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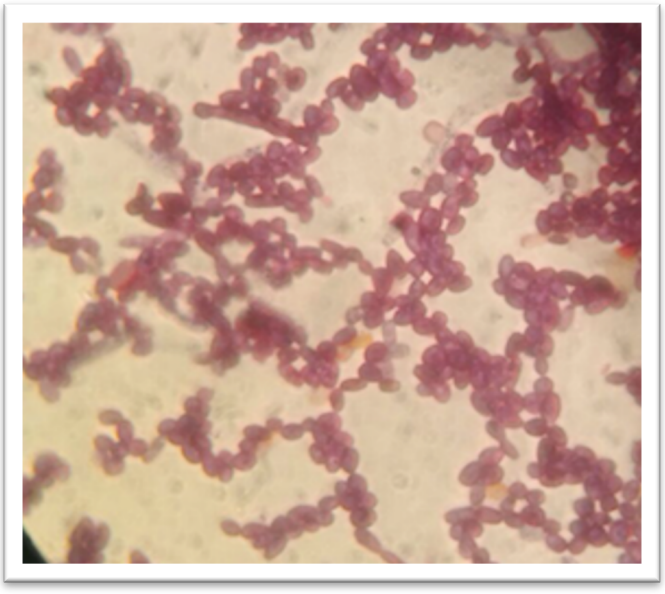
VARIABILITY IN THE RESPONSE OF SALMONELLA AND LISTERIA STRAINS TO DIFFERENT STRATEGIES FOR INACTIVATION

Doctorado en Técnicas Avanzadas en Investigación y Desarrollo Agrario y Alimentario



Marta Clemente Carazo

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UNIVERSIDAD POLITÉCNICA DE CARTAGENA

DEPARTAMENTO DE INGENIERÍA AGRONÓMICA

Tesis Doctoral

VARIABILITY IN THE RESPONSE OF *SALMONELLA* AND *LISTERIA* STRAINS TO DIFFERENT STRATEGIES FOR INACTIVATION

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Dirigida por

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DT-16

CONFORMIDAD DE SOLICITUD DEAUTORIZACIÓN DE DEPÓSITO DE TESIS DOCTORAL POR EL/LA DIRECTOR/A DE LA TESIS

Dª. Paula María Periago Bayonas Directora de la Tesis doctoral "*Variability in the response of Salmonella and Listeria strains to different strategies for inactivation*".

INFORMA:

Que la referida Tesis Doctoral, ha sido realizada por Dª. Marta Clemente Carazo, dentro del Programa de Doctorado Técnicas Avanzadas en Investigación y Desarrollo Agrario y Alimentario, dando mi conformidad para que sea presentada ante el Comité de Dirección de la Escuela Internacional de Doctorado para ser autorizado su depósito.

- ☐ X Informe positivo sobre el plan de investigación y documento de actividades de la doctoranda emitido por el Director/ Tutor (**RAPI**).

La rama de conocimiento en la que esta tesis ha sido desarrollada es:

- ☐ Ciencias
- ☐ Ciencias Sociales y Jurídicas
- ☐ X Ingeniería y Arquitectura

En Cartagena, a 14 de septiembre de 2020

LA DIRECTORA DE LA TESIS

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INFORMA:

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Tal y como dijo John Lennon, *"La vida es todo aquello que te va sucediendo mientras estás ocupado haciendo otros planes"*. Las etapas van pasando y no nos damos ni siquiera cuenta del momento, por tanto, hay que centrarse en ellas y aprender de lo bueno o malo que nos ofrecen. En este tiempo de realización de la tesis, he podido disfrutar y darme cuenta de que bonita puede ser tal experiencia que te hace crecer tanto física como psicológicamente. No obstante, un 50% de ella me ha hecho plantearme muchas veces, ¿qué hago?, ¿por qué estoy aquí?, ¿estaré perdiendo mi tiempo?, etc. Creo que la fuerza que he ganado durante este tiempo me ha hecho seguir y no frenar esta experiencia y por supuesto, ¡como no!, toda la gente (que no ha sido poca) que me ha animado y se ha alegrado en todo momento de lo que estoy haciendo.

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“La gente aprende cuando quiere, cuando encuentra una motivación”

Vivir es fácil con los ojos cerrados (2013)

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ABSTRACT

Food safety if still a great concern for modern societies. The European Union One Health 2018 Zoonoses Report notified a total of 5146 outbreaks of foodborne diseases in the European Union in 2018. The food industry plays an important role in ensuring the safety along the food chain, guaranteeing that its products are safe and of high quality for the consumer. Food production must account for a wide variety of pathogenic microbiological species that are capable of growing and surviving in food products, being a hazard for human health. As a result, an accurate risk assessment is essential in order to make design safe products. This needs the development of predictive models able to describe the microbial response to the processing treatments.

Among the foodborne pathogens, *Listeria monocytogenes* and *Salmonella* spp. are of great concern for the food industry. In the 2018 EFSA zoonoses report, it was reported that *Listeria monocytogenes* has the highest mortality and hospitalization rate among the foodborne pathogens, and *Salmonella* spp. is the foodborne pathogen with the highest number of outbreaks. Both microorganisms are characterized by being ubiquitous microbial species capable of living in relatively harsh conditions of pH, water activity and sodium chloride concentrations. As a result, the only mean to ensure that food products are free of these pathogens is by introducing a processing step able to inactivate them, reducing their concentration to innocuous levels. In order for these processes to be effective, mitigating over or under-processing, a description of the microbial response to stress is of utmost importance. This knowledge shall be reflected in predictive models, that could be used for process design and microbial risk assessment. This PhD thesis is framed within this need, being its main objective the study of different strategies for the inactivation of *L. monocytogenes* and *Salmonella* that could potentially be applied in the food industry.

In results chapter I, the heat resistance of four *L. monocytogenes* strains (Scott A, CECT 4031, CECT 4032 and 12MOB052) in two laboratory media (buffered peptone water [BPW] and pH 7 McIlvaine phosphate citrate buffer) and a food product (semi-skimmed milk) was studied. Experiments were carried out under isothermal and dynamic conditions. In isothermal treatments, the resistance of

the microorganisms was homogeneous between strains and between media. However, when the experiment was carried out under dynamic conditions that enabled the development of stress acclimation, important differences in resistance between strains and between media were observed. Strain CECT 4031 was able to increase its *D-value* by a factor of 10, whereas strain CECT 4032 was not able to develop stress acclimation. Among the media, the results in BPW were similar to the ones in semi-skimmed milk, observing higher acclimation than in the McIlvaine buffer. These results highlight, that the mechanisms describing variability in the microbial response to stress may be different under dynamic conditions than those observed under isothermal treatments.

Result chapters II and III apply combined treatments as a strategy to achieve microbial inactivation using milder processes than with the application of a single treatment. Chapter II evaluates the combination of an acid shock followed by a thermal treatment for the inactivation of *Salmonella* Senftenberg and *S.* Enteritidis. An acid shock at pH 4.5 was applied to both *Salmonella* serovars, followed by an isothermal treatment at four different temperatures at both pH 4.5 and 7.0. The results show that the effect of the acid shock on the resistance to the posterior treatments varies between both serovars. The application of an acid shock reduced the heat resistance of *S.* Senftenberg, whereas it did not affect or increase the heat resistance of *S.* Enteritidis. These results point out the added complexity of designing combined inactivation treatments. Bacterial cells are dynamic systems that can respond to stress increasing their resistance. In many cases, these mechanisms are shared between stresses, so the survivors of a mild treatment may have additional resistance to a posterior treatment (cross-resistance). Cross-resistances are especially relevant for the design of combined treatments, as they may make a combined treatment less effective than expected based only on the microbial response to each stress. Therefore, as highlighted in the results, the design of effective combined treatments require not just the description of the bacterial response to each stress, but also an evaluation of possible cross-resistances. Moreover, as shown in this research, cross-resistances may be affected by variability, adding additional complexity to this task.

Result chapter III of this PhD thesis proposed a second strategy for microbial inactivation based on combined treatments. In this case, the combination of pulse electric field (PEF) treatments with oregano essential oil (EO) as a natural antimicrobial on the inactivation of *Listeria* spp. was evaluated.

The combined treatment was applied following two strategies: simultaneous (the EO is added during the PEF treatment) and subsequent (the survivors of the PEF treatment are exposed to the EO). Whereas the simultaneous strategy did not improve microbial inactivation with respect to the PEF treatment, the subsequent one was an effective strategy for the inactivation of five of the six *Listeria* strains tested. However, the efficacy was strongly affected by the exposure time to the essential oil. An exposure of 20 minutes resulted in practically no improvement with respect to the PEF treatment, whereas an exposure of 60 minutes increased the inactivation in approximately 1 log cycle with respect to the PEF method. Therefore, this chapter identifies an effective combined strategy for the inactivation of *Listeria* spp. that could potentially be used by the food industry. Nevertheless, it also highlights the complexities of designing combined treatments, where many aspects of the process can affect inactivation.

To conclude, this thesis has explored different strategies for microbial inactivation that could reduce the intensity of treatments without hampering food safety. The results also highlight the additional complexities associated to these strategies. Namely, the strong impact of variability for thermal treatments and how it can be different from the one observed in isothermal experiments; as well as the added complexities of combined treatments associated to the development of cross-resistances and the impact of the process parameters. Therefore, the development of novel strategies for food processing should be based on a detailed scientific knowledge of the factors identified as relevant in this thesis.

RESUMEN

La seguridad alimentaria sigue siendo una gran preocupación para las sociedades modernas. El informe sobre zoonosis de One Health 2018 de la Unión Europea notificó un total de 5.146 brotes de enfermedades transmitidas por los alimentos en la Unión Europea en 2018. La industria alimentaria desempeña un papel importante en garantizar la seguridad a lo largo de la cadena alimentaria, asegurando que sus productos sean seguros y de alta calidad para el consumidor. En la producción de alimentos debe tenerse en cuenta una amplia variedad de especies microbiológicas patógenas capaces de crecer y sobrevivir en los productos alimenticios, lo que constituye un peligro para la salud humana. Por consiguiente, es esencial una evaluación precisa del riesgo para que el diseño de los productos sea seguro. Para ello es necesario elaborar modelos predictivos capaces de describir la respuesta microbiana a los tratamientos de elaboración.

Entre los patógenos transmitidos por los alimentos, *Listeria monocytogenes* y *Salmonella* spp. son de gran preocupación para la industria alimentaria. En el informe sobre zoonosis de la EFSA de 2018 se informó de que *Listeria monocytogenes* tiene la mayor tasa de mortalidad y hospitalización entre los patógenos transmitidos por los alimentos, y *Salmonella* spp. es el patógeno transmitido por los alimentos con el mayor número de brotes. Ambos microorganismos se caracterizan por ser especies microbianas ubicuas capaces de vivir en condiciones relativamente duras de pH, actividad del agua y concentraciones de cloruro de sodio. Por consiguiente, el único medio de garantizar que los productos alimenticios estén libres de estos patógenos es introducir una etapa durante la elaboración capaz de inactivarlos, reduciendo su concentración a niveles inocuos. Para que estos procesos sean eficaces, mitigando el exceso o la falta de procesamiento, es de suma importancia una descripción de la respuesta microbiana al estrés. Este conocimiento se reflejará en modelos de predicción, que podrían utilizarse para el diseño de los procesos y la evaluación de los riesgos microbianos. Esta tesis doctoral se enmarca en esta necesidad, siendo su principal objetivo el estudio de diferentes estrategias para la inactivación de *L. monocytogenes* y *Salmonella* que potencialmente podrían aplicarse en la industria alimentaria.

En el capítulo I de resultados se estudió la resistencia al calor de cuatro cepas de *L. monocytogenes* (Scott A, CECT 4031, CECT 4032 y 12MOB052) en dos medios de laboratorio (agua

de peptona tamponada [BPW] y tampón de citrato de fosfato de Mcllvaine de pH 7) y un producto alimenticio (leche semidesnatada). Los experimentos se llevaron a cabo en condiciones isotérmicas y dinámicas. En los tratamientos isotérmicos, la resistencia de los microorganismos fue homogénea entre las cepas y entre los medios. Sin embargo, cuando el experimento se llevó a cabo en condiciones dinámicas que permitieron el desarrollo de la aclimatación al estrés, se observaron importantes diferencias de resistencia entre las cepas y entre los medios. La cepa CECT 4031 pudo aumentar su valor *D* en un factor de 10, mientras que la cepa CECT 4032 no fue capaz de desarrollar una aclimatación al estrés. Entre los medios, los resultados en BPW fueron similares a los de la leche semidesnatada, observándose una mayor aclimatación que en el tampón de McIlvaine. Estos resultados ponen de relieve que los mecanismos que describen la variabilidad de la respuesta microbiana al estrés pueden ser diferentes en condiciones dinámicas que los observados en los tratamientos isotérmicos.

Los resultados de los capítulos II y III aplican tratamientos combinados como estrategia para lograr la inactivación microbiana mediante procesos más suaves en vez de la aplicación de un solo tratamiento. En el capítulo II se evalúa la combinación de un choque ácido seguido de un tratamiento térmico para la inactivación de *Salmonella* Senftenberg y *S.* Enteritidis. Se aplicó un choque ácido a pH 4,5 a ambos serovares de *Salmonella*, seguido de un tratamiento isotérmico a cuatro temperaturas diferentes tanto a pH 4,5 como a 7,0. Los resultados muestran que el efecto del choque ácido en la resistencia a los tratamientos posteriores varía entre ambos serovares. La aplicación de un choque ácido redujo la resistencia al calor de *S.* Senftenberg, mientras que no afectó ni aumentó la resistencia al calor de *S.* Enteritidis. Estos resultados señalan la complejidad añadida de diseñar tratamientos de inactivación combinados. Las células bacterianas son sistemas dinámicos que pueden responder al estrés aumentando su resistencia. En muchos casos, estos mecanismos son compartidos entre los estreses, de modo que los sobrevivientes de un tratamiento leve pueden tener una resistencia adicional a un tratamiento posterior (resistencia cruzada). Las resistencias cruzadas son especialmente relevantes para el diseño de tratamientos combinados, ya que pueden hacer que un tratamiento combinado sea menos eficaz de lo esperado basándose únicamente en la respuesta microbiana a cada estrés. Por lo tanto, como se destaca en los resultados, el diseño de tratamientos combinados eficaces requiere no sólo la descripción de la respuesta bacteriana a cada tensión, sino también una evaluación de las posibles resistencias cruzadas.

Además, como se muestra en esta investigación, las resistencias cruzadas pueden verse afectadas por la variabilidad, lo que añade una complejidad adicional a esta tarea.

El capítulo III de esta tesis doctoral proponía una segunda estrategia de inactivación microbiana basada en tratamientos combinados. En este caso, se evaluó la combinación de tratamientos de pulsos eléctricos de alto voltaje (PEAV) con aceite esencial de orégano (AE) como antimicrobiano natural en la inactivación de *Listeria* spp. El tratamiento combinado se aplicó siguiendo dos estrategias: simultánea (el AE se añade durante el tratamiento de PEAV) y secuencial (los supervivientes del tratamiento de PEAV se exponen al AE). Mientras la estrategia simultánea no mejoró la inactivación microbiana con respecto al tratamiento PEAV, la posterior fue una estrategia eficaz para la inactivación de cinco de las seis cepas de Listeria testadas. Sin embargo, la eficacia se vio fuertemente afectada por el tiempo de exposición al aceite esencial. Una exposición de 20 minutos no produjo prácticamente ninguna mejora con respecto al tratamiento PEAV, mientras que una exposición de 60 minutos aumentó la inactivación en aproximadamente un ciclo de 1 log con respecto al método PEAV. Por lo tanto, en este capítulo se identifica una estrategia combinada eficaz para la inactivación de *Listeria* spp. que podría ser utilizada potencialmente por la industria alimentaria. No obstante, también se destacan las complejidades del diseño de tratamientos combinados, en los que muchos aspectos del proceso pueden afectar a la inactivación.

Para concluir, esta tesis ha explorado diferentes estrategias de inactivación microbiana que podrían reducir la intensidad de los tratamientos sin obstaculizar la seguridad alimentaria. Los resultados también ponen de relieve las complejidades adicionales asociadas a estas estrategias. Teniendo en cuenta, el fuerte impacto de la variabilidad para los tratamientos térmicos y cómo puede ser diferente de la observada en los experimentos isotérmicos; así como las complejidades adicionales de los tratamientos combinados asociados al desarrollo de resistencias cruzadas y el impacto de los parámetros del proceso. Por consiguiente, el desarrollo de nuevas estrategias para la elaboración de alimentos debe basarse en un conocimiento científico detallado de los factores identificados como pertinentes en esta tesis.

List of abbreviations

AP: Apurinic or Apyrimidinic

ASP: acid shock protein

ATP: Adenosine triphosphate

ATR: Acid Tolerance Response

Aw: activity water

BPW: Buffer Peptone Water

EFSA: European Food Safety Authority

EO: Essential oil

EU: European Union

FA: Fatty acid

FBD: Food Borne Disease

FBO: Foodborne

FDA: Food and Drug Administration

GRAS: Generally Recognized as Safe

HACCP: Hazard Analysis and Critical Control Point

H_2O_2: Hydrogen Peroxide

HSPS: Heat-Shock Proteins

MIC: Minimum Inhibitory Concentration

MS: Member State

NaCl: sodium chloride

PCR: Polymerase Chain Reaction

PEF: Pulse Electric Field

pH_i: intracytoplasmic pH

QMRA: Quantitative microbial risk assessment

rRNA: ribosomal ribonucleic acid

RpoS: RNA polymerase S

RTE: Ready to Eat

S: Svedberg

SPIs: pathogenicity islands

UI: Uncertainty Interval

US: United States

w/v: weight/volume

YOPIs: Young, old, pregnant, immunosuppressed

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1.INTRODUCTION

According to the World Health Organization (WHO), at present there are 32 foodborne diseases transmitted by 31 food-borne agents that are still a relevant burden for society (WHO, 2015). Within the European Union, the European Food Safety Authority (EFSA) reported in the European Union One Health 2018 zoonoses report 5,146 cases of food-borne outbreaks (FBOs; including waterborne outbreaks) throughout 2018 in 26 Member States, as well as 48,365 cases of disease, of which 4,588 led to hospitalizations and 40 cases to deaths. On the other hand, 7 non-Member States (Bosnia and Herzegovina, Iceland, Montenegro, Norway, Republic of North Macedonia, Serbia and Switzerland) reported 143 FBOs with 2,434 illnesses and 213 hospitalizations (EFSA, 2019). With respect to 2017, outbreaks related to *Salmonella* have increased in 1,981 cases (a 20.6% increase). The number of hospitalizations due to outbreaks also increased slightly by 1% (47 more hospitalizations), as well as the number of deaths, with 7 more deceased with respect to 2017. Among the microorganisms, *Salmonella* spp. and *Listeria monocytogenes* are considered major threats, *Salmonella* for being responsible for the higher number of food-borne illnesses (284, 972 cases around the world in 2010) and *L. monocytogenes* for the severity, whose hospitalization rate is >90% and mortality between ~15% and ~30% for YOPIs (young, old, pregnant, immunosuppressed) (WHO, 2015).

The food industry plays one of the most important roles in the food chain, as it is part of the link between the agricultural sector, the distribution network and the last link in the chain, the consumers. In recent years, due to higher consumer demands for minimally processed products ("fresh-like"), food industries had to develop new processing technologies with a minimal impact on food quality, while retaining the safety of the product. This need has driven several innovations in the field of food engineering, focused on the development of novel non-thermal technologies, as well as on the reduction of the intensity of the thermal treatments. As well as through the development of new technologies, this has been achieved through the combination of different technologies, in order to allow the inactivation of microorganisms without modifying the organoleptic and sensory characteristics of the food (Manas & Pagán, 2005).

1.1. Food preservation treatments and their effects on microorganisms

1.1.1. Thermal treatment

Thermal treatments were formerly designed in ancient times as an indispensable tool for the food to be digestible and tasty. The technique was eventually applied further to enable the consumption of local products throughout the whole year. Later, the production of foods at an industrial scale generated a need for safe products, as the safety of the product could strongly influenced the image of the brand. Therefore, food companies implemented a "safety culture" to ensure that the products were safe for the consumer (Fryer, 1997).

It was not until the 18th century that the foundations of the application of thermal treatments were laid. However, the relevance of food safety for the functioning of a country was realized much earlier. Between 1803 and 1815, during the Napoleonic Wars in Europe, the French military was losing many soldiers to foodborne diseases and poor nutrition, reaching a higher percentage than casualties in battle. During this time, a chef named Nicolas Appert (1749) was exploring the use of fermentation, distillation and other methods for the preservation of a wide variety of foods. For instance, in 1804, Appert heated sugar syrups in champagne bottles, after modifying the neck to be able to fill them better. The bottles were kept for a long period of time unspoiled, extending the shelf life of the product. This success led to the application of similar methods in the French Navy with great success, increasing the safety and quality of the food consumed by the soldiers (Featherstone, 2012; Goldblith, 1972). For these advancements, Appert is known as "Father Canning", and heat sterilization is also known as "appertization" (Featherstone, 2012).

Later in 1862, the French chemist and microbiologist Louis Pasteur (1822-1895), dismantled the myth of spontaneous generation, demonstrating that fermentation was caused by the growth of microorganisms. He used flasks with swan neck or filters where only filtered air free of dust and other particles passed through. Then, he heated nutrient broth in these flasks, observing that microbial growth would not take place in the broth unless the flasks were opened. Based on this experiment, he concluded that microorganisms were present in airborne particles, and that they could contaminate to food products

causing deterioration or fermentation. This experiment by Pasteur was stepping stone for the development of modern methods for food preservation, focused in the inactivation of pathogenic or spoilage microorganisms. For this reason, inactivation treatments are usually named "pasteurization" (Goldblith, 1971).

The application of heat has been for decades the most common technology for food preservation, as it has proved its efficacy to inactivation a wide range of microorganisms. Some bacteria that can be very heat sensitive, such as *Aeromonas* or *Campylobacter,* while others can be more resistant, such as *Enterococcus faecium* or *Salmonella* Senftenberg 775w (Smelt & Brul, 2014; Sörqvist, 2003). On the other hand, the application of high temperatures can also have a negative impact on product quality, such as degradation of its nutrients and organoleptic losses (McMeekin et al., 2007). Therefore, there is a trade-off between safety (that demands stronger treatments) and product quality (that demands milder treatments). It is thus necessary for process optimization to know the microbial response to the treatment, as well as the one of the quality attributes of the product.

Besides temperature, many other factors affect microbial response to an inactivation treatment. Among them, the pH is of great relevance in many food products. Values of pH close to neutral result in resistance much larger than the observed at acidic pH (Juneja, 2002). For instance, Teo et al. (1996) reported the synergy found between the application of alkaline pH values and high temperatures to kill Gram-negative bacteria that were transmitted by contaminated food. Other attributes of the food matrix also influence the microbial resistance. Several studies have reported that the fat percentage influences the microbial resistance to thermal stress In the case of a minced beef contaminated by *S.* Typhimurium DT 104, to achieve an inactivation of 7 log CFU at 58°C with 7% fat it was necessary to apply a treatment for 53.5 minutes, while for 24% fat it was necessary to almost quadruple the treatment time (208 minutes) (Juneja & Eblen, 2000). Another study carried out by Donnelly & Briggs (1986), in this case, in different milks - skim milk and whole milk -, reported negligible differences between the *D-values* of *L. monocytogenes* after heat application. On the other hand, in another study, different milks from different animal species, sheep, cow and milk, were compared, and different *D-values* of *L. monocytogenes* were observed in this case, so this means that not only the amount of fat in the food matrix affects the heat resistance, but also other factors may have an influence (Juneja, 2002;

MacDonald & Sutherland, 1993). Sucrose is another solute commonly found in food matrices that can influence the thermal resistance of bacteria. In a study carried out on *S*. Typhimurium, the *D-values* were found to range from 0.29 to 40.2 min for a temperature of 65.6°C to a water activity (a_w) of the product between 0.98 and 0.83 respectively. For the same temperature, in *L. monocytogenes* the *D-value*s ranged from 0.36 to 3.8 min with an a_w between 0.98 and 0.90 (Sumner et al., 1991).

Besides these process factors and food attributes, the effectiveness of a thermal treatment depends largely on the microorganisms present in the food. Microbial spores require higher temperatures (>100ºC) for their inactivation than vegetative cells (~70ºC). Moreover, the heat resistance of microorganisms varies largely between species, with *Listeria monocytogenes* and *Staphylococcus aures* being more heat-resistant than *Salmonella* spp. (Amado et al., 2014; Aryani et al., 2015; Cebrián et al., 2007; Lianou et al., 2006). Furthermore, stress resistance also varies between strains or serovars of the save species. For instance, *Salmonella* Senftenberg 775w has a thermal resistance far superior to other serotypes of *Salmonella* spp. Therefore, variability plays a large role in the effectiveness of a heat treatment, and should be accounted for in microbial risk assessment and shelf life estimation (Koutsoumanis & Aspridou, 2017).

1.1.2. Pulsed electric field (PEF)

Electric pulses are a non-thermal technology that has been proposed as an alternative to heat in food preservation. It is based on the application of an electric field of varying intensity that can cause cell damage or death of both spoilage and pathogenic microorganisms, therefore, extending the shelf life and ensuring food safety. This technology has obtained promising results for the preservation in liquid products, such as fruit juices, liquid egg and milk (Barbosa-Canovas et al., 2004).

The origins of this technology can be found at the end of the 19th century, when a flow of electric current was passed through milk to heat it up and achieve its heat pasteurization. This technique, which actually resembled more ohmic heating than modern PEF, was called Electro-pure. Electro-pure had a bactericidal effect, mainly due to its thermal effects (Anderson & Finkelstein, 1919; Fetterman, 1928; Getchell, 1935). The first use of PEF as such was reported in 1949 by Flaumenbaum. It was not

used for food preservation, but for food processing, increasing fruit permeability and improving juice extraction (Heinz & Knorr, 2001). It was not until 1960 when a patent was first reported on the influence of electrical pulses on microbial inactivation (Doevenspeck, 1960, 1961). Seven years later, Sale & Hamilton (1967, 1968) published a series of studies in several microorganisms, showing that the microbial inactivation in PEF could be associated to the cell membrane losing its semi permeability (electroporation). This mechanism was studied in detail in the following years.

Under regular physiological conditions, it is crucial for cell survival that the cell transmembrane potential is maintained in order to carry out a series of actions: the protonmotive force to carry out energy reactions (entry of substrate against concentration gradient), regulate the impulse of the inverse flow of electrons in the respiratory chain, reduce the NAD and generate ATP (Murrell, 1991). However, when cells are exposed to electric fields, electrical charges are accumulated on both sides of the membrane, resulting in an increase in the cell transmembrane potential. When the transmembrane threshold potential is reached, pores begin to manifest (fig. 1). The electric field that has to be applied for this threshold transmembrane potential to be reached is called the critical electric field (Barbosa-Cánovas et al., 1999; Ho & Mittal, 1996; Raso, 2017; Tsong, 1990; Weaver & Chizmadzhev, 1996). The smaller the size of the cell, the larger the electric field that must be applied in order for the electroporation to take place on the membrane. It has been estimated that for large cells a field between 0.1-7 kV/cm is enough, while for the smaller ones it is necessary to apply up to 10-40 kV/cm (Donsì et al., 2010). This phenomenon can be reversible or irreversible. Reversible electroporation is used systematically in laboratories in many disciplines, such as genetic engineering, biotechnology or biomedicine, to introduce or extract molecules from target cells. Reversible means that the pores disappear, close when the electric field ceases and the viability of the cell is maintained. On the other hand, the application of more intense fields leads to irreversible electroporation and is considered as a food preservation method, since in this case it leads to cell inactivation (Murrell, 1991).

