Universitat Autònoma de Barcelona

MICROBIOLOGICAL SAFETY EVALUATION OF SOUS-VIDE TREATMENTS AT MILD TEMPERATURES APPLIED TO PORK LOIN

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Als meus pares, Joan Mº i Margarida

A en Jordi, la Marta i en Jaume

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Abstract

A raising interest has been detected nowadays in relation to the use of cooking techniques at moderate temperatures like *sous-vide* cooking. Given the possibility of using these techniques to prepare food in advance, and considering that data on behaviour of bacteria in the range of 40 to 60 °C is scarce, there is great concern in accurately define the microbiological safety of this food. The aim of this doctoral thesis was to characterize the effect of mild temperature vacuum cooking treatments on two main food pathogens, *L. monocytogenes* and *Salmonella* spp. inoculated in raw pork meat.

Challenge studies were conducted using two bacterial strains of *Salmonella (S. enterica* subsp. *enterica* serovar Enteritidis CECT 4300 and Senftenberg CECT 4565), and *Listeria monocytogenes* (CECT 4031 and Scott A), inoculated either individually or in combination. Pork loin pieces were inoculated, vacuum packed and cooked in a steam oven at two different temperatures (55 and 60 ºC) during 30, 60 or 90 minutes. Lethality caused by each treatment was determined just after cooking. Further recovery of injured cells was evaluated during storage at 4 and 8 ºC until the 30th day after treatments. Evolution of microbial counts were modelled using the predictive tools Bioinactivation FE for thermal inactivation and DMFit for growth through the storage time. The Monte Carlo method was applied in order to incorporate the variability observed between replicates in the predictive models of inactivation and growth.

The heterogeneity of results was important. One source of this variability was probably the steam oven performance between 55 and 60 ºC. At these cooking temperatures, *Salmonella* spp. was more heat resistant than *L. monocytogenes*, but it was less able to growth during cold storage. Microbiological safety evaluation of *sous-vide* mild heat treatments must be based both on lethality and on the capability of recovery during storage. Considering both factors, three different scenarios were obtained: complete inactivation, presence of viable cells and a growth/no growth interface. In case of a complete inactivation (*S.* Enteritidis and *L. monocytogenes Scott A* and CECT 4031 cooked at 60 ºC), inoculated strains were not detected in any replicate during all the storage period. In case of viability, all replicates showed counts above the quantification limit (5 CFU/g), whether growth was observed or not (*S.* Senftenberg, *L. monocytogenes* Scott A, cocktail of *L. monocytogenes* strains at 55 ºC, and cocktail of *Salmonella* spp. strains at 55 ºC during 30 min). Finally, in some experimental conditions a “growth/no

growth" behaviour, with different proportions of death, viable and injured cells, was observed. This circumstance took place in inoculated samples cooked at 60 ºC (S. Senftenberg, cocktail of *L. monocytogenes*) and 55 ºC (cocktail of *Salmonella* spp. and *L. monocytogenes* CECT 4031).

In conclusion, the microbiological safety of *sous-vide* mild heat processes have to be accurately assessed on a case-by-case basis. Heat treatments at 60 ºC for 90 min applied to pork loin could be considered reasonably safe and suitable for "cook-chill" systems in relation to *Salmonella* spp. and *L. monocytogenes* strains included in the essay, as long as no temperature abuse occurs during shelf-life. Treatments at 60 ºC during 30 and 60 minutes must be used only for "cook-serve" systems. *Sous-vide* cooking of pork loin at 55 ºC during 30, 60 and 90 minutes cannot be considered safe in relation to *S.* Senftenberg and *L. monocytogenes* Scott A due to the presence of survivor cells that can growth at 8 ºC. This treatment must be used only for "cook-serve" systems. This study provides useful data for future risk assessment studies applied to meat *sous-vide* cooked at mild temperatures.

Resum

Actualment s'observa un increment en la utilització de tècniques de cocció a temperatures moderades com la cuina al buit. Donada la possibilitat d'utilitzar aquestes tècniques per a preparar menjars amb antelació i considerant que les dades sobre el comportament microbià en el rang entre 40 i 60 ºC són escasses, hi ha un gran interès en definir la seva seguretat microbiològica. Aquesta tesi doctoral té com a objectiu avaluar l'efecte de la cocció al buit a temperatures moderades sobre *L. monocytogenes* i *Salmonella* spp. inoculades en llom de porc.

Es van realitzar assajos d'inoculació (*challenge test*) en llom de porc, amb dues soques de *Salmonella (S. enterica* subsp. *enterica* serovar Enteritidis CECT 4300 i Senftenberg CECT 4565) i *Listeria monocytogenes* (CECT 4031 i Scott A), avaluades de forma individual o combinada. Les porcions de carn inoculada es van envasar al buit i es van cuinar en un forn de vapor a dues temperatures (55 i 60 ºC) durant 30, 60 o 90 minuts. Es va determinar la letalitat i la recuperació microbiana durant l'emmagatzematge a 4 i 8 ºC fins al dia 30 post-cocció. L'evolució en el recompte es va modelitzar utilitzant les eines predictives Bioinactivation FE per a la inactivació tèrmica i DMFit pel creixement durant l'emmagatzematge. Es va aplicar el mètode Monte Carlo per incorporar la variabilitat observada en els models predictius.

L'heterogeneïtat dels resultats va ser important, i una de les seves fonts va ser probablement el funcionament del forn a les temperatures d'assaig. En general, *Salmonella* spp. es mostrà més termoresistent que *L. monocytogenes*, però el seu creixement durant l'emmagatzematge va ser més limitat. Per tant, l'avaluació de la seguretat microbiològica d'aquests tractaments moderats ha de considerar tant la letalitat com la capacitat de recuperació durant l'emmagatzematge. Tenint en compte tots dos factors, es van obtenir tres escenaris diferents. El primer vas ser d'inactivació completa (*S.* Enteritidis i *L. monocytogenes* Scott A i CECT 4031 a 60 ºC) és a dir, no es van detectar les soques inoculades en cap rèplica durant tot el període d'emmagatzematge. L'escenari de viabilitat es va observar en casos en què totes les rèpliques van mostrar recomptes superiors al límit de quantificació (5 CFU/g), amb o sense creixement (*S.* Senftenberg, *L. monocytogenes* Scott A, còctel de soques de *L. monocytogenes* a 55 ºC i còctel de soques de *Salmonella* spp. a 55ºC durant 30 min). Finalment, l'escenari de "creixement/no creixement", es va caracteritzar per diferents proporcions de mort,

viabilitat i lesió a nivell cel·lular. Aquesta circumstància es va produir en mostres inoculades cuinades a 60 ºC (*S.* Senftenberg, còctel de *L. monocytogenes*) i 55 ºC (còctel de *Salmonella* spp. i *L. monocytogenes* CECT 4031).

En conclusió, la seguretat microbiològica dels processos de cocció al buit a temperatura moderada ha de ser curosament avaluada cas per cas. El nostre estudi indica que els tractaments a 60 ºC durant 90 minuts aplicats al llom de porc es podrien considerar raonablement segurs, sempre que no es produeixi un abús de temperatura durant la vida útil. Els tractaments a 60 ºC durant 30 i 60 minuts es podrien utilitzar només en cas de servei immediat després de la cocció. La cocció de llom de porc a 55 ºC durant 30, 60 i 90 minuts no es pot considerar segura en relació a *S.* Senftenberg i *L. monocytogenes* Scott A, donat que les cèl·lules supervivents poden créixer a 8 ºC. Aquest tractament s'ha d'utilitzar només per a sistemes de servei immediat. Aquest estudi proporciona dades útils per a futures avaluacions del risc aplicades a la cocció al buit de carn a temperatures moderades.

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Abbreviations key

μmax	Maximum growth rate expressed in natural logarithm
λ	Latency/lag time
δ value	Time needed for the first decimal reduction at a particular temperature.
ANOVA	Analysis of variance
B_f	Bias factor
C_c	Protective or Critical components around or in each cell in the Weibull model
CFU	Colony-forming unit
CI	Confidence interval
CSPs	Cold shock proteins
D value	Decimal reduction time at a particular temperature
F value	Thermal death time or time required to cause a stated reduction in a population of microorganisms or spores at a particular temperature
Grmax	Maximum growth rate, expressed in decimal logarithm
HD	Heat Penetration testing
HSPs	Heat shock proteins
Lm 4031	*Listeria monocytogenes* CECT 4031
Lm cocktail	*Listeria monocytogenes* cocktail of strains
Lm Scott A	*Listeria monocytogenes* Scott A
min	minute
N_o	Initial count
N_{res}	Residual population density
p	Shape parameter of the Mafart model
QL	Quantification limit
RMSE	Root mean square error
RTE	Ready-to-eat
S. Enteritidis	*S. enterica* subsp. *enterica* serovar Enteritidis CECT 4300
S. Senftenberg	*S. enterica* subsp. *enterica* serovar Senftenberg CECT 4565
SD	Standard deviation
SE	Standard error of fit

PM	Predictive microbiology
TD	Temperature Distribution testing
z value	Temperature increment required for a ten-fold change of the log D value

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1. Literature review

1.1. *Sous-vide* cooking

Vacuum cooking or *sous-vide*, as it was originally called in France, is classically defined as "raw materials or raw materials with intermediate foods that are cooked under controlled conditions of temperature and time inside heat-stable vacuumized pouches" (Schellekens and Martens, 1993). *Sous-vide* cooking differs from traditional cooking methods in two fundamental ways: the raw food is vacuum-sealed in heat-stable, food-grade plastic pouches and the food is cooked using precisely controlled heating (Creed, 1998; Baldwin, 2012).

In the late 1960's, controlled heating at pasteurizing temperatures was industrially applied in order to incorporate a time buffer for foodservice and extend the shelf-life of processed foods. Different "cook-freeze" or "cook-chill" systems were developed, as the Nacka or the AGS systems. These methods were referred initially as "interrupted catering systems" (Sous Vide Advisory Committee, 1991; Ghazala, 2004). They were based on rapid chilling and portioning of cooked food, vacuum packing followed by chilled or frozen storage, reheating and serving (Creed, 1998). However, the real development of the *sous-vide* process, understood as enclosing food before cooking to prevent the escape of food juices and flavours during cooking is generally accredited to the French chef Georges Pralus (1985), who experimented with plastic film to minimize the cooking loss of *pâté de foie-gras*.

In recent years there has been an increased presence of *sous-vide* foods cooked at low-mild temperatures (from 42 to 60 °C) and the term *sous-vide* has been used to describe a much greater range of products than those covered by early definitions. Low temperature cooking is respectful with food's nutritional properties and makes the most of its qualities. Smell, color, and taste of food are usually more genuine and rich in nuance, and the textures are more tender than in traditional cooking (Roca and Brugués, 2003, 2016; Keller, 2008). However, this treatment allows the survival of microorganisms, mainly bacteria. The survival of bacteria and the extended shelf-life together are a concern for consumers in relation to the microbial safety of *sous-vide* foods (Baldwin, 2012; Stringer et al., 2012).

In *sous-vide* cooking, the ingredients can be either raw or can have received a previous preparation (marinating, cooking, grilling). After the assembly or mixture of ingredients, food is packaged in plastic pouches, usually without additives or preservatives, with the intention of keeping their image of freshness and minimal processing. Air is extracted from the package mechanically, immediately before sealing (vacuum packaging). The pack is then heat-processed. Heating usually is achieved by hot air or steam, or by immersion in large tanks of water, in which temperature can be controlled and monitored with accuracy, and adjusted to the desired organoleptic quality of the food. The final product appears to be draped by a plastic film, which assumes the same shape as that of the product. The application of *sous-vide* technology to foods has some advantages (Baldwin, 2012): 1) no contamination of the foods after packing, 2) heat transfer and the cooking of foods in their own juices are facilitated by the absence of an air layer, 3) losses of food flavours, aromas, and nutrients are low and 4) oxidation of the foods is prevented by the removal of air (99.9%), and therefore of O_2.

Figure 1 summarizes *sous-vide* development stages from a culinary perspective. Initial steps lead to the "cook-serve" and the "cook-chill" options, named also "direct" and "indirect" cooking, respectively (Roca and Brugués, 2003). These two possibilities depend on the food characteristics. Delicate products that require extremely mild temperatures and short cooking times are prepared during food services, since they are intended for immediate consumption. On the other hand, tough foods need higher cooking temperatures and times to soften. As certain degree of pasteurization is achieved, storage of these products under refrigerated conditions is possible. The "cook-chill" option includes low temperature long time heat treatments where ingredients are vacuum-sealed and heated at temperatures as low as 60 to 65 °C for 2–8 h. Shorter cooking times are also assigned to this group if tender foods like chicken breast have to be preserved. In this case, precise heating is even more important to avoid overcooking and to assure safety during shelf-life (Schellekens and Martens, 1993; Roca and Brugués, 2003, 2016; Botella, 2010; Agència de Salut Pública de Catalunya, 2012; Baldwin, 2012).

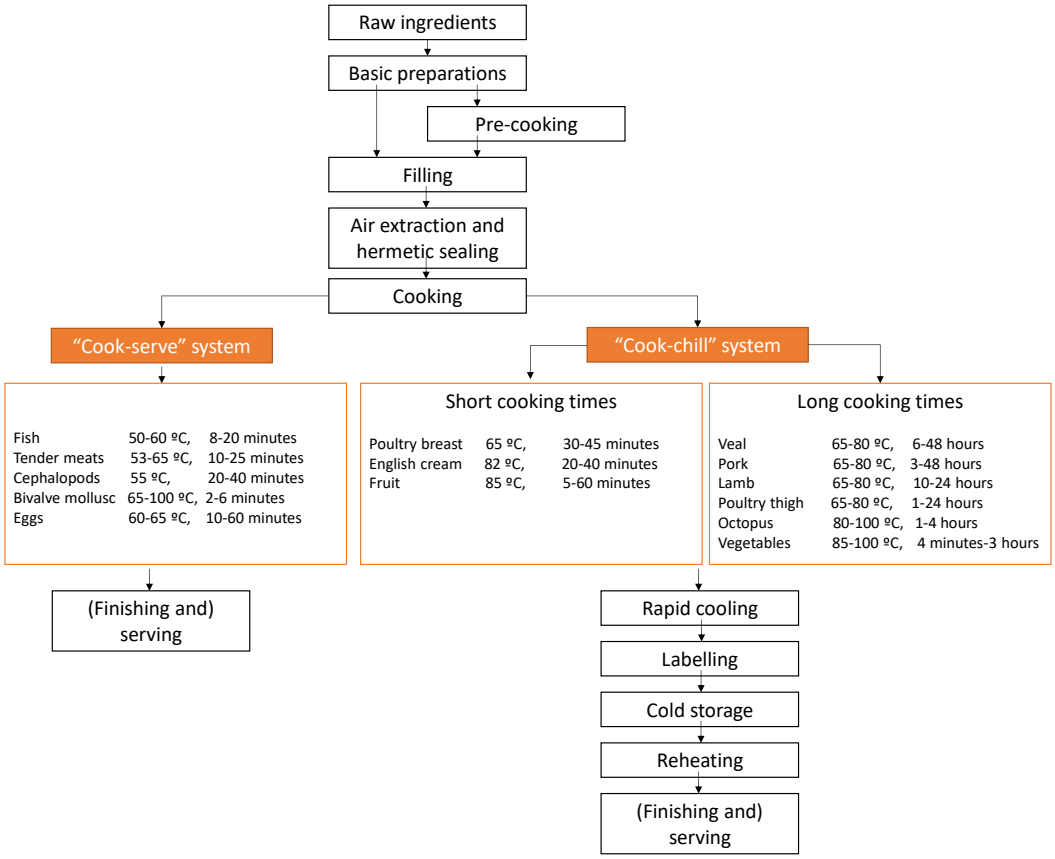


Figure 1. Flow diagram of *sous-vide* cooking (Schellekens and Martens, 1993; Roca and Brugués, 2003, 2016; Botella, 2010; Agència de Salut Pública de Catalunya, 2012; Baldwin, 2012).

In *sous-vide* cooking the combination of time and temperature during heat treatment and storage has to be established to guarantee microbiological safety at the end of self life. With this aim, monitoring of core's product temperature is used to estimate the degree of bacterial inactivation (Schellekens and Martens, 1993; Gould, 1999; Peck et al., 2006; Baldwin, 2012). Table 1 summarizes some time/temperature combinations for heat treatment and storage time (shelf-life) suggested by different authors, according to a specific microorganism lethality.

Table 1. Heat treatments and shelf-life recommended or suggested for cooked chilled foods.

Organism	Target log reduction	Core heat treatment		Shelf-life		Source
		ºC	min	days	ºC	
Listeria monocytogenes	6	65	15	10	4	(UAB and Fundació Alicia, 2012)
		70	2	4	8	(Gould, 1999)
				5	7	
				10	5	
		70	2	According to shelf-life studies		(Peck et al., 2006; Carlin, 2014)
Streptococcus faecalis	13	70	40	6	3	(Ministère de l'Agriculture, 1974, 1988)
	32.5	70	100	21	3	
	325	70	1000	42	3	
Non-proteolytic *Clostridium botulinum*	6	90	10	> 10	8-10	(Stringer et al., 1992; Gould, 1999; Peck et al., 2006)
		85	36			
		80	129			
		or equivalent lethality				

By the late 1990's, Gould (1999) stated that, considering the continued interest in the use of low heating temperatures for some of the more heat-sensitive types of food products, it would be useful to obtain a better definition of lethal rates, injury and recovery of microorganisms at temperatures below 90 °C. Nowadays, there is general consent with the premise of immediate consumption for any preparation cooked at temperatures below 65 ºC in order to avoid the risk of microbial growth. However, an increasing number of *sous-vide* foods are being cooked at mild temperatures that is, cooking treatments where there is not an equivalent combination time/temperature for a 6 log reduction in *Listeria monocytogenes*. A demand of scientific knowledge about microbiological safety of these products has emerged. Although there is a broad application of linear inactivation kinetics in food processing systems with a heating stage, their extended variability has been reported (Van Asselt and Zwietering, 2006; Duan et al., 2016; den Besten et al., 2018). Besides, it is well know that thermal inactivation variability increases with the decrease in temperature (Aguirre et al., 2009; Aryani et al., 2015b) and that care should be taken during mild heat treatments to check on the possible tailing of the inactivation curve

(Geeraerd et al., 2000). In addition, the Food Standards Agency (Stringer et al., 2012) highlights the lack of models describing the behaviour of microorganisms between 40 and 60 ºC. In particular, there are not enough data in the region between those described by growth and inactivation models, where the behaviour of microorganisms is more variable and uncertain. In other words, the authors exposed the need for further steps towards single-cell and molecular knowledge to complete the predictive capability of user-friendly predictive microbiology platforms like Combase (https://www.combase.cc/index.php/en/) of the University of Tasmania and the USDA Agricultural Research Service (USDA-ARS).

1.2. *Salmonella* spp. and *Listeria monocytogenes* as foodborne disease agents

Meat safety concerns, challenges and related issues may be divided into those associated with microbial pathogens and into other meat safety concerns like food additives, chemical residues, products of food biotechnology or genetically modified organisms (GMO), and intentional bioterrorism concerns. Major challenges are, however, and will remain into the future, microbial foodborne illnesses and outbreaks, associated product recalls, and related issues of regulatory compliance (Sofos, 2008).

Salmonella spp. and *L. monocytogenes* are two of the most important foodborne pathogens. Among all confirmed human zoonoses in the EU in 2017, salmonellosis is the second most commonly reported zoonosis after campylobacteriosis. Among *Salmonella* serovars, S. Enteritidis is the most commonly reported. Listeriosis was the most severe zoonoses with the highest hospitalisation and mortality rate (Table 2). *Listeria* infections were most commonly reported in the elderly population in the age group over 64 years and particularly in the age group over 84 years. Table 2 shows reported hospitalisation and case fatalities due to salmonellosis and listeriosis in confirmed human cases in the European Union (EU) during 2017.

Table 2. Reported hospitalisation and case fatalities in confirmed human cases in the European Union during 2017 (EFSA Panel on Biological Hazards, 2018a).

