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EVALUACIÓN DE LA IMPORTANCIA DE DIFERENTES FUENTES DE VARIABILIDAD EN LA SUPERVIVENCIA DE MICROORGANISMOS PATÓTENOS ALIMENTARIOS: ADAPTACIÓN AL ESTRÉS FRENTE A LA HETEROGENEIDAD GENÉTICA

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PhD THESIS

ASSESSING THE RELEVANCE OF DIFFERENT SOURCES OF VARIABILITY ON THE SURVIVAL OF FOODBORNE PATHOGENS: STRESS ADAPTATION AGAINST GENETIC HETEROGENEITIES

Presented by Leonidas Georgalis
to the Technical University of Cartagena in fulfilment of
the thesis requirement for the award of PhD

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Abstract

Microbial risk assessment is crucial for protecting public health and the food supply chain. Sources of variability in microorganisms, such as stress adaptation and genetic heterogeneities, can affect the survival, growth and virulence of microorganisms and their ability to cause disease or food spoilage. There are currently large knowledge gaps regarding variability of the microbial response and understanding it is essential for accurately estimating potential risks and to develop effective control measures. In light of this, this PhD thesis aims to compare and evaluate the importance of stress adaptation and genetic heterogeneities in microorganisms for the survival of bacteria to thermal treatments.

Chapter I discusses the thermal inactivation of two *Salmonella* strains (*Salmonella* Enteritidis CECT4300 and *Salmonella* Senftenberg CECT4565) under both isothermal and dynamic conditions. For isothermal treatments, *S.* Senftenberg was found to be much more resistant than *S.* Enteritidis (by approximately a factor of 10). We also observed qualitative differences, with the inactivation models used to describe the response of *S.* Senftenberg were weibullian, while the Bigelow model was successful in describing the isothermal response of *S.* Enteritidis. Models based on isothermal experiments were able to describe dynamic inactivation of *S.* Senftenberg, while *S.* Enteritidis required a dynamic model that considered stress acclimation. The study highlights that, besides quantitative, variability in microbial inactivation is also qualitative. This underlies importance of considering different model hypotheses for both isothermal and dynamic conditions.

Chapter II goes further in the thermal inactivation of *Salmonella* spp. focusing on the importance of phenotypic variability in microbial risk assessment, which refers to the

physiological differences of cells of the same bacterial species due to prior exposure to different environments. The impact of sub-optimal pre-culture conditions or the application of an acid shock on the thermal resistance of the same two *Salmonella* strains was studied, founding that phenotypic variability is also strain-dependent. For the highly resistant strain (*S.* Senftenberg), the conditions tested resulted in a reduction of thermal resistance with respect to optimal incubation conditions. On the other hand, sub-optimal incubation conditions had the opposite effect on the reference strain (*S.* Enteritidis), increasing its thermal resistance through the induction of cross-resistance mechanisms. The study suggests that phenotypic variability should be a main focus in predictive microbiology and risk assessment, and illustrates a hypothetical example of how this could be achieved in practice by linking pre-incubation conditions to the origin of bacterial contamination.

Chapter III uses a common model organism (*Bacillus subtilis*) to further study the differences between isothermal and dynamic bacterial inactivation. To link differences in the response to molecular mechanisms, experiments were made using both a wild type strain and a marker-free *sigB* null mutant. Survivor curves with an upward curvature were observed, which is often attributed to heterogeneity in thermal resistance (vitalistic hypothesis). However, a pretreatment resulted in log-linear survivor curves, indicating dynamic stress adaptation during the isothermal treatment as a possible explanation for the upward curvature. Based on this hypothesis, bounds were defined based on isothermal experiments to account for acclimation under dynamic conditions. The study provides an alternative interpretation for survivor curves, which can improve predictions of microbial response during pasteurization treatments.

Resumen

La evaluación de riesgos microbianos es crucial para proteger la salud pública, así como para que la cadena de suministro de alimentos sea eficiente. La evaluación debe considerar varias fuentes variabilidad en la respuesta microbiana, tales como la adaptación al estrés y las heterogeneidades genéticas, ya que éstas pueden afectar la supervivencia, el crecimiento y la virulencia de los patógenos alimentarios influyendo a su capacidad para causar enfermedades en humanos o alteraciones en los alimentarios. Actualmente existen grandes lagunas de conocimiento en cuanto a la variabilidad de la respuesta microbiana, por lo que su elucidación es esencial para una estimación precisa del riesgo, así como para el desarrollo de medidas de control eficaces. En base a esto, esta tesis de doctorado tiene como objetivo evaluar y comparar la importancia de la adaptación al estrés y las heterogeneidades genéticas en los microorganismos para la supervivencia de las bacterias a los tratamientos térmicos.

En el Capítulo I se estudia la inactivación térmica de dos cepas de Salmonella (*Salmonela* Enteritidis CECT4300 y Senftenberg CECT4565) tanto en condiciones isotérmicas como dinámicas. Para los tratamientos isotérmicos, se encontró que *S.* Senftenberg era mucho más resistente que *S.* Enteritidis (por un factor de aproximadamente 10). También observamos diferencias cualitativas: las curvas de supervivencia isotermas de la cepa de *S.* Senftenberg fueron de tipo Weibull, mientras que el modelo Bigelow (lineal) fue capaz de describir la respuesta de *S.* Enteritidis. Así mismo, los modelos basados en experimentos isotérmicos fueron capaces de describir la inactivación dinámica de *S.* Senftenberg, mientras que *S.* Enteritidis necesitó de un modelo dinámico que consideraba la aclimatación del estrés. El estudio destaca que, además de la variabilidad cuantitativa, la inactividad microbiana también

tiene una componente cualitativa. Esto enfatiza la importancia de considerar diferentes hipótesis de modelo para las condiciones isotérmicas y dinámicas.

El Capítulo II va más allá en la inactivación térmica de *Salmonella* spp. centrándose en la importancia de la variabilidad fenotípica en la evaluación del riesgo microbiano. Esta fuente de variabilidad incluye las diferencias en el estado fisiológico de diferentes células de las mismas especies bacterianas debido a una exposición previa a diferentes entornos. Se estudió tanto el impacto de las condiciones de pre-cultivo subóptimas en la resistencia térmica de las mismas dos cepas de Salmonella como el efecto de un choque ácido. Los resultados muestran que la variabilidad fenotípica también es dependiente de la cepa. Para la cepa de *Salmonella* altamente resistente (*S.* Senftenberg), se observó una reducción de la resistencia térmica con respecto a las condiciones óptimas de incubación. Por otro lado, las condiciones de incubación subóptimas tuvieron el efecto opuesto en la cepa de referencia (S. Enteritidis), aumentando su resistencia térmica a través de la inducción de mecanismos de resistencia cruzada. En base a estos resultados, el estudio sugiere que la variabilidad fenotípica debería ser un aspecto central en la microbiología predictiva y la evaluación de riesgos, e ilustra un ejemplo hipotético de incorporación de esta fuente de variabilidad en el análisis de riesgos vinculando las condiciones pre-cultivo al origen de la contaminación bacteriana.

El Capítulo III utiliza un organismo modelo (*Bacillus subtilis*) para profundizar en el estudio de las diferencias entre la inactivación bacteriana bajo condiciones isotermas y dinámicas. Con el objetivo de relacionar la respuesta microbiana observada a nivel poblacional con información a nivel molecular, se realizaron experimentos utilizando tanto una cepa silvestre como un mutante "*sigB null*". Se observaron curvas de supervivencia con una curvatura hacia arriba, que a menudo se atribuye a la heterogeneidad en la resistencia térmica

(hipótesis vitalística). Sin embargo, las curvas de inactivación tras la aplicación de un pretratamiento fueron log-lineales, indicando la adaptación dinámica al estrés durante el tratamiento isotérmico como una posible explicación de la curvatura observada. Esta interpretación de los resultados permite definir, en base a resultados isotermos, límites para la adaptación microbiana que se pueda desarrollar durante un tratamiento dinámico. Por lo tanto, este estudio proporciona una interpretación alternativa de las curvas de supervivencia bajo condiciones isotermas que podría llegar a mejorar nuestra capacidad de predecir la respuesta microbiana durante los tratamientos de pasteurización.

List of Abbreviations

ALOP: Acceptable Level of Protection

ATP: Adenosine Triphosphate

ATR: Acid Tolerance Response

Aw: activity water

CAC: Codex Alimentarius Commission

CCPs: Critical Control Points

COVID-19: Coronavirus Disease

DALYs: Disability Adjusted Life-Years

DNA: Deoxyribonucleic Acid

ECDC: European Centre for Disease prevention and Control

EFSA: European Food Safety Authority

FAO: Food and Agriculture Organization

FDA: Food and Drug Administration

FSMS: Food Safety Management System

GAP: Good Agricultural Practice

GMP: Good Manufacturing Practice

HACCP: Hazard Analysis Critical Control Point

HSF: Heat-Shock Factor

HSP: Heat-Shock Proteins

HSR: Heat-Shock Response

HTST: High Temperature Short Time

ICMSF: International Commission on Microbiological Specifications for Foods

ISO: International Organization for Standardization

LSODA: Livermore Solver for Ordinary Differential Equations

MRA: Microbial Risk Assessment

NaCl: sodium chloride

NACMCF: National Advisory Committee on Microbiological Criteria for Foods

ODE: Ordinary Differential Equations

pH: Intracytoplasmic pH

QMRA: Quantitative Microbial Risk Assessment

QMS: Quality Management System

RMSE: Root Mean Squared Error

RNA: Ribonucleic Acid

RNAP: RNA polymerase

RpoS: RNA polymerase S

rRNA: ribosomal ribonucleic acid

SigB: Alternative Sigma Factor B

TSA: Trypticase Soy Agar

TSB: Trypticase Soy Broth

UHT: Ultra-High Temperature

VBNC: Viable But Non-Culturable

w/v: weight/volume

WHO: World Health Organization

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1 Introduction

Unsafe food poses a global health threat that puts everyone at risk. In fact, every human being will become ill from foodborne illnesses several times throughout their lives (WHO, 2022). A global annual burden of 33 million disability-adjusted life years (DALY) and 420 000 premature deaths is caused by an estimated 600 million cases of foodborne illness, or about one in ten people worldwide (Havelaar et al., 2015; WHO, 2015). Food safety remains one of the main concerns in the European Union. In 2021, EU Member States reported 4,005 foodborne outbreaks affecting 32,534 people; this meant an increase of 29.8% compared with the previous year (3,086 in 2020). Human cases and hospitalisations also increased, by 62.6% (20,017 cases in 2020) and 49.0% (1,675 hospitalisations in 2020), respectively (EFSA & ECDC, 2022). Nevertheless, the above data should be treated with caution, since the measures associated to the COVID-19 pandemic also impacted the global food supply chain (WHO, 2020).

A foodborne disease outbreak occurs when at least two people become ill as a result of the same contaminated food or drink. Foodborne disease has long been a significant burden on public health and continues to pose a challenge to health-care systems around the world. Anyone can get a foodborne disease, but vulnerable populations like small children, the elderly, pregnant women, immunocompromised people and those living in poverty or food insecurity are especially sensitive (WHO, 2017). Modern food production has increased food variety and decreased food costs, but by centralizing the food supply, provides an opportunity for foodborne pathogens and toxins to infect and poison a substantial number of consumers (Garre, Fernandez, et al., 2019).

Foodborne illness is an important cause of morbidity in all countries and the number of cases remains relatively stable, despite impressive advancements in food science and

technology (Kopper et al., 2014). Due to the ability of microbes to grow and survive under stressful circumstances (Schimel et al., 2007), studying how bacteria and other microbes react to environmental stresses and how long they can survive under them has become a key component of food safety research (Spector & Kenyon, 2012a). For instance, *Salmonella* serovars are adept at surviving, growing and/or adapting to a wide variety of stressful environments, such as those with extracellular pH values as low as 3.99 and as high as 9.5, salt concentrations as high as 4% w/v NaCl and temperatures as high as 54 °C or as low as 2 °C (Doyle & Beuchat, 2007). As a result, the frequency and intensity of these stressors along the food supply chain will determine salmonellae's ability to survive during the preparation, processing and storage of food as well as their ability to transit through the host organism.

The science of evaluating risks, their likelihood of happening and their effects in the event that they do, is known as risk assessment. The procedure entails identifying and describing the risks, determining exposure and describing the risk level (Havelaar et al., 2008). As part of the process of producing safe foods, materials that enter the food chain must be carefully examined and evaluated. This involves conducting a thorough risk assessment of the materials, including assessing their potential for contamination with harmful pathogens, chemicals, or other contaminants. In addition, microorganisms must be taken into account when conducting risk assessment. Microorganisms sense and react to a wide range of signals, including temperature, pH and osmolarity, in order to adapt to the shifting environment, and the capacity of microorganisms to adapt rapidly to environmental change is crucial for their survival and virulence (Fang et al., 2016; López-García et al., 2022; Shen & Fang, 2012). Furthermore, it is important to understand that when microorganisms are exposed to stressors such as antimicrobial peptides, nitrosative and oxidative stress and nutritional scarcity, their adaptive responses can affect the risk assessment process.

To ensure the safety of food products, microbial growth must be suppressed, unit processing operations must be used to minimize or eliminate microbial burden through processing and post-contamination must be avoided (Bintsis, 2018). Various strategies can be employed to suppress microbial growth, including the use of preservatives, temperature control and packaging technologies (Cutter, 2002). Unit processing operations are a critical step in the production of safe food products and specific processing techniques must be used to minimize or eliminate microbial burden through processing (Artés-Hernández et al., 2013). It is important to note that the capacity of microorganisms to adapt to changing environments can affect the success of these strategies (Fang et al., 2016).

In conclusion, risk assessment in the food industry must take into account the potential risks posed by microorganisms and the ways in which they adapt to stressors. This knowledge can inform the development of new food preservation technologies and the optimization of existing ones to improve food quality, extend shelf life and enhance nutrient retention while ensuring high safety standards.

1.1 The state of food safety assurance in the 21st century

Food safety is a critical aspect of public health and it refers to the measures taken to ensure that food products are safe for human consumption. Food safety is achieved through the identification, assessment and management of hazards that may be present in food products (Borchers et al., 2010).There are several types of food contamination that can occur, which can lead to foodborne illness or other adverse health effects. These types of contamination include i) biological contamination that is caused by microorganisms such as bacteria, viruses and parasites. These microorganisms or their toxins can cause illness if they are present in food ii) Chemical contamination can occur when food is exposed to toxic substances such as pesticides,

heavy metals, or cleaning chemicals. These contaminants can cause illness or long-term health effects if consumed iii) Physical contamination occurs when foreign objects such as glass, metal, or plastic fragments are present in food. Consuming these objects can cause injury or illness iv) Allergen contamination occurs when foods that contain allergens, such as peanuts or gluten, come into contact with other foods. Consuming these allergens can cause allergic reactions, which can be severe in some cases (Borchers et al., 2010; H. Chen et al., 2020).

Among all those hazards, this PhD thesis is focused on the control of biological contaminants. The improvement of food safety involves taking steps to reduce their concentration in food products, as well as to prevent further contamination within the food chain. This includes proper handling, storage and preparation of food, as well as following food safety guidelines and regulations based on a detailed scientific knowledge of the microbial response to the environmental conditions typically encountered in the food supply chain.

1.1.1 Food safety management

Food is essential for sustaining life and can even be considered a part of enjoying it. At the same time, food can also be a vehicle of conveying dangers, causing disease and even death. Illness caused by contaminated food is possibly the most common transmissible health problem in the world today and it is a major cause of diminished economic productivity (WHO, 2022). The safety of our food can never be guaranteed in full. Testing every single item for every conceivable toxin, pollutant, adulterant or foodborne disease is simply not feasible; additionally, doing so would make our food prohibitively expensive (Borchers et al., 2010). It is important to note that most current testing methods, such as microbiological testing, are destructive. In such cases, it is essential to ensure that the amount of food tested is minimized

and that testing is only performed when necessary to ensure the safety and quality of the food supply (ICMSF, 2002).

Meeting the increasing demand for food supply while maintaining food safety standards is a complex challenge that requires balancing a range of factors, including population growth, changing dietary habits, climate change and technological advancements. As the global population continues to grow, the demand for food is increasing and it is expected to double by 2050 (Godfray et al., 2010). At the same time, consumers are becoming more concerned about food safety and there is a growing awareness of the risks associated with foodborne illnesses. To meet these challenges, the food industry needs to invest in food safety and quality management systems at every step of the food supply chain to help prevent, detect and respond to food safety risks. This includes implementing good agricultural practices (GAP), such as crop rotation and soil management, to reduce the risk of microbial contamination of food products. It also involves good manufacturing practices (GMP), ensuring that food is handled and processed in a way that minimizes the risk of contamination by pathogens or other harmful substances (FAO, 2022). However, meeting the need for food safety while also meeting the total food demand is challenging because, in some cases, food safety measures can increase the cost of food production and reduce the availability of certain products (Schmit et al., 2020).

Over time, the acceptable level of certain harmful substances in food has evolved and this has mainly been driven by concerns around food safety. For chemical hazards, the acceptable level of a substance refers to the maximum amount of that substance that can be present in food without posing a risk to human health. In the past, this level was often based on what was deemed to be acceptable from a sensory perspective, such as taste or appearance. However, as our understanding of food safety has improved, the focus has shifted to identifying and

controlling potential health risks associated with food (CAC, 1995). Regulatory bodies around the world now set acceptable low levels for various substances in food, such as microbial levels, contaminants, pesticides and food additives. These levels are typically based on scientific evidence and risk assessment, taking into account factors such as the toxicity of the substance, the amount that is likely to be consumed and the vulnerability of different population groups, such as infants or pregnant women (van der Meulen et al., 2022).

Nonetheless, the concept of a "safe concentration" is not generally applicable to bacterial hazards. Even a single cell may cause illness, so their acceptable level of protection (ALOP) is often based on estimates of the burden of disease for a particular hazard/food combination (van Schothorst et al., 2009). Hence, ALOPs vary depending on the specific type of microorganism, the food product in question and the intended use of the food. For example, certain types of raw meat or dairy products may naturally contain higher levels of bacteria, which is why they often require different handling and storage practices than other types of food (EFSA, 2012a).

Within the context of a structured management system, food safety policy is designed, operated, updated and integrated into overall management operations and offers the company and many parties connected to it the highest possible benefits (Panghal et al., 2018). The International Organization for Standardization (ISO) has developed two key standards related to food safety and quality management. ISO 22000 specifies the criteria for a Food Safety Management System (FSMS), while ISO 9001 outlines the requirements for a Quality Management System (QMS) specifically focused on the quality of food products. Both standards are designed to help organizations ensure the safety and quality of their food products through a comprehensive framework for managing food safety and quality (Arvanitoyannis & Kassaveti, 2009; Escanciano & Santos-Vijande, 2014).

Quality management systems advise organizations to regulate and coordinate quality by establishing objectives and implementing strategies for food quality assurance through a continuous improvement system (Chen et al., 2020; Varzakas & Arvanitoyannis, 2008). National food control systems, according to the Codex Alimentarius Commission (CAC/GL 82, (2013)), make an important contribution to food safety systems. The goal of a national food control system, as stated in "Principles and guidelines for national food control systems" (CAC, 2013) is "to protect the health of consumers and maintain fair practices in the food trade" (WHO, 2022).

As mentioned earlier, foodborne illnesses are a significant public health concern, with millions of cases reported worldwide each year. Many of these illnesses are caused by foodborne pathogens or their toxins when they are present in foods. These pathogens can be found in a wide range of food products, including meats, dairy products and fresh produce. Some of the most common foodborne pathogens are either ubiquitous or highly adapted to animals, making it difficult to control their spread (Barberán et al., 2014; Dudley, 2022). Examples of ubiquitous microorganisms that can cause foodborne illness include: i) *Salmonella* spp., which is commonly found in the intestinal tracts of animals, including birds, reptiles and mammals ii) *Listeria monocytogenes*, that is commonly found in soil and water, as well as in the digestive tracts of animals. Hence, it can contaminate a wide range of food products, including deli meats, soft cheeses and fresh produce (Dudley, 2022).

On the other hand, highly adapted microorganisms are those that have evolved to survive and thrive in specific environments, such as the digestive tract of animals. These microorganisms can be particularly difficult to control, as they are adapted to their host environment and can survive in adverse conditions. Examples of highly adapted

microorganisms that can cause foodborne illness include: i) *Escherichia coli* O157:H7 (one of the Shiga-like toxin–producing types of *E. coli*), which is commonly found in the digestive tracts of cattle, can contaminate food products such as ground beef and raw vegetables and cause illness in humans when consumed ii) *Campylobacter* spp., that is commonly found in the intestinal tracts of animals, including chickens, cattle and pigs. It can contaminate food products of animal origin (EFSA & ECDC, 2022).

Because these microorganisms are so widespread, it is often impossible to ensure their complete absence in food ingredients. Even with rigorous sanitation and hygiene practices, some pathogens may still be present in raw materials, such as meat, poultry, or produce. Therefore, it is important to implement measures to inactivate or inhibit the growth of potential pathogens during food processing. By implementing inactivation measures, food producers can significantly reduce the risk of foodborne illness and ensure that their products are safe for consumers (FAO & WHO, 2021).

Overall, the management of food microbiological hazards is critical to ensuring the safety and quality of food products. A comprehensive approach that combines multiple strategies can help minimize the risk of foodborne illness and protect public health. The management of microbiological hazards involves identifying potential sources of contamination and implementing measures to prevent, control, or eliminate them (Schirone et al., 2017; WHO, 2012). This can be achieved through a variety of measures, including:

1. Good hygiene practices: Introducing programs to ensure good personal hygiene of workers, cleaning and sanitizing equipment and surfaces and following appropriate food handling procedures can help prevent the spread of harmful microorganisms.

2. Hazard analysis and critical control points (HACCP): HACCP is a systematic approach to identifying potential hazards in the production process and implementing measures to control them.
3. Regular testing and monitoring: Regular testing and monitoring of food and water supplies can help detect and prevent the spread of harmful microorganisms.
4. Proper storage and handling: Proper storage and handling of food and other materials can help prevent the growth and spread of harmful microorganisms.
5. Education and training: Education and training for employees on the importance of good hygiene practices, proper food handling and food safety can help prevent the spread of harmful microorganisms.
6. Traceability: Traceability systems can help identify the source of any contamination and allow for rapid intervention to prevent the spread of harmful microorganisms.
7. Regulation and enforcement: Strong regulations and enforcement of food safety laws and standards can help prevent the spread of harmful microorganisms.

1.1.2 Microbial dynamics in the food production chain

The response of microorganisms in the food production chain is a crucial aspect of food safety. The fate of microorganisms in foods depends on intrinsic, extrinsic and external factors (Amit et al., 2017). Intrinsic factors refer to the inherent characteristics of the food, such as pH, water activity, nutrient content and antimicrobial compounds. Extrinsic factors refer to external environmental conditions, such as temperature, humidity and gas composition that can affect the growth and survival of microorganisms in foods. External factors refer to the conditions outside the food product that can also influence the fate of microorganisms, such as handling and processing practices, packaging and storage conditions. Control measures such as

sanitation, microbial testing and temperature control are necessary to minimize the risk of contamination by pathogenic microorganisms (Doyle & Beuchat, 2007).

Factors such as temperature, pH, water activity and nutrient availability play a critical role in the response of microorganisms in the food production chain. Temperature control is essential to prevent the growth of foodborne pathogens, which can thrive, depending on their physiology, in the danger zone of 4°C to 60°C. Similarly, pH can affect the growth and survival of microorganisms, with foodborne pathogens preferring a slightly acidic range of pH 4.5 to 7.0. Water activity (a_w), or the amount of available water in a food product, also plays a role, with microorganisms requiring a certain amount of water to grow. An a_w lower than 0.83 or a pH lower than 3.9 prevents the growth or production of toxins from food-based pathogenic microorganisms (EFSA, 2012b; NACMCF, 2010). Finally, nutrient availability is an important factor, with microorganisms requiring a source of nutrients to grow and reproduce.

Microorganisms require basic nutrients such as water, energy, nitrogen, vitamins and minerals for growth and metabolic functions. The type and amount of nutrients needed depend on the microorganism and they are present in varying amounts in different foods (Hamad, 2012). For example, meats have protein, lipids, minerals and vitamins, while plant foods have high concentrations of carbohydrates and varying levels of other nutrients (Ahmad et al., 2018). Milk is nutrient-rich and can support the growth of various microorganisms. Proper handling and storage of milk is important to prevent the growth of harmful bacteria like *E. coli* and *Staphylococcus aureus*. Nutrient availability also influences the growth of beneficial microorganisms like lactic acid bacteria in yogurt and cheese (Dash et al., 2022). In yogurt, a starter culture of lactic acid bacteria is added to lower the pH and inhibit harmful bacteria. In

cheese-making, the curd is salted and drained to lower the nutrient availability, making it less favourable for microbial growth (Hutkins, 2008; Kampen, 2014).

Microorganisms can derive energy from carbohydrates, fats and amino acids. Gram (+) bacteria are more fastidious in their nutritional requirements, while Gram (-) bacteria can derive their basic nutritional requirements from existing nutrients in food. The abundance of nutrients in most foods is sufficient to support the growth of a wide range of foodborne pathogens. Therefore, predicting pathogen growth or toxin production based on nutrient composition is difficult and impractical (Bonnet et al., 2020).

1.2 Typical food preservation treatments

The practice of applying treatments to extend the shelf life of foods is known as food preservation (Blackburn, 2006). This is often done by inactivating spoilage or pathogenic microorganisms, or modifying the physico-chemical attributes of the food to values that do not allow microbial growth. Food preservation has a long history, going back to the prehistoric era. Understanding how to preserve food was one of the most crucial steps in creating civilization because the possibility to store food for consumption during the winter enabled the development of stable human settlements. During the prehistoric era, the fundamental methods for food preservation were almost identical across cultures and locations (Eden, 1999). They also remained unchanged for centuries, to the point that it is often said that a Roman farmer would have been perfectly able to work in a 16th century English farm without any additional training.

Preservation treatments can be broadly classified into two main categories: those that prevent the growth of microorganisms and those that inactivate microorganisms. There is an

amalgamate of strategies and technologies that can be applied to either completely avoid, delay, or otherwise reduce food spoilage. All across the world, conventional food preservation methods like pasteurization, drying, freezing, chilling and chemical preservation are widely used. These techniques have evolved through time, especially since the 19th century (Shajil et al., 2018).

Treatments that prevent the growth of microorganisms, such as refrigeration, freezing and drying, are designed to create an environment that stops microbial proliferation. By reducing the temperature or water activity of the food, these treatments can slow down or stop the growth of microorganisms (Amit et al., 2017; Erkmen & Bozoglu, 2016).

Treatments that cause the inactivation of microorganisms are designed to kill or inactivate microorganisms that are already present in the food. Pasteurization consists of a treatment that is designed to inactivate most vegetative cells (active, growing cells) in food products. Sterilization, on the other hand, is a heat treatment that is designed to kill both vegetative cells and spores (dormant, highly resistant cells) that may be present in the food in order to avoid foodborne disease or spoilage during the shelf-life of the product (Chiozzi et al., 2022). Nevertheless, prions, heat-resistant viruses and thermophilic spores are all microorganisms that are known to be highly resistant to many of the commonly used methods of sterilization, such as heat or chemical disinfection (Ramesh, 2003).

Toxins in food can pose a serious health risk to humans if consumed in sufficient quantities. A possible route of contamination is the presence in the food of certain microorganisms able to produce toxins during product storage. Common types of food toxins include mycotoxins produced by moulds, bacterial toxins such as botulinum toxin and marine toxins such as ciguatoxins found in some types of fish (Burris & Stewart, 2012).

Preservation treatments can be effective in preventing the growth of toxin-producing microorganisms and reducing the risk. By creating an environment that does not allow microbial growth, preservation treatments such as refrigeration and drying can help to prevent or minimize the production of certain toxins in food products. Alternatively, pasteurization or sterilization treatments can kill or inactivate toxin-producing microorganisms, thereby reducing the risk of toxins being present in food at the moment of consumption. It is important to note, however, that most of these toxins are stable at high temperatures, so they cannot be inactivated even during sterilization treatments. Therefore, it is also essential to implement good manufacturing practices and proper storage and handling procedures to prevent contamination of food products with toxins (Fletcher & Netzel, 2020; Juneja & Sofos, 2009; Lattanzio, 2020).

There is currently a large variety of preservation strategies of technologies available. They are chosen depending on the food characteristics, the target microorganisms and the desired shelf life. This thesis studies two traditional strategies (heat and acidification) that, despite the emergence of novel methods during the last years, remain broadly used by food industries for many food products.

1.2.1 Application of heat for bacterial inactivation

Food preservation techniques have relied heavily on heat in diverse forms and levels to inactivate pathogenic and spoilage microorganisms. The first methods for preserving food date back thousands of years. Even if many of these procedures have evolved, their core principles remain unchanged and are still essential to our daily lives. The 1800s saw the popularization of pasteurization and canning, two significant procedures, that played a crucial role in the

development of food processing by enhancing food safety and accessibility (Tadini & Gut, 2022).

A French cook, named Nicolas Appert (1749-1841) began experimenting with heat, glass bottles, cork and wax to preserve food around 1810. La Maison Appert (The House of Appert) was the world's first food-bottling facility (Graham, 1981). Other innovators and businessmen expanded on this technology to create the tin can. With the start of World War I and the high demand for cheap, long-lasting, transportable food for soldiers, the tin can would become essential for nourishing soldiers in trenches for long periods of time (Featherstone, 2012). A second major historical advancement in food processing was the one by Louis Pasteur (1822-1895), a French microbiologist who developed the process of pasteurization in the 1860s. This procedure revolutionized the production of liquid products, particularly for milk, which is a staple of most diets and very sensitive to bacterial growth. Pasteurization inactivates germs by the application of heat to the food. Without this process, the history of food processing would have progressed much slower. Long-term food storage and transportation would have been highly limited around the world (Goldblith, 1971).

Thermal processing for commercial sterilization aims to apply enough heat to inactivate all potentially harmful bacteria that might be present in a specific food. Although this is the ultimate goal, a more accurate description is to lower the likelihood of survival and/or growth of microbes in a certain food to an acceptable level (Lund et al., 2000). Depending on their temperature and duration, heat treatments can result in either sterilization or pasteurization, with 100ºC often considered as a threshold temperature. Although sterilization results in increased safety, it can also lead to nutrient loss, changes in the texture, colour and food taste. The detrimental impact on quality depends on the type of product (Knorr & Augustin, 2021;

Ramesh, 2003). For example, some foods such as fruits, vegetables and some dairy products are too delicate to withstand the high heat and long processing times required for sterilization. In addition, some foods may develop an unpleasant taste or texture when subjected to heat, making sterilization an unsuitable preservation method (Rodrigo et al., 2016). Furthermore, sterilization can be more expensive and have a greater environmental impact than pasteurization because higher temperatures and longer processing times require more energy so sustainability can become an issue.

1.2.2 Acidification as a method of bacterial control

The ability of microbes to adapt to their immediate environmental conditions determines their ability to survive and reproduce. The local concentration of protons (hydrogen ions, H+), often expressed as the pH, is one of the most important environmental characteristics affecting bacterial growth and survival. In an acidic environment with high proton concentration, microorganisms may encounter several challenges that can impact their survival and growth. These challenges include denaturation of proteins, alteration of cell membrane properties, inhibition of metabolic pathways and disruption of DNA structure (Lund et al., 2020).

Acidity or basicity of an environment can significantly affect the growth and survival of organisms, as pH changes can alter the structure and properties of macromolecules. The optimum growth pH is the value that is best suited for the growth and metabolism of a particular organism. It can be considered as the pH range where the organism can maximize its growth and overall productivity. The minimum growth pH, on the other hand, is the lowest pH that an organism can tolerate and still grow, whereas the maximum growth pH is the maximum value enabling growth. It is worth noting that the optimum, minimum and maximum growth pH values are specific to each organism and are influenced by genetic and physiological factors,

as well as interactions with other environmental conditions (Jay et al., 2005). Besides affecting their ability to grow, changes in pH can also impact the activity of enzymes and other proteins, alter the structure and permeability of cell membranes and affect the stability and conformation of DNA and RNA. Therefore, understanding the effects of pH on microbial growth and metabolism is essential for optimizing processes that rely on microbial activity, such as bioremediation, bioprocessing and fermentation (Singh & Heldman, 2014).

In the context of food safety, bacterial survival often refers to the ability of microorganisms to remain viable and maintain their metabolic functions, including respiration and reproduction, in a given pH range. In acidic conditions, some microorganisms may have the ability to survive and maintain their viability, despite not being able to grow (Padan et al., 2005). Also in the context of food safety, inactivation refers to the loss of viability and reproductive ability of microorganisms due to exposure to pH conditions outside their tolerable range. In acidic or basic environments, microorganisms may exhibit different survival or inactivation responses depending on the species and their ability to adapt to changing pH conditions (Jin & Kirk, 2018). Growth, on the other hand, refers to the ability of microorganisms to actively multiply and increase in number under specific pH conditions. In acidic environments, only acid-tolerant microorganisms that have adapted to these conditions can grow and thrive. Acidic conditions can also prevent the growth of many pathogenic bacteria, such as *Salmonella* and *E. coli*, which prefer neutral pH conditions (Padan et al., 2005). Therefore, understanding the survival, growth, adaptation and inactivation of microorganisms based on pH conditions is important for designing effective food preservation strategies, developing microbial control measures and optimizing industrial processes that rely on microbial activity (Leistner & Gould, 2002).

Some products, such as fruits, fruit juices and dairy products, are naturally acidic (or become acidic after microbial fermentation) due to the presence of organic acids such as citric acid, malic acid, or lactic acid. These organic acids can create an environment that is favourable for the growth of certain microorganisms, while inhibiting the growth of others (Tamang et al., 2016; Tribst et al., 2009). For example, lactic acid bacteria (LAB) is a group of microorganisms that are commonly found in dairy products such as yogurt, cheese and fermented milk. These bacteria are able to grow and thrive creating acidic environments and they are responsible for the production of lactic acid during fermentation. The lactic acid produced by these bacteria lowers the pH of the product, which inhibits the growth of other types of bacteria that are not able to tolerate acidic environments (Zapaśnik et al., 2022).

The pH of food products can also be intentionally reduced to extend its shelf life. Fermentation, a process that naturally increases acidity, as well as acidification via direct addition of organic and/or suitable inorganic acids, have long been utilized for food preservation (Danyluk et al., 2012). Using acids to reduce the pH of foods can act as preservatives in acidification operations because they enhance the food's microbiological stability. In general, it is not possible to preserve all foods by adding enough acid to prevent the growth of bacteria, because most meals would be too acidic for consumption. Nevertheless, a sufficient amount of acid may be used to limit the growth of microbes, especially if it is paired with another method of preservation (e.g. drying and/or addition of salt or sugar) (Lund et al., 2000).

In this sense, the hurdle approach is a food preservation strategy that involves combining multiple, mild preservation methods, or "hurdles," to prevent the growth of microorganisms and extend the shelf life of food products. Each hurdle is chosen to target a specific aspect

affecting microbial growth, such as pH, water activity, temperature, or antimicrobial agents (Leistner, 1995; Leistner & Gould, 2002). The hurdle approach is based on the principle that the combination of multiple, mild preservation techniques can be more effective than a single, more severe technique. By using multiple hurdles, the aim is to create an environment that is inhospitable to microorganisms and prevent them from proliferating or even surviving in the product. This approach can also help to reduce the use of more aggressive preservatives or thermal processing methods that can have negative impacts on food quality, taste and nutritional value (Mahmoud et al., 2022).