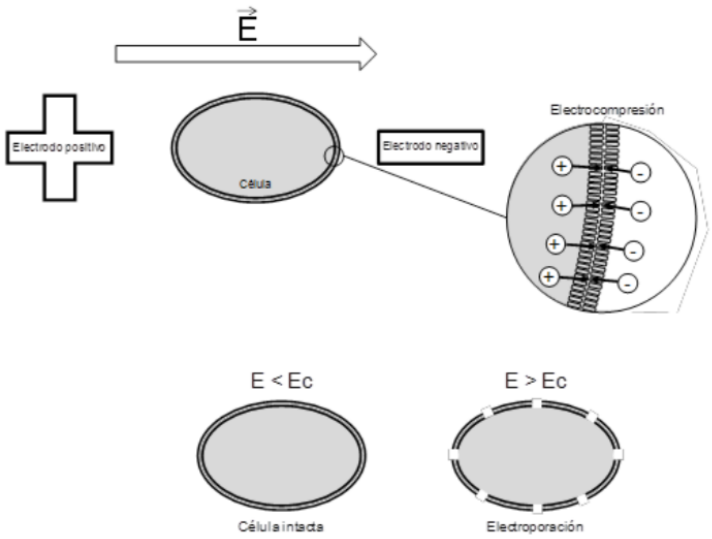

Figure 1 Phenomenon of electroporation by PEF according to the electromechanical models (Saldaña, 2011).

Another of the theories explaining the mechanisms of microbial inactivation by PEF was the one described by Zimmermann et al. (1974). The cell membrane simulates a storage of energy (capacitor), where the dielectric constant is much higher inside the cell than that of the medium surrounding the cell. This difference between the charges leads to a relocation of them at both surfaces of the membrane generating an electric potential difference, exerting force (electro-compression). As a result of this attraction force, the cell membrane becomes increasingly thinner, while viscoelastic forces appear that are opposite to those of compression. When the strength of the external electric field equals the transmembrane potential, reversible pores appear in the membrane, being more numerous and larger for higher intensity of the electric field. If the intensity is high enough, the pores become irreversible (Zimmermann, 1986). Alternative theories have been suggested to explain the mechanisms of inactivation by PEF, such the reorientation of phospholipids. This would create hydrophobic and hydrophilic pores in the lipid fraction of the membrane due to structural changes of the membrane, as long as the electric field exceeds the critical transmembrane potential (Tsong, 1989).

The process parameters that influence the level of inactivation were recently reviewed by Raso et al., (2016). One of the most important ones is the electric field strength (E, kV/cm), defined as the

force per unit load that is felt in the matrix between two electrodes. Therefore, the electric field strength will be defined as a function of the voltage and the arrangement of the electrodes (Eq. 1).

$$E = \frac{V}{d} \quad (1)$$

Where V is the applied voltage (kV) and d (cm) is the distance between electrodes.

Depending on the placement of the electrodes, they can be in parallel, causing the strength of the electric field to be uniform between both electrodes, but if these electrodes are arranged coaxially or collinearly, the homogeneity at all points of the chamber will not be the same, leading to parts where the electric field strength is higher. These areas are called "hot-spots", leading to an increase in temperature and in some cases to the appearance of electric arcs.

Regarding the shape of the pulse, at present, the most used pulses are either square-wave or exponential decay pulses, both unipolar and bipolar. Square-wave pulses are those that are generated in circuits with switches that have the ability to turn on and off and produce a rapid increase in voltage, staying high for a period of time and then falling rapidly (figure 2). Exponential decay pulses are characterized by a complete discharge of the energy of the system. The voltage increase is rapid and subsequently results in a progressive decrease in voltage. One advantage of square wave pulses over exponential decay pulses is that the maximum voltage is applied throughout the treatment. The field intensity in exponential decay pulses, on the other hand, is not constant through the treatment. Therefore, because electroporation shall only take place when the field is higher than a critical value, the tail of these pulses does not have a lethal effect on the cells, although it increases temperature.

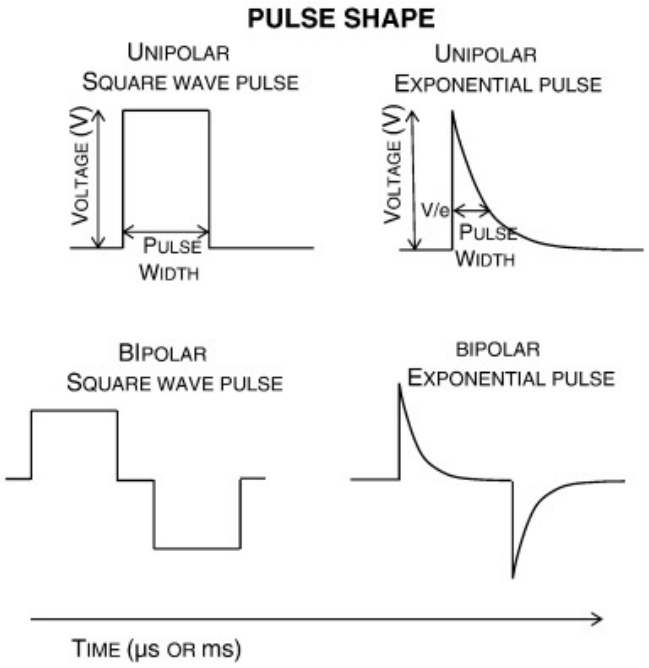

Figure 2 Pulse shapes commonly used in PEF treatments (Raso et al., 2016).

The pulse width (τ) refers to the duration of the pulse and usually is measured in µs. For the case of square wave pulses, the pulse width coincides with the pulse duration. In the case of exponential decay, it will be the time required to decrease 37% of the maximum voltage reached.

Treatment time (t_i) is referred to as the effective time to which the electric field is applied, which depends on the pulse width (τ) and the number of pulses applied (n) (Eq. 2) and is also measured in μs.

$$t_i = n \times \tau \tag{2}$$

The frequency refers to the number of pulses per time unit, whose unit of measure is Hz (pulses per second). The most commonly used frequencies vary between 1 and 500 Hz.

Temperature is a factor that can become critical, as it can condition the effectiveness of PEFs. The increase in temperature caused by PEF treatments increases the fluidity of the microbial cell membrane and as a consequence reduces its mechanical resistance, leaving the membrane more susceptible to electroporation and therefore achieving microbial inactivity (Lebovka et al., 2005; Raso

et al., 2016; Saldaña et al., 2010). However, PEF treatments are supposed to be non-thermal treatments, so any significant increase in the temperature during treatments should be avoided.

1.1.3. Application of acid as a method of bacterial control

The reduction of the pH by the direct addition of acids, or its induction by fermentation processes has been one of the methods of food preservation used from the antiquity. Nowadays, acidification is still one of the main techniques used in non-thermally treated RTE (Ready to eat) products, and can be due to inherent pH of food (in fruits), to fermentation (e.g. pepperoni, yogurt, sauerkraut) and to addition of organic acids (the most clear example are vinegar-based marinades) (Buchanan et al., 2002). With acidification, the growth of a wide range of microorganisms is inhibited, both pathogenic and spoilage, extending shelf life. Microbial inactivation due to acidification is usually associated to a reduction in the speed of certain enzymatic reactions. This leads to a decrease of the metabolic activity, inhibiting growth and, eventually, resulting in cell death. However, even at very low pH some microorganisms can still grow, since molds and yeasts have the ability to grow in very acidic environments of pH 2.0 and below, followed by lactic acid bacteria at pH close to 3.5. Nevertheless, for most pathogenic and spoilage microorganisms, the minimum pH of growth is close to 4.2.

One of the main advantages of using acidification as a method of food preservation, is its low economic cost and its ease of application. However, acidification can also have a negative impact on the quality of the product. One of the most emphasized disadvantages is that the lower pH causes significant sensory changes on the food, so the use of this method of preservation, is restricted to certain foods and processing methods (Cebrián, 2009). Moreover, several pathogenic microorganisms can survive for a relatively long time at low pH. There have been some outbreaks in acidic foods, where it has been found *Escherichia coli* O157:H7 in salami (Tilden Jr et al., 1996), yogurt (Morgan et al., 1993) and in apple cider without having received any heat treatment (unpasteurised) (Besser et al., 1993); *Salmonella* in unpasteurized orange juice and *E. coli* O111: H^- in mettwurst (Paton et al., 1996). As it has been shown there are pathogens that are capable of surviving and growing in acidic environments. Therefore, it is

important to understand what other factors influence on microbial survival and how they interact with the acid for better control of this complex technique (Buchanan et al., 2002).

The mechanism of inactivation of microorganisms by an acid is not yet completely clear. Some authors report that by reducing the pH of the medium, the enzymes of the cell envelope are denatured and therefore the cytoplasm of the bacteria becomes acidified, which causes the metabolism and the consequent cell multiplication to be affected (Booth et al., 1989; Brown & Booth, 1991; Foster, 2000; Rodríguez, 2007; Roe et al., 1998). There are two types of acids, strong and weak acids, which behave differently. The strong acids are usually dissociated in the medium, so they denature the enzymes and proteins found on the cell surface and envelope. This will result in a pH gradient between the external and internal part of the membrane which will lead to structural changes due to the increased permeability to protons by the lowering of the intracellular pH inside the cell (Beales, 2004). On the other hand, weak acids may be present in the medium in the undissociated form, depending on the pH of the medium and on the *pKa* of the acid. In some cases, the p*Ka* value is linked to the antimicrobial activity exerted by organic acids, since in order for these to cross the cell membrane they do not necessarily have to be completely undissociated. But in some other acids, such as citric acid, other more precise absorption mechanisms are necessary. Nevertheless, the particular mechanisms of inactivation can depend largely on the bacterial species being inactivated (Buchanan et al., 2002). Once inside the cell, they acid dissociates and acidify the cytoplasm, hindering microbial metabolism (Booth et al., 1989; Foster, 2000). Changes in the intracellular pH is very important, since the decrease of the intracellular pH, implies the reduction of the metabolic reactions necessary for the cellular survival, as it is the synthesis of ATP (Holyoak et al., 1996; McEntire et al., 2004; O'Sullivan & Condon, 1999; Serrano, 1984, 1988). Some studies also suggest that energy depletion is also one of the consequences by which environmental acidification influences cell death (Cotter & Hill, 2003; Davidson et al., 2012).

1.1.4. Natural antimicrobial agents

Essential oils are aromatic and volatile liquids that can be found in various parts of plants (leaves, barks, stems, roots, flowers, seeds and fruits). Many of them have antimicrobial effect, so they

are considered as antimicrobials of natural origin (Deans & Ritchie, 1987; Hammer et al., 1999; Sánchez et al., 2010). The first uses were in the field of medicine, but later in the nineteenth century began to be used as aroma and flavoring ingredients for food products. In the review by Burt (2004), she accounted for 3000 known types of essential oils, from which 300 were found in the market. EOs are usually extracted with steam and water distillation techniques, or increasingly, through supercritical fluids (Kalemba & Kunicka, 2003; Muñoz et al., 2010). Their chemical composition includes terpenic compounds (mono-, sesqui- and diterpenes), alcohols, acids, esters, epoxides, aldehydes, ketones, amines and sulphides (Calo et al., 2015).

The first study to evaluate the composition of essential oil vapors was done by Croix in 1881. He observed that they contain a complex mixture of compounds, including secondary metabolites and antibacterial agents (Burt, 2004). Because of this complex composition, EO have not just anti-bacterial characteristics (Deans & Ritchie, 1987; Oussalah et al., 2007), but they have a wide range of properties such as antiparasitic (George et al., 2009), insecticidal (Enan, 2001; Kim et al., 2003), antiviral (Astani et al., 2011), antifungal (Fitzgerald et al., 2003; Kalemba & Kunicka, 2003; Silva et al., 2011; Tserennadmid et al., 2011), and antioxidant (Brenes & Roura, 2010). The European Commission has accepted a wide variety of essential oils for use as flavorings in food, since they are products that are catalogued as not risking the health of the consumer. The Food and Drug Administration (FDA) of US has classified these substances as Generally Recognized As Safe (GRAS). Hence, clove, oregano, thyme, nutmeg, basil, mustard and cinnamon render crude oils are considered to be GRAS (Hyldgaard et al., 2012).

One of the main drawbacks of essential oils is that the same compounds responsible for the antimicrobial properties have a strong flavour and taste, so they can strongly affect the sensorial properties of the food. A study on vegetable products (lettuce, carrot, pumpkin, and dry coleslaw mix) had a poor acceptance, when applied thymol, oregano and rosemary EOs undiluted, with an apparently damaged visual appearance and an unpleasant smell (Scollard et al., 2013). A study by Uyttendaele et al. (2004), also obtained negative results on bell pepper, as the bell pepper tissues softened when thymol EOs were added. It is therefore necessary to know the minimum inhibitory concentrations (MIC) of each essential oil in the microorganisms to be tested, in order to achieve an appropriate balance between

sensory acceptability and antimicrobial capacity, through studies *in vitro* (Koutsoumanis et al., 1998, 1999; Tassou et al., 2000). Another drawback is their lipophilic character, which makes them difficult to mix with the hydrophilic part of foods. Nanoemulsions have emerged as a solution to this problem. They can increase the solubility of EO in an aqueous medium. As a result, they could increase the antimicrobial effect, decreasing the concentration of essential oils necessary to produce the same antimicrobial effect. Thanks to them, the physical stability of the active ingredients that make up EO is also improved and a better distribution of antimicrobial agents in the aqueous phase of the food matrix is achieved (Donsì et al., 2010; Donsì et al., 2011, 2014).

One of the EOs most commonly used for food preservation is the one extracted from oregano (*Origanum vulgare*), which belongs to the family *Lamiaceae*. It originates from western and south-western Eurasia and the Mediterranean region, yet it can nowadays be found on all five continents (Banchio et al., 2008). It is well known for its therapeutic properties – diaphoretic, carminative, antispasmodic, antiseptic and tonic- (Sağdıç et al., 2002; Şahin et al., 2004). Oregano is widely used in different industries, such as pharmaceuticals, cosmetics and culinary, as it is an herb that gives taste and smell in both food and alcoholic beverages (Aligiannis et al., 2001; Dorman & Deans, 2000). The main active compounds present in *O. vulgare* essential oil are carvacrol (68.1%) and p-cymene (15.9%). Other compounds also present, though at a lower concentration, are α-pynene and myrcene (Hyldgaard et al., 2012). Some studies have detected even more active compounds in the essential oil of *O. vulgare*, such as: γ-terpinene, thymol, γ-terpineol, sabinene, caryphyllene, germancrene and spathulenol (Daferera et al., 2003; Şahin et al., 2004; Velluti et al., 2003).

Several researcher works have proven the antimicrobial activity of *O. vulgare* EO (Baydar et al., 2004; Chun et al., 2005; Nostro et al., 2004; Skandamis & Nychas, 2001). However, it is hard to identify which antimicrobial compounds present in essential oil is responsible for microbial inactivation, as a mixture of more than 45 different components has been identified in the essential oil (Delaquis et al., 2002; Djenane et al., 2011; Espina et al., 2011). Essential oils rich in phenolic compounds, such as carvacrol, have been shown to have high microbial activity (Panizzi et al., 1993; Sivropoulou et al., 1996). These results were corroborated by an antimicrobial examination of active compounds by Aligiannis et al. (2001), about *Staphylococcus aureus*, *Staphylococcus epidermis* as, well as in Gram-

negative, such as *Escherichia coli*, *Enterobacter cloacae* and *Klebsiella pneumoniae*, but this effect was not seen in *Pseudomonas aeruginosa*. On the other hand, other components of oregano EO, such as *ρ*-cymene, did not produce any antimicrobial effect.

Many other authors have reported that the effectiveness of this EO on food matrices needs to be further investigated, as most studies have been done *in vitro* (Souza et al., 2007). Nevertheless, the efficacy of the oregano EO has been proven in several products. For instance, a study showed that the addition of oregano EO to cream cheese helped to prevent fat oxidation and fermentation, increasing the stability of the cheese flavors and extending shelf life (Olmedo et al., 2013).

The antimicrobial mechanisms of essential oils are still an active research topic. Skandamis & Nychas (2001) proposed that the mechanisms of action of EOs may be due to the capacity of penetration inside the bacterial membrane attributed to phenolic compounds, which leads to alterations in cell permeability and cytoplasmic damage. Moreover, it has been shown that essential oils may alter the development of metabolic chemical reactions, such us the energy consumption of the cell (ATP) and the driving force of protons, which would eventually lead to cell death (Baydar et al., 2004; Chun et al., 2005). Gram-positive bacteria have shown a higher susceptibility to essential oils than Gram-negatives, because the external membrane of Gram-negative bacteria is composed of hydrophilic lipopolysaccharides, which form a barrier to the entry of macromolecules and hydrophobic compounds (Nikaido, 1994; Nikaido, 2003; Trombetta et al., 2005). This highlights that the interaction between EOs and membrane lipids can be of high relevance for the antimicrobial effect of these compounds. Hence, changes in the structure and/or permeability of the membrane could be a good strategy for increasing the effectiveness of the EO, as it would ease the entry of a "foreign agent" capable of causing cell death (Friedly et al., 2009).

1.2. Sublethal injury and microbial response mechanisms

1.2.1. Sublethal injury.

Sublethal injury in bacteria is defined as the effect of one or more treatments, both by exposure to physical and chemical agents, which not causing bacterial death, lead to important damage (Hurst, 1977, 1984; Russell, 1984). The cells of a bacterial population, after being exposed to an inactivation treatment, can be divided in three categories: cells that have not been damaged and therefore can continue to grow and develop in both selective and non-selective media; damaged cells that are able to develop in non-selective media, but not in selective ones, and cells that have not withstood the treatment and are dead and under no condition are able to recover (Ray, 1989). The intensity of the treatment is the main factor determining the proportion of cells in each category, although other factors can also be relevant.

Sub-lethal damage can affect different parts of a microbial cell. Although some kinds of stress are associated to particular types of sublethal damages, most stresses damage the cell in different ways. The damage to the RNA is related to exposure to a low pH environment. This damage could be due to the loss of magnesium that is part of the ribosomal integrity (Hurst, 1984; Przybylski & Witter, 1979; Wesche et al., 2009). A number of physical stresses are related to membrane damage, such as freezing, cooling or heating (Boziaris & Adams, 2001; Mackey, 2000). Damage to the outer Gram-negative membrane causes the release of lipopolysaccharides, lipids, phospholipids, divalent cations that are necessary for the stability of lipopolysaccharides and periplasmic enzymes that alter the stability of membrane permeability (Boziaris & Adams, 2001; Hurst, 1977; Ray & Bhunia, 2007). Permeability regulation is also affected when temperatures are low, where the fluidity of the membrane decreases, as well as alkalinity (Virto et al., 2005) and chlorination (Sampathkumar et al., 2003) are two parameters that affect the permeability of the membrane (Graumann & Marahiel, 1996; Mackey, 1984). It has been seen that the outer surface layer of Gram-positive cells can be damaged due to the effect of freezing. This effect has been shown in some studies on the microbial species *Lactobacillus bulgaricus* (Mackey, 2000; Mathew & Ryser, 2002) and the magnesium and D-alanine are lost from the teichoic acid

polymers of the cell wall of *Staphylococcus aureus* when it is heated in phosphate buffer (Hurst et al., 1976). In relation to enzymes, dehydrogenases are very sensitive to heat and also glycolases and endonucleases, which are part of the DNA repair system. Heat treatments also inactivate catalase and superoxide dismutase in microorganisms such as *L. monocytogenes* and *S. aureus*, increasing their susceptibility to hydrogen peroxide (H_2O_2) and superoxide. As a consequence of this inactivation, the generation of hydroxyl radical increases (Andrews & Martin, 1979; Dallmier & Martin, 1988). The damage caused to the DNA of the cells has been proven in cells that have undergone drying during defrosting and by the application of acid (Mackey, 2000; Russell, 1984). There are theories that relate the stimulation of endonuclease activity to the breaking of the DNA chain during or after a heat treatment (Hurst, 1977; Russell, 1984). An example of this was reported in a study where the death of *E.coli* after a cold shock was related to the loss of magnesium, which is required by the DNA ligase for the subsequent synthesis and repair of DNA (Sato & Takahashi, 1970).

The different types of sub-lethal damage are reflected on the growth curves observed at the population level. For instance, it has been reported that lag times of heat-shocked *S.* Typhimurium cells varied largely between repetitions, ranging from 12 to 30 hours, whereas the variation of control cells was much smaller. Similar results were observed for *L. monocytogenes* cells after the application of nine different type of stress. This was attributed to the heterogeneity of the lag-times in the cells (Stephens et al., 1997).

From an experimental point of view, sub-lethal damage can be estimated by adding selective compounds to the culture media. Common selective compounds are NaCl, as well as bile salts, dyes, toxic chemicals, antibiotics and organic and inorganic acids (Wuytack et al., 2003). Recently, the development of modern image capture methods supported by Artificial Intelligence algorithms have enabled more detailed observations of sub-lethal damage. Several studies (Balomenos et al., 2017; Elfwing et al., 2004a) have coupled time-lapse pictures of microorganisms with machine learning algorithms to observe variations in shape, size and other cell attributes during microbial growth, survival or resuscitation. Moreover, these experiments have been combined with staining to observe the evolution of cell damage.

1.2.2. Heat Shock Response

In order to optimize the intensity of heat treatments, it is essential to understand how microbial cells response to high temperatures. Microbial cells are dynamic systems that undergo physiological changes after the application of a thermal stress, increasing their chance of survival. During heat shock, most microbial cells synthesize proteins that are specific to repair the cell and increase their resistance to the stress. These are known as heat-shock proteins (HSPs) (Farber & Brown, 1990; Lindquist, 1986; Schlesinger, 1986). This is especially relevant for food safety, as these adaptive microorganisms can reduce the effectiveness of inactivation treatments, hampering food safety.

Microbial inactivation by heat is usually not related to one single mechanism, but to a plethora of them. The fatty acid composition of the cytoplasmic membrane has been linked in several studies to heat resistance. The external membrane of Gram-negative bacteria becomes permeable due to heat treatments that lead to an increase in the perceptibility of various components - bile salts, lysozyme or some hydrophobic antibiotics (Boziaris & Adams, 2001; Cebrián et al., 2017; Mackey & Derrick, 1982). Permeation also results in the loss of periplasmic proteins (Cebrián et al., 2017; Halder et al., 2015; Tsuchido et al., 1985; Wesche et al., 2009). Other microbial structures and compounds are also affected by heat, such as components found in the cell wall, in the cytoplasmic membrane, as well as molecular mechanisms that are involved in cell metabolism (Earnshaw et al., 1995; Wu, 2008). Bacterial DNA is one of the cellular components with the highest thermal resistance, the damage caused leads to simple or double ruptures that leave it exposed to the action of endonucleases and subsequent denaturation (Kadota, 1978). Another cell component that can be affected by high temperatures is ribosomal RNA, which has been shown to denaturize at lower temperatures than bacterial DNA (Earnshaw et al., 1995). Bacterial proteins can also denaturize due to the application of high temperatures. Some have high sensitivity, such as the α and β units of RNA polymerase, LysS proteins (tRNA synthetase), Pgk protein (enzyme responsible for glycolysis) or MurA protein (involved in the cell wall system) (Kobayashi et al., 2003; Mogk et al., 1999). There are other labile proteins that, when denatured at high temperatures, lead to the loss of functionality, which is important for enzymes such as catalases or superoxide dismutases

and the alteration of essential cellular structures, such as the channels or transport pumps (Guillier et al., 2005; Métris et al., 2008; Smelt et al., 2002; Standaert et al., 2007).

1.2.3. Acid Shock Response (ASR)

The ability of microorganisms to resist environments with acidic pH is of high concern for the food industry (Gahan et al., 1996; Miller & Kaspar, 1994). It has been shown that both intrinsic and induced acidity can create adaptation of pathogenic bacteria on arrival in the stomach (Garcia-del Portillo et al., 1993; O'Driscoll et al., 1996). The following factors have been identified as inducing the development of tolerance/resistance to acid of pathogenic microorganisms (Buchanan et al., 2002):

- Previous exposure to acid media.
- If cells are grown in a glucose or acidogenic medium, resistance to acid is subsequently increased.
- The physiological state, since cells in the stationary phase are more resistant than those in the exponential phase.

Moreover, resistance to heat, salt, antimicrobials and ionizing and non-ionizing radiation have been associated with cross-protection resulting in tolerance to acid (Buchanan et al., 1999; Farber & Pagotto, 1992). It has been reported that exposure to a sublethal acid can induce acclimation of the cells, increasing their chance to survive posterior treatments at low pH. It is important to note that this mechanisms can occur naturally within the food chain (e.g. microorganisms present in acidic fruits), making it very relevant for food safety (Cebrián, 2009).

From a molecular point of view, several systems are related to the response to acidic environments. These are responsible for the synthesis of proteins (Acid Shock Proteins [ASP]) that are responsible for maintaining cell homeostasis, as well as cell repair (Foster & Hall, 1991; Gale & Epps, 1942; Raja, Goodson, Chui, et al., 1991; Raja, Goodson, Smith, et al., 1991). ASP include a wide range of proteins, among which we can find different subunits of the membrane ATPase, aminoacid-

decarboxylases, various chaperones and proteases and DNA repair enzymes. Moreover, the function of some ASP is still unknown (Cebrián, 2009).

As previously mentioned, pH homeostasis is a very important cellular process for cell survival. It has been shown that *L. monocytogenes* cells that have been pre-exposed to a pH between 5.0 and 5.5 during the exponential growth phase, had a higher cytoplasmic pH than non-adapted cells when they have been subjected to a lethal pH, due to the synthesis of specific proteins (Davis et al., 1996; Kroll & Patchett, 1992; O'Driscoll et al., 1996, 1997). In the case of amino acid decarboxylases, they are considered to be enzymes that are inducible after exposure to acid pH, since it has been proven that the lack of decarboxylases makes it more difficult for bacteria to grow at pHs below 6.0 (Becker,1967). These enzymes are primarily responsible for increasing resistance to acid pH in Gram-negative bacteria (Bearson et al., 1997; Foster, 2000). Dnak and GroEL chaperones, appear when other proteins have been damaged or denatured when bacteria have received an acid shock and are therefore related to the repair (Bore et al., 2007; Cotter & Hill, 2003).

