015; Ziuzina et al., 2015a,b). It is well known that cells within biofilms exhibit increased resistance to various disinfectant agents commonly used in the food industry (Pan et al., 2006). Similarly, cells in planktonic state show a higher sensitiveness towards NTAP than cells within biofilms. For instance, Jahid et al. (2014) reported for *A. hydrophila* that a treatment with an oxygen plasma for 15 seconds caused a 7 log reduction in planktonic cells, whereas only 3 log reductions were obtained for cells forming a biofilm, even when the treatment time was prolonged for up to 5 minutes. The increased NTAP resistance of cells within biofilms has been attributed to the protective effect exerted by the biofilm´s extracellular matrix, constituted mainly by polymeric substances, including polysaccharides, phospholipids, proteins, nucleic acids and teicoic acids (Shi and Zhu, 2009), which represents a physical barrier hindering the penetration of the chemical reactive species occurring in plasma, which would then require longer treatment times to exert their antimicrobial action (Vleugels et al., 2005).

Microbial resistance to various inactivation treatments is largely determined by growth conditions, especially temperature and pH (Alvarez-Ordóñez et al., 2008, 2009, 2010). However, little attention has been paid so far to the impact of such

environmental growth conditions on the effectiveness of NTAP treatments. Very few studies have assessed the effect of growth temperature on NTAP resistance. There exists some data for *S.* Typhimurium, *S*. Enteritidis, *L. monocytogenes* and *L. innocua*, grown in the range 10-45ºC (Fernández et al., 2013; Calvo et al., 2016, 2017), and for *A. hydrophila*, grown at temperatures between 4 and 30°C (Jahid et al., 2014). From these studies it can be concluded that no significant differences in NTAP resistance occur at growth temperatures close to the optimum growth temperature (20 to 45°C), while at temperatures below 20°C a minor sensitization of cells to plasma occurred. On the other hand, in the particular case of *A. hydrophila* cells forming biofilms, resistance to NTAP exponentially increased with increasing growth temperatures, with times required to reduce the population in 5 log cycles being 1.84 minutes at 4ºC and 25.33 minutes at 30°C (Jahid et al., 2014).

In relation to the influence of growth pH, our research team (Calvo et al., 2016, 2017) has recently demonstrated that the growth of *S.* Typhimurium, *S.* Enteritidis and *L. monocytogenes* in media acidified with different acids (acetic, ascorbic, citric, lactic, malic and hydrochloric) at low pH conditions (pH 6.5, 5.4 or 4.5) did not significantly influence the antimicrobial effectiveness of NTAP treatments. The short-term exposure of these microorganisms to acid, cold or heat stress shocks prior to NTAP treatments did not modify their resistance either. Since cross-resistance adaptive responses did not take place, these findings allowed us to conclude that NTAP may be a first-choice technology to be included into food processing schemes following a hurdles technology approach in combination with acidification, mild heating or refrigeration.

Several authors have shown that the effectiveness of NTAP treatments decreases when dealing with high microbial loads. This fact has been shown in various substrates and for different microorganisms, including *E. coli* (Yu et al., 2006; Burts et al., 2009; Miao y Yun, 2011; Bermúdez-Aguirre et al., 2013), *S. aureus* (Burts et al., 2009), *S.* Typhimurium (Fernández et al., 2012) and *S. cerevisae* (Lee et al., 2006). However, in the case of bacterial spores the results are not so clear. Indeed, while some authors did not find differences in NTAP effectiveness depending on the initial spore concentration for

different species of the genus B*acillus* (Purevdorj et al., 2001; Lee et al., 2006), in a recent study, Butscher et al. (2016) observed that when the initial load of *G. stearothermophilus* spores in wheat grains was reduced from 10^7 to 10^6 cfu/g the degree of inactivation achieved through a 10 min NTAP treatment increased from 1.02 to 1.82 log units.

Several authors have attempted to explain the loss of antimicrobial efficacy of NTAP on substrates with high microbial loads. Fernández et al. (2012) suggested that with high microbial loads the abundance of reactive species per cell would not be sufficient to cause their death. Even, these reactive species would react with already inactivated microorganisms or with their cellular components (e.g., proteins), which could act as sequestering agents (Kamgang-Youbi et al., 2008), thus protecting the remaining living cells. In this regard, Fernández et al. (2012) found that the addition of heat-inactivated cells significantly reduced NTAP effectiveness against *S.* Typhimurium. In addition, when high microbial loads are present, microorganisms are arranged in a stacked structure, in the form of multiple layers, with the upper microbial layers, even if already inactivated, constituting a physical barrier to the penetration of plasma, therefore protecting those microorganisms located in the inner layers. This distribution of microorganisms in layers has been observed through electron microscopy for *B. subtilis* spores (Deng et al., 2006), as well as through fluorescence microscopy for *S.* Typhimurium (Fernández et al., 2012). However, it should be taken into account that in all the studies mentioned above, contamination levels and microbial loads were much higher than those expected in naturally contaminated food.

1.3.3 Characteristics of the treatment medium or food

The most important factors related to the treatment medium properties determining microbial resistance to NTAP are the medium composition, in the case of liquids and liquid foods, and the topography and certain physical properties, in the case of solid foods.

For liquids, only those plasma-generated reactive species with a relatively long life, such as ozone, atomic oxygen, nitric oxide or hydrogen peroxide, would have the

capacity to diffuse through the medium and interact with microbial cells (Ziuzina et al., 2013). However, NTAP has been shown to be very effective in liquid media (Montenegro et al., 2002; Shi et al., 2011; Ziuzina et al., 2013; Surowsky et al., 2014), which has been attributed to the generation of new secondary reactive species through the interaction of the chemical species of plasma with each other or with water or other molecules present in the liquid or gaseous phase of the medium. For example, the coexistence of ozone and hydrogen peroxide in water results in highly reactive species such as hydroperoxides and hydroxyl radicals. In addition, atomic oxygen, upon reaction with water, can generate hydrogen peroxide and singlet oxygen (Surowsky et al., 2014; Takamatsu et al., 2015), and the interaction of nitric oxide and superoxide produces peroxynitrite, a highly reactive compound that can readily diffuse through cell membranes (Ercan et al., 2016).

Several authors have reported that NTAP treatment of water and other aqueous solutions causes an increase in both their electrical conductivity and oxidation-reduction potential (Tian et al. 2015; Xu et al., 2016). In addition, several studies have detected through various analytical techniques the presence of reactive oxygen species and reactive nitrogen species in plasma treated water (Ryu et al., 2013; Surowsky et al., 2014; Ercan et al., 2016). However, apart from the presence of these highly reactive species, the rapid acidification that NTAP treatment causes in non-buffered solutions, due to the dissociation of water and the formation of nitric acid and nitrous acid if nitrogen is available, is also believed to be responsible for the inactivation capacity of NTAP in liquid media. Several authors (Rowan et al., 2007; Chen et al., 2009; Naïtali et al., 2010; Ryu et al., 2013; Ma et al., 2015) have shown that the pH of plasma treated water decreases gradually with treatment time up to values close to pH 3.0. This same behavior has been observed in non-buffered saline solutions (Oehmigen et al., 2010; Ryu et al., 2013; Ziuzina et al., 2013), which would justify the greater antimicrobial effectiveness of NTAP in these media as compared to that observed in media containing substances with buffering capacity (Chen et al., 2009; Naïtali et al., 2010; Ryu et al., 2013; Ziuzina et al., 2013). However, several authors have observed that the level of microbial inactivation attained in NTAP treated media (reaching ~ pH 3.0) is much higher

than that observed for media acidified at pH 3.0 with different acidulants (Chen et al., 2009; Liu et al., 2010; Naïtali et al., 2010; Ercan et al., 2016). In order to explain this behavior, several authors have speculated about the possibility that low pH values could contribute to the stabilization of some of the reactive species generated in plasmas (Yost and Joshi, 2015) or to the formation of new compounds with antimicrobial potential (Naïtali et al., 2010). A possible synergistic effect of low pH and NTAP-derived reactive species on microbial inactivation has been also proposed (Oehmigen et al., 2010; Sun et al., 2012).

The influence of treatment medium composition on plasma effectiveness in liquid media has been scarcely studied, but some reports have shown that the presence of some salts, such as carbonates, phosphates and sodium chloride (Chen et al., 2009; Ryu et al. 2013), reduce the efficacy of NTAP treatments, through reacting with the chemically active species or due to their buffering capacity. The presence of organic matter can also influence the effectiveness of NTAP. Indeed, Rowan et al. (2007) observed a greater inactivation of various enteropathogenic microorganisms in poultry washing water than in distilled water, which was attributed to the formation of nitric acid and carbonic acid from fat and proteins present in the medium. On the other hand, the inactivation of molds and yeasts in complex media has been shown to be much less effective than in a saline solution or water (Ryu et al., 2013).

Regarding microbial inactivation on solid media, NTAP is a very efficient technology for surface decontamination of abiotic surfaces, such as stainless steels, various packaging materials, paper, glass and plastics of diverse nature (Muranyi et al., 2007; Leipold et al., 2010; Miao and Yun, 2011; Klämpfl et al., 2012; Patil et al., 2014; Butscher et al., 2016; Gabriel et al., 2016b). In addition, it is also highly effective for surface decontamination of a wide variety of foods, such as fruits, vegetables, spices, nuts, cereal grains, seeds, seaweed, meat and meat products, eggs, egg products, cheese slices and dried squid shreds (Table 2).

Table 2. Summary of major research articles assessing the level of microbial inactivation achieved and the quality changes occurring in foods subjected to non-thermal atmospheric plasma treatments.

Product	Gas	Maximal log reduction	Impact on quality attributes	Reference
Meat and meat products				
Pork loin	He or mixtures He+O_2; 10 slpm; 3 kV; 30 kHz; 3 mm distance	*Listeria monocytogenes* ~ 0.59 log *Escherichia coli* ~ 0.55 log, in 10 min	Loss of lightness. Significant reductions in sensory quality parameters (appearance, color, odor, acceptability).	Kim et al. (2013)
Pork	Mixtures of N_2 and O_2; 15 kHz; 2 W	*Listeria monocytogenes* ~ 2.0 log *Escherichia coli* O157:H7 ~ 2.5 log *Salmonella* Typhimurium ~ 2.7 log, in 10 min	Minor changes in color and taste. No effects in texture.	Jayasena et al. (2015)
Frozen and unfrozen pork	Air; 2.5 m/s; 20 kV; 58 kHz; 25 mm distance	*Listeria monocytogenes* ~ 1.0 log *Escherichia coli* O157:H7 ~ 1.5 log, in 2 min	Only significant impact on the sensory characteristics (color and appearance) of unfrozen pork	Choi et al. (2016)
Beef	N_2+O_2; 15 kHz; 2 W	*Listeria monocytogenes* ~ 1.9 log *Escherichia coli* O157:H7 ~ 2.6 log *Salmonella* Typhimurium ~ 2.6 log, in 10 min	Minor changes in color and taste. No effects in texture.	Jayasena et al. (2015)
Chicken meat and skin	He (5 L/min)+O_2 (100 mL/min); 16 kV; 30 kHz; 1 cm distance	*Listeria innocua* ~ 1.0 log on skin, in 8 min > 3.0 log on meat, in 4 min	Not assessed	Noriega et al. (2011)
Chicken breast and thigh	Air	*Salmonella enterica* ~ 2.5 log *Campylobacter jejuni* ~ 2.4 log, in 3 min	Not assessed	Dirks et al. (2012)
Beef jerky	Ar; 20,000 sccm; 200 W; 1 cm distance	*Staphycococcus aureus* ~ 1.8 log, in 8 min	No changes in texture and color	Kim et al. (2014)

(Continued)

Table 2. Summary of major research articles assessing the level of microbial inactivation achieved and the quality changes occurring in foods subjected to non-thermal atmospheric plasma treatments. Continued

Product	Gas	Maximal log reduction	Impact on quality attributes	Reference
Beef jerky	Air; 15 kHz	*Listeria monocytogenes* ~ 2.4 log *Escherichia coli* O157:H7 ~ 2.7 log *Salmonella* Typhimurium ~ 3.0 log *Aspergillus flavus* ~ 3.2 log, in 10 min	No significant changes in metmyoglobin content, shear force, and myofibrillar fragmentation index; changes in peroxides content and color parameters; negative effects on flavor, off-odor, and overall acceptability	Yong et al. (2017)
Cooked chicken breast	N_2 (7 L/min)+O_2 (0.07 L/min); 2 kV; 50 kHz; 4 cm distance	*Listeria monocytogenes* ~ 4.7 log, in 2 min	Not assessed	Lee et al. (2011)
Cooked ham	N_2 (7 L/min)+O_2 (0.07 L/min); 2 kV; 50 kHz; 4 cm distance	*Listeria monocytogenes* ~ 6.5 log, in 2 min	Not assessed	Lee et al. (2011)
Ham	He; 13.56 MHz; 150 W; 0,6 mm distance	*Listeria monocytogenes* ~ 1.7 log, in 2 min	Not assessed	Song et al. (2009)
Bacon	He (10 L/min)+O_2 (10 sccm); 13,56 MHz; 125 W; 3 mm distance	*Listeria monocytogenes* ~ 2.6 log *Escherichia coli* ~ 3,0 log *Salmonella* Typhimurium ~ 1.0 log, Total aerobic bacteria ~ 4.6 log, in 1,5 min	No physical damage on surface tissues. Higher lightness. Lipid oxidation was not observed	Kim et al. (2011)
Bresaola	Ar (70%)+O_2(30%); 27.8 kV; 15.5 W	*Listeria innocua* > 1.5 log, in 10 sec	Loss of redness, Induced lipid oxidation.	Rød et al. (2012)
Dairy products				
Cheese	He; 13.56 MHz; 150 W; 0,6 mm distance	*Listeria monocytogenes* ~ 8.5 log, in 2 min	Not assessed	Song et al. (2009)

(Continued)

Table 2. Summary of major research articles assessing the level of microbial inactivation achieved and the quality changes occurring in foods subjected to non-thermal atmospheric plasma treatments. Continued

Product	Gas	Maximal log reduction	Impact on quality attributes	Reference
Cheese	Air; 15 kHz; 250 W	*Escherichia coli* ~ 2.7 log, in 1 min *Salmonella* Typhimurium ~ 3.1 log, in 45 s	Not assessed	Yong et al. (2015a)
Cheddar cheese	Air; 15 kHz; 2 W	*Listeria monocytogenes* ~ 5.8 log *Escherichia coli* O157:H7 ~ 3.6 log *Salmonella* Typhimurium ~ 2.1 log, in 10 min	No changes in color and sensory appearance. Significant reductions in flavor and overall acceptance. Increased off-odor	Yong et al. (2015b)
Eggs				
Cooked egg white	N_2 (7 L/min)+O_2 (0.07 L/min); 2 kV; 50 kHz; 4 cm distance	*Listeria monocytogenes* ~ 6.7 log Total aerobic bacteria ~ 2.9 log, in 2 min	Loss of lightness. No impact on sensory attributes (color, flavor, texture, taste, acceptability).	Lee et al. (2012b)
Cooked egg yolk	N_2 (7 L/min)+O_2 (0.07 L/min); 2 kV; 50 kHz; 4 cm distance	*Listeria monocytogenes* ~ 7.1 log Total aerobic bacteria ~ 2.3 log, in 2 min	Loss of lightness. Significant reductions in flavor, taste and overall acceptability	Lee et al. (2012b)
Fish and seafood				
Dried squid shreds	Air; 2 L/s; 20 kV; 58 kHz; 25 mm distance	*Staphylococcus aureus* ~ 0.9 log Marine bacteria ~ 1.6 log Aerobic bacteria ~ 2.0 log Yeast and molds ~ 0.9 log, in 3 min	No significant changes in color characteristics and volatile basic nitrogen content; moisture and thiobarbituric acid reactive substances levels were altered; no significant impact on sensory characteristics (color, flavor, taste, texture, acceptability, overall acceptance)	Choi et al. (2017)

(Continued)

Table 2. Summary of major research articles assessing the level of microbial inactivation achieved and the quality changes occurring in foods subjected to non-thermal atmospheric plasma treatments. Continued

Product	Gas	Maximal log reduction	Impact on quality attributes	Reference
Fruit, cereals and vegetables				
Blueberries	Air; 4 cfm; 47 KHz; 549 W; 7.5 cm distance	Total aerobic count ~ 0.8 log Yeast and molds ~ 1.2 log, in 2 min	Significant reductions in firmness and anthocyanins; surface color was significantly impacted	Lacombe et al. (2015)
Strawberries	Air; 70 kV; 50 Hz; 140-160 mm distance	*Listeria monocytogenes* ~ 4.2 log *Escherichia coli* O157:H7 ~ 3.5 log *Salmonella* Typhimurium ~ 3.8 log, in 5 min	Not assessed	Ziuzina et al. (2014)
Pears	Air; 5 slm; 500 V	*Salmonella* spp. ~ 0.2 log, in 0.5 sec	Changes in physiochemical properties were within an acceptable range	Wang et al. (2012)
Apples	Ar (5 L/min)+O_2 (0.1%); 10 kV; 8 W; 17 mm distance	*Escherichia coli* ~ 4.7 log, in 1 min	Not assessed	Baier et al. (2014)
Cherry tomatoes	Air; 70 kV; 50 Hz; 140-160 mm distance	*Listeria monocytogenes* ~ 6.7 log, in 2 min *Escherichia coli* ~ 3.1 log, in 1 min *Salmonella* Typhimurium ~ 6.3 log, in 10 sec Aerobic mesophilic bacteria ~ 4.0 log, in 2 min Yeast and molds ~ 5.0 log, in 2 min	Not assessed	Ziuzina et al. (2014)
Tomato	Ar (5 L/min)+O_2 (0.1%); 10 kV; 8 W; 17 mm distance	*Escherichia coli* ~ 3.3 log, in 1 min	Not assessed	Baier et al. (2014)

(Continued)

Table 2. Summary of major research articles assessing the level of microbial inactivation achieved and the quality changes occurring in foods subjected to non-thermal atmospheric plasma treatments. Continued

Product	Gas	Maximal log reduction	Impact on quality attributes	Reference
Corn salad	Ar (5 L/min)+O_2 (0.1%); 10 kV; 8 W; 17 mm distance	*Escherichia coli* ~ 4.1 log, in 1 min	No changes in color were observed	Baier et al. (2014)
Cucumber	Air; 5 slm; 500 V	*Salmonella* spp. ~ 0.4 log, in 0.5 sec	Changes in physiochemical properties were within an acceptable range	Wang et al. (2012)
	Ar (5 L/min)+O_2 (0.1%); 10 kV; 8 W; 17 mm distance	*Escherichia coli* ~ 4.7 log, in 1 min	Not assessed	Baier et al. (2014)
Carrots	Air; 5 slm; 500 V	*Salmonella* spp. ~ 1.0 log, in 0.5 sec	Changes in physiochemical properties were within an acceptable range	Wang et al. (2012)
	Air (20 L/min); 2.45 Ghz; 1.2 kW	*Escherichia coli* ~ 4.5 log Mesophilic aerobic counts ~ 3.5 log, in 2 min	Significant effects on color. No impact on elastic properties	Baier et al. (2015)
Lettuce	Air; 4.5 com distance	*Aeromonas hydrophila* ~ 5 log, in 15 sec	Not assessed	Jahid et al. (2014)
Rapeseed seeds	Air (2 L/min); 20 kV; 58 kHz; 25 mm distance	*Escherichia coli* ~ 2.0 log *Bacillus cereus* ~ 1.2 log *Salmonella* spp. ~ 1.8 log Total aerobic bacteria ~ 2.2 log Yeast and molds ~ 2.0 log, in 3 min	Physicochemical (weight, length, moisture content) and sensory characteristics (appearance, color, flavor, taste, texture) were unaffected.	Puligundla et al. (2017)
Orange juice	Air; 20 kV; 60 kHz; 1.14 W/cm^2; batch	*Escherichia coli* ~ 5.0 log, in 8 sec *Staphylococcus aureus* ~ 5.0 log, in 12 sec *Candida albicans* ~ 5.0 log, in 25 sec	Almost no effect on vitamin C content, pH, turbidity or ºBrix	Shi et al. (2011)

(Continued)

Table 2. Summary of major research articles assessing the level of microbial inactivation achieved and the quality changes occurring in foods subjected to non-thermal atmospheric plasma treatments. Continued

Product	Gas	Maximal log reduction	Impact on quality attributes	Reference
Black peppercorns	Air (20 L/min) +Ar (14 L/min); 4 cm distance	*Salmonella* ~ 5.0 log, in 80 sec	Minimal changes in color	Sun et al. (2014)
Almonds	Air; 30 kV; 2 kHz	*Escherichia coli* ~ 5 log, in 30 sec	Not assessed	Deng et al. (2007)
	Air ; 47 kHz; 549 W; 6 cm distance	*Escherichia coli* O157:H7 ~ 1.34 log, in 20 sec	No gross changes in color, aroma and surface features	Niemira (2012)
Wheat grains	Ar (28 L/min); 8 kV; 10 kHz	*Geobacillus stearothermophillus* spores ~ 3.0 log, in 60 min	Functional wheat grain properties (falling number, gluten content) were not negatively affected.	Butscher et al. (2016)

slpm, standard liters per minute; sccm, standard cubic centimeters per minute; cfm, cubit feet meter.

However, microbial survival on different solids is conditioned by surface characteristics, which will determine its possible heating during NTAP treatment, the degree of microbial adhesion, as well as the level of formation and/or adsorption of active species. In particular, surface roughness, porosity and topography are of special relevance (Song et al., 2009; Noriega et al., 2011). A large number of studies have demonstrated a great variability in NTAP effectiveness against both vegetative cells and bacterial spores when identical treatments were applied onto different surfaces (Yun et al., 2010; Noriega et al., 2011; Yong et al., 2015a; Butscher et al., 2016). For example, Miao and Yun (2011) studied the inactivation of *E. coli* on PET (polyethylene terephthalate), PVC (polytetrafluoroethylene) and PTFE (polytetrafluoroethylene -teflon), and obtained the greatest lethal effect on PET, while the lowest inactivation was found on PTFE. These authors attributed the observed differences to differences in surface wettability of the materials, which was higher for PET, followed by PVC and, finally, PTFE. Fernández et al. (2013) obtained 2.7 log reductions in *S.* Typhimurium viability on polycarbonate filters after a 2 minutes NTAP treatment, while it took 15 minutes to reach 2.7; 1.8 and 0.9 log cycles of inactivation on lettuce, strawberries and cut potato, respectively. Similarly, Lee et al. (2011) and Butscher et al. (2016), comparing the effectiveness of NTAP for the inactivation of *L. monocytogenes* and *G. stearothermophilus* on abiotic surfaces and foods, observed that the highest antimicrobial activities were obtained on abiotic surfaces, such as agar plates or polypropylene grains. In addition, it has also been demonstrated that the effectiveness of NTAP as a food decontamination technique varies among different foods. Thus, inactivation of *E. coli* was more effective on tomato than on lettuce (Bermudez-Aguirre et al., 2013) or strawberries (Ziuzina et al., 2014), also being faster on carrots than on apples (Baier et al., 2015). For *L. monocytogenes* a greater lethal effect was also observed on cheese slices than on ham (Song et al., 2009), on sliced ham than on chicken breast fillets (Lee et al., 2011), or on tomato than on strawberries (Ziuzina et al., 2014). These results show that food topography should be taken into account when designing effective NTAP treatments. Additionally, differences can be also attributed to the fact that bacteria could have been in the form of planktonic cultures on the surface, biofilms or internalized in the food tissues, and it is well known

that this can show a very important influence on the inactivation effectiveness of NTAP (Ziuzina et al., 2015b; Berardinelli et al., 2016).

Overall, there is a unanimous agreement in that on smooth and polished surfaces the anti-microbial efficiency of NTAP is very high (Noriega et al., 2011; Bermudez-Aguirre et al., 2013; Fernández et al., 2013; Kim et al., 2014; Butscher et al., 2016; Cui et al., 2016a), whereas rough, porous and irregular surfaces, such as those of some foods, offer numerous places for microorganisms to fix and hide, thus avoiding the action of plasma (Fernández et al., 2012; Bermudez-Aguirre et al., 2013; Butscher et al., 2016; Cui et al., 2016a). In fact, some studies using electron microscopy have shown that inoculated microorganisms are capable of finding shelter within various food irregularities, such as cracks, grooves or gaps (Fernández et al., 2013; Jahid et al., 2014; Ziuzina et al., 2014; Cui et al., 2016a).

1.4 Applications of NTAP in the food industry

Once the mechanisms of microbial inactivation by NTAP and the factors that determine its lethal efficacy have been described, the following section of the review article will discuss the potential of this novel technology for specific applications within food processing industries.

1.4.1 NTAP as a decontamination technique

NTAP can be used as a decontamination technique for foods, packaging materials, equipment, and even the processing environment itself.

In relation to surface decontamination of foods, several studies have shown the potential of NTAP to improve the microbiological quality of a wide range of solid foods, including strawberries, tomatoes, chicken breast fillets, ham, cheese slices, carrots, melon, or lettuce, and liquid foods, such as milk, apple and orange juices, and coconut liquid endosperm (Table 2). Although microbial inactivation rates obtained widely vary among studies, surfaces and microbial species tested, promising results have been

reported. For example, with 2-minute treatments, 4 to 8 logarithmic reductions were obtained for *L. monocytogenes* in tomatoes, cooked chicken breast fillets and ham, cooked egg white and egg yolk, and cheese slices (Song et al., 2009; Lee et al., 2011; Lee et al., 2012b; Ziuzina et al., 2014), and 4.5 log units for *E. coli* in carrots (Baier et al., 2015a). Even treatments as short as 10 and 15 seconds have been able to reduce the population of *A. hydrophila* in lettuce in 5 log cycles (Jahid et al., 2014) and that of *S.* Typhimurium in tomatoes in 6 log units (Ziuzina et al. 2014). However, most studies have not evaluated the impact of NTAP treatments on the nutritional and sensory properties of such foods, despite the fact that plasma-generated reactive species could lead to organoleptic changes in the end product. Moreover, information available in the literature is very variable, with some authors identifying minor changes in color, texture, appearance, taste or aroma of foods, while others have reported important modifications in sensory attributes after NTAP treatment (Table 2). The observed discrepancies are probably due to the variability in equipment, plasma generation conditions or type of food used in validation studies. Indeed, several authors have shown how these factors influence the maintenance of food sensory attributes. For example, the application of similar NTAP treatments significantly modified the color of carrots (Baier et al., 2015) and the taste of cooked egg yolk (Lee et al., 2012b), but did not affect these quality attributes in apples and cooked egg white, respectively. Moreover, NTAP treatments using helium as working gas caused changes in pork loin color, whereas this quality attribute was not modified in treatments using a mixture of helium and oxygen (Kim et al., 2013). Baier et al. (2014) also reported changes in leaf lettuce color after a NTAP treatment, which only occurred when the distance between food and plasma generation source was low (5 mm). Furthermore, Rod et al. (2012) described that an increase in potency, treatment time and storage time was accompanied by an increased lipid oxidation and rancidity of bresaola. Similarly, a NTAP treatment of 5 to 10 minutes has been shown to induce lipid oxidation in cheese samples (Yong et al., 2015b). However, no significant changes in the fatty acid composition of beef jerky were detected after exposure to plasma for 5 minutes (Kim et al., 2014).