1.3 Typical microbial responses in the food supply chain

Through the food supply chain, microorganisms are exposed to a variety of stressors that challenge their ability to develop or their ability to survive. Examples of these environmental changes include nutrient deficiency, temperature, pH, osmolarity variations, radiation exposure and the presence of other hazardous substances including high levels of superoxides and heavy metals. Microorganisms use a remarkable variety of transcriptional regulatory circuits to monitor and convert extracellular stimuli into cellular signals, resulting in changed gene expression and protein activities, as a means of responding to and adapting to harmful environmental changes (Gao et al., 2011). The production of cysts and spores, alterations in cellular membranes, expression of repair enzymes for damage, creation of chemicals for stress reduction and other techniques are only a few of the ways that bacteria deal with stressful situations (Haruta & Kanno, 2015).

1.3.1 Sublethal injury and VBNC state

Microorganisms can survive in a variety of metabolic states and development phases depending on their environment and stressors, yet not all of these states include active cell

reproduction (Arvaniti & Skandamis, 2022; Davis, 2014). Although the lack of growth is not always a reliable indicator of the absence of microbial life, viability is typically defined as the presence of replication in culture media (Espina et al., 2016).

The presence or absence of growth in the culture media allows for multiple interpretations of the data. Colonies on a growing medium indicate that at least one viable cell was able to multiply. Additionally, it is possible for many viable cells to coexist in the same location and form a single colony, which could result in an underestimation of viable cells (Schottroff et al., 2018). The absence of colony growth on culture medium indicates that the sample contains no viable cells. However, another interpretation is the employment of the sub-optimal growth medium and conditions, i.e. temperature and time that, in the presence of injured or stressed cells are unable to form colonies. However, these cells may still be viable (Davey, 2011). In response to unfavourable environment conditions (heating, drying, setting under high osmotic pressure, contact with inhibiting chemicals), many bacteria have developed the unique survival strategy known as the viable but non-culturable (VBNC) state.

The VBNC population is a state of microbial dormancy that is characterized by a reduction in metabolic activity and the inability to grow on standard laboratory culture media, even though the microorganisms are still viable and capable of resuming growth and metabolism under appropriate conditions (Oliver, 2010). It is considered to be a survival strategy that microorganisms employ in response to environmental stresses, such as changes in temperature, pH, nutrient availability, or exposure to antimicrobial agents (Fakruddin et al., 2013). Microorganisms may enter the VBNC state in order to avoid death and they can remain in it for extended periods of time, potentially for months or even years. During the VBNC state, the microorganisms undergo significant physiological and morphological changes. These changes

may include alterations in cell membrane composition, decreased DNA replication and protein synthesis. As a result, the microorganisms become metabolically inactive and are unable to divide and form colonies on agar media (Ramamurthy et al., 2014; Zhao et al., 2017).

Despite their inability to grow on standard laboratory culture media, VBNC cells are still alive and can resuscitate under favourable conditions. Resuscitation can occur when the VBNC cells are exposed to conditions that promote their metabolism and growth. For example, VBNC cells may be resuscitated by changes in temperature, pH, nutrient availability, or exposure to certain chemicals or compounds that induce metabolic activity (Ramamurthy et al., 2014). Pathogenic bacteria in the VBNC state are seen as a potential risk to public health and food safety because conventional testing methods for food and water are unable to detect them (Ayrapetyan & Oliver, 2016).

1.3.2 Stress acclimation of bacterial cells

Throughout the food supply chain, foodborne bacterial pathogens are exposed to numerous stressors, particularly during food production, processing and cooking. Physical stresses include heat, pressure, or osmotic shock; chemical stresses include acids or detergents; and biological stresses include bacteriocins or other competitive strategies (Begley & Hill, 2015). Stress can cause bacterial cell injury, as well as damage to other cellular components including the cell wall, cell membrane, proteins, RNA and DNA (Wesche et al., 2009). The type of damage and its extent are influenced by both the nature and the severity of the stress.

Researchers have demonstrated that foodborne bacteria have evolved mechanisms to detect and respond to changing environmental conditions. One such mechanism is the regulation of gene expression, which allows bacteria to produce specific proteins in response to different environmental conditions (Helmann, 2002; Sourjik & Wingreen, 2012; Ulrich et al., 2005).

These stress response mechanisms may result in the production of proteins that repair damage, maintain cell homeostasis, or help the stress agent be removed. The adaptive or protective response is based on the idea that pre-exposure to mild (sublethal) levels of a particular stress protects the organism from subsequent harsh (usually lethal) levels of the same type of stress (Wiktorczyk-Kapischke et al., 2021).

The ability of bacterial cells to respond to environmental stressors increasing their possibility to survive inactivation treatments is of high relevance for food safety. This process is often called "stress acclimation" in this context, to separate it from stress adaptation (which often refers to long-term effects due to selective pressures). Stress acclimation has been broadly observed experimentally; for instance when the application of a pre-treatment (e.g., an acid or a heat shock) increased the resistance of the bacterial population to posterior treatments (Battesti et al., 2011). Interestingly, stress acclimation is not limited to a particular stressors. For instance, it is common for acid-shocked cells to have increased heat resistance (Clemente et al., 2021), a phenomenon often called "cross-resistances".

During the last years, the understanding of stress acclimation has been furthered through the study of this phenomenon during dynamic thermal treatments. Experimental studies have shown that stress acclimation may take place during the heating phase if the heating rate is low, increasing the resistance of the bacterial population to later (lethal) parts of the treatment (Hassani et al., 2006a; Valdramidis et al., 2006). Although initial studies only achieved a qualitative description, the development of microbial inactivation models that account for stress acclimation has enabled the comparison between different conditions aiding in the understanding of this phenomenon (Clemente et al., 2020; Garre et al., 2018c).

Despite the fact that the study of stress acclimation remains mostly empirical, it has been hypothesized that it can be related to the expression of bacterial stress response mechanisms. In particular, in bacteria, sigma factors are playing a crucial role in the regulation of gene expression by binding to RNA polymerase and directing it to specific promoters on the DNA molecule. The most relevant sigma factors responsible for recognizing different promoter sequences and regulating the transcription of specific sets of genes are often species dependent (Aertsen & Michiels, 2004). SigB is a specific sigma factor that is found in many Gram-positive bacteria, including *Staphylococcus aureus* and *Bacillus subtilis*. It is known to play a key role in the stress response and adaptation of these bacteria to a variety of environmental stressors, including acid stress, heat shock and oxidative stress (Piggot & Hilbert, 2004). Under conditions of stress, SigB is activated and triggers the transcription of a set of stress response genes, many of which are involved in protecting the cell from damage and promoting survival. For example, in acid stress conditions, SigB is activated in response to the low pH environment and directs the expression of genes involved in the maintenance of membrane integrity, the repair of damaged proteins and the production of compatible solutes to help the cell maintain osmotic balance (Andersson, 2016; Kazmierczak et al., 2005; Marmion et al., 2022; Mathis & Ackermann, 2016). Overall, the SigB is an important component of the bacterial stress response and plays a critical role in the adaptation and survival of bacteria in a range of different stress conditions (Marles-Wright & Lewis, 2007). Hence, it is reasonable to consider that its regulation would be closely related to the development of stress acclimation.

1.3.3 Heat shock response

When microorganisms are exposed to heat, their cellular structures and functions are affected in various ways. One of the first structures to be affected is the cell membrane, which becomes less permeable as the temperature increases (Alvarez-Ordóñez et al., 2009a). This

rigidity of the membrane makes it difficult for the microorganism to regulate its internal environment, leading to cellular stress, a loss of nutrients and an increase in waste accumulation (Álvarez-Ordóñez et al., 2009b).

Temperature has a profound impact on the functionality of enzymes, which are responsible for catalyzing biochemical reactions in cells. Beyond their optimal range, enzymes start to decline in activity and may eventually denature, leading to the permanent loss of function. The resulting impact on the microorganism's ability to perform essential metabolic processes can lead to inactivation or death (Smelt & Brul, 2014).

Protein denaturation is another key factor in thermal inactivation. Proteins are critical to many cellular functions and their three-dimensional structure is important to their activity. As the temperature increases, the thermal energy disrupts the weak interactions that hold the protein in its folded shape, leading to denaturation. This causes the protein to lose its activity and potentially form aggregates, which can be harmful to the cell. Denatured proteins can also interact with other cellular components, leading to further damage (Setlow & Setlow, 1998).

The heat-shock response (HSR) is an important protective mechanism for bacterial survival and adaptation to harsh environmental conditions. This response appears to be ubiquitous, as it has been detected in every bacterial species studied so far. It consists of a collection of well-coordinated responses and activities, largely involving the highly regulated creation of heat-shock proteins (HSPs). These, in turn, mostly consist of molecular chaperones and proteases, the intracellular abundance of which increases fast in response to a range of environmental stresses (Roncarati & Scarlato, 2017).

In order to counteract the detrimental effects on proteins brought on by stressors like high temperatures, oxidative stress and heavy metals, cells can respond to stress by producing more molecular chaperones. This process is known as the heat shock response (Morimoto, 1993). Because proteins are the primary functional components of a living cell, proteostasis (protein homeostasis) must be preserved (Balchin et al., 2016). In order to carry out their biological tasks, many proteins adopt a predetermined shape in a procedure known as protein folding. Critical functions may be impacted by the alteration of these structures, which could result in cell damage or death (Richter et al., 2010a). Under stress, HSPs can be expressed through the HSR, assist prevent or reverse protein misfolding and create an environment for correct folding (Weibezahn et al., 2005).

Chaperones are a diverse group of proteins that play a crucial role in maintaining protein homeostasis within the cell. One of their primary functions is to assist in the proper folding of nascent polypeptides, ensuring that they reach their native conformation and remain soluble in the cellular environment. Chaperones also help to prevent protein misfolding and aggregation by binding to exposed hydrophobic regions of proteins and stabilizing them until they can refold properly. Additionally, chaperones are involved in the degradation of damaged or misfolded proteins through interactions with the ubiquitin-proteasome system. The expression and activity of chaperones are tightly regulated by cellular stress, including the heat shock response (Richter et al., 2010a).

Chaperones are classified into numerous families, each with its own set of responsibilities (Beissinger & Buchner, 1998). Based on their measured molecular weights, heat shock protein chaperones are divided into Hsp60, Hsp70, Hsp90, Hsp104 and tiny Hsps (Bascos & Landry, 2019). Protein folding is difficult when environmental stresses denature proteins, causing even

more non-native folding to happen (Fink, 1999). If molecular chaperones are unable to prevent improper protein folding, the protein may be destroyed by the proteasome or autophagy to remove potentially hazardous aggregates (Cuervo & Wong, 2014). If left uncontrolled, misfolded proteins can aggregate, preventing the protein from adopting the right shape and eventually leading to a range of cellular malfunctions, including apoptosis, cell death and the development of various diseases (Tower, 2009).

While chaperones are the most well-known and studied molecules involved in the HSR, there are many other proteins and pathways that are also involved in this complex and highly coordinated response to stress. Heat shock factor (HSF) is a key player in the HSR and is responsible for activating the transcription of genes encoding HSPs. The regulation of HSF activity is a complex and highly coordinated process that involves several isoforms of HSF, each responding to different types of stressors (Chen et al., 2000). Co-chaperones are another group of molecules that play a role in the HSR. These proteins interact with HSPs and help to regulate their activity. There are many different co-chaperones that have been identified, each with a unique effect on HSP activity and specificity (Grallert & Buchner, 2001; Horwich et al., 2006). Finally, there are several other proteins that are involved in protein folding and degradation and are upregulated in response to stress. These include proteins involved in the ubiquitin-proteasome system, which is responsible for degrading damaged or misfolded proteins, as well as other chaperones and folding enzymes (Muratani & Tansey, 2003; Nandi et al., 2006).

1.3.4 Acid shock response

Microbes are exposed to acidic conditions in a variety of environments, including some soil types, acidic lakes and mines, geothermal sites and decomposing organic matter. They are

also exposed to acidic environments in a number of niches that are associated with humans, such as acidic food products, industrial fermentations, waste-treatment facilities, the gastrointestinal tract and other anatomical sites during infections (Lund et al., 2020). Microbes have developed a variety of tolerance or resistance mechanisms to survive at low pH, often included within the umbrella terms acid tolerance responses (ATRs) or acid resistance mechanisms (ARs) (Spector & Kenyon, 2012a).

ATRs include an amalgam of responses whose main goal is to avoid a dangerous drop in intracellular pH below a critical level. In general, three different tactics are utilized to stop such a crucial pH decline (Krulwich et al., 2011; Lund et al., 2014). First, protons are frequently consumed in enzyme-catalyzed processes in cells (decarboxylation). Second, to help counteract the low pH, cells can employ processes that result in basic compounds (e.g. the production of ammonia) (Pennacchietti et al., 2018). Third, many different types of microbial cells expel protons while utilizing Adenosine Triphosphate (ATP); e.g., protons can be effluxed from some bacteria using the F1Fo-ATPase (Krulwich et al., 2011; Russell, 2007). In addition to these short-term pH-maintenance mechanisms, cells have evolved defence mechanisms that provide a long-term resistance to acidic conditions. This includes the modification of the lipid composition of the cytoplasmic membrane to decrease the permeability to protons. Cyclopropane fatty acids are generated at higher rates in some bacteria under acid stress and they operate as a defence against acid pH by reducing membrane permeability to protons (Shabala & Ross, 2008).

The type and intensity of the stressor affect the stress response. For instance, low oxidative stress promotes cell proliferation, but moderate oxidative stress changes the physiology of the cell to boost its defences, making it more resilient to further assaults. The activation of

transcription factors and induced cell cycle arrest to enable DNA damage repair are two of the mechanisms of stress responses. Significant oxidative damage and cell injury, senescence and/or death are caused by extreme oxidative stress (Halliwell, 2000; Portt et al., 2011).

The effectiveness of the acid shock response in microorganisms depends on several factors, including the magnitude and duration of the pH shift, the type of acid stress (e.g., acute or chronic) and the specific mechanisms involved. Generally, the acid shock response is most effective in environments with a pH range between 4.5 and 5.5, as this is the range where most acid-tolerant bacteria can survive and grow. However, at extremely low pH values (below 3.0), it may not be effective, as the acid stress can be too severe for the bacteria to adapt quickly enough to survive. Additionally, other stress factors, such as high temperature or the presence of toxic compounds, can also affect the ability of microorganisms to respond to acid stress (Foster, 1995; Richard & Foster, 2003).

Combining stresses in food preservation (hurdle concept) is a common approach for ensuring food safety and quality (Leistner, 2000). However, the effects of cross-stresses on microbial survival and growth are not well understood and require experimental investigation. In some cases, the combined stresses can have a synergistic effect, resulting in higher microbial death rates than if they were applied separately (de Oliveira et al., 2017; Nguyen Huu et al., 2021). For example, the combined effect of heat and pressure has been shown to increase the lethality of the process and reduce the heat resistance of *B. anthracis* spores (Cléry-Barraud et al., 2004).

On the other hand, cross-adaptation can also occur when microorganisms are exposed to multiple stresses. In some cases, microorganisms may develop resistance to one stress in response to another stress. This can lead to decreased microbial death rates and pose a challenge

for food safety risk analysis. Studies have shown that microorganisms can adapt to heat and low pH conditions, which can lead to increased resistance to subsequent heat treatment and other stresses. For example, Leyer (1993) has found that *Salmonella* serovar Typhimurium can develop increased resistance to heat and salt after exposure to acidic conditions. This adaptation may be linked to the expression of certain acid shock proteins, which are also stimulated by heat shock. Specifically, about half of the acid shock proteins induced following exposure to acidic conditions have been found to be similarly induced by heat shock (Foster, 1995).

1.3.5 The link between genetic and phenotypic cell heterogeneities and population variability for risk assessment

Genetic differences between strains refer to variations in the genetic material, such as DNA, between different strains of the same microbial species (Rossum et al., 2020). These differences may arise due to several mechanisms such as mutations, genetic recombination, or genetic drift (Li et al., 2009). Such variations can result in changes in the physical and biochemical characteristics of the organism, such as differences in virulence, growth rate, or metabolic capabilities. These genetic differences can also affect the susceptibility of an organism to antimicrobial agents (Dijkshoorn et al., 2000).

On the other hand, differences between cells of the same strain refer to genetic or epigenetic variations between individual cells within a population of the same microbial strain. These differences may arise due to various factors, such as random mutations, gene expression changes, or environmental influences (Li et al., 2009). These differences can result in phenotypic diversity among cells in terms of their behaviour, morphology, or response to stimuli. For example, differences in gene expression patterns can lead to differences in the

production of certain enzymes or virulence factors, which can affect the ability of the organism to cause disease (Martin et al., 2022).

Individual microbial cells present differences in their genome that can make them respond differently to the same environmental conditions. This is known as microbial heterogeneity and it is a key factor in many biological processes (Levin, 2013). Microbial heterogeneity can arise from different levels. The first one is genetic variability within a population of cells. Genetic differences between cells can arise due to variations in the DNA sequence, such as mutations or genetic polymorphisms. These genetic differences can lead to differences in gene expression, protein function and cellular behaviour (Elowitz et al., 2002). For example, some cells may carry mutations that confer resistance to certain drugs or environmental stresses, while others may be more susceptible to these factors. Even within a clonal population of cells, mutations can arise that result in genetic differences between individual cells. In the context of microbial communities, genetic variability can contribute to microbial heterogeneity. For example, different strains or species of bacteria may have different genetic adaptations that allow them to occupy different niches within a given environment. These adaptations can include the ability to metabolize different nutrients, tolerate different environmental stresses, or interact with other microorganisms in different ways (Van den Bergh et al., 2018).

Phenotypic variability, another source of microbial heterogeneity, refers to the natural variability that exists in the behaviour and function of individual biological cells. This variability can be due to many factors, including epigenetic modifications, environmental factors and stochastic processes that occur within cells. At the molecular level, phenotypic variability can manifest as differences in gene expression, protein levels, or signalling pathways between individual cells (Ackermann, 2015). For example, a cell population may

contain some cells that are actively dividing while others are in a quiescent state, or some cells may be more sensitive to certain signalling molecules than others. Furthermore, these differences can lead to variations in cellular behaviour, metabolism and response to stimuli (Balaban et al., 2004). Even genetically identical microorganisms can display significant phenotypic variability. For example, some cells within a population may express different sets of genes, leading to differences in metabolism, stress response, or pathogenicity (Dhar & McKinney, 2007).

These heterogeneities in the response of individual cells to stress are reflected at the population level as "variability". The European Food Safety Authority (EFSA), and other regulatory agencies, have highlighted during the last years that a truthful representation of variability is essential for a robust risk assessment of foodborne hazards (Benford et al., 2018). In this context, it is also important to make a difference between variability and uncertainty. Variability is the reflection of these cell heterogeneities (regardless of their source), so it is an inherent part of the microbial response (Aspridou & Koutsoumanis, 2020). Furthermore, variability also includes the variation in other biotic and abiotic factors of the food chain that are inherently variable, such as differences in storage conditions between refrigerators or differences in disease susceptibility between individuals (den Besten et al., 2018b). On the other hand, uncertainty arises when there is a lack of understanding or incomplete information about the underlying system or when there is variability in the data or measurements. Uncertainty, unlike variability, is not an inherent part of the system and can potentially be reduced by gathering more and/or better information (Nauta, 2000).

The relevance of variability and uncertainty for microbiological risk assessment is partly due to the extremely low risk of most food products, which is often in the range of one disease

per billion servings (Rocourt et al., 2003). Despite these low risk levels, these illnesses are still relevant due to the large consumption of these items (Zwietering et al., 2021). Hence, the tails of the distribution, become the defining part of the outcome of a risk assessment model. Because the tails are defined by variability and uncertainty, quantifying them (while separating their contribution) is essential for the development of effective strategies for preventing the spread of foodborne diseases and controlling the growth of harmful microorganisms in industrial and environmental settings. This knowledge can also help in the optimization of current methods for food preservation, microbial control and biotechnological applications (Allison & Martiny, 2008).

1.4 Food microbiology and mathematical modelling

Food microbiology is the study of microbes that may colonize food, either intentionally added as part of food processing, or spoilers and pathogenic microorganisms that are undesirable in foods because they will reduce its shelf life. It is composed of the study of a wide range of microorganisms such as spoilage, probiotic, fermentative and pathogenic bacteria, molds, yeasts, viruses, prions and parasites. It deals with foods and beverages of various compositions, incorporating a wide range of environmental elements that may influence microbial survival and growth (Laranjo et al., 2019).

1.4.1 Predictive microbiology

Mathematical modelling is the process of developing a mathematical representation of a real-world scenario in order to make predictions or gain insight. Applying a formula and really creating a mathematical relationship are two different things (Langemann et al., 2018). Different types of mathematical models are nowadays routinely used to describe the behaviour of relevant microorganisms in food (pathogens or spoilers) during and after food processing

(Clemente et al., 2020; Garre et al., 2018c; Kapetanakou et al., 2019; Tesson et al., 2020; Valdramidis et al., 2008; van Boekel, 2002; Vega et al., 2016).

Predictive microbiology in foods is a subfield of food microbiology that develops mathematical models to anticipate microbial behaviour within the food supply chain (Baranyi & Roberts, 1995). As a common mathematical analysis, predictive microbiology can be used to predict the survival behaviours of microorganisms and mathematically model reproducible behaviour under certain environmental conditions, thereby contributing to the acquisition of information regarding bacterial responses such as survival (Bai et al., 2021; Whiting, 1995). Predictive microbiology uses mathematical models with a substantial empirical component, including some model parameters that must be determined from experimental data. Nonetheless, these parameters often have a biological interpretation (e.g., the growth rate), so the analysis of the parameter estimates often provides direct information on the microbial response.

The initial developments in the field date back to the 1920s, when heat resistance of microorganisms was explained using either the Arrhenius (1889) equation or the Bigelow model (1921). Nonetheless, most of the discipline's ideas and methods were developed considerably later, around the beginning of the 1990s, followed by the construction and description of microbiological models, as well as the generation of necessary databases and other software tools (Valdramidis, 2016). The development of predictive microbiology as a field was largely dependent on advances in computer technology. In the early days of microbiology, scientists relied on manual methods for model development and application, which limited their ability to accurately predict microbial behaviour. The advent of computers and the ability to process large amounts of data quickly allowed for the development of more

complex mathematical models and simulations, which were necessary for predictive microbiology (McMeekin & Ross, 2002).

Predictive models that depict microbial behaviour in foods are useful for food industries and regulatory agencies, as well as for education. Such models can be used by industry to aid in product and process design (Gkogka et al., 2013). Food industries can also use predictive models to analyze and simulate the behaviour of microorganisms in different food matrices, processing conditions and storage conditions. This information can be used to establish microbiological criteria for specific food products and to develop risk-based approaches to food safety management. Food safety authorities can use predictive microbiology models to estimate the likelihood and severity of foodborne illness caused by specific microorganisms and to identify the critical control points (CCPs) in the food production process where interventions can be implemented to reduce the risk of illness (Stavropoulou & Bezirtzoglou, 2019). This makes predictive microbiology a cornerstone of current methods for microbial risk assessment in foods (Messens et al., 2018).

1.4.2 Quantitative Microbial Risk Assessment (QMRA)

Risk analysis is the process of identifying, assessing and mitigating risks in a variety of contexts, including public health, food safety and environmental management. Overall, risk analysis for biological hazards is a complex and interdisciplinary process that involves drawing on knowledge from fields such as microbiology, epidemiology, toxicology and environmental science. It is an important tool for identifying and managing potential risks associated with exposure to biological agents and plays a critical role in protecting public health and safety (Stohlgren, 2006). The principles of risk analysis for food safety studies were defined by the

Codex Comission of the FAO as a structured process that consists of three main components: microbial risk assessment, risk management and risk communication.

Risk management involves the development and implementation of strategies to mitigate or control risks identified through risk assessment. This process can be divided into four stages: hazard control, risk reduction, risk transfer and risk avoidance. Hazard control involves implementing measures to reduce or eliminate the hazard. Risk reduction involves implementing measures to reduce the likelihood or severity of harm. Risk transfer involves transferring the risk to another party, such as through insurance or contractual agreements. Finally, risk avoidance involves avoiding the hazard or activity altogether (FAO, 2017).

Risk communication is the process of sharing information about risks with stakeholders, including the public, regulators and industry. This process can be divided into four stages: identifying the target audience for the communication, developing clear and accurate messages about the risk, selecting the most effective channels for communicating the risk to the target audience and receiving and responding to feedback from the target audience (Attrey, 2017).

The work presented in this thesis is focused on Microbial Risk Assessment (MRA). It involves identifying, evaluating and quantifying risks associated with a particular hazard or activity. It is a scientific evaluation that seeks to provide an estimate of a risk by taking into account the likelihood and severity of health consequences induced by a hazard in order to facilitate decision-making processes. MRA can be divided into four stages: hazard identification, exposure assessment, hazard characterization and risk characterization (FAO & WHO, 2021).

Hazard identification determines the pathogens of concern and the human health outcomes. In most cases, the health outcomes examined are infection or acute illness. The distinction between the two is operational: infection is defined as the presence of a pathogen in the human body, whereas disease is defined by specific symptoms (nausea, vomiting, etc.). **Exposure assessment** determines the exposure channels (air, food, etc.) and quantifies (using models) pathogen exposure dosages during defined exposure events. **Hazard characterization** describes the adverse effects that may occur as a result of ingesting a hazard, whether it be a bacterium or its toxin. Where possible, the hazard characterization should provide an assessment of the likelihood of causing an adverse health outcome as a function of dose for the population of interest. Ideally, this would take the form of a dose-response relationship. **Risk characterization** calculates the likelihood of the adverse health outcome (a.k.a., "risk") defined in hazard identification using the exposure doses from exposure assessment and the dose-response relationship from Hazard characterization.

The estimation of the probability of microbial risk can be qualitative or quantitative, based on the data and methods used. Quantitative microbial risk assessment (QMRA) is a method for quantitatively expressing risk in terms of infection, sickness, or fatality from microbial pathogens (Gerba, 2015). QMRA attempts to provide the most state-of-the-art information available to help people understand the nature of the potential effects of microbial exposure (Haas, 2014). Quantitative risk assessments are better suited for instances in which mathematical models, such as dose-response models, are available to describe phenomena and data are available to estimate model parameters. QMRA models are most often implemented as Monte Carlo simulations to account for variability and uncertainty in projected health risks. Monte Carlo simulations also enable sensitivity analysis, which can uncover risk factors

(environmental factors, exposure variables and so on) that have the greatest influence on risk (FAO & WHO, 2019, 2021) .

A qualitative MRA can give an initial assessment of the potential risk of foodborne pathogens, indicating the need for further analysis. This approach evaluates the likelihood and consequences of microbial hazards using expert judgment and existing data sources (FDA, 2010). Qualitative risk assessment may be suitable when data is limited, or when a hazard's potential consequences are well-known but the likelihood of occurrence is uncertain. Quantitative risk assessment, on the other hand, provides more precise risk estimates but requires more detailed data and expertise in mathematical modelling and statistics (Coleman & Marks, 1999). The choice between the two approaches depends on the specific needs and constraints of the risk assessment.

Both qualitative and quantitative risk assessments have advantages and disadvantages. Qualitative risk assessment is faster and less resource-intensive but may be subject to greater uncertainty and subjectivity in the evaluation of risks. Quantitative risk assessment provides more precise risk estimates but requires more detailed data and expertise in mathematical modelling and statistics. Thus, the selection of the appropriate approach for a food safety risk assessment must consider the available data, the level of uncertainty and the desired level of precision in the risk estimates (FAO & WHO, 2021).

1.4.3 Current challenges for the prediction of the microbial response to inactivation treatments

Mathematical models able to predict the reduction in the microbial concentration during the application of an inactivation treatment are nowadays a basic tool for the design and validation of food processing treatments. They are mathematical representations of the

inactivation process used to predict the outcome of various inactivation treatments, including heat, radiation and chemical treatments (Klotz et al., 2007). Despite their extensive use, the currently available models for microbial inactivation suffer from a number of drawbacks that compromise their accuracy and reliability for predicting the outcome of inactivation treatments (Garre et al., 2018a; Geeraerd et al., 2000). This is mainly due to the extreme complexity of the microbial inactivation process, making it impossible to define predictive models based on basic physical principles (Smelt & Brul, 2014). This PhD thesis advances current methodologies for modelling microbial inactivation focusing on three major limitations: the discrepancy between isothermal and dynamic experiments, the inclusion of variability in model predictions and the incorporation of the cell history in predictive models.

The first one is related to the type of experimental data used to build the model (Geeraerd et al., 2000). Most of the data currently available in the scientific literature was obtained using isothermal experiments, where the microorganisms are exposed to a constant temperature However, industrial processes are always dynamic (ingredients cannot be heated up instantly), meaning that temperature and other environmental factors change over time (Vicente & Machado, 2011).

In order to accurately model microbial inactivation under these conditions, it is important to use dynamic models that take these changes into account. By using dynamic models, it is possible to predict how microbial inactivation will occur under these changing conditions and to optimize treatment parameters to ensure effective inactivation (Garre et al., 2018c).

The availability of novel experimental methods has underlined the challenge of predicting microbial inactivation under industrial (dynamic) conditions using data gathered using isothermal experiments. This is due to some bacterial response mechanisms being relevant only

under dynamic conditions. An example is stress acclimation, which refers to an increase in bacterial resistance to heat under dynamic conditions with slow heating. This is due to the microorganisms dynamically adapting to the (sublethal) temperatures during the heating, in a similar way as observed after a heat shock (Hassani et al., 2006a; Valdramidis et al., 2006).

Classical predictive models are unable to account for stress acclimation reliably, as this process cannot be easily observed under isothermal conditions. Therefore, novel modelling approaches, based on the direct observation of the microbial response under dynamic conditions, are needed to more accurately describe inactivation under dynamic conditions (Garre et al., 2018b; Garre, González-Tejedor, et al., 2019).

A second limitation of current models of microbial inactivation is that they do not adequately account for variability. This is especially relevant for microbial risk assessment and can lead to erroneous predictions of inactivation kinetics and outcomes for various bacteria (den Besten et al., 2017). The failure of current microbial inactivation models to account for the combined effects of multiple sources of variability is a fundamental shortcoming. For instance, the susceptibility of a microorganism to inactivation may be affected by both the strain and the environmental conditions; models that do not account for the combined effects may not produce reliable predictions (Benford et al., 2018). In conclusion, the current models of microbial inactivation are limited in their ability to account for variability, which limits their accuracy and reliability in predicting the outcome of inactivation treatments.

A third limitation of current modelling methods addressed in this PhD thesis is the inclusion of the cell history on model predictions. It is well known that the physiology of bacterial cells has a degree of plasticity, adapting to the environmental conditions. Although these physiological changes are not well understood yet, they have proven their relevance for

microbial inactivation. For instance, the application of sub-lethal treatments will increase the chance of survival of bacterial cells to a posterior stress, even if the inactivating agent is of different nature (cross-resistance). Furthermore, the physiological state of the cell (exponential or stationary growth phase cells), the composition of the cultivation media or its water activity can also influence the heat resistance of bacterial cells (Aryani et al., 2015; Srisukchayakul et al., 2018; Tapia et al., 2020).

The impact of the cell history on microbial inactivation can be of high relevance for microbial risk assessment, as the food supply chain is likely to induce different types of adaptation. Microbial cells need to survive in food products or surfaces whose physicochemical attributes differ from the ones commonly used in laboratory protocols for cell cultivation (low pH, low water activity, presence of biocides, etc.). Considering the evidence for the impact of the cell history on the microbial response, it is feasible that the use of models based on laboratory media will introduce a relevant deviation in model predictions (den Besten et al., 2018b).

Finally, due to the numerous distinct microbial strains that have the potential to contaminate a good product prior to use for prediction, the microbial response is consequently described as a severe concern for microbial risk assessment (Hassani et al., 2006a). As a result, with the resources at hand today, risk assessment cannot be done for every conceivable strain. Instead, it is restricted to a few bacterial strains that have been found to be the most resilient and/or have the greatest potential for growth (Garre et al., 2018a).

1.5 Bacterial strains tested

1.5.1 *Salmonella* spp.

Salmonella is a genus of Gram-negative bacteria that are rod-shaped and belong to the family Enterobacteriaceae. *Salmonella* and *Escherichia coli* are thought to have developed from the same ancestor 160–180 million years ago, with some serovars of *Salmonella* adapting to mammals while others to reptiles (Mumy, 2014). *Salmonella enterica* and *Salmonella bongori* are the only two species of the genus, according to EFSA (EFSA & ECDC, 2022). Although there are only two known species of *Salmonella*, their importance should not be underestimated because *S. enterica* itself has six subspecies (*S. e. enterica, S. e. salamae, S. e. arizonae, S. e. diarizonae, S. e. houtenae, S. e. indica*) and over 2600 serovars (Gal-Mor et al., 2014; Ryan et al., 2017; L.-H. Su & Chiu, 2007). Based on surface antigens, the Kauffmann–White classification separates the genus *Salmonella* into serotypes (Grimont & Weill, 2007). It is given the names of Philip Bruce White and Fritz Kauffmann. Oligosaccharides coupled with lipopolysaccharide are utilised to define the "O" antigen type. The "H" antigen is then identified using flagellar proteins. Since Salmonella commonly displays phase fluctuation between two motile phenotypes, different “H” antigens may be expressed (Chattaway et al., 2021).

Salmonella species are non-spore-forming, primarily motile enterobacteria with peritrichous flagella and cell diameters between 0.7 and 1.5 μm (Khan, 2014). They are chemotrophs, drawing energy from organic sources through oxidation and reduction processes. Additionally, they are facultative anaerobes, able to produce Adenosine triphosphate (ATP) either anaerobically or aerobically depending on the available electron acceptors (Chen et al., 2021; Fàbrega & Vila, 2013).

Certain serotypes of *Salmonella* species are able to induce intracellular infections that cause disease. The majority of infections are caused by eating food contaminated with animal or human excrement, such as from a food-service worker in a restaurant (Jantsch et al., 2011).

Salmonella spp. can persist for a long time in food and other substrates and have fairly simple nutritional needs. It is affected by a variety of conditions, including temperature, pH, water activity and the presence of preservatives. The temperature range for growth is 5.2–46.2°C, with the optimal temperature being 35–43°C (ICMSF, 1996). Although freezing can be deleterious to the life of *Salmonella* spp., it does not guarantee the organism's inactivation/loss of viability. Due to the freezing damage, there is an initial rapid decline in the number of viable organisms at temperatures close to the freezing point. At lower temperatures, however, they are able to sustain long-term frozen storage (Hocking, 2003). A study by Strawn and Dayluk (2010) demonstrated that *Salmonella* can live on frozen mangoes and papayas for at least 180 days when stored at -20°C.