The application of acidic pH can also damage macromolecules like the DNA and RNA (Mackey, 2000; Madshus, 1988). Acidification leads to depurination and depyrimidation, which results in the generation of AP (apurimic or apyrimidinic) sites. This damage (which results in the loss of some purine or pyrimidine bases) can be repaired by AP-endonucleases (Smn), which are usually in high concentrations in acid-adapted cells. Another type of genetic damage is due to genes encoding proteins for DNA repair. Among them is the gene *polA1* encoding DNA polymerase I, responsible for correcting breaks in the DNA chains (Sinha, 1986); the gene rec*A* repairs replication forks (Quivey Jr et al., 2000) and lastly, uvr*A* is a gene that encodes the excinuclease responsible for removing damaged sections when they are very large (Kim et al., 2006; Raja, Goodson, Chui, et al., 1991; Sinha, 1986).

Although the response to stress regulation has not been studied in this thesis, it is interesting to note that there are several Gram-negative cell regulators, among them (Foster, 2000):

- Factor sigma σ^s and OmpR system, are responsible for regulating the acid shock in cells in the stationary phase.

• Factor sigma σ^S, regulatory protein Fur and system PhoPQ, that take care of the resistance control for cells in the exponential phase.

• The independent induction of a series of regulatory genes, aciA or aciB (Slonczewski, 1996).

Synthesis of ASPs is involved in repairing or preventing damage to macromolecules during ATR (Acid Tolerance Response) (Audia et al., 2001). Several regulatory genes involved in ASP have been studied in *S.* Typhimurium. Notable ones are the alternative iron-regulating sigma factor (RpoS), the signal translation system PhoP/PhoQ or the response regulator OmpR (Foster, 2000). The goal of most ASPs is the transcription and translation and cell regulation among many other (Bearson et al., 2006). The alternative sigma factor RpoS is responsible for the survival of *Salmonella* cells in the stationary phase as well as under other stress conditions and of course at the acidic pH (reviewed by Hengge-Aronis, 2002).

The fluidity of the cytoplasmic membrane is another mechanism involved in the regulation of ATR, since it is the first barrier of the cell defense. It is mainly composed of fatty acids (FA) that are responsible for maintaining the membrane fluidity. Several studies showed that a reduction in membrane fluidity increased the ability of *Salmonella* to survive lethal conditions of acid and heat, due to the adaptation of the membrane by the decrease of unsaturated FA and their conversion to saturated FA (Alvarez-Ordóñez et al., 2010; Álvarez-Ordóñez et al., 2008; De Jonge et al., 2003).

On the other hand, in the case of Gram-positive cells, no system of regulation of the resistance of the acidic environment common to all species has been found. The sigma factor σ^B, helps bacteria such as *Listeria monocytogen*es, *Streptococcus mutans* and *Bacillus subtilis* to develop some resistance to the acidic environment (Cotter & Hill, 2003). The efficacy of this factor has been demonstrated for *L. monocytogenes* during the stationary phase. It has also been seen that there are other cells of other bacterial species which, apart from requiring this sigma factor σ^B, require other regulatory systems if they have been previously exposed to a sublethal pH (Ferreira et al., 2003).

1.3. Mathematical modelling of microbial inactivation kinetics

Traditionally, the variation of the microbial count during an isothermal inactivation treatment has been assumed to follow a first order kinetics, where the specific inactivation rate is constant for isothermal treatments (Stumbo, 1973; Tomlins & Ordal, 1976) (equation 3). Over time, both regulatory agencies and the food industry have relied on the calculations provided by these first order kinetics, to take into account in their food safety regulations (McKee & Gould, 1988).

$$\log N_t = \log N_0 - \frac{1}{\mathrm{D}}\mathrm{t} \tag{3}$$

Where N_t refers to the number of surviving microorganisms after t minutes of treatment, N_0 to the initial number of microorganisms, and D to the *D-value* (treatment time required to reduce the microbial count a 90%)

Microbial inactivation kinetics are strongly dependent on the treatment temperature. This is reflected in the D-value. In predictive microbiology, it is common to assume a log-linear relationship between the *D-value* and temperature, as shown in Equation (4). This equation defines the *z-value*; the increase in the treatment temperature necessary to reduce the *D-value* by 90% (Juneja, 2002; Stumbo, 1973). This equation also introduces a reference temperature (T_{ref}), without a biological meaning but with an impact on parameter identifiability (4):

$$\log D(T) = \log D_{ref} - \frac{T - T_{ref}}{z} \tag{4}$$

Where T is the treatment temperature; D_{ref} is the reference *D-value* corresponding to the reference temperature T_{ref} (mostly expressed in °C); z= is the temperature increase that corresponds to a 10-fold reduction of the *D-value*.

However, several authors have observed deviations in the microbial concentration with respect to linearity. This can have a significant effect on the time required to achieve some level of inactivation (Geeraerd et al., 2005; Mafart et al., 2002; Peleg & Cole, 1998). Non-linear deviations taking place at the initial moments of the thermal treatment are usually called "shoulder effect". It refers to the time in which the microbial concentration is maintained at the approx. the same level as when it has been

inoculated. It may be due to poor heat transfer or to initial cell resistance to the treatment. Non-linear deviations at the end of the survival curves are defined as "Tailing effect". It can be due to part of the population having an exceptionally high resistance to stress, what makes them able to survive the treatment regardless of its duration (see figure 3) (Hansen & Riemann, 1963; Juneja, 2002; Stumbo, 1973).

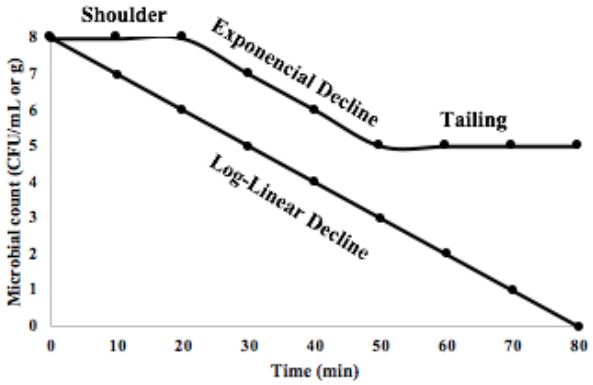

Figure 3 Different deviations in microbial inactivation. Adapted from (Juneja, 2002).

The frequent presence of these deviations has led some authors to propose alternative models to first order kinetics. The Mafart inactivation model assumes that the resistance of individual cells is not homogeneous but follows a Weibull distribution. It, thus, can describe survivor curves with upwards or downwards curvature. In accordance with Equation (5), the relationship between the microbial count (N) and the treatment time (t) depends on two parameters. The δ-value, which corresponds to the scale parameter of the Weibull distribution, can be interpreted as the treatment time required to reduce the microbial count to a 10% of the initial count, N_0. Furthermore (p-value) is the shape factor of the Weibull distribution and defines the curvature direction of the survivor curves: $p > 1$ results in survivor curves with downwards curvature and $p < 1$ in upwards curvature. For the particular case where $p = 1$, the Mafart model predicts log-linear inactivation (Fernández et al., 1999; Peleg & Cole, 1998).

$$\log \mathrm{N} = \log \mathrm{N}_0 - \left(\frac{\mathrm{t}}{\delta}\right)^p \qquad (5)$$

This model uses a secondary model similar to the Bigelow model, considering a log-linear relationship between the δ-value and temperature (Equation 6). The parameter p, on the other hand, is usually considered independent from temperature.

$$\log \delta(T) = \log \delta_{ref} - \frac{T - T_{ref}}{z} \tag{6}$$

Another non-linear inactivation model is the one proposed by Geeraerd et al (2000). The Geeraerd model is an extension of the log-linear inactivation model with hypotheses that enable the description of survivor curves with tail and/or shoulders. The model equations are shown in equation (7). This model introduces one parameter to describe each phase of the survivor curve. The exponential phase is described by parameter k_{max}, which equals the maximum inactivation rate during the thermal treatment. The shoulder is introduced in this model by the variable C_c, which corresponds to the period before inactivation begins. This variable follows a first order kinetics with the rate k_{max}. Lastly, in this model, it is assumed that a fraction of the population is able to survive the treatment, no matter its duration. This hypothesis is represented by the parameter N_{res}, which defines the minimum microbial count of the treatment (tail height) (Geeraerd et al., 2000). The Geeraerd model uses a secondary model similar to the one used in the Bigelow model, considering that the D-value can be calculated from k_{max} using the identity $D = \frac{\ln 10}{k_{max}}$.

$$\frac{dN}{dt} = -k_{max}\left(\frac{1}{1 + C_c}\right)\left(1 - \frac{N_{res}}{N}\right)N \tag{7}$$

$$\frac{dC_c}{dt} = -k_{max}C_c$$

These models were developed based on experimental data gathered under isothermal conditions. During the last decades, the development of novel experimental equipment has enabled the study of microbial inactivation under dynamic conditions. This has shown that some phenomena that are unique to dynamic treatments. One of this mechanisms is the development of stress acclimation. This concept refers to the development of a physiological response in the microbial cells during the heating phase of

a dynamic inactivation treatment that can increase its thermal resistance. This mechanism cannot be described by the above referenced models. For that reason, Garre et al (2018) proposed the inactivation model described by equations 8 and 9.

$$\frac{dN}{dt} = k(t,T)N(t) = k_1(T) \cdot k_2(t)N(t) \quad (8)$$

Where k_1, is an inactivation constant, which depends on the instantaneous environmental conditions (i.e. the temperature), and k_2, is another inactivation constant, which is a function of the thermal history of the cells.

$$\frac{dN}{dt} = \frac{\ln 10}{D(T_{ref}) \cdot 10^{-\frac{T-T_{ref}}{z}}} \cdot \frac{1}{1 + c \cdot p(t)} N(t) \quad (9)$$

Where $p(t)$ is related to time variation and c quantifies the maximum acclimation that the microbial cells can be potentially developed.

This model assumes that k_1, follows a linear logarithmic relationship with temperature, being equivalent to the assumptions of the Bigelow model. The coefficient k_2 is a correction factor that accounts for stress acclimation, taking values between 0 and 1 according to the logistic equation (9). The "amount" of acclimation is described by variable p(t), also defined between 0 and 1. If p=0 there is no acclimatization and the inactivation rate is equivalent to the one predicted by the Bigelow model. When p=1, the cells have developed their maximum capacity of resistance to the treatment, increasing its *D-value* by a factor 1+c. Therefore, parameter *c* quantifies the maximum acclimatization that the microbial cells can reach.

1.4. *Listeria* spp.

Listeria spp. is included within the phylum *Firmicutes* and belongs to the order *Bacillales*, can be aerobic and facultative chemoorganotrophs. They are composed of Gram-positive cocobacillus, positive catalases and facultative anaerobic that form chains of three to five cells (Buckley et al., 2015).

Within the genus *Listeria*, there are currently six recognized species -*L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi*- (Ryser & Marth, 1999), as well as two new species identified in 2009- *L. marthii* and *L. rocourtiae*- (Graves et al., 2010; Leclercq et al., 2010)

The genus *Listeria* is Gram-positive with a diameter of 0.5 µm and 1 to 2 µm in length, characterized by being a small, short and regular microorganism (Gutekunst et al., 1992). *Listeria* is not able to form spores or capsules (Bockemühl & Seeliger, 1968). *Listeria* is mobile at temperatures between 20-25°C, thanks to its flagella, although it loses mobility at higher temperatures (Galsworthy et al., 1990).

This microorganism is able to grow at refrigeration temperatures of 1-2°C and to withstand high temperatures of up to 45°C (Junttila et al., 1988; Sneath et al., 1986). This foodborne pathogen can survive in a large variety of products, such as those with low pH (between 4.5 and 9.2), high concentrations of sodium chloride (greater than 10% (w/v) NaCl) and is one of the few foodborne pathogens able to grow at a_w below 0.93 (Farber & Addison, 1994; George & Lund, 1992; Parish & Higgins, 1989; Petran & Zottola, 1989; Shahamat et al., 1980; Sneath et al., 1986).

Listeria colonies can reach a diameter of 0.2 to 0.8 mm on nutritive agar after 24-48 hours of incubation at 37°C. After 5 days, they can reach approximately 5 mm in diameter. In terms of visual appearance, they have a smooth, curved and somewhat pointed surface, and are usually translucent and blue-grey in colour. (Gray, 1957; Lachica, 1990; Moura et al., 1991). All the species of this genus are oxidase negative and catalase and squalene positive, so this is used in chromogenic media to differentiate them from other genera (Curtis et al., 1989). Carbon and nitrogen are the primary nutrients needed for the development and growth of *Listeria*. Glutamine and glucose are necessary, since *Listeria monocytogenes* grows through the presence of fermentable sugar (Daneshvar et al., 1989; Premaratne et al., 1991; Seeliger, 1961; Sneath et al., 1986).

L. monocytogenes is the only species in the genus that has been linked to cases of pathogenicity in humans. It is of great concern for the food industry due to its ubiquity in the environment, as it can be found in nature from soil to decaying plant matter (González-Tejedor et al., 2017; Kilcast and Subramaniam, 2011; Maza et al., 2019; Peng et al., 2017; Farber & Peterkin, 1991; Kathariou, 2000; Schuchat et al., 1991).

Some cases of infection in humans have been reported by *L. ivanovii*, but these have been very few. This species mostly affects domestic animals, of ovine production, causing abortions and neonatal septicemias and cases of ruminants have been found where this species is also considered pathogenic (Seeliger et al., 1984). Furthermore, *L. seeligeri* is not considered pathogenic, but it has been linked to some cases of meningitis (Rocourt et al., 1986).

1.4.1. Listeriosis as a foodborne disease

The main source of infection by *Listeria* is associated with food contamination, reaching a percentage of 99% (Gambarin et al., 2012; Mateus et al., 2013). The first cases of foodborne listeriosis were reported in the 1980s due to consumption of food that was in poor condition. The first case of listeriosis in animals was reported in 1925 in sheep in Germany (Cohrs, 1952). However, *L. monocytogenes* has been isolated from a wide range of animal species that have suffered from this food-borne disease (Ryser, 2016). The incubation period associated with this disease is relatively long, ranging from 1 to 70 days, with an average duration of 8 days. The symptoms of a person infected with listeriosis can range from mild to common flu-like, as well as nausea, vomiting, and diarrhea (Koutsoumanis et al., 2020). There is a part of the population that is more susceptible to listeriosis. This population ranges from newborns to the elderly, including immunosuppressed people (Gray & Killinger, 1966). Therefore, the precise infection dose of *L. monocytogenes* depends on several factors, especially the strain and the host. Nevertheless, the regulation establishes a limit of 100 CFU/g of *Listeria* as a food safety objective (EFSA, 2019).

The EFSA one health 2018 zoonoses report, showed an increasing trend in the number of people affected by listeriosis over the last 10 years (figure 4). In 2017, 2,480 cases were registered, while in 2018 this value increased by 1.37%, i.e. a total of 2,549 cases in the EU. EFSA (2019) reports an upward trend in confirmed human cases of listeriosis between 2014-2018 in nine MS (Germany, Estonia, Finland, Italy, Latvia, Lithuania, Poland, Romania and Spain), being Spain a significant country due to the increase in the improvement of the surveillance of this pathology. Seven other MS (Belgium, France, Greece, Hungary, Ireland, the Netherlands and Slovenia) that had an increase of cases during the years 2009-2014, decreased 5 years later being not significant from 2014 to 2019.

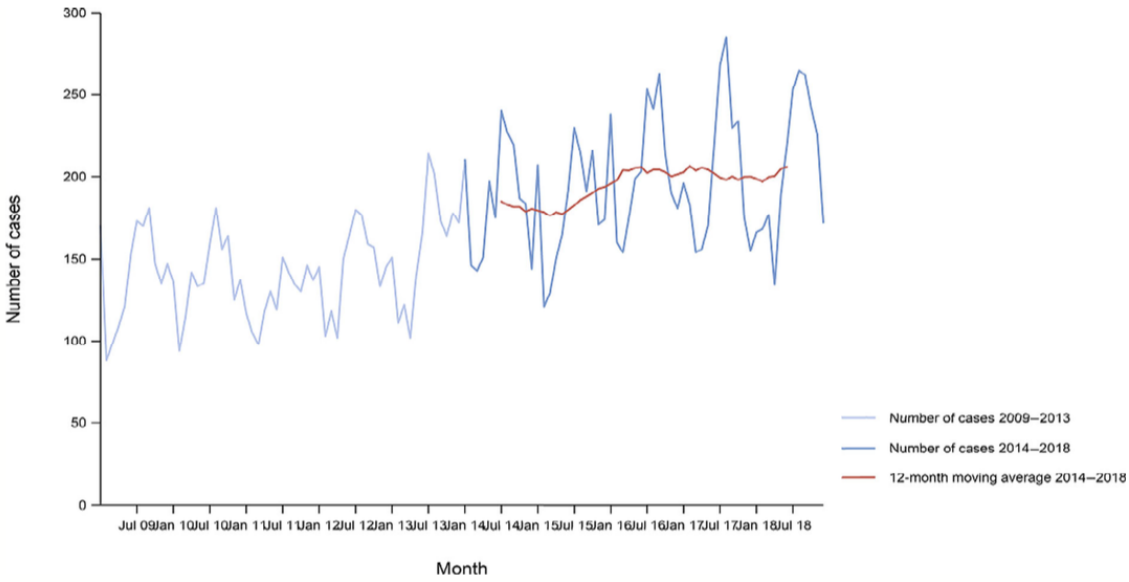

Figure 4 Confirmed human cases of listeriosis in the EU/EEA, by month, 2009-2018 (EFSA, 2019).

1.4.2. Presence of *L. monocytogenes* in food

L. monocytogenes has the capacity to grow and develop in different environments and food products. It has been isolated from a huge list of products for direct consumption: meat (lamb, turkey), milk, dairy products (pasteurized milk products, soft cheese, butter and made with fresh milk; homemade cheese), fish (smoked, marinated and carpaccio), seafood (shrimp, crabs, mussels) and in frozen products such as ice cream and fresh vegetables and fruits (corn, celery, cantaloupe) (Gambarin et al., 2012; Mateus et al., 2013; McIntyre et al., 2015; Schäfer et al., 2017). Scientific studies have shown that the prevalence of particular *Listeria* strains is depending on its origin. This has been associated to certain phenotypes. For instance, *Listeria* strains isolated from the intestines of animals have the ability to enter intestinal niches, which is directly related to virulence (Maury et al., 2019).

Ready-to-eat products are highly integrated into our daily intake, generating concern as the main ingredients that make up these products are raw vegetables or meat products with a mild cooking that make these products more susceptible to the possibility of the presence of pathogenic microorganisms such as *L. monocytogenes*. RTE milk products is the food categories with more samples examined, a

total of 59,313 in the 23 MS in the last year due to possible contamination of *L. monocytogenes* (EFSA, 2019).

The latest epidemiological report from Ministerio de Sanidad, Consumo y Bienestar Social of the Spanish Government, reported last summer 2019 one of the largest outbreaks of *Listeria monocytogenes* in shredded beef, with more than 200 people affected leaving three deaths and five miscarriages (Anonymous, 2019).

1.5. *Salmonella* spp.

The time *Salmonella* spp. was isolated dates from 1885, during a scientific study seeking the causative agent of cholera The doctor in veterinary medicine Daniel Elmer and his assistant Mr. Theobald Smith, isolated a new species called *Bacillus cholerasuis* which was later renamed as, *Salmonella enterica* serovar Cholerasuis (Doyle et al., 2019; Fàbrega & Vila, 2013). *Salmonella* spp. is a Gram-negative, anaerobic facultative rod belonging to the *Enterobacteriaceae* family. The rod shape is maintained, thanks to an actin-like protein that forms the bacterial cytoskeleton (Khan, 2014). They are 2-3 μm long and 0.5-1.5 microns wide, and their genome ranges from 4,460 to 4,857 kb. They are non-sporulated facultative anaerobes and generally non-encapsulated (Grimont & Weill, 2007; Khan, 2014; Koneman et al., 1998; Lennette, 1982; Malorny et al., 2011). *Salmonella* spp. includes over 2,579 serotypes (Abulreesh, 2012; Grimont & Weill, 2007; Jensen & Hoorfar, 2000; Malorny et al., 2011). In addition to the two species, *Salmonella bongori* and *Salmonella enterica*, in 2012 The Manual of the International Organization of Epizootics in its seventh edition, catalogued a third species, *S. subterranea* (Fernández, 2015). *S. enterica* is divided into six subspecies, which are distinguished by some biochemical characteristics and which have in common with other subgeneus: I enterica; II salamae; IIIa arizonae; IIIb diarizonae; IV houtenae and VI indica. The roman symbol V, is reserved for the serotypes of *S. bongori* (Tindall et al., 2005).

Salmonella enterica includes all the pathogen serovars of this genus affecting public health, such as *Salmonella* Typhi, causing typhoid fever, a systematic infection, which is common in developing

countries and, in some cases, *S*. Parathyphi (A, B and C), which causes clinical symptoms similar to the previous one, but of lesser severity. *S. bongori* is more closely related to animals, especially cold-blooded animals (Martelli & Davies, 2012).

The most used classification and one of the most representative schemes of this genus, is based on its biochemical and serological characteristics, using the scheme White-Kauffmann-Le Minor. However, with the support of molecular methods -PCR, hybridization, ribotyping, homology of the DNA, gel electrophoresis and multilocalized enzyme -, it has been possible to determine the current classification of this genus (Fernández, 2001). Still, the scheme of White-Kauffmann-Le Minor, is used by public health organizations worldwide for the determination of *Salmonella* serotypes based on antigen identification (Grimont & Weill, 2007). This classification of serotyping is a very useful way of monitoring diseases and detecting outbreaks (Fierer & Guiney, 2001).

Salmonella spp. is capable of growing at temperatures of 8-45°C (optimum temperature 37°C). Some strains grow at 54°C and at other at refrigeration temperatures (2-4°C), but do not survive at temperatures of 70°C. The pH enabling growth covers a wide range from acidic to alkaline, ranging from 4.0 to 9.5 (optimum range pH 6.5-7.0) (Doyle et al., 2019; Rönnqvist et al., 2018). This pathogen is associated with the risk of disease outbreaks caused by marine products or that are formulated with an a_w from 0.93, are able to survive between percentages of 0-4% NaCl, including in some cases in brines with up to 20% NaCl and resists acid pH, lower than 4.5 (Alvarez-Ordóñez et al., 2010; Flores Aguilar, 2003; Mattick et al., 2000; Zurera-Cosano et al., 2011).

Salmonella spp. is widely dispersed throughout nature, and is a pathogen that can be found in the gastrointestinal tract of humans, domestic and wild mammals, reptiles, birds, insects and rodents, causing in humans and animals a disease called salmonellosis (Flores Aguilar, 2003). The three most reported serovars in the last three years in Europe were *S*. Enteritidis, *S*. Typhimurium and monophasic *S*. Typhimurium, These represented 71% of the total 79,698 confirmed human cases in 2018 in Europe (EFSA, 2019).

1.5.1. Salmonellosis as a foodborne disease

Salmonellosis is a pathology caused by non-typhoid serotypes, which can be isolated from both humans and animals including cattle (Kurtz et al., 2017). Among the serotypes that are responsible for food borne disease are *S*. Typhimurium, *S*. Enteritidis, *S*. Newport or *S*. Heidelberg (Andino & Hanning, 2015). The infection in humans and animals occurs due to the intake of food and/or water contaminated by this pathogen, so it is considered that the environment is a very important transmission vector for *Salmonella* (Andino & Hanning, 2015; Kurtz et al., 2017; Wiedemann et al., 2015). For the development of salmonellosis, an infective dose of 10^6-10^8 cells is necessary. In some cases, a dose of 10 cells is sufficient to manifest the disease. The incubation time is 6-72 h and the disease does not last more than 7 days, depending on the infective dose. The most typical symptoms are nausea, fever, chills, joint and muscle pain, as well as cramps and loss of appetite (Antillón et al., 2017 ; Antunes et al., 2016; Fàbrega & Vila, 2013; Hald, 2013; Hung et al., 2017; Jarvis et al., 2016; Jiang et al., 2015). Once the symptoms have disappeared, *Salmonella* can remain in the intestines of an adult for 4 weeks, and a total of 7 weeks in the intestines of children (Octavia et al., 2015).

Among the most affected risk population are children under 4 years of age, who are infected with the most common serotypes, Enteritidis and Typhimurium. The US has the highest number of confirmed cases of salmonellosis per year worldwide (Anderson et al., 2016; Jiang et al., 2015), while countries on the European continent have a much lower incidence rate in this respect over the last few consecutive years (EFSA, 2019).

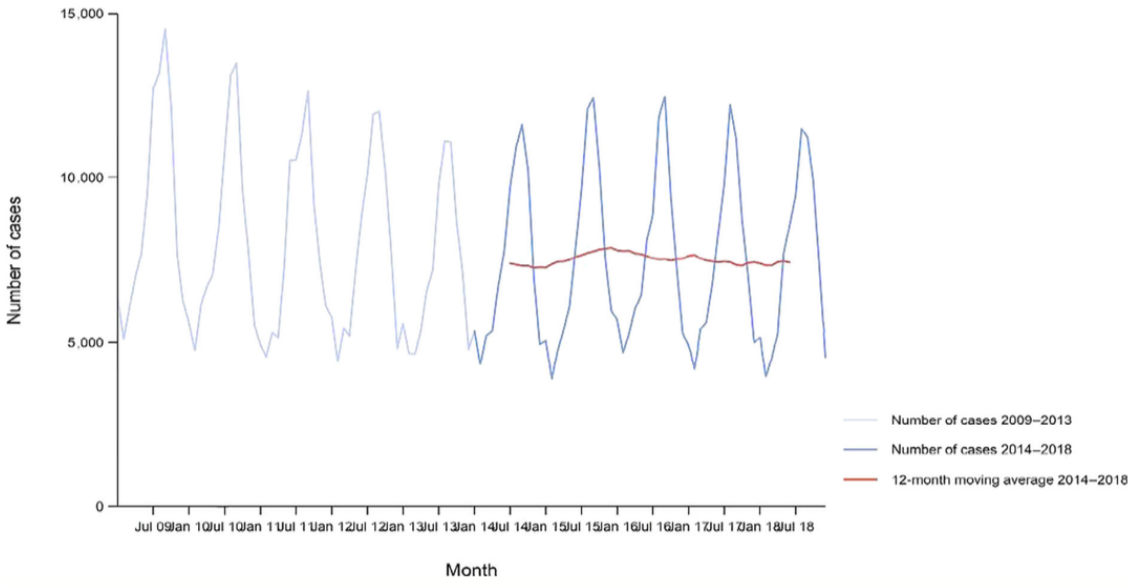

Figure 5 Confirmed human cases of salmonellosis in the EU/EEA, by month, 2009-2018 (EFSA, 2019).

As can be seen in Figure 5, the number of cases of *S.* Enteritidis in the EU remained constant between 2009 and 2018. Finland was the only member state that reported a significant fall in foodborne *S.* Enteritidis infection in the European Union between the years 2014-2018. Estonia, Ireland and Slovakia reported an important increase in the last five years (EFSA, 2019).