		Salmonellosis	Listeriosis
	Confirmed cases	91,662	2,480
	Notification rate per 100,000 population	19.7	0.48
Hospitalization	**Status available (%)**	43.1	40.4
	Reporting Member States	14	16
	Reported hospitalised cases	16,796	988
	Proportion hospitalised (%)	42.5	98.6
Deaths	**Outcome available (%)**	67.8	65.8
	Reporting Member States	17	18
	Reported deaths	156	225
	Case fatality (%)	0.25	13.8

Availability of descriptive information on the suspected food vehicle is higher for outbreaks than for sporadic cases. Regarding the food vehicle in strong-evidence food-borne outbreaks, the 19 % involved meat and meat products (i.e. including meat from poultry, pork, bovine, sheep, and other unspecified red meats and their products). 'Mixed food' and 'buffet meals', as well as 'other foods' including 'unspecified foods' were reported in almost 25 % of all strong-evidence outbreaks (EFSA Panel on Biological Hazards, 2018a).

Salmonella spp. is an important cause of foodborne disease in humans throughout the world and is a significant cause of morbidity, mortality and economic loss. Most of the infections are transmitted from healthy carrier animals to humans via contaminated food. Implicated foods are normally beef, pork, poultry, dairy products, eggs and fresh produce (Hald, 2013). The main reservoir of zoonotic *Salmonella* is the gastrointestinal tract of warm-blooded animals including food-producing animals. European Union monitoring data of *Salmonella* in pigs in 2017 shows that overall flock prevalence was 12.7% of the

sampled units. Among these, about 80% were collected at the slaughterhouse and 14.2% were positive (EFSA Panel on Biological Hazards, 2018a).

With respect to *Salmonella* process hygiene criteria monitoring data from pig carcasses, the proportions of *Salmonella* positive single samples from official control by competent authorities and from self-monitoring by food business operators were, respectively, 2.15% and 1.85%. In the other hand, the 1.6% of fresh pig meat samples and the 0.50% of the tested samples of RTE minced meat, meat preparations and meat products from pig meat were *Salmonella*-positive (EFSA Panel on Biological Hazards, 2018a) Data occurrence in pig carcasses was quite lower than those reported by the 2006-2007 Eropean Union-Wide Baseline Survey on the prevalence of *Salmonella* in slaughter pigs. Based on samples of lymph nodes of slaughtered pigs, *Salmonella* prevalence at the EU level was 10.3%, whereas carcass swabs showed that the prevalence of *Salmonella* was 8.3%. In both cases, data varied among member states (MS) (The EFSA Journal, 2008).

Following a request from the European Commission, the Panel on Biological Hazards was asked to deliver a scientific opinion on a Quantitative Microbiological Risk Assessment (QMRA) of *Salmonella* in slaughter and breeder pigs. The assessment would provide the input for a future cost/benefit analysis of setting a target for reduction in slaughter pigs at EU level. From the descriptive and comparable analysis of the serovar distribution in animal sources and humans, a cautious assessment would be that around 10-20% of human *Salmonella* infections in EU may be attributable to the pig reservoir. However, the use of this estimate necessitates caution due to the lack of MS-specific data on the distribution of serovars in humans. From the QMRA analysis it appears that an 80% or 90% reduction of lymph node prevalence should result in a comparable reduction in the number of human cases attributable to pig meat products (EFSA Panel on Biological Hazards, 2010).

L. monocytogenes is the only human pathogen of public health significance in the *Listeria* genus. Exposure to *L. monocytogenes* may manifest as mild gastroenteritis in healthy individuals, or as invasive listeriosis in high-risk host populations. Invasive listeriosis may lead to septicaemia, meningitis, encephalitis, as well as spontaneous abortions or still births in pregnant women (Chen and Nightingale, 2013).

L. monocytogenes is a psychrotrophic microorganism. Its lower limit for growth of in matrixes with a high content of nutrients and neutral pH is close to 0 ºC (ICMSF, 1996). In foods, the minimum growth temperature is between 3 and 4 ºC (Tienungoon et al., 2000), although growth at lower temperatures has been observed (Hudson et al., 1994). Cold storage temperatures generally found in the food productive and preservation environment (between 4 and 10 ºC), favour the adaptation and resistance of *L. monocytogenes* to other sources of stress (Lado and Yousef, 2007). The tolerance and ability to growth of *L. monocytogenes* to cold temperatures is one of the main problems for industry and retail establishments of ready-to-eat foods (RTE) (Bover and Garriga, 2014).

According to the European Union Summary Report on Trends and Sources of Zoonoses in 2017 (EFSA Panel on Biological Hazards, 2018a), there has been a statistically significant increasing trend of confirmed Listeriosis cases in the EU during the period 2008–2017. Factors considered likely to be responsible for the increasing trend in cases were the augmented population size of the elderly and susceptible population. The EU case fatality was 13.8% among the 1,633 confirmed cases with known outcome, a slight decrease compared with 2016. Listeriosis in animals is a relatively uncommon disease and reporting is not mandatory in the UE. Considering all different sampling units (‘animal’, ‘herd/flock’ or ‘holding’), the 1.3% were found to be positive. Most animals tested concerned domestic ruminants (cattle, sheep and goats). With respect to prevalence of *L. monocytogenes* in food, differences were seen as function of food category and type of establishment. At retail, the higher level of non-satisfactory results was obtained for soft and semi-soft cheeses, with the 5% of results having ≥ 100 CFU/g. At processing stages, worse results were obtained for meat products and minced meat intended to be eaten raw or ready-to-eat, and meat preparations like *pâté*, with the 4.2% of samples with positive detection (EFSA Panel on Biological Hazards, 2018a). The European Union-Wide Baseline Survey on *L. monocytogenes* carried out in 2010 and 2011 reported a prevalence at the end of shelf-life of 10.3% in fish samples, 2.1% in RTE meat products and 0.5% in cheese (EFSA, 2013).

A determinant factor in *L. monocytogenes* risk assessment studies is its ability to effectively persist in the food processing environment, so that total absence of *L. monocytogenes* cannot be expected. In a comprehensive study, over 2,200 environmental samples were collected following a harmonised sample scheme from 12 European food

processing facilities producing RTE foods of animal origin. Food processing environments (FPE) in each of the facilities were found positive at least once during the sampling period and the overall occurrence rate of *L. monocytogenes* was 12.6%. FPEs at meat-producing facilities were found to be positive at a fourfold higher rate than at milk-processing facilities (EFSA Panel on Biological Hazards, 2018b).

1.3. Predictive microbiology

1.3.1 Concept and history

The term Predictive Microbiology (PM) was first introduced by Roberts and Jarvis (1983) who brought into question the efficiency of traditional methodology and attitudes in food microbiology. They suggested the need to investigate on the measure of microbiological responses rather than on their detailed identity. They also proposed shared experiments between laboratories with standard techniques to facilitate mathematical analysis and build common data banks with predictable capabilities. They certainly established the conceptual basis of the modern PM.

PM is based upon the premise that responses of microorganisms to environmental factors are reproducible. Mathematical description of environments in terms of identifiable dominating constraints make it possible to predict, from past observations, the responses of those microorganisms (Ross and McMeekin, 1994). In the early years of PM, a reductionist approach was generally adopted and microbial responses were measured under defined and controlled conditions. The results were summarised in the form of mathematical equations that, by interpolation, could predict responses to novel sets of conditions, i.e. those not previously tested. For models based on data derived from non-food systems, assurance that the model is applicable to food systems is essential, i.e. after the model has been developed it must be tested against observations in real foods (McMeekin et al., 1993).

Mild food processing has become quite a challenge for PM because it frequently results in very low contamination levels of foods. The outcome of such contamination is greatly affected by the variability among single cells, and models based on average bacterial behaviour could result in incorrect predictions (Pin et al., 2013). *Sous-vide* cooking at mild temperatures is a particular case of mild treatment that can lead to low contaminated food with injured cells, presence of adaptive responses and/or cross protection against

other lethal exposures. To model this phenomena, more experimental data is necessary, as well as advances in the use of large datasets.

Actual trends in PM focus on developing an integrative view of bacterial responses. Whilst predictive modelling continues to rely heavily on studies of microbial population behaviour in foods (quantitative microbial ecology), integration of this knowledge with observations of attendant physiological events and with molecular approaches provides a basis for a holistic understanding at the cellular and subcellular level. Pin et al. (2013) show a top-down framework were trends in PM are placed in three stages: population level, single cell level and molecular level. The first level includes deterministic models that predict responses of homogeneous, relatively large, cell populations by means of kinetic parameters like the maximum growth rate (Gr_{max}, if expressed in decimal logarithm or μ_{max} if expressed in natural logarithm) or the latency/lag time (λ). The single cell level includes probabilistic models that take into account variability among single cells, being more precise in case of low contaminated food. Their outputs are distributions of kinetic and physiological parameters of single cells. And finally, system models that understand and imbed the molecular mechanisms underlying the cell response by using bioinformatics, system biology or neural networks.

PM has become a tool to improve food safety and quality. Some of its potential applications include Hazard Analysis and Critical Control Points (HACCP), Risk Assessment and Risk Management, shelf-life studies, innovation and development of new products, hygienic measures and temperature integration, education and experimental design of scientific studies (Pérez-Rodríguez and Valero, 2013).

Besides, PM is considered in the current EU Regulation on microbiological criteria for food (European Parliament and Council, 2005). According to that, food business operators shall ensure that foodstuffs comply with the microbiological criteria specified. When necessary, manufacturers of ready-to-eat (RTE) foods that are able to support the growth of *L. monocytogenes* shall conduct shelf live studies using tools of predictive mathematical modelling such as the DFMIT software of Combase. Many of the reference or guidance documents published since 2005 in order to help operators conducting shelf-life studies suggest the use of predictive models. These documents are promoted either by official authorities and/or private associations in the EU (European Commission, 2008; Chilled Food Association Ltd, 2010; Agencia Española de Seguridad Alimentaria y

Nutrición, 2011; Beaufort et al., 2014; Bover and Garriga, 2014; Food Safety Authority of Ireland, 2014) as well as in Canada (Health Canada, 2012) or Australia (The Australian and New Zealand Food Standards System, 2014). Over the past decade, the interest generated by shelf-life studies on *L. monocytogenes* in RTE foods has been reflected in many scientific publications describing practical aspects on their execution such as different methodological approaches or results interpretation (Uyttendaele et al., 2009; Vermeulen et al., 2011; Álvarez-Ordóñez et al., 2015).

1.3.2 Predictive models

The models used in predictive microbiology can be classified using different approaches (Ross and McMeekin, 1994; McKellar and Lu, 2004; Pin et al., 2013; Pérez-Rodríguez, 2014). A glance at these classification criteria is given in this section in order to introduce the models used in this thesis.

A general classification divides the models into empiric or mechanistic. Empiric models (also called descriptive or "black box" models) are those that fit a function to experimental data, with no consideration of the mechanism of action behind microbial behaviour. On the other hand, mechanistic models are those whose development comes from the understanding of the underlying biochemical and biological processes governing microbial phenomena. In these cases, model parameters are supposed to have a biological meaning. Due to the limited knowledge on these processes, most models tend to be of quasi-mechanistic nature (Pérez-Rodríguez, 2014).

According the type of structure and variables, models can be classified as primary, secondary and tertiary. Primary models are those reflecting the microbial load change with respect to time. They are also sub classified attending to their outcome. Kinetic models describe microbial behaviour by means of a growing or decreasing rate, while experimental outcomes in probabilistic models express dichotomy: growth/ no growth, sporulation/ no sporulation, toxigenesis /no toxigenesis. The use of probabilities requires the generation of many experimental data. Posada-Izquierdo et al. (2013) stated that almost 6 to 8 repetitions of each experimental condition are needed.

The model proposed by Baranyi and Roberts (1994) is one of most used with data of different food-borne microorganisms, such as *E. coli* (Pin and Baranyi, 2006; Posada-Izquierdo et al., 2013), *Salmonella* (Pin et al., 2011; Aspridou et al., 2018), or *Listeria*

(Métris et al., 2008; Aguirre and Koutsoumanis, 2016). In case of inactivation, linear exponential decay of counts with time can be explained by the Bigelow model (Bigelow, 1921). However, in many cases, the survival curve is not linear and presents a downward concavity (presence of a "shoulder") or an upward concavity (presence of a "tail"). Then, data need to be modelled with non-linear models, which represent the heterogeneity of the microbial population. The great variability of non-linear inactivation patterns has led the authors to take several modelling approaches. Weibull models (Mafart et al., 2002; Peleg, 2004) are built on the basis that the fraction of survivors can be described by the cumulative form of a Weibull distribution of temporal mortality events. Other approaches are the use of the biphasic model and models accommodating shoulder and tail, etc. as described in a study of Geeraerd et al. (2005).

Secondary models predict the changes in the parameters of primary models such as bacterial growth rate and lag time as a function of the intrinsic and extrinsic factors. Nonetheless, the latter is less frequent due to lag-specific secondary models being less accurate, since this kinetic parameter is strongly influenced by pre-culture conditions (Baty and Delignette-Muller, 2004; Swinnen et al., 2004; Aryani, 2016). Finally, tertiary models are implementations of primary and secondary models in software tools in order to provide estimates of microbial behaviour under specific conditions defined by users. Two of these tertiary models have been used in this thesis to obtain prediction kinetics of growth (DMFIT tool of Combase) and inactivation (*Bioinactivation* FE https://opada-upct.shinyapps.io/bioinactivationFull). Some review articles about PM list available tertiary models (Arroyo-López et al., 2014; Bover and Garriga, 2014; Pérez-Rodríguez, 2014). Even so, people interested in PM (food technologist, quality management personnel or food safety authorities) should be aware of continuous improvements or new developments in these user-friendly platforms. Among them, implementation of single-cell models to extend the scope of actual tools is required in performing quantitative risk assessment of mild treated products.

Table 3 shows a general classification of models in PM detailing the ones that will be used with the experimental data of this thesis. The following sections present the equations of each model. In case of inactivation models used in *Bioinactivation* FE, equations have been implemented in differential form, to be able to describe dynamic inactivation (Garre et al., 2017).

Table 3. Classification of predictive models used in this thesis depending on the microbiological event modelled and the type of outcome generated.

Variables	Event	Outcome	Models
Primary	Growth		Baranyi and Roberts
	Inactivation/ survival	Lineal	Bigelow
		Non-lineal	Mafart
			Geeraerd
	Growth / no growth	Probability models	
Secondary	Inactivation/ survival	Lineal	Bigelow
Tertiary			Combase
			Bioinactivation FE

1.3.2.1 Model of Baranyi and Roberts

The model of Baranyi and Roberts (1994) describes a sigmoid bacterial curve. The main difference between this model and other sigmoid curves is that the mid-phase is close to linear. Other sigmoid curves such as *Gompertz* or *Logistic* have a pronounced curvature there. This model is considered quasi-mechanistic because it assumes that during lag phase bacteria need to synthesize an unknown substrate critical for growth. Once cells have adjusted to the environment, they grow exponentially until limited by restrictions dictated by the growth medium. The model of Baranyi and Roberts is shown in equations (1) and (2) (Pérez-Rodríguez and Valero, 2013).

$$\frac{dx}{dt} = \frac{q(t)}{q(t)+1} \cdot \mu_{\max} \cdot \left(1 - \left(\frac{x(t)}{x_{\max}}\right)^{m}\right) x(t) \qquad (1)$$

$$h_0 = \ln\left(1 + \frac{1}{q_0}\right) = \mu_{\max}\lambda \qquad (2)$$

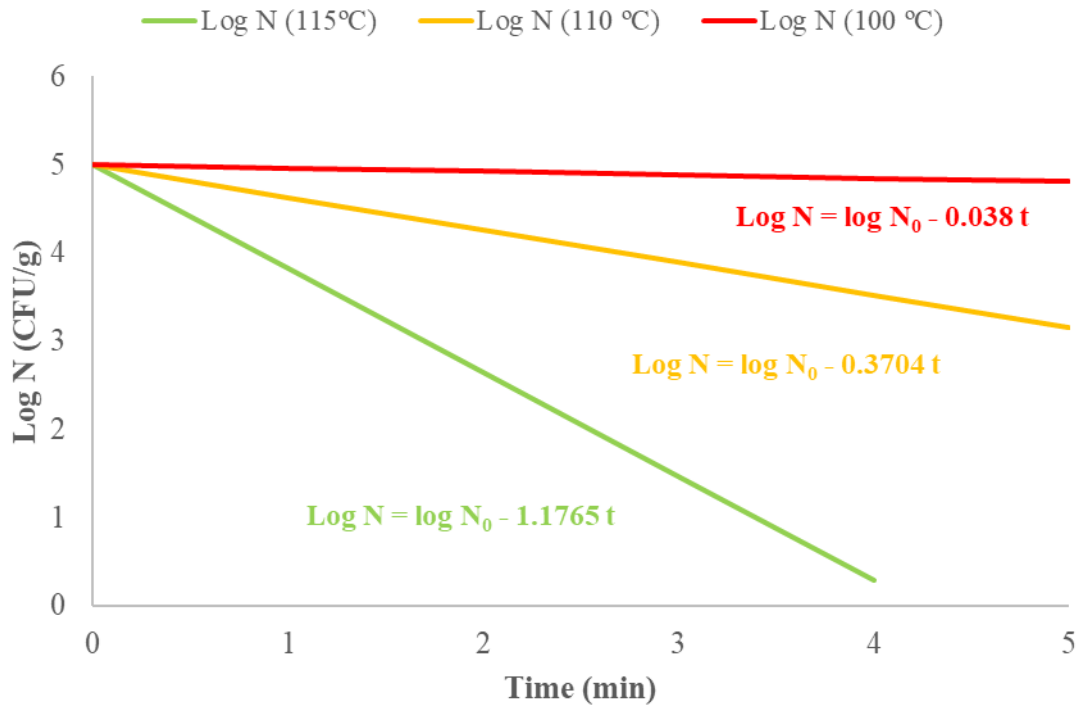
where x is the number of cells at time t, x_{max} the maximum cell density, and $q(t)$ is the concentration of the limiting substrate, which changes with time. The parameter m characterizes the curvature before the stationary phase. The initial value of q (q_0) is a measure of the initial physiological state of the cells. The parameter h_0 describes an interpretation of the lag and may be regarded as the 'work to be done' by the bacterial cells to adapt to their new environment before commencing exponential growth at the rate μ_{max} characteristic of the organism and the environment. λ is the lag phase.

The application of this model has been facilitated by its availability on Combase, by means of the DMFIT tool.

1.3.2.2 Log-linear model of inactivation

This model was first proposed to quantify microbial inactivation in the canning industry, assuming a first order kinetics under isothermal conditions (Bigelow, 1921; Esty and Meyer, 1922). The premise is that the number of surviving cells decreases exponentially. In other words, the $\log_{10}$ of microorganism concentration plotted against time of treatment is a straight line (Figure 2A). The log-linear primary inactivation model is shown in equation (3). According to this model, the microbial count (N(t)), decreases with time (t) so that the time required for one logarithmic reduction under constant temperature (T), is defined by a parameter called D value (D_T).