Heat resistance of *Salmonella* spp. in food is based on the 'composition, pH and water activity. 'Its tolerance to heat increases as the food's water activity declines. High-fat, low-moisture foods, such as peanut butter and chocolate, may have a protective effect against heat. *Salmonella* spp. Are less resistant to heat under conditions of low pH (Podolak et al., 2010; Shachar & Yaron, 2006). It can grow in a pH range from 3.8 to 9.5, with an optimal value of 7 to 7.5 (ICMSF, 1996). The lowest pH at which its species can grow depends on temperature, the concentration of salt and nitrite, and the type of acid present (Álvarez-Ordóñez et al., 2011). More bactericidal than organic acids such as lactic, citric and acetic acid are the volatile fatty acids. Outside the pH range for growth, cells may become inactive, however this is not

instantaneous and cells have been proven to persist viable for extended durations in acidic conditions (Bell & Kyriakides, 2001; Yousef & Abdelhamid, 2019).

The optimal water activity (a_w) for growth of *Salmonella* spp. is 0.99, while the minimum a_w is 0.93. It can survive for months or even years in low-a_w foods (such as black pepper, chocolate, peanut butter and gelatin) (Podolak et al., 2010). Its species Are susceptible to food preservatives in a manner similar to that of other Gram-negative bacteria, e.g. growth can be hindered by benzoic acid, sorbic acid, or propionic acid. *Salmonella* spp. can be inhibited more effectively by a combination of many preservation variables, such as the use of a preservative in conjunction with pH and lower temperature (Banerjee & Sarkar, 2004; Muhlig et al., 2014).

According to EFSA (2022) salmonellosis was the second most frequently reported foodborne gastrointestinal infection in humans, after campylobacteriosis. It is a significant source of foodborne outbreaks in EU MSs and non-MS nations. A total 60,050 confirmed cases of human salmonellosis were reported to the EU in 2021, which corresponds to a reporting rate of 15.7 per 100,000 population. This was a 14.3% rise compared to the incidence rate in 2020. The proportion of hospitalised patients was 38.1%, while the case fatality rate in the EU was 0.18 %, which was comparable to 2020. *S. enterica* ser. Enteritidis (54.6%), *S. enterica* ser. Typhimurium (11.4%), monophasic *S. enterica* ser. Typhimurium (8.8%), *S. enterica* ser. Infantis (2.0%) and *S. enterica* ser. Derby (0.93%) accounted for the majority of human illnesses caused by the different serovars. Regarding ready-to-eat food, over 73,238 sample units were tested, revealing a very low proportion of positive units (0.23%). The categories with the largest proportion of positives were pork and pork products (0.82%), followed by spices and herbs (0.7%). For non-ready-to-eat foods, 466,290 sampling units were collected and the percentage of positive samples was low (2.1%). The food categories with the largest

percentage of positive units were meat and meat products (2.2%), particularly those from broilers (4.4%) and turkeys (3.6%).

During 2012–2021, a seasonal pattern was identified for confirmed salmonellosis cases in the EU, with more cases reported in the summer (**Figure 1**). Comparing 2021 to 2020, a small rise in reported human cases was seen. In spite of this, the overall trend for salmonellosis from 2017 to 2021 showed no statistically significant differences. In the last five years, Denmark, Estonia, Finland, Ireland, Romania and Sweden showed a significantly declining trend ($p<0.05$) (2017–2021). In that period, only Malta reported a significantly growing trend ($p<0.05$) (EFSA & ECDC, 2022).

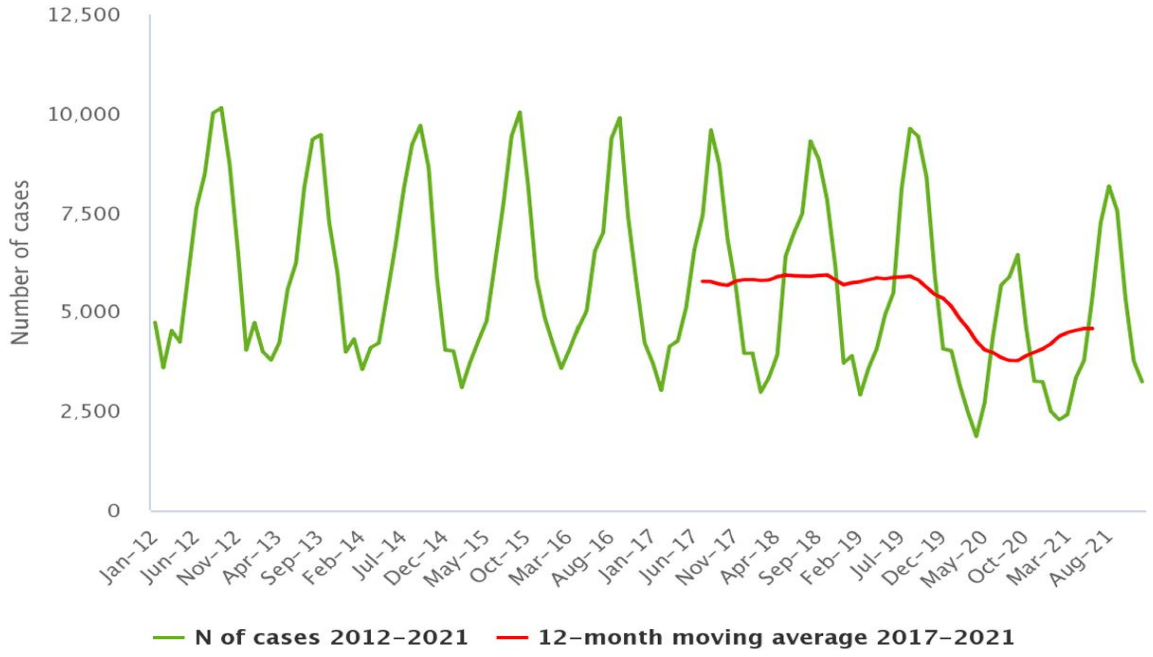

Figure 1. Trend in reported confirmed human cases of non-typhoidal salmonellosis in the EU by month, 2017–2021. (EFSA & ECDC, 2022). Source: Austria, Belgium, Cyprus, Czechia, Denmark, Estonia, Germany, Greece, Finland, France, Hungary, Ireland, Italy, Luxembourg, Latvia, Malta, the Netherlands, Poland, Portugal, Romania, Sweden, Slovenia and Slovakia.

1.5.2 *Bacillus* spp

Bacillus spp. is a genus composed of several spp, of which only *Bacillus cereus* is considered to be a foodborne pathogen hazard. Although the number of reported outbreaks is not high, it remains a relevant microorganism in heat processed foods. The mechanisms of adaptation and resistance are largely unknown, so it is important to consider better characterized surrogates where the mechanisms involved can be identified.

Bacillus subtilis is a Gram-positive, catalase-positive aerobic bacterium with rod-shaped cells that are typically 2–6 μm in length and slightly less than 1 μm in diameter. It can be found in soil and the gastrointestinal tract of ruminants, humans and marine sponges (Errington & van der Aart, 2020; Rahman et al., 2020). The optimal growth temperature is between 30 and 35 ºC, resulting in a doubling time of only 20 minutes. Under certain growth conditions, cells tend to form long chains joined by septal wall material that has not been cleaved. Under situations of starvation, the cells can undergo a complex-2 cell-differentiation process that results in the production of an endospore, which is then released by the lysis of the encapsulating mother cell (Earl et al., 2008). Endospores can remain viable for decades and are resistant to unfavourable environmental conditions such as drought, salinity, extreme pH, radiation and solvents (Higgins & Dworkin, 2012; Petrillo et al., 2020). Prior to sporulation, the cells may become motile by forming flagella, acquire DNA from the surrounding environment, or produce antibiotics. These responses are considered as attempts to get nutrition by seeking a more favourable environment, utilising new advantageous genetic material, or simply eliminating competition (McKenney et al., 2013; Tan & Ramamurthi, 2014).

B. subtilis is the most thoroughly investigated Gram-positive bacterium and a model organism for the study of bacterial chromosomal replication and cell differentiation. It is one of the bacterial champions in secreted enzyme synthesis and is utilised by biotechnology companies on an industrial basis (Errington & van der Aart, 2020). *B. subtilis* has been widely embraced as a model organism for laboratory investigations, particularly of sporulation, a simplified form of cellular differentiation, as it has proven to be very responsive to genetic manipulation (Su et al., 2020). In terms of popularity as a laboratory model organism, *B. subtilis* is frequently compared to *Escherichia coli*, a Gram-negative bacteria that has been extensively investigated and it can be considered a surrogate of *B. cereus*, a foodborne sporulated pathogen (Ruiz & Silhavy, 2022).

B. subtilis secretes various enzymes to breakdown a range of substrates, allowing it to thrive in an environment that is always changing (Earl et al., 2008). This species and some of its close relatives have an exceptional capacity for protein secretion, making them valuable hosts for the manufacture of therapeutic proteins and industrial enzymes (Westers et al., 2004; Zweers et al., 2008). For these reasons, it has been utilised extensively for the production of heterologous proteins (Cai et al., 2019; Cui et al., 2018; Hemilä & Sibakov, 1991; Zhang et al., 2017). In addition, its superior physiological properties and extremely adaptable metabolism make it simple to culture on inexpensive substrates. Consequently, *B. subtilis* grows rapidly and its fermentation cycle is shorter, typically around 48 hours, whereas *Saccharomyces cerevisiae's* fermentation cycle is roughly 180 hours (Aslankoohi et al., 2015). In addition, this organism has access to great expression methods with high genetic stability and it has no codon preference. *B. subtilis* possesses a single cell membrane, unlike *E. coli*, which promotes protein secretion, simplifies downstream processing and reduces process costs (Westers et al., 2004; Zweers et al., 2008). Lastly, this species is widely regarded as safe and has even been awarded

GRAS (Generally Recognized As Safe) status by the US Food and Drug Administration (FDA, 2016).

Therefore, it can be considered an ideal model microorganism to evaluate the mechanisms of heat resistance and adaptation in sporeforming bacteria, as there are well characterized mutants that can be used. The models and mechanisms can then be checked in other spp.

2 Justification and Objectives

Sources of variability in microorganisms, such as genetic heterogeneity and stress adaptation, play a crucial role in microbiological risk assessment. These sources of variability can affect the survival, growth and virulence of microorganisms, as well as their ability to cause disease or food spoilage. Understanding these sources of variability is important for accurately predicting the potential risks posed by microorganisms and determining appropriate control measures. By taking them into account, risk assessors can make better informed decisions and develop more effective risk management strategies to protect public health and the food supply chain.

Variability can be the outcome of different factors. A possible source of variability is stress acclimation, which refers to the ability of microorganisms to adapt to various stress factors, such as changes in temperature, pH, or nutrient availability. Stress acclimation can cause changes in the expression of genes and the metabolic pathways of microorganisms, allowing them to survive in harsh conditions and potentially increase their virulence.

Genetic heterogeneities, on the other hand, refer to the genetic differences within a population of microorganisms. They can result from mutations, recombination, or lateral gene transfer. Genetic heterogeneities can also impact the survival and virulence of microorganisms, as well as their response to stress factors and treatments.

Considering the importance of variability for microbial risk assessment, the main objective of this PhD thesis is to compare and evaluate the importance of different sources of variability in microorganisms (in particular, stress adaptation and genetic heterogeneities) for bacterial survival. This objective can be divided in the following partial objectives:

- To investigate whether there is strain-variability in stress acclimation of *Salmonella*

spp. during dynamic heat treatments.

- To quantify the impact of cell history (sub-optimal incubation) on the thermal resistance of *Salmonella* spp., investigating whether the effect is strain-dependent.

- To investigate the relationship between sigma B and stress acclimation during dynamic heat treatments using *Bacillus subtilis* as model organism.

3 Results and Discussion

3.1 Chapter I

Different model hypotheses are needed to account for qualitative variability in the response of two strains of *Salmonella* spp. under dynamic conditions

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Abstract

In this article, the thermal inactivation of two *Salmonella* strains (*Salmonella* Enteritidis CECT4300 and *Salmonella* Senftenberg CECT4565) was studied under both isothermal and dynamic conditions. We observed large differences between these two strains, with *S.* Senftenberg being much more resistant than *S.* Enteritidis.

Under isothermal conditions, *S.* Senftenberg had non-linear survivor curves, whereas the response of *S.* Enteritidis was log-linear. Therefore, weibullian inactivation models were used to describe the response of *S.* Senftenberg, with the Mafart model being the more suitable one. For *S.* Enteritidis, the Bigelow (log-linear) inactivation model was successful at describing the isothermal response.

Under dynamic conditions, a combination of the Peleg and Mafart models (secondary model of Mafart; t* of Peleg) fitted to the isothermal data could predict the response of *S.* Senftenberg to the dynamic treatments tested (heating rates between 0.5 and 10 ◦C/min). This was not the case for *S.* Enteritidis, where the model predictions based on isothermal data underestimated the microbial concentrations. Therefore, a dynamic model that considers stress acclimation to one of the dynamic profiles was fitted, using the remaining profiles as validation.

In light of this, besides its quantitative impact, variability between strains of bacterial species can also cause qualitative differences in microbial inactivation. This is demonstrated by *S.* Enteritidis being able to develop stress acclimation where *S.* Senftenberg could not. This has important implications for the development of microbial inactivation models to support process design, as every industrial treatment is dynamic. Consequently, it is crucial to consider different model hypotheses and how they affect the model predictions both under isothermal and dynamic conditions.

Keywords: Predictive microbiology, Pasteurisation, Process design, Dynamic models, Microbial risk assessment

Highlights:

- Strain variability causes quantitative and qualitative differences for microbial inactivation.
- *Salmonella* Enteritidis had linear isothermal curves and developed stress acclimation.
- *Salmonella* Senftenberg had non-linear isothermal curves and did not develop acclimation.
- Strain variability may imply not just different parameter values, but also model hypotheses.
- Model hypotheses must be carefully evaluated under isothermal and dynamic conditions.

1. Introduction

Access to safe food is a basic requirement for human health, while at the same time, food safety and security are becoming increasingly difficult. Although anyone may contract a foodborne disease, populations such as small children, elderly people, pregnant women, immunocompromised people and those living in poverty or who are food insecure are particularly vulnerable (FAO & WHO, 2021). According to the European Food Safety Authority (EFSA) One Health 2020 Zoonoses report, salmonellosis is the second most commonly reported gastrointestinal infection in humans and the main cause of food-borne outbreaks in the EU/EEA in 2020. In total, 52,702 confirmed cases of salmonellosis in humans were reported with an EU notification rate of 13.7 cases per 100,000 population. *Salmonella* caused 22.5% of all food-borne outbreaks and the vast majority (57.9%) of the salmonellosis food-borne outbreaks were caused by *S.* Enteritidis (EFSA & ECDC, 2022). Therefore, *Salmonella* spp. are one of the most relevant hazards to the food industry and are often a main aspect of quality control systems.

Over the past decades, food safety management has switched to a more risk-based approach to achieve food safety control (Koutsoumanis & Aspridou, 2016). In this sense, Quantitative Microbial Risk Assessment (QMRA), is currently the reference approach to ensure food safety and also the basis for decision-making (FAO & WHO, 2021). The risk associated with a given hazard is described using quantitative indicators (e.g. expected number of cases or probability of illness per serving). Therefore, the reliability of a QMRA is strongly dependent on the availability and quality of mathematical models able to describe the microbial response to the conditions encountered within the food chain.

In this study, mathematical models were selected to describe the inactivation of *Salmonella* spp. Although different technologies can be used for microbial inactivation (Mañas & Pagán, 2005), thermal treatment is still one of the most effective and easiest applied techniques to most food products (Peng et al., 2017). One of the main limitations of the predictive models currently available is that, due to experimental limitations, they were developed based on data gathered under isothermal conditions. Several scientific studies have questioned the validity of these models for dynamic treatments (i.e. with non-constant temperature), showing that models fitted to isothermal data often failed to predict the microbial response under dynamic conditions (Clemente et al., 2020; Garre et al., 2018c; Hassani et al., 2006; Stasiewicz et al., 2008; Valdramidis et al., 2007). One hypothesis to describe this deviation is stress acclimation, which is based on the concept that microbial cells respond to sublethal stresses by increasing their thermal resistance (Khan et al., 2022; Richter et al., 2010a). Therefore, if the heating phase is sufficiently long, it would increase the stress resistance in the microbial cells, thus increasing their chance to survive the treatment (Garre et al., 2018c).

A second main limitation of currently available microbial inactivation models is the impact of variability. In the context of microbial inactivation models, variability includes differences in the observed microbial response due to genetic and physiological differences between the cells. It is thus different to uncertainty, which includes experimental error and other sources of misinformation that can be reduced by gathering additional data with increased quality (Nauta, 2000). Several recent studies have attempted to assess the variability of microbial inactivation at different levels and to quantify its relevance (Aspridou & Koutsoumanis, 2015; den Besten et al., 2018a; Harrand et al., 2021). However, to the best of our knowledge, a single study has evaluated the relevance of strain variability under dynamic conditions (Clemente et al., 2020). Consequently, in this study, we advance in the understanding of variability in microbial

inactivation under dynamic conditions by comparing the responses of two strains of *Salmonella* spp. The two strains were considered in our study to evaluate the implications of variability in heat resistance in biological safety management. Strain selection was motivated by one being a reference strain commonly used in thermal resistance studies, while the other being a variant of remarkably high heat resistance. This approach will inform whether results obtained for reference strains are extrapolable for strains with extreme phenotypes, a question of high relevance for the study of variability in predictive microbiology.

2. Materials and Methods

2.1 Bacterial Culture and Media

Experiments were performed using *Salmonella enterica* serovar Enteritidis CECT 4300 and *Salmonella enterica* serovar Senftenberg CECT 4565. Both strains were provided by the Spanish Type Culture Collection (CECT, Valencia, Spain). They were selected due to their unique characteristics. *S.* Enteritidis is usually considered as a reference strain for this species, while *S.* Senftenberg is a well-known heat-resistant strain (Clemente et al., 2021; Guillén et al., 2020). The bacterial strains were stored at – 80 ± 2 ◦C (20% glycerol) until use. To perform experiments, fresh cultured plates were grown weekly in trypticase soy agar (TSA, Scharlau Chemie, Barcelona, Spain) for each strain. The fresh cultures were incubated for 24 h at 37 ± 1 ◦C in an incubator. Then, a single colony from the fresh culture plate was transferred to 10 mL of trypticase soy broth (TSB; Scharlau Chemie) and incubated at 37 ± 1 ◦C for 24 h. At this time, the cultures had already attained the stationary growth phase, with a concentration of approx. 10^9 CFU/mL.

2.2 Thermal treatments

Thermal treatments were carried out using a Mastia thermoresistometer (Conesa et al., 2009). Before starting the treatment, the vessel was filled with 400 mL of peptone water (10 g/L peptone from casein (Scharlau Chemie) and 5 g/L NaCl (Scharlau Chemie) as the standard heating medium, to avoid other effects, e.g. complex matrixes such as food items. In order to achieve a homogeneous temperature distribution, the vessel of the thermoresistometer was constantly stirred during the treatment. The heating medium was inoculated with 0.2 mL of the bacterial suspension in order to achieve approximately 10^6 CFU/mL.

Isothermal experiments were performed at different sampling times and temperatures for the two strains. Experiments with *S*. Enteritidis were carried out at 55, 57.5 and 60 °C. On the other hand, for *S.* Seftenberg, thermal treatment was performed at higher temperatures (60, 62.5, 65 and 67.5 °C) due to the heat resistance of the strain. Once the temperature in the vessel was stable, the bacterial suspension was inoculated. The heating medium was adjusted to pH 7.0 for both strains during treatments.

For dynamic conditions, five different temperature profiles were tested for each strain with varying heating rates (supp. Table 1). In each of them, the thermoresistometer was set to the initial temperature of the treatment. Once the temperature of the medium stabilised, it was inoculated with the cell suspension and the selected heating ramp was initiated.

The same procedure for both isothermal and dynamic profiles was followed for determining the viable cell count. Sterile test tubes were used to collect a sample of 3 mL at pre-set intervals and after appropriate serial dilutions in sterile 0.1% peptone water, they were

plated in TSA and incubated at 37 °C for 48 h. A minimum of two experiments were performed per condition, with freshly prepared cultures.

2.3 Modelling microbial inactivation under isothermal conditions

For the analysis of isothermal inactivation, the log-linear Bigelow model and the Mafart and Peleg inactivation models from the Weibull family were chosen. The Bigelow model assumes a log-linear relationship between the fraction of survivors (*S*) and the treatment time (*t*), as shown in Equation (1).

$$log_{10}\, S = -t/D(T) \qquad (1)$$

The slope of the inactivation curve is quantified by parameter *D(T)*; also known as the *D*-value, which is equal to the time required to reduce the microbial population tenfold. Its relationship with temperature (*T*) is supposed to be log-linear (Equation 2).

$$log_{10}\, D(T) = log_{10}\, D_{ref} - \frac{T - T_{ref}}{z} \qquad (2)$$

The sensitivity of the *D*-value to temperature changes is quantified by the *z*-value (*z*), equivalent to the temperature increase required to reduce the *D*-value by 90%. This model introduces a reference temperature (T_{ref}) without a biological interpretation but with an impact on parameter identifiability. In this equation, the value of *D* calculated at T_{ref} is represented by D_{ref}.

Under isothermal conditions, the primary model of the Mafart model (2002) is expressed as shown in Equation (3) where (*T*), usually called the δ-value at temperature *T*, can be interpreted as the time required for the first log-reduction of the microbial density for a treatment at temperature *T*. The p value corresponds to the shape factor of the Weibull

distribution and describes the concavity direction of the isothermal inactivation survivor curve. If p = 1, the shape of the isothermal survivor curve is log-linear and the results are equivalent to those obtained using the Bigelow model. When p is larger than one, the curve has a downward concavity, whereas when it is lower than one, there is a tail.

$$log_{10} S = -\left(\frac{t}{\delta(T)}\right)^{p} \quad (3)$$

Regarding the secondary model, the Mafart model hypothesises that the inactivation rate follows an exponential relationship with temperature (Equation 4), similar to the Bigelow model. The z-value (z) is the temperature change that is required to achieve a tenfold reduction in the δ-value. The parameter δ_{ref} represents the value of (T) estimated at the reference temperature.

$$log_{10} \delta(T) = log_{10}\delta_{ref} - \frac{T - T_{ref}}{Z} \quad (4)$$

The Peleg model (1998) uses a different, equivalent parameterization of the primary model based of *b(T)* instead of $\delta(T)$ (Equation 5). Furthermore, the shape factor is represented by n instead of *p*. Nonetheless, under isothermal conditions, both models are equivalent via the identity (T) = (1/(T))^{p}

$$log_{10} S = -b(T) \cdot t^{n} \quad (5)$$

On the other hand, the Peleg model uses a different secondary model than Mafart's. As shown in Equation (6), this model assumes a log-logistic relationship between $b(T)$ and temperature. If the temperature is much lower than the critical temperature (Tc), then *b(T)* equals zero and no inactivation takes place. When the temperature exceeds *Tc*, *b(T)* has a linear

relationship with temperature with slope *k*. In this model, there is a super-linear transition between both regimes.

$$b(T) = ln(1 + e^{k(T-Tc)}) \quad (6)$$

2.4 Modelling microbial inactivation under dynamic conditions

In this work, we used five different models to describe microbial inactivation under dynamic conditions: the Bigelow model, three Weibullian models and the acclimation model proposed by Garre et al.(2018c).

The Bigelow model can be extrapolated to dynamic conditions by calculating first derivatives with respect to time, assuming that the coefficients are constant (Equation 7)

$$\frac{d\ log_{10}S}{dt} = -1/D(T) \quad (7)$$

where the value of *D(T)* at any time point is defined by the secondary model (Equation 2).

According to van Zuijlen et al.(2010), the Mafart model can be used for dynamic conditions by calculating first derivatives of its primary model (Equation 3) considering that the coefficients remain constant. This results in the differential equation shown in Equation (8), where the symbols have the same interpretation as in Equation (3).

$$\frac{d\ log_{10}S}{dt} = -p \cdot (\frac{1}{\delta(T)})^p \cdot t^{p-1} \quad (8)$$

In this model, the value of $\delta(T)$ at any time point is given by the secondary model of the Mafart model (Equation 4).

The Peleg model adds an additional step to account for the fraction of the population that is already inactivated at any given time (Peleg & Penchina, 2000). This is accomplished by using an equivalent time $t^{\star} = -(\frac{log_{10}S(t)}{b(T)})^{1/n}$ instead of the treatment time, *t*. This results in differential Equation (9), where the symbols have the same interpretation as for the Peleg model under isothermal conditions (Equation 5).

$$\frac{d\ log_{10}S}{dt} = -b(T)\ n \cdot (\frac{-log_{10}S(t)}{b(T)})^{(n-1)/n} \qquad (9)$$

In a similar way as for the Mafart model, the value of *b(T)* at any time point is given by the secondary model (Equation 6).

Therefore, whereas for isothermal conditions the only difference between the Peleg and Mafart models is the secondary model, under dynamic conditions there is an additional difference: the use of t* in the Peleg model. Consequently, in this article, we also used an additional model (the Mafart/Peleg model in the rest of the manuscript) that introduces *t** into the Mafart model.

In the Mafart model, the equivalent time, *t**, can be calculated as

$$t^{\star} = (-\delta(T)^{p} \cdot log_{10}\ S)^{1/p} \qquad (10)$$

Then, substituting in (8), the differential equation of the Mafart/Peleg model would be

$$\frac{d\ log_{10}S}{dt} = -p \cdot (\frac{1}{\delta(T)})^{p} \cdot (-\delta(T)^{p} \cdot log_{10}\ S)^{(p-1)/p} \qquad (11)$$

Garre et al. (2018c) followed a different modelling approach to describe microbial inactivation under dynamic conditions. Their model is based on the hypothesis that sublethal stress during the heating phase of a dynamic treatment induces a physiological response of the microbial cells, increasing their resistance to the latter part of the heat treatment (stress acclimation). They proposed an extension of the Bigelow model, where the inactivation rate (k) of the microbial concentration (N(t)) is the product of two terms: $k=k_1 \cdot k_2$ (Equation 12).

$$\frac{dN}{dt} = -k_1 \cdot k_2 \cdot N(t) \qquad (12)$$

The first term, k_1, represents the effect of the instantaneous temperature on the inactivation rate under the same assumptions as the Bigelow model as shown in Equation (13), where z is the *z*-value, T_{ref} is a reference temperature without biological interpretation and D_{ref} is the *D*-value (time to reduce the microbial count in 1 decimal logarithm during an isothermal treatment) at the reference temperature.

$$k_1 = \frac{ln10}{D(T)} = \frac{ln10}{D(T_{ref})10^{-(T-T_{ref})/Z}} \qquad (13)$$

Stress acclimation is introduced in this model through the term k_2. This model uses the hypothesis that the physiological state of the cell can be described by a theoretical variable, *p(t)*. At the beginning of the experiment, *p(t)=0* indicating the lack of any stress acclimation. When the treatment temperature exceeds a stress-inducing temperature (T_{si}) the value of this variable changes through the treatment up to *p(t)=1*, indicating the maximum acclimation the microbial cell can develop. These hypotheses are included in the model through the empirical equation (14), where *a* and *E* are two rate parameters (Garre, Egea, et al., 2018).

$$\frac{dP}{dt} = \begin{cases} 0 & ;T < T_{si} \\ a(1-p)e^{E/(T-T_{si})} & ;T \geq T_{si} \end{cases} \quad (14)$$

Then, in this model, the effect of the acclimation on the inactivation rate is described by an empirical equation (Equation 15), where *c* is a model parameter that quantifies the relevance of the acclimation on the inactivation rate. In this model, the maximum acclimation results in an increase of the *D*-value by a factor of 1+*c*

$$k_2 = \frac{1}{1+C \cdot P(t)} \quad (15)$$

2.5 Numerical methods for model fitting and calculation of predictions

The models were fitted to the data obtained under isothermal conditions using the one-step approach with the *bioinactivation* package for R (Garre et al., 2017). We used the *fit_isothermal_inactivation* function, which uses nonlinear regression through the Newton-Raphson algorithm. For the models that use a reference temperature, this value was fixed to the medium of the temperature range as recommended by Peñalver-Soto et al. (2019).

The acclimation model was fitted in two steps using the same approach as in Garre et al.(2018a). First, the Bigelow model was fitted to the data under isothermal conditions using the one-step approach with *bioinactivation*. Then, the parameters of the acclimation model (*c*, *a*, *e*) were estimated from one dynamic experiment (with a heating rate of 0.5ºC/min) using the adaptive Monte Carlo algorithm (Haario et al., 2006) included in the *FME* package for R (Soetaert et al., 2010). The convergence of the algorithm was assessed following the usual conventions (Steve Brooks et al., 2011), needing 5,000 iterations with a burning length of 1,000 iterations and a covariance update every 500 iterations. For the fits, the value of *Tsi* was set to 37ºC. The data obtained for the other four dynamic profiles was used for model validation.

Predictions under dynamic conditions were estimated by numerical integration. For the Mafart and Peleg models, we used the *predict_dynamic_inactivation* function of *bioinactivation*, which uses the Livermore Solver for Ordinary Differential Equations (LSODA) algorithm (Hindmarsh, 1983). LSODA is a state-of-the-art numerical algorithm for solving ordinary differential equations (ODEs). Among other advancements with respect to older methods (e.g. Runge-Kutta), it includes adaptive stepsize or an automatic switch between a solver for stiff or non-stiff ODEs. The predictions for the Mafart/Peleg and acclimation models were estimated using the LSODA algorithm through the *deSolve* R package (Soetaert et al., 2010).

The goodness of the model fits and the predictions was evaluated based on the *n* residuals (e) using the Mean Error ($ME = \frac{1}{n}\sum_{i=1}^{n} e$) and Root Mean Squared Error ($RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n} e^2}$).

The RMSE quantifies the magnitude of the noise of the residuals, being defined between 0 and +infinite. On the other hand, the ME describes if there is a consistent bias between the model predictions and the observations, with negative values of ME indicating that the model predictions lay below the observations

All the calculations were implemented in R version 3.5.3. The code is available in the GitHub page of one of the co-authors (https://github.com/albgarre/acclimation-Salmonella).

3. Results

3.1 Inactivation of *Salmonella* Senftenberg under isothermal and dynamic conditions

The inactivation of *S.* Senftenberg observed under isothermal conditions is depicted in Figure 2. The data points have a clear curvature, so the Weibull model is more suitable than the Bigelow one for describing the survivor curves. The plot shows the fit of both the Peleg (blue) and Mafart (red) models to the data, showing that the Mafart model describes the data better under isothermal conditions (RMSE=0.35 log CFU/ml for Mafart; RMSE=0.51 log CFU/ml for Peleg). This can be attributed to the different secondary models used in both modelling approaches. Although both models use an equivalent primary model, the Mafart model assumes a log-linear relationship between δ and temperature, whereas the Peleg model assumes a log-logistic one. In view of the results illustrated in Figure 2, this assumption of the Peleg model is less suitable than the assumptions of the Mafart model for our data on the inactivation of *S.* Senftenberg, especially at low temperatures.

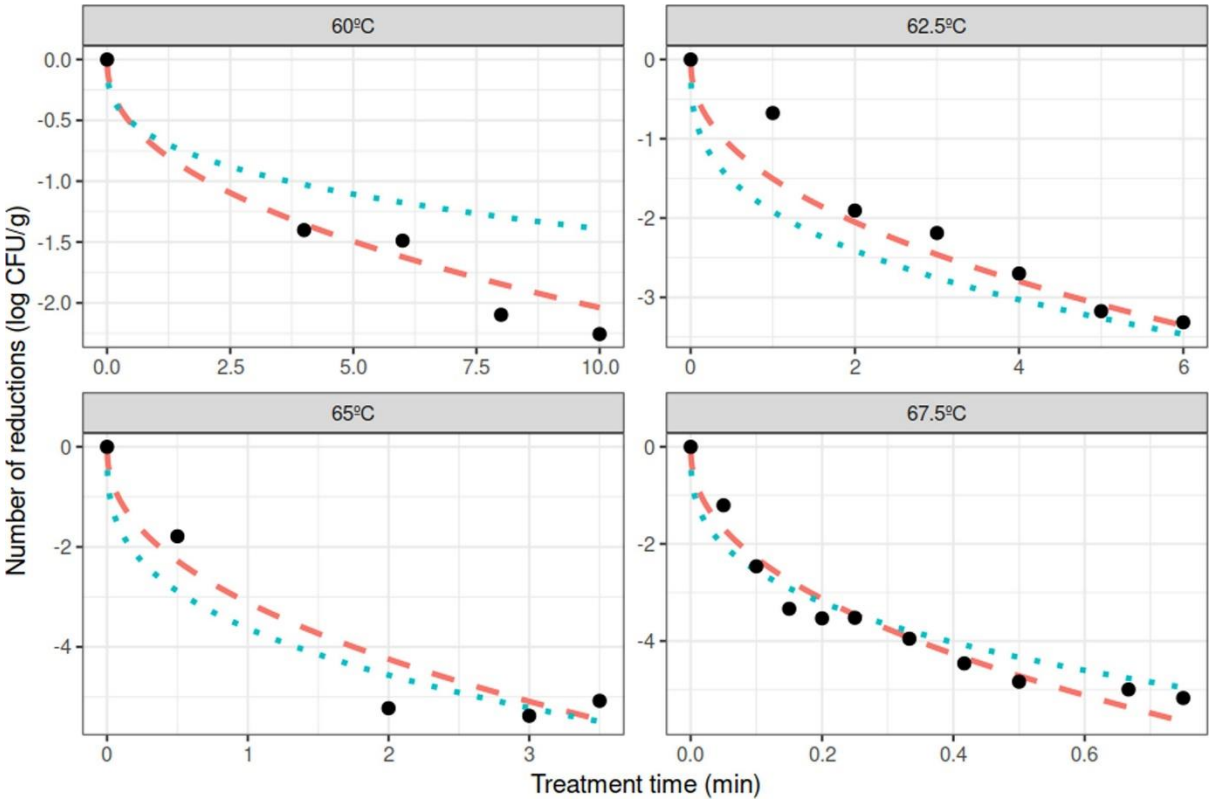

Figure 2. Isothermal inactivation of *S.* Senftenberg at 60, 62.5, 65 & 67.5 °C. The black dots represent the experimental data; the red dashed line is the fit of the Mafart model; the blue dotted line is the fitting of the Peleg model. Models were fitted using the one-step approach. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Consequently, the Mafart model with $\delta_{60} = 2.04 \pm 0.42 min$; $p = 0.45 \pm 0.03$; $z = 3.55 \pm 0.15ºC$ was used to predict the response of this strain under dynamic conditions. Figure 3 shows that these predictions have a clear bias with respect to the experimental data for every dynamic profile tested. This plot also shows that, in spite of its poor fit to the data gathered under isothermal conditions, the Peleg model fitted to isothermal data ($T_c = 60.12 \pm 0.37ºC$; $n = 0.33 \pm 0.04$; $k = 0.74 \pm 0.06\ 1/ºC$) is able to describe the overall trend of the observations. This observation is further confirmed in Table 1, where the ME of each prediction is reported, showing ME for the Mafart model close to 1 log CFU/ml for every dynamic profile and ME close to 0 log CFU/ml for the Peleg model.