1.5.2. Presence of *Salmonella* in food

European Union Regulation (EC) 2073/2005, considers *Salmonella* as a food safety criterium, setting a limit of "not detected in 25 g or mL" in five samples, for most food categories.

The main food where *Salmonella* can be found is in eggs or egg products and poultry. The EU Scientific Committee on Veterinary listed the public health hazards by category (SCVPH, 2003):

- "raw meat and some meat products intended to be eaten raw"
- "raw or undercooked poultry meat products"
- "eggs and products containing raw eggs"
- "unpasteurized milk and some milk products"

Among other products included too: germinated seeds, fruit juices that have not received any pasteurization treatment and dangerous as homemade mayonnaise, and vegetable products are also an important source of transmission of this pathogen to humans (Forshell & Wierup, 2006).

Meat products, is the category with the most food samples examined, due to the risk of contamination of *Salmonella* spp. A total of 417,886 sample units in the 28 MS, a higher amount compared to the last three years (EFSA, 2019).

2.JUSTIFICATION AND OBJETIVES

The presence of pathogenic microorganisms in foods is of great relevance for consumer health and a big concern for food producers. Traditionally, bacterial cells were inactivated by the application of thermal treatments. However, high temperatures can also have a detrimental effect on the quality of the food product. Hence, there is strong interest in the development of novel strategies alternative to traditional thermal processing that could reduce the impact on product quality. The implementation of these technologies requires an understanding of how bacterial cells respond to these stresses, as well as to the combination of different type of technologies.

The main objective of this PhD thesis was to **study different inactivation strategies for the inactivation of *Listeria* and *Salmonella* that could reduce the intensity of the treatments while ensuring food safety**. To carry out this purpose, the following secondary objectives were defined:

- To study the inactivation kinetics of *L. monocytogenes* during dynamic thermal treatments, highlighting the relevance of variability under isothermal and dynamic conditions.
- To analyze the effect of different liquid media on the inactivation kinetics of various strains of *L. monocytogenes* when exposed to isothermal and dynamic thermal treatments.
- To evaluate the combination of an acid shock and a thermal treatment for the inactivation of two *Salmonella* serovars, focusing on whether the treatment is synergistic or antagonistic.
- To test a strategy for the inactivation of *Listeria* spp. based on the combination of two technologies with potentially synergistic effect: pulsed electric fields and oregano essential oil.

3.RESULTS AND DISCUSSION

CHAPTER I

Variability in the heat resistance of *Listeria monocytogenes* under dynamic conditions can be more relevant than that evidenced by isothermal treatments

This chapter has been published as an article in the journal, Food Research International 137 (2020) 109538, in a special issue "Variability in behaviour of pathogens and spoilage organisms – impact of food safety and spoilage"

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Abstract

Heterogeneity in the response of microbial cells to environmental conditions is inherent to every biological system and can be very relevant for food safety, potentially being as important as intrinsic and extrinsic factors. However, previous studies analyzing variability in the microbial response to thermal treatments were limited to data obtained under isothermal conditions, whereas in the reality, environmental conditions are dynamic. In this article we analyse both empirically and through mathematical modelling the variability in the microbial response to thermal treatments under isothermal and dynamic conditions.

Heat resistance was studied for four strains of *Listeria monocytogenes* (Scott A, CECT 4031, CECT 4032 and 12MOB052), in three different matrices (buffered peptone water, pH 7 Mcllvaine buffer and semi-skimmed milk). Under isothermal conditions, between-strain and between-media variability had no impact in the heat resistance, whereas it was very relevant for dynamic conditions. Therefore, the differences observed under dynamic conditions can be attributed to the variability in the ability for developing stress acclimation. The highest acclimation was observed in strain CECT 4031 (10-fold increase of the *D-value*), while the lowest acclimation was observed in strain CECT 4032 (50% increase of the *D-value*). Concerning the different media, acclimation was higher in buffered peptone water and semi-skimmed milk than in Mcllvaine buffer of pH 7.0.

To the knowledge of the authors, this is the first research work that specifically analyses the variability of microbial adaptation processes that take place under dynamic conditions. It highlights that microbial heat resistance under dynamic conditions are sometimes determined by mechanisms that cannot be observed when cells are treated in isothermal conditions (e.g. acclimation) and can also be affected by variability. Consequently, empirical evidence on variability gathered under isothermal conditions should be extrapolated with care for dynamic conditions.

Keywords: predictive microbiology; food safety; pasteurization; foodborne pathogens, stress acclimation; stress response

Highlights

- Variability in *Listeria* heat inactivation was analysed using strains and 3 media.
- Variability were less relevant than uncertainty under isothermal conditions.
- Between-strain and between-media variability was significant for dynamic treatments.
- Variability can affect processes that are only observed under dynamic conditions.

1. Introduction

During the last years, the demand for ready-to-eat (RTE) food products has increased. This type of product is usually consumed in the same state as that in which it was sold and/or distributed, or may be just reheated, portioned, garnished or finished before being served (Lianou & Sofos, 2007).

Listeria monocytogenes is a foodborne pathogen of great concern in relation to RTE products. The reason for that are its ubiquity, as well as its ability to grow in a wide range of pHs (between pH 5 and 9), water activities (higher than 0.90), NaCl concentrations (up to 16%) and temperatures (between 0 and 45°C) (Blanco, 1994; Ryser & Marth, 1999). It is responsible for the disease called listeriosis (Gahan & Hill, 2005). Only in the European Union, 28 member states reported 2,549 cases of human listeriosis in 2018; i.e. 0.47 cases per 100,000 people (ECDC, 2019; EFSA, 2019). Although the number of cases of listeriosis is relatively low, it has a high severity when compared to other foodborne illnesses. In a recent outbreak in Spain, more than 200 people were affected by listeriosis, leaving three deaths and five miscarriages (Anonymous, 2019).

Regulation (EC) 2073/2005 on microbiological criteria for foodstuffs requires food business operators to conduct, as necessary, shelf-life studies, in particular in ready-to-eat foods that are able to support the growth of *L. monocytogenes*, in order to investigate compliance with the criteria throughout the shelf-life. As indicated in the same Regulation (EC) 2073/2005 "the studies shall take into account the inherent variability linked to the product, the microorganisms in question and the processing and storage conditions." Several challenge tests studies have been conducted since this Regulation entered into force in order to show the possibilities of this microorganism to grow under the regular storage conditions for specific ready-to-eat foods (Albert et al., 2005; Allende et al., 2007; Aryani et al., 2015; Augustin et al., 2011; González-Tejedor et al., 2018; Guillier et al., 2008). By contrast, challenge tests designed to determine the

probability of survival during food storage when the foods were exposed to preservation treatments in the professional part of the food chain are much less usual.

Heat sterilization and pasteurization processes have been traditionally used in the food industry due to its effectiveness to ensure microbiological stability. Its application can destroy or inactivate pathogenic microorganisms via damage on the cell wall, protein denaturation and/or affecting reactions related to cell function (Li & Gänzle, 2016). Therefore, they can reduce the microbial count to innocuous level to humans, guaranteeing microbiologically safe products. On the other hand, high temperatures can also have a negative impact on the nutritional and organoleptic properties of the product (Kilcast & Subramaniam, 2011; Maza et al., 2019; Peng et al., 2017). For instance, they can lead to denaturation of proteins and loss of nutrients and vitamins (Blake et al., 1995; González-Tejedor et al., 2017b; Lavigne et al., 1989). Consequently, it is necessary to understand the different factors that affect the microbial response to inactivation treatments, in order to design thermal treatments that are effective while having a minimum impact on product quality.

Besides environmental factors, biological variability can also be a very relevant factor determining the actual efficacy of an inactivation treatment (Den Besten et al., 2018; Koutsoumanis & Aspridou, 2017). In the context of predictive microbiology, variability refers to inherent variation of the system (e.g. due to biological differences between single cells). It is, thus, different to uncertainty, which is related to variation due to lack of knowledge and can be reduced by acquiring more data of better quality (Nauta, 2000). The analysis of variability and uncertainty has gained interest during the last years. This analysis has been tackled from two different directions. Several studies have applied a bottom-up approach, using numerical simulations to show the relevance of variability in the thermal resistance of cells (Den Besten et al., 2017;Koutsoumanis, 2008; Koyama et al., 2019). On the other hand, other analyses applied a top-down methodology, based on the statistical analysis of datasets of microbial responses obtained under similar conditions (e.g. the same strain) (Aryani et al., 2015; Den Besten et al., 2017). However, these empirical studies were limited to experimental data obtained under isothermal conditions. Indeed, to the knowledge of the authors, no empirical study has analysed

the variability under dynamic conditions, comparing it to the variability observed under isothermal conditions.

For historical reasons (especially the limitations of the laboratory equipment), most knowledge on microbial inactivation is based on data gathered under isothermal conditions. Several studies have highlighted difficulties to predict the microbial count for dynamic treatments using models built based on isothermal experiments (De Jonge, 2019; Dolan et al., 2013; Garre et al., 2019; Garre, Huertas, et al., 2018; Hassani et al., 2006; Huertas et al., 2016; Janssen et al., 2008; Smelt & Brul, 2014). One hypothesis to justify this deviation is that some physiological processes are only relevant under dynamic conditions and cannot be observed in isothermal treatments, where the heating of the microbial cells is instantaneous. An example of such process is stress acclimation. Microbial cells are able to develop a physiological response to temperatures above the maximum temperature for growth that can increase their resistance to posterior treatments (Hill et al., 2002; Li & Gänzle, 2016; Richter et al., 2010). As a consequence, microbial cells that were heat shocked can be more resistant to a posterior treatment than control cells (Bunning et al., 1990; Hassani et al., 2007; Lin & Chou, 2004b). Experimental evidence have shown that stress acclimation can also be developed during dynamic treatments with a low heating rate (Garre, Egea, et al., 2018; Garre, Huertas, et al., 2018; Hassani et al., 2005; Valdramidis et al., 2006). It is reasonable to hypothesize that the ability of microbial cells to develop a stress acclimation would be variable, in the same way that variability in thermotolerance to isothermal treatments has been proven to exist. Such variability could reside on the microbial strain, on the heating medium and/or even on the heating conditions, isothermal or dynamic. For example, it could happen that variability in stress acclimation could be independent from variability under isothermal conditions. However, to the knowledge of the authors, no article has been published exploring this possibility. Therefore, in this article we analyse the between-strain and between-media variability in the thermal resistance of *L. monocytogenes*. The study is done under isothermal and dynamic conditions, focusing on the relevance of variability in both situations and if there is a relationship between them.

2. Materials and methods

2.1. Bacterial culture and media

The strains studied were 12MOB052, provided by the National Food Centre (CNA, Madrid, Spain), and strains Scott A and CECT 4031 and CECT 4032, provided by the Spanish Type Culture Collection (CECT, Valencia, Spain). Strain 12MOB052 is a reference strain of the European Union Reference Laboratory for *Listeria monocytogenes* for conducting challenge tests, strain CECT 4032 was associated with a case of meningitis after eating soft cheese, strain Scott A was formerly isolated from milk and is a reference strain for conducting dairy product studies and finally strain CECT 4031 is the type strain of this species and was formerly isolated from rabbit. These strains were stored at -80°C (20% glycerol) until use. Subsequently, for the use of each strain, a loop of the cryopreserved culture was inoculated in Trypticase soy broth (TSB; Scharlau Chemie SA, Barcelona, Spain) supplemented with 0.6% yeast extract (YE, Scharlau Chemie SA) and incubated for 24 hours at 37ºC. A loop of this culture was sown on Trypticase soy agar (TSA, Scharlau Chemie SA,) supplemented with 0.6% YE and incubated for 24 hours at 37ºC Then, single colonies from fresh cultured plates were sown weekly in TSAYE. The fresh cultures were incubated for 24 hours at 37°C on an incubator.

For cultivation, tubes were prepared with TSBYE. For the production of the microbial pre-cultures, a single colony from a fresh culture plate was transferred to 5 mL of TSBYE and incubated overnight at 37°C. For obtaining the microbial cultures, flasks with 50 mL of TSBYE were inoculated with 1 mL of the pre-culture and incubated for 24 h at 37°C in agitation to obtain all cells in stationary phase with a concentration of approximately 10^9 CFU/mL.

2.2. Determination of the heat resistance of *Listeria monocytogenes*

Heat resistance determinations were carried out in a Mastia thermoresistometer (Conesa et al., 2009) using three different media. Two laboratory media, Buffered Peptone Water (BPW; Scharlau Chemie SA) and pH 7 Mcllvaine citrate-phosphate buffer (Dawson et al., 1969), and a food matrix, semi-skimmed milk, that was supplied by a Spanish local retailer.

For these experiments the vessel of the thermoresistometer was filled with 400 mL of the relevant heating medium and heated until the temperature was stable. Then, the heating medium was inoculated with 0.2 mL of the microbial suspension. Experiments were performed under isothermal at 57.5, 58.0, 60.0, 62.5 and 65.0ºC for 20, 10, 7, 1, and 0.5 min respectively and dynamic conditions. For dynamic conditions, experiments were performed using two bi-phasic temperature profiles where the heating media was heated from the initial temperature (30ºC) to the target temperature (57.5ºC) at two different heating rates (0.5ºC/min and 2ºC/min). In dynamic experiments, the heating medium was inoculated with 0.2 mL of the microbial suspension once the thermoresistometer had reached the initial temperature. Figures 6-9 illustrate these temperature profiles Samples were taken at predefined sampling points. They were recovered in TSAYE and they were incubated for 24 hours at 37°C. A minimum of 3 separate experiments were performed per condition.

2.3 Modelling of isothermal inactivation

The isothermal inactivation data of *Listeria monocytogenes* did not show shoulder or tail effects. Hence, it was analyzed using a log-linear primary inactivation model. The model equation is shown in Equation (12), where N(t) is the microbial density at time t. In this model, the exponential phase is described by parameter k_{max}, which equals the maximum specific inactivation rate.

$$\frac{dN}{dt} = -k_{max}N \tag{12}$$

As secondary model for the inactivation rate we use a log-linear relation derived from the Bigelow model (Bigelow, 1921). For that, we transform the inactivation rate to the *D-value* using the identity $D(T) = \frac{\ln 10}{k_{max}}$. This parameter equals the time required to cause a 90% reduction of the microbial count during an isothermal treatment at temperature T. In this model, the sensitivity of the cells to temperature changes is quantified by the *z-value* (z), which represents the temperature increase required to reduce the *D-value* a 90%. This equation defines a reference temperature (T_{ref}) without a biological meaning, but that can improve parameter identifiability (Dolan & Mishra, 2013; Peñalver-Soto et al., 2019). The parameter D_{ref} stands for the *D-value* calculated at the reference temperature. According to Peñalver-Soto et al. (2019), the reference temperature was set to the mean of the temperature range of the isothermal experiments (61.5ºC).

$$\log D(T) = \log D_{ref} - \frac{T - T_{ref}}{z} \quad (13)$$

The model parameters were estimated using a two-step approach. First, the log-linear model was fitted to the data obtained at each temperature, strain and media using GinaFit (Geeraerd et al., 2005). The Geeraerd model (Geeraerd et al., 2000) was also fitted to the isothermal data, validating that the shoulder and tails were not significant in these experiments. Then, the Bigelow secondary model (Equation 13) was fitted to the *D-values* using non-linear regression (the Newton-Raphson algorithm in the R programming language (R Core Team, 2016).

The relevance of variability with respect to uncertainty in regard to the variation of the *D-value* observed under isothermal conditions was determined using model selection. The experimental design of this study enables four different structures for the secondary model:

1. A global model where a single D_{ref} and z describe the relationship between the D-value and temperature for every strain and media. In other words, a model where variability does not affect the D-value.

2. A model assuming that between-strain variability is relevant, but not between-media variability. This model has 8 model parameters: 4 values for D_{ref} (one per strain) and 4 values for z (one per strain).

3. A model hypothesizing that between-media variability is relevant, but not between-strain variability. This model has 6 model parameters: 3 values for D_{ref} (one per media) and 3 values for z (one per media).

4. A model considering that both between-media and between-strain variability are relevant. This model has 24 model parameters: 12 values for D_{ref} (one per strain/media combination) and 12 values for z (one per strain/media combination).

In order to determine the most parsimonious model, all of them were fitted to the data using nonlinear regression (Gauss-Newton algorithm) and the models were compared using the Akaike Information Criterion (AIC) (Akaike, 1974). This index, based on information theory, accounts for model precision and model parsimony. Models with a lower AIC are preferred over models with higher AIC.

2.4 Modelling of microbial inactivation under dynamic conditions

The model's parameters obtained under isothermal conditions were used to predict the microbial count for non-isothermal experiments. For that purpose, the differential equations of the Bigelow model (Equations 14 and 15) were solved using the LSODA algorithm (Hindmarsh, 1983), using Bioinactivation (Garre, Clemente-Carazo, et al., 2018; Garre et al., 2017). This model does not consider any phenomena that may arise during non-isothermal treatments. For that reason, the acclimation model proposed by (Garre, Huertas, et al., 2018) has also been used. This model extends the first order inactivation model to account for the thermal history of the cells. It assumes that the specific inactivation rate (k) is the product of two independent term: k_1, which depends on the instantaneous environmental conditions (i.e. the temperature), and k_2, which is a function of the thermal history of the cells (Equation 14).

$$\frac{dN}{dt} = k(t,T)N(t) = k_1(T) \cdot k_2(t)N(t) \quad (14)$$

For k_1, this model assumes a log-linear relationship with temperature, similar to the one used in the Bigelow model. On the other hand, coefficient k_2 is a correction factor bounded

between 0 and 1 according to a logistic equation (Equation 15). The level of acclimation of the microbial cells is described in this model using the variable p(t), which is bounded between zero and one. When p=0, there is no acclimation and the predicted specific inactivation rate equals the one based only on the instantaneous temperature (k_1). Then, when p=1, it is assumed that the cells have developed their maximum capacity to resist the treatment and the *D-value* is increased by a factor of 1+c. Therefore, the parameter c quantifies the maximum acclimation that the microbial cells can potentially develop.

$$\frac{dN}{dt} = \frac{\ln 10}{D(T_{ref}) \cdot 10^{-\frac{T-T_{ref}}{z}}} \cdot \frac{1}{1 + c \cdot p(t)} N(t) \tag{15}$$

In this model, the acclimation is not static but changes through the treatment, described by the time variation of variable p(t). This model considers that for temperatures lower than a stress inducing temperature, T_{si}, no acclimation takes place and p(t) remains constant. This parameter was fixed to 30ºC, as in previous investigations for *Listeria* (Garre et al., 2019). For temperatures higher than T_{si}, p(t) grows with a rate described by parameters a and E, as shown in Equation (16). For temperatures much higher than T_{si} the first derivative of p with respect to time tends to a. Hence, higher values of a imply a higher development of stress acclimation. The temperature increase required to go from the regime with no development of acclimation ($T < T_{si}$) and maximum development ($T \gg T_{si}$) is defined by the parameter E. Lower values of this parameter imply that a shorter temperature increase is required for this transition. Further details on the kinetics of this variable can be found in (Garre, Egea, et al., 2018).

$$\frac{dp}{dt} = \begin{cases} 0, T < T_{si} \\ a \cdot e^{-\frac{E}{T-T_{si}}}(1 - p(t)), T \geq T_{si} \end{cases} \tag{16}$$

As suggested by Garre, Huertas et al. (2018), the *D* and *z-values* estimated from isothermal conditions were used in the dynamic model. Then, the model parameters of the acclimation model were estimated from the data obtained under dynamic conditions using the Adaptive Monte Carlo algorithm proposed by Haario et al.(2006), through the interface included in the R package FME (Soetaert & Petzoldt, 2010). The convergence of the Markov chain has been checked following usual procedures (Brooks, 2011; Plummer et al., 2006). Trace plots, pairs

plots and autocorrelation plots have been visually inspected to check the correct mixing and convergence of the Markov Chain. These visual tests have been complemented with Geweke's convergence test (Geweke, 1991), as well as the Heidelberger and Welch's diagnostic (Heidelberger & Welch, 1983). For every condition (strain/media) tested, the model has been fitted to the data obtained with a heating rate of 0.5ºC/min, using the data at 2ºC/min as validation. For every case, 5000 iterations were necessary after 1000 burning iterations with an update of the covariance matrix every 500 iterations were needed for the convergence of the Marko chain. The goodness of the fit was evaluated using the mean error ($ME = \frac{1}{n}\sum_{i=1}^{n} e_i$) and the root mean squared error ($RMSE = \sqrt{\frac{\sum_{i=}^{n} e_i^{\,2}}{n}}$).

3. Results

3.1 Inactivation under isothermal conditions

Supplementary Figure 10 illustrates the *D-values* obtained at each temperature for each isothermal experiment. The mean of the *D-values* estimated at 57.5ºC in laboratory media was 1.80 min. This result is comparable with the values reported by Casadei et al. (1998) and Foegeding & Stanley (1991) in similar conditions. In milk, the mean *D-value* at 57.5ºC was 3.04 min. The results obtained in this medium are comparable with those obtained by Rowan & Anderson (1998), who obtained a *D-value* of 4.26 min. The *z-value* estimated for the different conditions ranged between 3.7 and 8.0ºC. The large variability of these values is in the range of those reviewed by Doyle et al. (2001), who observed similar large variability in the values of this parameter reported in different studies. However, the results do not show an obvious trend (i.e. no strain or media has *D-value*s that are consistently higher or lower than the rest). Furthermore, the model comparison revealed that the global model (without disaggregating between strain or media) had a lower AIC than any other model combination. (AIC=12.62 for the global model, AIC=14.6 for the model by media, AIC=22.7 for the model by strain and AIC=29.1 for the model

by strain and media). Therefore, under our experimental design and conditions, under isothermal conditions between-strain and between-media variability were less relevant than uncertainty. Consequently, the *D* and *z-values* estimated under isothermal conditions using the general model were used to analyse the response under dynamic conditions; i.e. without differentiating between strains and/or media.

3.2 Inactivation under dynamic conditions

Figures 6, 7, 8 and 9 compare the observed microbial counts for strains 12MOB052, Scott A, CECT 4031 and CECT 4032, respectively, against the model predictions for the acclimation model (fitted to the data obtained with 0.5ºC/min) and the Geeraerd model fitted to isothermal experiments. For every case considered, the Geeraerd model predicts microbial count several log-cycles lower than observed. On the other hand, the stress acclimation model is able to describe the data obtained at both heating rates. This is confirmed in Table 1, where the ME and RMSE are shown for the different conditions and models. In every case, the ME of the stress acclimation model is bounded between ±0.7 log CFU/ml. This value is similar to the precision of the plate counting method, so it can be considered that the model predictions are unbiased. On the other hand, the ME of the Geeraerd model is larger in every case, having a maximum (absolute) value of 5.61 log CFU/mL. In every case, except for the experiments for strain CECT 4032 in McIlvaine buffer, where it takes the value 0.66 log CFU/mL, it is negative, indicating that the model underpredicts the microbial count, as can be observed in figures 6-9. Therefore, the deviations between the predictions of the Geeraerd model and the observations can be attributed to the stress acclimation that the microbial cells can develop during the heating phase that increases their thermal resistance with respect to what is expected based on isothermal experiments.

Table 1 Statistical indexes describing the deviation of the observations and the model fits/predictions.

Strain	Heating medium	Profile	Geeraerd model		Acclimation model	
			ME (log CFU/ml)	RMSE (log CFU/ml)	ME (log CFU/ml)	RMSE (log CFU/ml)
STCC4031	Buffered peptone water	0.5ºC/min	-3.91	5.43	-0.14	0.44
		2ºC/min	-4.60	-5.85	-0.15	0.27
	pH 7 McIlvaine buffer	0.5ºC/min	-4.04	5.31	-0.03	0.12
		2ºC/min	-2.55	3.28	-0.09	0.17
	Semi-skimmed milk	0.5ºC/min	-2.48	4.34	-0.31	0.47
		2ºC/min	-2.66	3.50	-0.21	0.30
STCC4032	Buffered peptone water	0.5ºC/min	-1.96	3.43	0.52	0.67
		2ºC/min	-5.00	6.56	-0.51	0.78
	pH 7 McIlvaine buffer	0.5ºC/min	0.66	0.98	0.62	1.01
		2ºC/min	-0.80	1.18	-0.57	0.91
	Semi-skimmed milk	0.5ºC/min	-1.87	3.03	0.08	0.47
		2ºC/min	-4.28	5.63	-0.37	0.62
12MOB052	Buffered peptone water	0.5ºC/min	-3.44	5.26	-0.21	0.41
		2ºC/min	-4.04	5.24	0.01	0.22
	pH 7 McIlvaine buffer	0.5ºC/min	-1.05	2.01	0.15	0.37
		2ºC/min	-1.33	-1.94	0.08	0.35
	Semi-skimmed milk	0.5ºC/min	-2.93	4.71	-0.00	0.22
		2ºC/min	-1.68	2.45	0.17	0.29
Scott A	Buffered peptone water	0.5ºC/min	-2.91	4.71	-0.04	0.24
		2ºC/min	-5.61	6.87	0.14	0.32
	pH 7 McIlvaine buffer	0.5ºC/min	-0.55	1.20	0.09	0.43
		2ºC/min	-1.67	2.42	-0.32	0.73
	Semi-skimmed milk	0.5ºC/min	-3.05	4.75	-0.13	0.19
		2ºC/min	-3.79	5.00	0.33	0.49

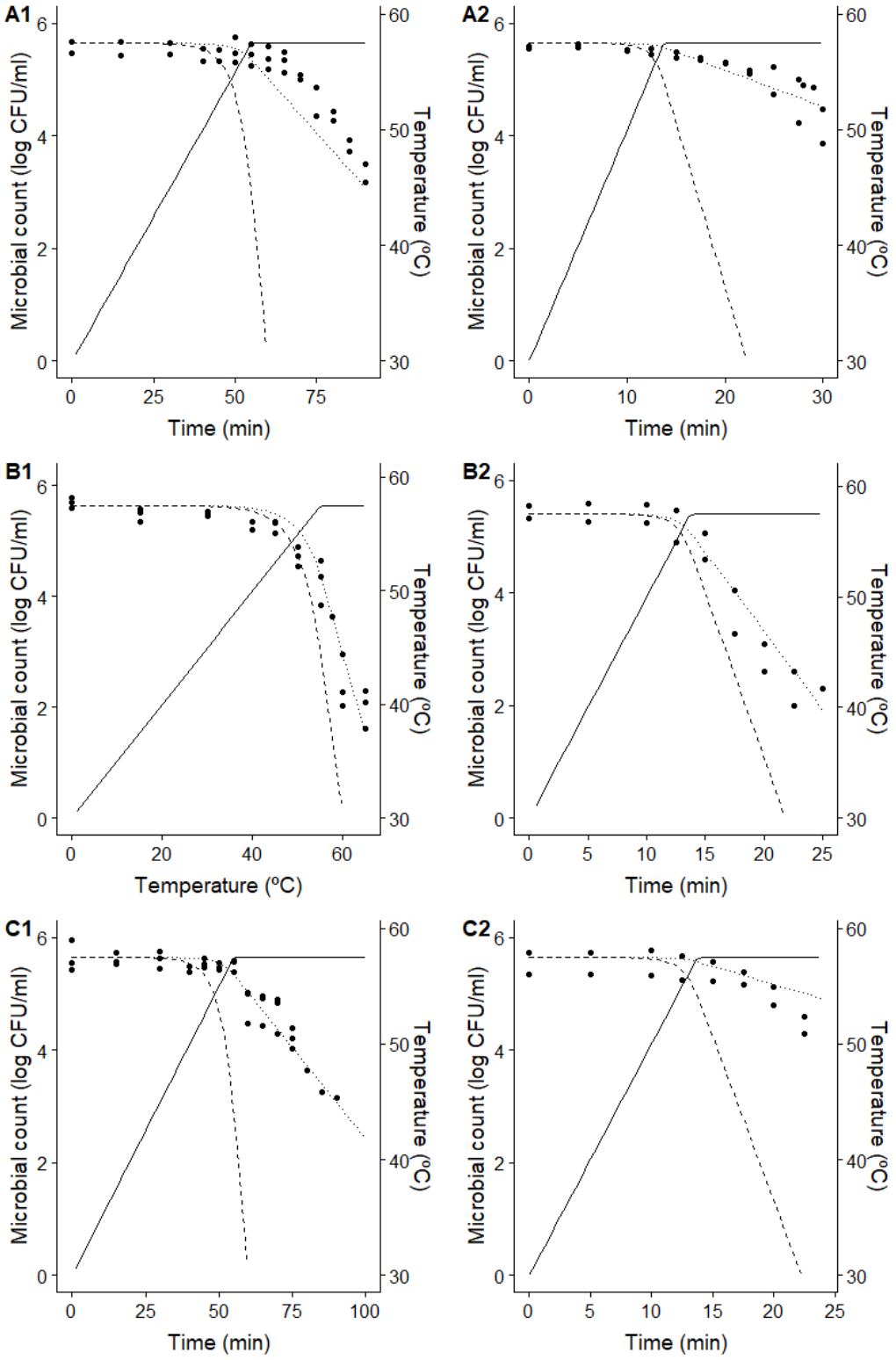

Figure 6 Comparison between the microbial counts (dots) for dynamic thermal treatments (-) and the predictions of the acclimatation model (..) and the predictions of the Geeraerd model based on isothermal experiments (−) for *L. monocytogenes* 12MOB052. Experiments were carried out in buffered peptone water (A), pH 7 Mcllvaine buffer (B) and semi-skimmed milk (C) and using two different thermal profile (1) & (2).