Although the vast majority of studies have focused on evaluating the potential of NTAP for the decontamination of solid foods, some authors have addressed its efficacy in liquid foods, obtaining also on some occasions promising results. Thus, up to 5 log reductions have been obtained for *E. coli* in orange and apple juice after a treatment of 8 and 40 seconds, respectively, and treatments of 12 and 25 seconds had a similar lethality effect for *S. aureus* and *Candida albicans*, respectively (Shi et al., 2011; Liao et al., 2018).

NTAP can be also used for the decontamination of food packaging materials. Currently, the most widely used methods with this aim are based on the employment of dry or wet heat or chemical agents, such as peracetic acid or hydrogen peroxide, which can be applied at relatively high temperatures, ranging from 65 to 80 °C, to increase their effectiveness. However, most polymers used as packaging materials do not withstand high temperatures. In addition, some chemical agents also present other drawbacks, such as their difficult handling, maximum limits allowed and the possible generation of toxic waste substances. NTAP is an attractive alternative to chemical decontamination agents, due to its effectiveness as a decontamination technique which successfully inactivates microorganisms, including bacterial and fungal spores, on abiotic surfaces (Muranyi et al., 2007, 2008, 2010; Klämpfl et al., 2012). For example, Muranyi et al. (2007), assessing the efficacy of NTAP for the inactivation of vegetative cells (*E. coli, S. aureus*, *S.* Mons and *D. radiodurans*), bacterial spores (*B. atrophaeus, B. pumilus, C. botulinum, C. sporogenes*) and fungi spores (*A. niger*) on PET, achieved, with treatment times as short as 1 second, between 5.6 and 6.9 log reductions for vegetative cells, between 5.1 and 6.1 log reductions for bacterial spores and 3 log reductions for *A. niger* conidiospores.

NTAP can even be used for the decontamination of food once packaged, using dielectric barrier discharge equipment (Fröhling et al., 2012a; Rød et al., 2012; Ziuzina et al., 2014; Jayasena et al., 2015). The reactive species generated in this case would simultaneously decontaminate both the packaging material and the food itself. Moreover, once the treatment concludes, the plasma species recombine, the

atmosphere returns to its initial composition, and recontamination of the end product after NTAP treatment is thus avoided. The use of NTAP for the decontamination of food once packaged, has been successfully studied in cherry tomatoes, strawberries, bresaola and pork and beef meat by several research groups (Fröhling et al., 2012a; Rød et al., 2012; Ziuzina et al., 2014; Jayasena et al., 2015).

Another potential application of NTAP in the food industry is the decontamination of food processing surfaces and equipment. In fact, a novel plasma system has been designed to decontaminate slicers, in which the cutting blade of the equipment constitutes one of the electrodes of the dielectric discharge system (Leipold et al., 2010). These authors obtained an effective inactivation of *L. innocua* inoculated on the cutting blade, representing a novel approach for decontaminating food processing equipment which could be applied to other surfaces, such as conveyors.

Recently, several authors have described the fact that water treated by NTAP, so-called plasma-activated water (PAW), has relevant antimicrobial activity, which persist over a long period of time (Kamgang-Youbi et al., 2009; Ercan et al., 2013; Zhang et al., 2013). This offers new possibilities for decontamination of both surfaces and food, through the treatment of water by NTAP, which will be then used for decontamination purposes. This approach may be a promising alternative to the chemical agents currently used for the sanitation of surfaces, equipment and minimally processed vegetables. In fact, the potential of plasma-activated water has recently been revealed for the decontamination of strawberries (Ma et al., 2015) and mushrooms (Xu et al., 2016). Indeed, a bactericidal effect similar to that obtained using sodium hypochlorite solutions (Issa-Zacharia et al., 2010), with no changes in color or firmness, was observed for strawberries treated with plasma-activated water, with log reductions for *S. aureus* ranging from 1.7 to 2.3 log depending on the exposure time (Ma et al., 2015). Log reductions achieved following a similar approach for treated mushrooms were between 1.5 and 0.7 log after a week of storage, and mushrooms maintained their initial quality attributes (Xu et al., 2016).

1.4.2 Other applications of NTAP in food processing

Apart from being used as a decontamination technique, NTAP may also represent an alternative food processing method which can be used, for example, to obtain the characteristic color of cured meat products without adding nitrites, extend the shelf-life of oils used for coating cookies, reduce the cooking time of cereals, improve the extraction of essential oils or modify the functional properties of flours.

As already mentioned, the interaction of plasma with water results in the generation of reactive oxygen and nitrogen species, including nitrates and nitrites (Oehmigen et al., 2010), reaching concentrations of up to 1,050 ppm (Hao et al., 2014). The generation of nitrites during plasma treatment may raise a flag since use of nitrites in food is restricted, with maximum allowed concentrations, due to their potential toxicological effects. However, Jung et al. (2015) manufactured frankfurters by replacing the nitrites of the curing salts by plasma-treated water and did not detect significant differences in their microbiological or organoleptic quality during 28 days of storage at refrigeration. Interestingly, the residual nitrite content was 30% lower in sausages processed with plasma-treated water. Similar results have been also obtained by directly treating the meat butter with NTAP at different stages of the sausage making process, and a patent for NTAP as a system to eliminate nitrites in meat products has been recently filed (Lim et al., 2015).

NTAP can be used to modify the surface properties of various materials, such as paper, polymers or electronic equipment, to confer them certain functional properties of interest. The idea of using this technology to achieve surface modifications in foods is innovative. NTAP has been used to modulate hydrophobicity or hydrophilicity of oil coatings on cookies, in order to improve palatability and appearance (Misra et al., 2014). These authors demonstrated that NTAP increased surface hydrophobicity, resulting in a greater extension of the incorporated oil and in a more accurate infiltration of oil, without affecting the color, odor and appearance of cookies. Thus, it allowed the preservation of the functionality of the sprayed oil, also decreasing the amount of oil required, which is interesting from a nutritional and economic point of view. A

modification of surface properties after NTAP treatments has also been observed for basmati rice (Thirumdas et al., 2015). In this case, NTAP increased the hydrophilicity and water absorption rate of the cereal, and decreased the cooking time, from 20 to 13 minutes. Electron microscopy images of the treated rice grains revealed the presence of cracks and depressions on their surface, which probably provide routes for faster absorption of water. Although this experiment was carried out under vacuum conditions, which does not allow a direct extrapolation of the results, similar results are expected at atmospheric pressure conditions. Similarly, NTAP is currently being also proposed as a promising technology for the physico-chemical modification of processing surfaces, such as those of food-contact materials, in a way to prevent microbial adhesion and formation of biofilms, therefore limiting episodes of food cross-contamination with persistent microorganisms colonizing food processing environments. These surface modification techniques are sometimes focused at changing electronegativity, hydrophobicity or morphology/topography of surfaces (Bazaka et al., 2015).

Another possible application of NTAP is its use to improve those processes that involve mass transfer. Indeed, exposure of lemon skins to plasma has been shown to increase the yield of extracted essential oils (Kodama et al., 2014). However, these results should be taken with caution, since essential oils can be oxidized to a certain extent, depending on the gas used for NTAP generation.

Finally, NTAP has been also evaluated as a methodology capable of inducing modifications in the functional properties of proteins. Misra et al. (2015) showed that the secondary structure of gluten became more stable when wheat flour had been treated with an air plasma, and observed significant changes in the rheological properties of the doughs obtained. These changes, both in viscosity and elasticity, depended on the treatment conditions, applied voltage and exposure time. Thus, NTAP can be used as an innovative strategy in order to modulate the functionality of wheat flour during processing of bread, pasta, noodles, cookies, and others.

1.5 Conclusions and future prospects

NTAP is a promising food decontamination technology capable of inactivating bacteria, yeasts, molds, fungal and bacterial spores both on abiotic surfaces (e.g., packaging materials, food processing environments and equipment) and on foods. In addition, NTAP has also other different innovative applications of great interest for food quality and safety improvement.

Although the exact mechanism of microbial inactivation by NTAP is not completely known yet, the cellular envelopes, DNA and proteins are recognized as potential targets, and damages produced to them are the result of the simultaneous action of the different components and reactive species occurring in plasma when the microbial detoxifying capacity is overcome, resulting in the accumulation of dysfunctional macromolecules that compromise cellular viability. The multi-target nature of NTAP could justify the great antimicrobial effectiveness of NTAP in comparison with other technologies that affect a single component or cellular structure. The relative contribution of each individual damage to the total lethal effect of NTAP is unknown yet, since, on the one hand, the composition of plasmas may be very different depending on the conditions of production and, on the other hand, the magnitude of the damage could be in turn dependent on the type of microorganism and the time of treatment (Muranyi et al., 2010; Fröhling et al., 2012b; Tseng et al., 2012; Lu et al., 2013 Han et al., 2016).

Inadequate reporting of experimental methodologies may hinder independent verification and validation of results among different laboratories and equipment units. It is therefore advisable to establish a set of recommended guidelines for conducting and reporting NTAP experiments which would help improve the reliability of future experimental data, thus facilitating process optimization for industrial implementation. Some suggestions on this regard are provided in Table 3.

Table 3. Suggested information to be provided in research studies related to non-thermal atmospheric plasma

Category	Variable and minimum description to be included
Process equipment	Type of equipment, type of discharge and electrode configuration
	Equipment model and vendor name
	Distance between the point of plasma generation and the sample
Processing conditions	Type of gas
	Gas flow rate and gas moisture content
	Energy supplied: voltage, power and frequency
	Processing time
	Composition of the generated plasma
Food or sample	Type of food or sample: detailed description
	Water activity, pH and composition of the sample
	Time – temperature history
Microbial factors	Genus, species and strain(s) used
	Initial microbial load
	Pre-history of the microbial inoculum
	Description of the procedure followed for preparing the inoculum and enumerating microorganisms

However, the industrial implementation of NTAP with these aims still requires substantial research efforts, which should be especially focused at (i) assessing the impact that NTAP shows on the nutritional and sensory quality of treated foods in order to facilitate the design of preservation regimes capable of inhibiting pathogenic and spoilage microorganisms while maintaining food quality attributes; (ii) identifying the plasma components and reactive species responsible for the antimicrobial activity of NTAP, which will allow a better selection of processing conditions; (iii) confirming the lack of toxicity of the chemical species generated during NTAP treatments; (iv) developing combined preservation treatments, within a hurdles technology concept, where other inactivation approaches showing a synergic effect when applied together with NTAP will be used; (v) and designing fit-for-purpose equipment susceptible to be

easily adopted in a processing line, being compact, energetically efficient, and cost-effective.

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2

Justificación y objetivos

Las nuevas tendencias de consumo de alimentos han incrementado la demanda de productos de elevada calidad nutritiva y sensorial, con características similares a las del producto fresco y libres de aditivos, lo que ha llevado, en los últimos años, al desarrollo de nuevas tecnologías no térmicas de conservación, con capacidad de inactivar microorganismos y enzimas presentes en los alimentos, pero sin alterar sensiblemente sus propiedades organolépticas, nutritivas y funcionales. Entre ellas, han adquirido especial relevancia las altas presiones hidrostáticas, los pulsos eléctricos de alto voltaje, los ultrasonidos, los pulsos de luz, los campos magnéticos oscilantes y, más recientemente, el plasma atmosférico no térmico (PANT).

El PANT se genera mediante la aplicación de una descarga eléctrica a un gas, lo que provoca fenómenos de ionización, disociación y excitación de sus átomos y moléculas. El PANT está por tanto constituido por electrones e iones positivos y negativos, radicales libres, átomos y moléculas en estado o no de excitación y fotones ultravioleta, que, al interaccionar con los microorganismos, incluyendo esporos, provoca su inactivación, al inducir lesiones importantes en sus envolturas celulares, ADN y proteínas.

Además de una elevada capacidad antimicrobiana, el PANT presenta, en comparación con otras estrategias de conservación de alimentos, una serie de ventajas muy importantes, como son el bajo coste de aplicación, la efectividad frente a esporos bacterianos y fúngicos a temperatura ambiente, el empleo de tiempos de tratamiento cortos, la posibilidad de tratar una amplia variedad de alimentos, incluso previamente envasados, y el ser una técnica medioambientalmente sostenible, por lo que se considera, en este momento, como una tecnología sumamente prometedora para mejorar la calidad microbiológica de los alimentos.

Sin embargo, la implementación industrial del PANT como una alternativa segura y eficaz a los métodos de conservación de alimentos actualmente utilizados requiere disponer no sólo de datos precisos acerca del grado, características y biovariabilidad de la resistencia de los microorganismos frente a esta tecnología, sino también identificar aquellos factores que condicionan su efectividad antimicrobiana con el fin de establecer

tratamientos que permitan maximizar su efecto letal limitando las repercusiones indeseables sobre las características de los alimentos. Además, el conocimiento de los mecanismos involucrados en la inactivación microbiana por acción del PANT permitirá desarrollar regímenes de tratamiento más eficientes, mediante el diseño de estrategias basadas en la tecnología de procesos combinados.

Esta Tesis Doctoral persigue evaluar la potencialidad del PANT como sistema alternativo de higienización de los alimentos. Aborda el estudio de su efectividad en la inactivación de algunos de los microorganismos patógenos de transmisión alimentaria más importantes: *Salmonella enterica* serovariedad Enteritidis, *Salmonella enterica* serovariedad Typhimurium, *Listeria monocytogenes* y *Escherichia coli* O157:H7. Además, investiga las bases moleculares del efecto letal del PANT.

En concreto, los objetivos específicos planteados en esta Tesis Doctoral son:

- Determinar la cinética de inactivación microbiana por acción del PANT.
- Estudiar la influencia de la composición y velocidad del gas de trabajo sobre la efectividad antibacteriana del PANT.
- Determinar el efecto de las condiciones de crecimiento (temperatura, pH y tipo de agente acidificante) de los microorganismos, y la exposición a diversos agentes estresantes (tratamiento térmico moderado, acidez y frío) sobre la eficacia letal del proceso.
- Evaluar la influencia de la matriz alimentaria en la resistencia microbiana frente a esta tecnología no térmica de conservación de alimentos.
- Caracterizar los mecanismos de inactivación microbiana por PANT, analizando los daños ocasionados en la composición y ultraestructura celular.

3

Resultados

Capítulo 1
Influence of processing parameters and stress adaptation on the inactivation of *Listeria monocytogenes* by Non-Thermal Atmospheric Plasma (NTAP)

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Abstract

This study evaluated the effectiveness of Non-Thermal Atmospheric Plasma (NTAP) treatments against *Listeria*. Firstly, the impact of gas composition and flow rate on *L. monocytogenes* and *L. innocua* (used as a surrogate) inactivation by NTAP was monitored. Secondly, the influence of stress adaptation (growth under suboptimal conditions, using a wide range of temperatures and media acidified up to pH 5.5 with citric, lactic, malic or hydrochloric acid, or short-term exposure to acid, cold or thermal shocks) on *L. monocytogenes* NTAP resistance was assessed. Survival curves obtained were concave upward. A mathematical model based on the Weibull distribution accurately described the inactivation kinetics. Both *L. monocytogenes* and *L. innocua* showed a higher sensitivity to plasma when the treatment was performed using air than when nitrogen was used. In fact, the use of nitrogen as working gas made the plasma treatment almost ineffective. The effect of gas flow rate on the effectiveness of the NTAP treatment depended on the type of gas used to generate plasma. Increases in flow rate from 5 to 10 L/min caused an acceleration of bacterial inactivation when air was used, while an additional increase of gas flow from 10 to 15 L/min had a minor impact on microbial inactivation. On the other hand, gas flow rate hardly affected NTAP treatment efficiency when nitrogen was used to generate plasma. *L. monocytogenes* growth under sub-optimal temperature or pH conditions or short-term exposure to acid, heat or cold stress conditions did not significantly modify its NTAP resistance. This suggests that temperature and pH stress adaptation does not induce a cross-protection response against NTAP treatments in *L. monocytogenes*, what makes NTAP an attractive technology for food decontamination within minimal processing strategies targeting this pathogenic microorganism

Introduction

Listeria monocytogenes is a Gram-positive food-borne pathogen, which causes sporadic and large outbreaks of human illness worldwide, with a mortality rate of about 30% in populations at risk, such as the elderly, pregnant women and immunocompromised individuals (Goulet et al. 2012). In 2014, 2,161 confirmed cases of listeriosis were reported in Europe, with a 15% of case fatalities. In the EU a statistically significant increasing trend of listeriosis has been observed in the 2008-2014 period and the highest notification rates during 2014 were observed in Denmark, Sweden, Finland and Spain (1.64, 1.30, 1.19 and 1.15 cases per 100,000 population, respectively) (EFSA, 2015). In the USA authorities reported an incidence of 0.26 cases per 100,000 population during 2013 (CDC, 2014).

Due to the highly adaptive physiology of *L. monocytogenes*, some hurdles used in the food industry during food processing and preservation to prevent bacterial growth in foods may not be sufficient to inhibit this pathogenic microorganism. In addition, *L. monocytogenes* is able to persist in food processing environments for long time periods attached to many different surfaces where it can form biofilms, in which cells become less susceptible to sanitizers and other antimicrobial agents (Moltz and Martin, 2005; Pan et al., 2006). *L. monocytogenes* has been frequently isolated from a wide variety of raw and processed foods, including ready-to-eat (RTE) foods. Indeed, a number of surveys of this microorganism in foods and food processing environments performed in recent years have revealed a variable prevalence of up to around a 20% (Lack et al., 1996; Leong et al., 2014; Jordan et al., 2015). Occurrence of *L. monocytogenes* in RTE products is a great public health threat, since these products are not subjected to a heat-killing step before consumption. Moreover, it constitutes an impediment for exportation of products to third countries applying a “zero tolerance” policy on *L. monocytogenes*, such as USA (Jordan et al., 2015).

Non-Thermal Atmospheric Plasma (NTAP) is an emerging and promising food decontamination and preservation technology. NTAP is a partially ionized gas,

generated by excitation of gas by electric discharges, which consists of ions, electrons, UV photons and reactive species, such as radicals, excited atoms and molecules, which are able to interact with microorganisms and damage them in a rapid and effective manner (Fernández and Thompson, 2012). As an antimicrobial strategy, NTAP offers many interesting advantages over more conventional methods in the food-processing sector, such as simpler design, lower process operational costs, shorter treatment times at near ambient temperature, lack of toxic effects and significant reductions of water consumption (reviewed by Guo et al., 2015).

NTAP has been described as an effective technology for the inactivation of *L. monocytogenes* in different foods, including RTE foods, with a minimum impact on their nutritional and sensory attributes. Indeed, inactivation of *L. monocytogenes* after NTAP treatments has been demonstrated on lettuce (Critzier et al., 2007), slices of cheese (Song et al., 2009; Yong et al., 2015), meat products such as ham, bacon, chicken breast, pork-butt and beef loin (Song et al., 2009; Kim et al., 2011; Lee et al., 2011; Jayasena et al., 2015), dragon fruit (Matan et al., 2015), and strawberries and tomatoes (Ziuzina et al., 2014), among others.

The direct comparison of NTAP inactivation data among studies is challenging due to the use of different plasma sources and the high variability in technical and experimental parameters. Nevertheless, some general conclusions can be drawn from the information available in the literature. The lethal effectiveness of NTAP treatments on *L. monocytogenes* depends on technological parameters, such as gas composition, input power/supply voltage and treatment time (Song et al., 2009; Yun et al., 2010; Kim et al., 2011; Lee et al., 2011; Kim et al., 2013; Gabriel et al., 2016), as well as on food intrinsic properties, including surface characteristics (Critzer et al., 2007; Song et al., 2009; Lee et al., 2011; Ziuzina et al., 2014; Smet et al., 2016). In general, available information shows that *L. monocytogenes* inactivation increases with an increase in energy and treatment time and decreases with an increase in complexity of food superficial characteristics. Regarding gas composition, oxygen presence seems to increase NTAP effectiveness. Indeed, Lu et al. (2014) have recently reported that the use

of a high oxygen gas mix, instead of a high nitrogen gas mix, resulted in a greater inactivation of *L. monocytogenes*.

No information is available yet on the effect of adaptation to stress conditions (including pH and temperature) on *L. monocytogenes* NTAP resistance, in spite of the fact that pH and temperature are by far the hurdles most widely applied by the food industry to control microbial growth. Nonetheless, it is well known that during growth under suboptimal conditions *L. monocytogenes* may alter its cellular physiology and become resistant to subsequent challenges (or food-processing stresses) (Hayman et al., 2007; Álvarez-Ordóñez et al., 2015; Teixeira et al., 2016). In fact, several studies have reported that growth temperature affects the sensitivity of *L. monocytogenes* to thermal (Juneja et al., 1998; Knabel et al., 1990; Pagán et al., 1999), high pressure (Bull et al., 2005; Hayman et al., 2007; Shearer et al., 2010; Juck et al., 2012) and pulsed electric fields treatments (Alvarez et al., 2002), and that adaptation of *L. monocytogenes* to acid conditions protects the pathogen against a variety of normally lethal conditions (Ryan et al., 2008; Patil et al., 2010). Short-term exposure to stress conditions can also have a similar protective effect against lethal treatments, as previously described for *L. monocytogenes* (Linton et al., 1990; Farber and Pagotto, 1992; Pagán et al., 1997; Jørgensen et al., 1999; Taormina and Beuchat, 2001).

The assessment of NTAP as an industrially valuable food decontamination strategy requires information on microbial plasma resistance after exposure to food-related environmental conditions simulating those prevailing during processing. The aims of this study were (i) to determine the impact of gas composition and flow rate on *L. monocytogenes* and *L. innocua* (used as a surrogate) inactivation by NTAP; (ii) and to assess the plasma inactivation kinetics of *L. monocytogenes* after its growth under suboptimal conditions, using a wide range of temperatures and media acidified up to pH 5.5 using different organic acids (citric, lactic and malic) and hydrochloric acid, or after its short-term exposure to acid, cold or thermal shocks.

Materials and methods

Bacterial strains and culture conditions

L. monocytogenes strain CECT 4301 and *L. innocua* strain CECT 910 were supplied by Colección Española de Cultivos Tipo (CECT) (Spanish Type Culture Collection). Freeze-dried stocks were revived in Brain Heart Infusion broth (BHI, Oxoid, England) at 37ºC for 24 h followed by streaking onto BHI agar (BHIA, Oxoid). BHIA plates were also incubated at 37ºC for 24 h and stored at 4ºC. Stationary-phase inocula were prepared by inoculating test tubes containing 10 mL of fresh BHI with a single colony, and incubating them at 37ºC for 24 h.

To study the effect of growth pH on NTAP resistance, tubes containing 10 mL of sterile BHI (pH 7.4), buffered BHI adjusted to pH 7.0 by addition of Sorensen buffer 0.2 M (bisodium [Merck, Germany]-monopotassium [Panreac, Spain] phosphate), and BHI acidified at pH values of 6.4 and 5.5 with several acids (citric [Sigma, USA], lactic [Merck], malic [Scharlau, Spain] and hydrochloric [Panreac]) were inoculated with the stationary-phase inocula to a final concentration of 10^3 cells/mL. These pH values were chosen since they are representative of the slightly acidic environments that this pathogenic microorganism can find in many foods. These cultures were then incubated at 37ºC for the time needed to reach the late stationary phase of growth: 24 h for buffered BHI, non-acidified BHI and BHI acidified at pH 6.4 and 48 h for BHI acidified at pH 5.5 (data not shown).

In order to study the effect of growth temperature on NTAP resistance, tubes containing 10 mL of BHI stabilized at 10, 25, 37, and 42ºC were inoculated with appropriate dilutions of the stationary-phase inocula to give a final concentration of 10^3 cells/mL. Tubes were then incubated for 24 h, except for cultures at 10ºC, which required 11 days to reach the late-stationary phase of growth.

For experiments of short-term exposure to stress conditions, aliquots (0.1 mL) of bacterial cultures grown at 37ºC for 24 h were exposed to acid stress (pH 4.5), heat stress

(45 ºC) or cold stress (0ºC) for different times (5 and 120 min) by inoculating them into 9.9 mL of peptone water 0.1% (w/v, Oxoid) acidified with hydrochloric acid, preheated to 45 ºC in a thermostated water bath or submerged in an ice-chilled bath, respectively. After the desired stress exposure time, samples were collected for NTAP treatments. In all cases, the level of water or ice in the baths was 3 cm above the level of peptone water in the tubes.

Determination of NTAP resistance

Plasma treatments were performed in a commercially available air plasma jet (CP121 Plasma Demonstrator, OMVE BV, Netherlands) as described elsewhere (Fernández et al., 2012). The NTAP system used is based on a copper wire electrode configured as a large bandwidth, high impedance voltage probe. Its electrical potential is perturbed by the afterglow of a jet of excited state processing gas produced by a high voltage discharge typically sampled at a rate of 1 kHz. The electrode is located 25 mm above the gas outlet. A grid near the electrode, is held at the same potential, to remove space charge. The system is operated at atmospheric pressure, and the temperature of the samples never exceeded 35ºC. Experiments were firstly performed using two different gas compositions (air and nitrogen) operated under three different flow rates (5, 10 and 15 L/min). Afterwards, experiments with stress adapted cells were carried out only with air plasma at a gas flow rate of 10 L/min (higher flow rates did not increase the microbial inactivation efficiency of the plasma generated). In all cases, experiments were performed with the power setting 3 (approx. 1 Watt output power). Information on plasma diagnostics for an equivalent NTAP treatment unit can be found in Mastwijk, et al. (2009) and Mols et al. (2013).