A



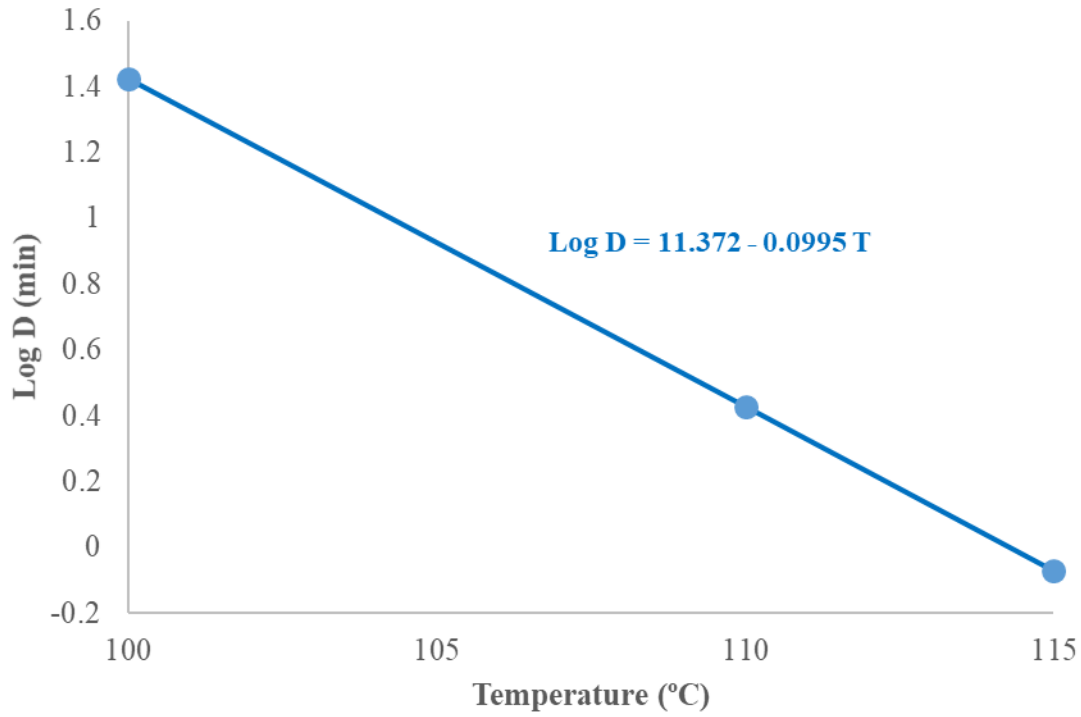


Figure 2. A) Log-linear survivor curve at different temperatures. B) Thermal Death Time plot. Based on Casp and Abril (2003)

Bigelow (1921) also concluded that the logarithm of the D value varied linearly with temperature. In fact, repeated experiments at isothermal conditions lead to a series of D values at different temperatures. Plotting $\log_{10}$ D values as function of temperature results in a straight line (Figure 2B). The reverse of the slope of this straight line is called *z* value, and is defined as the temperature increment required for a ten-fold change of the log D value. *z* value is a measure of the sensitivity of the D value to temperature changes. The equation of this straight line (4) constitutes the log-linear secondary inactivation model. T_{ref} is a reference temperature defined by the user according to the target microorganisms for each type of food.

$$\frac{d\log_{10}N(t)}{dt} = -\frac{1}{D_T} \tag{3}$$

$$\log_{10}D_T = \log_{10} D_{T_{ref}} - \frac{T - T_{ref}}{z} \tag{4}$$

The first aim of these models was to calculate the lethality of heat treatments. Ball and Olson (1957) introduced the so called Thermal Death Time (TDT or F value), that corresponds to the time required to cause a stated reduction in a population of micro-organism or spores. It is expressed as a multiple of the D value of a microorganism taken as reference. In the canning industry, the 12D concept has been safely applied for years to ensure virtual absence of proteolytic *C. botulinum*, meaning a survival rate of 1 spore in a population of 10^{12} spores. This log reduction is achieved by a treatment of 3 minutes at a reference temperature (T_{ref}) of 121.1 ºC. But heat processes take place at different temperatures. As the *z* value of *C. botulinum* spores is 10 ºC, equation (4), can be used to obtain the equivalent time of treatment at another temperature (T).

$$\log_{10}D_T = \log_{10} 3 - \frac{T - 121.1}{10}$$

Considering a temperature (T) of 115 ºC, the time (D_{115}) needed to obtain a 12D reduction of *C. botulinum* would be 12,22 minutes.

Equation 4 is mostly used to compare a real time / temperature combination with a treatment at a referenced temperature. For example, a heat treatment of 30 minutes at 100 ºC is equivalent to another treatment of 0.23 minutes at 121.1 ºC with respect to lethality of *C. botulinum* spores. Since F value is noted as F_z^{Tref}, it would be said that a heat treatment of 30 minutes at 100 ºC has a $F_{10}^{121.1}$of 0.23 minutes. Equation (5) shows a simple scale and log transformation of equation (4) that is mostly used to compare heat treatments of equivalent lethality.

$$F = t_{ref} = t \,.\, 10^{(T-Tref)/z} \qquad (5)$$

were t and T are known data of a heat treatment which lethality is equivalent to the obtained by another heat treatment of F minutes at the referenced temperature T_{ref}.

Since the temperature of the food during the heating process changes with time, F value is determined as the summation of the lethal rate or lethality obtained at each temperature interval. The most usual form for continuous F value computation is given in equation (6) (Holdsworth, 2004).

$$F = \int 10^{\,(T-Tref)/z} dt \qquad (6)$$

Nowadays, ovens or retorts are equipped with multipoint thermocouples that detect the slowest heat penetration curve in the food package. An internal computer calculates in real time the correspondent F value. These records obtained for each cooking cycle are integrated in the quality assurance system of the food operator.

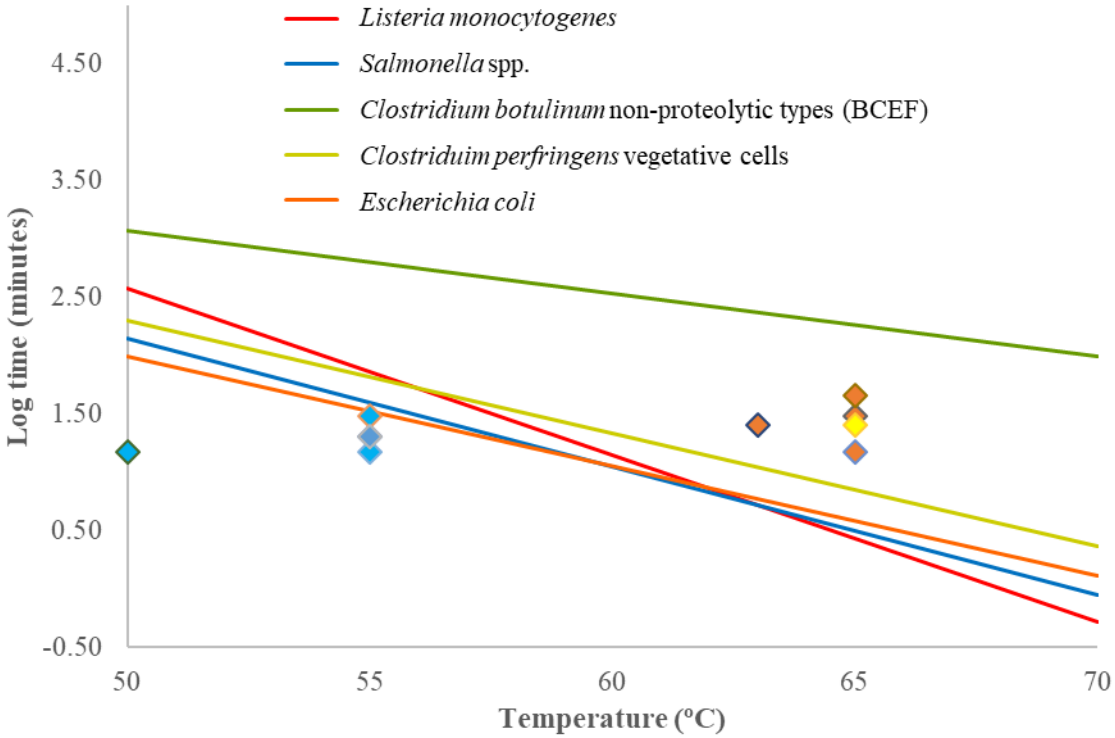
Log-linear model applied to *sous-vide* mild treatments

For “cook-serve” *sous-vide* products, the main pathogens of interest are the *Salmonella* species and the verotoxigenic strains of *E. coli*. There are, of course, many other food-borne pathogens like *Campylobacter* spp. This pathogen requires very few vegetative bacteria per gram to cause illness but is relatively heat sensible. For “cook-chill” *sous-vide*, *L. monocytogenes Yersinia enteocolitica* and non-proteolitc *C. botulinum* are the pathogens of interest due to their growing capacities at chill temperatures. Moreover, in

"low temperature long time" cooking, treatments take hours until meat can reach a final core temperature above 53 °C. Then, germination and growth of spore-forming bacteria, specially *C. perfringens,* is a concern.

Log D values are plotted versus temperature for the aforementioned microorganisms. Figure 3A shows the mean log D value while Figure 3B shows the 95% upper prediction interval given by Van Asset and Zwietering (2006). Coloured lines correspond to the reference value for a 6D reduction of the microorganism of concern. Blue, yellow and brown rhombus correspond to cooking time/temperature combinations for fish, eggs and meat receipts suggested by Roca and Brugués (2016). As can be seen, *Salmonella* spp. is the most heat resistant vegetative bacteria according to the 95 % confidence intervals of D values (Van Asselt and Zwietering, 2006), due to the effect of the matrix (e.g. it is especially heat resistant in chocolate). In addition, it can be noted that below 63 ºC, *Salmonella* spp. is always more heat resistant that *L. monocytogenes*.

A



B

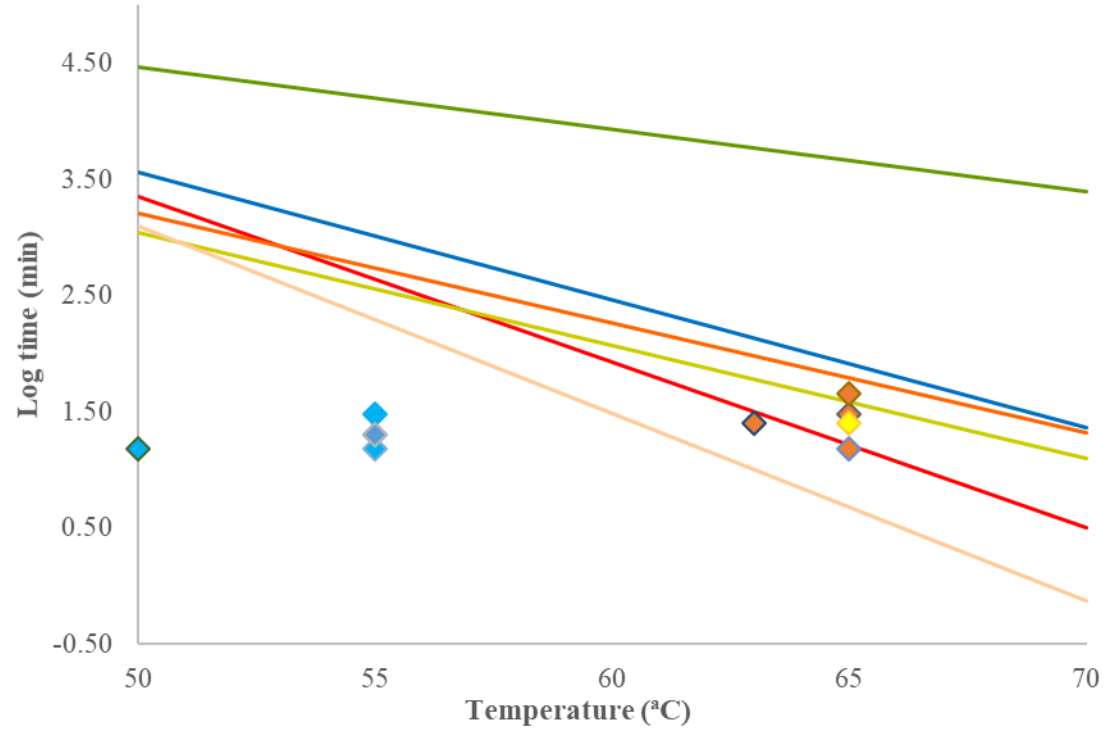


Figure 3. Heat resistance of pathogens of concern in *sous-vide* using the mean (A) and the 95% prediction upper level (B) based on Van Asselt and Zwietering (2006). Blue, yellow and brown rhombus correspond to cooking time/temperature combinations for fish, eggs and meat receipts, respectively, suggested by Roca and Brugués (2016).

1.3.2.3 Weibullian inactivation models

Weibullian inactivation models (Peleg and Cole, 1998; Mafart et al., 2002) are based on the hypothesis that microbial inactivation can be described as a failure phenomenon. Therefore, the time required to inactivate an individual cell follows a Weibull distribution. This distribution is defined by two parameters, the scale parameter α and the dimensionless shape parameter β. An upward concavity is described by β <1 while a downward concavity is described by β >1. If β=1, the survival curve is linear.

The model defined by Mafart *et al.* (2002) has 4 model parameters (*p, δ_{Tref}, z and T_{ref}*). *p* is the shape parameter, equivalent to β in the Weibull model. The scale parameter δ_T is the time of the first decimal reduction at a particular temperature. The Mafart model uses a secondary model for δ_T similar to the one devised by Bigelow for the D value (Equation 6), that includes the *z* value described before. The reference temperature (T_{ref}) is usually known by the user, reducing the number of model parameters to estimate to 3.

The model is described by the following system of differential and algebraic equations in *Bioinactivation FE*:

$$\frac{d\log_{10}N(t)}{dt} = -p\left(\frac{1}{\delta_T}\right)^p t^{p-1} \tag{7}$$

$$\log_{10}\delta_T = \log_{10}\delta_{T_{ref}} - \frac{T - T_{ref}}{z} \tag{8}$$

Peleg and Cole (1998) considered a different parameterization of the Weibull distribution. Nonetheless, its equation is equivalent to equation (7). However, they use a different secondary model that does not include the *z* value. The model of Mafart has obtained better fitting indexes in our experimental conditions.

1.3.2.4 Geeraerd model

The model described by Geeraerd *et al.* (2000) modifies Equation (3) to account for shoulder and tail effects, as shown in Equation (9).

$$\frac{dN}{dt} = -\alpha \cdot k_{max} \cdot \gamma \cdot N(t)$$

$$\frac{dC_c}{dt} = -k_{max_T} \cdot C_c \qquad (9)$$

$$\alpha = \frac{1}{1 + C_c}$$

$$\gamma = 1 - \frac{N_{res}}{N}$$

$$k_{max_T} = k_{max} 10^{-(T - T_{ref})/z} \qquad (10)$$

The description of the shoulder effect is based on the inclusion of a hypothetical substance C_c, which decays with a temperature at specific rate which maximum value is indicated as k_{maxT}. $N(t)$ is the number of microorganisms surviving at time t. The tail height is defined by the parameter N_{res}, that is residual population density. The relationship between k_{max} and temperature is described using Equation (10) similar to the one used in the Bigelow model.

This model has 4 model parameters (k_{maxT}, T_{ref}, z *and* N_{res}) and 2 variables (N and C_c). The reference temperature is usually set by the user, reducing the number of parameters to 3.

1.3.3 Goodness-of-fit model indexes

With the exception of the Bigelow model, mathematical models exposed previously are described by non-linear function(s) (i.e. functions where there is not a linear relationship between parameters and variables). The type of function included in the model affects both the fitting procedure and the statistical goodness-of-fit indexes that assess if the mathematical function fits well to the data points. In any case, it is necessary to test the normality of residuals in order to detect extreme observations (outliers).

In general, linear models can be adequately assessed by using the Coefficient of Determination (R^2), that is the ratio of the sum of squares due to regression to that of the

total sum of squares of the response variable around its mean. R^2 is an oft-used criterion appearing in the scientific literature. Similarly, another goodness-of-fit measure, the adjusted R^2 (R^2_{adj}) is based upon de variances (i.e. the mean squares) rather than upon the sum of squares. The R^2_{adj} attempts to penalize the inclusion of redundant parameters. However, the use of either of these measures for non-linear regression is inappropriate, usually leading to a rather overoptimistic view of the success of the modelling process (Ratkowsky, 2004; Geeraerd et al., 2005). Therefore, more suitable goodness-of-fit measures of non-linear models have been used. The root mean square error (RMSE), also called standard error of fit (SE) in Combase, is probably the most common index to test the goodness of fit of models to the data. Its simplicity and easy interpretation make it suitable for a first approach to the fitted model. Also, RMSE is a valid index for both linear and non-linear mathematical functions (Ratkowsky, 2004). A low RMSE value indicates a good fitting to data as a result of the closeness of the data points to the fitted model. In turn, a high RMSE value signals that the data points are far from the fitted models, that is, a poor fit to the data. The bias factor (B_f) is an overall average of the ratio of discrete model predictions to observations and assesses whether the model is fail-safe, fail-dangerous, or perfect. A value of 1 means that observations are equally distributed above and below predictions producing a perfect concordance, values < 1 mean a fail-dangerous model, and values > 1 indicate a fail-safe model. The acceptable B_f value for a predictive model can be 0.75–1.25 (Pérez-Rodríguez and Valero, 2013). Although the B_f was originally proposed by Ross (1996) as a step in model validation, a later article proposed a refined version (Baranyi et al., 1999), which is the one implemented in Bioinactivation FE. This new definition is consistent with established measures of goodness-of-fit and has similar meaning and utility to the meaning of the original B_f.

1.3.4 Relevant modelling issues

Considering the objectives and results of this thesis, there are some modelling issues of relevance: heterogeneity in microbial responses, non-linear inactivation kinetics, lag behaviour, growth/no growth models, stress, sublethal injury, adaptation and cross-protection, the generation of experimental data and detection of injured cells.

1.3.3.1 Heterogeneity in microbial responses: measure of uncertainty and variability

Population heterogeneity is an important component of the survival strategy of a microbial population. The totality of heterogeneities in an adapting population increases the chance that at least some individuals meet immediate or future challenges. Aryani et al. (2015a; b) studied different sources of variability in growth and inactivation kinetics of *L. monocytogenes*. Experimental variability was defined as the difference between duplicate experiments carried out in parallel at the same time on the same experimental day. Biological or reproduction variability was defined as the difference between independently reproduced experiments of the same strain performed on different experimental days from new pre-cultures and newly prepared media, and strain variability was defined as the difference between strains of the same species. In relation to thermal inactivation, strain variability was four times higher than the biological variability and ten times higher than the experimental variability. For all variables of growth kinetics, experimental variability was clearly lower than biological variability and strain variability and, remarkably, biological variability was similar to strain variability. In order to account for variations in growth and survival among strains, challenge studies should generally be conducted using an inoculum composed of multiple strains (i.e., a cocktail) of a given pathogen because it will help to encompass the variability among organisms and may reduce the number of required tests (NACMCF, 2010).

Variation found in experimental data plays an important role when modelling microbial responses. The sources of this variation can be categorised as variability or uncertainty. Variability represents a true heterogeneity of the population of subjects considered, and it is a consequence of the physical system and irreducible by additional measurements. It can be observed and quantified. One type of variability is stochasticity, where heterogeneity is a consequence of randomness. It should not be confused with uncertainty, that represents a lack of perfect knowledge and may be reduced by gathering additional knowledge, for example by further measurements. In principle, this lack of knowledge can be quantified based on some assumptions and beliefs. For example, the confidence interval that results from a statistical analysis usually serves as such a quantification of uncertainty. Other uncertainties, like a lack of representativeness, may be very hard to quantify, especially if data on a process step or a model parameter are missing. Nevertheless, uncertainty is an intrinsic aspect of microbiology, which should be acknowledged. Identification of the difference between variability and uncertainty may

be essential for a correct interpretation of predictive models. However, it is often difficult to dissociate variability and uncertainty, especially when both have the same order of magnitude. Mixing up uncertainty and variability, or improperly mixing up different sources of variability, may lead to a wrong interpretation of the results: if the probability distributions of the input parameter do not describe the same thing, it is not clear what the output distribution describes. This problem is complex, but can partly be solved by characterising the variability in results of repeated experiments (Nauta, 2007).

Monte Carlo simulation is one of the numerical techniques used for calculating these distributions of output parameters, and it is performed as follows. According to a first model fitting of experimental data (Figure 4b), the possible distribution of each input variable is defined (Figure 4d). Then, the probability distribution for the model output variables are constructed by randomly selecting values for input variables determined by their distribution (Figure 4e) and performing the operations on them according to the model's equation (Figure 4f). Then, the model is recalculated repeatedly (e.g., 10.000 times or iterations). Thus, 10.000 combinations of possible inputs are used to simulate 10.000 possible outcomes. When repeated many times, some output values are generated more often than the others because they result from the combinations of inputs that occur more often according to their probability distribution. The results of a Monte Carlo simulation are the likelihood of any outcome occurring and the ranges of possible outcomes that could occur, that is, distributions of possible outcomes. This range of possible outcomes is one of the major advantages of stochastic models because it allows risk managers to evaluate less likely events and decide whether their occurrences are acceptable or not (Vose, 2008; Ruzante et al., 2013).