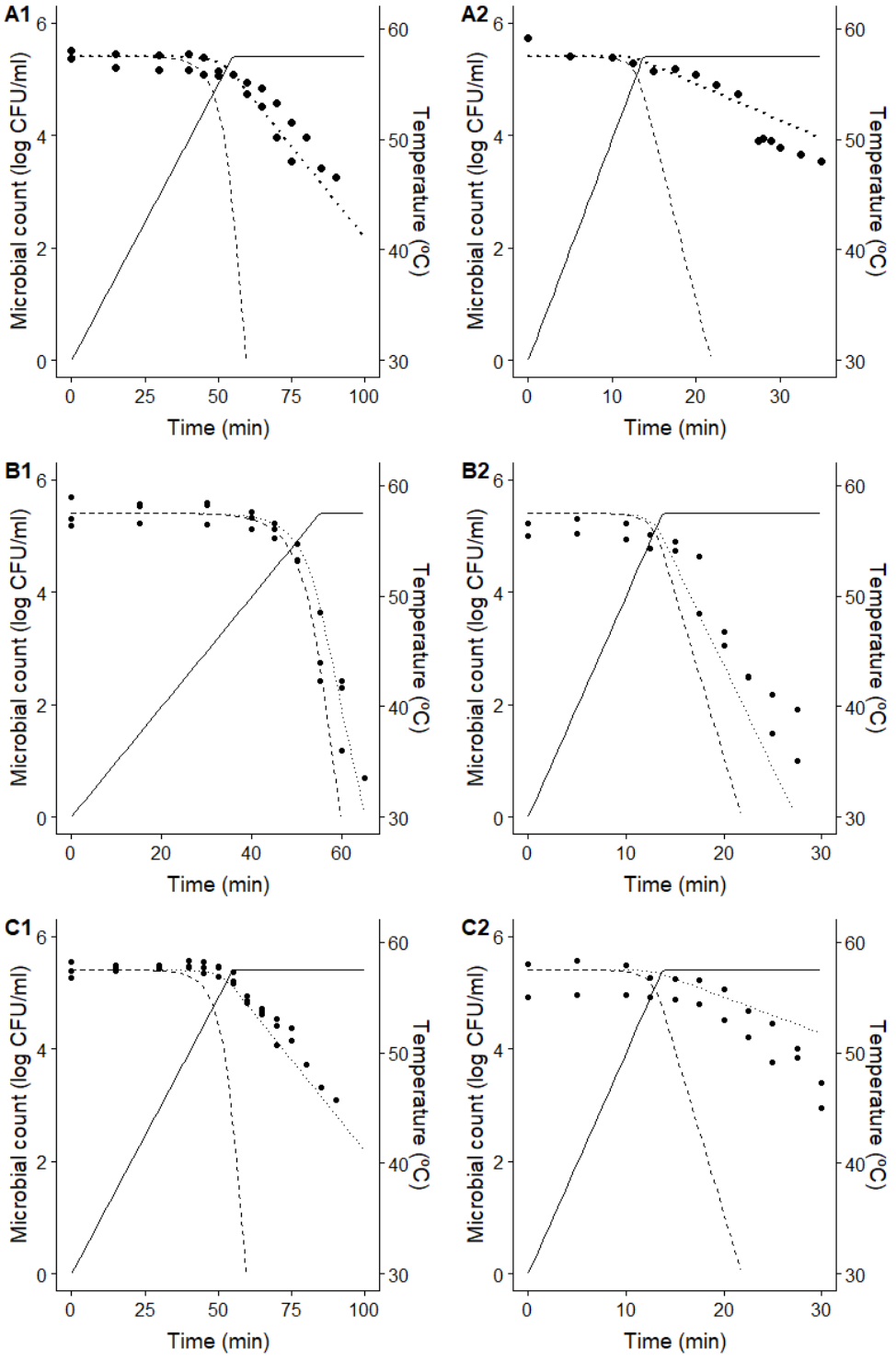

Figure 7 Comparison between the microbial counts (dots) for dynamic thermal treatments (-) and the predictions of the acclimatation model (..) and the predictions of the Geeraerd model based on isothermal experiments (−) for *L. monocytogenes* Scott A. Experiments were carried out in buffered peptone water (A), pH 7 Mcllvaine buffer (B) and semi-skimmed milk (C) and using two different thermal profile (1) & (2).

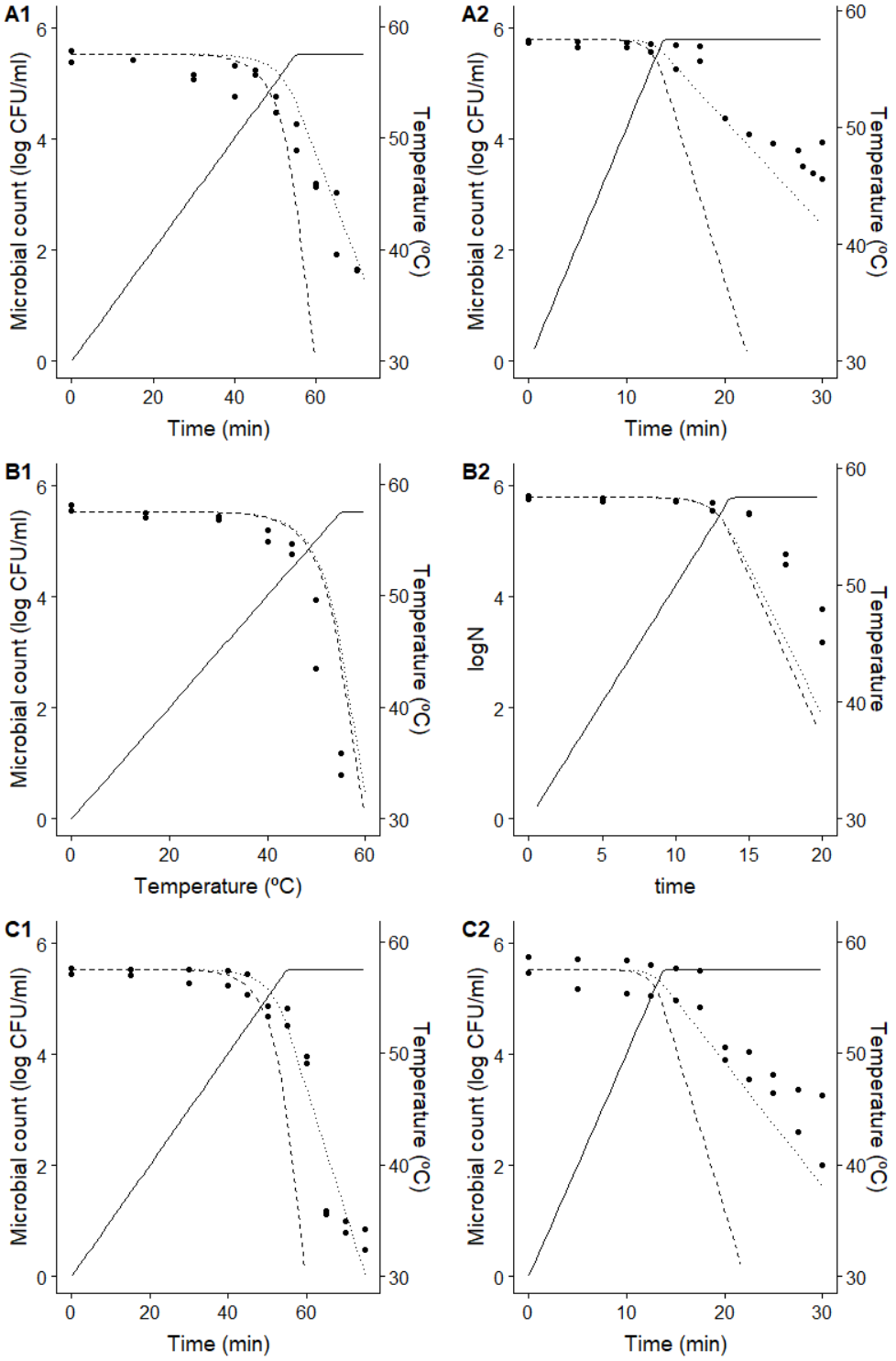

Figure 8 Comparison between the microbial counts (dots) for dynamic thermal treatments (-) and the predictions of the acclimatation model (..) and the predictions of the Geeraerd model based on isothermal experiments (−) for *L. monocytogenes* CECT 4031. Experiments were carried out in buffered peptone water (A), pH 7 Mcllvaine buffer (B) and semi-skimmed milk (C) and using two different thermal profile (1) & (2).

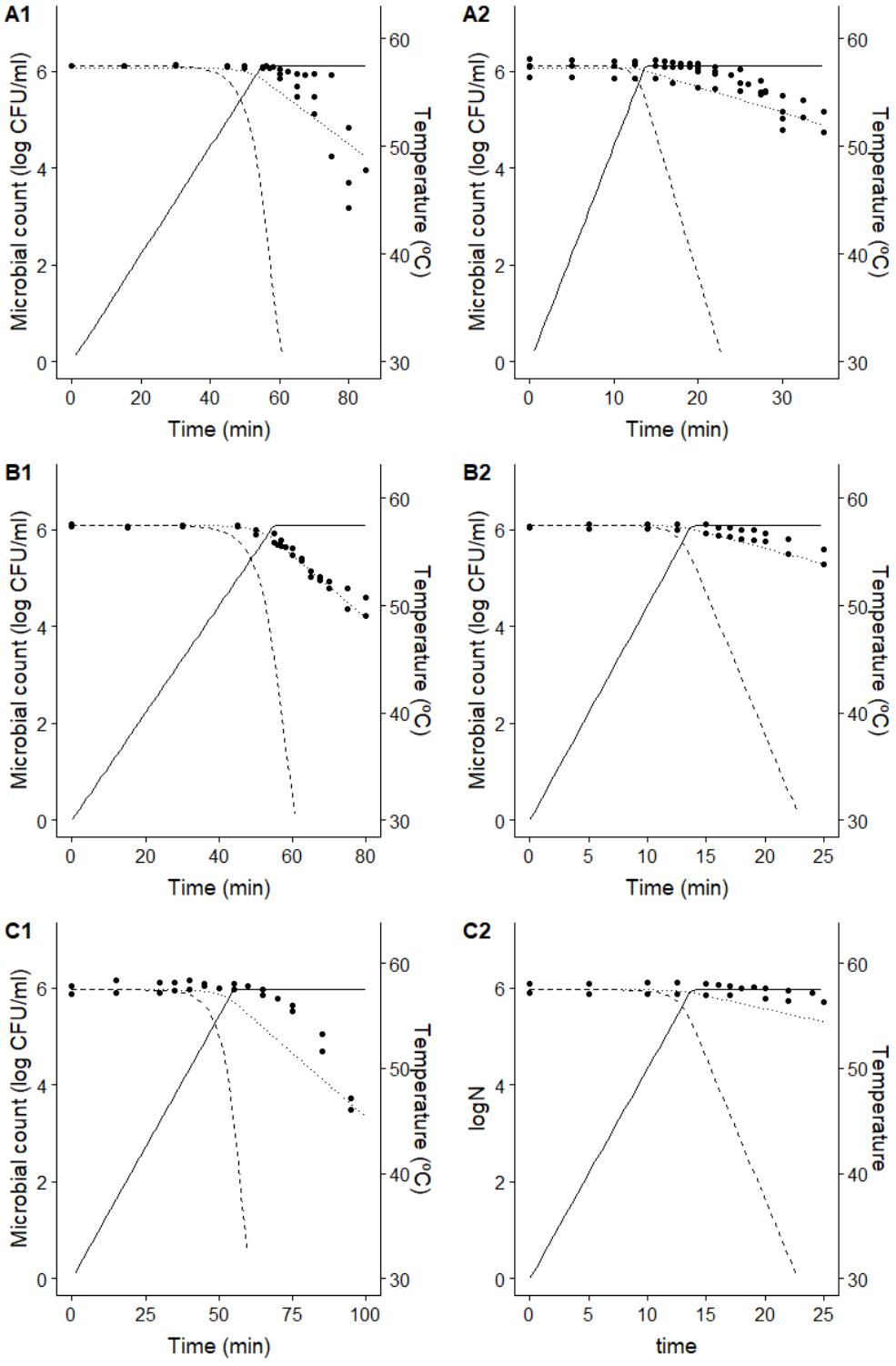

Figure 9 Comparison between the microbial counts (dots) for dynamic thermal treatments (-) and the predictions of the acclimatation model (..) and the predictions of the Geeraerd model based on isothermal experiments (−) for *L. monocytogenes* CECT 4032. Experiments were carried out in buffered peptone water (A), pH 7 Mcllvaine buffer (B) and semi-skimmed milk (C) and using two different thermal profile (1) & (2).

Table 2 reports the model parameters of the stress acclimation model estimated from the data obtained with a heating rate of 0.5ºC/min. The values obtained for parameters a and E are similar for all the conditions, indicating that the kinetics of the development of stress acclimation are similar in all the cases considered. However, there are large variations between-strain and between-media in the values estimated for parameter c. As mentioned before, this parameter quantifies the maximum acclimation that the microorganism can develop, being the *D-value* multiplied by a factor of 1+c. Therefore, we observe between-strain and between-media variability in the ability of the different strains tested to develop stress acclimation. The ability of the four strains to develop stress acclimation in BPW (c=8.41 for CECT 4031, c=2.28 for CECT 4032, c = 7.29 for B052, c = 6.92 for Scott A) was similar than the one observed in milk (c=8.99 for CECT 4031, c=1.74 for CECT 4032, c = 6.88 for B052, c = 6.95 for Scott A). On the McIlvaine buffer, on the other hand, only strain CECT 4031 was able to develop an acclimation (c = 6.70); whereas the other strains had a value of c much lower than the one observed for the other two media (c=0.42 for CECT 4032, c = 1.29 for B052, c = 0.76 for Scott A). The similarities between the results observed in BPW and milk can be explained based on their composition, since they are both rich and complex media, with a favourable pH value, where the bacteria may find suitable nutrients and conditions to build their acclimation response. McIlvaine buffer has no nutrients, so it can be expected that bacteria find difficulties to set such response. Strain CECT 4031 has probably other acclimation mechanisms, that are not dependent on the availability of nutrients on the surrounding medium.

Table 2 Model parameters of the stress acclimation model (value ± empirical standard error) estimated from the dynamic inactivation data obtained for a heating rate of 0.5ºC/min.

Strain	Heating medium	a (min^{-1})	E (ºC)	c (-)
STCC 4031	Buffered peptone water	0.24 ± 0.02	0.057 ± 0.004	8.41 ± 0.01
	pH 7 McIlvaine buffer	0.26 ± 0.02	0.055 ± 0.004	6.70 ± 0.03
	Semi-skimmed milk	0.24 ± 0.02	0.056 ± 0.004	8.99 ± 0.01
STCC 4032	Buffered peptone water	0.26 ± 0.01	0.055 ± 0.002	2.28 ± 0.02
	pH 7 McIlvaine buffer	0.38 ± 0.04	0.062 ± 0.003	0.42 ± 0.04
	Semi-skimmed milk	0.27 ± 0.03	0.059 ± 0.001	1.74 ± 0.02
12MOB052	Buffered peptone water	0.25 ± 0.02	0.054 ± 0.002	7.29 ± 0.03
	pH 7 McIlvaine buffer	0.23 ± 0.02	0.055 ± 0.001	1.29 ± 0.02
	Semi-skimmed milk	0.25 ± 0.01	0.056 ± 0.002	6.88 ± 0.04
Scott A	Buffered peptone water	0.30 ± 0.02	0.051 ± 0.001	6.92 ± 0.04
	pH 7 McIlvaine buffer	0.25 ± 0.02	0.057 ± 0.002	0.76 ± 0.02
	Semi-skimmed milk	0.27 ± 0.01	0.054 ± 0.002	6.95 ± 0.06

Table 2 also shows that there is variability at the between-strain level in the ability of cells to develop stress acclimation. The strain with the lowest ability for developing stress acclimation was CECT 4032, with a maximum value of c of 2.28. This means that, this strain can increase its *D-value* by a factor of 3.28 due to stress acclimation. This value is much lower than the ones observed for the other three strains tested. The strain with the highest ability to develop stress acclimation was strain CECT 4031, with a maximum value of c of 8.99 (an increase in the *D-value* by a factor of 9.99). Moreover, this strain is the only one able to develop substantial stress acclimation in the McIlvaine buffer, showing that this strain is the better adapted to develop stress acclimation. Finally, strains 12MOB052 and Scott A had a similar value of c (in the vicinity of c = 7), thus, closer to those observed for CECT 4031 than the ones of CECT 4032.

4. Discussion

History has an influence in the predictive models we are using today to describe microbial inactivation. The predictive models currently in use are the result of an iterative process, where available models were updated when new empirical evidence could not be described using the models available at the time. The first efforts in this direction date back to the works by Bigelow

and Esty in the 1920s (Bigelow, 1921; Bigelow & Esty, 1920; Esty & Meyer, 1922). Their observations resulted in the assumption of log-linearity in the microbial response and the definition of the *D-value* and its log-linear relationship with temperature. The availability of new empirical data showing that the log-linearity of the survivor curve was rather an exception than a rule led to the development of novel inactivation models that considered different types of curvature (Geeraerd et al., 2000; Mafart et al., 2002; Peleg & Cole, 1998).

A similar challenge is related to the prediction of the microbial inactivation under dynamic conditions based on information (models and data) gathered under isothermal conditions. There is empirical evidence showing that the microbial response under dynamic conditions is greatly affected by processes that cannot be observed under isothermal conditions. As a result, models developed based on isothermal conditions usually fail to predict the response under dynamic conditions (Corradini & Peleg, 2009; Garre, Egea, et al., 2018; Garre et al., 2019; Garre, Huertas, et al., 2018; Hassani et al., 2005, 2006; Huertas et al., 2016; Stasiewicz et al., 2008; Valdramidis et al., 2006, 2007). The limitations of these models to deal with microbial adaptative response under dynamic conditions were already discussed by Garre, Huertas et al (2018). A clear example of this adaptative response is stress acclimation. During isothermal treatments at relatively high temperatures (close to the temperature during the holding phase in the dynamic treatments in this study), the inactivation rate may be too fast for stress acclimation to develop. Therefore, it could not be observed. However, it can become relevant for dynamic treatments at the same temperature if the heating rate is slow. Therefore, the description of microbial inactivation under dynamic conditions require the definition of novel predictive models, where these mechanisms that can only be observed under dynamic conditions are included in model definition.

The lack of predictive models specifically designed for dynamic conditions explains why, although several studies in the literature have analysed stress acclimation of *Listeria*, most of them did only analyse the data qualitatively or used mathematical models that did not separate between thermal resistance and stress acclimation (e.g. by fitting a *D-value* to the complete curve). Therefore, the model parameters obtained in this study can only be compared to those

obtained by Garre et al. (2019). They performed similar experiments also using strain CECT 4032 of *L. monocytogenes*, but different heating media (Tryptic Soy Broth and skimmed milk). In their study, they estimated a value of the parameter c of 1.23±0.03. This result is comparable to those obtained in this investigation, where we have obtained a value of c of 1.74±0.02 for this same strain in semi-skimmed milk. The relatively small differences between these parameters can be attributed to between-media variability that, as shown in this research, can be very relevant for stress acclimation.

Although comments related to microbial variability have been included in legislative texts such as, for example Regulation (CE) 2073/2005 (where a reference to its consideration in shelf life studies is made), the interest in the scientific community in microbial variability and its relevance for risk assessment is relatively recent (Den Besten et al., 2018; Koutsoumanis & Aspridou, 2017). There is broad empirical evidence suggesting that variability is inherent to the microbial response to environmental conditions that enable growth or cause microbial inactivation (Aryani et al., 2015; Balomenos et al., 2017; Elfwing et al., 2004; Wells-Bennik et al., 2019). Our results under isothermal conditions somewhat contradict those of previous studies, which concluded that between-strain (Aryani et al., 2015) and between-media (Van Asselt & Zwietering, 2006) variability could be very relevant for isothermal inactivation of *L. monocytogenes*. However, those studies reached those conclusions after analysing the microbial response under a broad range of different conditions (20 strains and 40 products, respectively). It must be highlighted that, although between-strain and between-media variability can be very relevant for isothermal treatments, they may become less relevant and be indistinguishable from uncertainty in studies with a small sample size. Indeed, van Asselt & Zwietering found that, although between-media variability was relevant for the inactivation of Salmonella, the data could be grouped by some food products for which variability was not relevant. Therefore, the low impact of between-strain and between-media variability observed in our study under isothermal conditions can be attributed to the relatively low number of media and strains analysed compared to the experimental design of other studies that only analysed the microbial response under isothermal conditions.

However, most models have been developed without variability into consideration and are designed to make predictions as point-estimates (e.g. survivor curves), potentially missing an important factor that can be determinant for risk assessment. Variability must, then, be included post-hoc, usually through the definition of some probability distributions to describe the variability of one or more model parameters (Den Besten et al., 2017; Koutsoumanis, 2001; Koyama et al., 2019). Hence, variability is described by the variation in this model parameter. However, we must be aware of the large knowledge gaps that currently surround the variability of the microbial response. Further empirical data are required to assess whether, for instance, the definition of a probability distribution for the *D-value* can capture how variability affects microbial inactivation under isothermal conditions and what should be the parameterization of that distribution.

In this study, we have provided further empirical evidence to shed light on how variability affects microbial inactivation. We have demonstrated that variability can affect differently the microbial inactivation under isothermal and dynamic conditions. In the experiments gathered under isothermal conditions, no trend could be observed regarding the effect of the strain or the heating media on the inactivation rate (the *D-value*). Therefore, variability could not be distinguished from uncertainty in this case. However, the experiments obtained under dynamic conditions did show that the variability in strain and media had a very relevant impact on microbial inactivation. The reason for that can be attributed to the variability in stress acclimation observed under different conditions. Consequently, variability in microbial inactivation can affect differently the microbial response under isothermal and dynamic conditions.

5. Conclusions

Variability is inherent to the microbial response to thermal treatments and can be very relevant for the effectiveness of a thermal treatment. This is the first article that uses empirical evidence to analyse separately the impact of variability under isothermal and dynamic conditions.

We show that, even when variability is not relevant under isothermal conditions, between-media and between-strain variability can have a very important effect on the stress acclimation of microbial cells. This results in large differences in the microbial counts at the end of dynamic treatments.

Supplementary material

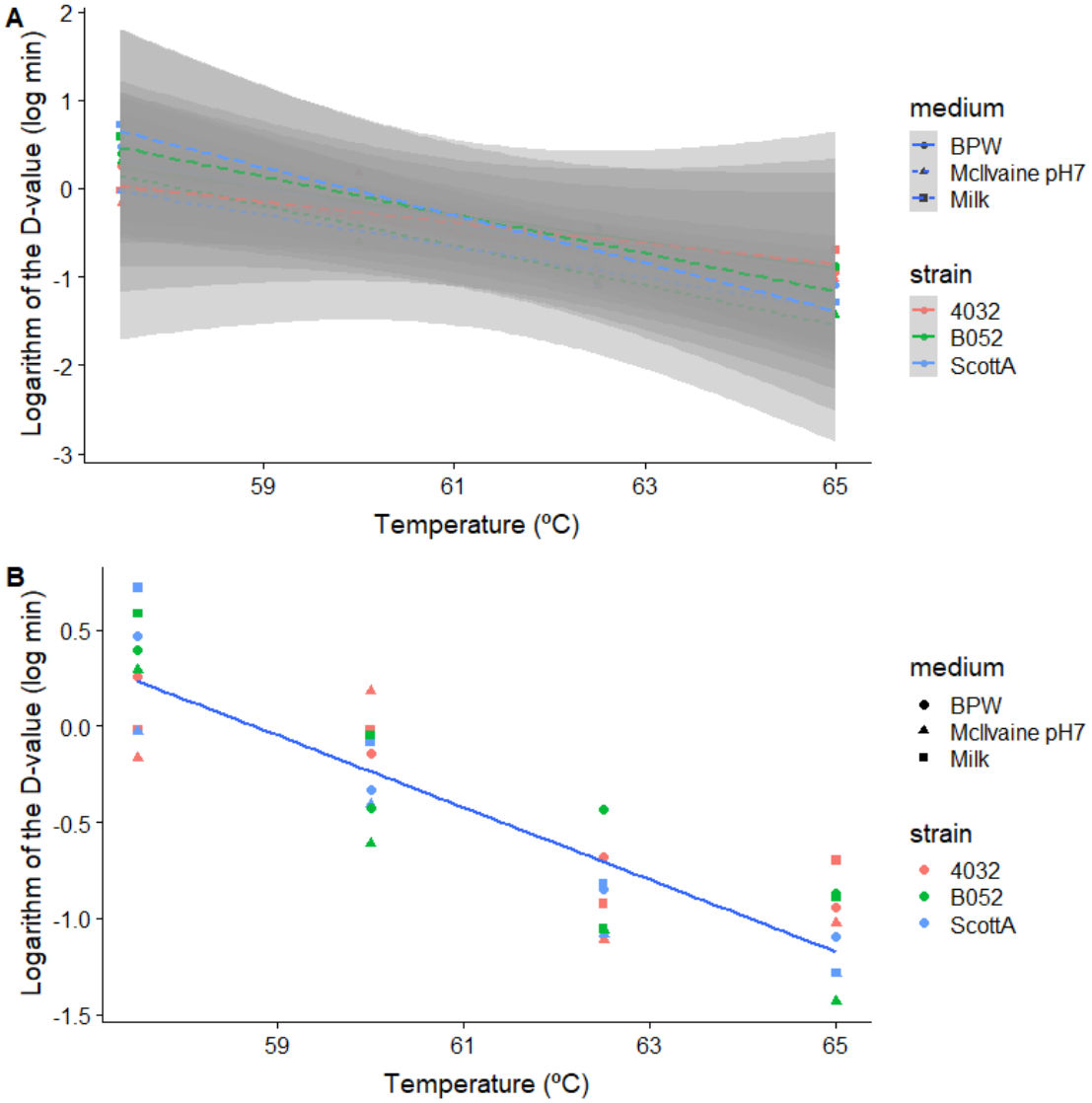

Figure 10 *D-value* of *Listeria monocytogenes* strains in different media.