Whatman polycarbonate membrane filters of 0.2 µpore retention, 25 mm diameter (Fisher Scientific, Loughborough, UK) placed on BHIA plates were inoculated with 30 µL of bacterial cultures grown in the different conditions, including control and stress-adapted suspensions (adjusted to approximately 10^7 CFU/mL). The inoculum was spread out on the entire upper surface of filters using a sterile loop. The filters were

then allowed to dry for 15 min in a laminar flow cabinet before plasma treatment. Afterwards, inoculated membrane filters were exposed to plasma conditions at predetermined times, for a period of up to 4 min. Following plasma treatment, cells were recovered from membrane filters by transferring them to sterile universal bottles containing 10 mL of peptone water and vortexing for 1 min. Series of decimal dilutions were prepared with peptone water and aliquots of 0.1-1 mL of appropriate dilutions were plated on BHIA plates in order to calculate the number of viable cells after incubation at 37ºC for 48 h (longer incubation times did not have any influence on the counts). All experiments were performed in triplicate using three independent bacterial cultures.

Resistance parameters and statistical analysis

In order to fit microbial survival curves, obtained by plotting the logarithm of the survivors fraction *vs* treatment time, the following mathematical function based on the Weibull distribution was used:

$$\log N_t/N_0 = -(t/\delta)^p$$

where t is the treatment time, N_t and N_0 are the population densities (CFU/filter) at time *t* and time 0, respectively, and δ and *p* are the scale and shape parameter, respectively.

The δ value represents the treatment time needed to reduce the first $\log_{10}$ cycle of the population. The *p* parameter accounts for upward concavity of a survival curve ($p < 1$), a linear survival curve ($p = 1$), or downward concavity ($p > 1$) (Mafart et al., 2002).

To fit the model to the experimental data and to calculate δ and *p* parameters, the least-squares criterion by the GraphPad PRISM software (GradhPad Software, San Diego, CA, USA) was used. The goodness of fit was indicated by R^2 and RMSE values, which ranged from 0.82 to 0.99 (in most cases >0.95) and from 0.01 to 0.63 (in most cases <0.2), respectively (data not shown). Student´s t-test was used to determine whether significant differences existed ($P<0.05$) between δ and *p* values obtained for

control and stress adapted *L. monocytogenes* cells (GradhPad Software, San Diego, CA, USA). x

Results and discussion

NTAP is a non-thermal food processing technology that has received considerable attention as a promising strategy to decontaminate and ensure the microbiological safety of a range of heat sensitive foods. Indeed, several studies have already reported successful application of NTAP for the inactivation of pathogenic and food spoilage microorganisms, including different *Salmonella* serovars, *E. coli* and *L. monocytogenes*, in a variety of foods (Critzer et al., 2007; Deng et al., 2007; Song et al., 2009; Lee et al., 2011; Ziuzina et al., 2014).

The real-life implementation of any alternative food preservation technology requires the assessment of solid scientific data on microbial behaviour under different operative scenarios. In particular, determination of microbial inactivation kinetic parameters and development of inactivation models are essential to design safe food preservation processes of industrial value. This study assessed the effect of six different NTAP operating conditions, i.e. two types of gases (air and nitrogen) at three different flow rates (5, 10 and 15 L/min), on the inactivation of *L. monocytogenes* and *L. innocua*. Moreover, the influence of growth temperature, growth pH and short-term exposure to acid, cold or heat shocks on the final NTAP tolerance was also monitored for *L. monocytogenes* in order to ascertain whether stress adaptation has an impact on the effectiveness of NTAP treatments.

Fig. 1 shows the survival curves obtained for *L. monocytogenes* and *L. innocua* under all gas composition and flow rate operating conditions studied. Although microbial inactivation increased with treatment time, the increment of the microbial lethality did not follow an exponential relationship with treatment time, which indicates that as the treatment continues it takes a progressively longer time to inactivate the same fraction of survival cells. For example, exposure of *L. monocytogenes* to air-based plasmas at 10 L/min for 15 seconds gave rise to ~1.5 log cycles of inactivation, whereas

a further increase in treatment time to 4 minutes only enhanced microbial inactivation by about an additional 1.0 log cycle units. This deviation from linearity has been frequently observed for microorganisms treated by NTAP (Critzer et al., 2007; Basaran et al., 2008; Niemira and Sites, 2008; Leipold et al., 2010; Kim et al., 2011; Fernández et al., 2012; Fröhling et al., 2012), including *L. monocytogenes* (Yun et al., 2010). Although the nature of this tailing effect is not exactly known, it has been attributed to the existence of a fraction of the population more resistant to plasma or protected against the lethal treatment by dead cells or different cellular metabolic compounds, such as certain proteins capable of quenching the active species of plasma (Kamgang-Youbi et al., 2008). Indeed, it has been recently demonstrated that addition of heat-killed cells protected *S*. Typhimurium from inactivation by NTAP (Fernández et al., 2012).

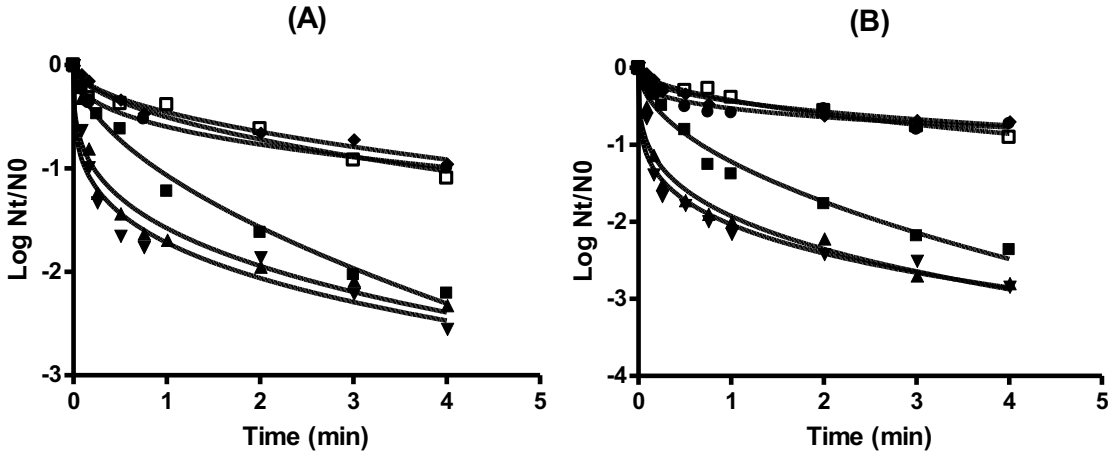

Fig. 1. Survival curves obtained after NTAP treatment of *L. innocua (*A) and *L. monocytogenes* (B) cells grown in BHI at 37ºC and treated using air at 5 L/min (■), air at 10 L/min (▲), air at 15 L/min (▼), nitrogen at 5 L/min (◆), nitrogen at 10 L/min (●) and nitrogen at 15 L/min (□). The symbols represent the experimental data obtained (one of three trials), while the lines were the result of the adjustment of the data through the mathematical model $\log N_t/N_0 = - (t/ \delta)^p$.

Several mathematical models have been proposed to fit concave upward survival curves, such as those observed in the current study. A mathematical model based on the Weibull distribution, which has been successfully used in the past to describe non-linear *L. monocytogenes* inactivation by thermal (Hassani et al., 2005; Fernández et al., 2007) and non-thermal treatments (Patil et al., 2010; Saldaña et al., 2010), was used in

this particular case. In order to quantify and compare plasma inactivation under all operating conditions, all survival curves were fitted to the cumulative form of frequency of the Weibull distribution of resistances, and the corresponding shape (*p*) and scale (δ) parameters were estimated (Table 1).

Table 1. Influence of operating parameters (gas composition and flow rate) on *p* and δ-values estimated from the fit of the mathematical model $\log N_t/N_0 = -(t/\delta)^p$ to the experimental data.

Microorganism	**Treatment conditions**		**Inactivation parameters**	
Species	**Gas**	**Flow rate (l/min)**	**δ-value**	**ρ-value**
L. monocytogenes	Air	5	0.68±0.16	0.76±0.04
		10	0.16±0.08	0.41±0.10
		15	0.12±0.06	0.42±0.03
	Nitrogen	5	8.57±1.86	0.43±0.06
		10	8.40±1.38	0.46±0.05
		15	7.56±0.52	0.55±0.03
L. innocua	Air	5	0.79±0.04	0.73±0.06
		10	0.26±0.05	0.44±0.09
		15	0.16±0.03	0.41±0.06
	Nitrogen	5	4.79±0.86	0.50±0.05
		10	4.14±0.46	0.36±0.03
		15	3.81±0.49	0.51±0.08

Plasma resistance depended on both type of gas and flow rate. Both *L. monocytogenes* and *L. innocua* showed a higher sensitivity to plasma when the treatment was performed using air. Significantly lower log reductions were achieved with nitrogen. In fact, the use of nitrogen as working gas made the plasma treatment almost ineffective. Indeed, less than 1 log cycle of inactivation was achieved for both microorganisms after 4 minutes of treatment, even when the highest gas flow (15 L/min) was used. In relation to the effect of gas flow rate on the effectiveness of the NTAP treatment, it depended on the type of gas used to generate plasma. Indeed, increases in flow rate from 5 to 10 L/min caused an acceleration of bacterial inactivation when air was used for the

generation of plasma, while an additional increase of gas flow from 10 to 15 L/min had a minor impact on microbial inactivation. On the other hand, gas flow rate hardly affected NTAP treatment efficiency when nitrogen was used to generate plasma. Information available in the literature regarding the impact of flow rate on microbial inactivation is controversial. Some authors have described that microbial inactivation increases with an increase in flow rate (Niemira and Sites, 2008; Lai et al., 2016), while others have not found any significant effect (Nishime et al., 2016) or have even observed a reduced inactivation at higher flow rates (Edelblute et al., 2015). The increase in *L. innocua* and *L. monocytogenes* inactivation observed at the highest flow rates (10 and 15 L/min) in air could be due to the fact that some short-living species may not reach the sample at low flow rates.

Plasma discharge results in the generation of a number of agents with antimicrobial activity, including reactive oxygen species (ROS), such as ozone, atomic oxygen, superoxide, hydroxyl radicals, nitric oxide, hydrogen peroxide and other free radicals, reactive nitrogen species (RNS), ultraviolet (UV) radiation, ions, and charged particles. Operating parameters, such as voltage, mode of plasma exposure, gas type, treatment time, and relative humidity (RH), influence the plasma chemistry and therefore change the composition and concentration of plasma species that react with the target sample (Kim et al., 2011; Lee et al., 2011; Lu et al., 2014). For instance, several studies have previously highlighted that the type of reactive species and their concentration depend on the type of gas used for plasma discharge, and have concluded that gas composition may accordingly affect microbial inactivation rates (Lee et al., 2011; Lu et al., 2014). The presence of oxygen in air may lead to the formation of larger quantities of these active species. Indeed, it has been previously reported that the addition of small percentages of oxygen to helium or nitrogen enhanced the anti-microbial properties of the resulting plasmas (Kim et al., 2011, 2013; Lee et al., 2011), which agrees with our findings on the higher antimicrobial effectiveness of air-based plasmas in comparison with nitrogen-based plasmas. Indeed, *L. monocytogenes* and *L. innocua* inactivation obtained in our study when nitrogen was used for the generation of plasma was very limited. However, nitrogen has been previously described as an

effective gas, able to provide a >4 log inactivation for *L. monocytogenes* in a two minutes treatment (Lee et al., 2011). In addition, these authors showed that nitrogen was more effective than helium for the inactivation of *L. monocytogenes* (Lee et al., 2011). Moreover, it has been even reported that nitrogen can be more effective than air or oxygen-based plasmas for the inactivation of other bacterial species, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Takamutsu et al., 2015).

Hereinafter, all experiments aimed at assessing the impact of stress adaptation on the subsequent NTAP resistance were performed using air-based plasma at a flow rate of 10 L/min and *L. monocytogenes* as a target microorganism. It is well known that environmental conditions prevailing during bacterial growth, such as temperature and pH of culture media, play a role in determining the resistance to different stress challenges and food processing technologies (Hayman et al., 2007; Álvarez-Ordóñez et al., 2015; Teixeira et al., 2016). However, information available so far on the effect of growth conditions on microbial NTAP resistance is scarce. As far as we know, only Fernández et al. (2013) and Jahid et al. (2014) have previously studied the effect of growth temperature on the subsequent NTAP resistance of *Salmonella* Typhimurium and *Aeromonas hydrophila*, respectively. Fernández et al. (2013) reported that *S.* Typhimurium growth in the range 20-45ºC did not significantly modified its subsequent susceptibility to plasma inactivation. Jahid et al. (2014) on the other hand described that NTAP treatments were more effective in reducing *A. hydrophyla* populations on lettuce at temperatures below 15ºC than at higher temperatures. In addition, Smet et al. (2016) have recently reported that *L. monocytogenes* cells grown planktonically were more easily inactivated than their counterparts grown on surface colonies.

L. monocytogenes is a psychrotrophic microorganism able to grow at a wide range of temperatures (4ºC-43ºC) and pH values (up to pH 4.4) (van der Veen et al., 2008; Hayman y col., 2007). In order to design safe anti-*Listeria* NTAP treatments in the food industry, it is essential to understand the behaviour of this microorganism when it is exposed to lethal plasma conditions after its growth or adaptation at different stress conditions. The effect of growth temperature on the subsequent survival of *L.*

monocytogenes against NTAP treatments was monitored using bacterial cultures grown up to stationary phase at 10, 25, 37 and 42ºC. In addition, to study the influence of growth pH on *L. monocytogenes* NTAP resistance, cells were grown in non-acidified BHI (pH 7.4), buffered BHI (pH 7.0) and BHI acidified with citric, lactic, malic and hydrochloric acids at pH 6.4 and 5.5. Microbial growth in BHI, which contains glucose, results in a drop in media pH and therefore can induce an acid adaptation response in the population. For this reason we have included in our experimental setup a control culture condition (BHI media buffered at pH 7.0), where pH did not fluctuate during *L. monocytogenes* growth (data not shown). An example of survival curves obtained for *L monocytogenes* temperature-adapted and pH-adapted cells is shown in Fig. 2, while *p* and δ-values, estimated after fitting survival curves to the Weibull distribution, are shown in Table 2. The shape parameter (*p* value) ranged from 0.33 to 0.52, indicating the presence in all cases of concave upward survival curves with tailing phenomena.

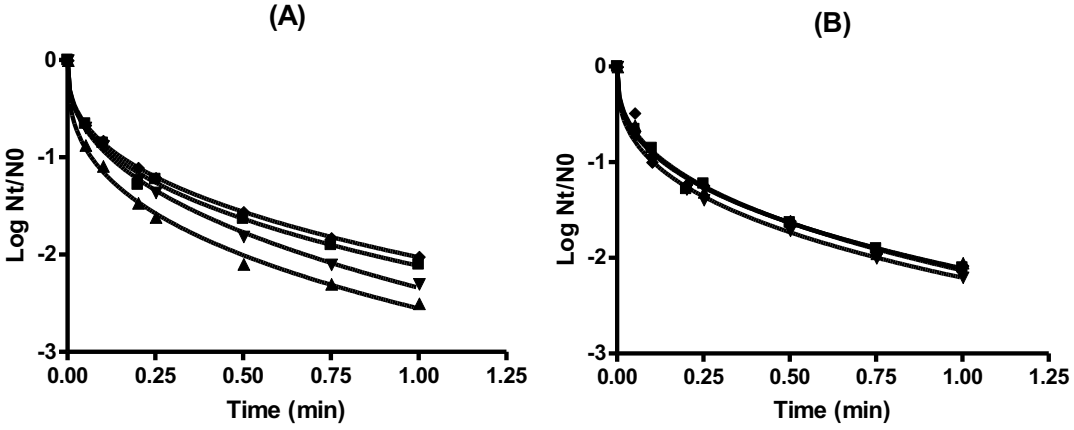

Fig. 2. Effect of growth temperature (A) and growth pH (B) on survival curves obtained after NTAP treatment of *L. monocytogenes* cells.

A. Survival curves obtained for *L. monocytogenes* cells grown in BHI at 10ºC (▲), 25ºC (▼), 37ºC (■) and 42ºC (♦). The symbols represent the experimental data obtained (one of three trials), while the lines were the result of the adjustment of the data through the mathematical model log $N_t/N_0 = - (t/ \delta)^p$.
B. Survival curves obtained for *L. monocytogenes* cells grown in BHI (■), buffered BHI pH 7.0 (▲), BHI acidified with lactic acid at pH 6.4 (▼) and BHI acidified with lactic acid at pH 5.5 (♦). The symbols represent the experimental data obtained (one of three trials), while the lines were the result of the adjustment of the data through the mathematical model log $N_t/N_0 = - (t/ \delta)^p$.

Table 2. Influence of growth temperature, growth pH and acid, heat or cold shock on p and δ-values estimated from the fit of the mathematical model log $N_t/N_0 = - (t/ \delta)^p$ to the experimental data.

Growth conditions			**Shock conditions**	**Inactivation parameters**	
Temp (ºC)	**Medium**	**Acidulant**		**δ-value**	**ρ-value**
37	BHI	-		0.16±0.08	0.41±0.10
10	BHI	-		0.07±0.01	0.33±0.08
25	BHI	-		0.13±0.04	0.43±0.14
42	BHI	-		0.15±0.06	0.38±0.13
37	Buffered BHI pH 7.0	-		0.15±0.06	0.38±0.04
37	BHI pH 6.4	Citric		0.14±0.02	0.52±0.08
		Lactic		0.14±0.05	0.44±0.04
		Malic		0.12±0.03	0.39±0.02
		HCl		0.12±0.01	0.50±0.02
37	BHI pH 5.5	Citric		0.12±0.03	0.47±0.15
		Lactic		0.16±0.08	0.45±0.07
		Malic		0.12±0.07	0.47±0.18
		HCl		0.16±0.04	0.51±0.06
37	BHI	-	HCl pH 4.5 5 min	0.18±0.05	0.44±0.10
		-	HCl pH 4.5 120 min	0.17±0.01	0.41±0.05
		-	0ºC 5 min	0.12±0.04	0.39±0.09
		-	0ºC 120 min	0.16±0.01	0.37±0.02
		-	45ºC 5 min	0.15±0.05	0.37±0.10
		-	45ºC 120 min	0.15±0.04	0.42±0.05

No statistically significant differences (P>0.05) were observed between *p* values obtained for non-adapted control cultures and *p* values obtained for temperature-adapted or pH-adapted cultures, i.e. stress type and stress intensity did not significantly modify the shape of the survival curves. Thus, in order to compare the plasma resistance of *L. monocytogenes* under the different growth conditions assayed the scale value (δ) of each survival curve was re-estimated after considering a mean *p*-value of 0.43±0.05 (Table 3).

Table 3. Influence of growth temperature, growth pH and acid, heat or cold shock on p and δ-values estimated from the second fit of the mathematical model $\log N_t/N_0 = -(t/\delta)^p$ to the experimental data considering a constant *p*-value of 0.43.

Growth conditions			**Shock conditions**	**Inactivation parameters**
Temp (ºC)	**Medium**	**Acidulant**		**δ-value**
37	BHI	-		0.17±0.06
10	BHI	-		0.11±0.04
25	BHI	-		0.12±0.03
42	BHI	-		0.17±0.04
37	Buffered BHI pH 7.0	-		0.17±0.05
37	BHI pH 6.4	Citric		0.12±0.02
		Lactic		0.13±0.03
		Malic		0.14±0.02
		HCl		0.10±0.01
37	BHI pH 5.5	Citric		0.11±0.01
		Lactic		0.15±0.05
		Malic		0.12±0.03
		HCl		0.14±0.04
37	BHI	-	HCl pH 4.5 5 min	0.18±0.03
		-	HCl pH 4.5 120 min	0.18±0.01
		-	0ºC 5 min	0.13±0.02
		-	0ºC 120 min	0.18±0.01
		-	45ºC 5 min	0.17±0.03
		-	45ºC 120 min	0.15±0.03

Control non-adapted cultures showed a δ value of 0.17±0.06 min, which was not significantly different ($P>0.05$) to δ values obtained for adapted cultures, which ranged from 0.10 to 0.17 min. These results demonstrate that neither the acidification of the growth medium up to pH 5.5, regardless of the acidulant used, nor changes in growth temperature, within the range 10-42ºC, significantly modified the survival of *L. monocytogenes* against NTAP treatments. Nevertheless, it is worth highlighting that in some particular cases (e.g. growth temperatures of 10 and 25ºC, growth pH 6.4 with

citric acid and HCl, and growth pH 5.5 with citric and malic acid) there was an apparent trend towards obtaining lower δ values for stress-adapted cultures.

In order to ascertain whether other forms of stress exposure could influence *L. monocytogenes* behaviour, NTAP resistance was also evaluated for cultures grown under control conditions (non-acidified BHI at 37ºC) and exposed for 5 min or 120 min to acid stress (pH 4.5), heat stress (45 ºC) or cold stress (0ºC). Results obtained are also shown in Tables 2 and 3. *p* values ranged from 0.37 to 0.44 and were not significantly different (P>0.05) to *p* values obtained for control cells. δ values re-estimated after considering a mean *p*-value of 0.43±0.05 ranged from 0.13 to 0.18 min. No significant statistical differences (P>0.05) were observed either between δ-values calculated for stress-shocked cells and control cells.

Food preservation technologies (e.g. thermal processing, high hydrostatic pressure, pulsed electric fields, radiation, refrigeration, drying, etc) impose a challenge to bacterial cells and can determine the fate of food-borne pathogens along the food chain. Consequently, bacteria have evolved a range of adaptive strategies to cope with them. Stress tolerance responses (e.g. the Acid Tolerance Response), which can be defined as the induced resistance to normally lethal stress conditions following growth or short-term exposure at mild sub-lethal stress conditions, are commonly mediated through activation of particular response regulators which control the expression of specific sets of genes and orchestrate very complex responses involving the induction of cellular enzymes, the synthesis of stress-shock proteins that protect or repair cellular proteins and DNA, and the modulation of the composition and physical properties of the cellular envelopes (Álvarez-Ordóñez et al., 2015). Bacterial stress tolerance responses can result in cross-protection against a wide variety of lethal exposures different from that inducing the adaptive response. Such cross-protection responses are particularly relevant when minimal processing of foods is considered, where the imposition of one sub-lethal stress may lead to the induction of multiple stress responses that may reduce the efficacy of subsequent treatments, and must be considered when implementing new preservation technologies or processing regimes (Abee and Wouters, 1999; Yousef

and Courtney, 2003; Wesche et al., 2009). It has been already established that growth or short-term exposure of *L. monocytogenes* at mild acid, heat or cold stress conditions modifies its physiology and increases its tolerance to a range of food preservation regimes, compromising the safety of industrial processing regimes (Pagán et al., 1999; Taormina and Beuchat, 2001; Hayman et al., 2007; Ryan et al., 2008; Patil et al., 2010; Shearer et al., 2010; Juck et al., 2012). Our results evidence that, on the contrary, acid, heat or cold adaptation/shock do not enhance *L. monocytogenes* NTAP resistance, what makes NTAP an attractive technology for food decontamination within minimal processing strategies targeting this pathogenic microorganism. Although, the knowledge on the mechanisms of bacterial death by NTAP is still very limited, it is generally considered that NTAP is a multitarget technology which causes injury to various cellular structures and macromolecules, such as the cellular envelopes, DNA and proteins (Surowsky et al., 2014; Yost and Joshi, 2015). Thus, it is likely that the adaptive responses induced in *L. monocytogenes* by exposure to temperature and pH stress would not be able to counteract all damages caused by NTAP.

In conclusion, the current study shows that NTAP is a promising food decontamination technology able to achieve *L. monocytogenes* inactivation with a minimal influence of pre-treatment conditions (i.e. growth pH and temperature, and stress adaptation/shock). The effectiveness of NTAP treatments was nevertheless influenced by operating conditions (i.e. gas composition and gas flow) and by the presence of concave upward survival curves or tailing phenomena. Further studies are needed in order to ascertain whether this behavior is also observed for other foodborne microorganisms and in food systems.

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Capítulo 2
Stress adaptation has a minor impact on the effectivity of Non-Thermal Atmospheric Plasma (NTAP) against *Salmonella* spp.

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Abstract

This study was aimed at studying the influence of gas composition (air and nitrogen) at different flow rates (5, 10 and 15 Lm^{-1}) and stress adaptation (growth under a wide range of temperatures [10-45ºC] and acid conditions [up to pH 4.5, using different organic acids] or short-term exposure to acid, cold or heat stress shocks) on the inactivation by Non-Thermal Atmospheric Plasma (NTAP) of *S.* Typhimurium CECT 443 and *S.* Enteritidis CECT 4300. Results obtained evidence that microbial inactivation was significantly higher when air was used for NTAP treatments. D-values obtained using air ranged from 0.86 to 2.43 min and 0.90 to 1.69 min for *S.* Typhimurium and *S.* Enteritidis, respectively, while those obtained using nitrogen ranged from 3.08 to 5.75 min and 2.28 to 5.54 min, respectively. Microbial inactivation also increased with increasing flow rates, although differences were not statistically significant in all cases. Growth temperature and pH or exposure to acid, cold or heat stress shocks had a minor impact on NTAP resistance. Indeed, D-values obtained under the different stress adaptation scenarios were not significantly different from those obtained for non-adapted control cultures (1.38±0.39 for *S.* Typhimurium and 1.23±0.36 for *S.* Enteritidis), with the exception of cells grown at 10ºC, which were significantly more sensitive to NTAP, with D-values of 0.68±0.11 and 0.45±0.10 min, respectively, for *S.* Typhimurium and *S.* Enteritidis. These findings suggest that adaptive responses triggered by exposure to acid, cold or heat stresses, already described in the past for these two *Salmonella* strains, do not provide protection against NTAP treatments, which allows us to conclude that NTAP may be a first-choice technology to be included into food processing schemes following a hurdles technology approach in combination with acidification, mild heating or refrigeration.