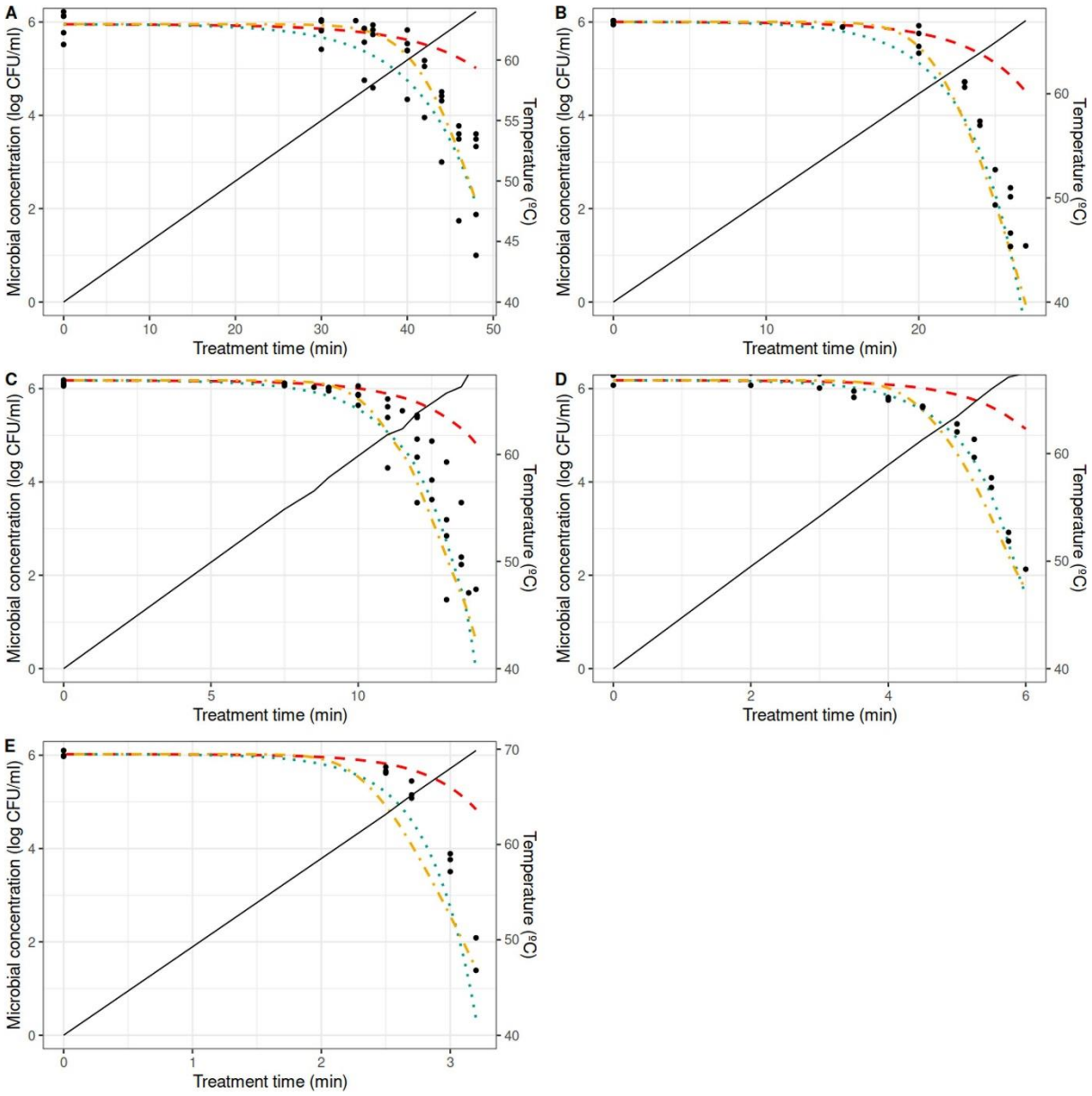

Figure 3. Comparison between predictions based on isothermal experiments and observed inactivation under dynamic conditions for *S*. Senftenberg. The temperature profiles included different heating rates: A) 0.5 °C/min, B) 1 °C/min, C) 2 °C/min, D) 5 °C/min, E) 10 °C/min. The black dots represent the experimental data; (-) prediction of the Mafart model based on isothermal data; (··) prediction of the Peleg model based on isothermal data; (·-) prediction of the Mafart/Peleg model based on isothermal data; (-) temperature profile.

Table 1. Statistical indexes evaluating the precision of the model predictions for *S.* Senftenberg for dynamic conditions based on the models fitted to data gathered under isothermal conditions.

	Mafart model		Peleg model		Mafart/Peleg model	
Heating rate (°C/min)	**ME (log CFU/ml)**	**RMSE (log CFU/ml)**	**ME (log CFU/ml)**	**RMSE (log CFU/ml)**	**ME (log CFU/ml)**	**RMSE (log CFU/ml)**
0.5	0.95	1.45	0.01	0.70	−0.31	0.75
1	1.56	2.02	−0.57	0.74	−0.63	0.80
2	1.09	1.59	−0.58	0.85	−0.42	0.79
5	0.88	1.29	−0.34	0.45	−0.12	0.24
10	1.16	1.59	−0.90	0.97	−0.81	0.90

The poor predictive power of the Mafart model under dynamic conditions can be attributed to the second difference between the Mafart and Peleg models: the introduction of t^* in the latter. The Peleg model is based on the hypothesis that the curvature of the survivor curves is due to a heterogeneous distribution of stress resistance within the population. Then, t* accounts for the fraction of the population that had already been inactivated, defining an "equivalent time" at the instantaneous temperature. Therefore, for treatments with constant temperature, t* has no effect on the model predictions. However, for dynamic profiles, this introduces an additional difference between the Peleg and Mafart models besides their different secondary models.

In order to obtain a unique model able to describe the inactivation of *S.* Senftenberg under both isothermal and dynamic conditions, we defined a "Mafart/Peleg model" that uses both a log-linear secondary model and introduces t* (Equation 10). Note that, because t* has no influence under isothermal conditions, this model has the same parameter estimates as the Mafart model ($\delta_{60} = 2.04 \pm 0.42 min;\ p = 0.45 \pm 0.03;\ z = 3.55 \pm 0.15ºC$). As illustrated in Figure 2, this model can predict the response of *S.* Senftenberg under dynamic

conditions based on isothermal data. This is further confirmed in Table 1, with ME for the predictions of this model closer to 0 log CFU/ml.

Doyle & Mazzotta (2000) gathered the *D*-values of *Salmonella* spp. reported in several publications. They observed that the *D*-value of *S.* Senftenberg at 60°C in laboratory media ranged between 0.62 and 6.3 minutes. Similar findings were reported for *S.* Enteritidis in a study by Clemente et al. (2021) where D_{60} was 0.08± 0.02. The *D*-values estimated in our study fall within the range of the literature and are comparable (*S.* Senftenberg D_{60}=2.04±0.42; *S.* Enteritidis D_{60}=0.08±0.03).

3.2 Inactivation of *Salmonella* Enteritidis under isothermal and dynamic conditions

The response of *S.* Enteritidis was very different to that of *S.* Senftenberg under both isothermal and dynamic conditions, emphasising the relevance of strain variability for microbial inactivation. As expected, this bacterial strain had lower thermal resistance than *S.* Senftenberg CECT4565. Furthermore, the survivor curves were also qualitatively different. Unlike for *S.* Senftenberg, the survivor curves of *S.* Enteritidis CECT4300 did not clearly deviate from linearity. Indeed, as illustrated in Figure 4, there is no difference between the model fits of Bigelow ($D_{57.5} = 0.52 \pm 0.02\ min;\ z = 2.92 \pm 0.06^{\circ}C$) and Mafart ($\delta_{57.5} = 0.47 \pm 0.05; p = 0.88 \pm 0.09;\ z = 2.86 \pm 0.08$).

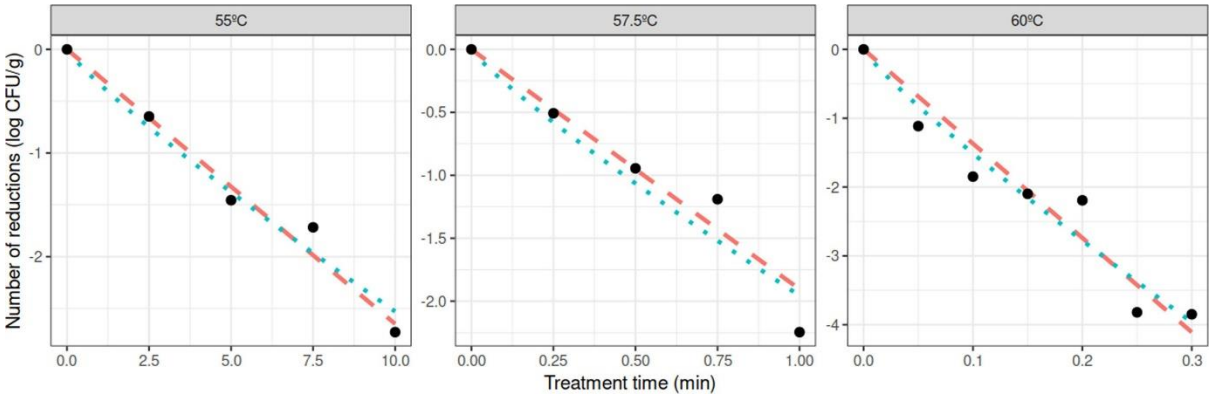

Figure 4. Inactivation of *S*. Enteritidis under isothermal conditions at 55, 57.5 & 60 °C. The black dots represent the experimental data; (--) fitting of the Bigelow model; (··) fitting of the Mafart model.

Therefore, the Bigelow model was used to predict the inactivation of *S.* Enteritidis under dynamic conditions. As illustrated in Fig. 5 and Fig. 6, these model predictions based on isothermal data are clearly biassed with respect to the observations under dynamic conditions, predicting lower microbial counts than observed. Although the observed dynamic response could potentially be described using a Weibullian model fitted directly to the dynamic data, this model would not be able to describe the isothermal inactivation because these are linear (Fig. 4). On the other hand, the acclimation model by Garre et al. (2018c) can be a good candidate to conciliate the microbial response observed for this strain under both isothermal and dynamic conditions. This model assumes that the earlier part of the dynamic treatment induces a physiological response that increases the stress resistance of the cell. This hypothesis is supported by the data, as the observations are higher than the predictions, indicating a higher stress resistance of the microbial cells. Furthermore, Table 2 shows that the *ME* of the model is much smaller for the profile with a heating rate of 10ºC/min than for slower heating, in line with the hypothesis of stress acclimation.

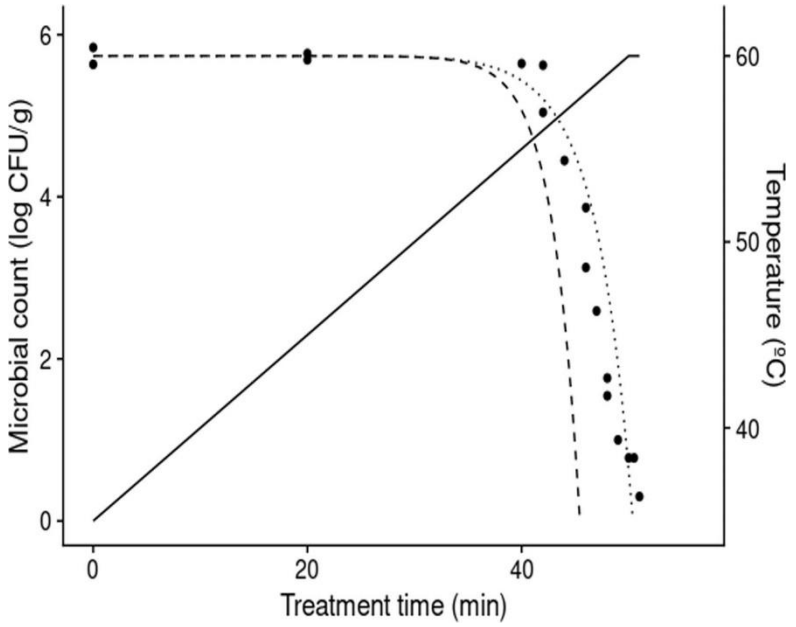

Figure 5. Fitting of the acclimation model to the data obtained for *S.* Enteritidis for dynamic thermal profile with a heating rate of 0.5 °C/min. The dots represent the experimental data. (-) thermal profile (secondary y-axis); (⋯) fitting of the acclimation model; (--) prediction of the Bigelow model based on isothermal data

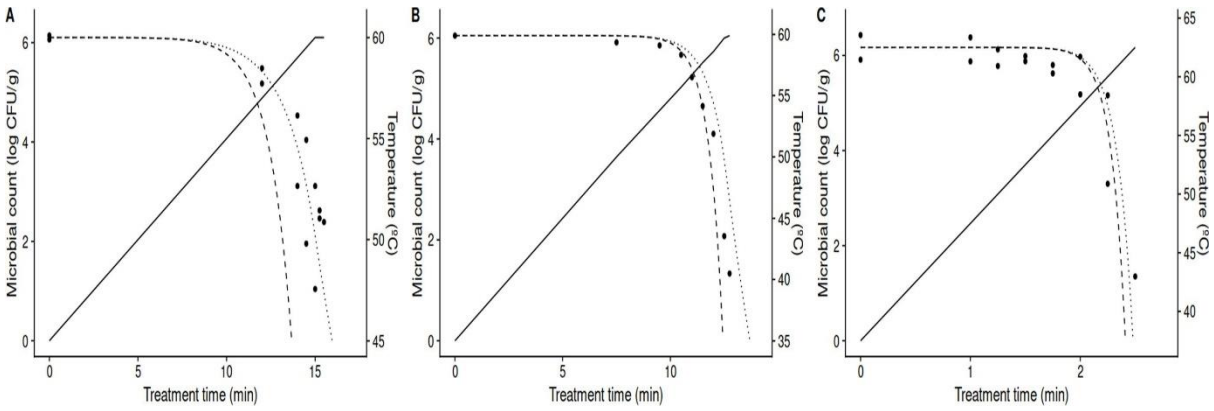

Figure 6. Comparison between experimental data and model simulations for the profiles of *S.* Enteritidis heat treated in peptone water at different heating rates (A for 1 °C/min, B for 2 °C/min, C for 10 °C/min). The dots represent the experimental data, (--) the dashed line is the prediction calculated by the Bigelow model whereas the (⋯) dotted line is the one of the proposed model. The solid line (-) represents the temperature profile (secondary y-axis).

Table 2. Statistical indexes evaluating the precision of the model predictions for *S*. Enteritidis for dynamic conditions based on the models fitted to data gathered under isothermal conditions.

	Bigelow model		**Acclimation model**	
Heating rate (°C/min)	**ME (log CFU/ml)**	**RMSE (log CFU/ml)**	**ME (log CFU/ml)**	**RMSE (log CFU/ml)**
0.5[1]	−9.08	13.07	0.19[1]	0.71[1]
1	−9.66	11.28	−0.58	1.01
2	−6.28	10.08	0.26	0.59
10	−0.36	2.17	−0.31	0.84

[1] Used to fit the acclimation model.

Hence, the acclimation model was fitted to the experimental results obtained for a dynamic profile with a 0.5°C/min heating rate. This profile was chosen because it had the longest heating time, allowing for a better observation of stress acclimation. Fig. 5 illustrates the fit of the model ($c = 8.97 \pm 0.26$; $e = 78.88 \pm 2.65$; $a = 10.36 \pm 1.63$), showing that the model could be fitted to the experimental data. Then, the model was validated by comparing the predictions of the acclimation model against independent experiments obtained for three different heating rates. As depicted in Fig. 6, for every temperature profile tested, the model was able to predict the overall response of the microbial population. This is further confirmed in Table 2, where the *ME* and *RMSE* of the model predictions are reported.

4. Discussion

4.1 On the relevance of the hypothesis of inactivation models under isothermal and dynamic conditions

The application of predictive models to describe industrial processing treatments currently faces an important dilemma. Every treatment is dynamic (ingredients must be heated up and cooled down), but the majority of scientific data obtained in recent decades was obtained under isothermal conditions. This empirical approach is reasonable considering the type of equipment available in most microbiology laboratories, but it also raises the question about the applicability of these models for the description of actual industrial processes. This is especially the case, considering the scientific evidence pointing out that models based on isothermal data may fail at predicting the microbial response under dynamic conditions (Clemente et al., 2020; Corradini & Peleg, 2009; Hassani et al., 2006; Stasiewicz et al., 2008; Valdramidis et al., 2007). Although some studies have been able to predict the microbial response under dynamic conditions (Milkievicz et al., 2021), it is questionable whether they are a rule or an exception. Consequently, the development of models able to describe the microbial response under both isothermal and dynamic conditions is today an active field of research.

If we wish to enhance microbial inactivation models, we must first realise that models are a collection of hypotheses and how those hypotheses vary between isothermal and dynamic situations. Microbial inactivation by heat is extremely complex, so population-level models used in predictive microbiology apply extreme simplifications. The simplest hypothesis that can be made is that the resistance of the cells within the population is homogeneous. Therefore, differences in the time that individual cells survive a treatment would not be due to genetic or physiological differences but just pure chance (Garre et al., 2021a). This hypothesis results in

the first-order kinetics model that, under isothermal conditions, predicts a log-linear relationship between the microbial concentration and the treatment time.

Plenty of scientific evidence has illustrated that, in most cases, the microbial response under isothermal conditions deviates from the log-linearity predicted by this simple model (van Boekel, 2002). A variety of models have used more complex hypotheses to describe this deviation (Aspridou & Koutsoumanis, 2020). One of the most common ones is the "vitalistic" approach, which considers that stress resistance is heterogeneous within the population. The most common vitalistic approach is based on the Weibull distribution (Peleg & Cole, 1998). Under isothermal conditions, this model hypothesis predicts survivor curves with an upwards or downwards curvature (Equation 5).

An alternative hypothesis to describe this curvature would be that stress resistance is homogeneous within the population but that it varies during exposure (e.g. due to a physiological response) according to a power law. Then, the *D*-value at any time point would be calculated as $D(t) = (t/\delta)^p$. Under isothermal conditions, this hypothesis results in predictions that are equivalent to those of the Peleg model. This does not imply that the primary models are the same, only that they are equivalent under that particular condition (in the same way that the Mafart model with $p=1$ is equivalent to the Bigelow model). Therefore, under isothermal conditions, any differences between these models are explained by the secondary models that describe how the inactivation rates are affected by the changes in the (constant) treatment temperature.

The differences between both model approaches become evident for dynamic conditions. In order to account for a heterogeneous population, the inactivation model must include a correction term, done in the Peleg model through the term $t^\star = (-\log_{10} S/b)^n$ (Peleg & Cole,

1998). This introduces an additional difference with respect to the Mafart model (van Zuijlen et al., 2010) that is only relevant under dynamic conditions. This can lead to situations where one modelling approach is more suitable for isothermal conditions (due to the secondary model), but the other one is more adequate for dynamic conditions (due to t^*). This is the case in our research, where the Mafart model fitted better the isothermal response of *S.* Senftenberg, but it failed at describing its dynamic response.

The availability of more advanced equipment during the last years has enabled the definition of novel models whose hypotheses are directly based on observations under dynamic conditions. An example of this novel approach is the acclimation model by Garre et al. (2018c), which uses a similar hypothesis to the Mafart model under dynamic conditions, assuming that the stress resistance within the population is homogeneous and dynamic. However, it adds a more mechanistic hypothesis, assuming that the sublethal parts of the dynamic treatment induce a physiological response of the microbial cells, increasing their resistance (stress acclimation). Accordingly, microbial inactivation under dynamic conditions would be a "race" between microbial inactivation and the development of stress acclimation, as illustrated in Figure 6. The application of a sublethal temperature would increase the resistance of the microbial cells (illustrated as % adaptation with a solid line in Fig. 7). If the heating is fast, the microbial cells are inactivated before they adapt. Consequently, there is barely any difference with respect to the predictions based on isothermal data (Fig. 7D). However, if the heating is slow, a significant level of acclimation takes place while the microbial population is still large, resulting in biassed predictions based on isothermal information (Fig. 7A).

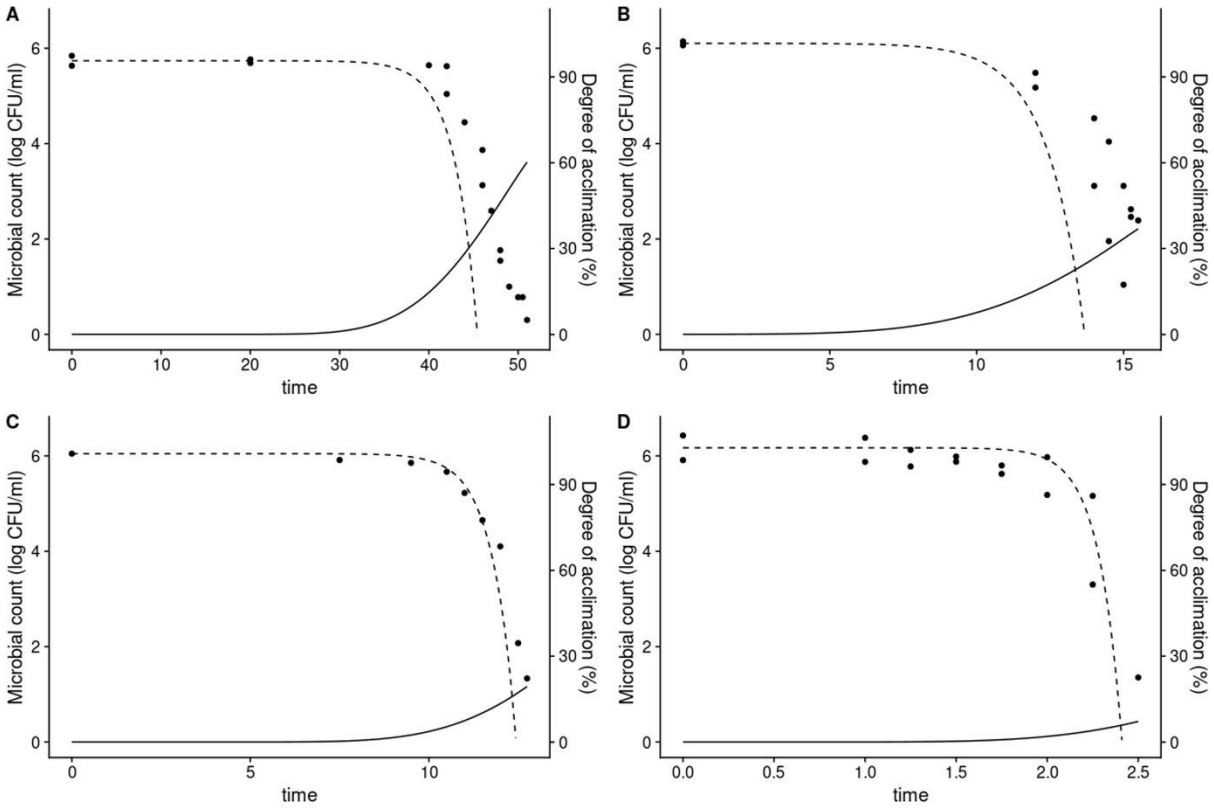

Figure 7. Percentage of acclimation (-, calculated as p(t)*100) for *S.* Enteritidis heat treated in peptone water under dynamic conditions at different heating rates (A for 0.5 °C/min, B for 1 °C/min, C for 2 °C/min and D for 10 °C/min). The observed microbial concentrations are illustrated as black dots. (--) predictions of the Bigelow model based on isothermal data.

It is of high importance to understand these different hypotheses. Uncovering the mechanisms by which individual cells process information and respond to changes is a major task in biological research. Differences in cell behaviour between individuals are always present to some degree in every population of cells and the overall behaviours of a population may not be representative of the individual behaviours (Altschuler & Wu, 2010). It has been demonstrated in a variety of cell types, ranging from bacteria to mammalian cells, that heterogeneity in cellular response can exist despite isogenicity (Abdallah et al., 2013).

4.2 Variability in microbial inactivation models is not just quantitative, but also qualitative

Several scientific studies have focused during the last decade in the study of variability of the microbial response, trying to quantify its impact on inactivation kinetics (Abe et al., 2020; Aryani et al., 2015; den Besten et al., 2018a; Garre et al., 2020; Guillén et al., 2020; Harrand et al., 2021; Luu-Thi et al., 2014; van Asselt & Zwietering, 2006). However, all these studies have applied the hypothesis that the effect of variability is only "quantitative". In other words, they have assumed that a unique model equation can describe the microbial kinetics and that strain variability can be described using different types of pooling (van Boekel, 2020). As demonstrated in this article, strain variability can also have a "qualitative" impact on microbial kinetics. In the case of *S.* Senftenberg CECT4565, the inactivation under isothermal conditions was nonlinear and the model fitted to this data was able to describe its response under dynamic conditions. *S.* Enteritidis CECT4300 had a totally different response: log-linear survivor curves under isothermal conditions and a significant deviation under dynamic conditions that could be attributed to stress acclimation. Therefore, our study shows that accounting for variability in microbial kinetics may require the use of different modelling approaches per strain, not just different model parameters.

The relevance of this fact may be small for isothermal conditions, as linear survivor curves are a particular case of the nonlinear models most commonly used (Weibullian models; Geeraerd model) (Aspridou & Koutsoumanis, 2020). However, it can be of high relevance for inactivation under dynamic conditions, due to the larger differences between the different modelling approaches. This was already indicated by Clemente-Carazo et al. (2020), who concluded that the relevance of variability under dynamic conditions may be different than under isothermal conditions. Consequently, future studies focused on the study of variability

should consider the possibility that variability may also have a qualitative effect on the models, not just quantitative.

This is also of high relevance for the interpretation of experimental data obtained using cocktails of strains. According to the results of this study, it is feasible that a cocktail of strains will include strains with qualitative differences in their responses (e.g. strains able to develop acclimation) that can affect which strain is more resistant under isothermal or dynamic conditions (Garre et al., 2018a). The use of a cocktail of strains would mask this qualitative variability, allowing only the observation of the response of the strain that is the most resistant in a particular situation. Therefore, this empirical approach can mask relevant information that can be helpful in the understanding of microbial inactivation.

On the other hand, food matrices very rarely have a unique bacterial strain. It is well known that strain variability impacts the thermal resistance of microbial cells requiring different values of the model parameters (den Besten et al., 2018a). Our study has shown that, besides that "quantitative variability", variability can also cause qualitative differences between bacterial strains. This raises a fundamental question for predictive microbiology. Most models were historically developed (and, in most cases, still are) with the goal of predicting the survivor curve of a single strain. Even when models were based on cocktails of strains, they would predict a single survivor curve that would correspond to an ideal, worst-case-scenario strain. Considering that we cannot predict the particular strain that will fall in a food product, how can we validate that any model will predict the response of that strain? This is still an open question in the field that will likely be a topic of scientific discussion in the future. Nonetheless, the first step towards resolving this question is the identification of the relevant sources of variability

under different scenarios. In this sense, our study provides additional insight, identifying an aspect of strain variability.

Apart from that, our results have shown that *S.* Senftenberg CECT4565, in spite of being an extremely resistant strain under static conditions, is not able to develop stress acclimation unlike *S.* Enteritidis CECT4300. This result is in-line with a recent study from our group, where we observed that the application of a heat shock would not induce an increased thermal resistance in bacterial cells of this strain (Clemente et al., 2021). This points out the possibility of an upper limit for the stress resistance of microbial cells that cannot be surpassed by the induction of a physiological response. This hypothesis can be of high relevance for microbial risk assessment because bacterial cells within the food chain are subject to a variety of sub-lethal stresses (desiccation, acidification, competition, etc.) that can affect their resistance to stress. Hence, a better understanding of how sublethal stress can affect stress resistance of bacteria and how it is affected by variability is a potential avenue for the improvement of microbial risk assessment models.

5. Conclusions

This article has illustrated that the effect of strain variability in microbial inactivation is not just quantitative but also qualitative. For *Salmonella* Enteritidis CECT4300, we observed log-linear survivor curves under isothermal curves and stress acclimation under dynamic conditions. This behaviour was largely different from that of *Salmonella* Senftenberg CECT4565. This especially resistant strain had non-linear survivor curves under isothermal conditions and did not show stress acclimation under dynamic conditions. This different response required the application of two different modelling approaches for each strain (Weibullian models for *S.* Senftenberg, acclimation model for *S.* Enteritidis). This qualitative

difference has not been described before in the context of dynamic microbial inactivation and emphasises the need to carefully evaluate different model hypotheses when describing variability in microbial inactivation.

6. Supplementary material Chapter I

Supp. Table 1. Parameters of the dynamic microbial inactivation experiments

	Heating rate (°C/min)	Starting temperature	Holding temperature (°C)
***S.* Senftenberg**	0.5	40	67.5
	1	40	67.5
	2	40	67.5
	5	40	67.5
	10	40	75.0
***S.* Enteritidis**	0.5	35	60.0
	1	40	60.0
	2	40	67.5
	5	40	67.5
	10	37.5	67.5

Authorship contribution statement

Leonidas Georgalis: Methodology, Formal analysis, Investigation, Visualization, Writing – original draft. **Anna Psaroulaki:** Project administration, Funding acquisition, Supervision. **Arantxa Aznar:** Supervision, Investigation, Data curation. **Pablo S. Fernández:** Conceptualization, Supervision, Project administration, Funding acquisition. **Alberto Garre:** Conceptualization, Formal analysis, Visualization, Software.

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3.2 Chapter II

Variability in cell history can be more impactful than biological variability in the survival to thermal treatments of some *Salmonella* strains

Abstract

The description of variability in the bacterial response to stress has received plenty of attention during the last years, partly due to its relevance to microbial risk assessment. Although the microbial response is affected by numerous variability sources, previous studies focused mostly on strain variability (inherent differences between strains of the same bacterial species). Here, we analyse a variability source relatively unexplored within microbial risk assessment: phenotypic variability. This refers to physiological differences of cells of the same species due to prior exposure to different environments.

In particular, we studied the impact that sub-optimal pre-culture conditions or the application of an acid shock have on the thermal resistance of two strains of *Salmonella* (a reference strain and a highly resistant one to heat). We observed that phenotypic variability is strain-dependent. For the resistant the conditions tested resulted in a reduction of thermal resistance with respect to control conditions. Conversely, they had the opposite effect for the reference strain, increasing its thermal resistance through the development of cross-resistance mechanisms: they induced a shoulder in the survivor curves and up to a 300% increase in the D-value. Considering that this increase in the same order of magnitude as the one typically attributed to strain variability, phenotypic variability should be a main focus on predictive microbiology and risk assessment. Using a simplified, hypothetical example, we illustrate how this could be attained in practice, by linking pre-incubation conditions to the origin of bacterial contamination.

Keywords: predictive microbiology; microbial risk assessment; microbial inactivation; pasteurization

Highlights:

- Microbiological variability includes genetic and physiological aspects
- The impact of both sources on thermal resistance can be comparable for some bacteria
- The magnitude of physiological variability is strain dependent
- The variability in physiological state could be linked to the sources of contamination in QMRA

1. Introduction

Risk assessment is a specialized area of applied science that entails analyzing scientific data in order to assess the risks related to certain hazards. Standards for food safety are established with the assistance of risk assessment (Cassini et al., 2016). Therefore, Microbial Risk Assessment (MRA) is a useful method for managing the risks brought on by food microbiological hazards. MRA is used to collect and analyse relevant information on newly discovered or uncharacterized foodborne pathogens. It may then lead on to risk management and communication with the goal of minimizing the negative impact that these pathogens have on human health (Brown & McClure, 2006).

With the aim of developing models to characterize and forecast the growth or inactivation of microbes under a variety of environmental conditions, the field of predictive microbiology blends microbiology, mathematics and statistics (Valdramidis, 2016). Quantitative microbial risk assessment (QMRA) is a mathematical modelling approach used to estimate the risk of infection when a population is exposed to microorganisms or their toxins. The process of QMRA involves identifying and characterizing the hazards, assessing exposure and offers the opportunity for a detailed risk characterization (EFSA, 2012b).

Uncertainty and variability are inherent to any microbiological risk assessment (Benford et al., 2018). The term "variability" refers to causes of variation that are inherent to the microorganism, the food supply chain or the environment (e.g. differences in the response of single cells or in the composition of the food media). It is then not the same as uncertainty, which refers to the sources of variation that can be tracked down to the use of partial or imperfect information (e.g. measurement errors or model misspecifications). Therefore, while it is possible to minimize uncertainty by amassing more data from experiments of a higher

standard, variability is an inherent element of the process and cannot be eliminated merely by gathering more and better data (Nauta, 2000).

Previous studies on the relevance of variability on microbiological risk were focused on genetic factors, especially on the differences between strains of the same species (den Besten et al., 2017, 2018a; Guillén et al., 2020; Lianou et al., 2017). However, there are other types of variability sources that could potentially have the same impact on the outcome of the calculations. For instance, Aryani et al.(2015) did a large screening of the thermal resistance of different strains of *Listeria monocytogenes*. When using cells on stationary phase for their experiments, they concluded that strain L6 was by far the most resistant among the ones tested. However, exponential-phase cells of L6 were more heat sensitive than stationary-phase cells of any other strain. Similar effects were observed in other studies (Ferreira et al., 2001, 2003; O'Driscoll et al., 1996). Therefore, in spite of receiving less attention during the last years, variability sources related to the history of the cells can be equally relevant as genetic differences between cells in some situations.

Consequently, this study focuses on the relevance for microbiological risk assessment of variability sources related to the physiological state of cells. To differentiate between both sources, in this article we use the term "strain variability" to refer to genetic differences between strains and "phenotypic variability" for variability related to the history of the cell (incubation conditions or the application of a sub-lethal stress), in line with Wagner and Altenberg (1996). Using *Salmonella* spp. as case study, we focus on the effect on thermal inactivation of three phenotypic variability sources: growth phase (stationary or exponential cells), the incubation under suboptimal pH conditions and the application of an acid shock before the treatment. The experiments were done with two different *Salmonella* strains, a

reference and an extremely heat resistant one to analyse if the magnitude of phenotypic variability was strain-dependent.

2. Materials and Methods

2.1 Bacterial culture and media

Experiments were performed using *Salmonella enterica* serovar Enteritidis CECT 4300 and *Salmonella enterica* serovar Senftenberg CECT 4565. Both strains were provided by the Spanish Type Culture Collection (CECT, Valencia, Spain). They were selected because *S.* Enteritidis is a reference strain, while *S.* Senftenberg is extremely heat-resistant (Guillén et al., 2020)**.**

The bacterial strains were stored at –80±2°C (20% glycerol) until use. To perform experiments, fresh cultured plates were grown weekly in trypticase soy agar (TSA, Scharlau Chemie, Barcelona, Spain) for each strain. The fresh cultures were incubated for 24 hours at 37±1°C in an incubator. Then, a single colony from the fresh culture plate was transferred to 10 mL of trypticase soy broth (TSB; Scharlau Chemie). In order to analyze the effect on the thermal inactivation kinetics of the growth phase (stationary or exponential), the cells were incubated at 37±1°C for 24 h (stationary phase) and at 37±1°C for 16-18h (exponential phase). The times were validated by a preliminary incubation experiment (data not shown).

Suboptimal pH conditions

For incubation under suboptimal pH conditions, a single colony from the fresh culture plate was transferred to 10 mL of trypticase soy broth (TSB; Scharlau Chemie) with suboptimal pH values (5.0, 5.5 and 6.0) adjusted with HCl (min. 37%, Sigma-Aldrich, Seelze, Germany). The cells were incubated to the selected growth phase (exponential or stationary) and immediately

centrifuged at 3600 rpm for 10 min at 4±1°C. Pellets were washed once with Peptone water (pH 7.2) and immediately heat treated. The pH was measured with a pH meter (Basic20, Crison; Alella, Cataluña, Spain), under strict aseptic conditions. Measurements were taken throughout the incubation process under suboptimal conditions, observing that the pH converged to 5.0-5.2 in every condition tested.