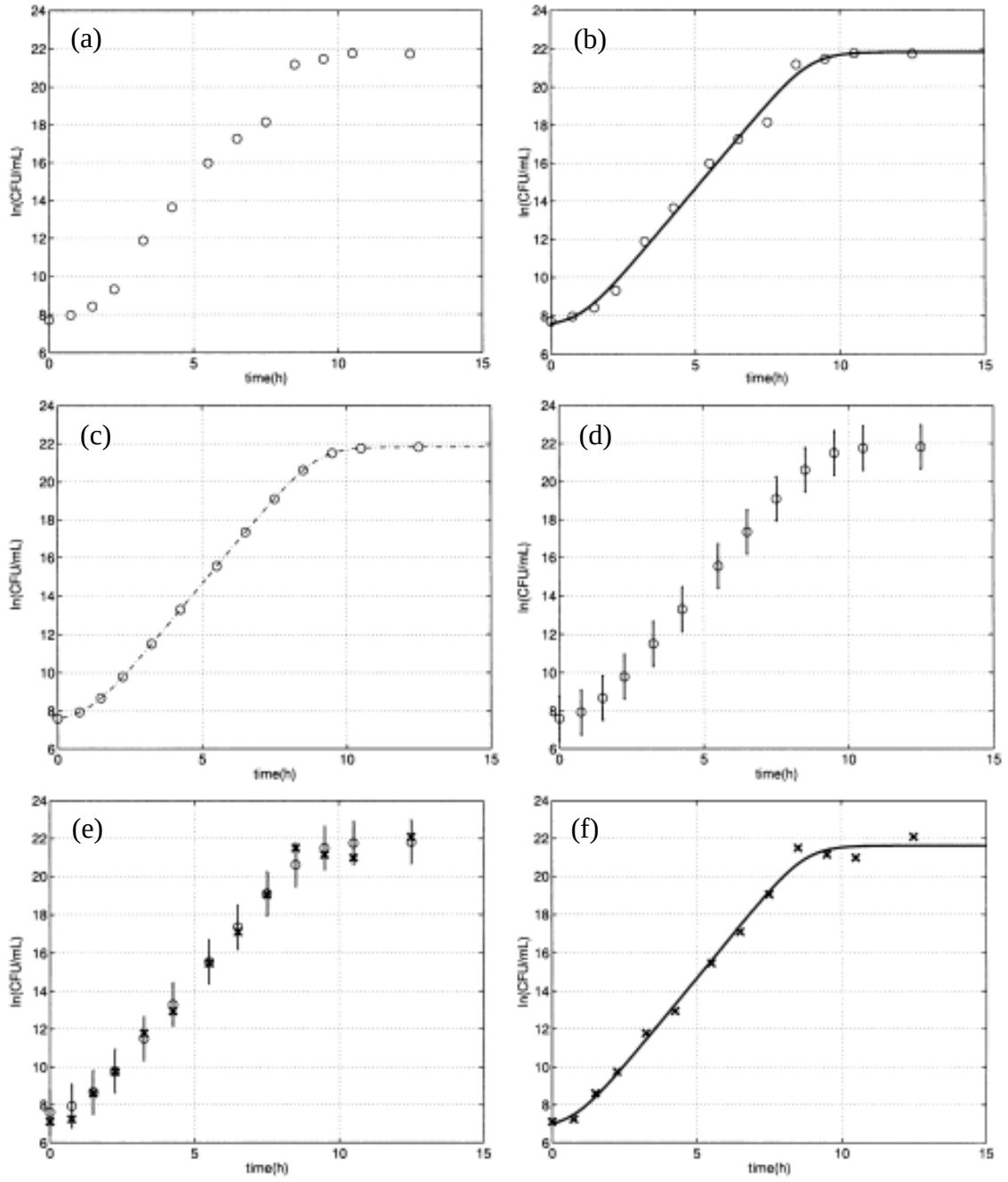


Figure 4. Monte Carlo procedure: (o): experimental data-set; (x): generated data-set. Reference is made to the text for the description of the different steps. Republished with permission of Academic Press, from "Monte Carlo analysis as a tool to incorporate variation on experimental data in predictive microbiology", F. Poschet, A.H. Geeraerd, N. Scheerlinck, B.M. Nicolaï, J.F. Van Impe, Food Microbiology 20 (2003); permission conveyed through Copyright Clearance Center, Inc.

Poschet et al. (2003) considered the overall variation on plate count data using Monte Carlo simulation in a methodological-oriented study. This method has been used in numerous studies to illustrate the propagation of the variation on experimental data (i) to the model parameters, and (ii) to the time predictions of microbial load. Posada et al.

(2013) used 6-8 replicates of the same inoculum and experimental conditions in each point analysis in a study contemplating biological variability in *Escherichia coli* O157:H7 in fresh-cut lettuce on agar counts. Koutsumanis and Lianou (2013) also used Monte Carlo simulation to conduct an stochastic approach in microbial growth of colonial growth dynamics of 220 measures on *S.* Typhimurium with time-lapse microscopy videos. Koyama et al. (2017) modelled stochastic variability in the numbers of surviving *S. enterica*, Enterohaemorragic *E. coli*, and *L. monocytogenes* cells at the single-cell level in different conditions of a desiccated environment by measuring turbidity. Nevertheless, Akkermans et al. (2018) concluded that the sigma point method leads to better predictions than Monte Carlo simulation. In the sigma point method, model inputs and parameters are not generated randomly but chosen in a systematic way.

1.3.3.2 Non-linear inactivation kinetics

In the 80's, Cerf (1977) published a review about tailing of survival curves of bacterial spores. Some years later, McKee and Gould (1988) showed that, for physiological reasons, the linearity of the D/z model is restricted to a limited range of temperatures. In fact, there are numerous reports of organisms whose semi logarithmic survival curves are clearly and characteristically non-linear, and it is unlikely that these observations are all due to a mixed population or experimental artefacts, as the traditional explanation implies. An alternative explanation is that the survival curve is the cumulative form of a temporal distribution of lethal events. According to this concept each individual organism, or spore, dies or is inactivated at a specific time. Because there is a spectrum of heat resistances in the population (some organism or spores are destroyed sooner or later than others), the shape of the survival curve is determined by its distribution properties. Thus, semi-logarithmic survival curves whether linear or with an upward or a downward concavity are only reflections of heat resistance distributions having a different, mode, variance, and skewness, and not of mortality kinetics of different orders (Peleg and Cole, 1998).

Upward concavity is a manifestation of the rapid elimination of the weak members of the population leaving progressively more heat resistant survivors, while downward concavity is an indication that accumulated damage sensitizes the survivors. A true linear semi-logarithmic survival curve would indicate that all the population members have the same probability of being inactivated at any given time. The important point here is that any mathematical survival model should be derived from the actual shapes of the

experimental survival curves, and not from the assumption that all inactivation processes obey a single universal law. One must also take into consideration that the general shape of the isothermal semi-logarithmic survival curves of the same organism do not have to remain fixed. Thus, a concavity inversion as the treatment's temperature increases or decreases is by no means unusual (Peleg, 2004; Muñoz-Cuevas et al., 2012).

1.3.3.3 Lag behaviour

" Because outgrowth of pathogens is unacceptable in a food product, there is a need to predict the lag time accurately" (Swinnen et al., 2004).

The lag phase observed at the population level can be defined via the geometry of the population growth curve, namely as the intersection between the inoculum level and the extension of the exponential phase (Pin et al., 2013). But model predictions obtained from data on large population sizes cannot be directly applied to small population sizes. In case of exposure to sublethal stress and/or presence of bacteria in very low concentration, the mean lag time is higher and the distribution becomes broader (increasing variability) (Elfwing et al., 2004; Koutsoumanis, 2008; Métris et al., 2008; Baranyi et al., 2009).

For predictive modelling this increased variance, which represents large uncertainty in a single event and a large variability in lag-times between (small) populations, may have considerable impact on the precision of model predictions for small population sizes. Stochastic models have been used to describe quantitatively the variability of single-cell colonial growth by introducing the kinetic parameters as probability distributions using Monte Carlo simulation in growth models. The output of the model is a stochastic growth curve in which the number of cells in the population at any time is a probability distribution (Nauta, 2007). As a general rule, the more stringent the stress either in the history in the contemporary environment, the longer and more spread the distribution of the lag times of a single cell (Pin et al., 2013).

Recent technological advances enable the study of microbial behaviour at the single cell level. Metris et al (2008) used optical density measurements to estimate the effect of heat treatments on the single-cell lag times of *Listeria innocua* fitted to a shifted gamma distribution. They proposed that the *F-value* concept can be also extended to sublethal injuries, but instead of the log- kill, the recovery time after the heat treatment should be used to quantify its effectiveness. Koutsumanis and Lianou (2013) used time-lapse

microscopy videos of *Salmonella* Typhimurium and found a highly heterogeneous behaviour. They created a stochastic model with initial count (N_0), latency (λ) and maximum growth rate (μ) characterized with probability distributions. Simulations of the model illustrated how the apparent variability in population growth gradually decreases with increasing initial population size (N_0). For bacterial populations with N_0 of >100 cells, the variability is almost eliminated and the system seems to behave deterministically, even though the underlying law is stochastic.

Aguirre and Koutsoumanis (2016) exposed a new approach on lag phase of microbial populations at growth-limiting conditions. They considered that stress environments divide population as function of its ability to growth. As the environment becomes more stressful, an increasing number of cells in the population is not able to grow. Then, the population lag is divided into a physiological lag (estimated by fitting only the growth data of the growing fraction) and the pseudo-lag, that is caused by the presence of the non-growing fraction of population (Figure 5). Population lag is equal to physiological lag when all cells are able to growth. Accordingly, pseudo-lag gets larger as the non-growing fraction increases.

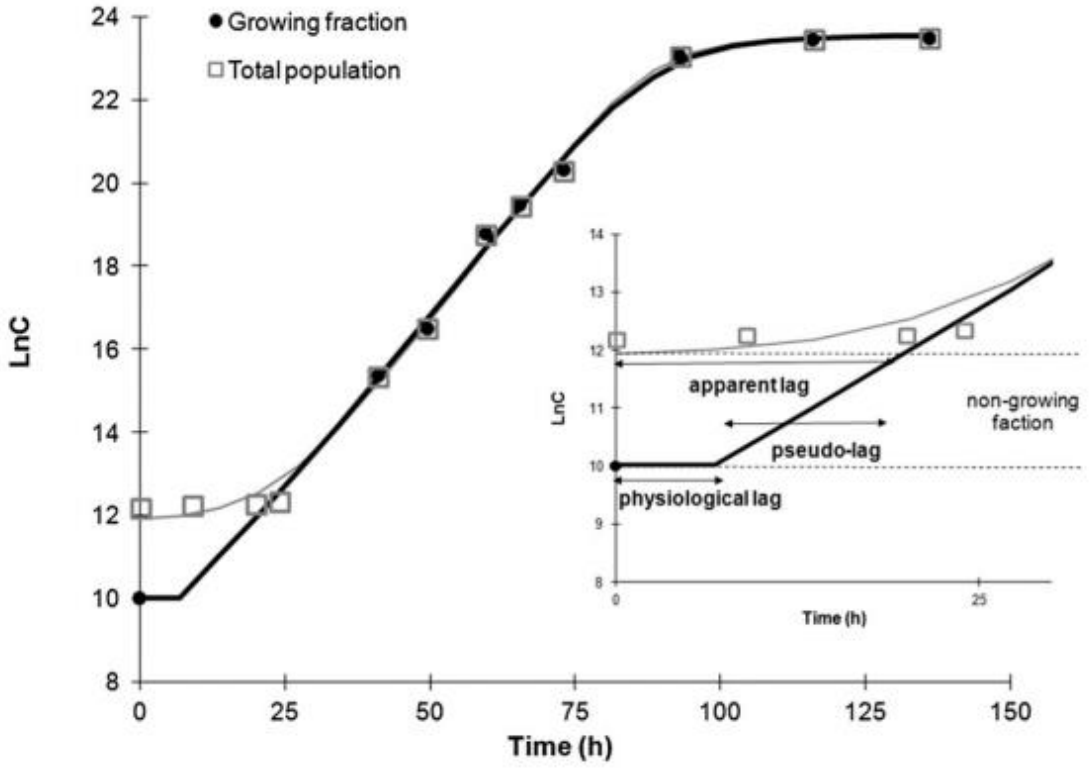


Figure 5. Fitting example of *Listeria monocytogenes* growth on TSA with a_w = 0.940 at 30 °C to the Baranyi and Roberts model. Inner graph shows a zoom in the lag phase. Republished with permission of Elsevier BV, from "Towards lag phase of microbial populations at growth-limiting conditions: The role of the variability in the growth limits of individual cells", J. S. Aguirre, K. P. Koutsoumanis, International Journal of Food Microbiology 224 (2016); permission conveyed through Copyright Clearance Center, Inc.

In their report about *L. monocytogenes* in RTE food, Bover and Garriga (2014) highlight the fact that the duration of the lag phase in shelf-life evaluation may range from 0 to infinity depending on the physiological state of the microorganisms, the magnitude of the shift in the environmental conditions, and the new environmental conditions themselves. Therefore, a cautious approach is preferred and the lag phase is not taken into account to demonstrate a "worst case scenario".

1.3.3.4 Growth/no growth models

Ross and McMeekin (1994) established that growth/no growth models are complementary to kinetic models. Once a significant growth is produced, predictive microbiology leads to growth kinetic models. However, when maximum growth rate approaches to zero and lag phase to infinite, the microbial behaviour should be quantified through growth/no growth conditions. The use of stochastic models has been proposed in these cases, through probability distributions that allow to take into account the variability

observed. Target values of growth probability reported in literature for growth/no growth are normally set at 0.1 (indicating inhibitory conditions), 0.5 (boundary zone), and 0.9 (high probability of growth) (Pérez-Rodríguez and Valero, 2013; Posada-Izquierdo, 2013). Growth/no growth can be used in case of microorganisms for which only their presence can represent a hazard (i.e., spores of *Clostridium* spp.), while kinetic models can be better applied for those non-pathogenic microorganisms or other microorganisms that can be dangerous when exceeding microbial limits, such as *Staphylococcus* spp. (Buchanan 1992).

Growth/no growth models have been widely used for designing formulations in minimally processed foods, by taking into account the *hurdle technology* concept (Leistner, 1992). This is a food preservation technique based on the application of a combination of generally mild treatments that act as 'obstacles' which microbiota must overcome to start to grow. Then, bacteria invest their energy in trying to maintain their homeostatic equilibrium instead of multiplying. Although the action mechanisms underlying these treatments are not fully understood, it is very useful to know their effect on bacteria cells as well as the extension of such effects (Carrasco et al., 2012).

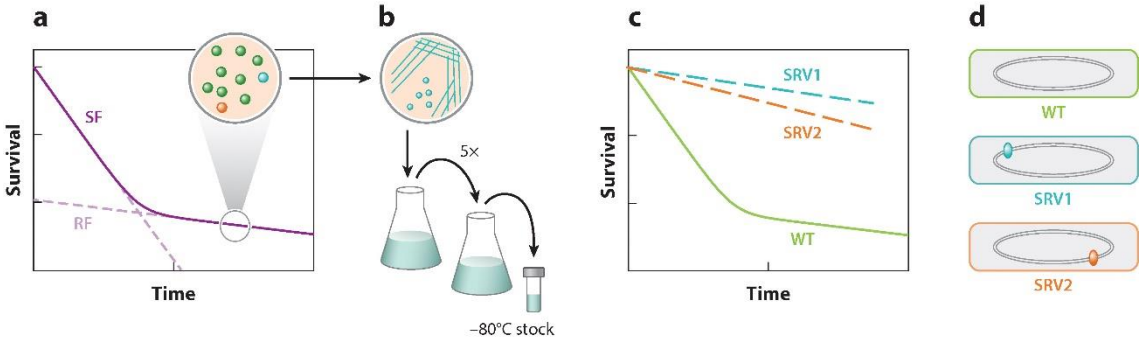
Growth/no growth models have been implemented to determine the combination of factors that just inhibit or allow growth at a specific probability level. The model of Kousoumanis et al. (2004) for *S.* Typhimurium and the model of Valero et al. (2009) for *Staphylococcus aureus* were both build from data in broth media. Pin et al. (2011) combined probabilistic and kinetic models to give predictions on the concentration of *Salmonella* spp. at any stage of the pork supply chain under fluctuating pH, a_w and/or temperature using the ComBase database. Another ComBase-derived tool including microbial G/NG data is the tertiary model developed by Koseki (2009) and named *Microbial Responses Viewer* (MRV). The software can be accessed at http://mrviewer.info. MRV provides useful information concerning growth/no growth boundary conditions and the specific growth rates of different microorganisms.

1.3.3.5 Stress, sublethal injury, adaptation and cross-protection

Bacterial injury may be defined simply as the effect of one or more sublethal treatment on a microorganism that may be transient or permanent, while the term stress is used in reference to the agents or treatments causing injury. Current literature pertaining to

microbial injury typically does not maintain this distinction, and the terms are often used interchangeably (Wesche et al., 2009). Sublethal stresses include physical treatments such as heat, pressure or osmotic shock; chemical treatments such as acids or detergents; and biological stresses such as bacteriocins.

Exposing microorganisms to these challenges may induce adaptation (stress acclimation) to subsequent lethal levels of the same type of stress or to multiple lethal stresses. In other words, microorganisms adapted to these stresses may survive or even proliferate under conditions that could have ordinarily eliminated them. The stress responses are global and complex systems of defence. They comprise networks to adapt to changing environments and to survive under adverse conditions. Enhanced survival of resistant subpopulations is reflected in tailing of the inactivation curve and may include the so-called persistent cells, that are resistant as a consequence of a transient phenotypic switch as well as cells that are resistant because of inheritable mutations (Figure 6). Furthermore, stress adaptation may alter the virulence properties of pathogens and can contribute to survival *in vivo* during infection (Yousef and Courtney, 2003; Juneja and Novak, 2005; Alvarez-Ordóñez et al., 2015; Begley and Hill, 2015; Abee et al., 2016).



Abee T, et al. 2016.
Annu. Rev. Food Sci. Technol. 7:439–56

Figure 6. Schematic presentation of the strategy used to isolate stable stress-resistant variants from the tail of the inactivation curve. (a) Upon exposure to stress, a sensitive wild-type (WT) fraction (SF) and a stress-resistant fraction (RF) can be identified, the latter composed of persister-type WT cells (green) and resistant variants (blue and orange). (b) Approximately 100 colonies are randomly selected from the tail and inoculated in a fresh medium followed by repeated propagation, which provides stock cultures that are stored in the freezer. (c) Stress exposure of cultures derived from the approximately 100 stocks enables the identification and quantification of the number of stable stress-resistant variants (SRVs; represented by SRV1 and SRV2) that show enhanced survival compared to WT. (d) Subsequent comparative genome analysis allows for identification of mutations in the SRVs. Republished with permission of Annual Reviews, from "Impact of Pathogen Population Heterogeneity and Stress-Resistant Variants on Food Safety", T. Abee, J. Koomen, K.I. Metselaar, M.H. Zwietering, and H.M.W. den Besten, Annual

In the case of heat treatments, the existence of a specific defence response, e.g., the heat shock response, has been long known. It is defined as a homeostatic protective mechanism triggered as a consequence of an increase in the extracellular medium's temperature. This signal is recognized by cellular sensors, and the transcription of the genes encoding for the HSPs (heat shock proteins) is induced. Most of the HSP are proteases and molecular chaperones, whose main function is to eliminate aberrant proteins and to aid in the correct folding of proteins. The direct consequence of their presence is the acquisition of a higher heat resistance, and also resistance to other agents such as ethanol or high hydrostatic pressure, which also induce the misfolding of cellular proteins (Richter et al., 2010; Cebrián et al., 2017). Thermal processes that include extended come-up phases, such as low-temperature pasteurization of eggs, slow roasting of certain meat products, or slowly rising temperatures in *sous-vide* can lead to surviving bacterial cultures injured to different extents thereby giving rise to different growth potentials. So, the shape of the dynamic thermal profile applied in heat treatments has to be considered in relation to microbial inactivation (Hansen and Knochel, 2001; Juneja and Marks, 2003; Wesche et al., 2009). Furthermore, the most resistant bacterial strain to a dynamic heating profile should not be identified based only on isothermal experiments (D and z value) but also considering the capacity for developing a stress acclimation (Garre et al., 2018b).