Introduction

Salmonella spp. continues to be one of the major biological hazards involved in foodborne outbreaks caused by the consumption of contaminated foods. The total number of cases of foodborne illnesses caused by *Salmonella* spp. in the European Union (EU) in 2015 was 94,625, resulting in a notification rate of 21.2 cases per 100,000 population (EFSA, 2016). Although non-typhoid human salmonellosis can be caused by more than 2,500 different serovars of *Salmonella enterica*, in most cases, only five have been implicated, *S.* Typhimurium, *S.* Enteritidis, *S.* Newport, *S.* Heidelberg and *S.* Javiana (CDC, 2002). In the EU, *S.* Enteritidis and *S.* Typhimurium are the two serovars most frequently associated with outbreaks of disease, representing 45.7% and 15.8%, respectively, of all reported serovars in confirmed cases of infection (EFSA, 2016).

Thermal processing is an efficient and reliable approach capable of reducing the risks associated with major foodborne pathogens, including *Salmonella* spp. However, thermal treatments adversely affect organoleptic, nutritional and functional properties of foods. The need to fulfill current consumers´ demands for high-quality fresh products without compromising their microbial food safety has led to the development of the so-called non-thermal or low-temperature preservation technologies, which include high hydrostatic pressures, pulsed electric fields, ultraviolet light, ultrasounds and, more recently, non-thermal atmospheric plasma (NTAP). NTAP is generated by applying an electric or electromagnetic field to a gas, and is basically constituted by excited and non-excited atoms and molecules, positive and negative ions, free radicals, electrons and ultraviolet radiation, which have the ability to inactivate a wide range of microorganisms, including bacteria, molds, yeasts, spores and even viruses, parasites and prions (Lackmann & Bandow, 2014; Liao et al., 2017; Mai-Prochnow, Murphy; McLean; Kong, & Ostrikov, 2014). Interestingly, microbial inactivation is achieved at ambient temperature and no hazardous chemical residues are generated in the process. In addition, costs of operation and reported treatment times are low and water consumption is reduced, leading to an important decline in effluents, which makes NTAP an economic and environmentally friendly technology (Alkawareek, Gorman, Gram, &

Gilmore, 2014; Kayes et al., 2007; Lee, Paek, Ju, & Lee, 2006; Niemira & Sites, 2008; Vleugels et al., 2005).

NTAP has been reported as an effective technology able to inactivate different pathogenic serovars of *Salmonella enterica* on different food packaging materials (Puligundla, Lee, & Mok, 2016), liquid foods (Gabriel et al., 2016; Kim et al., 2015), and food surfaces, such as meat and meat products (Jayasena et al., 2015; Kim et al., 2011; Yong et al., 2017), eggshells (Cui, Ma, Li, & Lin, 2016; Ragni et al., 2010), cheese slices (Yong et al., 2015a; Yong et al., 2015b), raw nuts (Niemira, 2012a), sprouts (Puligundla, Kim, & Mok, 2017), black peppercorns (Sun, Anderson, & Keller, 2014) and fresh produce (Critzer, Kelly-Wintenberg, South & Golden, 2007; Fernández, Noriega & Thompson, 2013; Matan, Puangjinda, Phothisuwan, & Nisoa, 2015; Min et al., 2016; Niemira & Sites, 2008; Ziuzina, Patil, Culle, Keener, & Bourke, 2014).

From all these studies it can be concluded that both processing parameters, substrate characteristics and microbial-related properties have an impact on *Salmonella* spp. behavior against NTAP. Regarding processing parameters, the presence of oxygen in gas or the addition of small percentages of oxygen to helium leads to a greater inactivation of both *S.* Enteritidis and *S.* Typhimurium (Kim et al., 2011; Marsili, Espie, Anderson, & MacGregor, 2002; Niemira, 2012a). High humidity levels (Ragni et al., 2010), flow rates (Niemira & Sites, 2008) of the gas and input power (Cui et al., 2016; Kim et al., 2011), also seem to increase the effectiveness of NTAP. Regarding substrate characteristics, the liquid media composition and surface topography of solids are considered the most important factors influencing *Salmonella* spp. NTAP resistance. In general, bacteria on abiotic surfaces are more susceptible to NTAP inactivation than bacteria on food surfaces (Fernández et al., 2013; Yong et al., 2015a; Yong et al., 2015b). Moreover, the efficiency of NTAP treatments is reduced when the superficial structure of the substrate becomes more complex (Fernández et al., 2013). Less information is available in the literature in relation to the influence of the microbial physiological status. To the best of our knowledge only two research groups have addressed this issue (Fernández, Shearer, Wilson, & Thompson, 2012; Fernández et al., 2013; Smet et al.,

2016). Fernández and co-authors monitored the influence of load contamination, growth temperature and growth phase (early and late exponential phase, and stationary phase) on the inactivation of *S.* Typhimurium and found that whereas neither growth phase nor growth temperature, within the range 20-45ºC, modified the NTAP resistance of the resulting cells, inoculum size influenced the efficiency of the treatment when it was applied to surfaces. Indeed, the inactivation efficacy decreased at high contamination levels which was attributed to the shelter provided by bacteria in the upper layers to those located in inner layers. Smet et al. (2016) studied the efficacy of helium-oxygen plasma in various food structures against *S.* Typhimurium cells grown under osmotic (0, 2, 6% NaCl) or acid stress (pH 5.5, 6.5, 7.4) and concluded that the presence of high salt concentrations or suboptimal pH values in the growth medium induced stress hardening, creating cells resistant towards the subsequent NTAP treatment.

Microbial adaptive stress reponses are complex systems of defense which aid bacteria to cope with rapidly changing environmental conditions and ensure their survival in adverse conditions (Alvarez-Ordóñez, Broussolle, Coli, Nguyen-The, & Prieto, 2015). Therefore, they may compromise the efficacy and safety of food processing technologies and represent a challenge for food industries when developing minimal processing regimes based on the imposition of mild inactivation treatments. In a recent study carried out by our research group it was observed for *Listeria monocytogenes* that NTAP was able to achieve microbial inactivation with a minimal influence of pre-treatment conditions (i.e. growth pH and temperature, and stress adaptation/shock) (Calvo, Alvarez-Ordóñez, Prieto, González-Raurich, & López, 2016), which suggested that stress adaptation did not induce a cross-protection response against NTAP. This would make NTAP an attractive technology for food decontamination within minimal processing strategies. Nevertheless, in order to confirm these findings, further studies using strains with well characterized stress response mechanisms are required.

This study was aimed at determining the influence of (i) gas composition (air and nitrogen) at different flow rates (5, 10 and 15 Lm^{-1}), (ii) growth under a wide range of

temperatures (10-45ºC) and acid conditions (up to pH 4.5 using different organic acids - acetic, ascorbic, citric, lactic and malic- and hydrochloric acid), (iii) short-term exposure to acid, cold or heat stress shocks, and (iv) recovery temperature on the inactivation by NTAP of *S.* Typhimurium CECT 443 and *S.* Enteritidis CECT 4300, two strains whose stress adaptive responses to acid stress and temperature shifts have been extensively studied in our laboratory in the past (Alvarez-Ordóñez, Fernández, López, Arenas, & Bernardo, 2008; Alvarez-Ordóñez, Fernández, Bernardo, & López, 2009; Alvarez-Ordóñez, Fernández, Bernardo, & López, 2010a; Alvarez-Ordóñez, Fernández, Bernardo, & López, 2010b; Alvarez-Ordóñez, Halisch, & Prieto, 2010c).

Materials and Methods

Bacterial strains and culture conditions

Salmonella enterica serovar Typhimurium CECT 443 *and Salmonella enterica* serovar Enteritidis CECT 4300 were obtained from Colección Española de Cultivos Tipo (CECT). For revitalization, freeze-dried bacteria were transferred to Brain Heart Infusion broth (BHI, Oxoid) and incubated at 37ºC for 24 h followed by streaking onto BHI agar (BHIA, Oxoid) plates. Plates were then incubated under the same conditions to obtain single colonies, and were stored at 4ºC for routine use. Microbial overnight cultures were prepared by inoculating test tubes containing 10 mL of fresh BHI with a single colony from BHI agar plates, with subsequent incubation at 37ºC for 24 h.

In order to obtain acid adapted cultures, the overnight cultures were used to inoculate tubes containing 10 mL of BHI acidified at pH values of 6.4, 5.4 and 4.5 with acetic acid (Prolab), ascorbic acid (Merck), citric acid (Sigma), lactic acid (Merck), malic acid (Scharlau) and hydrochloric acid (Panreac). In parallel, tubes containing 10 mL of sterile BHI (pH 7.4) and buffered BHI adjusted to pH 7.0 by addition of Sorensen buffer 0.2 M (bisodium [Merck]-monopotassium [Panreac] phosphate), which allow to maintain the pH value within ± 0.1 pH units during the incubation period, were also inoculated and served as non-acid adapted control cultures, as described by Alvarez-

Ordóñez et al. (2009). Both non-acid adapted and acid adapted cultures were then incubated at 37ºC for the time needed to reach the late stationary phase of growth: 24 h for buffered BHI, non-acidified BHI and BHI acidified at pH 6.4 and 48 and 72 h for BHI acidified at pH 5.4 and 4.5, respectively (Alvarez-Ordóñez et al., 2009).

In order to study the effect of growth temperature, tubes containing 10 mL of BHI stabilized at 10, 25, 37, and 45ºC were inoculated with the overnight inocula and incubated at the corresponding temperature for 24 h, except for cultures at 10ºC, which required thirteen days to reach the late stationary phase of growth (Alvarez-Ordóñez et al., 2010a).

For short-term stress exposure experiments, aliquots (0.1 mL) of overnight cultures were exposed to acid stress (pH 4.5), heat stress (45 °C) or cold stress (0 °C) for different time periods (5 and 120 min) by inoculating them into 9.9 mL of peptone water 0.1% (w/v, Oxoid) acidified with hydrochloric acid, preheated at 45 °C in a thermostated water bath or submerged in an ice-chilled bath, respectively. Samples were collected at the desired stress exposure times and subjected to NTAP treatments, as explained below. In all cases, the level of water or ice in the baths was 3 cm above the level of peptone water in the tubes.

NTAP treatments

NTAP treatments were performed in a commercially available air plasma jet (CP121 Plasma Demonstrator, OMVE BV, Netherlands) following the methodology described in Calvo et al. (2016). Information on plasma diagnostics for an equivalent NTAP treatment unit can be found in Mastwijk, Wichers, van Dijk, & Schuten (2009) and Mols, Mastwijk, Nierop Groot, & Abee (2013). The unit is based on a copper wire electrode configured as a large bandwidth, high impedance voltage probe. The electrical potential is perturbed by the afterglow of a jet of excited state air produced by a high voltage discharge sampled at a rate of 1 kHz. The electrode is located 25 mm above the gas outlet. In order to remove space charge, a grid is held at the same potential near the electrode. The unit works at atmospheric pressure and sample temperature never

exceeded 35ºC. Experiments were initially performed using two different gas compositions (air and nitrogen) under three different flow rates (5, 10 and 15 L/min). Then, experiments with stress adapted or stress shocked cells were carried out only with air plasma at a flow rate of 10 L/min (higher flow rates did not significantly increase the inactivation efficiency of the treatment). Experiments were performed in all cases with the power setting 3 (approx. 1 Watt output power).

For NTAP treatments, Whatman polycarbonate membrane filters of 0.2 µpore retention, 25 mm diameter (Fisher Scientific, Loughborough, UK) were used. Thirty µL of bacterial cultures obtained under the different experimental conditions (diluted to ca. 10^7 CFU/mL) were spread out on the entire upper surface of filters using a sterile loop. Filters were then allowed to dry for 15 min in a laminar flow cabinet and placed on BHI agar Petri dishes which were finally exposed to plasma conditions at predetermined times, for a period of up to 12 min. Following plasma treatment, membrane filters were transferred to sterile universal tubes containing 10 mL of peptone water and survivors were recovered through vigorous vortexing for 1 min. Then, aliquots of appropriate serial dilutions were plated on BHI agar plates, which were incubated at 37ºC for 48 h, and colonies were enumerated (longer incubation times did not influence the counts). For experiments carried out to ascertain the effect of recovery conditions on NTAP inactivation, survivors plated on BHI agar plates were also incubated at 10, 25 and 45ºC for 336, 72 and 48 h, respectively. All experiments were performed in triplicate using three independent bacterial cultures.

Resistance parameters and statistical analysis

Survival curves were obtained by plotting the logarithm of the survivors fraction *versus* treatment time. The line that best fitted survivor plots was determined by linear regression and D-values (min) were calculated as the negative reciprocal of the slope. R^2 values ranged from 0.80 to 0.99 (in most cases R^2 values were above 0.95; data not shown). Student´s t-test was used to determine significant differences ($p<0.05$) between D-values under the different experimental conditions (IBM SPSS Statistics 21.0).

Results

The inactivation of *S.* Typhimurium CECT 443 *and S.* Enteritidis CECT 4300 by NTAP was studied using two gas compositions (air and nitrogen) under three different flow rates (5, 10 and 15 L/min). At a given flow rate, microbial inactivation was significantly higher when air was used for NTAP treatments (Fig. 1; Table 1).

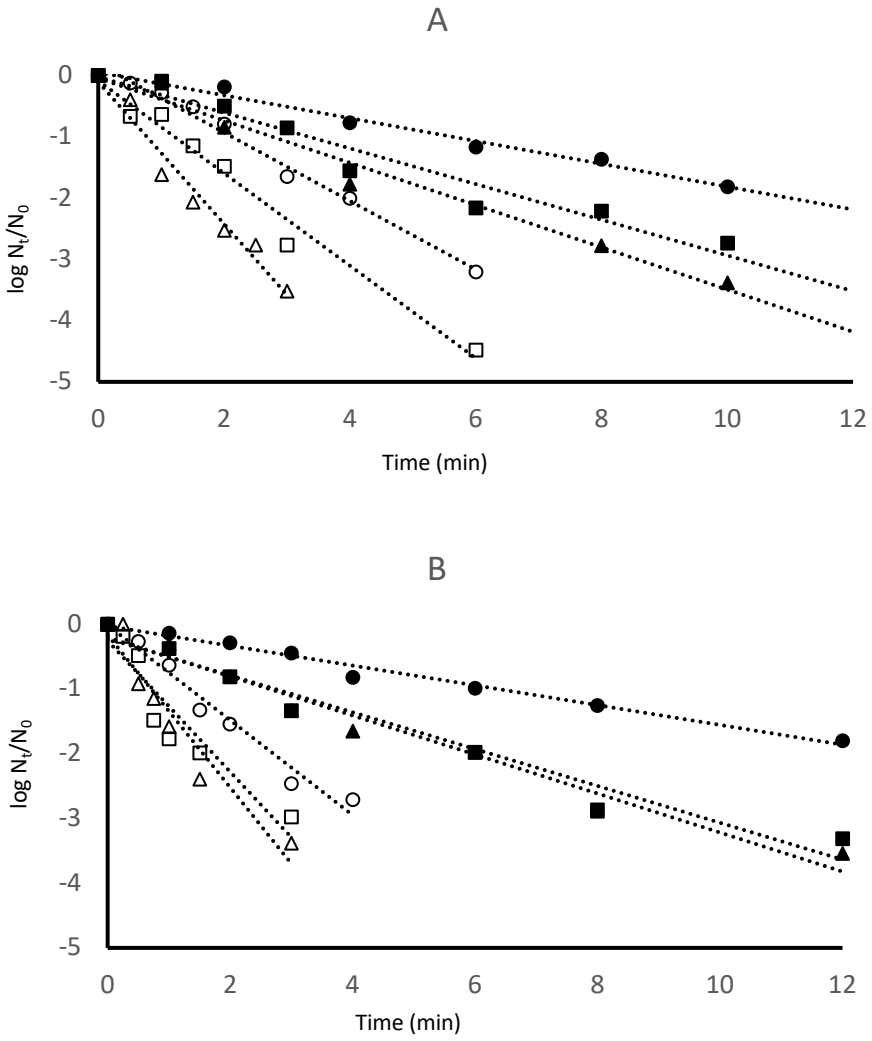

Fig. 1. Survival curves (one of three trials) obtained after NTAP treatment of *S*. Typhimurium (A) and *S*. Enteritidis (B) cells grown in BHI at 37 °C and treated using air at 5 (○), 10 (□), and 15 (Δ) L/min or nitrogen at 5 (●), 10 L/min (■) and 15 (▲) L/min.

Indeed, D-values obtained using air ranged from 0.86 to 2.43 min and 0.90 to 1.69 min for *S.* Typhimurium and *S.* Enteritidis, respectively, while those obtained using

nitrogen ranged from 3.08 to 5.75 min and 2.28 to 5.54 min, respectively. In addition, for each working gas, microbial inactivation increased with increasing flow rates, although differences were not statistically significant in all cases and increases in flow rate from 5 to 10 L/min caused a more dramatic enhancement in inactivation than increases from 10 to 15 L/min, which had a minor impact on microbial inactivation.

Table 1. Influence of operating parameters (gas composition and flow rate) on D-values (min) obtained for *S*. Typhimurium and *S*. Enteritidis.

Microorganism	**Treatment conditions**		**Inactivation parameters**
	Gas	**Flow rate (L/min)**	**D-values (mean ± SD)**
S. Typhimurium	Air	5	2.43±1.09^{a,b,d}
		10	1.38±0.39^{a,e}
		15	0.86±0.21^{a}
	Nitrogen	5	5.75±1.20^{c}
		10	3.18±0.88^{b,d}
		15	3.08±0.61^{b}
S. Enteritidis	Air	5	1.69±0.46^{d,e}
		10	1.23±0.36^{a,e}
		15	0.90±0.12^{a}
	Nitrogen	5	5.54±0.68^{c}
		10	2.68±0.46^{b,d}
		15	2.28±0.39^{b,d}

$^{a–d}$: D-values (mean of three experiments ± SD) with different superscript in the same column are significantly different ($P<0.05$).

Experiments focused at investigating the influence of stress adaptation and stress shocks on *S.* Typhimurium and *S.* Enteritidis NTAP resistance were only performed using air plasma at a flow rate of 10 L/min (Table 2; Table 3). An example of survival curves obtained for temperature-adapted and pH-adapted cells of both microorganisms is shown in Fig. 2. In general, inactivation rates obtained under the same experimental conditions were slightly higher for *S.* Enteritidis, with D-values ranging from 0.45 to 1.55 min, than for *S.* Typhimurium, with D-values ranging from 0.68 to 1.67 min.

Table 2. Influence of growth temperature, recovery temperature, growth pH and acid, heat or cold shock on D-values (min) obtained for *S*. Typhimurium.

Growth conditions			Shock conditions	Recovery conditions	Inactivation parameters
Temp (ºC)	**Medium**	**Acidulant**			**D-values (mean ± SD)**
37	BHI	-	-	BHIA - 37ºC	1.38±0.39
10	BHI	-	-	BHIA - 37ºC	0.68±0.11*
25	BHI	-	-	BHIA - 37ºC	1.08±0.35
45	BHI	-	-	BHIA - 37ºC	1.23±0.48
37	Buffered BHI pH 7.0	-	-	BHIA - 37ºC	1.52±0.29
37	BHI pH 6.4	Acetic	-	BHIA - 37ºC	1.64±0.13
		Ascorbic	-	BHIA - 37ºC	1.66±0.18
		Citric	-	BHIA - 37ºC	1.46±0.22
		Lactic	-	BHIA - 37ºC	1.63±0.18
		Malic	-	BHIA - 37ºC	1.67±0.24
		HCl	-	BHIA - 37ºC	1.55±0.17
37	BHI pH 5.4	Acetic	-	BHIA - 37ºC	1.28±0.13
		Ascorbic	-	BHIA - 37ºC	1.54±0.31
		Citric	-	BHIA - 37ºC	1.52±0.22
37	BHI pH 5.4	Lactic	-	BHIA - 37ºC	1.43±0.23
		Malic	-	BHIA - 37ºC	1.26±0.07
		HCl	-	BHIA - 37ºC	1.48±0.38
37	BHI pH 4.5	Ascorbic	-	BHIA - 37ºC	1.16±0.37
		Citric	-	BHIA - 37ºC	1.52±0.17
		Malic	-	BHIA - 37ºC	1.49±0.43
		HCl	-	BHIA - 37ºC	1.65±0.34
37	BHI	-	HCl pH 4.5 5 min	BHIA - 37ºC	1.23±0.16
		-	HCl pH 4.5 120 min	BHIA - 37ºC	1.43±0.34
		-	0ºC 5 min	BHIA - 37ºC	1.34±0.29
		-	0ºC 120 min	BHIA - 37ºC	1.52±0.17
		-	45ºC 5 min	BHIA - 37ºC	1.48±0.32
		-	45ºC 120 min	BHIA - 37ºC	1.30±0.18
37	BHI	-	-	BHIA - 10ºC	1.41±0.16
		-	-	BHIA - 25ºC	1.66±0.26
		-	-	BHIA - 45ºC	1.55±0.17

*D-values significantly different from those obtained for control cells (grown in non-acidified BHI at 37ºC, non-exposed to stress shocks, treated by NTAP and recovered on BHIA plates at 37ºC) are marked with an asterisk.

Table 3. Influence of growth temperature, recovery temperature, growth pH and acid, heat or cold shock on D-values (min) obtained for *S*. Enteritidis.

Growth conditions			Shock conditions	Recovery conditions	Inactivation parameters
Temp (ºC)	**Medium**	**Acidulant**			**D-values (mean ± SD)**
37	BHI	-	-	BHIA - 37ºC	1.23±0.36
10	BHI	-	-	BHIA - 37ºC	0.45±0.10*
25	BHI	-	-	BHIA - 37ºC	0.73±0.12
45	BHI	-	-	BHIA - 37ºC	0.83±0.16
37	Buffered BHI pH 7.0	-	-	BHIA - 37ºC	0.98±0.17
37	BHI pH 6.4	Acetic	-	BHIA - 37ºC	1.23±0.34
		Ascorbic	-	BHIA - 37ºC	1.25±0.30
		Citric	-	BHIA - 37ºC	1.06±0.07
		Lactic	-	BHIA - 37ºC	0.99±0.04
		Malic	-	BHIA - 37ºC	1.28±0.28
		HCl	-	BHIA - 37ºC	1.12±0.27
37	BHI pH 5.4	Acetic	-	BHIA - 37ºC	1.27±0.19
		Ascorbic	-	BHIA - 37ºC	1.22±0.31
		Citric	-	BHIA - 37ºC	1.19±0.22
		Lactic	-	BHIA - 37ºC	1.17±0.14
		Malic	-	BHIA - 37ºC	1.39±0.46
		HCl	-	BHIA - 37ºC	1.38±0.33
37	BHI pH 4.5	Ascorbic	-	BHIA - 37ºC	1.44±0.42
		Citric	-	BHIA - 37ºC	1.00±0.17
		Malic	-	BHIA - 37ºC	1.55±0.38
		HCl	-	BHIA - 37ºC	1.46±0.41
37	BHI	-	HCl pH 4.5 5 min	BHIA - 37ºC	1.33±0.22
		-	HCl pH 4.5 120 min	BHIA - 37ºC	1.15±0.30
		-	0ºC 5 min	BHIA - 37ºC	1.42±0.27
		-	0ºC 120 min	BHIA - 37ºC	1.43±0.15
		-	45ºC 5 min	BHIA - 37ºC	1.44±0.22
		-	45ºC 120 min	BHIA - 37ºC	1.38±0.37
37	BHI	-	-	BHIA - 10ºC	0.92±0.12
		-	-	BHIA - 25ºC	1.11±0.08
		-	-	BHIA - 45ºC	1.04±0.11

*D-values significantly different from those obtained for control cells (grown in non-acidified BHI at 37ºC, non-exposed to stress shocks, treated by NTAP and recovered on BHIA plates at 37ºC) are marked with an asterisk.

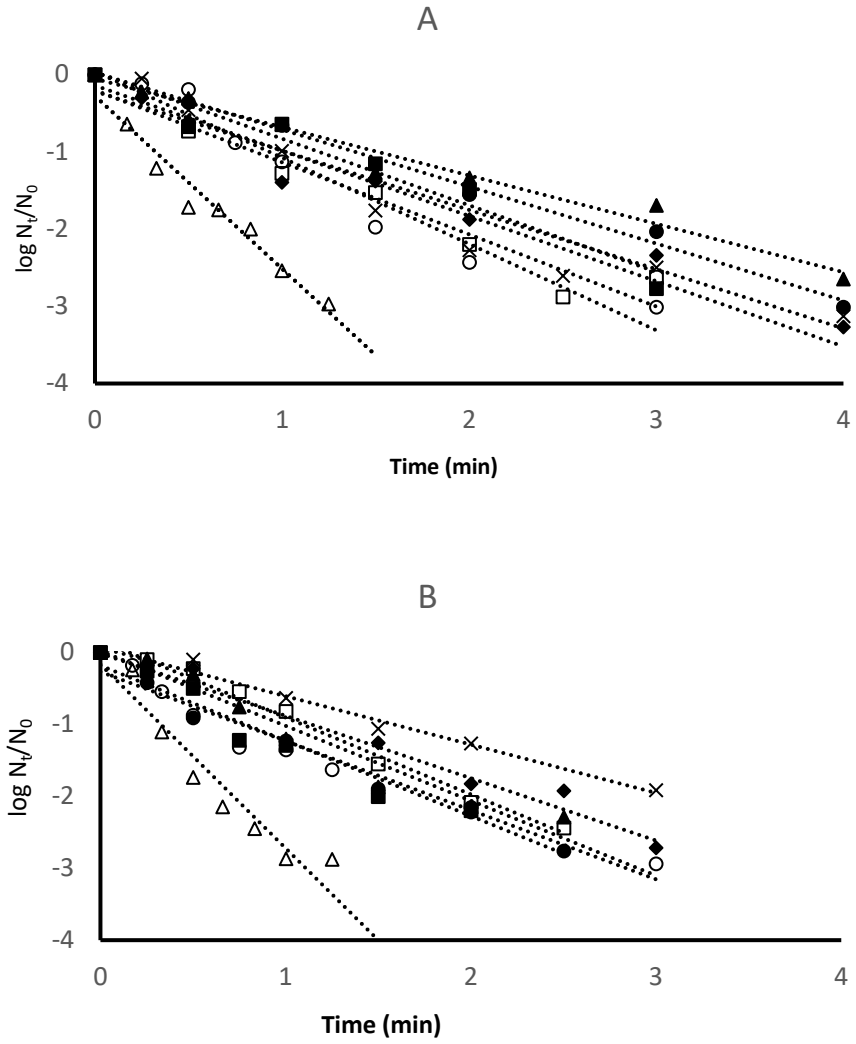

Fig. 2. Effect of growth temperature and growth pH on survival curves obtained after NTAP treatment of *S*. Typhimurium (A) and *S.* Enteritidis (B) cells.
Survival curves (one of three trials) obtained for cells grown in BHI at 10 (Δ), 25 (○), 37 (■) and 45ºC (□), or at 37ºC in buffered BHI pH 7.0 (●) or BHI acidified with malic acid at pH 6.4 (▲), 5.4 (♦), or 4.5 (x).