Author contributions

Marta Clemente-Carazo: Conceptualization, Investigation, Writing. **Guillermo Cebrián**: Conceptualization, Investigation, Writing, Supervision. **Alberto Garre**: Conceptualization, Formal Analysis, Writing, Supervision. **Alfredo Palop**: Conceptualization, Writing, Supervision.

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CHAPTER II

The different response to an acid shock of two *Salmonella* serovars marks their resistance to thermal treatments

The contents of this chapter have *been submitted for publication in the journal Foods*, in a special issue "New Insight into the Mechanisms of Microbial Resistance/Adaptation to New and Traditional Technologies for Food Preservation"

Abstract

Microbial cells are not static systems. Instead, microorganisms may develop a physiological response to certain stresses that may increase their resistance to other posterior treatments, either of the same or of different nature, improving their chance to survive. One example is the response to the application of an acid shock (Acid Shock Response; ASR), which has been observed in various microbial species to increase the cell resistance to different types of stresses, including thermal treatments. The ASR is thus of relevance for food safety, as it can occur naturally during the farm-to-fork chain of acidic products, or during processing in products that are acidified before further treatment. Hence, it is important to unravel the conditions where ASR may take place and how microorganisms may be capable of developing a physiological response that may increase their stress resistance. This knowledge would enable a reduction of the uncertainty linked to the risk assessment for this kind of food products.

In this study, we studied the ASR of two serovars of *Salmonella* spp. (*S.* Enteritidis and *S.* Senftenberg). Microbial cells previously exposed to an acid shock at pH 4.5 were subjected to heat treatment under isothermal conditions 52.5, 57.5, 60ºC for *S.* Enteritidis and 57.5, 60, 62.5, 65ºC for *S.* Senftenberg in peptone water at two different pH levels (4.5 and 7.0). The results were compared against those obtained for control cells without an acid shock. We observed that the two strains tested had different ability to develop ASR. The acid shock reduced the resistance of *S.* Senftenberg to posterior thermal treatments. For instance, the treatment time required to reduce the microbial count in 3 log cycles was reduced for a treatment at 60.0ºC and pH 7.0 in more than one fifth from 10.75 to 1.98 min. However, acid shocked cells of *S.* Enteritidis did not show higher sensitivity. On the contrary, they increased their heat resistance for a treatment at low pH, making them practically insensitive to the pH of the medium. These observations have been complemented by differential platting with addition of NaCl, supporting the hypothesis that the differential response between serovars is related to sub-lethal damage in the cell membrane. Our results provide empirical evidence that different serovars of the same species may have a different

ability to develop ASR. This factor should be properly characterized to reduce uncertainty in risk assessment of food products with acidic pH.

Keywords: Foodborne pathogens; acid shock; pasteurization, cross-resistance; stress adaptation

Highlights

- Acid Shock Response differed between *S.* Enteritidis and *S.*Senftenberg.
- The acid shock reduced the heat resistance of S. Senftenberg with respect to controls.
- Acid-shocked cells of *S.* Enteritidis had higher resistance to acid plus heat treatment than controls.
- The results can be explained based on the sub-lethal damage on the cell membrane.

1. Introduction

Food products can be a potential health hazard for consumers because pathogenic microorganisms may enter the farm-to-fork chain and survive or multiply until consumption (FAO, 2011). According to the FAO, at present there are 32 foodborne diseases transmitted by 31 food-borne agents (WHO, 2015). Although governmental agencies and other institutions have made intensive efforts towards developing laws and guidelines to ensure food safety, there are still large knowledge gaps in the field. Among them, *Salmonella* spp is one of the main pathogens of concern for the food industry. It is the causative agent of salmonellosis, the second most common foodborne disease in humans in the European Union (Pires et al., 2019). In 2018, 91,857 human cases of salmonellosis were reported in the European Union. *Salmonella* Enteritidis and *Salmonella* Typhimurium are the serovars most commonly isolated in the salmonellosis outbreaks (Manas et al., 2001). Both serotypes are able to grow in a wide range of NaCl concentrations (between 0 and 4%), acid pH (down to 4.5), water activities (minimum 0.93) and temperatures (between 10 and 45ºC) (Alvarez-Ordóñez et al., 2010; Mattick et al., 2000; Zurera-Cosano et al., 2011). European Union Regulation (EC) 2073/2005, considers *Salmonella* as a food safety criterium, setting a limit of "not detected in 25 g or mL" in five samples, for most food categories.

Salmonella can be found in all warm-blooded organisms and the environment, so it can potentially be present in the raw materials used for food production (EFSA, 2019). Hence, some step(s) of food production are usually designed to ensure that the microbial count of this microorganism is below a threshold concentration. The most common technology for food preservation is the application of high temperatures. Heat treatments are not only effective to ensure microbiological stability and inhibit alterations caused by pathogens, but it can also lead to enzymatic inactivation (Peng et al., 2017). However, high temperatures can also have a negative impact on the quality of the product, reducing its organoleptic and nutritional attributes (González-Tejedor et al., 2017a; Kilcast & Subramaniam, 2011; Maza et al., 2019; Peng et al.,

2017). For this reason, industries seek a reduction of the intensity of the processing treatments, delivering safe products with a minimal quality loss (minimally processed products).

The application of other technologies to substitute or complement thermal treatments has been suggested as a strategy to reduce their impact on food quality. Leistner (1978) proposed the so-called "hurdle technology", where mild treatments of different nature are applied in sequence or simultaneously. According to this approach, food safety can be ensured while reducing the intensity of each individual treatment, improving food quality and reducing economical cost. On the other hand, due to the combination of several treatments, the hurdle technology requires a deeper knowledge of the process, and a more detailed, science based, knowledge of the microbial response to each of the processing treatments (Santos & Silva, 2008). For instance, the application of a treatment that combines high temperature and low pH requires an understanding on how the microbial cells respond to both the high temperature and the acidic environment.

The principles of the hurdle technology assume that cells that are able to survive the first "hurdle" will be destroyed by the next "hurdles". However, it is important to take into account that bacterial resistance to stress is affected by the previous history of the cells (Garre et al., 2019; Manas & Pagán, 2005): sub-lethal damage can alter cellular structures in a reversible or irreversible way, altering their stress resistance (Gilbert, 1984; A. Hurst, 1984; Yousef & Courtney, 2003). In the particular case of physiological responses to an acid shock, they are denominated Acid Shock Response (ASR) (Leyer et al., 1995; Miller & Kaspar, 1994; Zhao et al., 1993). There are several reports of ASR increasing the resistance of microbial cells to posterior stress. For instance, it has been shown that exposing *Salmonella* Typhimurium to mild acidic conditions during a short period of time, can increase its resistance to posterior stresses (Bang et al., 2002; Kwon & Ricke, 1998; Rishi et al., 2005). Álvarez-Ordóñez et al. (2012) observed that, although pathogenic microorganisms were unable to grow in fruit juice, incubation in this medium improved their resistance to acidic conditions, showing the potential relevance of ASR in an actual food product. Due to the combination of different stresses, food processing based on the hurdle technology can also induce ASR. For instance, it is common for some products to be washed in an acidic solution before they are processed further. This can result in an ASR in the

surviving cells that could induce and increased resistance to stress (Hurst, 1984; Wesche et al., 2009). For that reason, treatments based on the hurdle technology should be designed from an understanding on how the combined treatments affect the cells. In this article we advanced in that understanding, analyzing the ASR of two different *Salmonella* serovars (*S.* Senftenberg and *S.* Enteriditis), and how it can induce an increase in the stress resistance of the surviving cells.

2. Materials and methods

2.1 Bacterial culture and media

The bacteria studied in this research were *Salmonella enterica* serovar Entereditis CECT 4300 (type strain) and *Salmonella enterica* serovar Senftenberg CECT 4565. Both were provided by the Spanish Type Culture Collection (CECT, Valencia, Spain). *S.* Enteritidis was selected because it is usually considered as a reference strain for this species. *S.* Senftenberg was studied because it is a well-known heat-resistant serovar.

The bacteria were stored at – 80±2°C (20% glycerol) until use. Subsequently, for the use of each strain, fresh cultured plates were sown weekly in trypticase soy agar (TSA, Scharlau Chemie, Barcelona, Spain) supplemented with 0.6% yeast extract (YE, Scharlau Chemie). The fresh cultures were incubated for 24 hours at 37±1°C in an incubator.

A single colony from a fresh culture plate was transferred to 5 mL of trypticase soy broth (TSB; Scharlau Chemie) supplemented with 0.6% YE and incubated overnight at 37±1°C. Flasks with 50 mL of TSBYE were then inoculated with 1 mL of the pre-culture and incubated for 24 h at 37±1°C in agitation to obtain all cells in stationary phase, which usually are more resistant to thermal stresses, with a concentration of approx. 10^9 CFU/mL.

2.2 Determination of minimum pH for growth

Sterile TSBYE was prepared at different pH (3.75, 4.00, 4.25, 4.50 and 7.00) by the addition of 1 M citric acid (Panreac, Barcelona, Spain). The pH was measured after sterilization with a pH meter (Basic20, Crison; Alella, Cataluña, Spain), under strict aseptic conditions, to check that the pH value had not changed.

Then, the growth of both *Salmonella* serotypes in the acidified TSBYE was determined in a Bioscreen C (LabSystems Helsinki, Finland) at a wavelength of 600 nm. A total of 25 repetitions per pH value were performed, of which 5 were left uninoculated and used as negative control. Samples at pH 7.00 were used as positive control.

2.3 Induction of the acid tolerance response (acid shock)

A volume of 1 mL of cells in the stationary growth phase (10^9 CFU/mL) of each serotype was centrifuged at 60000 g for 10 min at 4±1°C. Pellets were resuspended in pH 7.0 TSBYE and centrifuged two more times and resuspended in fresh medium. After the last centrifugation, pellets were resuspended in pH 4.5 TSBYE and were incubated at 37±1°C for 30 min in an incubator and immediately heat treated.

2.4 Determination of the heat resistance

The determination of the heat resistance of the microorganisms was carried out in a Mastia thermoresistometer (Conesa et al., 2009). The heating medium for every experiment was peptone water (10 g/L peptone from casein (Scharlau Chemie) and 5 g/L NaCl (Scharlau Chemie). For the experiments that were conducted in acidic media, peptone water was acidified with 1M citric acid to pH 4.5 and measured with a pH meter.

The heating medium was inoculated with 0.2 mL of the bacterial suspension (acid-shocked and control) approximately 10^6 CFU/mL. Experiments were performed under isothermal

condition. The temperatures and sampling times were set according to the thermal resistance of each microorganism and conditions. For *Salmonella* Enteritidis experiments were performed at 52.5, 57.5 and 60.0ºC at both pH 7.0 and pH 4.5. For *Salmonella* Typhimurium experiments were carried out at higher temperatures (57.5, 60.0, 62.5 and 65.0°C) at both pH values (7.0 and 4.5) because of the higher thermal resistance of this serovar.

Survival counting was performed, from appropriate dilutions in peptone water, in TSAYE or TSAYE + NaCl (for the determination of sublethal damage; see section 2.5). A minimum of three experiments were performed per condition.

2.5 Assessment of sublethal damage after treatments

The concentration of cells with sub-lethal damage after the acid shock was estimated by differential plating in TSAYE and TSAYE supplemented with the maximum non-inhibitory concentration of NaCl and incubation at 37±1ºC for 48 h (Ray, 1989; Wuytack et al., 2003). The maximum non-inhibitory concentration of NaCl was determined preparing TSAYE with different percentages of NaCl, from 1 to 5%. Then, cells of both serovars of *Salmonella* spp. were plated in Petri plates and incubated at 37±1ºC for 48 h. The maximum non-inhibitory concentration determined as the one that reduced the microbial count in more than 20% with respect to cells grown in TSAYE (without the addition of NaCl). This concentration resulted in 1% NaCl for *S.* Enteritidis and 3% for *S.* Senftenberg.

2.6 Inactivation modelling and data analysis

The isothermal inactivation data were analysed using the Mafart inactivation model (Mafart et al., 2002). This model extends the log-linear inactivation model assuming that the resistance of individual cells is not homogeneous but follows a Weibull distribution. It thus can describe survivor curves with upwards or downwards curvature. According to Equation (17), in

this model the relationship between the microbial count (*N*) and the treatment time (*t*) depends on two parameters: δ and β. The former (also called δ-value) corresponds to the scale parameter of the Weibull distribution and can be interpreted as the treatment time required to reduce the microbial count to a 10% of the initial count, N_0. The latter (also called β-value) is the shape factor of the Weibull distribution and defines the curvature direction of the survivor curves: $\beta > 1$ results in survivor curves with downwards curvature and $\beta < 1$ in upwards curvature. For the particular case where $\beta = 1$, the Mafart model predicts log-linear inactivation. The primary inactivation model was fitted using the Excel add-in GinaFit (Geeraerd et al, 2005).

$$\log N = \log N_0 - \left(\frac{t}{\delta}\right)^{\beta} \quad (17)$$

Because the β-value of the survivor curves varied between conditions, in order to compare between them, we calculated the treatment time required to reduce the microbial count 3 log-cycles according to the Weibull model ($t_{3\delta}$). It was calculated as shown in Equation (18).

$$t_{n\delta} = \delta \cdot n^{1/\rho} \quad (18)$$

3. Results

Tables 3 and 4 report the parameters of the Weibull model estimated for each isothermal treatment. They confirm the high resistance of *S.* Senftenberg to thermal treatments compared to the reference strain. As an illustration, for the experiments at 60.0ºC at pH 7.0 without a prior acid shock, a value of *10.75* min was estimated for coefficient $t_{3\delta}$ for *S.* Senftenberg, whereas a value of *0.30 min* was estimated for *S.* Enteritidis (36 times smaller). This difference in the resistance between both serovars is also illustrated in Figure 11, where the survivor curves observed at 60.0ºC for both microorganisms are depicted. The remaining survivor curves are provided as supplementary material to this article. The inactivation rates are in the order of similar

studies found in the literature. Doyle and Mazzotta (2000) reviewed several studies observing that the *D-value* of *S.* Senftenberg at 60ºC in laboratory media ranged between 0.62 and 3.06 min. The results are also similar to those by Shah et al. (1991), who observed *D-value*s of *Salmonella* Enteritidis between *9.98* min at 54.4°C and *0.05* min at 62.8°C.

Table 3 Parameters of the Weibull model estimated from the survival curves of *S.* Senftenberg heat treated in peptone water without a prior acid shock (control cells) and after an acid-shock at pH 4.5.

	pH	Temperature (°C)	δ (min)	β (·)	$t_{3\delta}$ (min)	RMSE (log CFU/ml)
Control cells	4.5	57.5	1.53±0.55	0.66±0.12	8.08	0.31
		60.0	0.61±0.12	0.77±0.08	2.54	0.27
		62.5	0.04±0.01	0.46±0.04	0.44	0.29
		65.0	0.11±0.03	1.01±0.16	0.33	0.45
	7.0	57.5	6.61±2.61	0.70±0.15	31.75	0.32
		60.0	1.67±0.51	0.59±0.08	10.75	0.27
		62.5	0.37±0.08	0.51±0.04	3.19	0.17
		65.0	0.16±0.05	0.70±0.11	0.77	0.33
Acid-shocked cells	4.5	57.5	0.65±0.15	0.55±0.04	4.79	0.21
		60.0	0.33±0.09	0.69±0.08	1.62	0.31
		62.5	0.01±0.01	0.30±0.04	0.39	0.31
		65.0	0.05±0.01	0.73±0.09	0.23	0.23
	7.0	57.5	1.42±0.29	0.60±0.06	8.86	0.17
		60.0	0.24±0.05	0.52±0.04	1.98	0.20
		62.5	0.08±0.03	0.47±0.05	0.83	0.26
		65.0	0.0016±0.0013	0.24±0.04	0.16	0.16

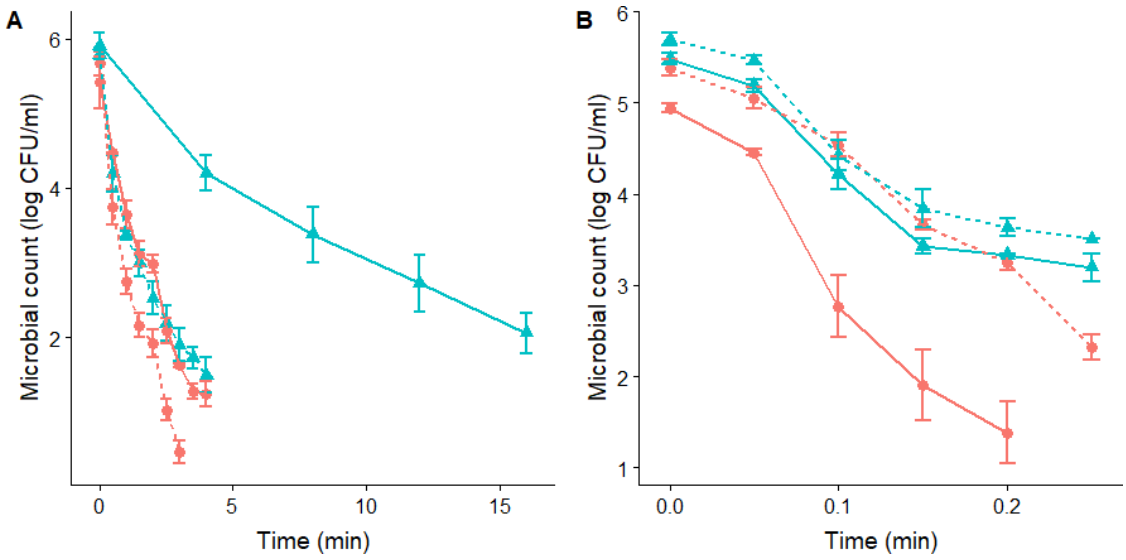


Figure 11 Survivor curves of *S.* Senftenberg (A) and S. Enteritidis (B) during isothermal treatments at 60ºC in peptone water. The pH of the heating media was set at pH 7.0 (▲) and pH 4.5 (●). Experiments were made for cells with a previous acid shock (--) and control cells, without acid shock (-).

Regarding the curvature direction of the inactivation curves, they differed between serovars. *S.* Senftenberg (Figure 11A; Table 3) presented a tail phenomenon (upwards concavity) in all the treatments applied, reflected in β-values lower than one (for a temperature of 60.0ºC: $\beta=0.69\pm0.08$ for pH 4.5 after acid shock, $\beta=0.52\pm0.04$ for pH 7.0 after acid shock, $\beta=0.77\pm0.08$ for pH 4.5 without acid shock, and $\beta= 0.59\pm0.08$ for pH 7.0 without acid shock). On the other hand, *S.* Enteritidis showed β-values close to one (Figure 11B and Table 4), thus having survivor curves close to log-linear (for a temperature of 60.0ºC: $\beta=1.34\pm0.10$ for pH 4.5 after acid shock, $\beta=0.82\pm0.15$ for pH 7.0 after acid shock, $\beta=0.98\pm0.18$ for pH 4.5 without acid shock, $\beta= 0.83\pm0.16$ for pH 7.0 without acid shock).

Table 4 Parameters of the Weibull model estimated from the survival curves of *S.* Entereditis heat treated in peptone water without a prior acid shock (control cells) and after an acid-shock at pH 4.5.

	pH	Temperature (°C)	δ (min)	β (·)	$t_{3\delta}$ (min)	RMSE (log CFU/ml)
Control cells	4.5	52.5	2.44±0.43	0.72±0.05	11.22	0.26
		57.5	0.14±0.03	0.76±0.08	0.59	0.24
		60	0.05±0.01	0.98±0.18	0.15	0.38
	7.0	52.5	6.32±1.84	0.65±0.09	34.26	0.28
		57.5	0.16±0.04	0.62±0.07	0.94	0.28
		60	0.08±0.02	0.83±0.16	0.30	0.29
Acid-shocked cells	4.5	52.5	5.82±2.12	0.76±0.14	24.70	0.43
		57.5	0.36±0.04	0.98±0.07	1.10	0.19
		60	0.11±0.01	1.34±0.10	0.25	0.13
	7.0	52.5	18.9±0.6	2.15±0.12	31.52	0.18
		57.5	0.34±0.05	1.24±0.18	0.82	0.27
		60	0.08±0.02	0.82±0.15	0.31	0.27

As expected, the combination of an acidic media and a heat treatment had a significant impact on the inactivation rate of both serovars. As shown in Table 3, *Salmonella* Senftenberg the time for 3 log-reductions at pH 4.5 was between two and seven times shorter than at pH 7.0 ($t_{3\delta}$ values at pH 4.5 of *8.08*, *2.54*, *0.44* and *0.33* min at 57.5, 60.0, 62.5 and 65.0°C respectively, compared to $t_{3\delta}$ values at pH 7.0 of *31.75*, *10.75*, *3.19* and *0.77* min at 57.5, 60.0, 62.5 and 65.0°C respectively). *S.* Enteritidis was less sensitive to thermal treatment under acidic pH (Table 4), showing a time to reach 3 log-reductions at pH 4.5 between only two and three times shorter than at pH 7.0 ($t_{3\delta}$ values at pH 4.5 of *11.22, 0.59* and *0.15* min at 52.5, 57.5 and 60.0ºC respectively, compared to $t_{3\delta}$ values at pH 7.0 of *34.26, 0.94* and *0.30* min at 52.5, 57.5 and 60.0ºC respectively) (table 5).

It was concluded that the minimum pH of growth for both serovars of *Salmonella* spp. was between 4.25 and 4.50 (see supplementary material).

The application of an acid shock prior to the thermal treatment had a different impact on the thermal resistance of both serovars. For *S.* Senftenberg, the survivor curves at pH 7.0 after acid shock showed a significantly faster inactivation rate than the one of control cells. The application of the acid shock reduced $t_{3\delta}$ at 60ºC from *10.75* min for control cells to *1.98* min

for acid shocked cells. Notably, the value of $t_{3\delta}$ observed for acid-shocked cells at pH 7.0 (1.98 min) was about the same as that observed at pH 4.5 without any previous acid-shock (2.54 min). This similarity between inactivation under both conditions is clearly illustrated in Figure 11A. Therefore, the application of an acid-shock prior to a treatment at pH 7 had a similar impact on the inactivation kinetics as a combined thermal treatment at pH 4.5. Regarding the effect of an acid shock before a thermal treatment at pH 4.5 for *S.* Senftenberg, although the stress resistance was reduced, its magnitude was not as large as for the treatments at pH 7. The value of $t_{3\delta}$ was reduced from 2.54 to 1.62 min at 60ºC. Similar results were obtained at all other temperatures tested (Table 3). In summary, the application of a heat shocked to *S.* Senftenberg reduced its thermal resistance. Indeed, the inactivation kinetics of heat shocked cells at a pH of 7.0 was similar to the one of control cells at pH 7.0. For treatments with a pH of 4.5, the thermal resistance of acid shocked cells was further reduced.

Conversely, *S.* Enteritidis had a very different response to the application of an acid shock. As shown in Table 4, acid-shocked cells of *S.* Enteritidis had a resistance to the thermal treatment in both acidic and neutral media equivalent to the one observed for control cells at pH 7 ($t_{3\delta}$ at 60.0°C of *0.30* min for control cells at pH 7.0, *0.31* min for acid-shocked cells treated at pH 7.0, and *0.25* min for acid-shocked cells treated at pH 4.5). These similarities are illustrated in Figure 11B, where the survivor curves obtained at 60.0ºC are illustrated. This plot shows how the survivor curves for acid-shocked cells for a treatment pH of 4.5 and 7.0 is practically the same as the one observed for control cells for a treatment pH of 7.0. This result can indicate that the acid shock causes an ASR that induces an increase resistance of *S.* Enteritidis cells, making them insensitive to changes in the acidity of the heating medium in posterior treatment.

The data obtained using differential plating with NaCl provide further evidence to support the hypothesis that ASR is responsible for the different results observed between the two serovars. Figure 12 compares the microbial counts observed in TSAYE and TSAYE supplemented with NaCl. For *S.* Senftenberg, we observed microbial counts between 1 and 2 log CFU/mL lower in TSAYE+NaCl than in TSAYE at both pH levels (7.0 and 4.5). This is an indicator of sub-lethal damage in the microbial cells that could justify our results where acid-shocked cells had a lower

heat resistance than control cells (Figure 12A). On the other hand, we did not observe any difference between the microbial counts recovered in TSAYE and TSAYE+NaCl for *S.* Enteritidis, indicating the absence of sub-lethal damage for this serovar. This could enable the development of ASR of the cells that could induce an increase thermal resistance, justifying our results (Figure 12B).