There is also a considerable interest in the cold adaptation of food-related bacteria, including starter cultures for industrial food fermentations, food spoilage bacteria, and foodborne pathogens. Mechanisms that permit low-temperature growth involve cellular modifications for maintaining membrane fluidity, the uptake or synthesis of compatible solutes and the maintenance of the structural integrity of macromolecules and macromolecule assemblies, such as ribosomes and other components that affect gene expression. A specific cold response that is shared by nearly all food-related bacteria is the induction of the synthesis of so-called cold shock proteins (CSPs), which are involved in protein synthesis and/or freeze protection (Wouters et al., 2000) In addition, CSPs are able to bind RNA and it is believed that, like HSP, these proteins act as RNA chaperones, thereby reducing the increased secondary folding of RNA at low temperatures (Nyachuba and Donnelly, 2005)

1.3.3.6 The generation of experimental data

Enumeration methods of microorganisms should be considered because accuracy in data collection is essential in model development. Total viable count (TVC) and optical density (OD) are used in population essays. Both methods are imprecise due to their drawbacks. TVC is extremely laborious and time consuming. Therefore, experimental design becomes relevant because quantity and positioning of data are determinant. A detection threshold in the range of 10^6–10^7 bacteria /mL is the main disadvantage for OD. Besides, OD is an indirect method that does not offer information on the cell vitality and should not be used in the development of inactivation or survival studies (Arroyo-López et al., 2014). On the other side, measuring individual cell behaviour is extremely important in case of stochastic models development with low inoculum size. Serial dilutions of the original inoculum measured by OD, methods based on microscopy and image analysis or flow cytometry have been reported (Elfwing et al., 2004; Konstantinos P Koutsoumanis, Lianou, and Gougouli, 2016; Métris et al., 2008; Carmen Pin and Baranyi, 2008).

1.3.3.7 Detection of injured cells

A large proportion of the survivors from any treatment will often carry some structural or metabolic injury which will impede their capacity for normal growth and metabolism. This is, by definition, a reversible state from which sublethally injured organisms can resume their usual phenotype, given conditions suitable for them to effect repair. Procedures to detect pathogens within the mixed microbiota of foods invariably employ selective agents that will restrict the growth of competitors. In this regard, the situation could lead to an overestimation of the lethality of treatments, or to the failure in the detection of pathogens during routine quality control of foods or outbreak investigations. It is important therefore that, if they are to be detected, injured cells have an opportunity to repair before they are exposed to selective agents. For this reason, a resuscitation step in a nutritionally complex medium, free of any selective agents, is a primary step in the standard isolation and identification protocols for several bacterial pathogens, particularly those causing foodborne infections where the infectious dose may be low (Adams, 2005). Also, sublethal injury has been characterized in some studies by comparing enumerations on nonselective and selective media (Hansen and Knochel, 2001; Yuste et al., 2004;

Wesche et al., 2009; Noriega et al., 2013; Alvarez-Ordóñez et al., 2015; Wang et al., 2017).

2. Objectives

2.1 Statement of the problem

This project started as a final project of a master's degree in Food Safety in 2010. In this research, three independent batches of chicken breasts were inoculated with S. *enterica* subsp. *enterica* serovar Enteritidis CECT 4300 and with *L. monocytogenes* CECT 4031. Inoculated chicken breasts were vacuum packed and cooked in a water bath at 65ºC for 75 min. Lethality and recovery capacity were determined for control and inoculated samples during 21 days of storage at 4 and 10 ºC. Even though a lethality higher than 5 log CFU/g was obtained, both pathogens were able to survive and growth during the storage period, especially at 10 ºC. A high biological (reproduction) variability among the experiments was already observed in this previous study. This variability was attributed to heterogeneity of the food matrix and to the cooking method (number and position of bags into the water bath, that could interfere in heat transmission). During the following two years, some studies were carried out by our group, considering other variables like the food matrix or pre-treatment (freezing), microorganism of concern, cooking temperature and equipment or storage temperature.

Nowadays, there has been general consent with the premise of immediate consumption for any preparation cooked at temperatures below 65 ºC in order to avoid the risk of microbial growth. However, an increasing number of *sous-vide* foods are being cooked at mild temperatures. Besides, it is well know that thermal inactivation variability increases with the decrease in temperature (Aguirre et al., 2009; Aryani et al., 2015b) and that care should be taken during mild heat treatments to check on the possible tailing of the inactivation curve (Geeraerd et al., 2000). In addition, the Food Standards Agency (Stringer et al., 2012) highlights the lack of models describing the behaviour of microorganisms between 40 and 60 ºC. Moreover, The Catalan Food Safety Agency (ACSA) promoted in 2010 the edition of a Good Hygiene Practices Guide on Vacuum Cooking, with great interest in defining safe time/temperature combinations to allow further storage of *sous-vide* preparations. Our group in CIRTTA-UAB took part in the redaction team (Agència de Salut Pública de Catalunya, 2012). A conclusion of this guide was that experimental data were required to get further knowledge on bacterial kinetics before the mild heat stress applied to these products. Then, a demand of scientific knowledge about microbiological safety of these products has emerged (Stringer et al., 2012)

2.2 General objective

The overall aim of this Doctoral Thesis was to characterize the effect of mild temperature vacuum cooking treatments on two main food pathogens, *L. monocytogenes*, relevant in RTE foods and *Salmonella* spp., highly present in raw pork meat. The degree of lethality and recovery of injured microorganisms and the evolution of the surviving microbiota in optimal and abuse temperature of conservation conditions was determined.

2.3 Specific objectives

a) To evaluate the suitability of steam-convection ovens in *sous-vide* cooking.
b) To characterize the lethality and inactivation kinetic of *Salmonella* spp. and *L. monocytogenes* inoculated in pork loin samples vacuum cooked at 55 and 60 ºC.
c) To characterize the survival and recovery of *Salmonella* spp. and *L. monocytogenes* inoculated in pork loin samples vacuum cooked at 55 and 60 ºC during storage at optimal (4ºC) and abuse (8ºC) temperature conditions.
d) To evaluate the microbiological safety of *sous-vide* treatments at 55 and 60 ºC applied during 30, 60 and 90 minutes to pork loin in relation to *L. monocytogenes*, and *Salmonella* spp.

3. Materials and Methods

3.1. Work plan

Preliminary experiments were carried by our group, considering different variables, like the food matrix, pre-treatment processes (e.g. freezing), microorganism of concern, cooking or storage temperature and cooking equipment. Considering their results, the experimental procedure was established as follows. Challenge studies were conducted using two bacterial strains of *Salmonella* or *Listeria*, either individually or in combination. Inoculated pork loin fillets were portioned in regular pieces of 30 g and vacuum packed. After an overnight storage period at 4 ºC, they were cooked in a steam oven at two different temperatures (55 and 60 ºC) during 30, 60 or 90 min. Lethality was determined at day 0 as well as further recovery during storage at 4 and 8 ºC until day 30. Pathogen enumeration and detection test in samples with counts below the quantification limit were carried. Three replicates were used in each analysis point.

In general, experiments were performed from a same production batch. Batches had in common that pork loin meat samples were purchased the same day. Moreover, the same inoculum, pre and post cooking treatment and cold storage was applied. A total of 240 samples were analysed of each bacterial strain (individually or combined). The only exception to this definition of batches were for *S.* Enteritidis and *S.* Senftenberg essays. In these cases, heat treatments were performed in different years. Therefore, samples cooked at 55 ºC and 60 ºC of these two strains were different biological replicates (i.e. different meat batches, inoculums and cold storage period). A schematic representation of the experimental design is showed in Figure 7.

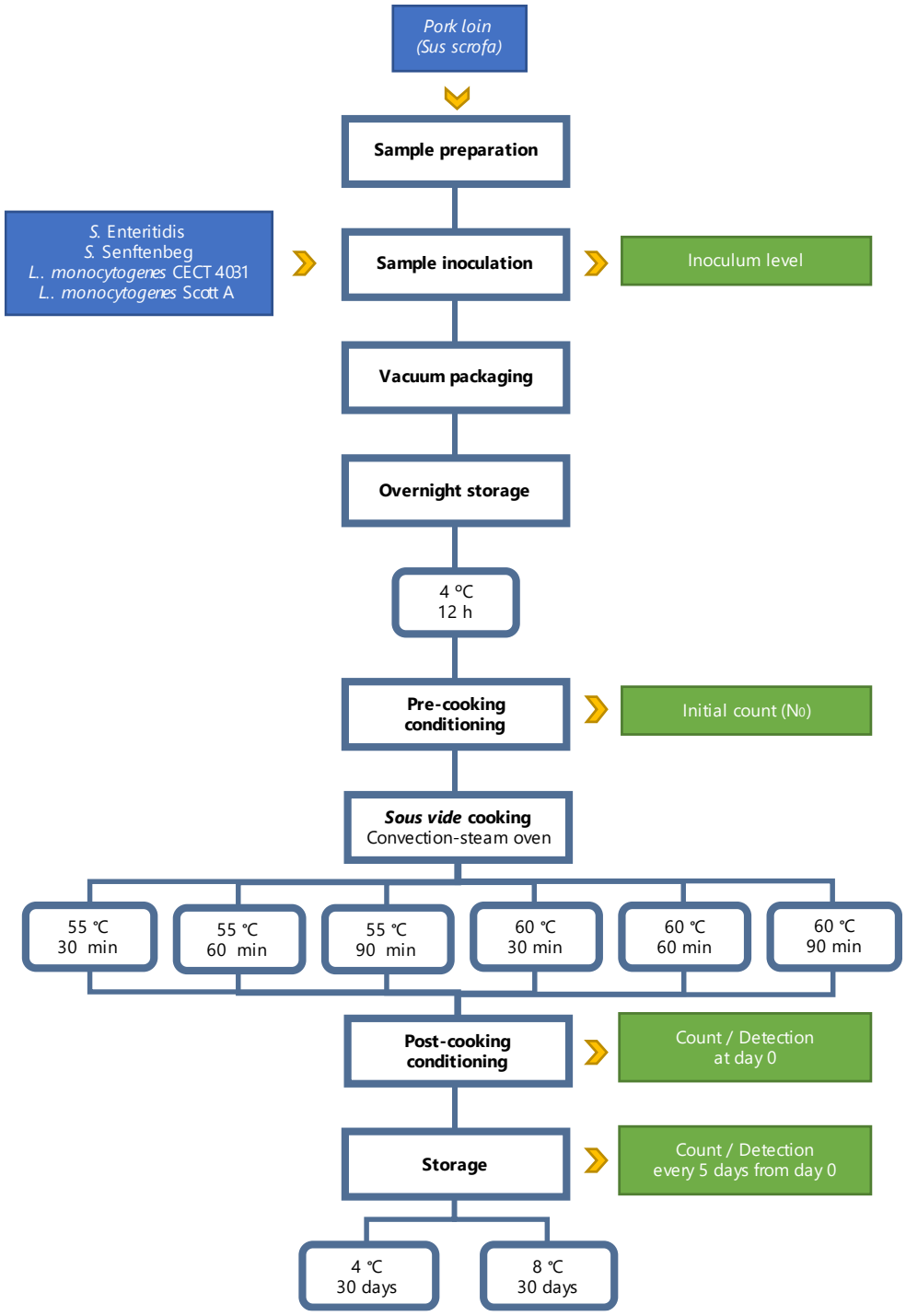


Figure 7. Work plan to evaluate the microbiological safety of *sous-vide* treatments at mild temperatures applied to pork loin.

3.2. Bacterial strains and inoculum preparation

Strains of *S. enterica* subsp. *enterica* serovar Enteritidis CECT 4300, *S. enterica* subsp. *enterica* serovar Senftenberg CECT 4565 and *L. monocytogenes* CECT 4031 (serotype

1/2a) were obtained freeze-dried from the Spanish Type Culture Collection (CECT) (University of Valencia, Valencia, Spain). *L. monocytogenes* Scott A (serotype 4b) was obtained from the National Institute of Agricultural and Food Research and Technology (INIA, Madrid, Spain). The strains were rehydrated in 1 mL of trypticase soy broth (TSB, Oxoid, Basingstoke, Hampshire, United Kingdom) for 30 min and subsequently were transferred to 10 mL of TSB broth and incubated for 24 h at 37 °C. Afterwards, a loopful from the inoculated broth was streaked onto plates of trypticase soy agar (TSA, Oxoid) and incubated at 37 °C for 24 h. Then, isolated colonies were streaked in TSA slants and incubated at 37 °C for 24 h. Working cultures were maintained on TSA (Difco) slants at 4 ºC for a maximum period of 9 weeks.

In order to guarantee a similar physiological status (stationary phase) of the initial inoculum, working cultures were consecutively subcultured twice in TSA plates at 37 ºC for 24 h. From the second subculture, cell suspensions were prepared in 10 mL of 0.85% sodium chloride solution (Panreac, Montcada i Reixac, Barcelona, Spain) to obtain 8.0 log CFU/mL. Turbidity of each bacterial suspension was measured with a densitometer (DENSIMAT, bioMérieux Marcy-l'Etoile, France), until a value of 1.0 McFarland units (UMcF) for *L. monocytogenes* and 1.5 UMcF for *Salmonella* spp. strains. A 1/10 dilution was prepared from this suspension in 100 mL of working saline solution. In case of cocktail suspensions, the 1/10 dilution was prepared in 100 mL of working saline solution, mixing 5 mL of the suspension of each strain of *Salmonella* or *Listeria*. The working saline solution was used to inoculate fillets of pork loin to reach a final concentration of 4 to 5 log CFU/g.

3.3. Pork loin preparation and inoculation procedure

Pieces of pork loin (*Longissimus dorsi*) were purchased in a local supermarket in Sant Quirze del Vallès (Spain) and transported to the laboratory within a maximum of 1 h. Meat was stored at 4 ºC for no more than 24 h. Afterwards, it was cut in fillets of 80-100 g and 1 cm thick with a domestic slicer (Bosh MAS9001, BSH Hausgeräte GmbH, Spain). Each fillet was in depth inoculated from the working saline solution by puncture with a 64-needle array specifically designed for this application. Two uniform meat pieces of 3 cm x 3 cm x 1 cm (length, width and height) and between 25-30 g were obtained from each fillet. Finally, these pieces were vacuum packed with a vacuum packing machine (Tecnotrip EVT-7-VT, Terrassa, Spain), in thermostable plastic bags (Lore 90 cook, Vac-

BCN, Rubi, Spain) of 150 x 200 mm (length, width), made from coextruded polyamide and high density polyethylene (PA/HDPE 20/70) of 90 µm thick. Raw samples were stored for 12 h at 4 ºC.

3.4. *Sous-vide* heat treatments

An agreement was established in 2012 with RATIONAL Iberica Cooking Systems S.L. (Cornellà de Llobregat, Spain) that assigned the Special Research Center Plant of Food Technology (CERPTA, Bellaterra, Spain) one of their steam-convection ovens, of 0.5 m^3 of capacity (SelfCookingCenter® 5 Senses, Rational Aktiengesellschaft, Landsberg am Lech, Germany). It was used to define the experimental design and to carry out the first experimental trials. Since 2014, *sous-vide* cooking was performed in the RATIONAL's demonstration centre in Cornellà de Llobregat, with two steam-convection ovens of 2 m^3 of capacity. The homogeneity in heat distribution throughout the cooking chamber of the ovens was verified using three sensors (TELID®251 device, Microsensys iID, Swizertland) placed inside the oven during a cooking cycle, in different shelves and as far as possible from each other. The air temperature, obtained with thermocouple sensors connected directly to the oven electronics was also considered. Standard deviation of all these records during the holding phase of a cooking cycle was calculated.

Ovens were programmed at 55 and 60 ºC to hold internal temperature for 30, 60 and 90 min. Different sources of information were consulted to establish the time/temperature cooking profiles. Treatment temperatures were proposed by some cook professionals. These temperatures are usually used to cook *sous-vide* meats like chicken breast or tenderloin in packs of individual portions. Treatment duration was based on the mean D value provided by Van Asselt and Zwietering (2006). According to this source, treatment of 55 ºC during 30, 60 and 90 min should cause almost 2, 4 and 6 log-reduction in counts (log CFU/g) of *L. monocytogenes.*

Internal or core temperature was monitored with a thin and sharp thermal probe incorporated in the oven's electronics and inserted through a foam septum to prevent losing of vacuum packaging conditions. The probe was introduced in the centre of a non-inoculated sample located in the coldest area of the oven. After a preheating phase, samples were introduced in the oven. Then, the heating phase began and lasted until the internal temperature reached the target temperature (55 or 60 ºC). Duration of this heating

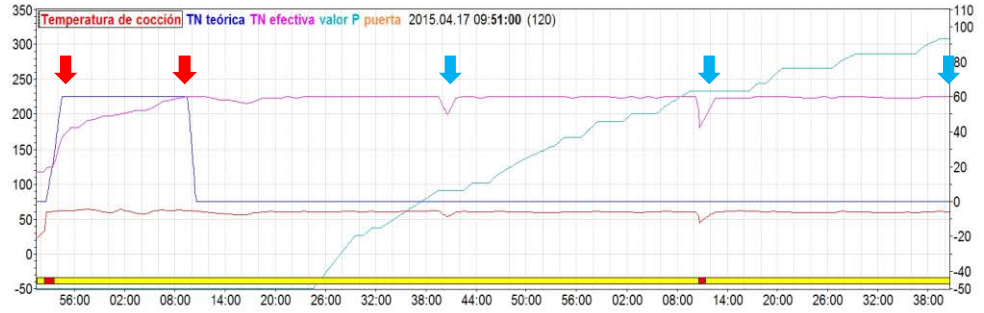
stage could vary, according to the heating kinetic of the monitored sample. Considering all cycles, the heating phase lasted between 10 and 33 min, with a mean value of 21.14 min. Thereafter, the holding phase at 55 or 60 ºC for 30, 60 or 90 min began. Once each treatment was finished, the samples were quickly cooled in an iced-water bath for approximately 30 min, until internal temperature below 10 ºC was reached. Subsequently, the samples were cold stored at 4 and 8 ºC for 30 days.

Figure 8 shows an HACCP display of a batch with information of the temperature records of the cooking program in graph and table form (Software SelfCookingCenter®, Version 07-00-07.1). After the preheating phase, the heating phase was initiated measuring the internal or core temperature. This step finished when the product reached the established cooking temperature, in this case, 60 ºC at the minute 18. Thereafter, during the cooking phase at 60 ºC, samples were removed out of the oven at the minute 30, 60 and 90 (see downward peaks in the ambient and product temperature). Although the HACCP software calculates the P-value (pasteurization value), the specific z value for *Salmonella* spp. and *L. monocytogenes* were not parametrized in the software. For that reason, it was not taken into account. However, this value was calculated on a spreadsheet based on product's core time/temperature records. Data will be commented with more detail in the Results and Discussion section.