Acid adaptation (i.e. growth in media acidified with different acidulants at pH values up to 4.5) did not significantly modify *S.* Typhimurium and *S.* Enteritidis NTAP resistance. For *S.* Typhimurium, D-values obtained for cells grown in buffered BHI and non-acidified BHI were 1.52±0.29 and 1.38±0.39 min, respectively, while the mean D-value obtained for acid adapted cells was 1.50±0.15 min. Similarly, D-values obtained for *S.* Enteritidis cells grown in buffered BHI and non-acidified BHI were 0.98±0.17 and

1.23±0.36 min, respectively, while the mean D-value obtained for acid adapted cells was 1.25±0.16 min (Tables 2 and 3; Fig. 3).

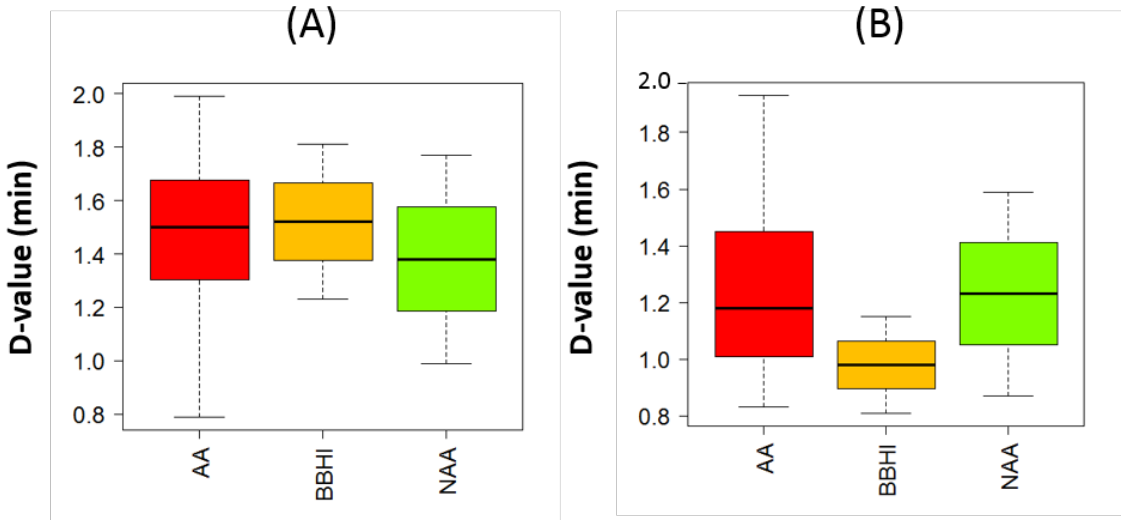

Fig. 3. Distribution of D-values (min) among *S.* Typhimurium (A) and *S.* Enteritidis (B) cells grown in buffered BHI (BBHI), non-acidified BHI (NAA) or acidified BHI (AA).

Regarding growth temperature, fluctuations in the range 25 to 45ºC did not significantly influence the microbial inactivation achieved, but growth of both *S.* Typhimurium and *S.* Enteritidis at 10ºC enhanced the antimicrobial effectiveness of NTAP treatments, with D-values of 0.68±0.11 and 0.45±0.10 min, respectively, which were significantly lower ($P<0.05$) than those obtained for cells grown at 37ºC.

Short-term exposure to acid (pH 4.5), cold (0ºC) and heat (45ºC) stress shocks of *S.* Typhimurium and *S.* Enteritidis cells grown in BHI broth at 37ºC did not influence the effectiveness of NTAP treatments either. Indeed, D-values obtained for stress shocked *S.* Typhimurium and *S.* Enteritidis cells ranged from 1.23 to 1.52 min and 1.15 to 1.44 min, respectively, and were not significantly different from those obtained for control bacterial cultures.

Finally, the influence of recovery conditions on *S.* Typhimurium and *S.* Enteritidis inactivation by NTAP was studied using different recovery temperatures (10, 25, 37 and 45ºC) for incubation of BHI agar plates obtained after treatment of cultures grown in

BHI broth at 37ºC. No statistically significant differences ($P > 0.05$) were observed between D-values obtained under the different recovery temperatures tested either (Table 2; Table 3).

Discussion

NTAP is a promising non-thermal food processing technology which is been increasingly investigated as a tool to sanitize delicate, heat-sensitive foods (Mir, Shah, & Mir, 2016; Niemira, 2012b). Its effectiveness for the inactivation of a range of spoilage and pathogenic microorganisms has been already shown in previous studies (Gabriel et al., 2016; Rowan et al., 2007; Yong et al., 2017). In the particular case of *Salmonella* spp., information available in the literature shows that NTAP treatments are effective for the elimination of various *Salmonella* serovars on solid surfaces (Fernández et al., 2012; Puligundla et al., 2016), liquid media (Rowan et al., 2007) and foods (Critzer et al., 2007; Cui et al., 2016; Fernández et al., 2013; Jayasena et al., 2015; Kim et al., 2011; Matan et al., 2015; Min et al., 2016; Niemira, 2012a; Niemira & Sites, 2008; Puligundla et al., 2017; Ragni et al., 2010; Yong et al., 2015a; Yong et al., 2015b; Yong et al., 2017; Ziuzina et al., 2014).

Results obtained in this study confirm that NTAP treatments successfully inactivate *S.* Typhimurium and *S.* Enteritidis cells and evidence that whereas processing parameters can significantly affect the inactivation rate achieved, minor variability is caused by changes in the microbial physiological status.

Lethality obtained with air plasmas was higher than that observed for nitrogen plasmas. This finding agrees with previous reports using *Salmonella* spp. and other microbial species (Marsili et al., 2002; Niemira, 2012a). Several authors have concluded that gas composition influences microbial inactivation due to changes in the type and concentration of reactive species generated (Lee et al., 2011; Lu, Patil, Keener, Cullen, & Bourke, 2014). Some of these, such as reactive oxygen species (ozone, atomic oxygen, superoxide, hydroxyl radicals, nitric oxide, hydrogen peroxide) and reactive nitrogen species, have antimicrobial activity and their presence in higher or lower proportions in

the plasma can influence the final effectiveness of the NTAP treatment. The higher antimicrobial efficacy of air based plasmas may be due to the presence of oxygen, since previous studies have shown that the addition of small amounts of oxygen to helium or nitrogen enhanced the antimicrobial activity of the resulting plasmas (Kim et al., 2011; Kim, Yong, Park, Choe, & Jo, 2013; Lee et al., 2011).

For both air and nitrogen based plasmas, the inactivation of *S.* Typhimurium and *S.* Enteritidis in general increased as the gas flow rate increased from 5 to 10 L/min. A further increase in flow rate from 10 to 15 L/min caused a minor increase in microbial inactivation. While some reports in the literature describe a reduced microbial inactivation at high gas flow rates (Edelblute, Malik, & Heller, 2015) or no effect of the flow rate in microbial inactivation (Nishime et al., 2017), it is generally acknowledged that lethality increases with an increase in flow rate (Lai, Cheung, Wong, & Li, 2016; Niemira & Sites, 2008). This is likely caused by the fact that certain reactive species with a very short shelf life may not reach the substrate at low flow rates (Calvo et al., 2016).

Bacterial stress responses are adaptive strategies generally consisting of changes in the pattern of gene expression which allow microbes to maintain viability under harsh conditions (Abee & Wouters, 1999). These adaptive systems coordinate a series of events leading to the activation of homeostatic systems, the synthesis of stress proteins involved in the repair of DNA and proteins, and the induction of compositional and functionality modifications in cellular envelopes (Alvarez-Ordóñez et al., 2015). Furthermore, stress tolerance responses are often accompanied by cross-protection against a range of stresses different from the one inducing the adaptive response. Stress induced cross-protection responses must be taken into account when designing and implementing minimal processing regimes, since the imposition of a sub-lethal stress may trigger adaptive responses leading to a reduction of the effectiveness of otherwise safe food preservation technologies (Wesche, Gurtler, Marks, & Ryser, 2009; Yousef & Courtney, 2003). One of the most well-characterized stress responses is the Acid Tolerance Response (ATR) of *Salmonella* spp., induced following growth at low pH (acid adaptation) or short-term exposure to mild acid conditions (acid shock) (Alvarez-

Ordóñez, Prieto, Bernardo, Hill, & López, 2012). The ATR of *S.* Typhimurium CECT 443 has been intensively studied by our research team, who has demonstrated that growth of *S.* Typhimurium CECT 443 in media acidified with different acids (acetic, ascorbic, citric, lactic, malic and hydrochloric) at low pH conditions (pH 6.5, 5.4 or 4.5) not only increased the subsequent resistance to acid challenges at extreme acid conditions (pH 2.5 and 3.0) but also enhanced survival against lethal thermal and alkaline treatments (Alvarez-Ordóñez et al., 2008; Alvarez-Ordóñez et al., 2009; Alvarez-Ordóñez et al., 2010a; Alvarez-Ordóñez et al., 2010b; Alvarez-Ordóñez, Fernández, Bernardo, & López, 2011). In addition, the main cellular systems involved in *S.* Typhimurium CECT 443 ATR were unveiled in by our research team in previous studies, which showed that *S.* Typhimurium CECT 443 induced various homeostatic amino acid dependent decarboxylase systems (arginine and lysine decarboxylase systems) and modulated its membrane composition and fluidityupon adaptation to acid environments, adaptations which are commonly accompanie by increased tolerance to different stresses and food processing technologies, such as thermal treatments and high pressure processing (Alvarez-Ordóñez et al., 2008, 2010b, 2010c; Casadei et al., 2002; Sampathkumar et al., 2004).

Stress tolerance responses, including the ATR, represent a challenge to the food industry, especially when designing minimal processing regimes relying in the hurdles technology concept, where several stresses or hurdles, are combined at mild conditions to control spoilage and pathogenic microorganisms (Leistner, 2000). The design and implementation of safe preservation systems based on the hurdles technology requires the accurate selection of processing technologies capable of guaranteeing inactivation of target microorganisms regardless of their physiological or stress-adaptation status. There is scarce information available in the literature regarding the effectiveness of NTAP against stress-adapted microorganisms. However, a recent report by Calvo et al. (2016) described that stress-adaptation to acid or low temperature conditions or short-term exposure to acid, cold and heat shocks did not modify *L. monocytogenes* NTAP resistance and concluded that NTAP could be an attractive technology for food decontamination within minimal processing strategies due to the absence of stress-

induced cross-protection responses. Results obtained in the present study, using two *Salmonella* spp. strains with well-characterized stress tolerance systems, confirm that NTAP is capable of inactivating stress-adapted or stress-shocked cultures at the same rate that non-adapted control cultures. Thus, no significant differences were observed among D-values obtained for non-acid adapted and acid adapted *S.* Typhimurium and *S.* Enteritidis cells, or non-shocked and acid, cold or heat shocked cultures, while growth temperature had a minor effect on NTAP resistance, with cells grown at low temperatures (10ºC) being even more sensitive than cells grown in the range 25-45ºC. These findings suggest that adaptive responses triggered by exposure to acid, cold or heat stresses and already described in the past for these two *Salmonella* spp. strains or for other related strains (i.e. modulation of membrane fatty acid composition and membrane fluidity, or synthesis of acid-, cold- or heat-shock proteins) do not provide protection against NTAP treatments. This allows us to conclude that NTAP may be a first-choice technology to be included into food processing schemes following a hurdles technology approach in combination with acidification, mild heating or refrigeration.

Acknowledgements

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Capítulo 3

Effect of Non-Thermal Atmospheric Plasma on food-borne bacterial pathogens on ready-to eat foods: morphological and physico-chemical changes occurring on the cellular envelopes

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Abstract

Currently, there is a need for new technological interventions to guarantee the microbiological safety of ready-to-eat (RTE) foods. Non-thermal atmospheric plasma (NTAP) has emerged as a promising strategy for inactivating microorganisms on thermo-sensitive foods, and the elucidation of its mechanisms of action will aid the rational optimization and industrial implementation of this technology for potential applications in the food industry. In this study, the effectiveness of NTAP for inactivating strains of *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Escherichia coli*O157:H7 and *Listeria monocytogenes* contaminating the surface of different sliced RTE foods ("chorizo", salami, bacon, smoked salmon, tofu and apple) was investigated. In addition, to further assess the bacterial inactivation mechanisms of NTAP, the morphological and physico-chemical damages in bacterial cells were analyzed. NTAP was effective for the surface decontamination of all products tested and, especially, of cut apple, where the microbial populations were reduced between 1.3 and 1.8 log units for the two *Salmonella* strains and *E. coli* O157: H7, respectively, after 15 min of exposure. In the rest of foods, no significant differences in the lethality obtained for the *E. coli* O157:H7 strain were observed, with inactivation rates of between 0.6 and 0.9 log cycles after a 15-min treatment. On the other hand, the strains from the rest of pathogenic microorganisms studied were extremely resistant on tofu, where barely 0.2–0.5 log units of inactivation were achieved after 15 min of plasma exposure. *S.* Enteritidis cells treated for 10 min exhibited noticeable morphological and structural changes, as observed by transmission electron microscopy, which were accompanied by a loss in membrane integrity, with an increased leakage of intracellular components and uptake of propidium iodide and marked changes in regions of their FTIR spectra indicating major alterations of the cell wall components. Overall, this indicates that loss of viability was likely caused for this microorganism by a significant damage in the cellular envelopes. However, the plasma-treated cells of *L. monocytogenes* did not show such obvious changes in morphology, and exhibited less marked effects on the integrity of their cytoplasmic membrane, what suggests that the death of this pathogenic microorganism upon NTAP exposure is more likely to occur as a consequence of damages in other cellular targets.

1. Introduction

Because of changes in consumers' lifestyle, the demand for ready-to-eat (RTE) foods has increased considerably in the last few years. As these food products meet the specific needs of convenience, most of them are consumed without any culinary preparation to eliminate microbial loads. As a result, outbreaks of foodborne illnesses associated with the consumption of RTE foods have been recurrently reported [1]. Indeed, many studies have documented the presence of foodborne bacterial pathogens, including *Salmonella enterica* serovar Enteritidis, *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes* and verocytotoxigenic *Escherichia coli* in different RTE foods [2,3,4,5]. Therefore, there is a need for new technological interventions at the end of food processing in order to mitigate the risks associated with these products while also maintaining the organoleptic and other quality traits desired by consumers. Although several strategies are being explored for this purpose, Non-Thermal Atmospheric Plasma (NTAP) has been the focus of increased research in recent years due to the advantages it presents in comparison to the other microbial inactivation methods, such as its effectiveness at ambient temperature, lack of residual toxicity, low costs of operation and the possibility of food processing inside the package, among others [6,7,8]. Recent studies have shown that NTAP can reduce the microbiological risk of different RTE foods, including both raw and processed foods from animal and vegetable origin, while preserving their quality attributes [9,10,11,12,13].

NTAP is generated by subjecting a gas to an electric discharge under ambient temperature and atmospheric pressure conditions. This leads to the partial ionization of the gas molecules and the formation of large quantities of microbicidal active agents, such as excited and non-excited atoms and molecules, positive and negative ions, free radicals, electrons and ultraviolet radiation. Despite much research efforts, the main cellular targets and the underlying mechanisms of action of NTAP are far from clear. The contribution of ultraviolet (UV) radiation to the antimicrobial effect of NTAP obtained at atmospheric pressure is controversial, while most authors consider that reactive chemical species generated through gas ionization are responsible for the bacterial

inactivation by causing severe damages on various cellular structures and macromolecules, including cellular envelopes, DNA and proteins [6,7,14,15,16,17,18,19,20,21]. The cell envelopes are under investigation as the primary target of NTAP treatments [22,23,24,25,26]. The plasma charged particles could cause mechanical damages to the cell envelopes, either through their physical impact [27] or their accumulation in certain parts of the cell surface resulting in cytoplasmic membrane electroporation [22]. However, reactive oxygen species and reactive nitrogen species are generally considered the NTAP key components that most contribute to the loss of cell viability, especially by oxidizing the polyunsaturated fatty acids of the cytoplasmic membrane [14,28], which are very susceptible to lipid peroxidation phenomena. In fact, it has been reported that lipid peroxidation can compromise the cell viability by modifying membrane properties, decreasing its permeability or even its integrity [6,14,17,19]. To the best of our knowledge, no publication so far has focused simultaneously on the effects of NTAP in the cell structure as shown by of Infrared spectra, electronic microscopy, UV spectrophotometry and flow cytometry.

The objective of this study was to evaluate the effectiveness of NTAP for the inactivation of *Salmonella enterica* serovar Enteritidis, *Salmonella enterica* serovar Typhimurium, *Escherichia coli* O157:H7 and *Listeria monocytogenes* on different sliced foodstuffs, including fermented sausages, such as “chorizo” and salami, bacon, smoked salmon, tofu and freshly cut apple, which might become contaminated with these pathogenic microorganisms through manipulation by food handlers and/or contaminated equipment including cutting boards, knives, and working surfaces. In addition, in order to gain insights into the mode of action of this non-thermal technology, the morphological and physico-chemical changes occurring on the cellular envelopes of these pathogenic microorganisms after NTAP treatments were also determined by monitoring the uptake of propidium iodide (PI), the loss of intracellular contents, the morphological alterations as revealed by transmission electron microscopy (TEM) analysis, and the global changes in cellular biochemical features as revealed by Fourier transform infrared (FTIR) spectroscopy.

2. Materials and Methods

2.1. Microorganisms and Culture Conditions

Salmonella enterica serovar Enteritidis (CECT 4300), *Salmonella enterica* serovar Typhimurium (CECT 443), *Escherichia coli* O157:H7 (ATCC 43895) and *Listeria monocytogenes* (ATCC 15313) were purchased from Colección Española de Cultivos Tipo (CECT, Spanish Type Culture Collection) or American Type Culture Collection (ATCC). Bacteria were revitalized in tubes containing 10 mL of Brain Heart Infusion broth (BHI, Oxoid, UK) at 37 °C for 24 h. These bacterial cultures were streaked onto BHI agar plates which, after their incubation under the same conditions, were stored at refrigeration temperature (4 ± 1 °C) as stock cultures. The experimental cultures were obtained by transferring a single colony from the stock cultures into a sterile tube with fresh BHI, which was incubated at 37 °C for 24 h, entering the stationary phase, with a final cell concentration of approximately 10^8–10^9 cfu/mL.

2.2. Preparation and inoculation of Samples

Commercial vacuum-packed slices of “chorizo”, salami, bacon and smoked salmon, as well as apples (“Fuji” variety) and heat-treated tofu (presented in orthogonal blocks and packed in an aqueous solution) were purchased in a local supermarket. These foods were selected due to their great variability in composition, especially in sodium chloride content, and physicochemical properties (pH, a_w and pH). Tofu and apples were cut to obtain slices of approximately 6 mm thick. Afterwards, slices of all products were cut into 20 mm diameter disks. Prior to inoculation of pathogenic bacteria, all food products, except apple and tofu samples, were subjected to a pre-decontamination step. For this purpose, sliced chorizo, salami, bacon and smoked salmon were pressurized (900 MPa for 5 min) in a model FPG 7100:9/2C Series Foodlab (Standsted Fluid Power Ltd., Essex, UK). After pressurization, the number of microorganisms detected on those samples were below 10 cfu/g, as this is our limit of detection of the methodology used. To inoculate food samples, from bacterial cultures, ten-fold serial

dilutions were prepared for each bacterial strain in sterile 0.1% (*w*/*v*) peptone water (Oxoid). Then, 30 µL of the appropriate dilutions were inoculated onto the surface of foods in order to achieve a final concentration of 10^3–10^4 cfu per disk.

For the assessment of the cellular membrane integrity (PI uptake and measurement of intracellular leakage) and the morphological alterations occurring on the cellular envelopes of *S.* Enteritidis and *L. monocytogenes* (TEM analyses) after NTAP treatments, Whatman polycarbonate membrane filters of 0.2 µ pore retention and 25 mm diameter (Fisher Scientific, Loughborough, UK) were inoculated with each bacterial culture to achieve a cell density of ~10^8–10^9cfu/filter. After spreading out the inoculum on the entire upper surface of filters and food disks, samples were air-dried for 15 min in a laminar flow cabinet (Telstar, model BV-100, Bristol, PA, USA) before NTAP treatments were conducted.

2.3. NTAP Treatments

For the NTAP treatment, a specially designed lab-scale plasma jet (CP121 Plasma Demonstrator OMVE BV, Netherlands) was used following the methodology described by [29]. Further details regarding plasma diagnostics for an equivalent NTAP treatment unit can be found elsewhere [30,31]. The equipment operates at atmospheric pressure and at a temperature below 40 °C [32]. The 0 bias position (oxidoreduction conditions), the 2 mm hole nozzle, air as working gas at a flow rate of 10 L/min, and a voltage difference of 2 kV between electrodes were used. The equipment operated with an output power of 1 watt.

Inoculated food disks were exposed to NTAP at predetermined time intervals, for a period of up to 15 min, whereas inoculated membrane filters were plasma treated for 10 min (an exposure time which was previously shown to reduce the bacterial populations in ca. 1 log reduction. Once treatments were completed, cells were recovered from food disks and membrane filters by transferring them to sterile Falcon tubes containing either 10 mL of 0.1% peptone water or 10 mL of PBS (Merck, Darmstadt, Germany), respectively, and vortexing for 2 min. For the enumeration of

survivors, aliquots of 1 mL were inoculated into petri dishes and 20 mL of molten BHI agar tempered at a temperature of about 45 °C was then added. Once solidified, these agar plates were incubated for 48 h at 37 °C and, subsequently, the number of colonies was counted. Longer incubation times did not increase the number of recovered survivors. The bactericidal effectiveness of NTAP was evaluated via the calculation of Log reductions, by using the formula below:

$$\text{Log reduction} = \text{Log}\ (N_{control}/N_{treated})$$

where:

- $N_{control}$ = number of microorganisms in untreated control samples (CFU)
- $N_{treated}$ = number of microorganisms in NTAP-treated samples (CFU)

In all cases, three independent experiments were performed in different days, using a different bacterial culture in each trial. Also in the experimental set up were included controls, which consisted of inoculated food samples and membrane filters not exposed to NTAP. In each trial, one food sample and membrane filter were tested.

2.4. Fourier Transform Infrared (FTIR) Spectroscopic Analyses

The bacterial cultures obtained as described in *Microorganisms and Culture Conditions* were centrifuged at 11,000 rpm for 20 min at 4 °C. The pellet was resuspended in 1 mL of Ringer solution, inoculating 50 µL on a window of Zn-Se (zinc-selenium), which was dried in an oven (30 min at 37 °C). Then the window was NTAP-treated for 10 min. In parallel, a control consisting of cells not subjected to the action of the plasma was prepared. These experiments were carried out in triplicate.

The infrared (IR) spectrum was obtained using a FT-IR spectrophotometer equipped with a MIRTGS detector (Perkin-Elmer System 2000 FT-IR, Whaltman, MA, USA). The measurements were collected in the wave number range of 3500 to 700 cm^{-1} with an interval of 1 cm^{-1}. The spectral resolution was 4 cm^{-1}. The final spectrum of the samples was obtained by averaging 20 scans. The digitalized IR spectra (comprising a

total of 2800 points) were digitally stored and mathematically transformed, including normalization (0 for absorption at 1800 cm^{-1}, 1 for maximum absorption, located around 1650 cm^{-1}) and the second derivative (Savitzky-Golay algorithm). Afterwards, transformed spectra were recorded in ASCII format and, for calculation purposes, divided into five spectral windows: w_1 (3000–2800 cm^{-1}, influenced by functional groups of membrane fatty acids); w_2 (1800-1500 cm^{-1}, affected by amide I and amide II groups belonging to proteins and peptides); w_3 (1500-1200 cm^{-1}, mixed region influenced by proteins, fatty acids and phosphate-carrying compounds); w_4 (1200–900 cm^{-1}, providing information mostly for carbohydrates and polysaccharides in the cell wall); and w_5 (900–700 cm^{-1}, called “true fingerprint”, because of very specific spectral patterns) [33].

A reproducibility analysis of the triplicates of the IR measurements was performed. Reproducibility measurements (measuring the internal variability) were obtained averaging three independent measures of the Pearson correlation coefficient (between replicates 1–2, 2–3 and 1–3), according to the equation, and this value was expressed as the Differentiation Index, *Di* [34].

$$r_{y1y2} = \frac{\sum_{i=1}^{n} y1_i y2_i - n\,\overline{y1}\,\overline{y2}}{\sqrt{\sum_{i=1}^{n} y1_i^2 - n\overline{y1}^2}\,\sqrt{\sum_{i=1}^{n} y2_i^2 - n\overline{y2}^2}}$$

Where:

- $y1_i$, $y2_i$: individual values of absorbance of the two spectra to be compared.
- n: number of points in the spectrum range to be compared.
- $\overline{y1}$ & $\overline{y2}$: arithmetic means values y1 and y2.
- (r_{y1y2}): value of the correlation coefficient. From this value the Differentiation Index Dy1y2 is defined, according to the equation:

$$D{\cdot}y1{\cdot}y2=(1-ry1{\cdot}y2){\cdot}1000$$

Di can have values between 0 and 2000. It will be zero when the values in the spectral ranges are identical; 1000 when the values represent an inverse correlation, and 2000 for spectra with negative correlation, i.e., uncorrelated spectra.