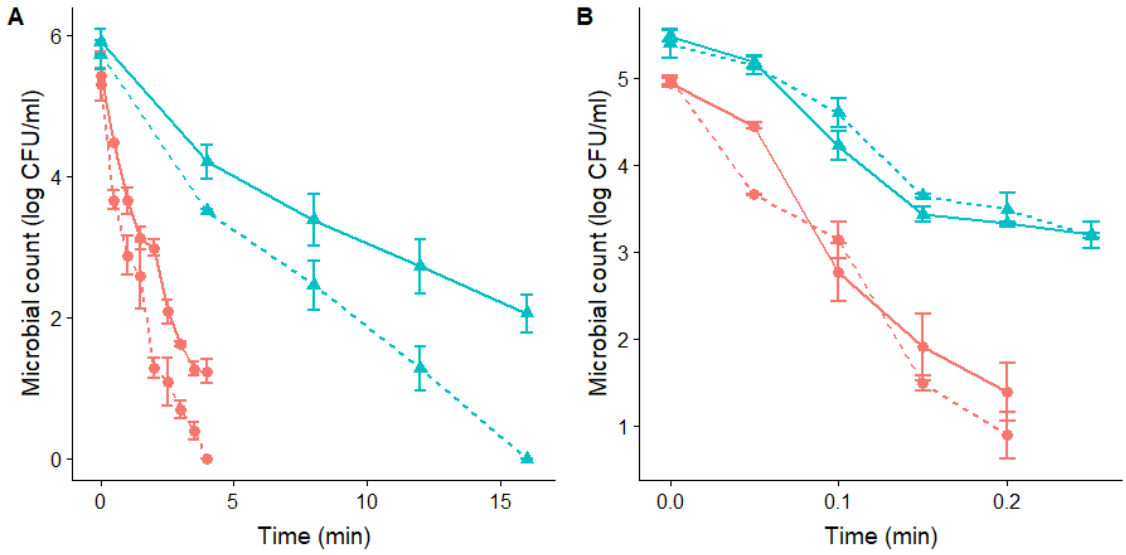


Figure 12 Survivor curves of *S.* Senftenberg (A) and *S.* Enteritidis (B) during isothermal treatments at 60°C in peptone water and recovered in TSAYE (-) and TSAYE with NaCl(⋯) for experiments performed at pH 7.0 (▲) and pH 4.5 (●).

4. Discussion

The addition of chemicals (e.g. salts, acids and oxidizers) or the application of physical treatments (e.g. heat and pressures) for food conservation induce a variety of environmental stresses that can inactivate pathogenic and spoilage microorganisms present in the food product. However, current processing treatments are not flawless, evidenced by the fact that a number of foods with an acidic pH have been implicated in outbreaks. Indeed, some studies have questioned the effect of acid on the effective control of some microorganisms (Evans et al., 1999; Jain et al., 2009; Parish, 1997; Rodriguez-Romo et al., 2005; Vojdani et al., 2008). Furthermore, previous

studies have demonstrated that acidic conditions may improve the resistance of some strains of *Salmonella* spp. An example is the work by Perez et al. (2010), who showed that incubation in acidic condition led to an increase in the survival of *S*. Enteritidis in gastrointestinal liquids, due to prior adaptation to an acidic pH. The ability of *Salmonella spp.* to survive this type of stresses and their presence in a wide variety of foods makes it a high-risk pathogen (Rychlik & Barrow, 2005). As a result, an accurate risk assessment for this microorganism requires to understand the physiological state of the cells and the potential for a stress adaptation (Álvarez-Ordóñez et al., 2013). This is especially relevant as several strains of *Salmonella* can develop an increased stress resistance when exposed to acidic conditions, including *S.* Typhimurium, *S*. Enteritidis and *S.* Senftenberg (Álvarez-Ordóñez et al., 2012; Greenacre et al., 2003; Hsin-Yi & Chou, 2001; Koutsoumanis & Sofos, 2004; Koutsoumanis et al., 2003; Skandamis et al., 2009; Stopforth et al., 2007).

In the current study, we have observed a differential response in two *Salmonella* serovars when exposed to an acid shock. Acid shocked cells of *S.* Enteritidis hadn´t increased resistance to stress with respect to control cells, since the value of $t_{3\delta}$hardly changed, whereas the same acid shock reduced the resistance of *S.* Senftenberg to posterior treatments. Indeed, acid-shocked cells of *S.* Enteritidis were practically insensitive to changes in the pH of the heating medium in our experiments. These results are in-line with those by Malheiros et al. (2009), who observed that at temperatures of 52.0 and 56.0ºC *S*. Enteritidis was able to grow, showing higher adaptation capabilities than other serovars of this species in a heating medium in the presence of glucose at pH 4.5. A similar physiological response (albeit for a different microorganism) was reported by Haberbeck et al. (2017). In their study, they observed that 48 *Escherichia coli* strains were able to adapt after incubation at pH 5.5, increasing their thermal resistance to treatments at 58.0°C. Not all the studies confirm these results, since other authors showed an increased sensitivity to heat when exposed to low pH in this same pathogen of *E.coli* (Lee and Kang, 2009; Parry-Hanson et al., 2010). From our study, it can be concluded that there is variability in the ability of different serovars of a microorganism to develop an increased stress resistance after being subject to an

acid shock. Hence, some of the differences in the results reported in the literature for *E. coli* could be potentially attributed to variability between serovars of that microorganism.

Conversely to *S.* Enteritidis, we observed that for *S.* Senftenberg the application of an acid shock increased the sensitivity of the bacteria to posterior thermal treatments. Based on our observations using differential plating, this result could be attributed to sub-lethal damage in the cytoplasmic membrane of *S.* Senftenberg cells. This result is in disagreement with those reported by Alvarez-Ordonez et al. (2009a), who observed that acid-shocked cells of *S.* Senftenberg would develop increased stress resistance. However, these authors used food matrices as heating media (orange juice and apple juice), whereas in our study we used laboratory media. There is empirical evidence showing that food matrices can have a protective effect on microbial cells (Maté et al., 2017; Verheyen et al., 2019, 2020). Hence, it is a plausible hypothesis that the food matrix shall also influence the development of increased stress resistance. Nevertheless, this hypothesis needs to be validated using experimental data.

During the last decades, the application of modern technologies (e.g. genomics) have shed light on the genes involved in the microbial response to stress, and the molecular mechanisms that intercede in the cellular response to acidic conditions. Due to low pH environments, protons are expelled from the cytoplasm to regulate pH levels within the cell. This is carried out with antiporter pumps potassium-proton and sodium-proton, keeping constant the gram-negative intracellular pH (Foster, 2000). Previous studies have also shown that through the synthesis of acid shock proteins (ASPs) and regulatory proteins, the cell is able to avoid or repair the damage caused by the acid stress. These molecular mechanisms can explain the empirical evidence supporting the hypothesis that microbial cells are able to recover from the damage caused by an acid stress (Audia et al., 2001).

5. Conclusions

Our empirical results evidence that the response to an acid shock of *Salmonella* cells can vary between serovars of this species. The application of an acid shock strongly reduced the heat resistance of *S.* Senftenberg, whereas the same shock did not induce an increased stress resistance in *S.* Enteritidis. Therefore, we provide evidence of the existence of variability at the between-serovar level in the ASR of microbial cells. This result can advance risk assessment, reducing the uncertainty associated to the microbial response in acidic food products.

Supplementary material

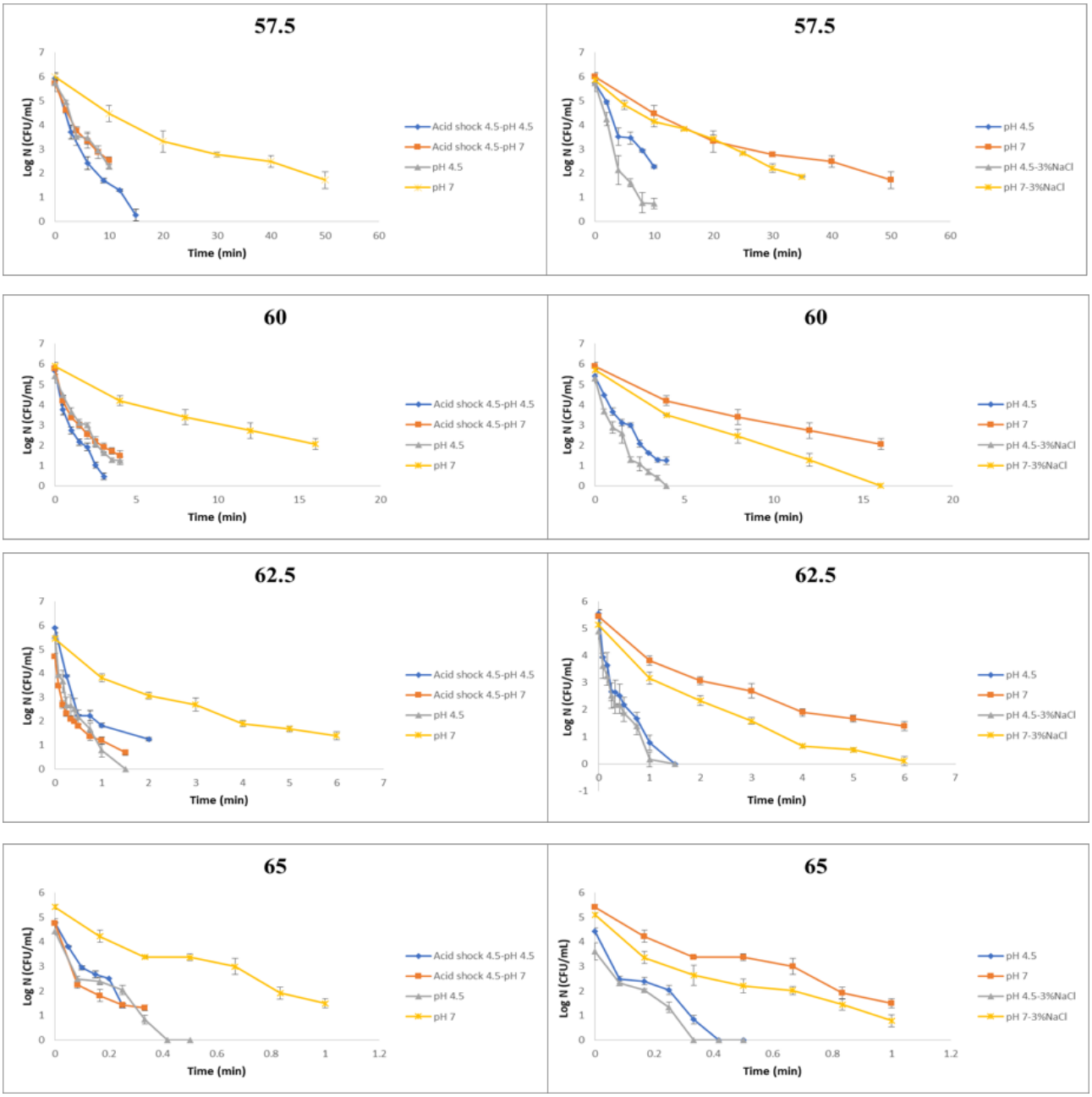


Figure 13 Inactivation kinetics at different temperatures and determination of sublethal damage with TSA+NaCl for *Salmonella* Senftenberg.

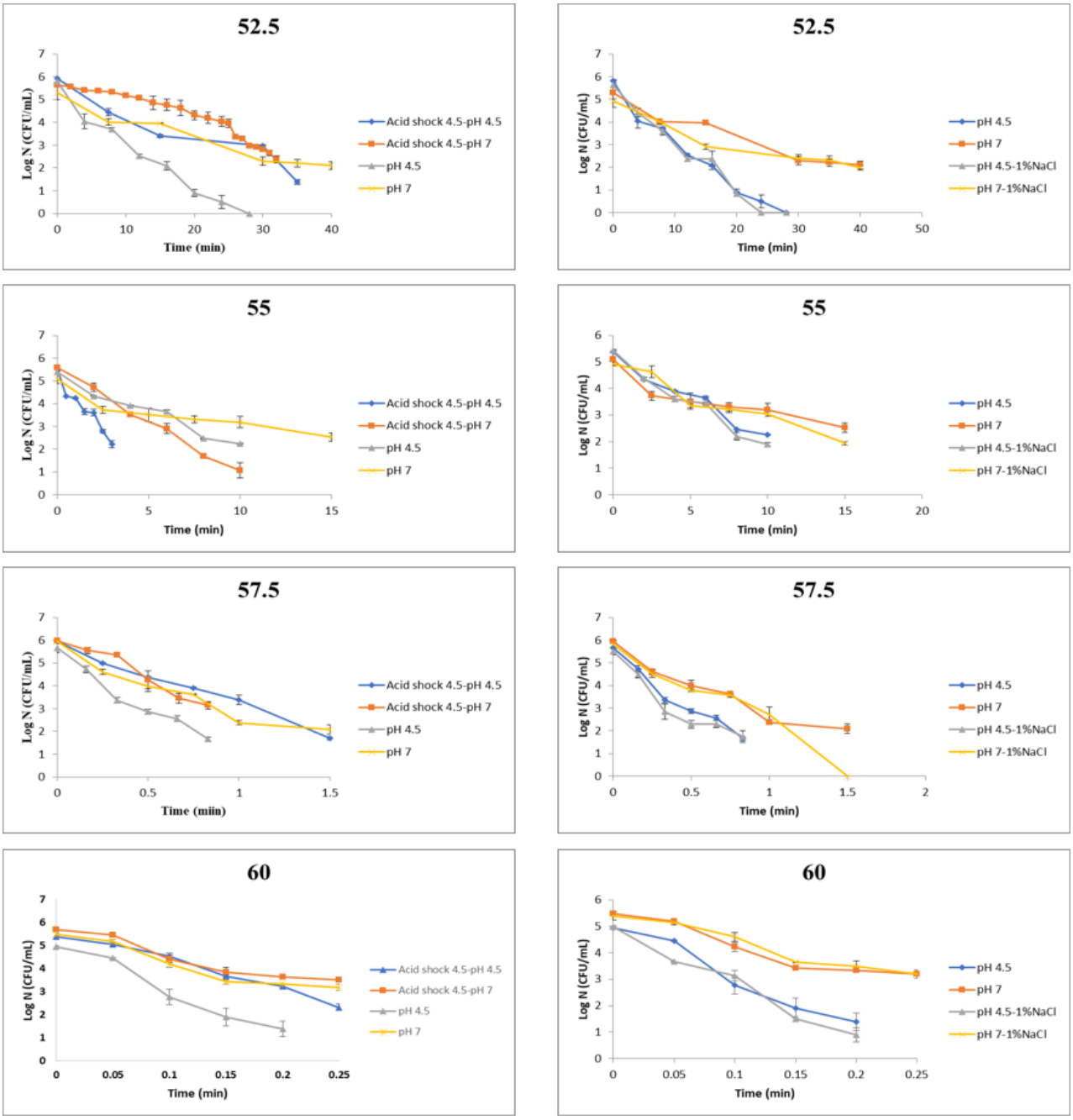
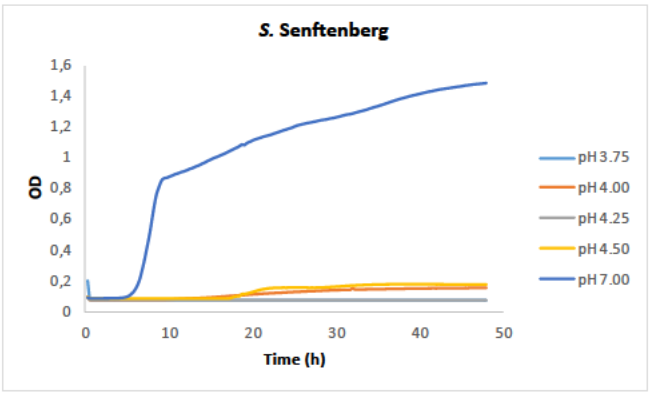


Figure 14 Inactivation kinetics at different temperatures and determination of sublethal damage with TSA+NaCl for ***S****almonella* Enteritidis.



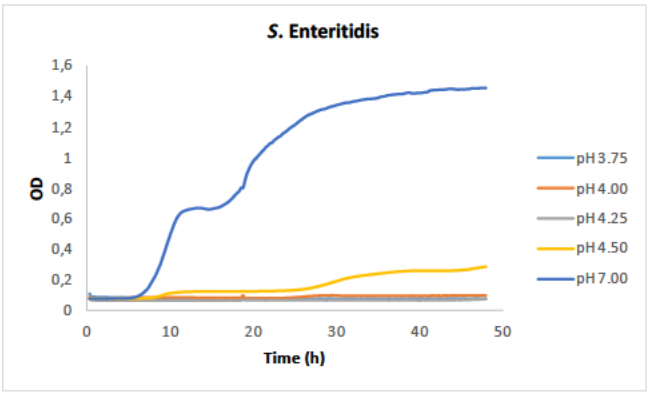


Figure 15 Determination of the minimum growth pH for both serovars

Author contributions

Marta Clemente-Carazo: Conceptualization, Investigation, Formal Analysis, Writing. **José Juan Leal**: Conceptualization, Investigation. **Juan-Pablo Huertas Baquero:** Conceptualization, Supervision. **Alberto Garre**: Conceptualization, Formal Analysis, Writing, Supervision. **Alfredo Palop**: Conceptualization, Writing, Supervision. **Paula M. Periago:** Conceptualization, Writing, Supervision.

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CHAPTER III

Development of a combined strategy based on pulsed electric fields and oregano essential oil for the inactivation of *Listeria monocytogenes* in liquids

This chapter corresponds to the following article, which is being prepared for publication in a journal included in the Science Citation Index:

Abstract

In the present work, the effect of oregano essential oil (EO) and pulsed electric fields (PEF) between 5-20 kV/cm was evaluated for the inactivation of six strains of *Listeria* spp. Additionally, the combinations of PEF and oregano essential oil as a prospective combined treatment was assayed. The simultaneous application of PEF (20 kV/cm, 50 pulses, 20 µs pulse width and 1 Hz) and oregano EO (2000 ppm) showed no significant difference ($p > 0.05$) with respect to PEF applied alone in most of the strains tested, being only significant in two strains. On the other hand, suspending the survivors in the antimicrobial solution for 60 minutes after the PEF treatment, i.e. subsequent treatment, resulted in significantly greater inactivation than PEF treatment applied alone or even simultaneously to EO ($p < 0.05$). Therefore, this work identifies an effective strategy for the inactivation of *Listeria* spp. based on the combination of PEF and oregano EO. This approach could be used to improve the decontamination of foodstuffs with characteristics similar to those tested in vitro and replace conventional technologies.

Keywords: *Listeria* spp., Pulsed electric field, Oregano, Essential oil, Combined processes, Microbial Inactivation

Highlights

- Combined treatment of PEF and Oregano EO showed similar results.
- Increasing the intensity of the electric field resulted in greatest inactivation.
- Application of EO after PEF was more effective than simultaneous application.

1. Introduction

One of the major causes of morbidity in society are foodborne diseases, which are mainly caused by bacteria, parasites and viruses, causing several deaths a year worldwide and resulting in socioeconomic issues (Scallan et al., 2011; Tauxe et al., 2010; WHO, 2015). It is therefore important for the relevant authorities to make efforts to achieve food safety (Hathaway, 2013). Among the foodborne pathogens, *Listeria monocytogenes* is a big concern for food industries, especially for ready-to-eat (RTE) products (Gahan & Hill, 2005). In 2018, a total of 2,549 cases of listeriosis were registered in the 28 member states of the European Union, i.e., 0.47 cases per 100,000 inhabitants (ECDC, 2019; EFSA, 2019). Despite its relatively low impact rate, the control of this disease is considered of major importance, since it affects mainly risk populations such as pregnant women, children, elderly people and finally people with previous pathologies (Gerner-Smidt, 2007). The ubiquity of this pathogen, linked to the wide temperature ranges (between 0-45°C), NaCl concentrations (over 16%), water activities (higher than 0.90) and pHs (between 5 and 9) in which it can develop (Blanco, 1994;Ryser & Marth, 1999), make it a threat for the food industry.

Last century several non-thermal technologies have emerged as an alternative to heat treatments. A promising example are pulsed electric fields (PEFs), which has been applied for the inactivation of various pathogenic and spoilage microorganisms (Álvarez et al., 2003; Amiali et al., 2007; Grahl & Märkl, 1996; Mosqueda-Melgar et al., 2008; Wan et al., 2009). The first studies with PEFs were reported by Sale and Hamilton (Sale & Hamilton, 1967), who identified the process parameters most relevant for microbial inactivation: the electric field strengths, the pulse frequency and the pulse length. The mechanisms of action of PEFs on bacteria are based on electromechanical forces. The electric field strength leads to the accumulation of electric charges on both sides of the cell membrane, which causes its compression. When the attraction forces exceed the viscoelastic strength of the membrane, pores are created, increasing membrane permeability (Zimmermann et al., 1974). This can lead to an irreversible loss of the

semipermeable function of the membrane, eventually resulting in cell death (Sale & Hamilton, 1968). An alternative theory was later proposed, naming this mechanism electroporation (Tian Yow Tsong, 1989).

Although PEF have been successfully applied in several research works, there are still technical limitations to overcome before it can be applied at an industrial scale. In order to increase the efficiency of PEF technology, it has been proposed to apply it in combination with other technologies such as heat (Saldana et al., 2011; Saldaña et al., 2012) or essential oils (Espina et al., 2014; Monfort et al., 2012, 2013). The use of essential oils (EOs) as an alternative to synthetic antimicrobials is a growing trend today. They represent an interesting source of natural antimicrobials for food preservation (Burt, 2004). A wide variety of EOs have been permitted by the European Commission as flavorings and the Food Drug Administration of United States (FDA) has classified them as Generally Recognized as Safe (GRAS) substances (FDA, 2017; Hyldgaard et al., 2012).

This research work explores a novel strategy for inactivation of Listeria spp. based on the combination of natural antimicrobials (essential oils) and PEF. It is hypothesized that the electroporation induced by the PEF treatment shall increase the effect of the antimicrobial due to the higher membrane permeability. Therefore, this strategy has the potential to take advantage of a synergistic effect between both technologies.

2. Materials and methods

2.1. Bacterial culture and growth media

Six Listeria strains were used in the susceptibility studies. *L. monocytogenes* NCTC 11994 was provided by the National Collection of type cultures (operated by Public Health England) and was associated with a case of meningitis after eating soft cheese (Bannister, 1987). The remaining strains (two *L. welshimeri* (*Lw*1 and *Lw*3) and three *L. monocytogenes* (*Lm*5, *Lm*6 and *Lm*7), were isolated from retail skin-packed raw cod and salmon by Pedrós-Garrido et al.

(2020) and genes were sequenced by16S rRNA. These bacteria were stored at -80°C (20% glycerol) until use. Subsequently, fresh cultured plates were sown weekly in Trypticase soy agar (TSA; Oxoid, Basingstoke, UK) supplemented with 0.6% yeast extract (YE; Oxoid, Basingstoke, UK).

Listeria suspensions were prepared using a protocol described by Haughton et al. (2012) with some modifications. A single colony from a fresh culture plate was transferred to 5 mL of Trypticase soy broth (TSB; Oxoid, Basingstoke, UK) supplemented with 0.6% yeast extract (YE; Oxoid) and incubated overnight at 37±1°C. For obtaining the microbial cultures, flasks with 50 mL of TSBYE were inoculated with 1 mL of the pre-culture and incubated for 24 h at 37±1°C in agitation to obtain all cells in stationary phase with a concentration of approximately 10^8 CFU/mL.

2.2. Determination of minimum inhibitory concentration (MIC)

Oregano oil (CAS 8007-72-0) was provided by Sigma-Aldrich (Sigma-Aldrich Ireland Ltd., Wicklow). The minimum inhibitory concentration (MIC) for each *Listeria* strain was determined by microdilution, according to the protocol proposed by McDermott et al. (2005) and slightly modified by by Pedrós-Garrido et al. (2020), with emulsions that had been made with ultrasound at different concentrations, between 0-0.8%. In order to carry out the protocol, 100 µL of a culture of the cells of the *Listeria* spp. strains to be tested were required and placed in microtiter plates and the latter were left to incubate at 37 °C with gentle agitation at 150 rpm (Friedman, Henika, & Mandrellm, 2002) for 24 hours, after which time the growth was assessed visually and MIC defined as the lowest concentration of compound without visible growth (Isabel Clemente et al., 2016; Lambert et al., 2001). The wells in which there was no growth were taken 100 microliters and placed on brain heart infusion agar (BHI, Oxoid, Basingstoke, UK) and after incubation the number of colonies was counted. The pH of the oregano oil solutions was measured, using a digital pH-meter (Crison Instruments, Barcelona, Spain).

2.3. PEF treatments

2.3.1. Preparation of Listeria suspensions for treatment

A volume of 1 mL of *Listeria* suspensions was transferred to an Eppendorf tube and centrifuged for 5 min at 13000 rpm. Pellets were washed in maximum recovery diluent (MRD; Oxoid, Basingstoke, UK) and re-centrifuged twice before final resuspension in 10 mL Mcllvaine buffer pH 7.0 and the conductivity was adjusted to 6.0 µS/cm (Dawson et al., 1969). The concentration of cell suspensions was approx. 10^6 CFU/mL. Other samples were prepared for use as controls.

2.3.2. PEF equipment

ELCRACK HVP5 (German Institute for Food Technologies (DIL), Quakenbrück, Germany) was the PEF equipment used in this study. This equipment has a maximum output voltage of 25 kV, a pulse width of 4–32 µs, a pulse repetition rate of up to 1000 Hz, a pulse power of 3 MW and square shaped pulse with alternating polarity. The batch chamber used for inactivation trials in the liquid was designed to accommodate an electroporation cuvette with 0.2 cm gap and electrode surface area of 2.76 cm^2 (ELG300-202G, Fischer Scientific, Dublin, Ireland) in which liquid samples were placed for treatment, and whose capacity in the cuvette has a maximum of 1.5 mL of sample (I. Clemente et al., 2020).

2.3.3. PEF treatments applied alone

A suspension of 100 µL of each plus 900 µL de pH 7 buffer Macllvaine, was treated in a cuvette using the following PEF settings: 50 pulses of 20 kV/cm electrical field strength, 20 µs pulse width at a frequency of 1 Hz. All time the temperature was controlled during PEF treatment by a fiber optic temperature probe into the cuvette treatment (Luxtron One Probe fitted to a Luxtron 812 temperature monitor, Luxtron Corporation, Santa Clara, California, USA). It was ensured that the samples did not exceed 20°C, so that temperature would not interfere with

microbial inactivation (Saldaña et al., 2012). After the treatments, the relevant serial dilutions were made with the samples treated in MRD (Maximum Diluent Recovery), they were plated in a 0.1 mL in Petri plates and they were incubated at 37±1°C for 24-48 hours.

2.3.4. Combined PEF- antimicrobial treatments

For PEF treatments, 100 µL of *Listeria* suspensions plus 900 µL de pH 7 buffer Macllvaine were placed in the treatment cuvette and a range of PEF treatments were applied (to 800, 600, 300 and 300 pulses for electrical fields strength of 5, 10, 15 and 20 respectively and 20 µs pulse width at a frequency of 1 Hz). After that, 100 µL of each sample was plated with TSAYE and incubated at 37±1°C for 24-48h.

For simultaneous treatments, plus 880 µL de pH 7 buffer Macllvaine and 20 µL of oregano EO, MIC (2000 ppm) were placed in the treatment cuvette and PEF treatments were applied for the same conditions at the PEF treatments described above. After the treatment, samples were incubated at 37±1°C for 24-48h.

For sequential treatments, 100 µL of *Listeria* suspensions plus 900 µL de pH 7 buffer Macllvaine, were placed in the treatment cuvette and PEF treatments for the same conditions described above were applied. Subsequently, 100 µL of suspension cells of *Listeria* were re-suspended in an Eppendorf tube with 880 µL (pH 7 buffer Mcllvaine) and 20 µL of oregano EO for 20, 40- and 60-min. After the sequential treatment, samples were incubated at 37±1°C for 24-48h. All assays were performed at least in triplicate.