HACCP data

17.04.2015 16:47:01

Número de lote:	2264	Versión soft. Aparato:	SCC-06-00-06
Número de lote del cliente:		Versión cadena:	
Modelo:	SCC101	Fecha de realización:	17.04.2015 09:51:26
Número de serie:	E11SH13102374547		17.04.2015 11:40:31
Nombre del programa:	UAB 60		



Hora	Tiempo	Puerta	Modo de cocción	T. cocción	TNteórica	TNefectiva	Unid.	Opc. energ.	1/2 Energía	Valor P
17/04/2015 9:51:26	00:00:00	CERRAR		22.0	0.0	17.0	°C	0	0	0.0
17/04/2015 9:51:26	00:00:00	CERRAR		22.0	0.0	17.0	°C	0	0	0.0
17/04/2015 9:51:27	00:00:01			22.0	0.0	17.0	°C	0	0	0.0
17/04/2015 9:52:26	00:01:00			58.0	0.0	18.0	°C	0	0	0.0
17/04/2015 9:52:33	00:01:07	ABRIR		60.0	0.0	19.0	°C	0	0	0.0
17/04/2015 9:52:33	00:01:07	ABRIR		60.0	0.0	19.0	°C	0	0	0.0
17/04/2015 9:52:34	00:01:08			61.0	0.0	19.0	°C	0	0	0.0
17/04/2015 9:52:34	00:01:08			61.0	0.0	19.0	°C	0	0	0.0
17/04/2015 9:52:35	00:01:09			62.0	60.0	19.0	°C	0	0	0.0
17/04/2015 9:53:26	00:02:00			61.0	60.0	27.0	°C	0	0	0.0
17/04/2015 9:54:26	00:03:00			62.0	60.0	37.0	°C	0	0	0.0
17/04/2015 9:55:26	00:04:00			62.0	60.0	42.0	°C	0	0	0.0
17/04/2015 9:56:26	00:05:00			63.0	60.0	42.0	°C	0	0	0.0
17/04/2015 9:57:26	00:06:00			64.0	60.0	46.0	°C	0	0	0.0
17/04/2015 9:58:26	00:07:00			63.0	60.0	47.0	°C	0	0	0.0
17/04/2015 9:59:26	00:08:00			60.0	60.0	49.0	°C	0	0	0.0
17/04/2015 10:00:26	00:09:00			59.0	60.0	49.0	°C	0	0	0.0
17/04/2015 10:01:26	00:10:00			64.0	60.0	50.0	°C	0	0	0.0
17/04/2015 10:02:26	00:11:00			61.0	60.0	51.0	°C	0	0	0.0
17/04/2015 10:03:26	00:12:00			58.0	60.0	52.0	°C	0	0	0.0
17/04/2015 10:04:26	00:13:00			57.0	60.0	52.0	°C	0	0	0.0
17/04/2015 10:05:26	00:14:00			62.0	60.0	54.0	°C	0	0	0.0
17/04/2015 10:06:26	00:15:00			63.0	60.0	57.0	°C	0	0	0.0
17/04/2015 10:07:26	00:16:00			62.0	60.0	58.0	°C	0	0	0.0
17/04/2015 10:08:26	00:17:00			63.0	60.0	59.0	°C	0	0	0.0
17/04/2015 10:09:26	00:18:00			62.0	60.0	60.0	°C	0	0	0.0
17/04/2015 10:09:27	00:18:01			62.0	0.0	60.0	°C	0	0	0.0
17/04/2015 10:10:26	00:19:00			61.0	0.0	60.0	°C	0	0	0.0
17/04/2015 10:11:26	00:20:00			60.0	0.0	60.0	°C	0	0	0.0
17/04/2015 10:12:26	00:21:00			59.0	0.0	59.0	°C	0	0	0.0
17/04/2015 10:13:26	00:22:00			58.0	0.0	58.0	°C	0	0	0.0
17/04/2015 10:14:26	00:23:00			57.0	0.0	58.0	°C	0	0	0.0
17/04/2015 10:15:26	00:24:00			56.0	0.0	57.0	°C	0	0	0.0
17/04/2015 10:16:26	00:25:00			56.0	0.0	56.0	°C	0	0	0.0
17/04/2015 10:17:26	00:26:00			59.0	0.0	57.0	°C	0	0	0.0
17/04/2015 10:18:26	00:27:00			60.0	0.0	59.0	°C	0	0	0.0
17/04/2015 10:19:26	00:28:00			61.0	0.0	59.0	°C	0	0	0.0

Figure 8. Example of a batch record that shows the temperature profile of the cooking program and a detailed profile in table format. a) chamber temperature in red, b) core temperature in pink, c) P value in green, c) programmed core temperature in blue. Red arrows in the graph and the table indicate the initial and final time point of the heating phase. Blue arrows in the graph indicate minute 30, 60 and 90 of the holding phase.

3.5. Sampling and microbiological analysis

For microbiological analysis, pieces of pork loin (around 25-30 g) were placed into a sterile filter blender bag (Interscience, Saint-Nom-la-Bretêche, France). A 1/5 dilution with buffered peptone water (Oxoid) was prepared using a gravimetric dilutor (Delta Dilutor, IUL Instruments, GmbH, Königswinter, Germany). The mixture was homogenized for 2 min in a paddle blender (Masticator; IUL micro, Barcelona, Spain). In case of *L. monocytogenes* inoculated samples, incubation at room temperature was performed during 1 hour according to the UNE-EN ISO 11290-2 procedure (AENOR, 2005). Culturable cells were enumerated by plating out appropriate dilutions using surface or spiral plate method on SM2 (BioMérieux) for *Salmonella* and ALOA (BioMérieux) for *Listeria monocytogenes* with 24 h incubation at 37 ºC. If after 18–24 h no suspect colonies were evident, plates were re-incubated for an additional period of 24 h. Plates were counted manually or in an automated colony counter (IUL Flash and Go; IUL micro, Barcelona, Spain). When microbial counts were below the quantification limit (5 log CFU/g), buffered peptone water incubation (enrichment culture) continued at 37 °C for a total of 24 h, to allow *Salmonella* and *Listeria* strains growth to levels above the quantification limit. Afterwards, the enrichment culture was streaked on ALOA or SM2 in order to determine presence of *Listeria* or *Salmonella* spp. The lethality of each treatment was calculated as the difference between the logarithms of colony counts of treated (N at day 0) and untreated samples (N_0).

3.6 Statistical analyses

Counts of the three experimental replicates obtained at each time point for each condition tested were log transformed. One-factorial analysis of variance (ANOVA) was used to compare lethality obtained after applying different thermal treatments. The Tukey test was used to obtain paired comparisons among sample means. Bacterial counts obtained during the storage period that could not be modelled (see next section), were transformed into three categorical variables: no detection, detection below the QL and detection above the QL. The chi-squared (χ^2) test and analysis of contingency tables were applied to study the dependence between discrete variables. Statistical analyses were performed with the SPSS version 23 (IBM SPSS Statistics; IBM corporation, Armonk, NY, USA). In all analyses, p-values < 0.05 were considered to be significant.

3.7. Mathematical modelling of microbiological kinetics

Points below the quantification limit (5 CFU/g) were not included in the fitting procedures, since real value was unknown.

Decreasing counts through the heating time were modelled using the non-isothermal fitting module of Bioinactivation FE (Garre et al., 2018a) (https://opada-upct.shinyapps.io/bioinactivationFull/). This software supplies a user-friendly interface to selected functions of the model fitting of non-isothermal experiments and generates prediction intervals of these functions. The first step was the input of the temperature profile and the microbial counts obtained during the experiment. Then, an inactivation model between the ones implemented in the tool was chosen (in concrete, the Bigelow, the Mafart or the Geeraerd models). The definition of initial guesses of the model parameters, based on literature, were required. Furthermore, several settings of the fitting algorithm were defined. A figure of the fitting result was shown, indicating the temperature profile, the experimental points and the predicted survivor curve. Bioinactivation FE provided estimates of the values and standard deviations of the model parameters, as well as confidence intervals at the 95% confidence level. Furthermore, several indexes evaluating the goodness of the fit and the convergence of the fitting algorithm were output. Besides, Bioinactivation FE generates prediction intervals of the microbial response using the variance of the model parameters estimated using the non-isothermal data. For that reason, the temperature profile was input again, and several settings of the algorithm for the calculation of prediction intervals were defined. Once it was done, the results were shown in another figure.

Bacterial growth modelling was performed as long as two of the three replicates showed a sustained increase in two consecutive time-periods. The primary growth model of Baranyi and Roberts (1994) was fitted, and the estimates of three kinetic parameters, i.e. latency time (lag, expressed in days), maximum growth rate (Grmax, expressed in log CFU/g/day) and maximum cell numbers (yend, expressed in log CFU/g) were calculated using the DMFIT software of the Combase tool (https://www.combase.cc/index.php/en/). Confidence intervals for the estimated kinetic parameters (Grmax and lag) as well as confidence bands for the fitted growth model were computed with a software tool created *ad-hoc* (Poschet et al., 2003; Posada-Izquierdo et al., 2013). The confidence intervals define the uncertainty range on the estimated regression parameters (Grmax and lag),

while confidence bands define graphically the uncertainty range on the best-fit growth model (i.e. growth curve). This software is based on the Monte Carlo method using the standard error (SE) reported initially by the DMFit software. To compute the confidence intervals, a normal distribution was defined with mean equal to 0 and standard deviation (SD) equal to the SE, which accounts for the range of error in the estimated concentration (log CFU/g) associated with the best-fit estimate for the model of Baranyi and Roberts (1994). Then, the normal distribution applied to each time point were simulated by using Monte Carlo method with 10.000 iterations for each one, resulting in a set of new 10.000 growth curves. The Baranyi model was fitted to each new growth curve obtained by the Monte Carlo method, obtaining different estimates of the kinetic parameters for each growth curve. The 95% confidence interval for each kinetic parameter was obtained based on 2.5th and 97.5th percentiles for each set of simulated kinetic parameter values. Similarly, the confidence bands (95%) for the best-fit model were estimated based on 2.5th and 97.5th percentiles of the concentration predicted at each time by the growth models defined by the 10.000 simulated kinetic parameters.

Two goodness-of-fit measures of non-linear models have been used. The Root Mean Squared Error (RMSE) is the square root of the residual mean square. The RMSE may be viewed as the average of the discrepancy between the observed data, transformed if necessary, and their predicted values. On the other hand, the Bias Factor (B_f) is an estimate of the reliability of the model that compares predictions and independent observations. A low RMSE value means better adequacy of the model to describe data, and an acceptable B_f value for a predictive model can range from 0.75 to 1.25 (Ross, 1996; Pérez-Rodríguez and Valero, 2013). These indexes can be calculated by using the equations given in Table 4.

Table 4. Goodness-of-fit measures of the models.

Root Mean Squared Error (RMSE)	$\sqrt{\frac{\sum_{i=1}^{n}(Y_i - \hat{Y}_i)^2}{n}}$
Bias Factor (B_f)	$10^{\left[\sum \log\left(\frac{gpred}{gobs}\right)/n\right]}$

Observed value (Y_i)
Predicted value ($\hat{Y}_i$)
Predicted value ($\bar{Y}_i$)
Total number of data (n)
Observed generation time (*gobs*)
Predicted generation time (*gpred*)

4. Results and discussion

4.1. Process monitoring

4.1.1. Validation of inoculation procedure

A preliminary assay to validate homogeneity on the inoculation procedure of raw samples was performed. Ten samples were inoculated in depth with *L. monocytogenes* Scott A (Lm Scott A) resulting a mean count of 5.52 ± 0.18 log CFU/g. This value was similar to other studies in which lethal effect caused by technological processes was also evaluated (Posada-Izquierdo et al., 2013; Mataragas et al., 2015). Therefore, the inoculation method was definitively approved.

Counts of different strains of *Salmonella* and *L. monocytogenes* inoculated in raw pork loin used for *sous-vide* cooking treatments at 55 and 60 ºC are shown in Table 5. Mean initial counts were 4.86 ± 0.54 log CFU/g for *Salmonella* spp. and 5.53 ± 0.16 log CFU/g for *L. monocytogenes*. Standard deviation of individual experiments ranged between 0.05 and 0.68 log CFU/g.

Table 5 Initial counts (log CFU/g ± standard deviation) of different strains of *Salmonella* and *Listeria monocytogenes* inoculated in pork raw meat loin used in *sous-vide* cooking treatments at 55 and 60 ºC

Treatment	*Salmonella* strains	Initial Counts	*Listeria monocytogenes* strains	Initial Counts
60 ºC	**Cocktail**	4.64 ± 0.14	**Cocktail**	5.52 ± 0.10
	Enteritidis	4.89 ± 0.05	**CECT 4031**	5.69 ± 0.10
	Senftenberg	5.31± 0.23	**Scott A**	5.58 ± 0.15
55 ºC	**Cocktail**	4.46 ± 0.09	**Cocktail**	5.62 ± 0.10
	Enteritidis	4.20 ± 0.68	**CECT 4031**	5.58 ± 0.13
	Senftenberg	5.65 ± 0.64	**Scott A**	5.23 ± 0.19

4.1.2 Validation of the heating process

Food companies that elaborate in-container heat processed products use two different temperature measurement systems to validate its safety: Temperature Distribution Testing

(TD) and Heat Penetration Testing (HP). TD aims to prove that the processing system (water bath, oven or autoclave) delivers heat to the product in a uniform way. In HP testing, probes are inserted into the food as a means of measuring temperatures that are converted to integrated process value. The aim of a HP study is to determine the heating and cooling behaviour of a specific product in order to establish a safe thermal process regime and to provide the data to analyse future process deviations. In recent years, temperature measurement hardware and analysis software have been developed to allow measurements of heat treatments that have emerged in response to the consumer demands for less processed foods (Tucker, 2004).

TD and HP testing were conducted to provide the relationship between the oven instrumentation and both the temperature of the oven chamber and the samples. Results are shown in the following sections.

Temperature Distribution Testing

Standard deviation of records obtained with four temperature sensors placed in different locations inside the oven chamber during the holding phase of a heat treatment were of 0.20 and 0.47 ºC for the ovens of 0.5 and 2 m^3, respectively. Such deviations were considered correct since it is a common practice to start measuring the time into the holding phase by which the temperature distribution has stabilised around 1 ºC (Tucker, 2004). Figure 9 shows records of this verification.

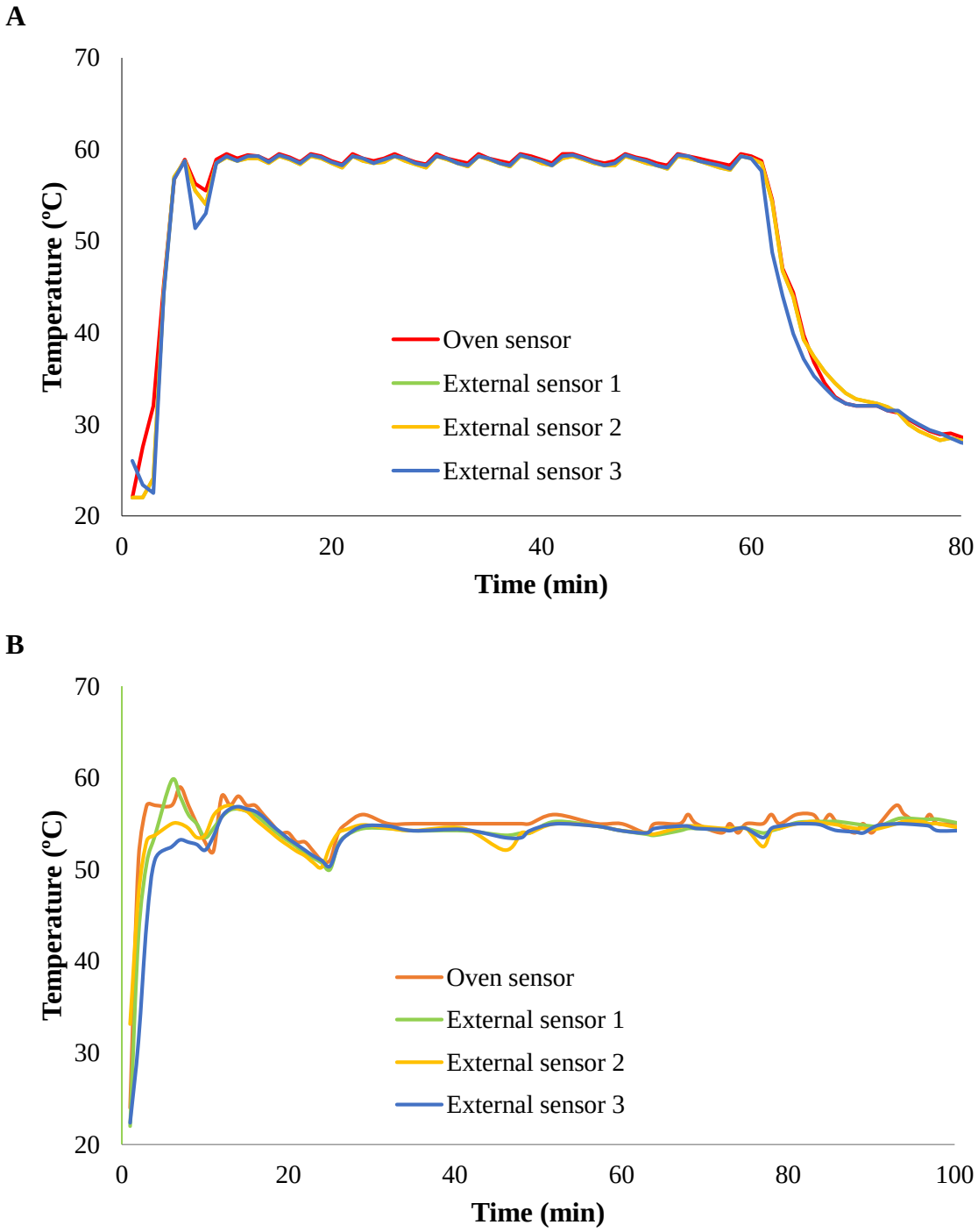


Figure 9 Records of the temperature distribution testing of the oven of 0.5 m^3 (A) and 2 m^3 (B)

Heat Penetration Testing

As well as ambient heating conditions, product core temperature was also monitored using a non-inoculated sample placed in the same location at each heat treatment. A total of 6 replicate runs were compared, one for each type of inoculum and cooking temperature. Downward peaks displayed on temperature records were caused by the opening of the oven door to remove the cooked samples at min 30, 60 or 90 (**Figure 10**). These peaks were not considered when inactivation kinetic was evaluated.

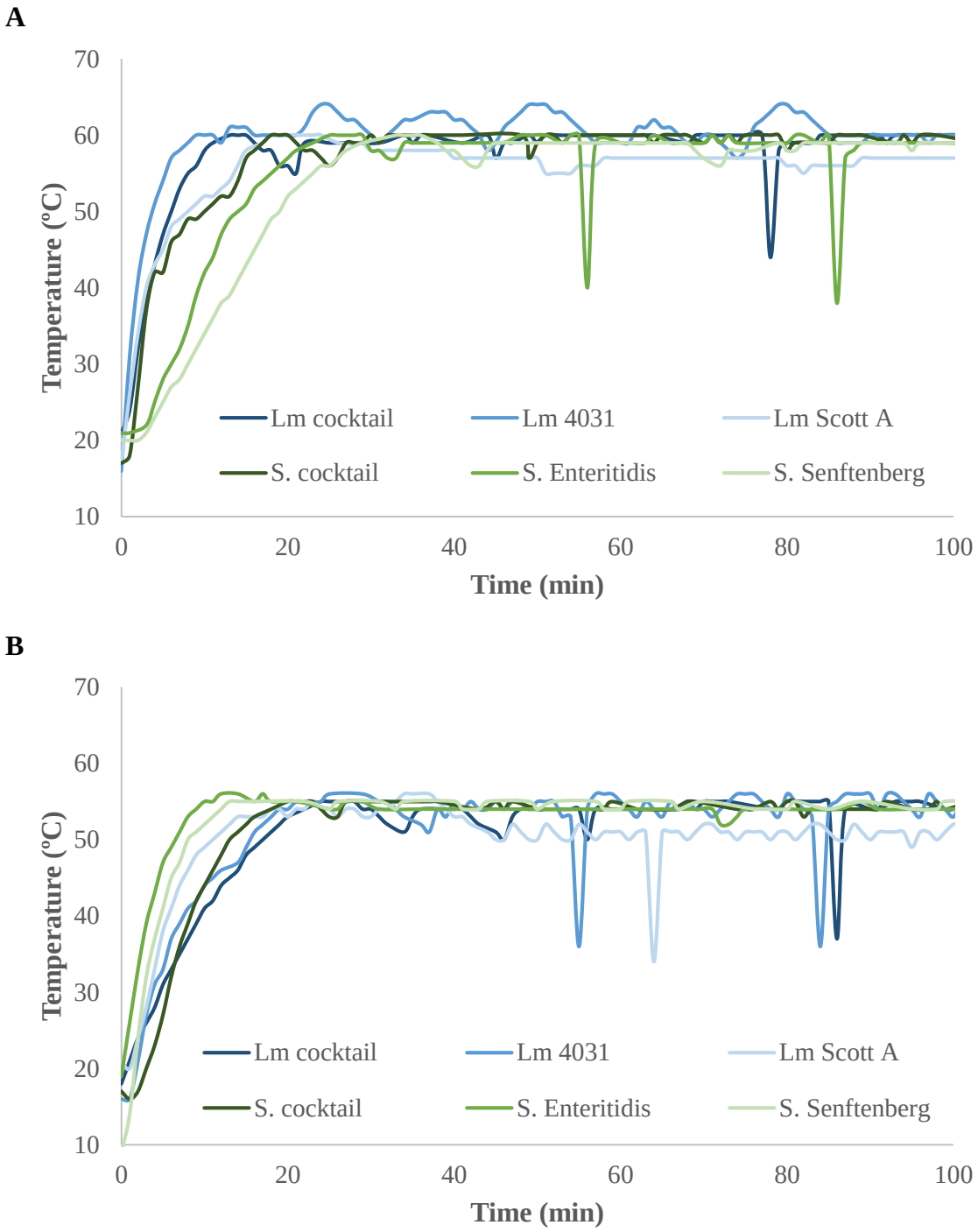


Figure 10. Heat penetration curves of cooking cycles at 60 ºC (A) and 55 ºC (B) for inoculated pork loin samples.