The calculation of *Di* was obtained for the full spectrum range (ranges 3000–2800 and 1800–700 cm^{-1}) and independently for the five spectral windows described above. A hierarchical clustering analysis was performed on the IR spectra obtained, using the Pearson's correlation coefficient as a measure of similarity between spectra. The final grouping was obtained using the Ward algorithm, checking different spectral windows. All calculations (including calculation of coefficients, grouping of variables, and graphs) were performed with the Statistica for Windows v. Program. 7 (Statsoft Inc., Tulsa, OK, USA).

2.5. Transmission Electron Microscopy (TEM) Analyses

Untreated and 10-min NTAP treated cells of *S*. Enteritidis and *L. monocytogenes* were harvested by centrifugation, fixed in 2.5% glutaraldehyde (TAAB Laboratories Ltd., Aldermaston, Berks, UK)-PBS for 3 h at 4 °C and washed three times with PBS. Cells were then fixed with osmium tetroxide (TAAB Laboratories)-1% PBS for 45 min at room temperature in the darkness. The fixed cells were washed again three times with PBS, pelleted in bacteriological agar (Oxoid, Hampshire, UK), dehydrated by passage through a graded series of etanol solutions and embedded in an epoxy resin (Epon 812; Tousimis, Rockville, MD, USA), which was polarized through its incubation for 48 h at 60 °C. Following this, ultrathin sections were collected onto a copper grid and stained with uranyl and lead. TEM micrographs were taken on at least ten different microscope fields using a JEOL 1010 microscope (JEOL Ltd., Tokio, Japan) at 80 kV.

2.6. Membrane Integrity Tests

2.6.1. Assessment of Propidium Iodide (PI) Uptake

After recovering control untreated and NTAP-treated cells, bacterial suspensions were incubated with 1 µL of PI 1 mg/mL solution (Molecular Probes, Life Technologies, Grand Island, NY, USA) for 10 min at room temperature in the dark in order to allow for its internalization into the permeabilised cells. Afterwards, samples were centrifuged at 3220× *g*and 10 °C for 15 min in order to remove the excess PI. The supernatants were

then discarded and the cellular pellets were resuspended in PBS. Flow cytometry experiments were carried out using a CyAn-adp flow cytometer (Beckman Coulter, Brea, CA, USA). Samples were excited using a 488-nm air-cooled argon-ion laser. The instrument was set up with the following configuration: forward scatter (FS), side scatter (SS) and red fluorescence (613/20 nm) for PI. The cell population was selected by manually gating in a FS *vs* SS dot plot, which allowed for the exclusion of aggregates and cell debris. Fluorescence histograms were represented in single-parameter histograms. Data were analysed with Summit version 3.1 software (Cytomation, Fort Collins, CO, USA).

2.6.2. Measurement of the Leakage of Intracellular Nucleic Acids and Proteins

Bacterial suspensions (3-mL) prepared as described in *NTAP Treatments* were filtered through a 25-mm-diameter, 0.22 µm pore size, Millex-GS syringe filter (Millex-GS, Millipore Co, Billerica, MA, USA) to remove bacteria. Then, the release of nucleic acids and proteins was estimated by measuring the absorbance of the filtrates at 260 and 280 nm, respectively, with a UV/Vis UV-3100PC spectrophotometer (WWR International, Radnor, Pennsylvania).

2.7. Statistical Analysis

The analysis of statistical differences in log reductions and optical density values was carried out through the analysis of variance (ANOVA-one way) after applying the Kolmogorov-Smirnov's test with Lilliefors correction and Levene's test to confirm that the experimental dataset followed a normal distribution and the variances were homogeneous, respectively. Statistically significant differences ($p < 0.05$) between means were detected using a post-hoc Tukey's test. All statistical analyses were carried out using the statistical software IBM SPSS Statistics 21 (Armonk, NY, USA).

3. Results and Discussion

3.1. Effectiveness of NTAP on Bacterial Inactivation

The effectiveness of NTAP for reducing *S.* Enteritidis, *S.* Typhimurium, *E. coli* O157:H7 and *L. monocytogenes* on "chorizo", salami, bacon, smoked salmon, tofu and fresh-cut apple was evaluated after 4, 8 and 15 min of NTAP treatment. The results showed that this non-thermal technology was effective for the inactivation of all the studied foodborne pathogens (Table 1). However, the lethality achieved depended on the three variables tested, i.e., treatment time, type of food and bacterium assayed. Although in all cases an increase in exposure time led to more efficient bacterial inactivation, the increment of the lethality did not follow a first order inactivation kinetic, indicating that the remaining bacterial population may potentially became more tolerant to plasma. The exact nature of this phenomenon is poorly understood but it is frequently observed for various microorganisms after NTAP treatment, including *S*. Typhimurium [32], *E. coli* [35] and *L. monocytogenes* [29,36].

Overall, NTAP treatments were more effective for the microbial decontamination of apple samples, with 1.30, 1.34, 1.47 and 1.79 log reductions being achieved after a 15 min-treatment for *S.* Enteritidis, *S.* Typhimurium, *L. monocytogenes* and *E. coli* O157:H7, respectively. However, no significant differences ($p < 0.05$) in the lethal effects obtained on the rest of food products were observed for *E. coli*, reaching 0.30–0.41, 0.45–0.55 and 0.57-0.89 log reductions after NTAP treatments of 4, 8 and 15 min, respectively. A similar behaviour was observed for *L. monocytogenes* when treated for 4 min (0.29–0.37 log reductions) or 8 min (0.40–0.71 log reductions), whereas a reduced effectiveness of NTAP for the decontamination of tofu was found at the longest treatment time (15 min), with only 0.42 log cycles of inactivation being achieved on this foodstuff for *L. monocytogenes*. The lowest reduction in the population of both *S.* Typhimurium and *S.* Enteritidis was also obtained in tofu, with only 0.46 and 0.22 log reductions being detected, respectively, even with treatment times of up to 15 min. In addition, similarly to the findings for other species, no marked differences were found

in the log reductions attained for each serovar of *Salmonella* among the different foods of animal origin. For example, in the case of *S.* Enteritidis, treatments of 4, 8 and 15 min caused 0.30–0.58, 0.51–0.77 and 0.82–1.09 log reductions, respectively.

Table 1. Log cycles of inactivation obtained for *S.* Enteritidis, *S*. Typhimurium, *L. monocytogenes* and *E. coli* O157:H7 for different NTAP treatment times on different ready-to-eat foods

Treatment Time	Food	Microorganisms			
		***S*. Enteritidis**	***S*. Typhimurium**	***E. coli*O157:H7**	***L. monocytogenes***
4 min	"chorizo"	$0.58 \pm 0.05_{c}^{1,2}$	$0.81 \pm 0.10_{d}^{2}$	$0.36 \pm 0.07_{a}^{1}$	$0.37 \pm 0.13_{a}^{1}$
	salami	$0.37 \pm 0.01_{b}^{1}$	$0.62 \pm 0.13_{cd}^{2}$	$0.32 \pm 0.08_{a}^{1}$	$0.32 \pm 0.08_{a}^{1}$
	bacon	$0.30 \pm 0.04_{ab}^{1}$	$0.34 \pm 0.07_{a}^{1}$	$0.33 \pm 0.04_{a}^{1}$	$0.38 \pm 0.10_{a}^{1}$
	smoked salmon	$0.40 \pm 0.05_{bc}^{1}$	$0.39 \pm 0.06_{abc}^{1}$	$0.30 \pm 0.05_{a}^{1}$	$0.29 \pm 0.01_{a}^{1}$
	tofu	$0.16 \pm 0.06_{a}^{1}$	$0.36 \pm 0.04_{ab}^{1}$	$0.41 \pm 0.0_{a}^{2}$	$0.34 \pm 0.06_{a}^{2}$
	apple	$0.59 \pm 0.15_{c}^{1}$	$0.60 \pm 0.12_{bcd}^{1}$	$1.31 \pm 0.18_{b}^{2}$	$0.89 \pm 0.08_{b}^{1}$
8 min	"chorizo"	$0.77 \pm 0.13_{bc}^{1,2}$	$1.03 \pm 0.07_{d}^{2}$	$0.55 \pm 0.08_{a}^{1}$	$0.71 \pm 0.14_{a}^{1}$
	salami	$0.58 \pm 0.18_{b}^{1}$	$0.75 \pm 0.15_{bcd}^{1}$	$0.48 \pm 0.04_{a}^{1}$	$0.45 \pm 0.09_{a}^{1}$
	bacon	$0.51 \pm 0.09_{b}^{1}$	$0.53 \pm 0.15_{ab}^{1}$	$0.48 \pm 0.04_{a}^{1}$	$0.56 \pm 0.12_{a}^{1}$
	smoked salmon	$0.70 \pm 0.07_{bc}^{2}$	$0.71 \pm 0.09_{bc}^{2}$	$0.45 \pm 0.04_{a}^{1}$	$0.49 \pm 0.01_{a}^{1}$
	tofu	$0.20 \pm 0.07_{a}^{1}$	$0.39 \pm 0.04_{a}^{2}$	$0.50 \pm 0.07_{a}^{2}$	$0.40 \pm 0.06_{a}^{2}$
	apple	$0.96 \pm 0.10_{c}^{1}$	$0.87 \pm 0.11_{cd}^{1}$	$1.50 \pm 0.11_{b}^{2}$	$1.12 \pm 0.26_{b}^{1,2}$
15 min	"chorizo"	$1.01 \pm 0.10_{bc}^{1}$	$1.20 \pm 0.12_{b}^{1}$	$0.89 \pm 0.18_{a}^{1}$	$1.04 \pm 0.17_{bc}^{1}$
	salami	$1.09 \pm 0.10_{bc}^{1}$	$1.10 \pm 0.24_{b}^{1}$	$0.65 \pm 0.13_{a}^{1}$	$0.80 \pm 0.25_{ab}^{1}$
	bacon	$0.82 \pm 0.24_{b}^{1}$	$1.00 \pm 0.35_{ab}^{1}$	$0.64 \pm 0.14_{a}^{1}$	$1.00 \pm 0.20_{bc}^{1}$
	smoked salmon	$0.86 \pm 0.14_{b}^{1,2}$	$1.02 \pm 0.16_{b}^{2}$	$0.57 \pm 0.08_{a}^{1}$	$0.57 \pm 0.10_{ab}^{1}$
	Tofu	$0.22 \pm 0.03_{a}^{1}$	$0.46 \pm 0.03_{a}^{2}$	$0.57 \pm 0.06_{a}^{2}$	$0.42 \pm 0.10_{a}^{2}$
	apple	$1.30 \pm 0.20_{c}^{1}$	$1.34 \pm 0.11_{b}^{1}$	$1.79 \pm 0.06_{b}^{2}$	$1.47 \pm 0.19_{c}^{1,2}$

$^{a–d}$ Mean values for each microorganism at the same treatment time with different superscript are significantly different ($p < 0.05$). $^{1–2}$: For each food, mean values with different superscript in the same row are significantly different ($p < 0.05$)

A great variability in NTAP effectiveness against both vegetative cells and bacterial spores has been reported in the literature when identical treatments were applied onto different surfaces (microstructure) [27,36,37]. This suggests that some surface-associated factors, such as surface roughness, adsorption of diffusing plasma species, and moisture might affect the survival of microorganisms on food. For example,

higher rates of inactivation have been described for seven bacterial species, including *S.* Enteritidis, *S.* Typhimurium, *L. monocytogenes* and *E. coli* O157:H7, on agar than on smoked salmon, suggesting the need to account for surface characteristics [36]. The effectiveness of NTAP as a food decontamination technique has been reported to vary among different foods. Thus, inactivation of *E. coli* was more effective on tomato than on lettuce [38] or strawberries [39], or on carrots than on apples [40]. A greater lethal effect was also observed on cheese slices than on ham [16], on sliced ham than on chicken breast fillets [41], or on tomato than on strawberries [39] for *L. monocytogenes*. Similarly, inactivation of *S.* Typhimurium on lettuce was greater than on strawberries and potato [37]. Overall, it is generally acknowledged that complex food surfaces, such as those with increased roughness and presence of irregularities, may provide numerous sites for microorganisms to fix and hide, thus decreasing the bactericidal effect of NTAP treatments. In fact, some studies have shown that inoculated microorganisms are capable of finding shelter within various food irregularities, such as cracks, grooves or gaps [37,39].

Of particular interest in our results is the fact that no clear differences in the rates of inactivation were observed between Gram-negative (*S.* Enteritidis, *S.* Typhimurium, *E. coli* O157:H7) and Gram-positive (*L. monocytogenes*) bacteria when identical NTAP treatments were applied on the different types of foods studied. Thus, although *S.*Enteritidis exhibited a significant ($p < 0.05$) higher resistance than *E. coli* and *L. monocytogenes* on tofu, minor or no significant inter-specific differences in plasma susceptibility were observed when NTAP treatments were applied to decontaminate slices of “chorizo”, salami, bacon and smoked salmon. For example, no significant differences among species ($p < 0.05$) were found in the log reductions achieved on bacon, regardless of the treatment time. Likewise, 8 and 15 min NTAP treatments caused similar log-reductions on salami, regardless of the bacterial species. Interestingly, despite the minor differences observed, *L. monocytogenes* was not generally more resistant to NTAP than Gram-negative bacteria. Gram-positive bacteria are generally considered to be more resistant to NTAP treatments than Gram-negative bacteria [39]. This is attributed to the presence of a thicker peptidoglycan layer on their

cellular wall, which would provide a greater rigidity and resistance to the diffusion of plasma reactive species through the bacterial cell wall. However, some comparative studies testing the inactivation by NTAP of different bacterial species under identical treatment conditions have found similar resistance for particular species from both Gram-positive and Gram-negative groups [7,25,42,43]. There exist even some reports of strains of *L. innocua* [10], *L. monocytogenes* [44], *B. cereus* [45], *S. aureus* and *E. faecalis* [46] being more sensitive to NTAP than strains of *E. coli, S.* Typhimurium, *S*. Enteritidis, *Vibrio parahaemolyticus* and *Pseudomonas aeruginosa*, respectively. Results obtained in the present study could contribute to explain these apparently contradictory results.

The levels of inactivation obtained in the current study when NTAP treatments were applied on a range of RTE foods are probably not enough to assure, on its own, the safety of the products, especially when complete absence of some microorganisms, such as *Salmonella* spp., is seeked. Therefore, this non-thermal technology should be combined with other surface decontamination strategies in order to increase its antimicrobial effectiveness and achieve the desired microbial reductions. A recent study has described the potential of NTAP to be incorporated within a hurdles technology approaches [36]. An intelligent design of minimal processing strategies requires reliable knowledge on their mechanisms of action. Although recent investigations have tried to elucidate the mechanisms of microbial inactivation by different non-thermal plasmas obtained under atmospheric conditions, the specific mode of action is until date still unknown. Taking into account the large number of different reactive species present in plasma, it is considered that several structures and macromolecules in the cell are likely affected [17,18,21]. However, it has been speculated that the main target are the cellular envelopes [6,17,18,21,22,23,24,25,26]. In this study, several analytical techniques have been applied to characterize the morphological and physico-chemical changes occurring in bacterial cells after their exposure to NTAP for 10 min.

3.2. Bacterial Chemical Changes Induced by NTAP

A study of the infrared spectrum of control-untreated and 10 min-NTAP treated *S.* Enteritidis, *S.* Typhimurium, *E. coli* O157:H7 and *L. monocytogenes* cells was carried out using FT-IR spectroscopy. FT-IR spectroscopy is a physical-chemical method used to interpret the chemical composition of cells, reflecting the composition of cellular components (proteins, polysaccharides, fats and nucleic acids), and constitutes a suitable technique for the study of the molecular changes after exposure to stress [47,48,49]. The analysis of the IR spectra requires a previous processing that allows to minimize the methodological variability and amplify the spectral differences due to chemical variations. A mathematical transformation was carried out that allowed generating much more marked differences between the spectral characteristics of treated and untreated cells. The most important feature of this transformation is the use of derivations to reduce the variability due to the incubation and preparation of the samples.

The IR spectrum of the untreated cells for the four microorganisms studied (controls) was similar to IR spectra obtained in previous studies for *E. coli* O157:H7 [50], *S.* Typhimurium, *S.* Enteritidis [48]. The reproducibility was studied in detail for the five previously defined ranges (w_1 to w_5) and for the full spectrum. Three replicates per strain (NTAP-treated and controls) were processed in independent tests under the same experimental conditions. There are environmental factors that influence the reproducibility of the spectra (such as the manufacturing batch, the preparation of the culture medium, the temperature and the incubation time), as well as the conditions of measurement of the spectrum (the sample preparation, the calibration of the spectrometer and the conditions of measurement) [49]. To obtain classification schemes and perform correct identifications, the intra-replication variability must be minimized to the maximum, which is achieved using a careful standardization of the laboratory protocol and an adequate mathematical pre-treatment of the spectra.When the differentiation index (D_I) is used to evaluate the reproducibility, global values between 7 and 10 are considered adequate when analyzing the replicates of samples prepared in independent trials, and the D_I values can be up to 300 when comparing strains of

different genera [49]. However, these values may vary depending on the types of microorganisms and the differences in chemical composition between them. Figure 1 presents the DI and the global standard deviation calculated for the four microorganisms: *S.* Enteritidis, *S.*Typhimurium, *E. coli* O157:H7 and *L. monocytogenes*, globally (complete IR spectrum) and for the five spectral windows.

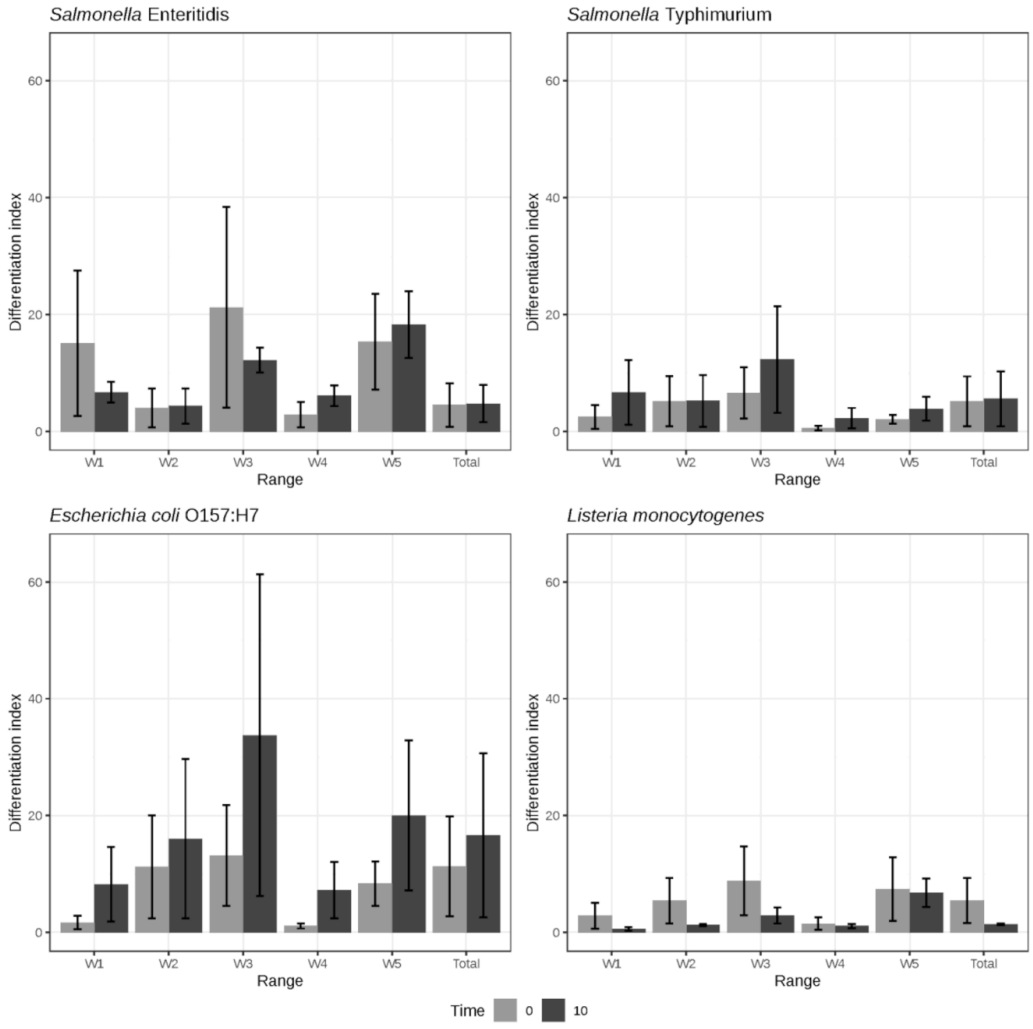

Figure 1. Differentiation index (Di) and global standard deviation calculated for *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Escherichia coli* O157:H7, and *Listeria monocytogenes* for the full IR spectrum and for the five spectral windows.

The results indicate that a good standardization of the conditions of incubation, treatment and processing of the sample, and of the spectroscopic analysis was obtained. Thus, according to the quality criteria for the D_I index previously discussed [49], *S.* Enteritidis, *S.* Typhimurium and *L. monocytogenes* obtained adequate D_I values (in the

case of *S.* Enteritidis the appropriate values were only found in some windows, among them the w_4 used in the cluster analysis, Figure 2).

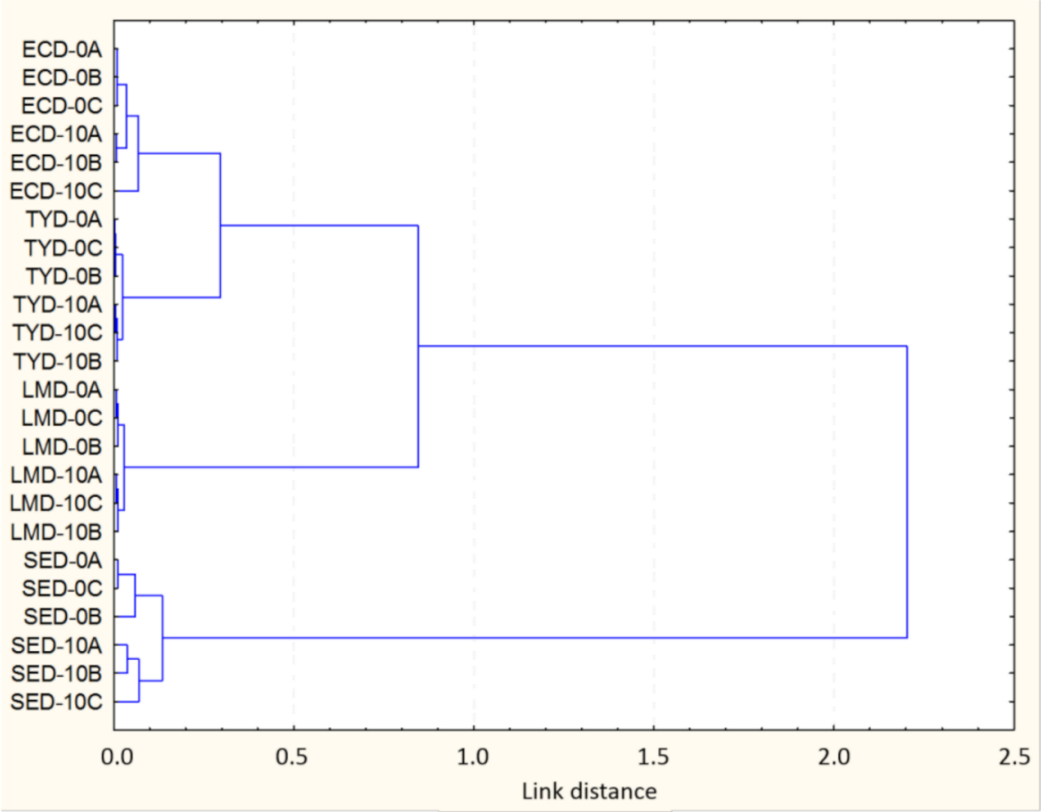

Figure 2. Clustering after the calculation of similarities (Pearson's correlation coefficient) and cluster analysis (Ward method) of the window w_4 of the IR spectrum of *Salmonella* Enteritidis (SED), *Salmonella* Typhimurium (TYD), *Escherichia coli* O157:H7 (ECD), and *Listeria monocytogenes* (LMD) after treatment by NTAP. The numbers indicate the treatment time (0 and 10 min) and the final letter (A, B, C), the replicate.

On the other hand, D_I values were higher, indicating a lower reproducibility, for *E. coli* O157:H7. These results show that the D_I value is a very reliable indicator of the quality of the spectra, since the results are similar to those found with the cluster analysis (Figure 2). Statistically significant differences ($p < 0.05$) were obtained between the different microorganisms. The replicas belonging to *L. monocytogenes* and *S.* Typhimurium offered the lowest $D_I$values, both in the individual ranges and, logically, in the global measure. These data agree with the arrangement and grouping of the replicas observed in the cluster (Figure 2), which are described below. The w_4 range generally obtained the lowest D_I values for both processed and unprocessed samples (0.60 for S.

Typhimurium, 1.13 for *E. coli* O157:H7, 1.53 for *L. monocytogenes*, 2.84 for *S.* Enteritidis). In contrast, windows w_3 and w_5 generally showed the highest $D_I$values (33.74 for *E. coli* O157:H7 in w_3 and 21.22 for *S.* Enteritidis). In the analysis of the D_I values obtained for the NTAP-treated samples vs the control samples, NTAP-treated samples from *S.* Typhimurium and *E. coli* O157:H7 obtained greater variability compared to the untreated ones, in all the windows. However, for *L. monocytogenes*, control samples showed greater variability, and no clear trends were observed for *S.* Enteritidis.