Application of acid shock

A volume of 1 mL of cells of each strain (in stationary and exponential growth at pH 7.0) was centrifuged at 3600 rpm for 10 min at 4±1°C. Pellets were washed with TSB (pH 7.0) and resuspended in acidified TSB (pH 4.5, 5.0, 5.5, 6.0) adjusted with HCl (min. 37%, Sigma-Aldrich, Seelze, Germany). Then, the cell suspension was incubated at 37±1°C for 60 min in an incubator. Afterwards, the pellets were washed with Peptone water (pH 7.2) by centrifuging them at 3600 rpm for 10 minutes at 4±1°C. Following the wash, the pellets were immediately subjected to heat treatment. The pH was measured with a pH meter (Basic20, Crison; Alella, Cataluña, Spain), under strict aseptic conditions.

2.2 Thermal treatments

A Mastia thermoresistometer was used to conduct the thermal treatments (Conesa et al., 2009). A volume of 400 mL of sterile peptone water (10 g/L peptone from casein, Scharlau Chemie) and 5 g/L NaCl (Scharlau Chemie's normal heating medium) were added to the vessel before the treatment began. The thermoresistometer's vessel was continuously stirred throughout the procedure to provide a uniform temperature distribution. A volume of 0.2 mL of the bacterial suspension was inoculated into the termoresistometer as an inoculum, reaching an initial concentration of approximately 6 $\log_{10}$ CFU/mL.

S. Enteritidis heat resistance experiments were conducted at 55 and 60 °C. Due to its higher resistance, *S.* Seftenberg underwent thermal treatment at 60 and 65°C. The bacterial suspension was inoculated once the vessel's temperature had stabilized. The duration of the experiment was adapted for each condition to reach approx. 4 log reductions of the initial bacterial concentration. For both strains, the pH of the heating medium was set to 7.0.

For determining the viable cell counts at each data point, different sterile test tubes were used to collect a sample of 3 mL at pre-set time intervals and after appropriate serial dilutions in sterile 0.1% peptone water, they were plated in TSA and incubated at 37 °C for 48 h. A minimum of two experiments were performed per condition.

2.3 Mathematical modelling and statistical analysis

Depending on the conditions, survivor curves with linear or non-linear shapes were observed. For *S.* Enteritidis we obtained survivor curves that were either linear or with a stable phase (shoulder) followed by linear decay. For the former case, we used the log-linear inactivation model (often called “Bigelow model” for his pioneering works (Bigelow, 1921) to explain the variation in the microbial concentration (N) with time (t) with respect to the initial one (N_0). In this model, represented in Equation (16), the inactivation rate is described by the D-value (D), which represents the treatment time required to reduce the microbial concentration by a ten-fold.

$$log\ N = log\ N_0 - \frac{t}{D} \qquad (16)$$

For cases with a shoulder followed by linear decay, we used the Geeraerd model without tail (Geeraerd et al., 2000) as shown in Equation (17), where *SL* stands for the duration

of the shoulder length and *k* is the inactivation rate, related to the *D*-value by the identity $k = \ln(10)/D$.

$$N = -N_0 \cdot e^{-k \cdot t} \cdot \frac{e^{k \cdot SL}}{1 + (e^{SL} - 1) \cdot e^{-k \cdot t}} \quad (17)$$

For the curves that had a smooth nonlinear shape, without a clear tail or shoulder, the Mafart inactivation model was used (Mafart et al., 2002), shown in Equation (18). In this model, the parameter δ (δ-value) represents the time to the first log reduction. The nonlinearity is defined by parameter β (β-value) with values of $\beta < 1$ indicating an upwards curvature in the survivor curve and $\beta > 1$ indicating a downwards one.

$$log\ N = log\ N_0 - \left(\frac{t}{\delta}\right)^{\beta} \quad (18)$$

We estimated different values of β depending on the condition tested, making it impossible to compare between conditions only based on the δ-value. Hence, we compared the time to reach four log-reductions (t_{4D}) according to Equation (19). Calculations were also done for 3 and 5 log-reductions, reaching the same qualitative results (not shown).

$$t_{4D} = \delta \cdot 4^{1/\beta} \quad (19)$$

The models were fitted by nonlinear regression using the web version of *bioinactivation* (Garre et al., 2017), currently available at https://foodlab-upct.shinyapps.io/bioinactivation4/. The goodness of the fit was evaluated qualitatively by visually comparing the fitted curves against the observations and quantitatively using the Root Mean Squared Error ($RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n} e^2}$; with *n* the number of data points and *e* the residuals).

3. Results and Discussion

3.1 Relevance of phenotypic variability in the heat resistance of *Salmonella* Enteritidis

3.1.1 Cells in the stationary growth phase

Figures 8 and 9 show, respectively, the D-values (of the Bigelow or Geeraerd model) and shoulder lengths (of the Geeraerd model) estimated for each condition tested for *S.* Enteritidis. The numeric values are included in Supp. Table 2 and the survivor curves in Supp. Figures 1 through 4. The results under control conditions (incubation at optimal pH without any acid shock; cells in early stationary phase) are similar to the ones reported previously for *S.* Enteritidis in a previous study by our group (Georgalis et al., 2022). Our results show that the heat resistance was substantially affected by the pre-culture conditions, indicating that phenotypic variability is very relevant for this strain. When compared to control cells (incubated at pH 7), cells of *S.* Enteritidis in stationary phase pre-adapted to low pH or acid-shocked exhibited a significant increase in their heat resistance (Fig. 8), in agreement with previous studies on *S.* Enteritidis (Alvarez-Ordóñez et al., 2010; Clemente et al., 2021; Leyer & Johnson, 1993).

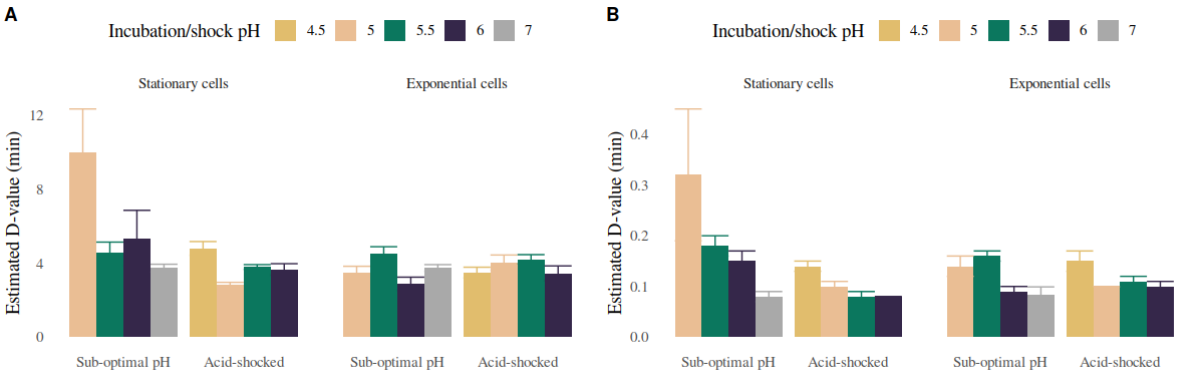


Figure 8. D-values of *Salmonella* Enteritidis (either from the Bigelow or Geeraerd models) for isothermal heat treatments at 55ºC (A) and 60ºC (B). Phenotypic variability was studied performing experiment for control cells (stationary phase cells incubated at pH 7) and cells incubated at suboptimal pH (5, 5.5 and 6) or subject to an acid shock (1h at pH 4.5, 5, 5.5 or 6).

For these conditions, we tested cells on both the stationary and exponential growth phases. Error bars illustrate the standard error of the regression

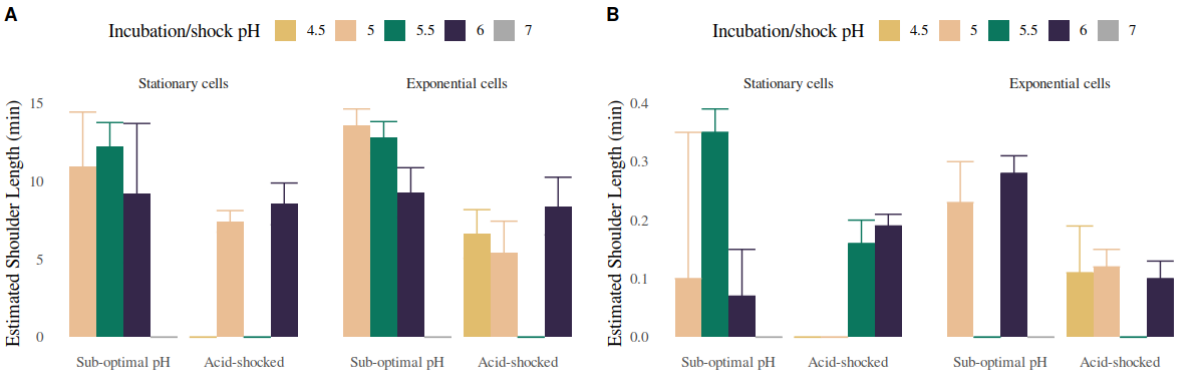


Figure 9. Shoulder length (*SL* of the Geeraerd model) of *Salmonella* Enteritidis for isothermal heat treatments at 55ºC (A) and 60ºC (B). Phenotypic variability was studied performing experiment for control cells (stationary phase cells incubated at pH 7) and cells incubated at suboptimal pH (5, 5.5 and 6) or subject to an acid shock (1h at pH 4.5, 5, 5.5 or 6). For these conditions, we tested cells on both the stationary and exponential growth phases. Conditions without a shoulder (i.e., linear inactivation) are shown as a horizontal line on the x-axis. Error bars illustrate the standard error of the regression.

Our experimental design, consisting of several time points per experiment (Supp. Figure 1&2), enables a detailed analysis of the pre-culture conditions on the D-value (Figure 8) and the linearity of the survivor curve (Figure 9). Although the survivor curves under control conditions were linear, the application of a pre-treatment (pre-adaptation or acid shock) introduced a shoulder in the microbial response in almost every case (Figure 9), although the effect of the acid shock is more irregular. This implies that the deviation from optimal pre-treatment conditions (a sub-optimal incubation pH, or the application of an acid shock) increases the bacterial resistance, resulting in the thermal stress being unable to immediately cause microbial inactivation. Instead, the microbial cells are able to resist the stress for a given time. This result is in-line with previous studies that observed the emergence of cross-resistances to thermal stresses after the application of acidic conditions (Alvarez-Ordóñez et al., 2008, 2009a; Spector & Kenyon, 2012b).

We observe that the effect of the previous environmental conditions of the population on its inactivation kinetics depends both on the type of deviation (sub-optimal pH or acid shock) and the pH level. For our experimental conditions, we observed that incubation under sub-optimal conditions has a larger impact on the emergence of cross-resistances than the application of an acid shock. The largest effect on the D-value was observed at the lowest incubation pH tested (pH 5), which caused an increase of the D-value between 167% (at 55ºC) and 300% (at 60º). This is in accordance with similar findings of Koutsoumanis and Sofos (2004), who discovered that the pH range in which habituation resulted in increased acid resistance was 4.0-5.0. Regarding the SL, the largest effect is observed for pre-incubation at pH 5.5. It is interesting that the SL observed after pre-incubation at pH 5.5 is longer than at pH 5. This could be attributed to two reasons. The first one would be that they are fundamentally different responses (the shoulder is an initial resistance that results in no inactivation, whereas the D-value represents the resistance to a lethal stress). Therefore, different pH values would favour either response (through natural selection of different variants, or activation of different molecular pathways). The second explanation is due to poor parameter identifiability, as the estimated SL after pre-incubation at pH 5 had relatively large error bars at both temperatures tested. Therefore, to determine the generality of this result, additional data is needed.

Although acid shock had a lesser effect than pre-incubation conditions, it still increased resistance at the lowest pH tested (4.5), suggesting that our acid shock conditions (one hour at pH 4.5) do not cause significant sublethal damage to the bacterial cells. Alternatively, the level of sublethal damage may have masked the cross-resistance effect. At pH 4.5, acid shock activated stress response mechanisms that resulted in cross-resistance to thermal stress. However, this effect was pH-dependent and at both temperatures tested, only the lowest pH

(4.5) resulted in a relevant increase in the D-value, while intermediate pH values resulted in longer shoulders in some cases

3.1.2 Cells in exponential phase

Figures 8 and 9 depict the kinetic parameters estimated for thermal resistance of microbial populations in exponential growth phase. We observed no difference in the thermal resistance between stationary-phase and exponential-phase cells incubated at pH 7, as both showed linear inactivation without significant differences in D-value. However, the effect of cell history (such as incubation at sub-optimal pH or application of an acid shock prior to thermal treatment) on thermal inactivation kinetics differed for exponential-phase and stationary-phase cells

The effect of acidic incubation conditions on the D-value of cells in the exponential growth phase was generally lower than for stationary cells (maximum increase between 20 and 96%). Furthermore, the trend of the response was also different. Whereas for stationary-phase cells lower pH values resulted in higher D-values, for exponential-phase cells we observed higher D-values for a pH 5.5 than for pH 5. Regarding the shoulder length, we observe a different response for the two temperatures tested. At 55ºC, exponential-phase cells incubated at acidic pH had a shoulder length comparable to the one observed for stationary-phase cells. Moreover, we observed a clear trend, with lower pH resulting in longer shoulders. However, the results at 60ºC were not so consistent, with two conditions (pH 5 and pH 6) showing a shoulder, but not the intermediate one. Nonetheless, the shoulder represents the treatment duration that the bacterial population can withstand without inactivating and is very sensitive to the experimental conditions. Therefore, it is reasonable that there is more noise in the shoulder than in the D-value.

The effect of acid shock on exponential-phase cells is comparable to that observed for stationary-phase cells. At 55ºC, we did not observe any significant difference with respect to the control conditions. At 60ºC, only the lowest pH level tested resulted in a significant increase in the D-value. We also observed a similar effect on the shoulder length as for stationary-growth-phase cells. Every experiment, except for the heat shock at pH 5.5, induced a shoulder in the survivor curve, with a duration generally lower than for cells incubated under sub-optimal pH conditions.

3.2 Relevance of phenotypic variability in the heat resistance of *Salmonella* Senftenberg

3.2.1 Stationary phase cells

The survivor curves for *S.* Senftenberg showed a smooth non-linearity, without a clear shoulder and/or tail. Hence, they were described using the Mafart model. Figure 10 depicts the treatment time for 4 log-reductions according to models fitted for each condition. The parameter estimates are provided in Supp. Table 3 and the survivor curves in Supp. Figures 5 through 8. As expected, this strain was much more heat resistant than the *S.* Enteritidis strain, with a D-value at 60ºC ~20 times larger (δ_{60}=2.62±1.85 min for *S.* Senftenberg; D_{60}=0.08±0.01 min for *S.* Enteritidis). Furthermore, whereas the survivor curves for *S.* Enteritidis under control conditions were linear, the ones for *S.* Senftenberg had a significant curvature (β=0.63±0.25), in agreement with our previous results (Georgalis et al., 2022).

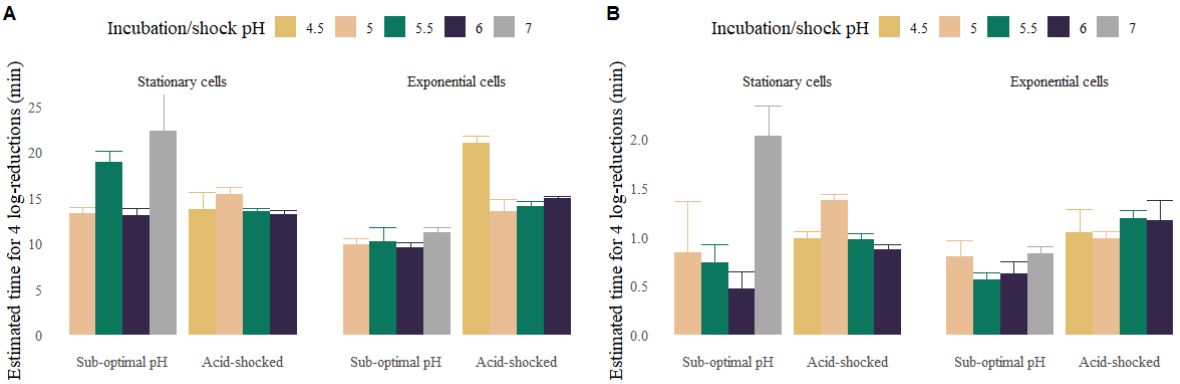


Figure 10. Time to reach 4 log-reductions in the concentration of *Salmonella* Senftenberg according to the parameters of the Mafart model fitted for an isothermal treatment at 60ºC (A) and 65ºC (B). Phenotypic variability was studied performing experiment for control cells (stationary phase cells incubated at pH 7) and cells incubated at suboptimal pH (5, 5.5 and 6) or subject to an acid shock (1h at pH 4.5, 5, 5.5 or 6). For these conditions, we tested cells on both the stationary and exponential growth phases.

Phenotypic variability related to the history of the bacterial population (sub-optimal incubation conditions or an acid shock) had a relevant impact on its resistance to the thermal treatment. However, whereas for *S.* Enteritidis we observed an increased resistance (cross-resistance), we observed the opposite effect for *S.* Senftenberg. Both sub-optimal incubation conditions and acid shock resulted in reduced thermal resistance for all conditions tested. In particular for acidic incubation conditions, the reduction in the time for 4 log-reductions ranged between 20% (pH 5.5) and 46% (pH 6) for the treatment at 60ºC, and between 49% (pH 5) and 74% (pH 6). The application of an acid shock resulted in a reduction in a similar range (34 to 44% at 60ºC; 33 to 58% at 65ºC). This result in is in-line with those reported previously for these strains in a previous work from our work (Clemente et al., 2020).

The observation that the magnitude (and direction) of phenotypic variability is strain-dependent is of high relevance for the interpretation of the bacterial response to stress. The *S.* Senftenberg strain studied has an extremely high heat resistance (this investigation; (Guillén et al., 2020). However, whereas *S.* Enteritidis can adapt to acidic conditions, resulting in cross-resistances to the thermal stress, *S.* Senftenberg cannot. Instead, any deviation from optimal

growth conditions resulted in a reduction of its resistance (Figure 10). This result can be compared to our previous investigation (Georgalis et al., 2022), that studied the heat resistance of these two strains under dynamic conditions. We concluded that *S.* Enteritidis was able to adapt to the low temperatures at the beginning of a dynamic treatment, increasing its stress resistance if the heating rate was too low, whereas the resistance of *S.* Senftenberg remained unchanged. These results may point out that there might be a trade-off between the "static" stress resistance of a bacterial strain and its ability to adapt and increase that resistance, a concept that has not yet been explored in depth. Hence, it is possible that relatively weak bacterial strains under optimal incubation conditions become relatively resistant when exposed to conditions that enable bacterial adaptation. Although this was not the case due to the extreme phenotype of the *S.* Senftenberg strain, it can result in phenotypic variability being more relevant than initial strain variability and some situations (Garre et al., 2018a). Nonetheless, this concept has not yet been explored in detail in the scientific literature, so additional data is needed to prove or disprove this hypothesis.

3.2.2 Cells in exponential phase

Compared to acid shock cells, pre-adapted cells in the exponential phase displayed substantially lower stress resistance (Figure 10). For cells pre-incubated at pH 7, we observed a 52% reduction in the time for 4 log-reductions at 60ºC and a 60% reduction at 65ºC with respect to stationary-phase cells. Unlike for stationary-phase cells, the application of a heat shock increased the thermal resistance of exponential-phase cells of *S.* Senftenberg. Namely, we observed a 20 to 87% increase of the time for 4 log-reductions at 60ºC, and a 19 to 44% increase at 65ºC. This result can be interpreted in a similar way as those obtained for *S.* Enteritidis. For our experimental conditions, the application of an acid shock, rather than causing sublethal damage, induces a stress response on the bacterial cells that results in a cross-

resistance to the thermal stress. It is somewhat remarkable that this effect is observed for exponential-phase cells, whereas the opposite one is observed for stationary phase cells (where we see a decrease in resistance). This can be attributed to the exponential and stationary growth phases being essentially different physiological states for cells. There is a limit to how much the stress resistance can be inversed.

Regarding incubation under sub-optimal pH, exponential-phase cells of *S.* Senftenberg had a similar response to stationary cells of this strain, with a reduced stress resistance with respect to cells incubated at pH 7. In particular, we observed a reduction between 1 and 31% at 65ºC, and between 8 and 14% at 60ºC. This result emphasizes that, in spite of being based on the same agent, the bacterial response to the application of an acid shock is essentially different from the response to acidic incubation conditions. This is because under acidic incubation conditions, the cells are able to grow and, despite the stress, they focus on growth rather than on stress

The acid shock is a short-term event where the cells are exposed to an acidic condition potentially causing a combination of lethal and sublethal damage, and activation of stress response mechanisms. On the other hand, incubation under sub-optimal conditions is a long-term event that can alter population balances due to natural selection and affect the bacterial pathways responsible for growth. According to the SPANC hypothesis, in certain environments, natural selection may favour nutritional ability over stress resistance, potentially resulting in modifications to the network by decreasing or abolishing RpoS function (Stoebel et al., 2009). Laboratory studies on *E. coli* have shown that selection against RpoS activity occurs (Finkel, 2006; King et al., 2006; Notley-McRobb et al., 2002) and natural populations of *E. coli* and *Salmonella enterica* have been found to have low- or null-activity rpoS alleles

(King et al., 2004; Robbe-Saule et al., 2003). Massey et al. (1999) provides evidence for the SPANC hypothesis, which suggests that natural selection in certain environments may favour nutritional ability over stress resistance. The authors discuss how this can result in modifications to the genetic network by decreasing or abolishing the activity of the RpoS protein. They report that in their experiments, mutations that led to decreased RpoS activity were beneficial for growth under nutrient-limited conditions, but resulted in reduced stress resistance. These findings suggest that the trade-off between nutritional ability and stress resistance is an important factor in the evolution of Salmonella and other microbes in changing environments.

Our results indicated that this fundamental difference is very relevant for the bacterial response to posterior stress and can be of high relevance for microbial risk assessment. An acid shock would be similar to an acidic wash of the food product (e.g., with peracetic acid) whereas the incubation under sub-optimal conditions would be closer to the microbial population thriving in an environment (e.g., a food ingredient) whose physicochemical conditions deviate from the optimal conditions used in the laboratory for culture preparation.

3.3. Implication of risk assessment of *Salmonella*

Microbiological sources of variability have been identified as one of the most relevant factors for microbiological risk assessment of foods (Aspridou & Koutsoumanis, 2015; den Besten et al., 2017; Pouillot & Guillier, 2020). Previous studies were mostly focused on the microbiological variability associated with genetic differences (mostly) between different strains of the same bacterial species and/or between different cultures of the same species (Aryani et al., 2015; Garre et al., 2020; Guillén et al., 2020; Papagianeli et al., 2022). On the other hand, differences in the microbial response due to the physiological state of the cells

(which includes their "history") are not often included in risk assessment models, despite scientific evidence demonstrating its relevance (Clemente et al., 2020; den Besten et al., 2017; Garre et al., 2018a). This could be due to the complexity of microbial systems and the difficulty of controlling for all variables make it challenging to investigate these differences comprehensively. However, ongoing research in this field is gradually shedding light on the impact of microbial history on cellular behaviour and response to various stimuli.

Our study agrees with these studies, emphasizing the relevance of physiological sources of variability in inactivation kinetics. As illustrated in Figure 8, stress acclimation at low pH during incubation can increase the resistance of the *S.* Enteritidis strain tested up to a 3-fold factor. This increase is similar to the difference observed between *Salmonella* strains in the screening by Guillén et al. (2020), proving that physiological sources of variability are as relevant as the genetic ones for bacterial survival to pasteurization treatments. Therefore, these sources should be incorporated into QMRA models.

Furthermore, physiological sources of variability could be easier to implement than genetic ones into QMRA models, in some scenarios. Although some pioneering studies provided evidence for specialised bacterial phenotypes in some products (Liao et al., 2021; Maury et al., 2019) that could be used to reduce the uncertainty of QMRA models, this information is still scarce, costly to obtain (requires very extensive sampling) and sensitive to any bias in the sampling.

On the other hand, the physiological sources of variability identified in this study could be more easily mapped to different contamination routes. As argued above, the pre-adaptation experiments would represent bacterial cells that have survived for a long time into an acidic environment. As an illustration, we will use a theoretical smoothie with a combination of figs

(pH 5), leek (pH 5.5), asparagus (pH6) and water (pH7). If we assign to each of these products the inactivation models fitted for each pre-incubation condition (illustrated in Figures 8-9), we would calculate different survival curves for *S.* Enteritidis depending on the origin of the contamination, as illustrated in Figure 11. This implies that there would be a strong link between the ingredient responsible for the introduction of the pathogen and its survival to the thermal treatment (e.g., contaminated figs would be more relevant than contaminated water). Although this is a hypothetical illustration, obtaining data on prevalence and bacterial concentrations on each ingredient is relatively simple (indeed, it is part of the quality assurance plan of most companies), so it is feasible to adapt the model predictions to "real" industrial data. This could largely contribute to reduce the uncertainty of the risk assessment and lead to more effective food safety interventions targeted at the most relevant product considering not only the prevalence of bacterial pathogens, but also its impact on bacterial survival.

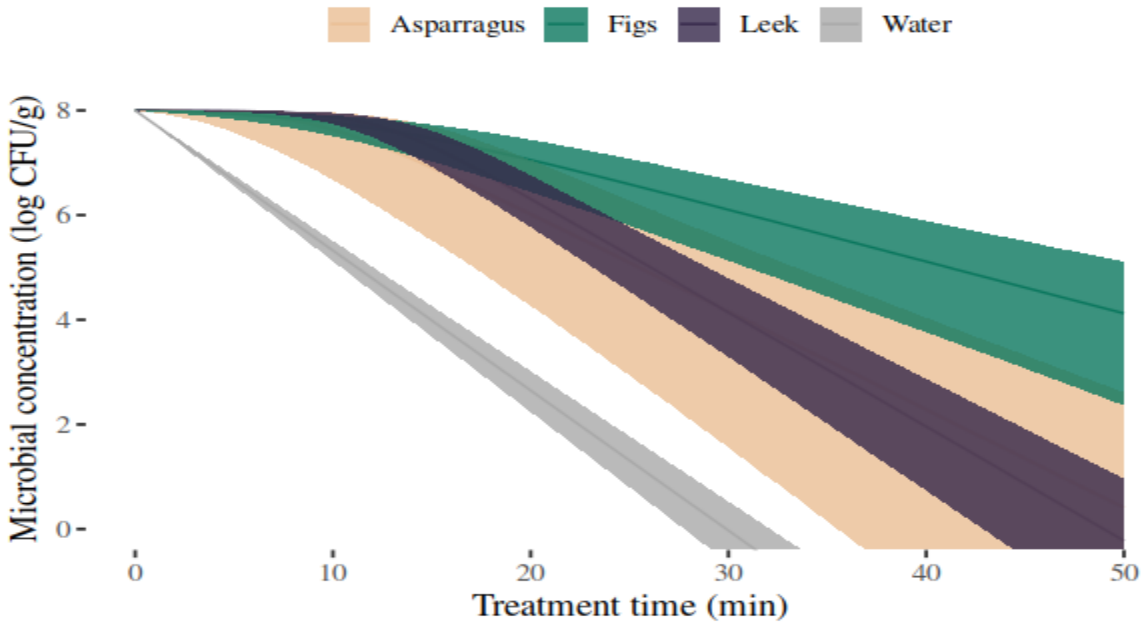


Figure 11. Theoretical illustration of the reduction of *S.* Enteritidis at 55ºC, in a smoothie with a combination of cherries (pH 4.5), figs (pH 5), leek (pH 5.5), asparagus (pH6.0) and water (pH7) based on the Geeraerd model fitted for each condition (supp. Figure 1). This scenario considers that *S.* Enteritidis has survived for a long time into an acidic product.

4. Conclusions

Variability sources for the bacterial response to thermal treatments can appear in many forms. In this study we analysed phenotypic variability (differences among cells of the same strain linked to different history) of *Salmonella* spp. In particular, we studied the effect of acidic pre-incubation conditions or the application of an acid shock on the thermal resistance of two *Salmonella* strains. For the *S.* Enteritidis one (a reference strain), we observed that pre-incubation at pH 4.5 led to a 3-fold increase in D-value with respect to control conditions and the emergence of shoulders in the survivor curves. This increase is of the same order of magnitude as previously reported for strain variability. On the other hand, the thermal resistance of the *S.* Senftenberg strain (a highly resistant variant) remained constant (or was reduced) when incubated at acidic conditions or after an acid shock. Therefore, the magnitude (and direction) of phenotypic variability would be strain-dependent. Finally, we illustrated that phenotypic variability would be relatively simple to implement in microbial risk assessment models (e.g., different incubation conditions could be linked to the contamination from different ingredients). Therefore, considering its relevance and its feasibility to include in the analysis, phenotypic variability should become a main focus in predictive microbiology.

5. Supplementary material Chapter II

Supp. Table 2. *S.* Enteritidis numeric values. Phenotypic variability was studied performing experiment for control cells (stationary phase cells incubated at pH 7) and cells incubated at suboptimal pH (5, 5.5 and 6) or subject to an acid shock (1h at pH 4.5, 5, 5.5 or 6). For these conditions, we tested cells on both the stationary and exponential growth phases.

Exponential phase *S.* Enteritidis at 55°C

	Control	Pre-adaptation			Acid -Shock			
Value	pH 7	pH 5	pH 5.5	pH 6	pH 4.5	pH 5	pH 5.5	pH 6
D	3.75	3.48	4.51	2.89	3.5	4.04	4.22	3.46
D (SE)	0.18	0.36	0.39	0.36	0.29	0.41	0.25	0.4
shl D	0	13.59	12.83	9.27	6.62	5.41	0	8.41
Shl D (SE)	0	1.05	1	1.6	1.56	2.03	0	1.85

Stationary phase *S.* Enteritidis at 55°C

	Control	Pre-adaptation			Acid -Shock			
Value	pH 7	pH 5	pH 5.5	pH 6	pH 4.5	pH 5	pH 5.5	pH 6
D	3.74	9.99	4.58	5.36	4.81	2.86	3.8	3.67
D (SE)	0.21	2.37	0.57	1.51	0.37	0.11	0.13	0.31
shl D	0	10.93	12.27	9.23	0	7.43	0	8.55

Shl D (SE)	0	3.51	1.5	4.48	0	0.69	0	1.34

Exponential phase *S.* Enteritidis at 60°C

	Control	Pre-adaptation			Acid -Shock			
Value	pH 7	pH 5	pH 5.5	pH 6	pH 4.5	pH 5	pH 5.5	pH 6
D	0.08	0.14	0.16	0.09	0.15	0.1	0.11	0.1
D (SE)	0.01	0.02	0.01	0.01	0.02	0	0.01	0.01
shl D	0	0.23	0	0.28	0.11	0.12	0	0.1
Shl D (SE)	0	0.07	0	0.03	0.08	0.03	0	0.03

Stationary phase *S.* Enteritidis at 60°C

	Control	Pre-adaptation			Acid -Shock			
Value	pH 7	pH 5	pH 5.5	pH 6	pH 4.5	pH 5	pH 5.5	pH 6
D	0.08	0.32	0.18	0.15	0.14	0.1	0.08	0.08
D (SE)	0.01	0.13	0.02	0.02	0.01	0.01	0.01	0
shl D	0	0.1	0.35	0.07	0	0	0.16	0.19
Shl D (SE)	0	0.25	0.04	0.08	0	0	0.04	0.02

Supp. Table 3. *S.* Senftenberg numeric values. Phenotypic variability was studied performing experiment for control cells (stationary phase cells incubated at pH 7) and cells incubated at suboptimal pH (5, 5.5 and 6) or subject to an acid shock (1h at pH 4.5, 5, 5.5 or 6). For these conditions, we tested cells on both the stationary and exponential growth phases.