Oscillations recorded in core product temperature during the holding phase of each heat treatment were considered a source of variability. As a measure of the variability of each cooking cycle, the standard deviation of core temperature was calculated, and presented values between 0.5 and 1.7 ºC. As a measure of variability between cooking cycles, the standard deviation of mean core temperatures obtained for each cooking cycle was

calculated, resulting in a value of 1.0 ºC for treatments at 60 ºC and 0.3 ºC for treatments at 55 ºC.

The length and rate of the heating phase of treatments were considered another source of variability between cooking cycles. An example of differences in the heating phase is showed in Figure 11 Despite samples inoculated with Lm Scott A and Lm cocktail reached a final core temperature of 55 ºC (see vertical dotted lines), time elapsed since the beginning of the holding phase was shorter for samples with Lm cocktail (24 min) than for samples with Lm Scott A (33 min). However, the heating rate until 50 ºC was faster for samples with Lm Scott A (2.7 ºC/min) than for Lm cocktail samples (1.9 ºC/min). This fact could explain differences in lethality values between Lm cocktail and Lm Scott A. Impact of this variability on results will be discussed later.

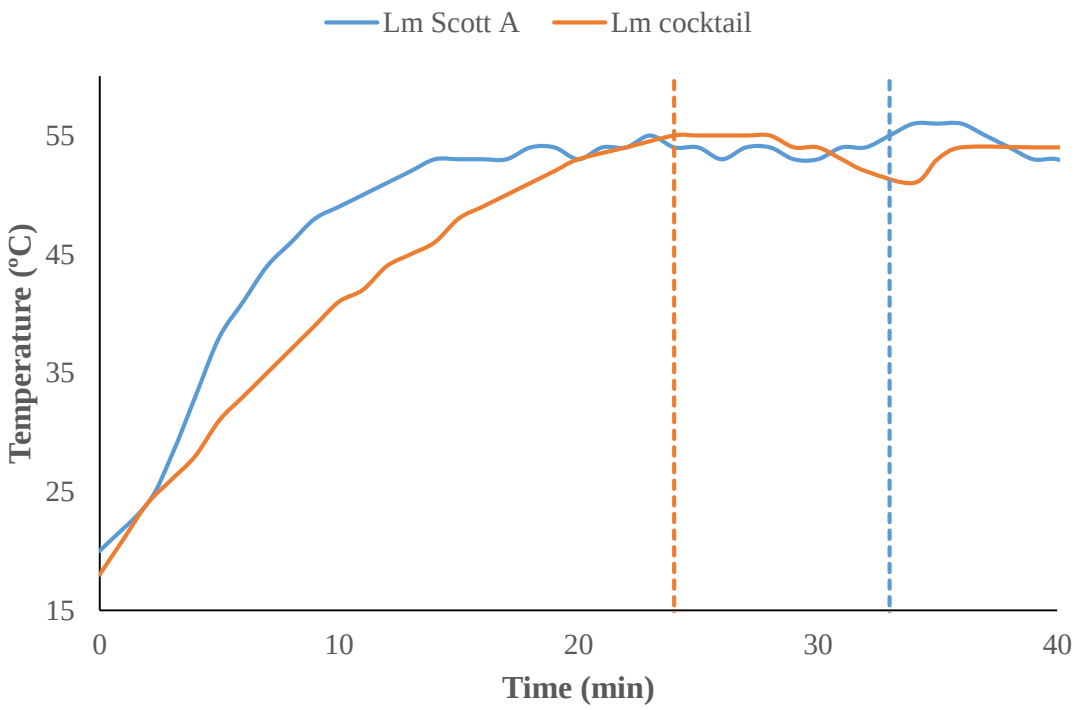


Figure 11. Heat penetration curves of cooking cycles at 55 ºC of pork meat samples inoculated with *Listeria monocytogenes* cocktail (orange) and *Listeria monocytogenes* Scott A (blue). Time at which core temperature reached 55 ºC is indicated with dotted lines.

Baldwin (2012) affirms that variation in core product temperature during the holding phase of each cycle or in the heating rate between cycles is a result of the relatively poor distribution of steam at temperatures below 100ºC, and the oven dependence on condensing steam as the heat transfer medium. In contrast, circulating water baths heat very uniformly

and typically have temperature swings of less than 0.1ºC. In this case, it is important that the pouches are completely submerged and are not tightly arranged or overlapped.

Thermal treatments begin at sublethal temperatures. If the heating rate of the food substrate is not fast enough, physiological changes may occur in the bacterial cells, increasing their resistance to posterior stresses (stress acclimation). This fact is a potential food safety risk because a larger number of pathogenic bacterial cells than expected may survive the heat treatment (Garre et al., 2018c). Several authors have reported significant changes in the ability of cells to survive a heat treatment as function of the rate at which the organism is heated. Juneja and Marks (2003) determined that the estimated D values of *Salmonella* spp. in *sous-vide* cooked beef at 58 ºC were significantly larger for longer come-up heating times. Hansen and Knochel (2001) observed that although processing at slowly rising temperatures may slightly increase the survival of *L. monocytogenes* 13-249 in cooked beef at 60 ºC, there seem to be no indication of an increase in subsequent growth potential of the surviving cells. Garre et al. (2018b) accounted for a model that describes stress acclimation of *E. coli* CECT 515 under non-isothermal conditions (heating rates below 2 °C/min). They concluded that stress acclimation may be relevant for microbial risk assessment respecting the choice of the microorganism of concern.

4.1.3 Initial lethality considerations (F value)

Many food-processing systems contain a heating step to reduce the number of bacteria in a product. This enhances food safety and increases the shelf-life of a product. To achieve these goals, the required time/temperature combinations are set, usually based on challenge tests, legislation and experience. In order to assess the adequacy of a heating step, log reductions of bacteria can be estimated using the D/z concept. This concept assumes a log linear inactivation during time. Due to its broad applicability, the D/z concept is appropriate to obtain a first impression on the performance of an inactivation process (Van Asselt and Zwietering, 2006). D value is defined as the time needed to reduce the number of organisms by a factor of 10 at a certain temperature and z value (resistance coefficient) is defined as the temperature (°C) increase required to reduce D value by a factor of 10 (Van Doornmalen and Kopinga, 2009). For example, considering a z value of 7 ºC for *L. monocytogenes* (Van Asselt & Zwietering, 2006), a pasteurization process of 70 ºC during 2 min designed to

obtain a 6-logarithm reduction (Baldwin, 2012), would be equivalent to a process at 77 ºC during 0.2 min or to another process at 63 ºC during 20 min.

In pasteurisation and sterilisation treatments, process heating conditions are designed to achieve the desired performance safety standards based on the Thermal Death Time (TDT or F). F value is a measure of the equivalence of the changing temperature integrated lethal effect to an isothermal process at a reference temperature (Peleg, 2004). The F value (also called P value for pasteurisation processes or C value for cooking processes) has been traditionally applied in canning industry. The availability of cooking systems equipped with appropriate sensors of temperature and computers able to elaborate the data recorded during the cooking cycles has extended the use of the F concept to commercial restoration (Pittia et al., 2008).

Although cooking cycles were programmed as function of a target temperature (e.g. to maintain 60 ºC for 30 min), particular settings of each oven used in this study caused differences in the heating process. As a first approach, F value was computed to quantify microbial heat inactivation. The cooking temperature (55 or 60 ºC) was considered the reference temperature and the z value applied was 7 ºC for *Salmonella* and 9 ºC for *L. monocytogenes* (Van Asselt and Zwietering, 2006). F values obtained can be used to quantify differences in heat penetration curves between the *sous-vide* cooking treatments applied (Table 6).

If temperature would have been stable around 55 or 60 ºC, F values would have had D values around 30, 60 and 90 min. However, F values (mean ± standard deviation) of processes of 30, 60 and 90 min were 30.76 ± 6.08 min, 55.08 ± 15.78 min and 77.44 ± 23.61 min, respectively. As can be seen, processes of 60 and 90 min showed lower F values than expected. Besides, standard deviation of F value was larger at longer heat treatment times. Both facts were caused by temperature oscillations produced during heat treatments.

Table 6. Mean temperature (ºC) during the holding phase and estimated F value of pork meat loin submitted to *sous-vide* cooking treatments at 55 and 60 ºC.

Treatment temperature (º C)	Microorganisms	Mean Temperature (º C)			F value (min)		
		t30[a]	t60	t90	t30	t60	t90
	***Salmonella* spp.**						
	***S*. cocktail**	58.95	58.95	58.95	30.58	59.62	85.82
60	***S*. Enteritidis**	59.14	59.14	59.14	30.77	54.63	78.79
	***S*. Senftenberg**	58.64	58.64	58.64	31.48	52.42	75.54
	***S*. cocktail**	54.50	54.42	54.36	33.42	58.50	83.41
55	***S*. Enteritidis**	54.75	54.55	54.23	28.75	54.81	78.76
	***S*. Senftenberg**	55.33	54.64	54.58	35.54	64.10	86.70
	L. monocytogenes						
	Lm cocktail	58.75	59.16	58.96	27.14	55.55	80.11
60	**Lm 4031**	61.10	61.08	60.82	48.50	100.31	139.62
	Lm Scott A	58.30	57.39	57.17	21.89	32.41	42.58
	Lm cocktail	53.44	53.77	53.71	24.78	48.93	74.44
55	**Lm 4031**	54.36	54.13	54.05	30.07	56.66	82.97
	Lm Scott A	51.90	51.18	51.05	28.54	34.02	42.16

[a] t30, t60 and t90: *sous-vide* cooking treatments for 30, 60 and 90 min

Despite the worldwide use of the D/z model, especially in the canning industry, a lot of deviations have been observed (particularly at mild temperatures and for vegetative cells) (Geeraerd et al., 2005). The first reason is that the assumption of a linear relationship between log D and T, gives an inappropriate weight to low temperatures at the expense of high temperatures, where most of the inactivation actually takes place (Peleg, 2004; Muñoz-Cuevas et al., 2012; Stringer et al., 2012). Especially at low temperatures, semi-logarithmic survival curves of many microorganism and spores are not lineal. Therefore, forcing a straight line through the curved experimental survival data may result in an over- or under-estimation of processing, increasing the safety risk in this late assumption.

Another objection to microbial inactivation models based on the D/z concept is their need to be obtained under isothermal conditions. These models are based on experiments where microbial culture is heated and cooled in a capillary or a narrow metal tubes in order to shorten the come-up and cooling times as much as possible. However, heat treatments applied in the food industry typically have complex profiles far from isothermal conditions. In view of this fact, mathematical models to describe dynamic inactivation profiles have been developed by extending the ones used for isothermal treatments. However, experimental observations have shown that predictions using model parameters estimated from isothermal experiments usually overpredict microbial inactivation (Valdramidis et al., 2006; Hassani et al., 2007; Garre et al., 2018c). Furthermore, these tools can only be used at the tested temperature range and cannot be extrapolated (Garre et al., 2017). For example, ComBase (www.combase.cc) includes thermal death models for *Salmonella* spp., *Listeria monocytogenes/innocua* and *E. coli* that cover the range 54.5 – 65 °C, 60 – 68 °C and 54.5 - 64.5 °C, respectively (Stringer et al., 2012).

Indeed, non-isothermal conditions are relevant in the heating phase, where acclimation of the microbial cell to thermal stress should be considered (Juneja and Novak, 2005; Garre et al., 2018a). In our study, all cooking cycles have been performed at heating rates below 10 °C/min. The mean value in ºC/min was 2.20 ± 1.02, within the range of 1.03 – 4.40 ºC/min. For such low heating rates, inactivation is probably too slow and microbial cells could develop effective acclimation and subsequent thermal resistance.

4.2 *Salmonella* spp.

4.2.1 Lethality

Almost all heat treatments at 60 ºC achieved a reduction of 4 log CFU/g. Values ranged from 4.30 to 4.64 log CFU/g for 30 min treatments, from 3.98 to 4.64 log CFU/g for 60 min treatments and from 3.74 to 4.89 log CFU/g for 90 min treatments. No statistical differences were found between different heat treatment times for any strain (Table 7).

After the heat treatment at 55 ºC, lethality of *S.* Enteritidis was higher than 4 log CFU/g, and all samples at day 0 showed counts lower than the quantification limit (QL), that was 5 CFU/g. However, *S.* Senftenberg presented the lowest lethality value, in accordance with the higher heat resistance of this strain (Doyle and Mazzota, 2000; Mañas et al., 2003; ICMSF,

2005; O'Bryan et al., 2006; Van Asselt and Zwietering, 2006). Samples inoculated with *S.* cocktail showed an intermediate lethality, as can be seen in Table 7. Lethality of *S.* Senftenberg and *S.* cocktail increased with duration of heat treatment. Lethality was similar at min 30 for *S.* Senftenberg and *S.* cocktail, while significant differences were observed at min 60 and 90.

Table 7. Lethality of *Salmonella* strains inoculated in pork meat samples *sous-vide* cooked at 55 and 60 ºC for 30, 60 and 90 min (mean ± standard deviation, log CFU/g).

Temperature (ºC)	***Salmonella* spp. strain**	**Lethality (log CFU/g)**		
		Heating time (min)		
		30	**60**	**90**
60	***S.* Enteritidis**	4.50 ± 0.68^{a}	3.98 ± 1.58^{a}	4.89 ± 0.00^{a}
	***S.* Senftenberg**	4.30 ± 0.91^{a}	4.18 ± 0.98^{a}	3.74 ± 1.42^{a}
	***S.* cocktail**	4.64 ± 0.00^{a}	4.64 ± 0.00^{a}	4.64 ± 0.00^{a}
55	***S.* Enteritidis**	4.20 ± 0.00^{a}	4.20 ± 0.00^{a}	4.20 ± 0.00^{a}
	***S.* Senftenberg**	0.16 ± 0.45^{bx}	0.22 ± 0.13^{cx}	1.53 ± 0.06^{cy}
	***S.* cocktail**	0.62 ± 0.59^{bx}	2.17 ± 1.24^{by}	3.80 ± 0.65^{by}

$^{a-c}$ Different letters on the same column indicate statistically significant differences ($p < 0.05$) between groups.
$^{x-y}$ Different letters on the same row indicate statistically significant differences ($p < 0.05$) between groups.

4.2.2 Modelling of thermal inactivation

Taking into consideration the drawbacks of applying isothermal models, thermal inactivation was modelled with the non-isothermal fitting module of Bioinactivation FE. This on-line open-source tool was specially designed for the characterization of model parameters from non-isothermal experiments, and for the generation of prediction intervals of the inactivation curve. As suggested by Aryani et al. (2015b) points below the quantification limit (5 CFU/g) were not included in the fitting procedures, since real value was unknown. For this reason, heat inactivation was characterized only for meat samples inoculated with *S.* Senftenberg cooked at 55 and 60 ºC, and for *S.* cocktail samples cooked at 55 ºC. The best fit to experimental data were obtained for Mafart and Geeraerd models (Figures 12, 13 and 14 and Table 8).

Statistical summary of the model fitting is showed in Table 8, including the estimated value, standard deviation and 95 % confidence intervals (CI) of seven model parameters fitted. The δ and p values in the Mafart model could be interpreted as the time required for the first log-reduction under constant temperature conditions and a shape factor of the Weibull distribution, respectively. In the Geeraerd model, N_0 is defined as the initial microbial concentration, N_{res} as the residual (final) microbial count and Cc is related to the physiological state of the cells. This last parameter describes the shoulder effect and is based on the hypothesis of the presence of a pool of protective or critical components around or in each cell (Geeraerd et al., 2005).

In relation to the goodness-of-fit measures of the models used, an often used criterion appearing in the scientific literature for judging whether or not a model fits well is the coefficient of determination (R^2), that is the ratio of the sum of squares due to regression to that of the total sum of squares of the response variable around its mean. Similarly, another goodness-of-fit measure, the adjusted R^2 (R^2_{adj}) is based upon de variances (i.e. the mean squares) rather than upon the sum of squares. The R^2_{adj} attempts to penalize the inclusion of redundant parameters. The use of either of these measures for non-linear regression is inappropriate, usually leading to a rather overoptimistic view of the success of the modelling process (Ratkowsky, 2004; Geeraerd et al., 2005). Therefore, more suitable goodness-of-fit measures of non-linear models have been used. The Root Mean Squared Error (RMSE) is defined as a measure of the discrepancy between observed and predicted values, and the Bias Factor (B_f) as an estimate of the reliability of the model that compares predictions and independent observations. A low RMSE value means better adequacy of the model to describe data, and an acceptable B_f value for a predictive model can range from 0.75 to 1.25 (Ross, 1996; Pérez-Rodríguez and Valero, 2013).

Table 8. Statistical indexes and parameter estimation (mean, standard deviations and their 95% confidence intervals) of the inactivation model used for *Salmonella* strains.

	Units	*S.* Senftenberg at 60 ºC	*S.* Senftenberg at 55 ºC		*S.* cocktail at 55 ºC
Model	-	Geeraerd	Mafart	Bigelow	Mafart
RMSE	-	1.26	0.68	2.13	4.33
B_f	-	1.02	0.87	1.03	0.91
z ± SD	ºC/log min	9.55 ± 0.58	9.01 ± 0.58	9.31 ± 0.58	8.55 ± 0.59
z (95% CI)	ºC/log min	8.08 – 9.95	8.03 – 9.55	8.05-9.95	8.04 – 9.95
log N_0 ± SD	log CFU/g	5.40 ± 0.14	5.59 ± 0.31	-	4.28 ± 0.23
log N_0 (95% CI)	log CFU/g	5.01 – 5.49	4.95 – 6.06	-	4.01 – 4.76
D_{ref} ± SD	min	5.11 ± 0.55	-	90.25 ± 8.78	-
D_{ref} (95% CI)	min	5.06 – 6.89	-	90.74-119.40	-
δ_{ref} ± SD	min	-	85.97 ± 4.37	-	55.03 ± 7.26
δ_{ref} (95% CI)	min	-	85.56 – 99.56	-	50.30 – 77.70
p ± SD	-	-	3.81 ± 0.86	-	2.36 ± 0.33
p (95% CI)	-	-	0.95 – 3.98	-	2.34 – 3.45
log N_{res} ± SD	log CFU/g	1.85 ± 1.64	-	-	-
Log N_{res} (95% CI)	log CFU/g	1.27 – 2.29	-	-	-
C_c ± SD	-	32.07 ± 1.41	-	-	-
C_c CI	-	30.09 – 34.85	-	-	-

Parameter estimations of z values were similar to those reported by Van Asselt and Zwietering (2006). D values and δ values generated by Bioinactivation FE were also similar to the mean and the 95% CI value estimated by these authors at the same reference temperature (between 6.58 and 173.10 min at 55ºC and between 1.86 and 48.85 min at 60 ºC). Lower z and D values at similar temperatures were reported in previous thermal inactivation studies performed with *Salmonella* spp. in a food matrix (Murphy et al., 2004; Osaili et al., 2007; Karyotis et al., 2017) or in culture broth or agar (Wang et al., 2017). This fact indicates that, in our experimental conditions, *Salmonella* spp. is more resistant to the lethal effect of heat than in the previously reported literature. Furthermore, Velasquez et al.