The results show a great difference in the reproducibility observed between windows, which is attributed to changes in the chemical compounds of the sample that are reflected in the IR spectrum. Despite the standardization, the windows w_2, w_3 and w_5 were the most variable. On the other hand, the window w_4 showed a high degree of chemical stability as revealed by low D_I values. These results agree with those previously obtained by us [34], in which values lower than 10 were obtained for the worst reproducibility window (w_5) and lower than 1 for w_4, with intermediate values of 4.58 and standard deviation of 3.05. The D_I value obtained from the replicas can function as a quality parameter of the IR spectra allowing a priori knowledge of the quality and taxonomic or quimiotaxonomic significance of the subsequent cluster analysis. In this way, replicas that do not reach defined values would be discarded automatically.

To represent the existence of differences between strains treated and not treated by PANT, a study was carried out on the changes suffered in the IR spectrum. The hierarchical classification obtained using a cluster analysis of the second derivative of the IR spectra revealed differences between the treated and untreated cells, for the five spectral regions (w_1–w_5), although the w_4 region, with a global distance of 2.3, was the most discriminating region (Figure 2). The results obtained for this spectral region showed that the conveniently transformed average IR spectrum is able to discriminate between intact (controls) and damaged cells. The resulting cluster presented four sub-clusters that grouped separately the strains belonging to each species, with taxonomic significance. Within each cluster, the strains were grouped separating the treated from the untreated (controls) samples. It should be noted that the global link distance was

2.3, intermediate value that indicates that the differences between clusters are adequate, although those present within each cluster are small and limited to small ranges of the window used (w_4). These results agree with those obtained by [51] that show not only the potential of FT-IR spectroscopy to discriminate between intact and damaged cells, but also the ability of the technique to study the molecular aspects of the bacterial response to stress. Interestingly, while it was possible to discriminate treated from untreated cells for all the four microorganisms tested, the differences were more marked for Gram-negative bacteria, and especially for *E. coli* O157:H7 and *S.* Enteritidis, than for Gram-positive ones (*L. monocytogenes*) (Figure 2).

3.3. Bacterial Morphological Changes Induced by NTAP

Figure 3 shows the morphology of cells of *S.* Enteritidis and *L. monocytogenes*, as representative of Gram negative and Gram positive bacteria, respectively, obtained from TEM analysis before and after a 10-min NTAP treatment.

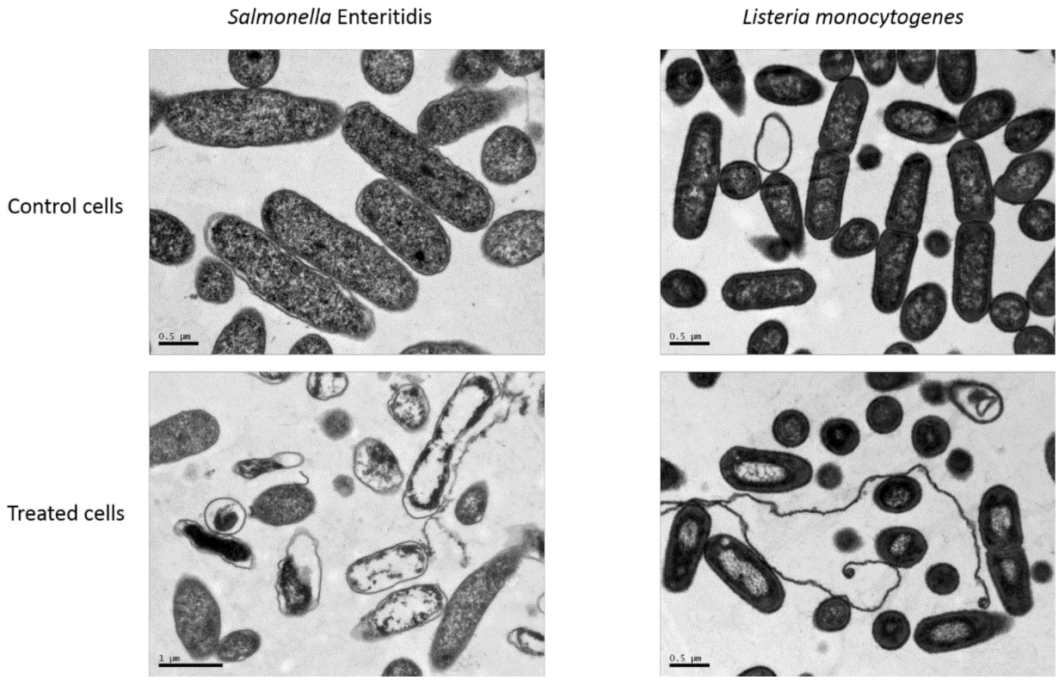

Figure 3. Representative transmission electron micrographs for cells of *Salmonella* Enteritidis and *Listeria monocytogenes* untreated and after NTAP treatment.

The untreated bacteria showed a typical spherical or elliptical shape, a smooth surface, and an intact cell wall and cell membrane. However, NTAP-treated cells of *S.* Enteritidis showed morphological and structural changes with notable shrinkage, deformation of the external shape, disruption or detachment from the cytoplasm of the cellular envelopes, and unevenness of the intracellular content. On the other hand, *L. monocytogenes* did not exhibit such obvious changes in morphology after NTAP treatment, although cells showed some blank spaces in their cytoplasm and condensation of the cytoplasmic material in amorphous compacted regions.

3.4. Damage in Bacterial Membrane Integrity Induced by NTAP

To further study the effects of NTAP, membrane integrity was assessed by measuring the UV absorbing intracellular components (i.e., the nucleic acids and proteins absorbing at 260 and 280 nm, respectively) of cell-free filtrates obtained from untreated and treated samples (Figure 4). While for *L. monocytogenes* no significant ($p > 0.05$) increases in the release of intracellular components were observed after exposure to NTAP, the treatment of *S.* Enteritidis cells markedly increased the content of both nucleic acids and proteins present in the cell-free filtrates in comparison with those obtained in the corresponding filtrates for the untreated control cells.

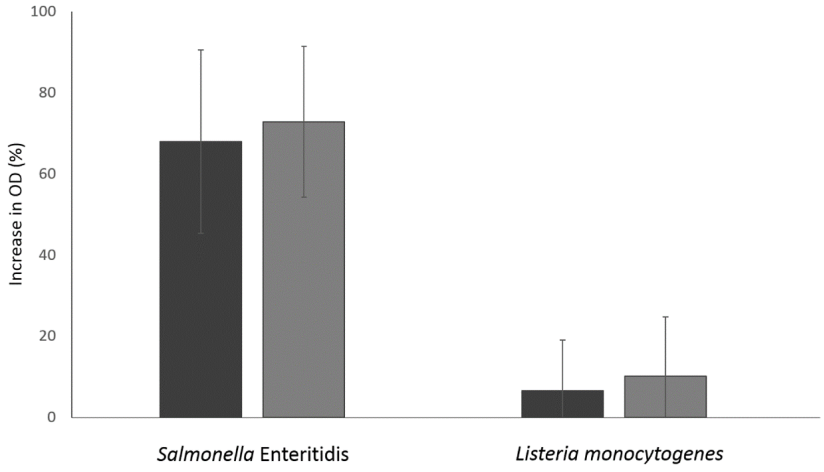

Figure 4. Optical density at 260 (black) and 280 (grey) nm of the cell-free filtrates of *Salmonella* Enteritidis and *Listeria monocytogenes* after their exposure to the action of NTAP. The graph represents the increase, expressed as percentage, in the optical density obtained for the treated cells in relation to that calculated for the untreated cells.

Membrane integrity was also assessed by measuring the intake of PI, a small fluorescent probe that binds to intracellular single- and double-stranded nucleic acids yielding red fluorescence, but cannot passively traverse into cells with intact cytoplasmic membranes [51]. For both *S.* Enteritidis and *L. monocytogenes*, less than 5% of untreated cells were PI stained (3.6% and 1.7%, respectively). However, after a 10-min NTAP treatment, whereas near 100% of *S.*Enteritidis cells (96%) were PI-positive, only a subpopulation of 44% of *L. monocytogenes* cells showed a damaged cytoplasmic membrane (Figure 5). Interestingly, the presence of this fraction of cells with an undamaged membrane correlates well with the concave upward survival curves previously observed for this pathogenic microorganism under identical experimental conditions [29]. It is noteworthy that the inactivation of *S.* Enteritidis followed, on the contrary, an exponential decay with time under the same experimental conditions [52].

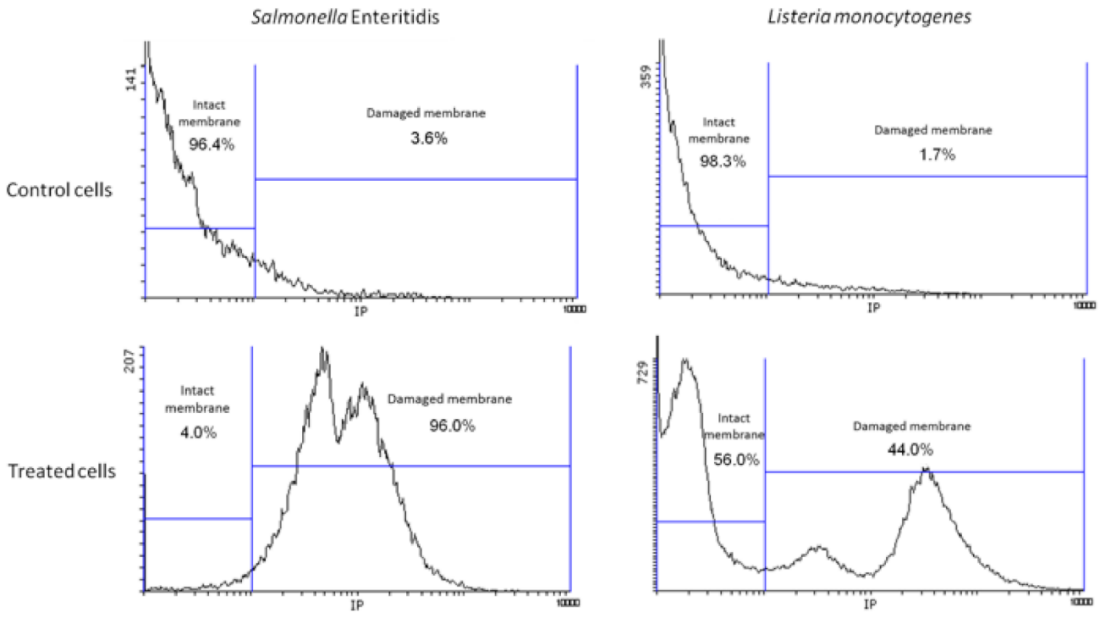

Figure 5. Flow cytometry histograms (red fluorescence) showing the uptake of propidium iodide (PI) by untreated and NTAP- treated cells of *Salmonella* Enteritidis and *Listeria monocytogenes.*

Taking into account the results obtained for *S.* Enteritidis, i.e., the leakage of intracellular nucleic acids and proteins, the intake of PI, the visualization of broken cells and the higher discrimination between untreated and treated cells obtained for this

bacterium in the w_4 window in the FTIR spectroscopic analyses, it can be concluded that NTAP exerts its bactericidal effect in this pathogenic microorganism through the disruption of the cellular envelopes.

4. Conclusions

Results obtained confirm that NTAP is an effective technology in inactivating *S.* Enteritidis, *S.* Typhimurium, *E. coli*O157:H7 and *L. monocytogenes* on the surface of different RTE foods, reducing the microbial population up to 1.8 log units after a 15-min treatment. Although the cell membranes are considered the main target of NTAP, since they represent the first contact barrier with the reactive chemical species generated in plasma, the results obtained in the current study, through the application of different analytical techniques, suggest that the antimicrobial mechanism of action of NTAP could be different for Gram positive and Gram negative bacterial species as had been previously hypothesized [53]. Thus, the damages to the cell envelopes would be the main cause of viability loss for Gram negative bacteria, which would facilitate the action of some antimicrobial agents that, under normal conditions, are ineffective against this bacterial group due to their inability to pass through the outer membrane. This behavior opens the possibility of developing combined processes that would allow reducing the intensity of the treatments and/or increasing the antimicrobial effectiveness of NTAP, thus reducing its possible adverse effects on the quality attributes of foods. However, the inactivation of Gram positive bacteria is probably a consequence of the lesions caused in other cellular targets, such as the DNA and/or enzymes, given the ability of some of the chemical species present in plasma to cross the cell membranes and exert their action at intracellular level, as has been previously demonstrated [14,21,54]. All these aspects require a more in-depth study, since the knowledge on the molecular bases of the mechanisms involved in microbial inactivation would allow progress in the design of more effective treatments and particularly in the use of combined processes for food preservation.

Author Contributions

Conceptualization, T.C.; M.P., A.A.-O. and M.L.; Methodology, M.P., A.A.-O. and M.L.; software, T.C. and M.P.; Validation, T.C., M.P. and A.A.-O.; Formal Analysis, T.C. and M.P.; Investigation, T.C., MP, A.A.-O., M.L.; Resources, M.P., A.A.-O. and M.L.; Data Curation, T.C., M.P. and A.A.-O.; Writing—Original Draft Preparation, T.C., M.P. and A.A.-O.; Writing—Review & Editing, M.P., A.A.-O. and M.L.; Visualization, M.L.; Supervision, M.P., A.A.-O. and M.L.; Project Administration, M.L.; Funding Acquisition, M.P., A.A.-O. and M.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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4

Discusión general

En los últimos años, los alimentos listos para el consumo (en inglés “ready to eat”, RTE) han experimentado un aumento considerable en su demanda en los países desarrollados, motivado, fundamentalmente, por los nuevos hábitos de los consumidores y la gran oferta existente. Su preparación implica operaciones de troceado, loncheado, dosificación, envasado y otras manipulaciones que incrementan el riesgo de contaminación accidental por bacterias patógenas, incluyendo diversas serovariedades de *Salmonella* (principalmente Enteritidis y Typhimurium), *Escherichia coli* O157:H7 *y Listeria monocytogenes*, lo que resulta especialmente preocupante dado que la mayoría de estos alimentos se consumen sin ningún tipo de preparación culinaria que permita eliminar su carga microbiana. De hecho, se han notificado de forma recurrente casos de toxiinfecciones asociados con el consumo de este tipo de productos (EFSA, 2019). Es por ello necesario continuar desarrollando nuevas alternativas o nuevos procesos que puedan ser aplicados en las etapas finales de procesado o en el producto envasado y que permitan su higienización y, al mismo tiempo, mantener los atributos organolépticos y de calidad deseados por los consumidores.

Recientemente se ha conseguido la obtención, a presión atmosférica, de plasma frío, también conocido como plasma atmosférico no térmico (PANT), con capacidad para inactivar microorganismos. Esta nueva tecnología abre un abanico de posibilidades para su uso en la industria alimentaria con el fin de mejorar la calidad microbiológica de los alimentos. Es destacable el hecho de que además de su efecto letal, presenta otras ventajas como el bajo coste de aplicación y el empleo de tiempos de tratamiento cortos. Además, su naturaleza no tóxica y la significativa reducción del consumo de agua y agentes químicos se traduce en una reducción de efluentes que resulta beneficiosa no solo desde un punto de vista económico sino también ambiental.

En estos últimos años se han realizado numerosos trabajos sobre la inactivación microbiana por PANT orientados, especialmente, a conocer los principales factores que determinan su eficacia letal, y estudiar la cinética de inactivación y los mecanismos involucrados en la muerte celular. En esos estudios se ha demostrado que los tratamientos de PANT resultan eficaces frente a una amplia variedad de

microorganismos, que incluirían mohos, levaduras, bacterias e, incluso, esporos y virus, al provocar lesiones importantes en sus envolturas celulares, ADN y/o proteínas, aunque la diana celular que conduce a la pérdida de viabilidad, por el momento, se desconoce. En cualquier caso, en líneas generales, parece que las levaduras y mohos toleran mejor la acción del plasma que las bacterias (Klämpfl y col., 2012; Tseng y col., 2012; Takamatsu y col., 2015), siendo las células vegetativas bacterianas más sensibles que los esporos, aunque también influye la especie (Klämpfl y col., 2012). Sin embargo, en algunos estudios se ha comprobado que la forma de las gráficas de inactivación depende no sólo de la especie bacteriana, sino también del gas empleado (Lee y col., 2011; Patil y col. 2014; Takamatsu y col., 2015). La información disponible acerca la efectividad de los tratamientos en la descontaminación superficial pone de manifiesto que algunos factores desempeñan un papel importante en el grado de inactivación alcanzado. Entre ellos destacan el nivel de contaminación y ciertas propiedades específicas de los alimentos, como porosidad, rugosidad y presencia de irregularidades (Song y col., 2009; Noriega y col., 2011), así como las condiciones de procesado, la configuración del equipo empleado, la energía aplicada y la composición y velocidad de flujo del gas de trabajo (Niemira y Sites., 2008; Ragni y col., 2010; Noriega y col., 2011). Sin embargo, y a pesar de la gran cantidad de estudios desarrollados, aún es necesario realizar un importante esfuerzo investigador en este campo para la implementación de la tecnología de PANT a nivel industrial con garantías de éxito, debido, fundamentalmente, a la dificultad para interpretar los datos obtenidos por los diferentes autores, que utilizan equipos y condiciones de operación muy diversas, lo que se traduce en plasmas muy diferentes en cuanto a sus propiedades y, en consecuencia, de muy distinta efectividad antimicrobiana.

El objetivo de esta Tesis Doctoral ha sido evaluar la aplicación del PANT como estrategia de higienización superficial de alimentos termosensibles, determinando (i) la cinética de inactivación, en diferentes condiciones, de algunos de los microorganismos patógenos de transmisión alimentaria más importantes, (ii) las condiciones óptimas de generación de plasma en relación al tipo de gas precursor y su velocidad de flujo con el fin de lograr el máximo efecto letal, (iii) las repercusiones que el historial previo de los

cultivos bacterianos tiene sobre el efecto letal obtenido, (iv) el impacto que presentan las características de los alimentos sobre la eficacia antimicrobiana del plasma y (v) el mecanismo de inactivación bacteriana por acción del PANT.

Resulta importante destacar que, con el fin de que todos los resultados fueran directamente comparables, en el desarrollo de esta investigación se ha utilizado el mismo equipo de generación de PANT, CP121 Plasma Demonstrator OMVE, que además de haber sido validado y diseñado para crear un plasma bien caracterizado (Mastwijk y col., 2009; Mols y col., 2013), está construido en un formato que, con ligeras modificaciones en su diseño, podría ser usado a escala industrial.

4.1 Cinética de inactivación microbiana por PANT

Los estudios existentes en la bibliografía encaminados a evaluar la resistencia de los microorganismos, tanto alterantes como patógenos, frente a la acción del PANT se han llevado a cabo por dos procedimientos: (i) el método del punto final, o (ii) a través de la obtención de gráficas de supervivencia. En el método del punto final la resistencia se estima a partir de dos únicos valores, el número inicial de microorganismos y el de supervivientes tras un tiempo fijo de tratamiento. Este sistema, aunque simple, no permite predecir la evolución de la muerte de los microorganismos durante el tratamiento ni conocer la existencia de desviaciones del orden logarítmico asumido en la inactivación microbiana. Por el contrario, el método de las gráficas de supervivencia, en el que se representa el logaritmo del número de supervivientes (o el logaritmo de la fracción de supervivientes) en función del tiempo de exposición a un determinado tratamiento tecnológico, permite conocer el ritmo real de inactivación durante el tratamiento y estudiar detalladamente la evolución de la muerte microbiana, facilitando, por tanto, la comprensión de los distintos fenómenos que van a conducir a la misma. En el desarrollo de esta Tesis Doctoral se obtuvieron en las diferentes condiciones experimentales ensayadas las correspondientes gráficas de supervivencia para *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Listeria monocytogenes*, *Listeria innocua* y *Escherichia coli* O157:H7, encontrándose que éstas presentaban dos perfiles

claramente diferenciados. Cuando el PANT se aplicó para la descontaminación superficial de la superficie abiótica estudiada (filtros de policarbonato), independientemente de las condiciones de generación del plasma (composición y velocidad de flujo del gas precursor del plasma) e historial previo de los microorganismos (temperatura de incubación, tipo de agente acidificante y pH del medio de crecimiento), las curvas de supervivencia de *S*. Typhimurium y *S*. Enteritidis siguieron la tradicional cinética de primer orden, mientras que las exhibidas por *L. monocytogenes* y *L. innocua* presentaron un perfil cóncavo. Sin embargo, las curvas de supervivencia obtenidas cuando los microorganismos fueron inoculados en la superficie de distintos alimentos exhibieron en todos los casos un perfil cóncavo. Esta desviación de la linealidad, caracterizada por la aparición de retrasos en la inactivación al final de las gráficas de supervivencia, indica que a medida que avanza el tiempo de exposición al plasma se requieren tratamientos progresivamente más prolongados para inactivar la misma fracción de células supervivientes.

Aunque no se conoce con exactitud la naturaleza de esta desviación de la linealidad, conocida como "cola", descartando posibles artefactos metodológicos (presencia de agregados, mezcla de células vegetativas y esporos, etc.), se puede atribuir a la existencia en la población de una fracción de microorganismos mucho más resistentes al plasma o a que éstos resulten protegidos frente al efecto letal del tratamiento a medida que avanza el mismo. La existencia de microorganismos con diferente resistencia al PANT en una población determinaría que al comienzo del tratamiento se inactivarían más rápidamente los más sensibles y, a continuación, cuando sólo quedasen los más resistentes, se observaría un cambio en la pendiente de la gráfica de supervivencia. La segunda posibilidad está relacionada con la escasa capacidad de penetración del plasma y la protección originada por ciertas características superficiales de los alimentos (rugosidad, porosidad, presencia de irregularidades,..) e incluso proporcionada por las células muertas o por diferentes componentes metabólicos celulares, tales como ciertas proteínas capaces de secuestrar las especies citotóxicas del plasma (Kamgang-Youbi y col., 2008), protegiendo así a los microorganismos viables. En este sentido, se ha demostrado que la adición de células

inactivadas térmicamente puede proteger a *S.* Typhimurium de la acción del PANT (Fernández y col., 2012). Al margen de estas hipótesis, algunos autores que consideran que la radiación UV-C, a través de su interacción con las especies químicas reactivas generadas, juega un papel de cierta importancia en la inactivación bacteriana por PANT, han postulado que la rápida velocidad de inactivación observada durante los primeros tiempos de tratamiento es debida a los efectos de los fotones UV sobre el ADN celular y que la segunda fase de inactivación, más lenta, se debe a la acción de especies químicas reactivas (Moisan y col., 2001; Dasan y col., 2016). No obstante, independientemente del mecanismo responsable de este fenómeno, se ha observado repetidamente la aparición de "colas" en las gráficas de supervivencia obtenidas para diversos microorganismos expuestos a la acción del PANT (Muranyi y col., 2008; Jung y col., 2010; Fernández y col., 2013; Akan y Çabuk, 2014; Heo y col., 2014; Jahid y col., 2014; Flynn y col., 2015; Reineke y col., 2015; Dasan y col., 2016; Lunov y col., 2016; Smet y col., 2016). Para describir este tipo de gráficas de supervivencia se han propuesto diferentes modelos matemáticos, siendo el basado en la distribución de Weibull (Mafart y col., 2002) uno de los más utilizados, al permitir el ajuste de gráficas de supervivencia con desviaciones de la linearidad distintas.

En consecuencia, para comparar en las diferentes condiciones estudiadas la resistencia de las células al PANT o definir el tiempo requerido para conseguir un determinado efecto letal se utilizaron, respectivamente, el valor D (tiempo necesario para reducir a la décima parte la población microbiana), que se calculó cuando existía una relación lineal entre el logaritmo de la fracción de supervivientes y el tiempo de tratamiento, o los parámetros cinéticos del modelo matemático basado en la distribución de Weibull (parámetro de forma (ρ); parámetro de escala (δ)) cuando las gráficas exhibieron un perfil cóncavo.

4.2 Influencia de las condiciones de generación del plasma, gas precursor y velocidad de flujo, sobre la efectividad antimicrobiana del PANT

En numerosos estudios se ha comprobado que la naturaleza del gas y la velocidad de flujo utilizadas para la obtención del PANT determinan marcadamente las especies químicas producidas y su proporción y, en consecuencia, las propiedades antimicrobianas de los plasmas resultantes. Sin embargo, aunque en alguna ocasión se había descrito que los plasmas de nitrógeno (Lee y col., 2011; Takamutsu y col., 2015) y aire (Marsili y col., 2002; Niemira, 2012) eran de los más efectivos en la inactivación de diversos microorganismos patógenos y alterantes, no existía un acuerdo sobre cuál de ellos resultaba el más eficaz. En relación a la influencia ejercida por la velocidad de flujo, las conclusiones de los diferentes autores que habían evaluado este parámetro resultaban contradictorias. Mientras que en algunos casos las tasas de inactivación conseguidas aumentaban con la velocidad de flujo (Niemira y Sites, 2008; Lai y col., 2016), en otros, un incremento de ésta iba acompañado de una pérdida de eficacia (Edelblute y col., 2015) e incluso también en algún caso se había observado la existencia de una velocidad de flujo óptima (Miao y Jierong, 2009).

En esta Tesis se ha evaluado la efectividad frente a *S.* Enteritidis, *S.* Typhimurium, *L. monocytogenes* y *L. innocua* de los plasmas obtenidos, en condiciones atmosféricas, a partir de aire y nitrógeno a tres velocidades de flujo (5, 10 y 15 l/min), con el fin de definir las condiciones óptimas de generación de PANT. En todos los casos, las mayores tasas de inactivación se consiguieron al emplear aire como gas de trabajo. La mayor efectividad de los plasmas a base de aire podría estar relacionada, probablemente, con la presencia de oxígeno y la consiguiente mayor formación de especies reactivas de oxígeno. El uso de nitrógeno como gas de trabajo hizo que el tratamiento con plasma fuera casi ineficaz frente a *L. monocytogenes* y *L. innocua*, encontrando tasas de inactivación inferiores a 1 ciclo logarítmico para ambos microorganismos al aplicar 4 min de tratamiento, incluso cuando se utilizó el flujo de gas más alto (15 l/min). Los tiempos de reducción decimal obtenidos para S. Typhimurium y S. Enteritidis al emplear aire, que oscilaron, respectivamente, entre 0,86 y 2,43 min y entre 0,90 y 1,69 min, también se

vieron incrementados hasta valores comprendidos entre 3,08 y 5,75 min y 2,28 y 5,54 min, respectivamente, al utilizar nitrógeno como gas precursor.