Exponential phase of *S.* Senftenberg at 60°C

	Control	Pre-adaptation			Acid -Shock			
Value	pH 7	pH 5	pH 5.5	pH 6	pH 4.5	pH 5	pH 5.5	pH 6
δ	1.48	1.25	1.06	1.17	6.19	2.91	4.05	4.99
δ (SE)	0.11	0.24	0.45	0.18	0.59	0.87	0.52	0.22
p	0.68	0.67	0.61	0.66	1.14	0.91	1.12	1.27
p (SE)	0.04	0.05	0.09	0.04	0.11	0.15	0.1	0.05

Stationary phase of *S.* Senftenberg at 60°C

	Control	Pre-adaptation			Acid -Shock			
Value	pH 7	pH 5	pH 5.5	pH 6	pH 4.5	pH 5	pH 5.5	pH 6
δ	2.62	2.39	3.4	2.26	2.83	3.98	4.26	4.1
δ (SE)	1.85	0.38	0.6	0.4	1.04	0.56	0.29	0.37
p	0.63	0.81	0.81	0.79	0.88	1.03	1.2	1.19
p (SE)	0.25	0.06	0.09	0.07	0.18	0.1	0.06	0.08

Exponential phase of *S.* Senftenberg at 65°C

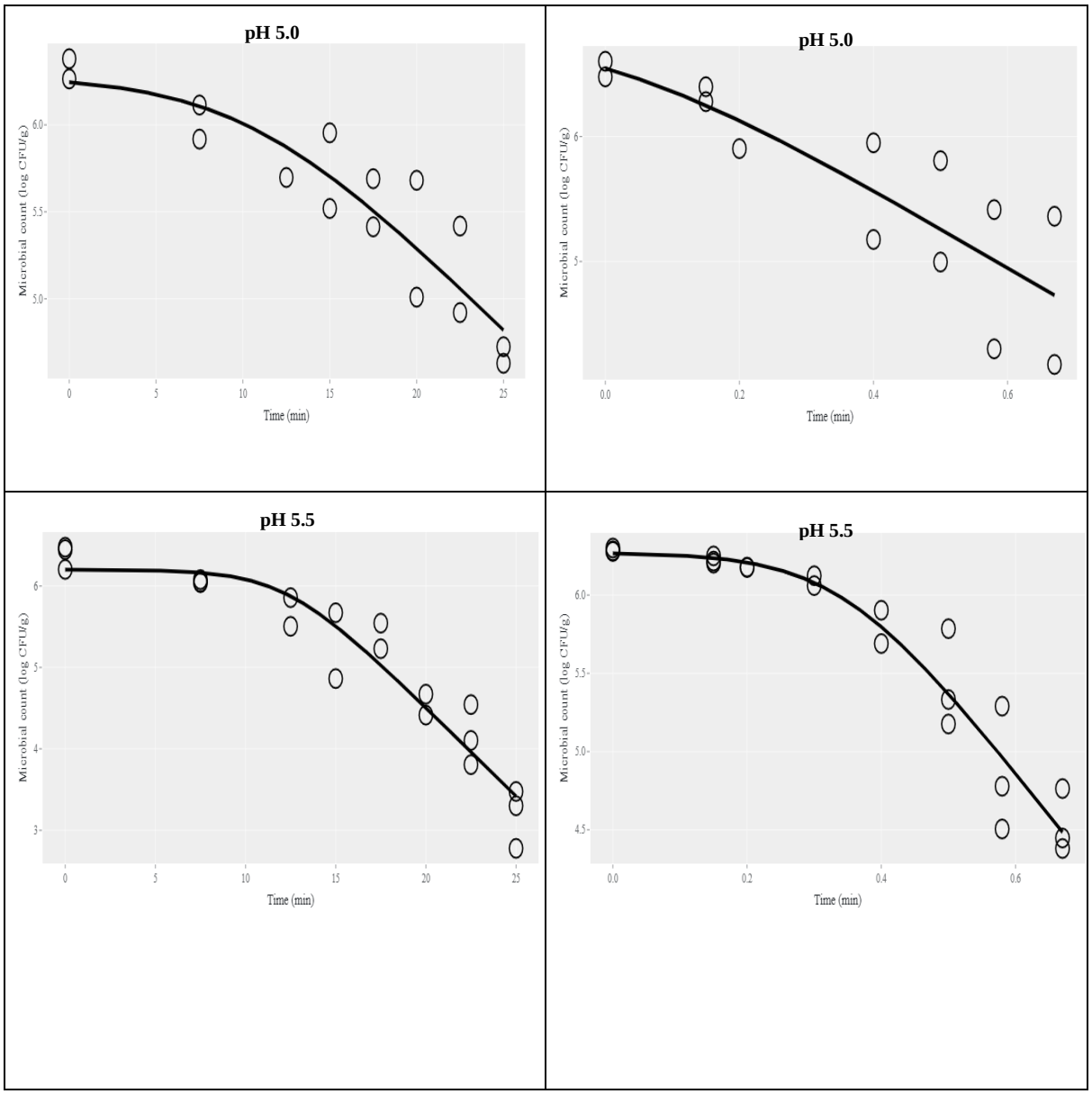
Value	Control	Pre-adaptation	Acid -Shock

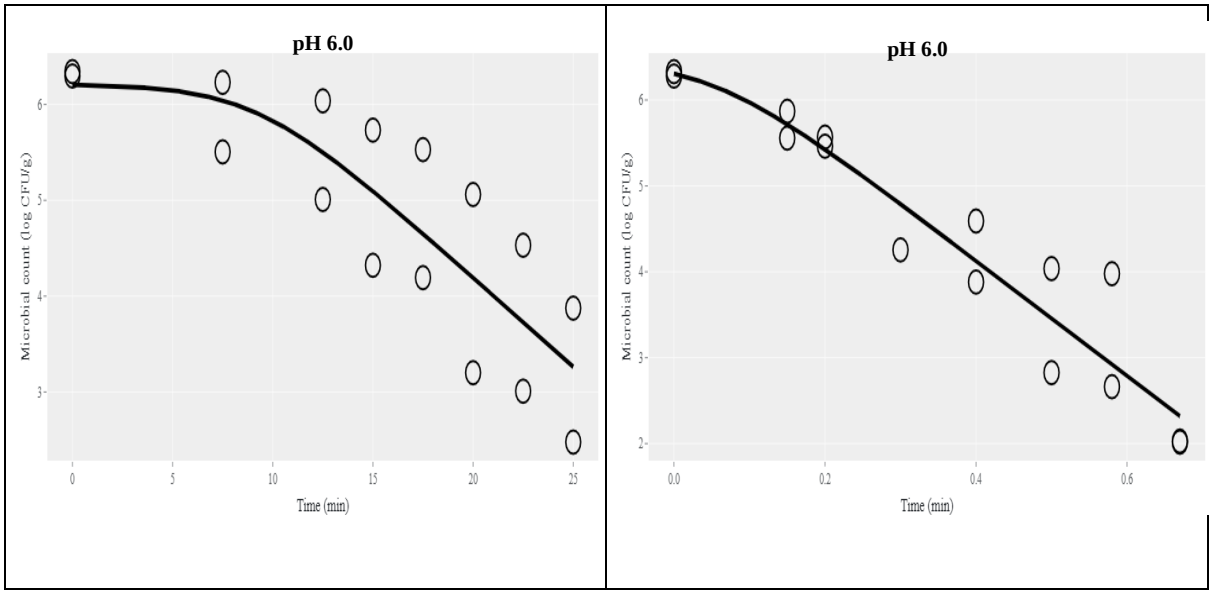
	pH 7	pH 5	pH 5.5	pH 6	pH 4.5	pH 5	pH 5.5	pH 6
δ	0.09	0.03	0.05	0.02	0.1	0.12	0.15	0.13
δ (SE)	0.02	0.02	0.02	0.01	0.07	0.03	0.03	0.07
p	0.62	0.42	0.57	0.4	0.59	0.66	0.67	0.63
p (SE)	0.04	0.05	0.06	0.06	0.12	0.05	0.05	0.11

Stationary phase of *S*. Senftenberg at 65°C

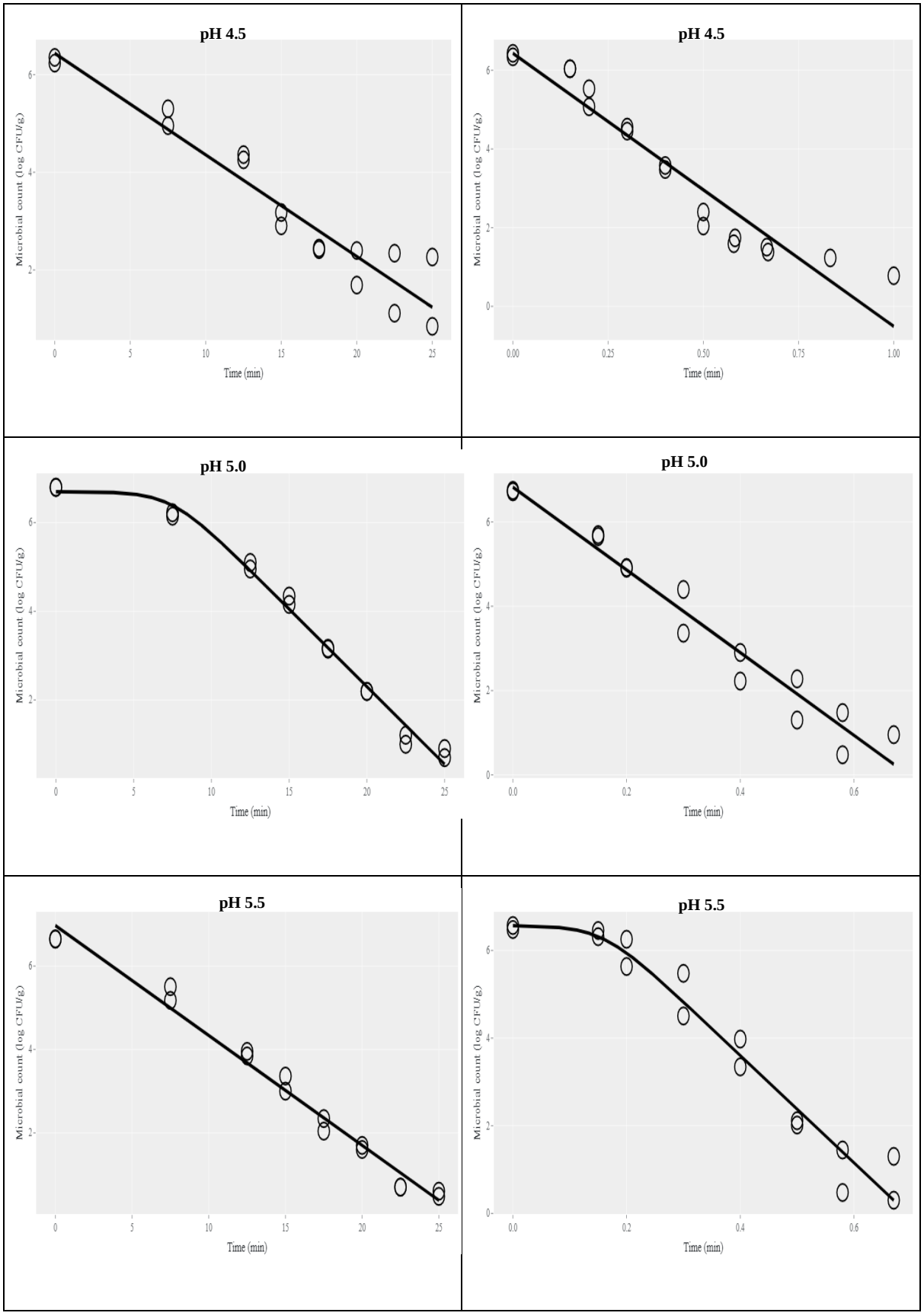
	Control	Pre-adaptation			Acid -Shock			
Value	pH 7	pH 5	pH 5.5	pH 6	pH 4.5	pH 5	pH 5.5	pH 6
δ	0.18	0.03	0.04	0.01	0.12	0.2	0.2	0.14
δ (SE)	0.08	0.05	0.03	0.01	0.03	0.03	0.04	0.03
p	0.57	0.39	0.47	0.35	0.66	0.72	0.88	0.76
p (SE)	0.08	0.14	0.08	0.07	0.05	0.04	0.08	0.05

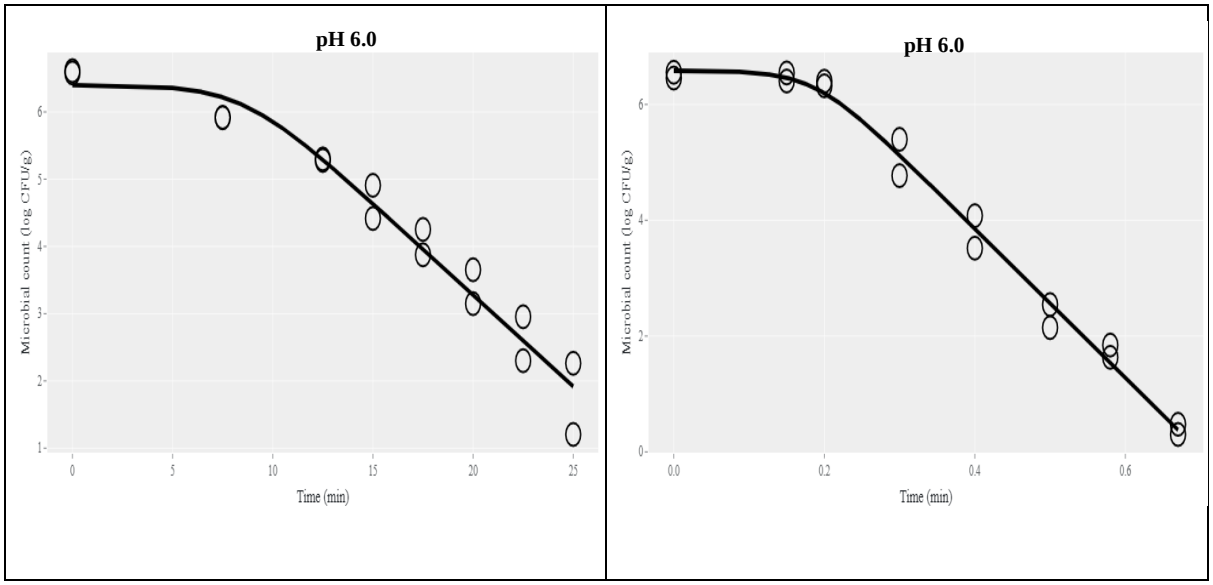
Supplementary Figures



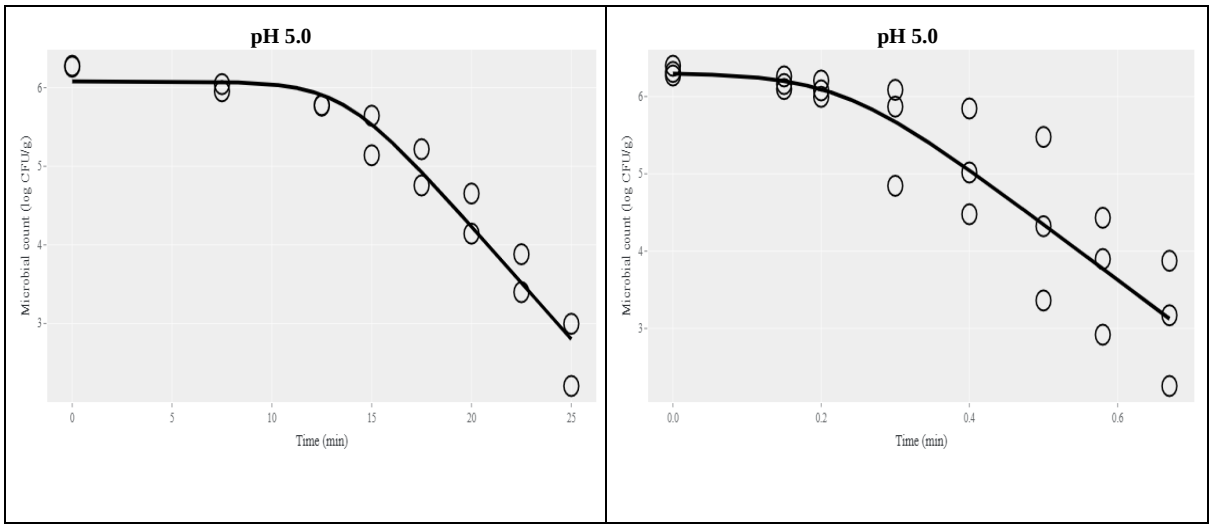


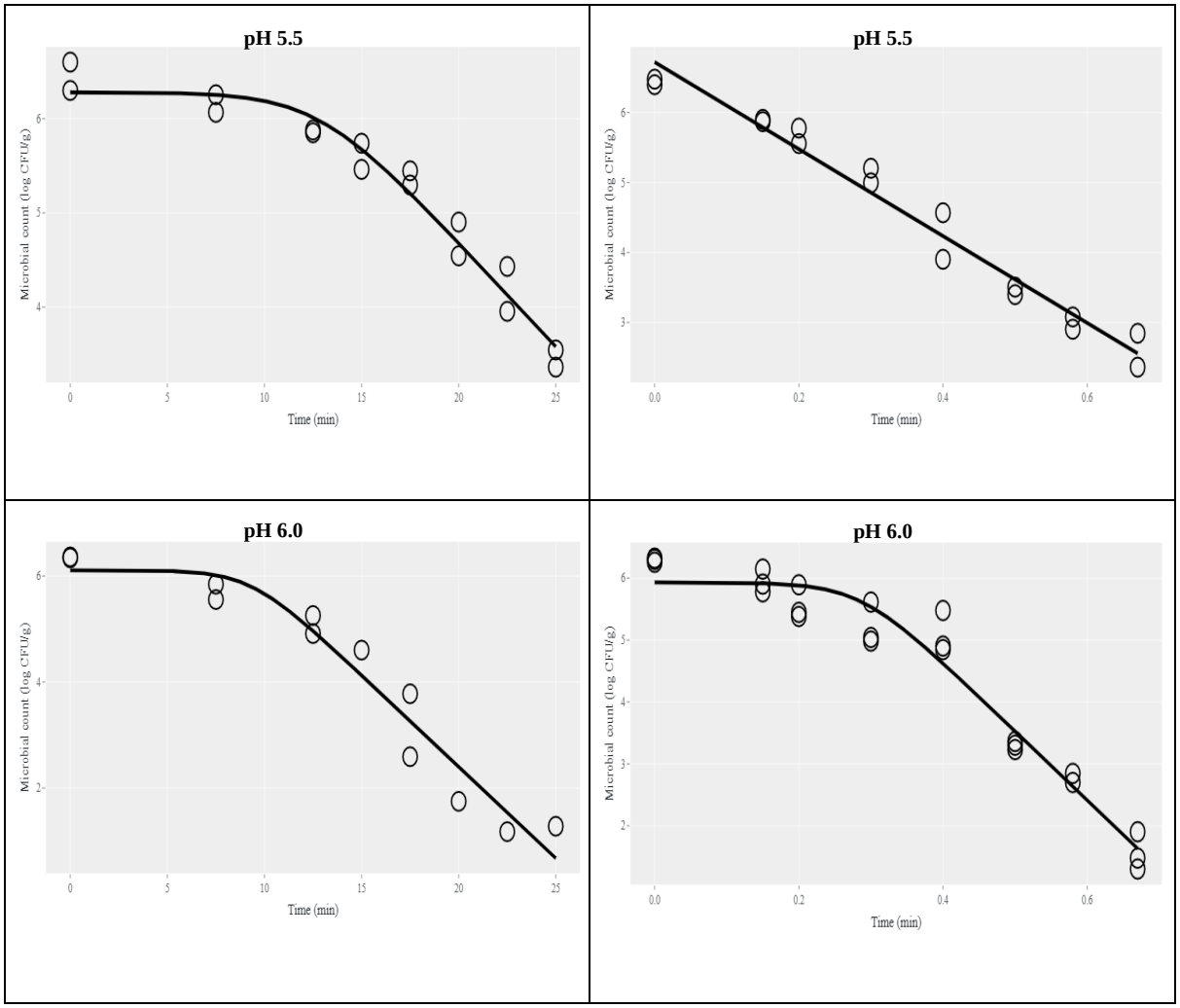
Supp. Figure 1. Survivor curves of *S*. Enteritidis stationary phase at 55°C (left side) and 60°C (right side) for ***pre-adapted cells***, using the Geeraerd model.


pH 4.5
Microbial count (log CFU/g)
Time (min)
pH 4.5
Microbial count (log CFU/g)
Time (min)
pH 5.0
Microbial count (log CFU/g)
Time (min)
pH 5.0
Microbial count (log CFU/g)
Time (min)
pH 5.5
Microbial count (log CFU/g)
Time (min)
pH 5.5
Microbial count (log CFU/g)
Time (min)

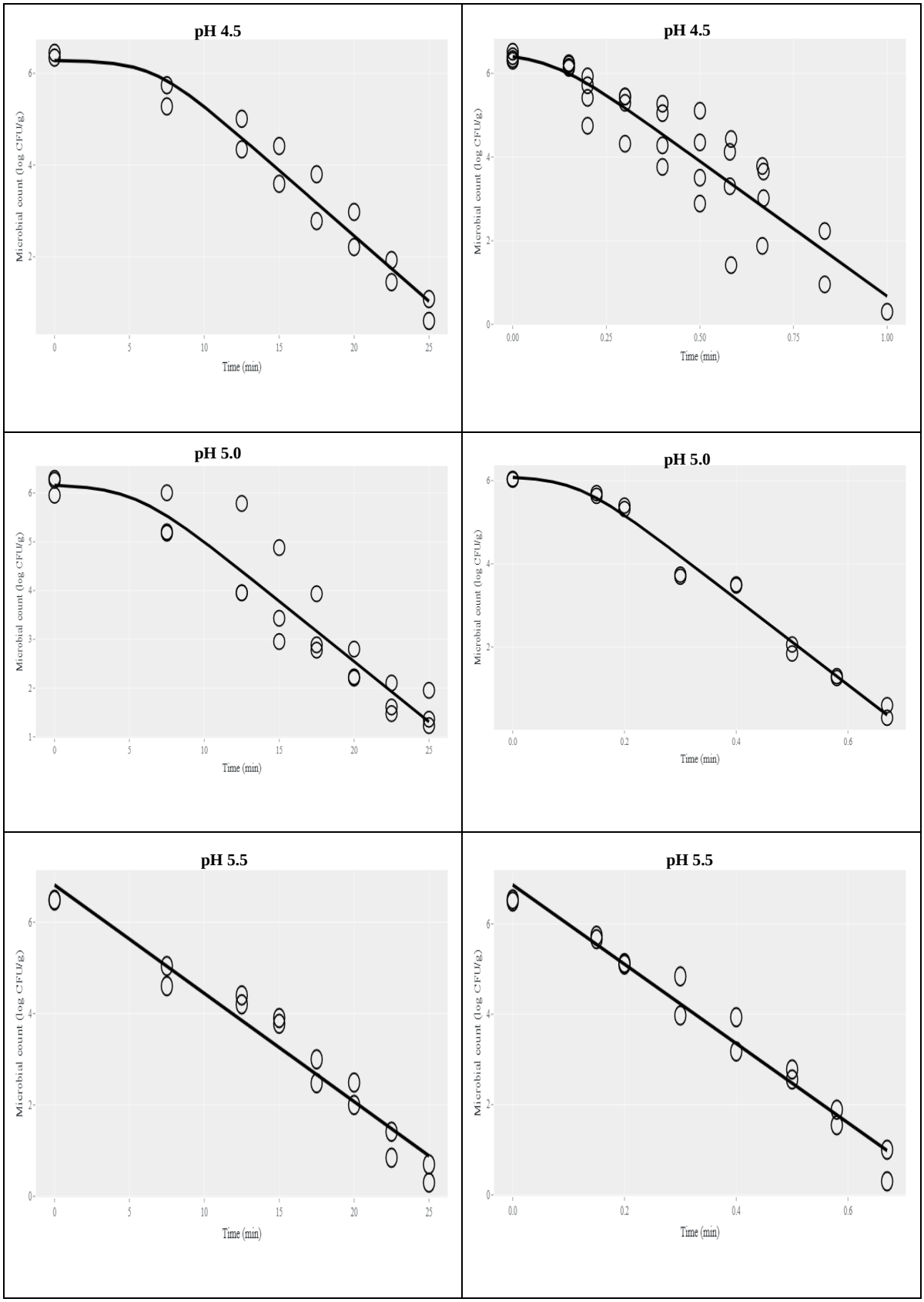


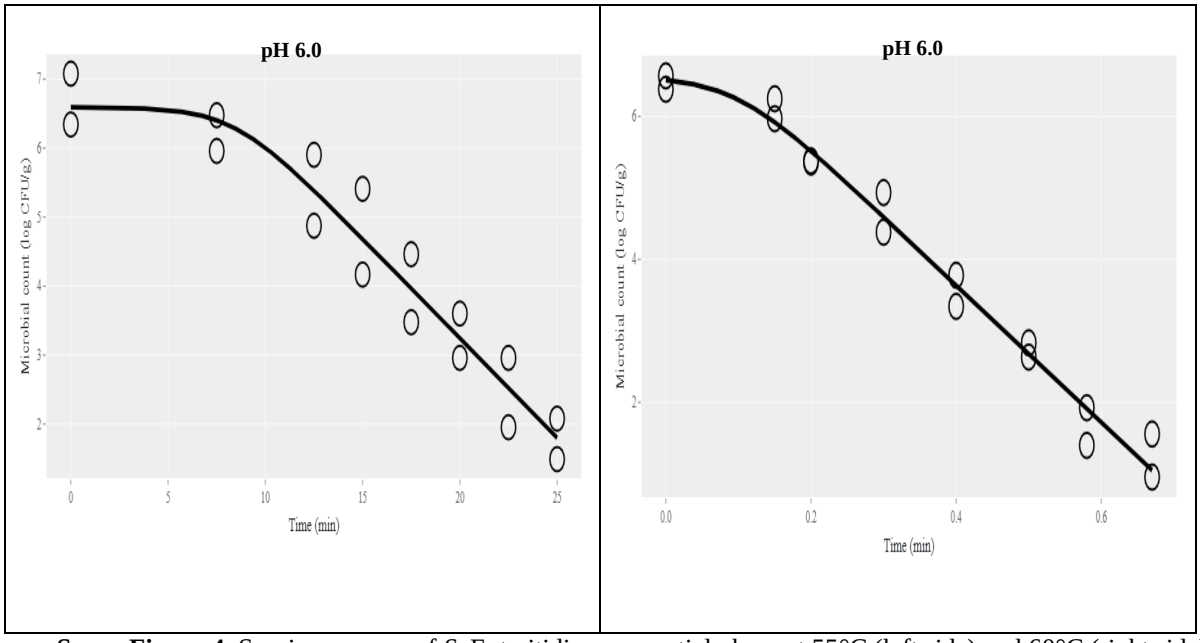
Supp. Figure 2. Survivor curves of *S.* Enteritidis stationary phase at 55°C (left side) and 60°C (right side) for ***acid-shocked cells***, using the Geeraerd or Bigelow model.



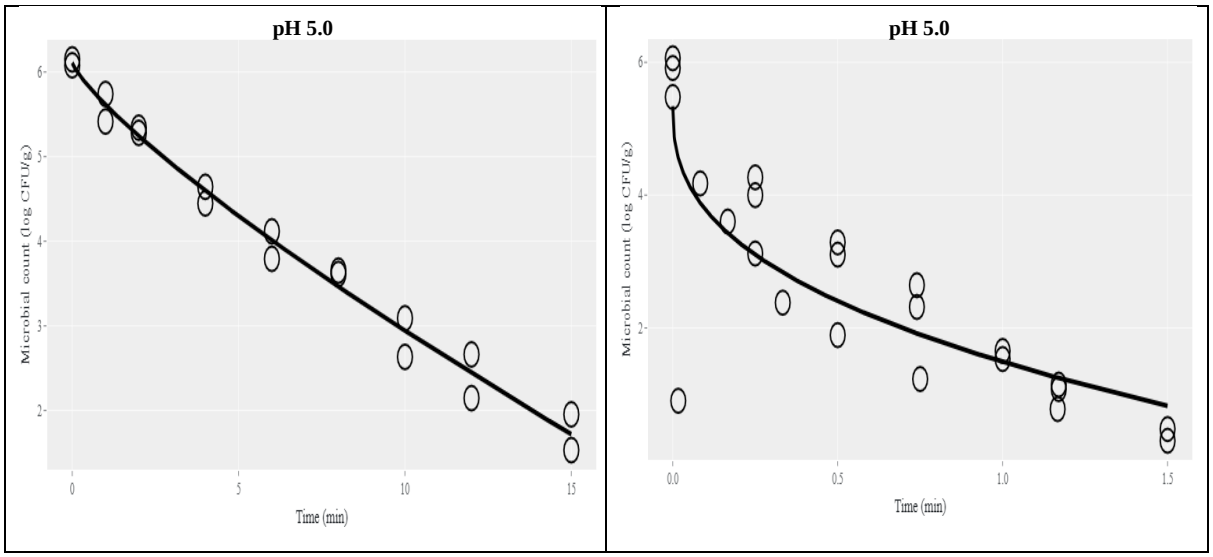


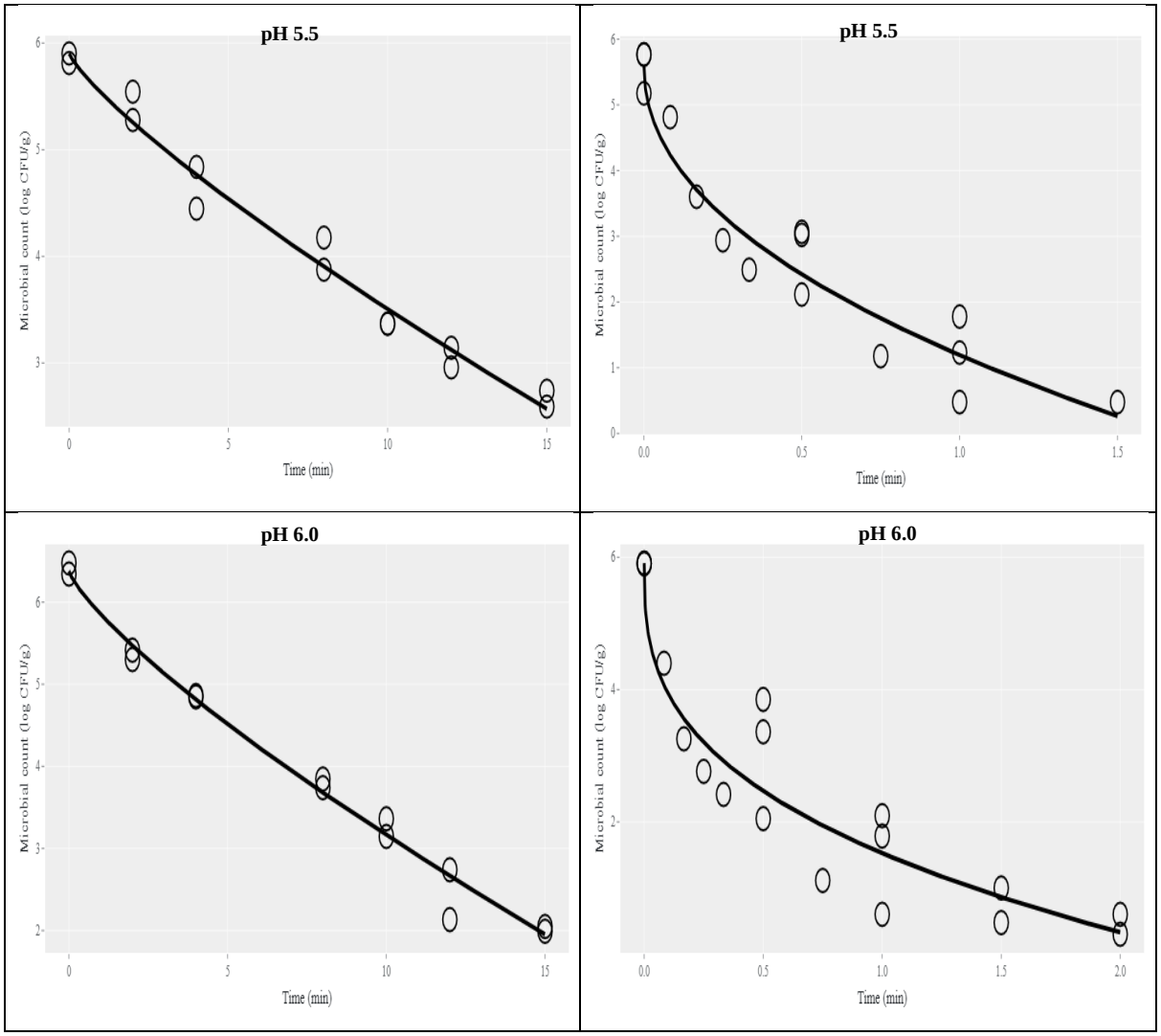
Supp. Figure 3. Survivor curves of *S*. Enteritidis exponential phase at 55°C (left side) and 60°C (right side) for ***pre-adapted cells***, using the Geeraerd or Bigelow model.


pH 4.5
Microbial count (log CFU/g)
Time (min)
pH 4.5
Microbial count (log CFU/g)
Time (min)
pH 5.0
Microbial count (log CFU/g)
Time (min)
pH 5.0
Microbial count (log CFU/g)
Time (min)
pH 5.5
Microbial count (log CFU/g)
Time (min)
pH 5.5
Microbial count (log CFU/g)
Time (min)

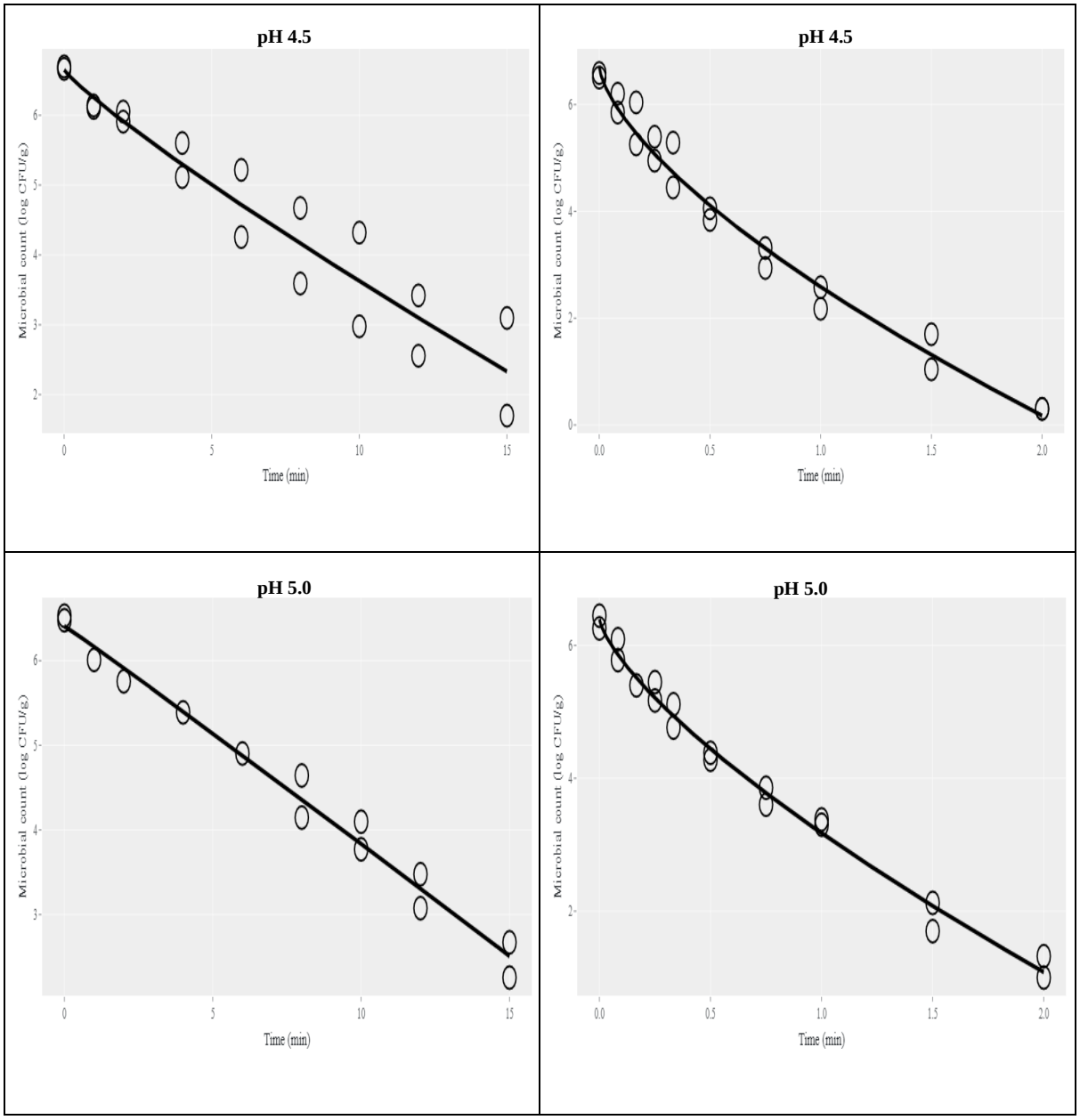


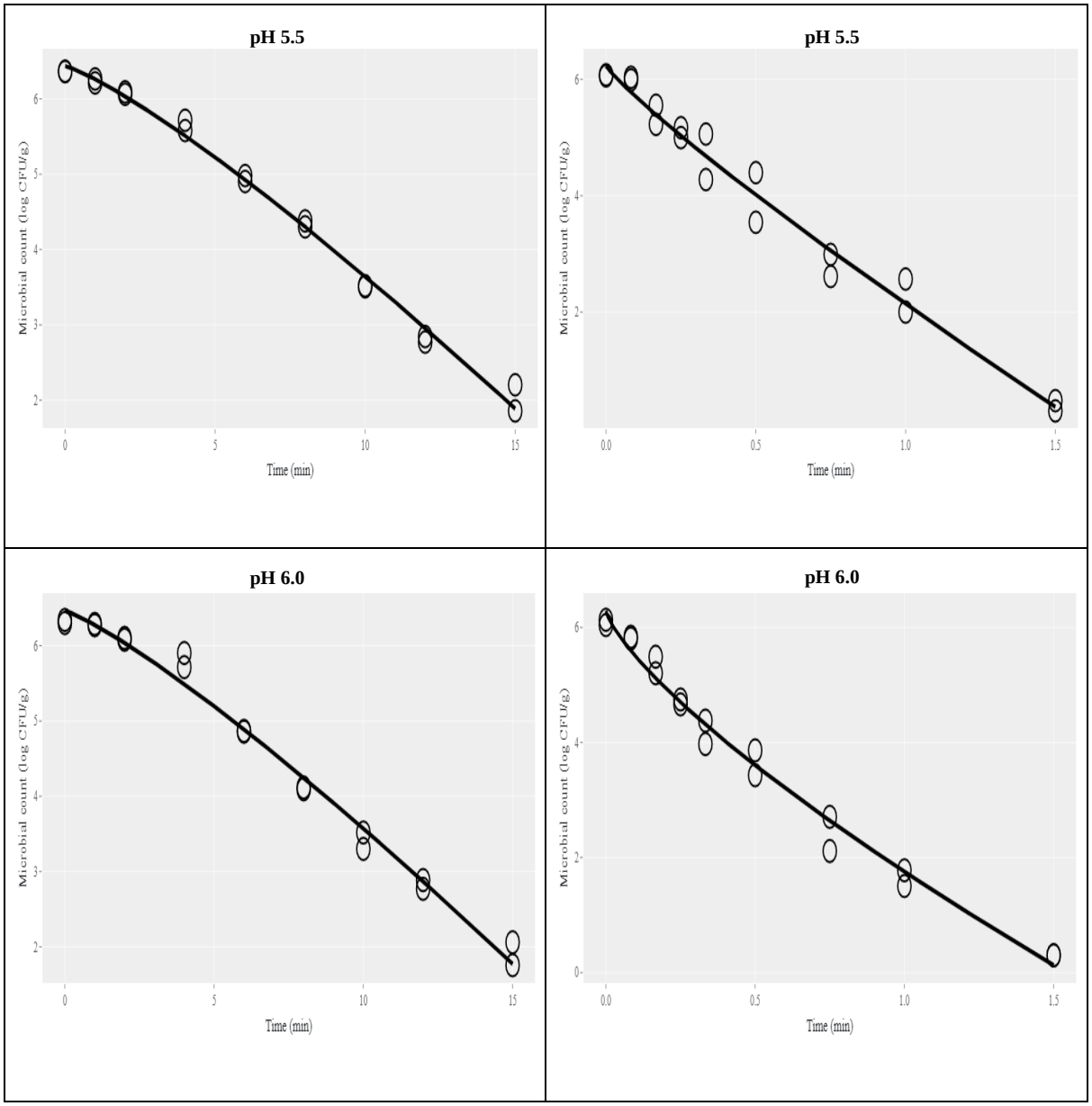
Supp. Figure 4. Survivor curves of *S.* Enteritidis exponential phase at 55°C (left side) and 60°C (right side) for ***acid-shocked cells***, using the Geeraerd or Bigelow model.