2.4. Statistical analysis

All experiments were repeated at least in triplicate. Results were analysed by t-test and ANOVA followed by Tuckey's test. In all cases significance was defined as $p < 0.05$. Error bars in the figures correspond to the standard error of the mean. Data was evaluated using GraphPad PRISM 5.0 software (GraphPad Software, Inc., San Diego, CA, USA).

3. Results and discussion

3.2. Assessment of the resistance of *Listeria* spp. to PEF treatments

Figure 16 shows the inactivation levels achieved in a FEP treatment for the six strains tested in this study. All strains showed similar resistance against PEF treatment for the conditions evaluated (50 pulses, 20 µs pulse width and 20 kV/cm and a frequency of 1 Hz). The inactivation levels of strains NCTC 11994, *Lw*1, *Lw*2 and *Lm*5, *Lm*6 were 2.45, 2.72, 2.66, 2.65 and 2.98, respectively, without significant differences between them according to a Tukey test ($p>0.05$). Therefore, the resistance to PEF treatments of these five strains can be considered homogeneous. Strain *Lm7*, on the other hand, had an inactivation (3.35 $\log_{10}$ cycles) significantly higher than the one of three of the strains tested (NCTC 11994, *Lw2* and *Lm5*). However, the observed inactivation for this strain had also the highest standard error. Therefore, considering that the experiments were done in triplicate, it is possible that the higher inactivation observed is due to experimental error, not due to a lower resistance.

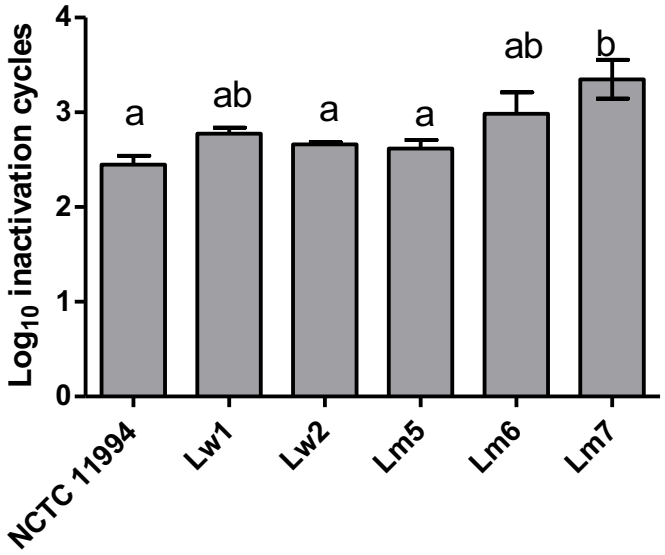

Figure 16 PEF resistance of different *Listeria* spp. after 50 pulses, 20 µs pulse width and 20 kV/cm and a frequency of 1 Hz, expressed as Log_{10} inactivation cycles. Letters refer to significant differences ($p \leq 0.05$) between samples.

3.3. Combined PEF and antimicrobial compound treatments

To test the effectiveness of PEF combined with natural antimicrobials, two strategies were carried out: adding oregano simultaneously to PEF (PEF treatment and EO oregano at the same time) or subsequently (the survivors of the PEF treatment are suspended in oregano EO). The concentration of antimicrobial used in the experiments was the MIC (2000 ppm) that was added to the pH 7 Mcllvaine buffer. The conditions of the PEF treatment were 50 pulses with a 20 μs pulse width and 20 kV/cm and a frequency of 1 Hz. Table 5 shows the level of inactivation for PEF treatments and in different combinations with the antimicrobial (simultaneously and subsequently, with different incubation times). For the simultaneous treatment, this combination did not improve the inactivation levels of PEF treatments. Indeed, in several cases, the PEF treatments was more effective than the combined treatment. Among the strains tested, the ones with the highest inactivation were *Lm6* and *Lm7*, with 2.985 and 3.350 $\log_{10}$ cycles respectively. There were also, no significant differences ($p \geq 0.05$) between the resistance of the strains of *Listeria* spp. (PEF treatment at the same time as oregano EO), highlighting the homogeneous resistance of the strains to the treatment. Similar results were reported by Espina et al. (2014), who studied the combination of a range of essential oils together with PEF at 25 kV/cm, without observing an increase in the lethality of PEF treatments on *Listeria monocytogenes*. In a different study, Ait-Ouazzou et al. (2013) observed a synergistic effect between 1.3 mM of carvacrol and 20 pulses of 30 kV/cm PFE, reaching the inactivation of 5 $\log_{10}$ cycles of *E. coli* O157:H7 in different juices. Similar results showed the effect on skimmed milk supplemented with three different concentrations of cinnamon (1-5%), increasing the inactivation levels on *Salmonella* Typhimurium when it was treated with PEF, 0.17 and 0.98 Log_{10} cycles in an electric field of 10 and 30 kV/cm respectively (Pina-Pérez et al., 2012). These variation in the results of different studies demonstrate that the effect of the combined treatment EO+PEF depends on several factors, both intrinsic parameters of the microorganisms and the parameters of the process. For that reason, the effect of a subsequent strategy, where the EO is applied after the PEF treatment was also assayed in this study.

Table 5 Inactivation levels at different treatments for all *Listeria* spp. strains studied at 50 pulses, 20 kV/cm electric field strength, 20 μs pulse width and 1 Hz frequency.

Strain	PEF	PEF+OR	PEF+20'	PEF+40'	PEF+60'
NCTC 11994	2.45 ± 0.23^{1A}	2.32 ± 0.07^{12A}	1.43 ± 0.07^{23A}	1.21 ± 0.00^{3A}	1.59 ± 0.07^{23A}
*Lw*1	2.72 ± 0.17^{1AB}	2.90 ± 0.36^{1A}	3.26 ± 1.04^{12BD}	3.99 ± 0.71^{23B}	4.57 ± 0.25^{3B}
*Lw*2	2.66 ± 0.05^{1A}	2.24 ± 0.05^{1A}	$2.41\pm0.28_{1}^{BC}$	2.52 ± 0.07^{1C}	3.68 ± 1.31^{2BC}
*Lm*5	2.66 ± 0.23^{12A}	2.66 ± 0.51^{12A}	1.85 ± 0.27^{1AC}	3.30 ± 0.00^{23BC}	3.85 ± 0.00^{3BC}
*Lm*6	2.99 ± 0.25^{1AB}	2.32 ± 0.18^{2A}	3.54 ± 0.24^{13D}	4.02 ± 0.34^{3B}	3.85 ± 0.43^{3BC}
*Lm*7	3.35 ± 0.20^{1B}	2.69 ± 0.12^{2A}	2.78 ± 0.40^{12BCD}	2.46 ± 0.23^{2C}	3.54 ± 1.16^{1C}

Results expressed as CFU/mL, mean ± SD (standard deviation). Letters refer to significant differences ($p\leq0.05$) between strains within treatments, and numbers refer to significant differences ($p\leq0.05$) between treatments within strains. PEF+OR, simultaneously (PEF treatment at the same time thar oregano EO) and subsequently (PEF treatment re-suspended in oregano EO at different time; 20-40-60 minutes).

Table 5 shows the levels of inactivation a subsequent strategy for the combined treatment, where the survivors of the PEF treatment (same pulse width, pulse number and frequency as in the simultaneous treatment) were suspended in oregano EO (2000 ppm; MIC) for 20, 40 or 60 minutes. The results show that a subsequent strategy is effective to increase the total inactivation, although the efficacy depends on the duration of the exposition to the EO and is variable between strains. For an exposure of 20 minutes, the inactivation was on the same range as for the PEF treatment, attaining higher inactivation for 2 strains (*Lw1* and *Lm6*) and lower for 4 strains (NCTC 11994, *Lw2, Lm5, Lm7*). Therefore, this exposure time would not improve the inactivation of *Listeria* spp. with respect to the attained in the PEF treatment. However, an exposition of 60 minutes to the EO significantly increase the inactivation with respect to the one other treatments. Strains *Lw*1, *Lw*2, *Lm*5, *Lm*6 and *Lm7* increased their inactivation with respect to the PEF treatment by 1.85, 1.02, 1.19, 0.86 and 0.19 $\log_{10}$ cycles respectively; and with respect to the treatment with an exposure of 20 minutes by 1.31, 1.27, 2.00, 0.31 and 0.75 $\log_{10}$ cycles for *Lw*1, *Lw*2, *Lm*5, *Lm*6 and *Lm7,* respectively. These results show that a combined treatment combining a PEF treatment with exposition of the survivors to oregano EO for 60 minutes is an effective

strategy for the inactivation of these *Listeria* strains. These results are in line with those reported by Clemente et al. (2020), who also obtained increased inactivation levels on *Campylobacter jejuni* when the survivors of the PEF treatment were resuspended in oregano EO, although in their study the higher inactivation was observed for an exposure of 20 minutes. Therefore, based on these results, it can be deduced that the oregano EO needs a relatively long time (>20 minutes) to have an effect on microbial inactivation.

However, an increased inactivation was not observed for strain NCTC 11994. This strain seems to be insensitive to the oregano EO tested in this study, as shown by the fact that the inactivation attained is practically the same for the three exposure times tested (20, 40 and 60 minutes). Indeed, every combined treatment resulted in lower inactivation than the treatment single treatment with PEF. The different behavior of this strain could be associated to between-strain variability in the response of *Listeria* spp. to stress that has been reported for thermal (Clemente-Carazo et al., 2020; Garre et al., 2020) and acidic (Metselaar et al., 2013) treatments. However, it is not clear from our results whether this strain is specially resistant to PEF, being less susceptible to electroporation; better prepared for repairing the damage of the PEF, closing the pores before the EO can affect the cell; or more resistant to the oregano EO.

3.4. Effect of the intensity of the electric field on the inactivation of *Listeria* spp.

In order to further analyze the effect of the intensity of the PEF, additional experiments were done using strain *Lm5*. It was selected because it is a pathogenic strains isolated from a product that can be a high risk for listeriosis. The effect of the intensity of the electric field is illustrated in Figure 17, where the survivor curves for the PEF (A) and PEF+20 (B) treatments are shown for different intensities of the electric field. As illustrated in Figure 17A, the level of inactivation is higher as the electric field strength increases. The treatment at 20 kV/cm reached almost 6 $\log_{10}$ cycles of inactivation, a value that can be considered sufficient for inactivation of *L. monocytogenes*. After 60 pulses, this intensity caused 2.01 $\log_{10}$ cycles, whereas a treatment at

15 kV/cm needed 300 pulses to reach the same inactivation. The treatments at 5 or 10 kV/cm practically did not result in inactivation of *L. monocytogenes*. These results are in agreement with those by Maza et al. (2019), who associated the formation of pores in the microbial cytoplasmic membrane to fields higher than 15 kV/cm, because from this electric field strength the transmembrane threshold potential is exceeded. On the other hand, a study by Álvarez et al. (2003) observed no inactivation for an intensity of 15 kV/cm, requiring higher values (28 kV/cm) to observe inactivation of almost 5 $\log_{10}$ cycles. These results may indicate that there is a threshold value of the intensity of the electric field below which there is no inactivation, and that this threshold may vary between strains.

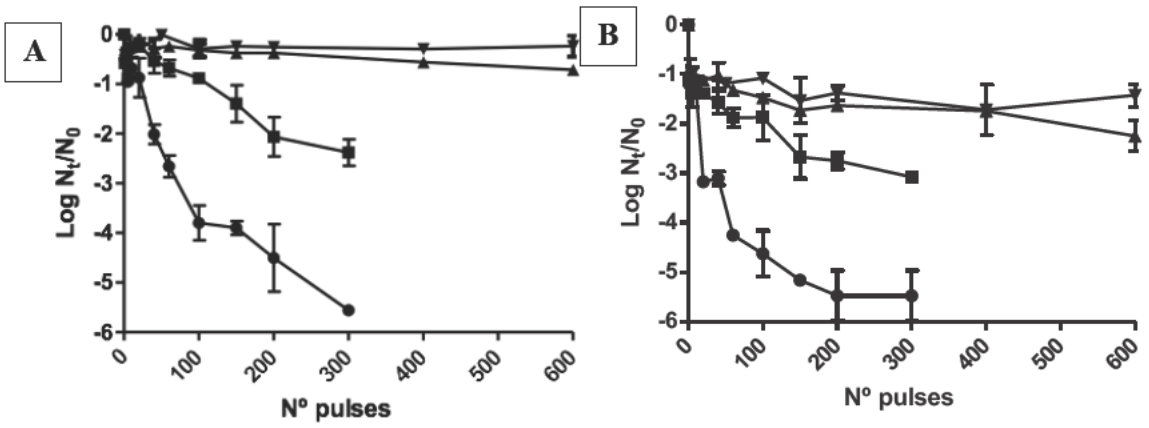

Figure 17 Influence of electric field strength on the inactivation on *Listeria monocytogenes* 5; 5 kV/cm (▼) ,10 kV/cm (▲), 15 kV/cm (■) and 20 kV/cm (●).

Fig. 17B, shows the survivors curves for *Lm5* when oregano (2000 ppm) is subsequently added to the recovery solution (pH 7 buffer McIlvaine) for 20 minutes to the survivors of the PEF treatment. Our results show a sharp drop of 1 log cycle at the beginning of the treatment. This could be associated to the effect of the oregano EO, which is able to inactivate part of the population regardless of whether there is a sublethal damage. This sharp drop was also observed by Saldaña et al. (2010) on *Staphylococcus aureus* when oregano essential oil was added to the

media. Apart from that, the number of survivors at the end of the treatment are similar to those observed in the PEF treatment (Figure 17A).

Generally, gram-positive bacteria are more susceptible to essential oils than gram-negative. The mechanisms of action of EO for microbial inactivation are not yet well defined, although it is known that penetration related to phenolic compounds can cause damage at the cytoplasmic level (Skandamis & Nychas, 2001). Baydar et al. (2004) and Chun et al. (2005) theorized that the inactivation mechanisms is associated to the EO impairs some of the metabolic reactions taking place inside the cell. One of the most important of which is the deterioration of the proton motive force, which would result in a reduction of protein synthesis and, eventually, cell death. Particularly for oregano EO, its main active compounds is carvacrol, whose ability to reduce cellular respiration and change the cytoplasmic pH has already been demonstrated (Burt, 2004).

3. Conclusions

This investigation identified a combined strategy based on the combination of PEF and oregano EO that can be effective for the inactivation of *Listeria* spp. However, the efficacy of the technique depends largely on the mode of application. The application of both treatments at the same time resulted in practically no improvement with respect to the application of the PEF treatment alone. On the other hand, the suspension of the survivors of the PEF treatment in an oregano EO solution increased microbial inactivation in 5 of the 6 strains tested. This could be due to the sub-lethal damage caused by the PEF treatment on the cell membrane easing the entry of the antimicrobial compound in the cell. Moreover, the time of exposure to the EO was decisive for the total inactivation, being practically ineffective for 20 minutes and observing substantial inactivation for an exposure of 60 minutes. Therefore, this article proposes an innovative strategy for the inactivation of *Listeria* spp. that can be of interest for food industries as an alternative to conventional heat treatments.

Author contributions

Marta Clemente-Carazo: Conceptualization, Investigation, Formal Analysis, Writing. **Virginia Sánchez-Jimenez**: Investigation. **Isabel Clemente:** Conceptualization, Formal Analysis, Writing, Supervision. **Santiago Condon-Abanto**: Conceptualization, Formal Analysis, Writing, Supervision. **James G. Lyng**: Conceptualization.

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4.GENERAL DISCUSSION

The availability of safe and nutritious food for the entire population is one of the main concerns of modern societies (WHO, 2015). However, the environment that we inhabit is shared with a large number of microbial species. These microbes are present in the raw materials used for food production, as well as the surfaces of food industries. Some of these microorganisms have been used for centuries in food production, improving the quality of the final product; a typical example being the use of the yeast *Saccharomyces cerevisiae* for beer production. However, some of the microbial species present in the environment can spoil foods, and/or can cause an illness in humans after consumption of the products. Because these microorganisms are ubiquitous, they may enter the product at different points of the food chain. Therefore, the introduction of some type of processing step to inactivate these microorganisms to innocuous levels for humans is necessary for food safety.

Currently, thermal treatments are probably the most applied technology for food preservation. The application of high temperatures can damage the cell membrane, denaturize some key proteins for cell functioning, or impair cell metabolism; resulting in cell inactivation (Smelt & Brul, 2014). However, the application of high temperatures can also enable or accelerate chemical reactions that reduce the nutritional or organoleptic properties of the food product. Typical examples of this are the inactivation of beneficial compounds (e.g. vitamins) or an alteration of the flavor (e.g. "cooked" flavor in milk). Furthermore, this thermal treatments have a high energy consumption that contributes to the overall environmental impact of the food production system. Consequently, research efforts are currently being dedicated towards designing more efficient processing treatments. One direction is the development of a better understanding of thermal inactivation, which may result in milder treatments without a reduction in food safety. A different research line is the development of novel inactivation technologies that can substitute or complement thermal treatments.

Regarding thermal treatments, it is important to highlight that most of the empirical data available on microbial inactivation has been gathered under isothermal conditions. During the last decades, the availability of novel empirical methods has enabled the performance of inactivation experiments under dynamic temperature conditions in the laboratory; conditions that are closer to

those actually applied in food industries. These experiments have concluded that, under dynamic conditions, some phenomena may rise that cannot be observed under isothermal conditions. One example is the development of stress acclimation by the microbial cells. This term refers to a physiological response of the cells during a slow heating that increases their resistance to posterior stresses. This phenomenon can be of high relevance for many industrial processes where the food ingredients are heated from room temperature to the target temperature for microbial inactivation. As shown in Chapter I of this thesis, slow heating rates can induce stress acclimation of *Listeria*, reducing the effectiveness of the posterior thermal treatment. Therefore, this phenomenon can be of relevance for the safety of food products.

Moreover, previous scientific studies on stress acclimation had been limited to a single bacterial strain. However, empirical evidence shows that stress resistance can vary between strains of the same microorganisms, or even between cells of the same strain (Den Besten et al., 2018). Hence, because it is not possible to predict without uncertainty the microbial strain that may contaminate a food product, the effect of variability shall be included in risk assessments and shelf life estimation. Indeed, this is already reflected in food safety regulation. As stated in the annex II of the Regulation (CE) 2073/2005, shelf life studies performed by food business operators should have into account the variability inherent to the product, the microorganisms or the processing and storage conditions. Although the studies referred in Reg. 2073/2005 are shelf life studies (focused mainly on food storage conditions), these recommendations could also apply for food processing. Moreover, these studies are especially applicable to RTE foods in which *L. monocytogenes* can develop, so it is worth to extend the studies to the processing conditions. In this same line, challenge test that have been developed for this shelf life studies should include a minimum of three microorganisms in order to comply with this microbial variability requirement (see for example ISO 20976-1 or EURL-Lm Technical guidance document for conducting shelf life studies on *Listeria monocytogenes* in ready to eat foods).

Chapter I of this thesis has combined both types of analysis: the study of variability and the study of stress acclimation. We performed dynamic inactivation experiments that enabled the development of stress acclimation using four different *Listeria* strains and three different media.

This experimental design is highly innovative, as previous studies had studied variability only under isothermal conditions or analyzed stress acclimation using a limited number of strains or media. The data was analyzed using the inactivation model proposed by (Garre, Huertas, et al., 2018), which can separate the contribution of the instantaneous temperature and the stress acclimation to the inactivation kinetics. In our results, we did not observe variability between-media or between-strains under isothermal conditions. However, under dynamic conditions, variability at the between-strain and between-media levels was very relevant. These differences can be attributed to variability in the development of stress acclimation. This result implies that the relevance of variability in inactivation kinetics under dynamic conditions may be different than the one observed under isothermal conditions. Therefore, it shall be studied whether results obtained using isothermal experiments can be extrapolated to inactivation treatments under dynamic conditions.

A different approach to reduce the intensity of heat treatments is their combination with other kind of stresses. This approach was proposed several decades ago, and is usually called the "hurdle technology" (Leistner, 1995). At present more than 60 hurdles have already been described, the most important of which are: temperature (high or low), water activity (a_w), acidity (pH), redox potential (Eh), preservatives (e.g., nitrite, sorbate, sulfite), and microorganisms (e.g., lactic acid bacteria). The principle behind this technology as the each "hurdle" will inactivate a fraction of the bacterial population, causing sub-lethal damage to the remaining cells. Then, the next hurdle should be more effective as the cell will have lower resistance (e.g. due to a damaged membrane or loss of cell homeostasis). This would result in a synergistic effect, where the combined treatment is more effective than the simple addition of the individual hurdles. Consequently, a combined treatment could accomplish the same level of inactivation than a single treatment using milder individual treatments.

The most common combined treatment applied nowadays in industrial setting is the combination of heat inactivation with acidification. From a practical point of view, both "hurdles" can be applied at the same time by acidifying the food product while the heat treatment is applied. Alternatively, they can be applied subsequently, by first washing the food product with an acidic

solution and later applying the heat treatment. In principle, both approaches should have a similar impact on the total microbial inactivation. However, it is important to realize that bacterial cells are not static systems. When a microbial cell is under stress, it develops a complex physiological response that can increase its resistance. For instance, there is empirical evidence of changes in cell membrane permeability after application of a sub-lethal thermal stress. Several of these adaptive microorganisms are shared between different stresses. Consequently, a cell that has adapted to some type of stress may develop an additional resistance to a stress of a different type (cross-resistance). This is of high relevance for the development of combined heat treatments, as cross-resistances may result in an antagonistic effect of the combined treatment, resulting in lower inactivation than the one that would be accomplished applying each treatment individually. Therefore, the design of combined treatments based on the "hurdle approach" requires a detailed understanding not only of the microbial responses to each individual treatment, but also to the possible antagonistic or synergistic effects between technologies.

The importance of analyzing whether a combined treatment is synergistic or antagonistic has been demonstrated in Chapter II of this thesis. In this research, we have studied the thermal inactivation of two *Salmonella* serovars (Enteritidis and Senftenberg) after the application of an acid shock. In the case of *S.* Senftenberg, the acid shock reduced the cell resistance to thermal stresses. Therefore, in this case, the application of an acid shock (e.g. by a wash with an acid solution) would be an effective way to reduce the intensity of a posterior thermal treatment. However, for *S.* Enteritidis the acid shock did not reduce the resistance of the survivors to the posterior heat treatment. Hence, the combined treatment would not be effective in this case, requiring a more intense thermal treatment to cause the same microbial inactivation. Consequently, combined treatments should be designed with special care, as the effectiveness of the treatment can vary between members of the same bacterial species.

Besides traditional treatments, during the last years several novel technologies have been proposed for food processing. With the exception of high hydrostatic pressure that is currently being implemented at the industry level for some products, most of them are rarely used as the only mean for microbial inactivation. One reason for this may be the lack of technological

development, making their application for microbial inactivation cost-ineffective. However, several research works have shown that the combination of several novel technologies, or the combination of novel technologies with thermal treatments, can be effective for microbial inactivation. This approach is especially interesting, as the adaptive response of microbial cells can vary between stresses, potentially resulting in synergistic effects of the combined treatment. Chapter III of this thesis reports an example of such strategy. In this research work, Pulsed Electric Fields (PEF) have been applied in combination with oregano essential oil for the inactivation of *Listeria monocytogenes*. Our results show that the combination of these technologies can be effective for the inactivation of *Listeria* spp. because PEF causes electroporation of the cell membrane, increasing the cell permeability and making it more susceptible to the entry of external agents. Hence, it increases the effect of the oregano essential oil on cell inactivation, as these molecules can more easily enter the cell. However, our experimental results also shown that the mode of application influenced the effectiveness of the combined treatment. When the essential oil was applied after the PEF treatment, the effect was much higher than when it was applied at the same time. This result highlights that the design of combined treatments has an added complexity with respect to the design of single treatments. The design of safe combined treatments require an understanding of not just the microbial response to each individual stress, but also to the possible cross-resistances or sensitizations that may arise during the combined treatment.

Closing up, there is currently a strong interest in the development of different processing strategies that can result in safe food products with a minimal impact on product quality. This strategies range from the optimization of existing thermal treatments to the application of novel technologies in combination or in substitution of thermal treatments. However, either approach requires further scientific understanding in order to be applied safely. Regarding thermal treatments, there are still several knowledge gaps about the microbial response, especially when it comes to dynamic inactivation treatments. With respect to combined treatments, promising results have been reported in literature where strong effects have been observed between different technologies. However, some studies report the opposite effect, where the application of a

combined treatment resulted in lower inactivation than the application of each treatment individually. Therefore, the mechanisms of stress response to different stresses should be better understood to design effective treatments. Moreover, variability in the microbial response to stress has a very strong impact on the effectiveness of inactivation treatments. Although several advances have been done during the last years, there is still a need to develop methods (experimental and computational) to quantify it and to include it in predictive models. Therefore, although promising results are reported in this thesis, as well as in other works from the scientific literature, further understanding of the underlying mechanisms of microbial response to stress is required in order to design more effective strategies for microbial inactivation and food safety.

5.CONCLUSIONS

1. **Variability in the microbial response to thermal treatments may be different under dynamic conditions than under isothermal treatments.** The D-value of *L. monocytegenes* strain CECT 4031 developed stress acclimation during dynamic treatments, increasing its D-value 10-fold, while strain CECT 4032 only increased its D-value a 50%. Under isothermal conditions, no differences were observed in the D-value of these strains.
2. **Acclimation of *Listeria monocytogenes* depends on the heating medium, being more pronounced in rich and complex heating media.** In dynamic experiments, the highest acclimation was observed in buffered peptone water and semi-skimmed milk and the lowest in pH 7 Mcllvaine buffer. This could be attributed to the higher content in nutrients of these media.
3. **The thermal resistance of bacterial cells after an acid shock can vary between serovars of the same species.** An acid shock reduced the resistance of *S.* Senftenberg to a posterior heat treatment (time to reduce 3 $\log_{10}$ cycles in a pH 7 treatment medium at 60°C decreased from 10.75 to 1.98 min). On the contrary, an acid shock did not affect the resistance of *S.* Enteritidis to posterior heat treatments (in this case the time necessary to reduce 3 $\log_{10}$ cycles in a pH 7 treatment medium at 60°C was of 0.30 min before the acid shock and of 0.31 min after). This could be attributed to the acid-shock causing a sub-lethal damage on *S.* Senftenberg but not in *S.* Enteritidis.
4. **A strategy combining pulsed electric fields (PEF) and oregano essential oil can be effective for the inactivation of *Listeria monocytogenes*.** This could be attributed to the fact that PEF causes the electroporation of the cell membrane, facilitating the application of the essential oil. However, the mode of application influenced the efficacy of the combination treatment: when the essential oil was applied after PEF treatment, the effect was much greater than when it was applied simultaneously. The exposure time to the essential oil was also decisive for microbial inactivation, requiring 60 minutes for a noticeable effect.

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