Estos resultados concuerdan con los obtenidos por otros autores que encontraron un mayor efecto letal para los plasmas obtenidos a partir de oxígeno (Rowan y col., 2007), o que la adición de pequeñas cantidades de oxígeno a algunos gases nobles, como helio (Gweon y col., 2011; Kim y col., 2011; Lee y col., 2011, 2012a; Noriega y col., 2011; Galvin y col., 2013), argón (Surowsky y col., 2014) y nitrógeno (Lee y col., 2011, 2012b), mejoraba la inactivación microbiana tanto de bacterias esporuladas como de células vegetativas, incluyendo *L. innocua*, *L. monocytogenes*, *S.* Typhimurium y *S.* Enteritidis, lo que era atribuido a una mayor formación de radicales hidroxilo e hidroperoxilo, oxígeno atómico, peróxido de hidrógeno, oxígeno singlete y ozono. En cualquier caso, el hecho de que los microorganismos muestren una mayor sensibilidad a los plasmas a base de aire presenta una ventaja evidente desde el punto de vista económico y logístico.

En relación al efecto ejercido por la velocidad de flujo del gas sobre la efectividad de los tratamientos por PANT se pudo comprobar que, en general, un aumento de ésta mejoró las propiedades antibacterianas de los plasmas resultantes, probablemente debido a que muchas de las especies reactivas con una vida corta no alcanzarían a los microorganismos a bajas velocidades de flujo. En el caso concreto del aire, un incremento de 5 a 10 l/min mejoró marcadamente el efecto bactericida logrado, aunque al aplicar velocidades superiores a 10 l/min el incremento ocasionado en las tasas de inactivación no resultó tan marcado. Sin embargo, cuando se utilizó nitrógeno para generar el plasma la velocidad de flujo apenas afectó a la eficacia del tratamiento frente a *L. monocytogenes* y *L. innocua*. Este conjunto de resultados contribuiría a explicar las conclusiones aparentemente contradictorias descritas en la bibliografía en cuanto a la influencia de este parámetro sobre la eficacia antimicrobiana de esta tecnología.

En base de los resultados obtenidos en este ámbito, los experimentos sobre la influencia ejercida por el historial previo de los microorganismos y las características de la superficie en la que éstos se encuentran durante el tratamiento por PANT en la

efectividad antibacteriana del mismo, así como las investigaciones acerca del mecanismo de acción de esta tecnología, fueron llevados a cabo utilizando plasmas de aire obtenidos a una velocidad de flujo de 10 l/min.

4.3 Influencia de las condiciones de exposición de los microorganismos sobre su posterior resistencia al PANT

Aunque la resistencia microbiana frente al calor y las tecnologías no térmicas de inactivación está determinada en gran medida por las condiciones de crecimiento de los microorganismos y por su exposición previa a situaciones adversas (Knabel y col., 1990; Juneja y col., 1998; Pagán y col., 1999; Alvarez y col., 2002; Bull y col., 2005; Hayman y col., 2007; Alvarez-Ordóñez y col., 2008, 2009, 2010; Shearer y col., 2010; Juck y col., 2012; Álvarez-Ordóñez y col., 2015; Teixeira y col., 2016), estos aspectos han sido poco estudiados en el caso del PANT, a pesar de que su conocimiento resulta imprescindible para establecer tratamientos efectivos. En consecuencia, en esta Tesis se ha evaluado la influencia ejercida por la temperatura de incubación y el pH del medio de cultivo, así como por la exposición de los microorganismos, durante diferentes períodos de tiempo (5 y 120 min), a diferentes condiciones subletales de calor (45ºC), acidez (pH 4,5) y frío (0ºC) sobre la efectividad del PANT en la inactivación de *L. monocytogenes*, *S.* Typhimurium y *S.* Enteritidis.

En los experimentos encaminados a estudiar el efecto de la temperatura de crecimiento de los microorganismos se utilizaron cultivos bacterianos en fase estacionaria incubados en un amplio rango de temperaturas (10, 25, 37 y 42ºC para *L. monocytogenes* y 10, 25, 37 y 45ºC para *S*. Tyhimurium y *S.* Enteritidis). Los resultados obtenidos pusieron de manifiesto que, en ningún caso, un cambio de la temperatura en el intervalo entre 25 y 45ºC modificaba significativamente ($p>0,05$) la efectividad antimicrobiana del PANT, hallándose para *L. monocytogenes* valores δ (tiempo necesario para inactivar el primer ciclo logarítmico de la población microbiana) comprendidos entre 0,12 ± 0,03 y 0,17 ± 0,06 minutos y para *S*. Tyhimurium y *S.* Enteritidis valores D que oscilaron entre 1,08 ± 0,35 y 1,38 ± 0,39 min y entre 0,73 ± 0,12

y 1,23 ± 0,36 min, respectivamente. Sin embargo, la incubación a 10ºC dio lugar a células de *S*. Tyhimurium y *S.* Enteritidis ligeramente más sensibles al plasma, con valores D, respectivamente, de 0,68 ± 0,11 y 0,45 ± 0,10 min. Tras una exhaustiva revisión bibliográfica, tan sólo hemos encontrado dos trabajos en los que se ha estudiado el efecto de la temperatura de incubación sobre la resistencia microbiana al PANT, referidos a *S.* Typhimurium para el intervalo 20-45ºC (Fernández y col., 2013) y *Aeromonas hydrophila* entre 4 y 30ºC (Jahid y col., 2014), con resultados cualitativamente semejantes. Para ambas bacterias, temperaturas de incubación próximas a la óptima de crecimiento, entre 20 y 45ºC, tuvieron escasa influencia en la resistencia de las células, aunque la incubación a temperaturas inferiores a 20ºC iba acompañada de una ligera sensibilización de *A. hydrophila* al tratamiento.

Para la determinación de la influencia ejercida por el pH del medio de cultivo sobre la efectividad del PANT frente a *L. monocytogenes*, *S.* Typhimurium y *S.* Enteritidis, se utilizaron células en fase estacionaria tras su incubación a 37ºC en caldo Brain Heart Infusion (BHI) acidificado hasta valores de pH de 4,5 con diversos ácidos orgánicos comúnmente empleados por la industria alimentaria (acético, ascórbico, cítrico, láctico, y málico), así como con ácido clorhídrico (células adaptadas a la acidez). Asimismo, las cepas de *L. monocytogenes*, *S.* Typhimurium y *S.* Enteritidis también fueron crecidas en dos condiciones control, en BHI no acidificado (células no acidificadas) y BHI tamponado a pH 7,0 con tampón Sorensen (células no adaptadas a la acidez). Los valores D medios obtenidos para las células no adaptadas y adaptadas a la acidez de *S*. Typhimurium (1,52 ± 0,29 min *vs* 1,50 ± 0,15 min) y *S.* Enteritidis (0,98 ± 0.17 min *vs* 1,25 ± 0,16 min), así como los correspondientes valores δ calculados para las células no adaptadas (0,17 ± 0,05 min) y adaptadas (0,13 ± 0,01 min) a la acidez de *L. monocytogenes* mostraron que la acidificación del medio de crecimiento, independientemente del agente acidificante empleado, no originaba modificaciones significativas ($p>0,05$) en la eficacia de esta tecnología para la inactivación de los patógenos estudiados.

El hecho de que la efectividad antimicrobiana del PANT se vea mínimamente afectada por las condiciones a las que previamente han estado expuestos tres de los

patógenos de transmisión alimentaria más importantes supondría una ventaja adicional para la implantación de esta tecnología a nivel industrial. Son numerosos los autores que han comprobado que el crecimiento en condiciones subóptimas y/o la exposición de diversas especies bacterianas, incluyendo *S.* Enteritidis, *S.* Typhimurium y *L. monocytogenes* (Shearer y col., 2010; Juck y col., 2012; Álvarez-Ordoñez y col., 2012, 2015; Teixeira y col., 2016; Smet y col., 2016), a condiciones ambientales adversas, puede ocasionar el desarrollo de respuestas adaptativas, lo que puede traducirse en un aumento de su posterior resistencia, no sólo frente al agente inductor de las mismas sino también frente a otras condiciones de estrés o tratamientos tecnológicos, entre los que se incluirían los diferentes procedimientos de conservación de los alimentos. En este sentido, se ha demostrado previamente que el crecimiento de las células de la misma cepa (CECT 443) de *S.* Typhimurium utilizada en esta Tesis en presencia de ácido acético, ascórbico, cítrico, láctico, málico y clorhídrico hasta un valor de pH de 4,5 incrementaba su posterior capacidad de supervivencia tanto frente a ambientes ácidos (pH 3) y alcalinos (pH 11) extremos como frente a un tratamiento térmico (58ºC) (Álvarez-Ordoñez y col., 2008, 2009, 2010, 2011, 2012). Asimismo, el aumento de la temperatura de crecimiento, desde 10 a 45ºC, conducía a la obtención de células más tolerantes al calor, con valores D_{58} de hasta 10 veces más altos (Alvarez-Ordóñez y col., 2008). Estos estudios previos demostraron que en estas respuestas adaptativas estaba implicada la inducción de ciertos sistemas homeostáticos de reparación de macromoléculas, de mantenimiento del pH intracelular y/o de modulación de la fluidez de las membranas celulares. Sin embargo, todos estos cambios fisiológicos no parecen proporcionar un efecto protector frente a la acción del PANT, permitiendo, en consecuencia, optimizar los tratamientos con una menor incertidumbre.

Además, los resultados obtenidos tras exponer los microorganismos patógenos estudiados a los distintos agentes estresantes empleados, a los que frecuentemente se ven sometidos durante la producción y distribución industrial de los alimentos y que se sabe que reducen la efectividad de otros métodos de conservación, evidenciaron la incapacidad de estos microorganismos para desarrollar respuestas de protección cruzada frente al PANT, por lo que esta tecnología podría ser de primera elección para

su inclusión en procesos combinados que incorporen un proceso de acidificación, calentamiento moderado o refrigeración.

4.4 Influencia de las características de las superficies en la efectividad antimicrobiana del PANT

La efectividad antimicrobiana del PANT está marcadamente determinada por las características superficiales del sustrato en el que se encuentran las bacterias durante el tratamiento. De hecho, resultó más eficaz para la descontaminación de superficies abióticas que para la de los alimentos estudiados (chorizo, salami, beicon, salmón ahumado, tofu y manzana). Por ejemplo, un tiempo de exposición tan corto como 30 segundos a la acción de un plasma generado a partir de aire a una velocidad de flujo de 10 l/min logró reducir la población de *S.* Enteritidis, *S.* Typhimurium y *L. monocytogenes* en filtros de policarbonato entre 0,6 y 1,6 ciclos logarítmicos, mientras que tan sólo se conseguían entre 0,16 y 0,89 ciclos logarítmicos de inactivación en los alimentos, incluso tras prolongar el tiempo de tratamiento hasta 4 minutos.

Diversos autores (Chen y col., 2009; Yong y col., 2015) han observado la gran influencia que ejercen las características superficiales del sustrato en el que se encuentran los microorganismos en la efectividad del PANT. Aunque la comparación directa de los resultados obtenidos en estos estudios resulta muy difícil debido a las diferencias en la metodología, equipos, condiciones de operación para generar el plasma, microorganismos y matrices utilizadas, en general, de los estudios existentes en la bibliografía se desprende que, en superficies lisas y pulidas el PANT resulta muy eficaz en la inactivación tanto de células vegetativas como de formas esporuladas (Niemira y Sites, 2008; Perni y col., 2008; Song y col., 2009; Noriega y col., 2011; Bermúdez-Aguirre y col., 2013; Fernández y col., 2013; Kim y col., 2014; Butscher y col., 2016; Cui y col., 2016), mientras que las superficies rugosas, porosas y con presencia de irregularidades, como son las de los alimentos, proporcionan numerosas oportunidades para que los microorganismos eviten la exposición directa a los componentes citotóxicos del plasma (Bermúdez-Aguirre y col., 2013; Butscher y col., 2016). De hecho, se ha observado por

microscopía electrónica cómo los microorganismos inoculados en diversos alimentos son capaces de introducirse en diversas irregularidades, grietas, ranuras, oquedades, etc. (Bermúdez-Aguirre y col., 2013; Fernández y col., 2013; Jahid y col., 2014; Ziuzina y col., 2014; Hertwing y col., 2015; Yong y col., 2015; Butscher, 2016). Así, por ejemplo, Fernández y col. (2013) lograron una reducción de la viabilidad de *S.* Typhimurium de 2,7 unidades logarítmicas en filtros de policarbonato tras un tratamiento de 2 minutos, mientras que se requerían 15 minutos para alcanzar 0,9 ciclos logarítmicos de inactivación en patata cortada. Resultados similares han sido obtenidos con otros microorganismos. Lee y col. (2011) al comparar la inactivación de *L. monocytogenes* en agar y en pechuga de pollo, obtuvieron 3 ciclos logarítmicos menos de inactivación en el alimento tras un tratamiento de 2 minutos. Butscher (2016) también observó que el número de esporos viables de *Geobacillus stearothermophilus* se reducía rápidamente en polipropileno, logrando, tras 10 minutos de tratamiento, 5 unidades logarítmicas de inactivación, mientras que en granos de trigo tan solo se obtenían 3 ciclos logarítmicos, incluso tras prolongar el tiempo de tratamiento hasta 60 minutos.

Por otra parte, también se detectó la existencia de una gran variabilidad en las tasas de inactivación conseguidas para *S.* Enteritidis, *S.* Typhimurium, *E. coli* O157:H7 y *L. monocytogenes* cuando tratamientos idénticos por PANT, de 4, 8 y 15 minutos de duración, eran aplicados a diferentes alimentos, tanto de origen animal (chorizo, salami, bacón y salmón ahumado) como vegetal (tofu y manzana), resultando esta tecnología más eficaz en la descontaminación de la manzana cortada, en la que se logró reducir la población de los patógenos entre 1,3 (para *S.* Enteritidis) y 1,8 (para *E. coli* O157:H7) unidades logarítmicas tras 15 minutos de exposición. Aunque no se detectaron diferencias significativas ($p \geq 0,05$) en el efecto letal obtenido frente a *E. coli* O157:H7 cuando el patógeno fue inoculado superficialmente en el resto de los alimentos estudiados, con tasas de inactivación comprendidas entre 0,6 y 0,9 ciclos logarítmicos tras un tratamiento de 15 min, las dos serovariedades de *Salmonella* y *L. monocytogenes* fueron altamente resistentes en tofu, donde apenas se lograron 0,2-0,5 unidades logarítmicas de inactivación tras un tiempo similar de exposición al plasma. Estos resultados concuerdan con los observados en otros estudios en los que se ha

evidenciado una gran variabilidad en las tasas de inactivación logradas para una determinada especie microbiana cuando se aplicaban idénticos tratamientos a distintos alimentos. En el caso concreto de *E. coli*, se ha descrito que su inactivación resulta más efectiva en tomate que en lechuga (Bermúdez-Aguirre y col., 2013) o en fresas (Ziuzina y col., 2014), siendo, asimismo, más rápida en zanahorias que en manzana (Baier y col., 2015). También se encontró un mayor efecto letal frente a *L. monocytogenes* en lonchas de queso que en jamón loncheado (Song y col., 2009), en lonchas de jamón que en filetes de pechuga de pollo (Lee y col., 2011) o en tomate que en fresas (Ziuzina y col., 2014). De manera similar, la inactivación de *S.* Typhimurium en lechuga fue mayor que en fresas y patatas (Fernández y col., 2013). Todo este conjunto de resultados sugiere que algunos factores asociados a la superficie de los alimentos, como la rugosidad, la adsorción de especies reactivas del plasma, o la humedad, podrían afectar a la supervivencia de los microorganismos en los alimentos, lo que debería considerarse a la hora de diseñar tratamientos eficaces por PANT.

Otro hallazgo interesante fue el hecho de que a pesar de que se apreciaron pequeñas diferencias en las tasas de inactivación conseguidas para los distintos patógenos estudiados cuando se aplicaron tratamientos idénticos a los diferentes tipos de alimentos, *L. monocytogenes* no resultó el microorganismo más resistente frente al PANT. En general, suele considerarse que las bacterias Gram positivas exhiben una menor susceptibilidad al plasma que las Gram negativas debido a que presentan una capa más gruesa de peptidoglicano en su pared celular, proporcionando una mayor resistencia a los daños mecánicos causados por el PANT y dificultando la difusión de las especies reactivas a través de las membranas (Lee y col., 2006; Fröhling y col., 2012; Ziuzina y col., 2014, 2015; Edelblute y col., 2015; Jayasena y col., 2015; Yong y col., 2015; Lunov y col., 2016; Pasquali y col., 2016; Smet y col., 2016; Puligundla y col., 2016, 2017). Sin embargo, algunos autores que compararon la resistencia al plasma, en condiciones idénticas de tratamiento, han encontrado una resistencia similar para determinadas especies de bacterias Gram-positivas y Gram-negativas (Lee y col., 2006; Tseng y col., 2012; Klämpfl y col., 2012; Takamatsu y col., 2015) e incluso se ha observado en algunas ocasiones que *L. innocua* (Baier y col., 2014), *L. monocytogenes* (Gabriel y col., 2016)*, B.*

cereus (Marsili y col., 2002), *S. aureus* (Mok y col., 2015) y *E. faecalis* (Nishime y col., 2017) eran más sensibles que *E. coli, S.* Typhimurium, *S.* Enteritidis*, Vibrio parahaemolyticus* y *Pseudomonas aeruginosa,* respectivamente. Los resultados obtenidos en la presente Tesis Doctoral podrían contribuir a explicar estos resultados aparentemente contradictorios descritos en la bibliografía.

4.5 Mecanismo de inactivación microbiana por el PANT

Con el fin de contribuir a elucidar el mecanismo de inactivación por el PANT en esta Tesis Doctoral se han evaluado, aplicando diferentes técnicas, los cambios morfológicos y físico-químicos que tienen lugar en las células tras su exposición durante 10 minutos a la acción del PANT.

Los cambios en la composición química celular se determinaron por espectroscopía de infrarrojos con transformada de Fourier (FTIR). La espectroscopía FTIR es un método físico-químico usado para estudiar la composición química celular, que aporta información sobre los grupos funcionales de los principales componentes celulares: proteínas, polisacáridos, grasas y ácidos nucleicos (Curk y col., 1994; Álvarez-Ordoñez y col., 2010; Álvarez-Ordoñez y col., 2012). Para detectar la existencia de cambios químicos en las células de *S.* Enteritidis, *S.* Typhimurium y *L. monocytogenes*, se realizó un estudio sobre las modificaciones inducidas en los espectros FTIR, que exigió un procesado previo de los mismos que minimizara la variabilidad metodológica y amplificara las diferencias espectrales debidas a las variaciones químicas. Para ello, se llevó a cabo una transformación matemática que permitió generar diferencias mucho más marcadas entre las características espectrales de células tratadas y no tratadas. El rasgo más importante de esta transformación es el empleo de derivatizaciones para reducir la variabilidad debida a las condiciones de incubación y preparación de las muestras. Asimismo, se desarrolló una clasificación jerárquica empleando un análisis de clusterización de la segunda derivada de los espectros FTIR, lo que reveló la existencia de diferencias entre las células tratadas y no tratadas para las cinco regiones espectrales (w_1-w_5) estudiadas, aunque la región w_4 (región del espectro FTIR que se caracteriza por

la absorción de radiación infrarroja por parte de algunos grupos funcionales característicos de los polisacáridos) fue la más discriminante. Además, cabe destacar que las diferencias resultaron más marcadas para las bacterias Gram negativas, especialmente para *E. coli* O157:H7 y *S.* Enteritidis, que para *L. monocytogenes*.

Las imágenes obtenidas mediante microscopía electrónica de transmisión de células tratadas por PANT de *S.* Enteritidis y *L. monocytogenes*, como representativas de las bacterias Gram negativas y Gram positivas, respectivamente, evidenciaron que el tratamiento inducía importantes cambios en la morfología y estructura de las células de *S.* Enteritidis, que mostraban una notable contracción, deformación de su forma externa, aparición de un espacio entre la membrana citoplasmática y la pared celular e irregularidad del contenido intracelular. Sin embargo, las células de *L. monocytogenes* no exhibieron tras el tratamiento modificaciones tan intensas, aunque también se observaron células que mostraban algunos espacios vacíos en su citoplasma y condensación de material citoplasmático en regiones compactas amorfas.

Asimismo, se evaluó la integridad de la membrana celular cuantificando, tras la exposición de ambos microorganismos al plasma, la liberación de material intracitoplasmático por espectrofotometría, determinando el contenido en proteína y ácidos nucleicos, a 280 y 260 nm, respectivamente, así como cuantificando mediante citometría de flujo la penetración del Ioduro de Propidio (IP, fluorocromo que no es capaz de penetrar en células con membranas intactas, pero puede hacerlo cuando éstas están comprometidas, tiñendo componentes intracelulares específicos). Tras la exposición de las células bacterianas a la acción del plasma, sólo en el caso de *S.* Enteritidis se observó un aumento en la liberación de proteínas y ácidos nucleicos intracelulares, resultando además el 96% de la población celular IP positiva, mientras que tan sólo una subpoblación del 44% de las células totales de *L. monocytogenes* mostraron una membrana citoplasmática dañada.

A pesar de que las membranas celulares se consideran la principal diana celular del PANT, dado que representan la primera barrera de contacto con las especies químicas reactivas generadas en el plasma (Muranyi y col., 2010; Miao y Yun, 2011;

Tseng y col., 2012; Tian y col., 2015), los resultados obtenidos, mediante la aplicación de diferentes técnicas analíticas, sugieren que el mecanismo de acción antimicrobiano del PANT podría ser diferente en especies bacterianas Gram positivas y Gram negativas. Así, los daños originados en las envolturas celulares sería la principal causa de la pérdida de viabilidad de las bacterias Gram negativas, lo que facilitaría la acción de algunos agentes antimicrobianos que, en condiciones normales, resultan ineficaces frente a este grupo bacteriano por su incapacidad de atravesar su membrana externa. Este comportamiento abre la posibilidad de desarrollar procesos combinados que permitan reducir la intensidad de los tratamientos y/o incrementar la efectividad antimicrobiana del PANT, reduciendo, en consecuencia, sus posibles efectos adversos sobre los atributos de calidad de los alimentos. Sin embargo, la inactivación de las bacterias Gram positivas probablemente sea consecuencia de las importantes lesiones originadas en otras dianas celulares, como el ADN y/o enzimas, dada la capacidad de algunas de las especies químicas presentes en el plasma de atravesar las membranas celulares, como ha sido previamente demostrado (Joshi y col., 2011; Ziuzina y col., 2015; Xu y col., 2020). Todos estos aspectos requieren un estudio más profundo, dado que el conocimiento de las bases moleculares de los mecanismos involucrados en la inactivación microbiana por acción del PANT permitiría avanzar en el diseño de tratamientos más eficaces y, particularmente, en el uso de procesos combinados para la conservación de los alimentos.

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Conclusiones

1. Los plasmas generados a partir de aire muestran una mayor efectividad antibacteriana que los obtenidos al emplear nitrógeno como gas precursor, lo que representa una ventaja desde el punto de vista económico y logístico. Además, el efecto letal de los plasmas de aire se puede potenciar incrementando la velocidad de flujo del gas.

2. La efectividad del PANT en la inactivación de *L. monocytogenes*, *S.* Typhimurium y *S.* Enteritidis se vio mínimamente afectada por las condiciones de crecimiento (temperatura de incubación, pH del medio de crecimiento, tipo de agente acidificante) de los microorganismos, hecho que permitirá optimizar los tratamientos con una menor incertidumbre.

3. La incapacidad de estos microorganismos patógenos para desarrollar respuestas de protección cruzada frente al PANT tras su exposición a la acción de diferentes agentes estresantes (calentamiento moderado, acidez, frío) a los que frecuentemente se ven sometidos durante la producción y distribución industrial de alimentos y son conocidos por reducir la efectividad de otros métodos de conservación, evidencia que esta tecnología podría ser de primera elección para su inclusión en procesos combinados que incorporen un proceso de acidificación, calentamiento moderado o refrigeración.

4. Aunque el PANT resulta menos eficaz en la descontaminación microbiológica de los alimentos que en la de superficies abióticas, en cada uno de los alimentos estudiados, tanto de origen animal (chorizo, salami, beicon y salmón ahumado) como vegetal (tofu y manzana), la aplicación de tratamientos de una determinada duración origina tasas de inactivación prácticamente similares para *S.* Enteritidis, *S.* Typhimurium, *E. coli* O157:H7 y *L. monocytogenes*. En consecuencia, el diseño de estrategias de conservación por PANT no está tan condicionado, como en otras tecnologías térmicas y no térmicas, por la especie bacteriana patógena contaminante.

5. La información obtenida a través de diferentes técnicas analíticas (citometría de flujo, espectrofotometría de luz ultravioleta, espectroscopía de infrarrojos con transformada de Fourier, microscopía electrónica), con diferente fundamento, en relación a la evaluación de los daños ocasionados en las membranas de las células de *S.* Enteritidis y *L. monocytogenes* expuestas a la acción del PANT sugiere que, aunque estas estructuras constituyen una de las dianas celulares de las especies químicamente reactivas generadas en el plasma, su implicación en la pérdida de viabilidad podría variar entre las bacterias Gram positivas y Gram negativas. La inactivación de las especies Gram negativas podría deberse principalmente a las importantes lesiones originadas en las envolturas celulares que conducirían a la pérdida de su integridad, mientras que la inactivación de las bacterias Gram positivas estaría causada esencialmente por daños irreparables de componentes intracelulares, como el ADN y/o sistemas enzimáticos.