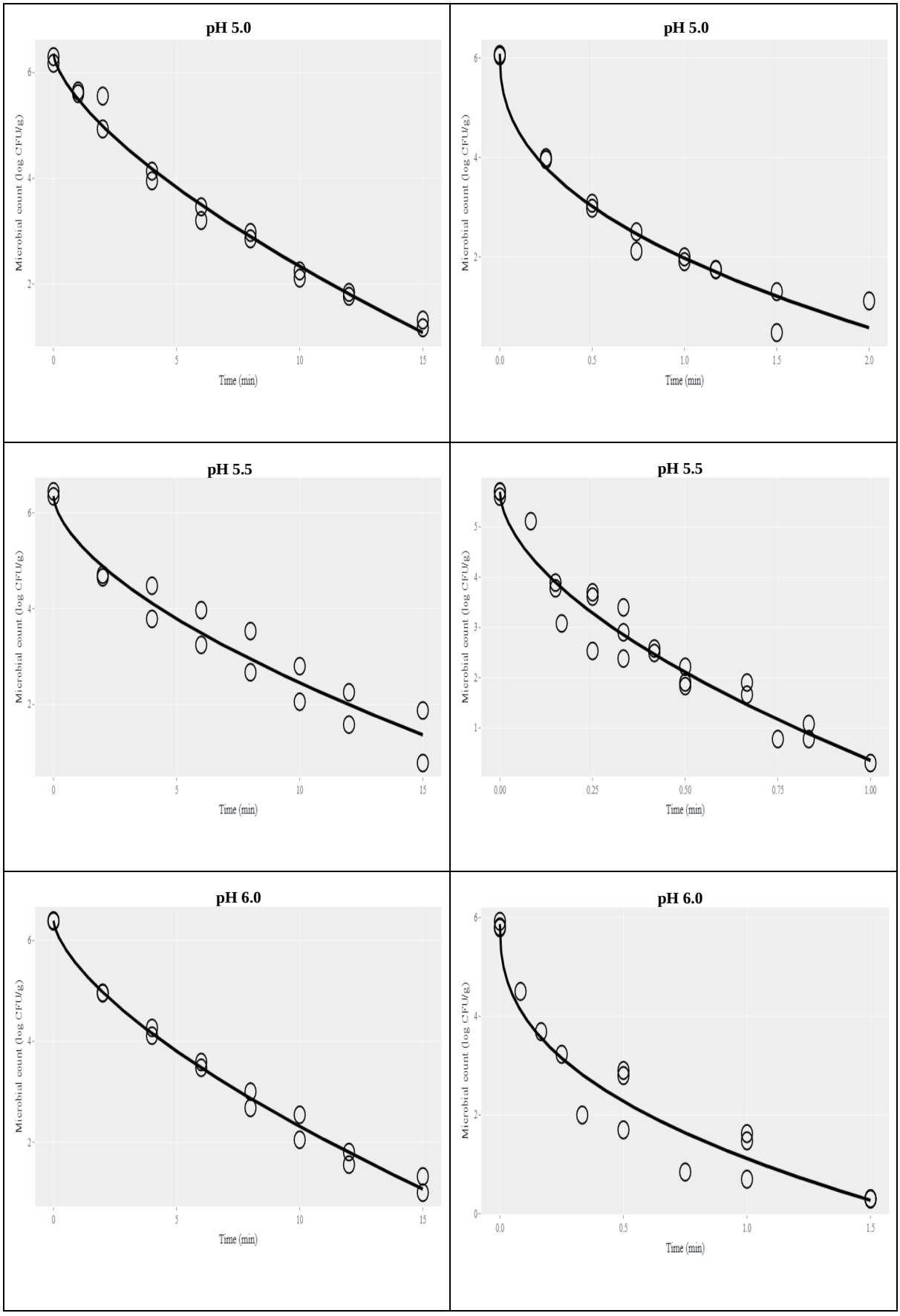


Supp. Figure 5. Survivor curves of *S.* Senftenberg stationary phase at 60°C (left side) and 65°C (right side) for ***pre-adapted cells***, using the Mafart model.

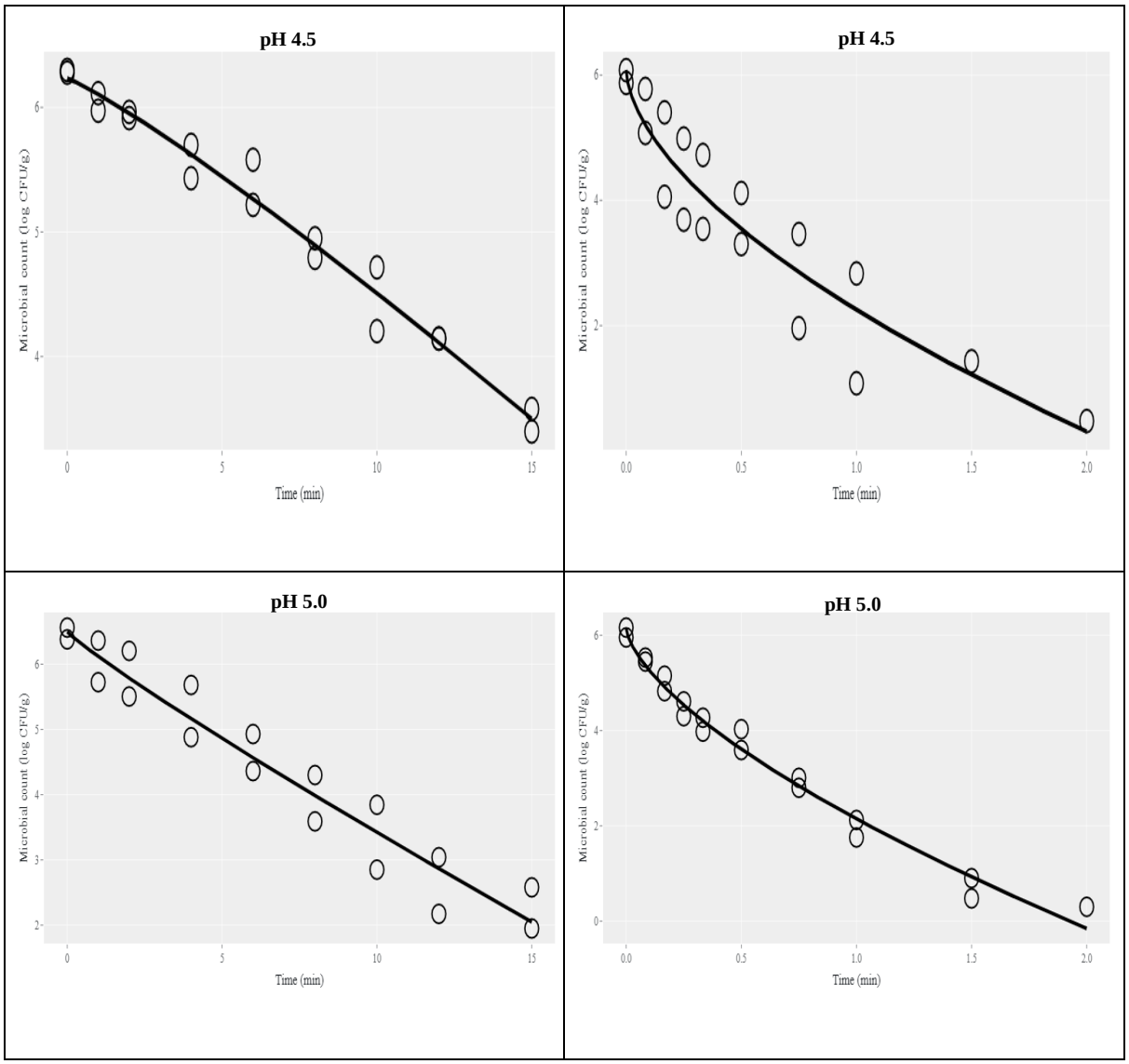

pH 4.5
Microbial count (log CFU/g)
Time (min)
pH 4.5
Microbial count (log CFU/g)
Time (min)
pH 5.0
Microbial count (log CFU/g)
Time (min)
pH 5.0
Microbial count (log CFU/g)
Time (min)

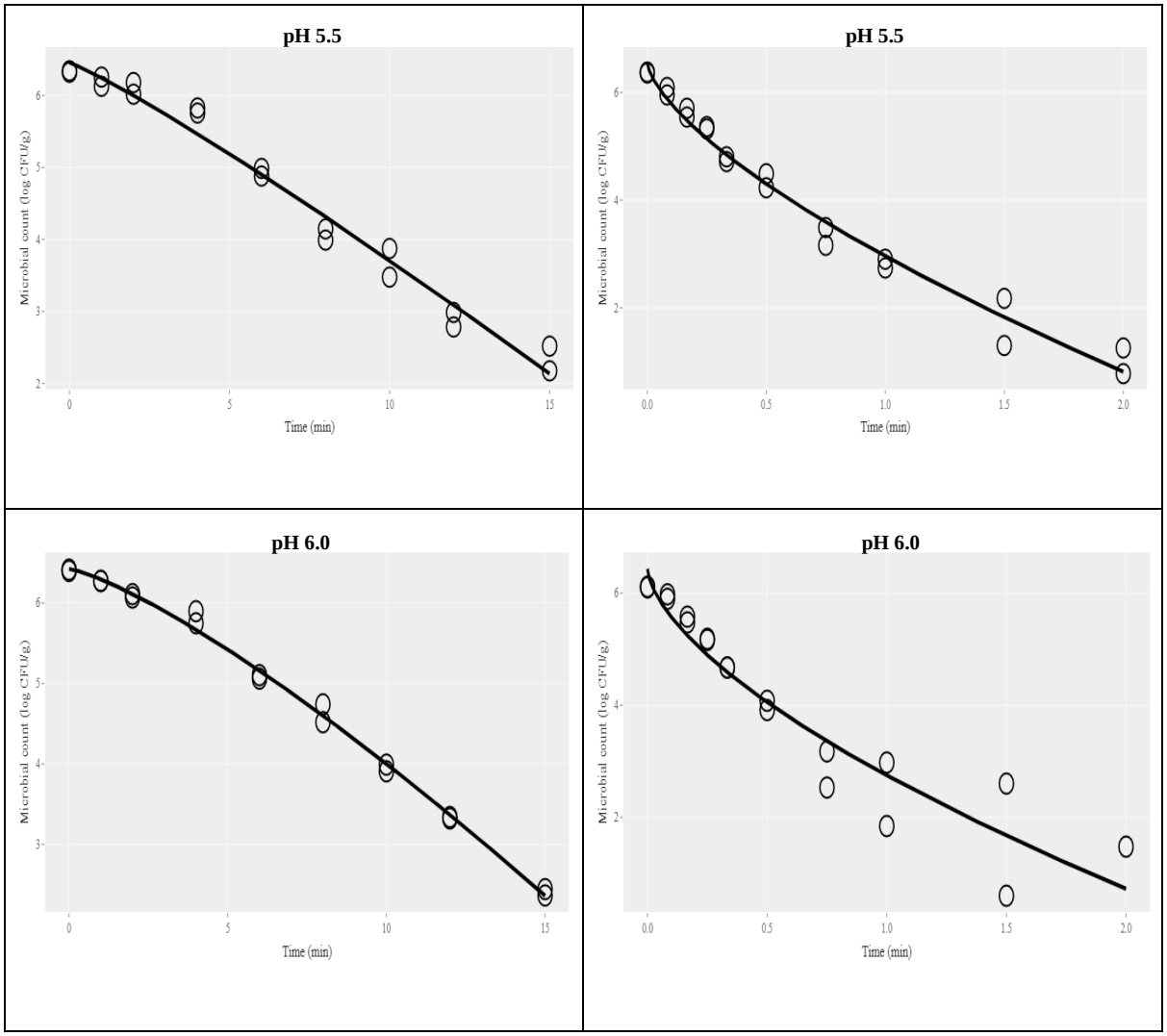


Supp. Figure 6. Survivor curves of *S.* Senftenberg stationary phase at 60°C (left side) and 65°C (right side) for ***acid-shocked cells***, using the Mafart model.


pH 5.0
Microbial count (log CFU/g)
Time (min)
pH 5.0
Microbial count (log CFU/g)
Time (min)
pH 5.5
Microbial count (log CFU/g)
Time (min)
pH 5.5
Microbial count (log CFU/g)
Time (min)
pH 6.0
Microbial count (log CFU/g)
Time (min)
pH 6.0
Microbial count (log CFU/g)
Time (min)

Supp. Figure 7. Survivor curves of *S.* Senftenberg exponential phase at 60°C (left side) and 65°C (right side) for ***pre-adapted cells***, using the Mafart model.





Supp. Figure 8. Survivor curves of *S.* Senftenberg exponential phase at 60°C (left side) and 65°C (right side) for ***acid-shocked cells***, using the Mafart model.

Acknowledgments

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3.3 Chapter III

Unravelling the causes for nonlinearities in bacterial survival curves - initial heterogeneities or dynamic stress adaptation?

Abstract

In this study, we analysed the thermal resistance of vegetative cells of *Bacillus subtilis* under isothermal (51, 52.5 and 55ºC) and dynamic conditions, using both a wild type strain and a marker-free *sigB* null mutant. At 52.5 and 55ºC, we observed survivor curves with an upwards curvature. This common deviation from log-linearity is most often considered an upshot of an initial heterogeneity in the thermal resistance of cells within the population (vitalistic hypothesis). However, the application of a pretreatment (48ºC/5 min) resulted in loglinear survivor curves, an outcome hardly compatible with vitalistic assumptions. Survivor curves at 51ºC were initially log-linear, with the pretreatment inducing a shoulder, again a result hardly compatible with vitalistic hypotheses.

Using predictive microbiology, we observed that the inactivation was biphasic at 52.5 and 55ºC, with the inactivation rate during the second phase being practically the same as the one obtained after the application of a pretreatment. Hence, our results were interpreted based on stress adaptation. We hypothesised that lethal treatment temperatures induced an adaptive response in the cell similar to the one taking place with the pretreatment at 48ºC. Then, if bacterial cells survive the lethal treatment for long enough (due to chance, not due to an induced higher heat resistance), an increase in their thermal resistance resulting in an upwards curvature in the survivor curve was observed. Similar arguments can explain the results obtained at 51ºC.

Due to the similarities of these assumptions with those supporting stress acclimation under dynamic conditions, we defined bounds that would account for acclimation based on the isothermal data. These bounds include the microbial inactivation observed for a heating rate of 10ºC/min and 5ºC/min, but concentrations at 1ºC/min are evidently higher than expected. This would imply that the impact of stress acclimation would be higher during slow heating than for

isothermal conditions. In conclusion, our study provides an alternative interpretation for bacterial survivor curves. This can advance our understanding of microbial inactivation by heat, improving our ability to predict the microbial response during pasteurisation treatments.

Keywords: predictive microbiology; microbial inactivation; *Bacillus subtilis*; pasteurisation; variability, SigB

Highlights:

- Nonlinear survivor curves for isothermal treatments can be interpreted using different hypotheses
- Dynamic stress adaptation was identified as a potential cause of nonlinear survivor curves
- We use isothermal results to define bounds for potential adaptation during dynamic treatments
- The effect of dynamic stress acclimation seems larger than adaptation under isothermal conditions

1. Introduction

Thermal processing has traditionally been the main preservation technique used in the food industry for most products to improve food safety and extend the product's shelf life. In this type of process, pathogens and spoilage microorganisms are inactivated due to the application of heat at specific temperatures and for a specific time (Van Impe et al., 2018). However, these conventional thermal treatments can negatively influence the sensorial properties (flavour, taste), as well as the nutritional value of the food. Considering the growing consumer demand for products that are more "natural" (Battacchi et al., 2020), there is a current shift towards milder heat treatments in the food industry (Peng et al., 2017).

Mathematical models from predictive microbiology are a useful tool for the design of mild heat treatments (Allende et al., 2022; Alvarenga et al., 2022). These models aim to predict the microbial inactivation attained for a combination of treatment temperature and time. Although this question has been a matter of scientific study for roughly a century, there are still large knowledge gaps in the field, such as the high complexity of the molecular mechanisms responsible for microbial inactivation (Richter et al., 2010b; Smelt & Brul, 2014). As a result, predictive models cannot be defined based on fundamental knowledge; they are empirical models with (interpretable) parameters that are estimated from experimental data (Perez-Rodriguez & Valero, 2013).

Hence, microbial inactivation experiments are an essential part of the model definition. If every bacterial cell in a population had the same probability of surviving a given heat treatment (i.e., the same heat resistance) and their resistance remained unchanged, survivor curves observed empirically under isothermal conditions would always be log-linear (Garre et al., 2021b). However, deviations from linearity are relatively common (van Boekel, 2002). There

are two main hypotheses to justify this deviation (Aspridou & Koutsoumanis, 2020). The *vitalistic* approach assumes that there are initial differences between the cells of the bacterial population. Hence, nonlinear survivor curves would be a representation of the weaker members of the population (either a subpopulation or a smooth distribution) being inactivated earlier. On the other hand, the *mechanistic* approach introduces two different hypotheses. The first one is that microbial inactivation would be a multi-hit process. Consequently, every cell in the bacterial population would be able to resist the treatment for a given time, resulting in survivor curves with an initial horizontal phase (shoulder). The second one defines that tailing in the survivor curves (a maximum number of reductions in the bacterial concentration that cannot be surpassed regardless of the treatment duration) would be due to the existence of extremely resistant cells in the population (i.e., with similar arguments as in the vitalistic approach).

Although the vitalistic and mechanistic hypotheses are generally accepted for the interpretation of bacterial survivor curves under isothermal conditions, the emergence of laboratory equipment able to apply thermal treatments under dynamic conditions has emphasised the challenges associated with extrapolating these hypotheses to non-isothermal treatments. Empirical studies have shown that models based on isothermal data often fail at predicting microbial inactivation under dynamic conditions (Dolan et al., 2013; Hassani et al., 2006; Janssen et al., 2008). This is most likely due to the emergence of phenomena that are specific to dynamic heating conditions and cannot be observed in isothermal experiments. This is of great concern because industrial heat treatments are always dynamic (ingredients cannot be heated up immediately).

Stress acclimation is an example of that type of dynamic phenomenon. It considers that bacterial cells are not static but have a series of stress response mechanisms to increase their

probability of survival. If the heating rate is slow, these mechanisms may activate during the initial part of a dynamic treatment, resulting in higher stress resistance to the sublethal part of the treatment than expected based on isothermal experiments. In previous studies by our group, we have been able to quantify the relevance of stress acclimation for different bacterial species using a dynamic model, estimating that it may increase the *D*-value by up to a 10-fold factor (Clemente et al., 2020; Garre et al., 2018a; Georgalis et al., 2022). Therefore, stress acclimation can be of high relevance for the processing of some products, where the desired reduction of the microbial load would not be attained due to the ability of bacterial cells to dynamically adapt to the heat treatment.

One of the main challenges for the study of stress acclimation is that its quantification requires specific equipment and complex mathematical methods (dynamic models). In this study, we propose that these restrictions could be circumvented by exploiting the potential influence of stress acclimation on inactivation under isothermal conditions. Although stress acclimation has been mostly associated with heating at sub-lethal temperatures, the bacteria respond in a similar way to the lethal temperatures used in mild pasteurization treatments. Therefore, if the intensity of the (lethal) treatment is low (i.e., low temperature), stress acclimation may emerge before microbial inactivation, resulting in survivor curves with an upwards curvature. Although this effect has only been suggested hypothetically (Garre et al., 2022), it could provide an alternative explanation of isothermal inactivation to the mechanistic and vitalistic ones.

Hence, the goal of this study is to evaluate the potential relevance of stress acclimation in bacterial survivor curves under isothermal conditions and how they differ from inactivation under dynamic treatments. We compared such differences using the vegetative cells of the

Bacillus subtilis wild-type strain and the Δ*sigB* null mutant that lacked the master regulator of stress (SigB). The knockout of *sigB* caused the missing regulation on the SigB regulon, which is one of the largest general stress adaptation mechanisms in *B. subtilis* when exposed to environmental stress, such as heat (Rodriguez Ayala et al., 2020).

2. Materials and Methods

2.1 Bacterial culture and media

Experiments were performed using the *Bacillus subtilis* strains 168-wild type (wt) of Marburg origin as indicated in Yeak et al. (2023) and the Δ*sigB* marker-free mutant BY47. The Δ*sigB* (mutant) was constructed following the exact same procedures as described in detail in Yeak et al. (2023). Briefly, the *sigB* gene in strain 168 wt was knockout using the long flanking homology recombination method with a chloramphenicol marker and the chloramphenicol cassette was excised in a second step via the *cre*-recombination plasmid to create a marker-free mutant, and renamed to BY47. The culture conditions and medium used are as described in Yeak et al. (2023) with slight modifications. Briefly, 100µl of bacterial stock was transferred to 10 ml of Luria-Bertani (LB) broth medium (Scharlab Chemie S.L., Spain) and then incubated at 30°C for less than 12 hours, with constant stirring at 200 rpm. This prevented sporulation of *B. subtilis* (checked visually). After the overnight incubation, a washing step was followed. The culture was centrifuged for 10 minutes at 4°C and at 3600rpm and then, the supernatant was removed, and 1ml peptone water (Scharlab Chemie S.L., Spain) was added to the cell pellet. This procedure was followed for both strains.

2.2 Preadaptation experiments

Some experiments included a pre-adaptation step of the bacterial population to high temperatures. The fresh culture was introduced in tubes and immersed in a water bath

(Memmert, Germany) at 48ºC for 5 minutes. After the pre-treatment, the heating medium was inoculated with the cell suspension (0.2 mL) and the heating ramp selected was initiated (51, 52.5 and 55ºC for isothermal experiments and 1, 5, 10ºC/min increase for the dynamic profiles)

2.3 Thermal treatment and enumeration of survivors

Thermal treatments were carried out using a Mastia thermoresistometer (Conesa et al., 2009). Before starting the treatment, the vessel was filled with 400 mL of peptone water (10 g/L peptone from casein (Scharlab Chemie) and 5 g/L NaCl (Scharlab Chemie) as the heating medium. In order to achieve a homogeneous temperature distribution, the vessel of the thermoresistometer was constantly stirred during the treatment. The heating medium was inoculated with 0.2 mL of the bacterial suspension to achieve a concentration of approximately 10^7 CFU/mL.

Isothermal experiments were performed at 51, 52.5 and 55ºC. The thermoresistometer was set to the target temperature and, once the temperature in the vessel was stable, the bacterial suspension was inoculated. For dynamic conditions, monophasic profiles with an initial temperature of 35ºC and different heating rates (1, 5, 10ºC/min) were tested. Once the temperature of the medium stabilised, it was inoculated with the cell suspension and the heating ramp selected was initiated. The temperature in the vessel was recorded during the experiment to ensure there were no deviations with respect to the target one. A minimum of three experiments were performed per condition, with freshly prepared cultures. In every case, the treatment duration was adjusted to attain a reasonable number of log-reductions for model fitting.

The same procedure for both isothermal and dynamic profiles was followed for determining the viable cell count. Sterile test tubes were used to collect a sample of 3 mL at pre-set intervals and, after appropriate serial dilutions in sterile 0.1% peptone water, 1 ml samples were plated in duplicate in LB agar and incubated at 37 °C for 24 h. Colonies were counted and results were expressed as CFU/ml.

2.4 Predictive microbiology and numerical methods

2.4.1 Analysis of microbial inactivation under isothermal conditions

Survivor curves under isothermal conditions were either log-linear or biphasic. In the former case, the relationship between the (decimal) logarithm of the microbial concentration (log N) and the treatment time (t) was described using the Bigelow model (log-linear inactivation). In this model (Equation 20), the inactivation rate is quantified by the D-value (D), which stands for the treatment time required to cause the microbial concentration to reduce tenfold. The initial microbial concentration is given by N_0.

$$\log N = \log N_0 - t/D \qquad (20)$$

On the other hand, biphasic survivor curves were described using a bilinear model. The model, described in Equation (21), has a continuous transition at an unknown transition time (t_{tr}). Both phases present log-linear inactivation with unknown D-values (D_1 and D_2, respectively). Hence, the model is defined by three model parameters (t_{tr}, D_1 and D_2) as well as the initial concentration, N_0.

$$\log N = \log N_0 - t/D_1; \quad \text{if } t < t_{tr} \qquad (21)$$

$$\log N = \log N_0 - t_{tr}/D_1 - (t - t_{cr})/D_2; \quad \text{otherwise}$$

The relationship between the *D*-value and the treatment temperature (*T*) was described using the log-linear model commonly used in predictive microbiology (Equation 22), where the *z*-value (*z*) represents the temperature increase required to reduce the *D*-value in one log-unit. This equation introduces a reference temperature (T_{ref}) without a biological interpretation but with an impact on parameter identifiability. To improve parameter identifiability, it was set at an intermediate value within the experimental design (Peñalver-Soto et al., 2019). Hence, the secondary model is defined by parameter *z* and by the *D*-value at the reference temperature (D_{ref}).

$$\log D = \log D_{ref} - (T - T_{ref})/z \qquad (22)$$

Models were fitted by least squares using the Levenberg-Marquardt algorithm (Moré, 1978). The fitting was done in R version 4.2.1 (R Core Team, 2022) using the interface provided by the *FME* package (Soetaert et al., 2010). The R code is available from the GitHub page of one of the co-authors [https://github.com/albgarre/bioinactivation_FE].

2.4. Analysis of microbial inactivation under dynamic conditions

Microbial inactivation under dynamic conditions was predicted based on data gathered under isothermal conditions using the dynamic version of the Bigelow model. This model (Equation 23) describes the variation in the microbial concentration (*N*) as an ordinary differential equation. Then, the *D*-value varies through the treatment as a function of the instantaneous treatment temperature according to the secondary inactivation model (Equation 22).

$$dN/dt = - \ln (10)/D(T) \cdot N(t) \qquad (23)$$

Model predictions were calculated numerically using the LSODA algorithm (Hindmarsh, 1983), through the implementation included in version 1.2.3 of the *bioinactivation* package for R (Garre et al., 2017). The R code is available in the GitHub page of one of the co-authors [https://github.com/albgarre/bioinactivation_FE].

3. Results and Discussion

3.1 Thermal inactivation of *B. subtilis* under isothermal conditions – limitation of the vitalistic approach

Figure 12 illustrates the isothermal inactivation of the wild-type strain of *Bacillus subtilis* at the three temperatures tested (51, 52.5 and 55ºC). Survivor curves at 51ºC under standard incubation conditions (grey in Figure 12) are log-linear, experiments at 52.5 and 55ºC had a clear deviation from log-linearity. As described in the introduction, this type of deviation is most often justified based on the vitalistic or mechanistic hypotheses. In this case, there are no clear shoulders or tails, so the data would most often be described using the vitalistic arguments: an initial heterogeneity in the bacterial population, with some cells being more heat resistant than others. This heterogeneity can either be continuous based on a probability distribution (van Boekel, 2002) or be the outcome of two or more sub-populations with marked differences (Coroller et al., 2006).

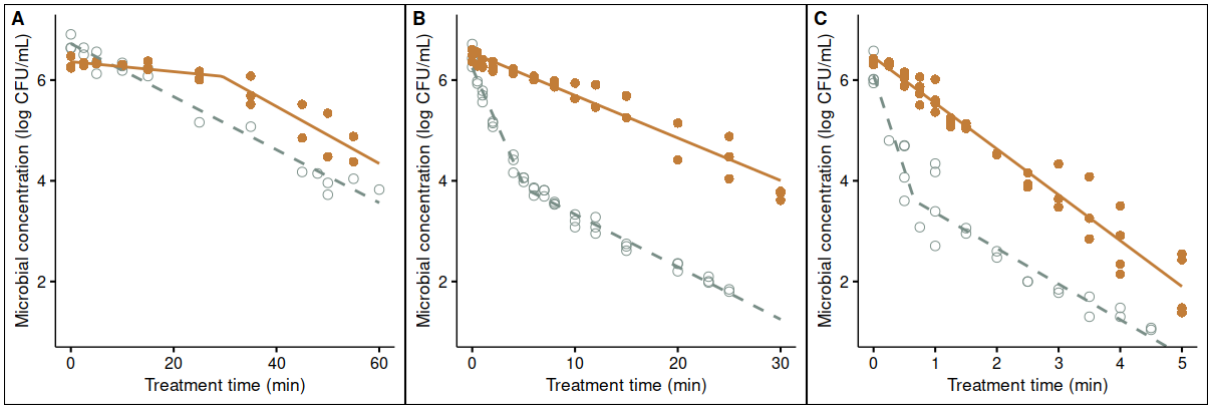


Figure 12. Thermal inactivation of *Bacillus subtilis* 168 at 51 (A), 52.5 (B) and 55ºC (C). Experiments were done for cells without pre-adaptation (grey, open dots) and after a pre-adaptation of 48ºC for 5 min (orange, closed dots). The lines show the fitted models (dashed grey lines for the experiments without pre-adaptation; solid orange lines for pre-adaptation).

Due to the use of an experimental design with a relatively high number of time points, we can compare both vitalistic hypotheses (two separate sub-populations or a continuous heterogeneity) for the treatments at 52.5 and 55ºC. The results show a sharp transition between an initial phase of higher inactivation to a second phase of higher stress resistance (especially visible in Figure 12B). This result supports the hypothesis that the initial microbial population is divided into two subpopulations, against a smooth distribution (e.g., Weibull). Accordingly, both populations would have log-linear inactivation kinetics if isolated, but with a different *D*-value. This is a relatively common interpretation in predictive microbiology, with some predictive models that represent that hypothesis already available (Coroller et al., 2006).

Figure 12 also illustrates the survivor curves obtained when a preadaptation step (48ºC for 5 min) is applied before the isothermal treatment. For every temperature studied, the preadaptation impacted the survivor curves both qualitatively and quantitatively. In the case of isothermal treatments at 52.5 and 55ºC, the survivor curves became log-linear (Figures 12B and 12C). This result is hardly compatible with the general interpretation of the vitalistic

hypothesis of population heterogeneity. If the intensity of the pretreatment was enough to inactivate the less resistant subpopulation (i.e., selecting the most resistant one) we would observe log-linear survivor curves with a clear reduction in the initial concentration with respect to the standard experiment. However, that is not the case, as there are no relevant differences in the initial concentration between both experiments (t-test; $p > 0.05$). Hence, we can conclude that the pretreatment at 48°C is not inactivating the weaker population, but rather increased the heat resistance of the overall population when exposed to higher temperatures at 52.5°C or 55°C (Figures 12B and 12C)

Regarding the experiment at 51ºC (Figure 12A), the survivor curve obtained after the pre-treatment is again qualitatively different from the ones obtained at 52.5 and 55ºC. In this case, the application of the pretreatment resulted in a bilinear survivor curve, with an initial phase of practically no inactivation followed by a phase with faster inactivation. This change from linear inactivation to a biphasic inactivation is also hard to justify using vitalistic hypotheses, highlighting the limitations of this approach to describe our data.

The BY47 Δ*sigB* mutant strain was less heat resistant than the 168 wt strain, with faster microbial inactivation at every condition tested (Supp. Figure 9). Nonetheless, from a qualitative point of view, the response of this strain was very similar to the one observed for the wild-type strain. We also observed log-linear survivor curves at 51ºC and nonlinear curves with a sharp transition at 52.5 and 55ºC (Figure 13).

The pretreatment condition at 48ºC for 5 min provided a similar heat resistance effect for both Δ*sigB* mutant and the 168 wt at all three tested temperatures (Figure 13). Namely, it induced a shoulder for the treatment temperature of 51ºC and a log-linear response at 52.5 and 55ºC. These results can be supported by the knowledge of the *B. subtilis* adaptive stress

response mechanism. The pretreatment would lack the intensity to cause a relevant microbial inactivation (t-test; $p > 0.05$), but would be able to induce a physiological cell response that increases their ability to survive heat treatments at higher temperatures, so-called the general stress response (GSR) (Haldenwang, 1995).

In wt cells, the exposure to 48°C would cause the induction of the *sigB* gene, which leads to the production of SigB, and the activation of the GSR in *B. subtilis* (Petersohn et al., 2001; Voelker et al., 1995). SigB then subsequently would regulate the production of many general stress proteins that can help *B. subtilis* to fight environmental stress, such as heat. Recently, SigB is reported to regulate > 500 direct and indirect genes and/or proteins in the SigB regulon of *B. subtilis* (Rodriguez Ayala et al., 2020; Yeak et al., 2023). Therefore, this GSR in wt explained the better survival of the pre-adapted wt cells compared to the non-preadapted wt cells. In Δ*sigB* cells, the deletion of the global stress regulator caused the failure of the mutant cells to activate the GSR and the production of many general stress proteins in the pre-adapted conditions and thus the overall survival of Δ*sigB* cells was lower when compared to wt cells. However, the pre-adapted Δ*sigB* cells still survived better than the non-preadapted Δ*sigB* cells due to the presence of the heat shock stimulon in *B. subtilis*, which include other heat shocks regulons, such as those regulated by HrcA, CtsR, HtpG and the CssRs (Schumann, 2003). Overall, this would explain the increased resistance both after the pre-treatment and dynamically during the isothermal treatment, as well as the lower resistance in Δ*sigB* mutant compared to wt.

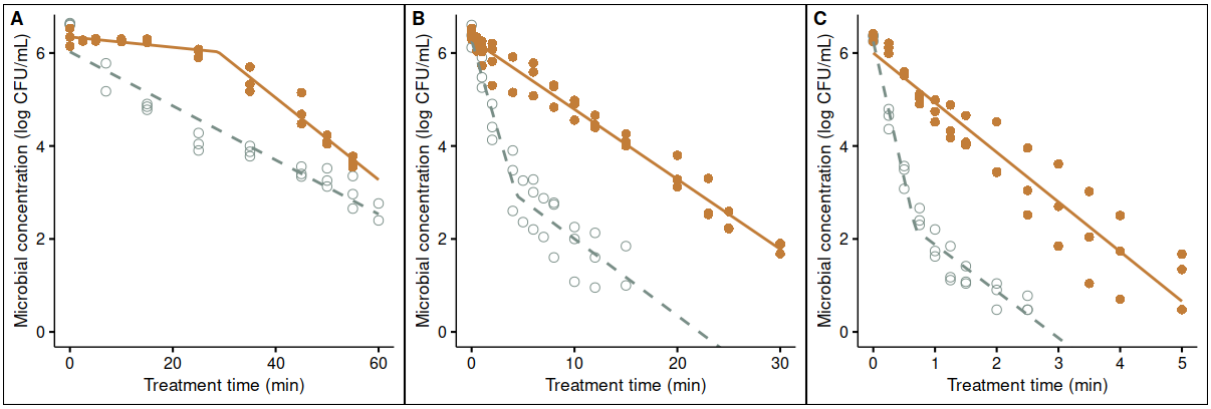


Figure 13. Thermal inactivation of the Δ*sigB* mutant *Bacillus subtilis* strain at 51 (A), 52.5 (B) and 55ºC (C). Experiments were done for cells without pre-adaptation (grey, open dots) and after a pre-adaptation of 48ºC for 5 min (orange, closed dots). The lines show the fitted models (dashed grey lines for the experiments without pre-adaptation; solid orange lines for pre-adaptation).

3.2 Quantitative analysis of the thermal inactivation of *B. subtilis* under isothermal conditions – alternative interpretation based on stress adaptation

The application of predictive microbiology to quantify the different inactivation rates provides an alternative interpretation of the experimental data. As illustrated in Figures 12 and 13, we fitted log-linear or bi-linear models to the survivor curves, whose parameters are included in supp. Table 4. Figure 14 compares the *D*-values estimated for each condition at 52.5 and 55ºC. Considering that this parameter quantifies the heat resistance of the population, this figure clearly illustrates the higher heat-resistance in the second phase of the inactivation, with roughly a 5-fold increase in the *D*-value for every condition tested.

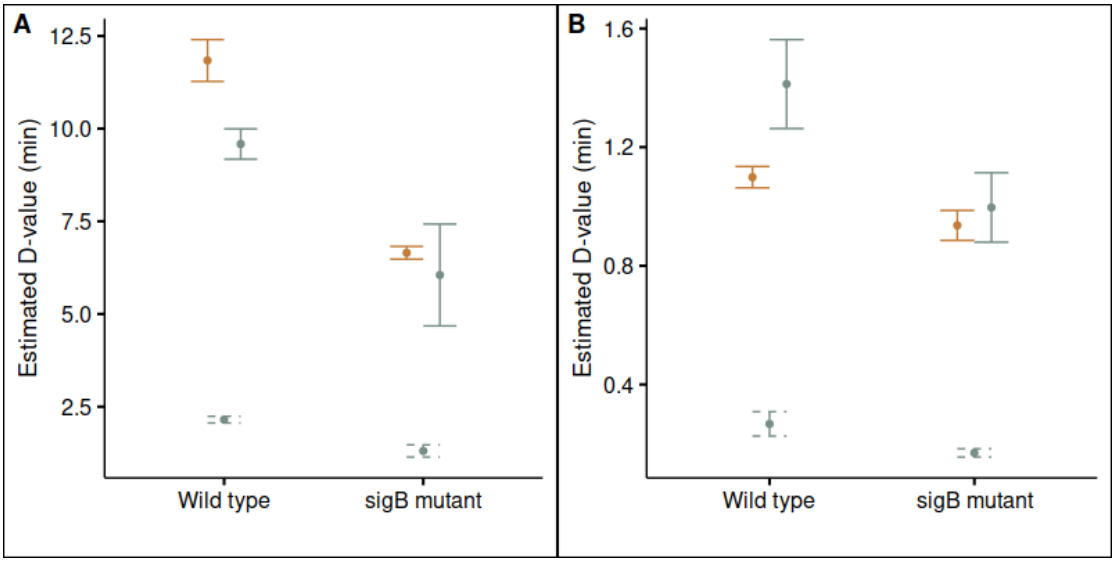


Figure 14. Estimates of the *D*-value for the thermal inactivation of both *Bacillus subtilis* strains at 52.5 (A) and 55ºC (B). The dots represent the estimated values and the error bars are their standard errors. For the conditions with pre-adaptation (orange) a single error bar is shown because survivor curves were log-linear (Figure 12-13). Experiments without pre-adaptation (grey) show two error bars as the inactivation was biphasic (Figure 12-13): a dashed bar for the initial phase and a solid one for the second phase.

Remarkably, the *D*-value obtained during the second part of the standard experiment is very close to the one observed after pre-adaptation. This is also evident in Figures 12 and 13, where both survivor curves are practically parallel. This result strongly supports an interpretation of the results based on stress adaptation, which serves as an alternative to the vitalistic hypotheses. Accordingly, the bacterial population would initially be composed of cells with homogeneous stress resistance. The application of a lethal temperature would cause the inactivation of some bacterial cells, with the difference between surviving and inactivated cells being defined not by biological differences but by chance (Garre et al., 2021). The surviving cells would activate their stress response mechanisms and, if they survive the treatment long enough, would see their heat resistance increased. This dynamic adaptation

during the treatment would result in an upward curvature of the survivor curve, as observed in Figures 12 and 13 for 52.5 and 55ºC.

Regarding the pretreatment, the application of sublethal temperatures (48ºC in our case) would induce a similar stress response, albeit without causing any microbial inactivation. If the duration of the pretreatment is long enough to enable the complete bacterial population to develop stress adaptation, inactivation would be log-linear, albeit with a higher *D*-value than during the initial phase of an experiment without preadaptation. Note that the population would still be homogeneous, so the difference between inactivation or survival would again be defined by chance. This prediction is in-line with the results obtained for 52.5 and 55ºC after the preadaptation (Figures 13 and 14). Considering this interpretation and that the *D*-values estimated after the pretreatment are practically the same as the *D*-value of the second phase of the standard isothermal treatment (Figure 14) implies that, regardless of the way the adaptive response of *B. subtilis* is triggered, the final heat resistance of the (surviving) cells would likely be the same.

This interpretation based on stress acclimation can also explain the results obtained at 51ºC. Although it is possible that there is a stress acclimation during the earliest part of the treatment, the intensity of the treatment is too low to observe any noticeable inactivation before ~10 min. As observed for the in the other conditions tested, the application of 48 (increased resistance after the pre-treatment), 52.5 or 55ºC (nonlinearity of the survivor curves) during 5 min induces stress adaptation in the population. Hence, the log-linear results obtained at 51ºC would represent the resistance of a homogeneous population adapted to that temperature before the first time point can be taken.

Regarding the results obtained at 51ºC after the pretreatment, shoulders are often interpreted as the bacterial cell being able to withstand the treatment for a given time until the accumulated damage is large enough to cause inactivation. Therefore, the pretreatment would induce a bacterial stress response increasing their resistance. Then, these preadapted cells could withstand the thermal treatment that cells incubated under standard conditions could not, resulting in a shoulder of the survivor curve.

The interpretation of stress acclimation being responsible for the nonlinearity of the survivor curves has interesting implications for the interpretation of bacterial response to stress. The first one is related to the observation of a very clear jump in bacterial resistance, with two marked linear phases (e.g., Figure 12B). This would indicate the two distinct states in bacterial stress adaptation. During the heat treatment, the bacteria may upregulate chaperone and other heat shock proteins to help stabilize cellular structures from being damaged by heat, such as Sigma A-dependent heat shock response pathways (Hecker et al., 1996). However, such regulation involves the production of heat-stress proteins that do not occur immediately after the activation of a heat-stress response. Depending on the type of protein, it may take minutes up to an hour for its production. This may explain why the cells in the first phase were rapidly inactivated (first inactivation state) and appeared slightly more heat-resistant afterward (second inactivation state).

Another interesting aspect of our results is related to the transition time. Although (as expected) the Δ*sigB* mutant had lower *D*-values than the wild-type strain, the transition between the initial and adapted phase occurred at the same time. Namely, at 52.5ºC, we estimated a transition at 4.36 ± 0.59 min for the mutant strain and 5.18 ± 0.23 min for the wild type; whereas at 55ºC we estimated transitions at 0.69 ± 0.05 and 0.67 ± 0.11 min, respectively.

This result may indicate that different stress response mechanisms may share similar timescales. That is, even if their overall effect on stress resistance has a different magnitude, the time they need to be effective may be roughly the same. To our knowledge, no previous study had studied this effect, so additional data is needed to confirm it.

3.3 Extrapolation of isothermal inactivation to dynamic heating conditions

The interpretation of the isothermal data as the outcome of stress adaptation could potentially improve our ability to define predictive models for microbial inactivation under dynamic conditions using isothermal data. This could be of great interest because, currently, the only way to include stress acclimation in predictive models is by performing dynamic experiments, a method that requires specific equipment unavailable in most laboratories.

As illustrated in Figure 15, the *D*-values estimated for our experimental design provide an envelope of the possible microbial response to the heat treatment depending on whether or not cells are adapted. Therefore, in order to account for the potential effect of stress adaptation on the *D*-value, we fitted a model for the lowest resistance observed (initial inactivation phases at 52.5 and 55ºC; inactivation after shoulder at 51ºC) and a second model for the *D*-values that represent the higher level of adaptation (shoulder at 51ºC, *D*-value after adaptation at 52.5ºC, second inactivation phase at 55ºC).

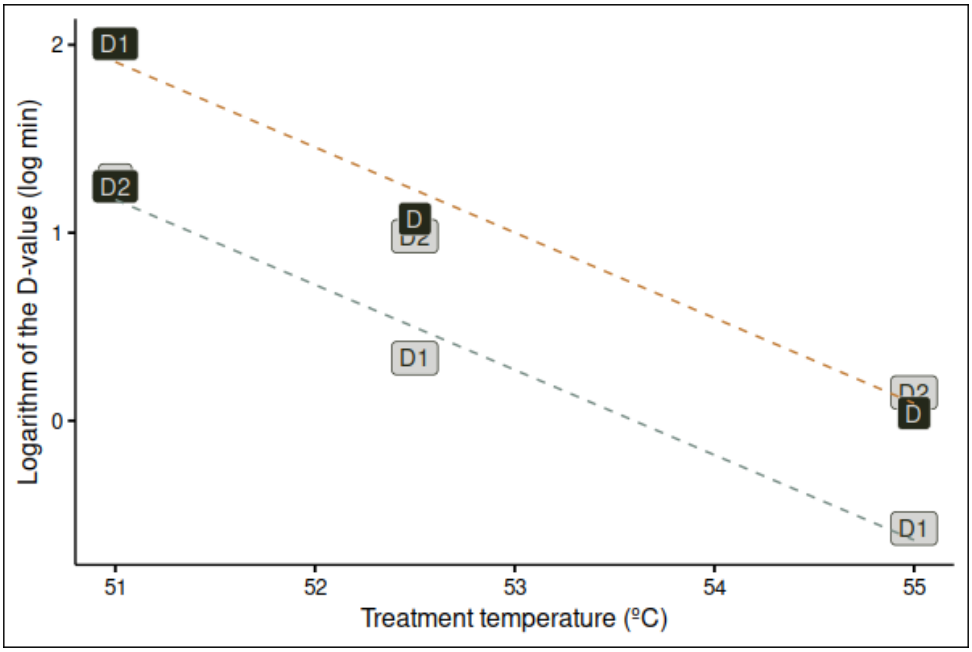


Figure 15. Secondary models (dashed lines) fitted to describe the relationship between the *D*-value and the treatment temperature for *B. subtilis* based on the data observed under isothermal conditions (squares). The plot shows two models, one representing the lowest heat resistance (grey) and a second one representing the maximum stress adaptation observed under isothermal conditions (orange). The *D*-values were obtained without pre-adaptation (light squares) or after pre-adaptation (dark-squares)

Table 3 includes the values of the parameter estimates for the secondary models. As illustrated in Figure 15, the models described the general trend of the data. It is remarkable that both secondary models have practically the same *z*-value (2.21 and 2.20ºC). This indicates that the effect of temperature changes on the *D*-value is practically constant, regardless of whether the cells have adapted or not. A different, equivalent interpretation is that the ~5-fold increase of the *D*-value observed experimentally would be temperature independent (Figure 15).

Table 3. Values (estimate ± std. error) of the secondary model fitted to the *D*-values of *B. subtilis* strain 168 under isothermal conditions. An independent model was fitted to the upper and lower bounds of the *D*-value, according to the stress adaptation observed under isothermal conditions. A reference temperature of 53ºC was used for the fit

	log D_{ref} (log min)	***z* (ºC)**
Lower bound	0.27 ± 0.12	2.21 ± 0.35
Upper bound	1.00 ± 0.11	2.20 ± 0.32

Please note that these *z*-values should not be compared against the ones often calculated in predictive microbiology. Although the parameters in Table 3 also describe the relationship between the *D*-value and the treatment temperature, they only describe a linear part of a nonlinear survivor curve (Figure 12). Hence, they are fundamentally different from the common *z*-value in predictive microbiology, which describes a complete survivor curve – often ~5ºC for vegetative cells, roughly twice of what is reported here.

These secondary models were used to predict upper and lower bounds for the inactivation of *B. subtilis* under dynamic heating conditions. Based on our previous studies on other bacterial species (Garre, González-Tejedor, et al., 2019; Georgalis et al., 2022), we expected the lower bound to be representative of bacterial inactivation for high heating rates (that do not allow stress acclimation), whereas the upper bound would describe low heating rates (that allow stress acclimation). However, as illustrated in Figure 16, our initial hypotheses were only partly true. The experiments for a heating rate of 10ºC/min fell reasonably within the envelope defined by the two secondary models, indicating that the impact of stress acclimation for this heating rate would be lower than the one observed under isothermal conditions (either after a preadaptation or during dynamic adaptation to the isothermal treatment). The data obtained at 5ºC/min is also in line with our initial hypotheses, with the experimental results being close to

the upper bound predicted. This could be interpreted as the increase in thermal resistance for this dynamic temperature profile being similar in magnitude as the one observed under isothermal conditions.

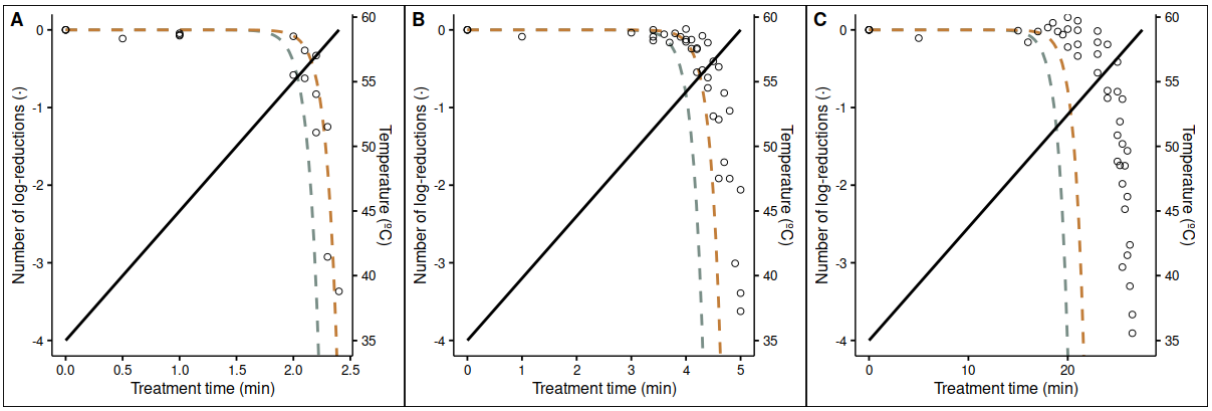


Figure 16. Comparison between microbial inactivation (o) observed for dynamic thermal treatments with a heating rate of 10 (A), 5 (B) and 1ºC/min (C), for *B. subtilis*. The solid, black line represents the temperature profile during the experiment, whereas the dashed lines show the prediction of the Bigelow model using the secondary model for adapted cells (orange) and for non-adapted ones (grey) (models shown in Figure 15)

However, the microbial concentrations observed for a heating rate of 1ºC/min are clearly larger than defined by the upper bound predicted based on the adaptation observed under isothermal conditions. There are two possible reasons for this deviation. The first one would be that the magnitude of the impact of stress acclimation on microbial inactivation is larger than the one of pre-adaptation or dynamic inactivation during isothermal experiments. This is in-line with previous results from our group for vegetative cells of other species. Namely, we estimated (based on parameter *c* of the acclimation model (Garre et al., 2018c)) up to a 9-fold increase in the *D*-value due to stress acclimation (Clemente et al., 2020; Garre, González-Tejedor, et al., 2019; Georgalis et al., 2022), which is larger than the 5-fold increase observed for isothermal treatments (Figure 15). This would imply that, although the mechanisms behind both processes would be similar, stress acclimation due to slow heating would result in a larger

increase in thermal resistance than adaptation to a pretreatment or the one developed during an isothermal treatment.

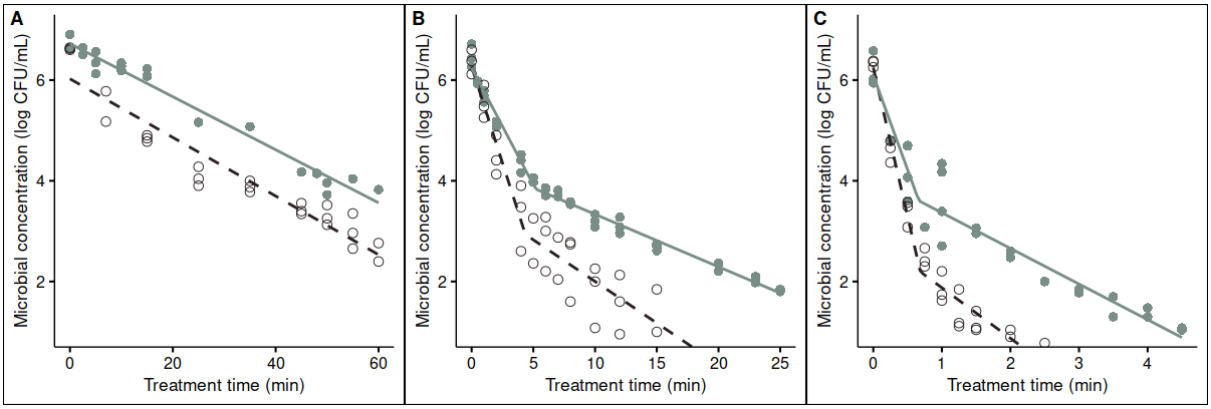
The second explanation for the deviation between the upper bound and the empirical observations for the lowest heating rate is related to the limitations of our experimental and data analysis methods. The secondary model was defined using only three temperatures (Figure 15). Hence, although the standard errors are small due to the low number of degrees of freedom (Table 3), there are large model uncertainties that were not included in the model prediction. Furthermore, the secondary model has been fitted to the parameter estimates of the primary model, so the parameter uncertainty of the *D*-values was not propagated to the secondary model. Nonetheless, these simplifications are reasonable considering the innovative aspects of this research, as this is the first study that infers a phenomenon that can only be observed under dynamic conditions (stress acclimation) in a model fitted to isothermal experiments. Therefore, these limitations shall be circumvented in future studies that build upon the methodology developed here.

4. Conclusions

The deviations from log-linearity that are often observed in bacterial survivor curves can be explained using different arguments, both biological and experimental. In this study, we demonstrated that the generally accepted vitalistic arguments (initial heterogeneities in the stress resistance of the cells in the population) may fail to describe microbial inactivation in some situations. In this sense, we showed how dynamic stress acclimation during an isothermal treatment provides an alternative explanation for survivor curves with an upwards curvature. We also provided an innovative experimental approach based on preadaptation experiments to evaluate which hypothesis is more suitable for the bacterial response. Furthermore, we used

our experimental results to define bounds for the possible stress acclimation that may take place during dynamic treatments, concluding that the magnitude of stress acclimation may be larger for dynamic treatments than for isothermal experiments. We demonstrated the contribution of the SigB general stress system to heat resistance by comparing the heat survival of wt and the Δ*sigB* mutant. Both strains survived better in 51, 52.5 and 55°C when cells were pre-adapted at 48°C, compared to non-pre-adapted cells. However, Δ*sigB* was less robust than wt due to the missing SigB general stress system. Although these conclusions were based on *B. subtilis* as a model organism, our innovative methodology will also be applicable for further studies on bacterial inactivation, ultimately improving our ability to predict bacterial survival to thermal treatments.

5. Supplementary material Chapter III



Supp. Figure 9. Thermal inactivation of *Bacillus subtilis* strain 168 (greenish closed dots) and the Δ*sigB* mutant (grey open dots) at 51 (A), 52.5 (B) and 55ºC (C) without pre-adaptation. The lines show the fitted models (dashed black lines for the Δ*sigB* mutant and solid greenish lines for *B. subtilis* strain 168)

Supp. Table 4. Parameters of the fitted log-linear or bi-linear models, with respective standard deviation in parenthesis, to the survivor curves of isothermal microbial inactivation for *B.subtilis* 168 and Δ*sigB* mutant at the three temperatures tested (51, 52.5 and 55 ºC). Experiments were done for cells without pre-adaptation (normal cells) and after a pre-adaptation of 48ºC for 5 min. D value represents the decimal reduction time for the log-linear model; D1 and D2 represent the decimal reduction time for the bi-linear model; $logN_0$ is the initial population at the start of the isothermal inactivation; t_crit is the time of a sharp transition between an initial phase of higher inactivation to a second phase of higher stress resistance (initial microbial population is divided into two subpopulations)

Temperature 51 ºC		Log-linear model		Bi-linear model			
Strain	Cell state	D	$logN_0$	D1	D2	$logN_0$	t_crit
B.subtilis 168	Normal	18.99 (0.74)	6.72 (0.06)				
Δ*sigB* mutant	Normal	17.18 (1.08)	6.03 (0.14)				
B.subtilis 168	Preadapted			101.26 (59.04)	17.64 (2.75)	6.37 (0.07)	29.44 (4.22)
Δ*sigB* mutant	Preadapted			89.93 (36.69)	11.34 (0.82)	6.34 (0.06)	28.79 (1.99)

Temperature 52.5 ºC		Log-linear model		Bi-linear model			
Strain	Cell state	D	$logN_0$	D1	D2	$logN_0$	t_crit
B.subtilis 168	Normal			2.15 (0.09)	9.59 (0.41)	6.24 (0.06)	5.18 (0.23)
Δ*sigB* mutant	Normal			1.31 (0.16)	6.05 (0.37)	6.27 (0.22)	4.36 (0.59)

B.subtilis 168	Preadapted	11.84 (0.56)	6.54 (0.06)				
Δ*sigB* mutant	Preadapted	6.65 (0.17)	6.29 (0.05)				

Temperature 55 ºC		Log-linear model		Bi-linear model			
Strain	Cell state	D	$logN_0$	D1	D2	$logN_0$	t_crit
B.subtilis 168	Normal			0.27 (0.04)	1.41 (0.15)	6.09 (0.20)	0.66 (0.11)
Δ*sigB* mutant	Normal			0.17 (0.01)	1.00 (0.12)	6.25 (0.16)	0.69 (0.05)
B.subtilis 168	Preadapted	1.10 (0.04)	6.45 (0.07)				
Δ*sigB* mutant	Preadapted	0.94 (0.05)	6.00 (0.14)				

Acknowledgments

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4 General Discussion

“If it isn’t safe, it isn’t food”. This statement by the FAO emphasizes well the significance of food safety. Foodborne infections can cause significant health difficulties and even death, which can result in costly hospital expenses, lost wages and extensive loss of healthy life time to consumers, reducing their life expectancy. Therefore, food processing technologies used in food pasteurisation or commercial sterilisation to eliminate or inactivate harmful microorganisms in food are a key element of the food supply chain. The food is subjected to high temperatures for a predetermined amount of time in order to eliminate or reduce both pathogenic and spoilage microorganisms.

The current Doctoral Thesis has focused on comparing and evaluating the significance of various sources of variability for the survival of microbial population to thermal pasteurization treatments. Specifically, it focused on stress adaptation and initial genetic heterogeneities. The primary objective was addressed through several strategies outlined in each chapter.

In Chapter I, we highlighted the link between predictive microbiology models and different hypotheses regarding variability. This can be used to identify whether a particular source of variability is relevant in some scenarios, thus improving our understanding of how the stress resistance of different bacterial strains is affected by variability.

This chapter is focused on microbial inactivation during dynamic heat treatments. While most scientific data are obtained under isothermal conditions, it is questionable whether these models are applicable for actual industrial processes because models based on isothermal data often fail to predict microbial response under dynamic conditions. This raises the need to develop models that can describe microbial response under both isothermal and dynamic conditions.

Chapter I discussed how the hypotheses of microbial inactivation models vary between isothermal and dynamic situations. By studying the inactivation of two strains of *Salmonella* under isothermal and dynamic conditions, we concluded that variability in microbial inactivation is not just quantitative, but also qualitative. Strain variability can have a qualitative impact on microbial kinetics, requiring different modelling approaches per strain, not just different parameter values. More specifically, we studied the inactivation of two strains of *Salmonella* under isothermal and dynamic conditions. We observed log-linear survivor curves under isothermal conditions and stress acclimation under dynamic conditions with slow heating rates for *S.* Enteritidis CECT4300. This behaviour was significantly different to that of *S.* Senftenberg CECT4565, where, this strain displayed non-linear survivor curves under isothermal conditions and no stress acclimation in dynamic conditions. This distinct response required the use of 2 separate modelling approaches for each strain (Weibullian models for *S.* Senftenberg, acclimation model for *S.* Enteritidis). This qualitative distinction has not been previously described in the context of dynamic microbial inactivation and underlines the need to analyse model hypotheses carefully when defining variability in microbial inactivation.

In Chapter II, we studied a different source of variability related to bacterial adaptation: the impact of pre-culture conditions. Namely, we studied the effect of incubation at sub-optimal pH and the application of an acid shock, on the heat resistance of *S.* Enteritidis and *S.* Senftenberg. Microorganisms initiate a series of responses to protect themselves against acidic conditions, such as modifying the membrane permeability, adjusting their metabolism and producing protective proteins. As a side effect, these changes can often also increase the cell resistance to thermal treatments, resulting in cross-resistances. Therefore, for an effective risk assessment, it is essential to understand whether an acidic treatment will damage the cell

(reducing its resistance to a posterior heat treatment) or induce cross-resistances (increases its probability to survive).

In a similar way as in Chapter I, the results varied qualitatively between the two strains tested. For *S.* Enteritidis, pre-adaptation to low pH or acid shock increased heat resistance, resulting in longer survivor curves with a shoulder in almost every case. The effect was more pronounced for sub-optimal pH incubation and was most significant at the lowest pH level tested. In contrast, for *S.* Senftenberg, deviations from optimal growth conditions resulted in reduced heat resistance in every condition tested, regardless of whether the cells were pre-incubated at sub-optimal pH or subjected to an acid shock.

This result emphasizes the difference between phenotypic and genetic variability when interpreting the relevance of strain variability for bacterial survival. The *S.* Senftenberg strain tested is extremely resistant within Salmonella spp., having a D-value approximately 10 times larger than other strains within this species. However, it seems that this strain would not be able to increase that resistance through stress adaptation. Considering that the other Salmonella strain tested was able to develop stress adaptation through different means, this result may indicate the existence of a trade-off between its static stress resistance and its ability to adapt and increase resistance. This concept has not been explored in depth in the scientific literature and requires additional data to prove or disprove the hypothesis.

Pre-adaptation experiments can represent bacterial cells that have survived for a long time in an acidic environment. Phenotypic variability was found to be strain-dependent and could be relatively simple to implement in microbial risk assessment models. Obtaining data on prevalence and bacterial concentrations on each ingredient could reduce the uncertainty of the risk assessment and lead to more effective food safety interventions. This could help target the

most relevant product, considering not only the prevalence of bacterial pathogens, but also its impact on bacterial survival. Therefore, considering its relevance and feasibility, phenotypic variability should become a main focus in predictive microbiology.

The results of Chapter II highlight the importance of considering phenotypic variability in microbial risk assessment, as deviations from optimal growth conditions can significantly impact the heat resistance of bacterial populations. The findings also suggest that different pre-culture conditions can have fundamentally different effects on the bacterial response to stress, which may have implications for food safety management. One advantage of physiological sources of variability is that they could be easier to implement into QMRA models than genetic ones, particularly in scenarios where information on specialized bacterial phenotypes in different products is limited. For instance, the study demonstrates how pre-adaptation experiments could be used to map bacterial survival to different contamination routes in hypothetical scenarios involving different food products

Chapter III uses a different approach to the analysis of variability, focusing on the relevance of variability for microbial inactivation under dynamic conditions. This type of study is more complex than isothermal experiments because it requires specialized equipment and complex mathematical methods. For that reason, this chapter proposes an alternative approach that exploits the potential influence of stress acclimation on inactivation under isothermal conditions.

The study proposes that, in some cases, the upwards curvature observed in survivor curves under isothermal conditions could be the outcome of stress acclimation. This alternative interpretation of the experimental data can be used to estimate the potential relevance of stress acclimation under dynamic conditions. Although stress acclimation has only been associated

with the initial heating phase of a dynamic treatment, bacteria respond in a similar way to mild heat treatments. As a result, treatments at low temperature may induce stress acclimation before microbial inactivation, resulting in survivor curves with an upwards curvature.

Based on this interpretation, the study estimated bounds for the stress acclimation that may take place under dynamic conditions based on isothermal data. Although the validation studies clearly showed the inability of this method to predict dynamic microbial inactivation for heating rates lower than 1ºC/min, the approach shows great potential. Currently, most laboratories cannot produce microbial inactivation data under dynamic conditions due to the lack of specific equipment. This study can be the first step towards the development of a novel methodology able to estimate dynamic effects using only isothermal experiments. This would improve the models developed within the predictive microbiology community, improving our ability to predict microbial inactivation during industrial treatments, which are always dynamic.

5 Conclusions

The primary objectives of this PhD thesis were to investigate the impact of stress adaptation and genetic heterogeneities on microbial risk assessment and to evaluate the behaviour of bacterial populations under dynamic heat treatments. To achieve these objectives, we compared the impact of these sources of variability on the assessment of risks posed by different bacterial species, with a focus on two strains of *Salmonella* spp. We further examined the implications of variability in heat resistance in biological safety management and investigated the behaviour of *B. subtilis* 168-wild type and its ΔsigB mutant under isothermal and non-isothermal heating conditions.

This thesis highlights the need for models that can describe microbial response under both isothermal and dynamic conditions, as models based on isothermal data often fail to predict microbial response under dynamic conditions. It also emphasizes the importance of considering phenotypic variability in microbial risk assessment, as deviations from optimal growth conditions can significantly impact the heat resistance of bacterial populations. Our findings indicate that both stress adaptation and genetic heterogeneities can have significant implications for microbiological risk assessment and considering these sources of variability is crucial for ensuring the safety of the food supply and public health.

Furthermore, our study defines the heat adaptation of *B. subtilis* 168-wild type and its ΔsigB mutant caused by exposure to mild heat stress and compares the impact of their genetic differences. The findings suggest that different pre-culture conditions can have fundamentally different effects on the bacterial response to stress, which may have implications for food safety management. Finally, our study calls for additional data to explore the hypothesis of a trade-off between static stress resistance and the ability to adapt and increase resistance.

Overall, our research provides valuable insights into the significance of different sources of variability for the survival of microbial populations to thermal pasteurization treatments. Our findings have important implications for microbial risk assessment and food safety management and emphasize the importance of considering both stress adaptation and genetic heterogeneities in these assessments.

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FE DE ERRATAS

Información incorporada al documento entregado para su defensa

Tesis doctoral: ***Assessing the relevance of different sources of variability on the survival of foodborne pathogens: stress adaptation against genetic heterogeneities***

Evaluación de la importancia de diferentes fuentes de variabilidad en la supervivencia de microorganismos patótenos alimentarios: Adaptación al estrés frente a la heterogeneidad genética

Doctorando: Leonidas Georgalis

	Donde pone	**Debería poner**
Page 107, Chapter II Results	there is an error in the number of the Figure. The number should be changed to Figure 9, instead of Figure 10. "… introduced a shoulder in the microbial response in almost every case (Figure 10), although the effect of the acid shock is more irregular."	**The new correct text is**:"… introduced a shoulder in the microbial response in almost every case (Figure 9), although the effect of the acid shock is more irregular."
Page 85, Chapter I results, Figure 6	The label of Figure 6 has an error. There is a misunderstanding between the dotted and the dashed lines and which model represent what.	**The correct label should be the following:** "**Figure 3**. Comparison between experimental data and model simulations for the profiles of *S.* Enteritidis heat treated in peptone water at different heating rates (A for 1 °C/min, B for 2 °C/min, C for 10 °C/min). The dots represent the experimental data, (**--**) the dashed line is the prediction calculated by the Bigelow model whereas the (‥) dotted line is the one of the proposed model. The solid line (**-**) represents the temperature profile (secondary y-axis)."
Page 145, Chapter III, Materials and Methods	There is a mistake in the numbering of an equation. In the text of the last paragraph of the page, the number of the equation in the parenthesis should be 22 and not 3, since it	"Then, the *D*-value varies through the treatment as a function of the instantaneous treatment temperature according to the secondary inactivation model (**Equation 22).**"

	is referring to a previous equation **Old text:** "Then, the *D*-value varies through the treatment as a function of the instantaneous treatment temperature according to the secondary inactivation model (Equation 3)."	
Page 151, Figure 14	There is an Error in figure legend. In the parenthesis it says (Figure 1-2) and should be changed to Figures 12-13	
Page 117, Chapter II, Conclusions	There a typo in the line: "For the *S.* Enteritidis one (a reference strain), we observed that pre-incubation at **pH 4.5** led to a 3-fold increase in D-value with respect to control conditions".	"For the *S.* Enteritidis one (a reference strain), we observed that pre-incubation at **pH 5** led to a 3-fold increase in D-value with respect to control conditions".