(2010) noted that *Salmonella* was more heat resistant in whole muscle pork than in ground pork. These studies emphasize the need to consider both the presentation of pork meat being cooked as well as the final internal temperature necessary to inactivate *Salmonella* in determining cooking conditions (Jarvis et al., 2016). When applying outcomes from models based on laboratory media and condition to foods it is important to validate these models carefully and take into account differences that might occur due to other factors of the food matrix and indigenous competing microbiota, such as composition, texture and physico-chemical characteristics (Wang et al., 2015).

Figures 12, 13 and 14 show experimental counts, temperature profile, fitted curve and 90% prediction intervals (PI) generated by Bioniactivation FE in each case. In meat samples inoculated with *S.* Senftenberg treated at 60 ºC for 30, 60 and 90 min, one of the three replicates counts were below the QL (data was not included in the inactivation model) (Figure 12). The survival curve obtained was non-linear and displayed tailing. Tail subpopulation was detected from min 30 to min 90 of treatment, with counts between 1.30 and 2.76 log CFU/g. This kinetic has been described for *Salmonella* spp. by other authors at similar temperatures (Humpheson et al., 1998; Noriega et al., 2013; Wang et al., 2015). The Geeraerd model estimates a narrow 90% PI for surviving cells, although some of the experimental points were out of this range. Moreover, as can be seen in Table 8, N_{res} the residual population density, showed a great uncertainty. In the next section, cell recovery and growth of these cells will be discussed.

The tailing phenomenon is important from a practical point of view, since the possible presence of a small percentage of cells capable of surviving a thermal treatment represents a risk to food safety and stability. In many cases, tails are attributed to methodological causes such as a heterogeneity of heat distribution; however, factors related to cell physiology may likewise explain the presence of tails. These basically include the existence of a distribution of intrinsic thermotolerance within the bacterial population, the presence of microbial adaptation phenomena occurring during treatment, even at lethal temperatures (Mackey and Derrick, 1987), and the existence of multitarget inactivation phenomenon (Cebrián et al., 2017).

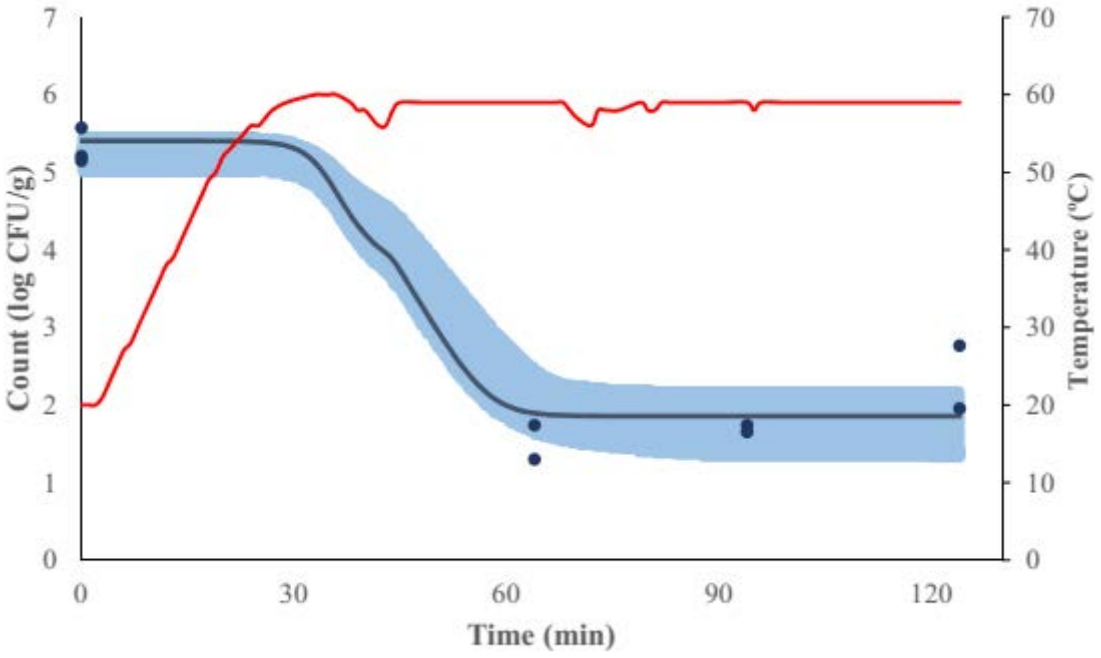


Figure 12. *Salmonella* Senftenberg inactivation data in inoculated samples cooked for 30, 60 and 90 min at 60 ºC. Experimental counts (blue dots), temperature profile recorded during the experiment (red line) and the survivor curve generated by Bioinactivation FE by fitting the Geeraerd model (blue line), including the 90 % prediction interval of the microbial reduction (blue band).

In meat samples inoculated with *S.* Senftenberg treated at 55ºC lethality obtained was lower than 2 log CFU/g. Most *Salmonella* cells were not affected by heat treatment and maintained its growth capability as it will be shown later (Figure 13). The Mafart model estimated a δ value between 85.56 and 99.56 min. Similarly, a D value of 90.26 min was obtained after fitting the Bigelow model to experimental data. However, the Mafart model was chosen because it showed better goodness-of-fit indexes (Table 8).

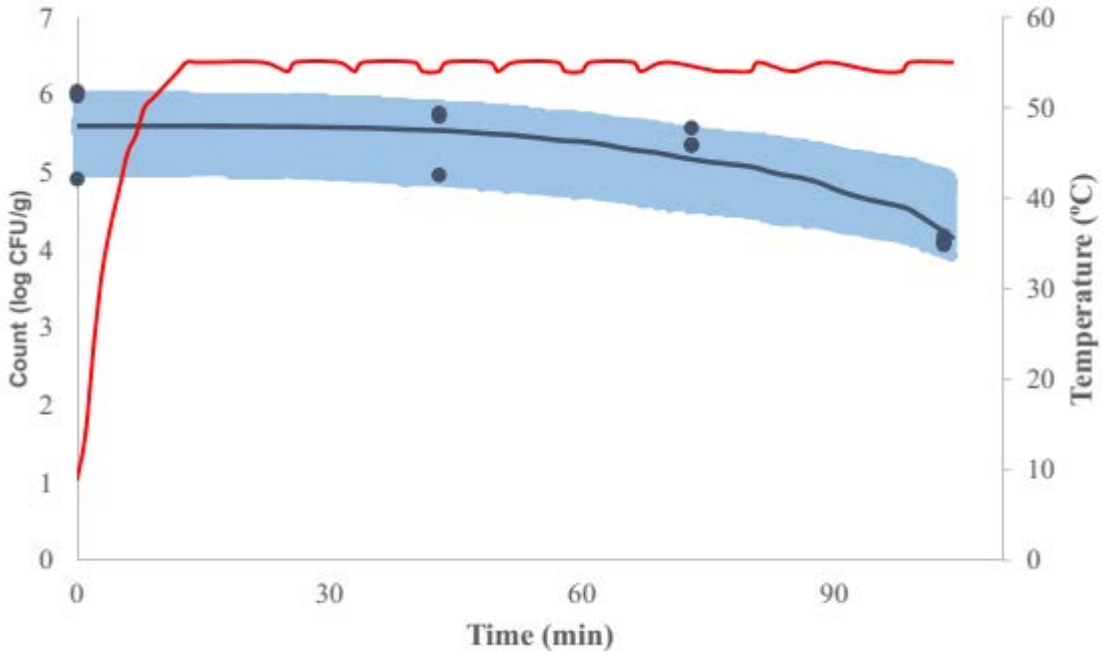


Figure 13. *Salmonella* Senftenberg inactivation data in inoculated samples cooked for 30, 60 and 90 min at 55 ºC. Experimental counts (blue dots), temperature profile recorded during the experiment (red line) and the survivor curve generated by Bioinactivation FE by fitting the Geeraerd model (blue line), including the 90 % prediction interval of the microbial reduction (blue band).

In meat samples inoculated with *S.* cocktail cooked at 55 ºC during 90 min, one of the three replicates had counts below the QL. Survival curve was adjusted by the Mafart model (Figure 14). The Bigelow model generated a similar prediction interval but with worse goodness-of fit indexes. Fitting indexes and prediction interval were affected by the high variability obtained between experimental data with differences greater than 1 log CFU/g (Figure 14). Nevertheless, Bioinactivation FE offers a general view of the population inactivation tendency. The 95% confidence interval estimated for the δ value was between 50.30 and 77.70. This is a reflect of the limited lethality caused by this heat treatment. In any case, more data would be required to reduce model uncertainty.

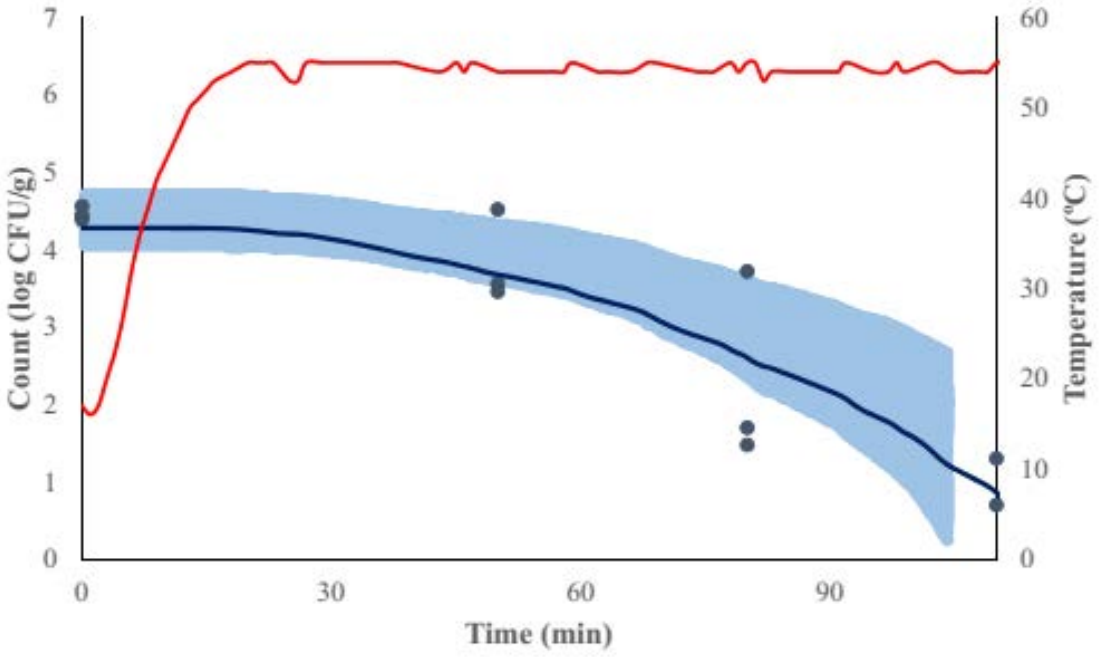


Figure 14. *Salmonella* cocktail inactivation data in inoculated samples cooked for 30, 60 and 90 min at 55 ºC. Experimental counts (blue dots), temperature profile recorded during the experiment (red line) and the survivor curve generated by Bioinactivation FE by fitting the Mafart model (blue line), including the 90 % prediction interval of the microbial reduction (blue band).

4.2.3 Effect of the cooking treatment on the survival and recovery of *Salmonella*

Effect of cooking treatment on the damage degree of *Salmonella* spp. was evaluated during cold storage of meat samples at 4 and 8 ºC. As a result of the high heterogeneity showed by *Salmonella* spp. counts, data were processed considering the individual values of triplicates. Results are shown in Tables or Figures depending on how cooking treatment or cold storage affected the behaviour of different strains, since growth, survival and death of *Salmonella* spp. can occur simultaneously after heat and cold stress. Simultaneous growth, survival and death of *Salmonella enterica* serotype Agona under osmotic stress has been recently described by Aspridou et al. (2018) confirming that, at conditions approaching the boundaries of growth, high heterogeneity of individual cell responses takes place and, consequently, complexity in the behaviour of microbial populations increases.

4.2.3.1 *Salmonella* spp. behaviour in pork loin samples cooked at 60 ºC

Salmonella spp. inoculated in pork samples, heat treated at 60 ºC and cold stored, showed a bimodal behaviour (survival/death). The effect of heat treatment was severe, as counts below the quantification limit were obtained for all samples inoculated with *Salmonella* spp. since day 5 at any temperature of storage. Nevertheless, live *Salmonella* spp. cells could be detected in some samples during the storage period. In summary, *S.* Senftenberg showed the highest % of survival, followed by *S.* cocktail and *S.* Enteritidis. Table 9 shows the % of survival in samples over the storage time as function of the strain, storage temperature and treatment time. Cells in green correspond to treatments where a complete inactivation of the bacteria was achieved, while treatments where *Salmonella* spp. was detected in all three replicates appear as red coloured cells. Cells coloured in orange mean that *Salmonella* spp. was detected in at least one of the replicates of these treatments.

S. Enteritidis inoculated in pork loin samples showed a high sensibility to heat and cold stress This bacterium was only detected on day 0 after 30 and 60 min treatments at 60 ºC. In meat samples inoculated with *S.* cocktail, as in meat samples with *S.* Enteritidis, survival of *Salmonella* cells was not detected immediately after treatments of 90 min. However, a high heterogeneous behaviour during cold storage was observed. Survival of *Salmonella* spp. in samples inoculated with the cocktail of *Salmonella* strains was detected in a high number of samples cooked for 30 min, especially when stored at 4 ºC. These detections can be attributed to the *S.* Senftenberg component of the cocktail of strains. In samples treated during 60 and 90 min detection was more variable, possibly due to the heterogeneity of cell sensibility to stress conditions. Immediately after heat treatment, counts in 2 up to 3 samples inoculated with *S.* Senftenberg were above the QL, describing a tail in the inactivation kinetic, as mentioned in the previous section. No more samples presented counts above the QL and survival of this bacteria decreased throughout the storage time. *S.* Senftenberg was detected until the 10^{th} day in samples treated during 30-60 min, and only until the 5^{th} day in samples treated at 90 min, possibly due to a greater heat damage caused.

Table 9. Percentage of survival of *Salmonella* spp. during cold storage at 4 and 8 ºC in pork loin samples cooked at 60 ºC for 30, 60 and 90 min.

Strains	Storage Temperature (º C)	Time of heat treatment (min)	Storage time (day)						
			0	5	10	15	20	25	30
S. Enteritidis	4	30	100[1]	0	0	0	0	0	0
		60	33	0	0	0	0	0	0
		90	0	0	0	0	0	0	0
	8	30	100	0	0	0	0	0	0
		60	33	0	0	0	0	0	0
		90	0	0	0	0	0	0	0
S. cocktail	4	30	33	33	100	33	66	66	33
		60	33	0	0	0	0	0	0
		90	0	0	33	0	0	0	0
	8	30	33	33	33	0	33	0	0
		60	33	0	0	0	33	0	0
		90	0	0	0	0	0	0	0
S. Seftenberg	4	30	100	100	100	0	0	0	0
		60	66	66	100	66	0	0	0
		90	66	66	0	0	0	0	0
	8	30	100	100	100	0	0	0	0
		60	66	66	0	0	0	0	0
		90	66	33	0	0	0	0	0

[1] Cells in green correspond to complete inactivation of the bacteria. Cells are red coloured if *Salmonella* spp. was detected in all three replicates. Cells are in orange if *Salmonella* spp. was detected in one or two replicates.

Except for *S.* cocktail in 30 min treatments, no significant differences were obtained in results between samples stored at 4 ºC and 8ºC. Therefore, the overall results obtained during storage at 4 and 8 ºC were combined to evaluate *Salmonella* spp. survival probability as function of duration of heat treatment and strain (Figure 15). As expected, the longer the time of heat treatment, the lower the *Salmonella* spp. probability of survival. Significant differences in survival behaviour were observed between strains as function of the duration of treatment. *S.* Enteritidis had a low survival probability (< 0.08) and no significant differences were observed between treatments. On the other hand, any heat treatment applied to *S.* Seftenberg, was able to reduce the probability of survival under 0.13 during the storage period. Moreover, significant differences were observed between treatments of 30 or 60 min and treatments of 90 min. S. cocktail showed an intermediate probability of survival, and significant differences were observed between treatments of 30 min and the longer ones. Anyway, *S.* Seftenberg is not totally representative of strains reasonably expected to be present in pork loin (NACMCF, 2010). In view of these results, heat treatment of 90 min can be considered reasonably safe as long as any temperature abuse occurs during storage, distribution, retail, sale or handling of meat samples.

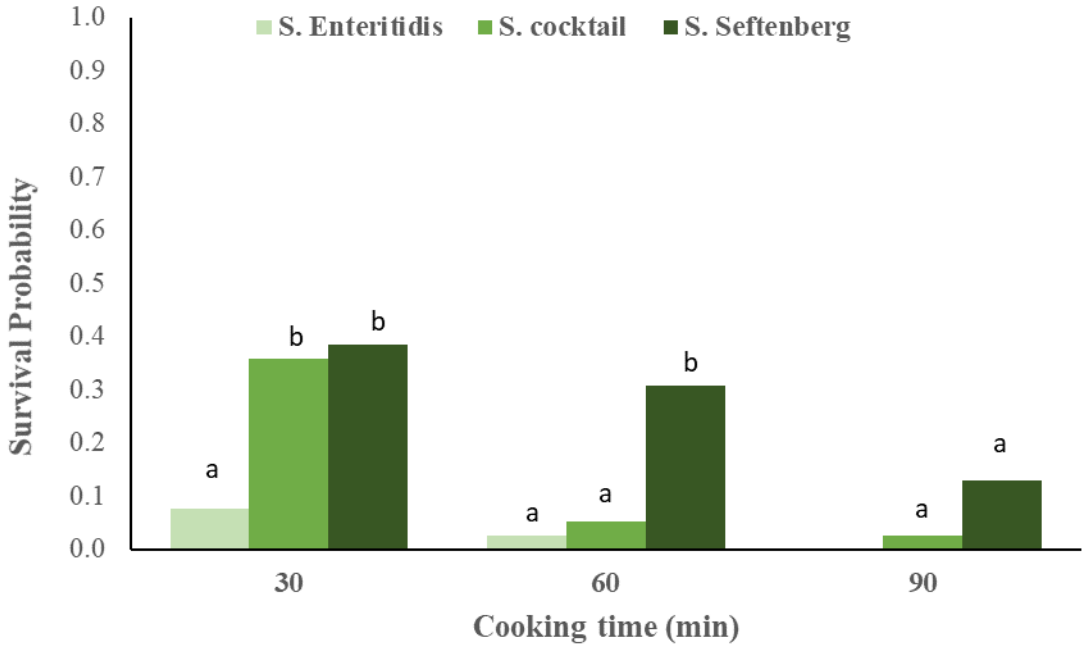


Figure 15. Survival probability during cold storage of *Salmonella* spp in pork loin samples *sous-vide* cooked at 60 ºC for 30, 60 and 90 min. Different letters indicate statistically significant differences ($p < 0.05$) between strains and treatments.

4.2.3.2 *Salmonella* spp. behaviour in pork loin samples cooked at 55 ºC

***Salmonella* Enteritidis**

A high heterogeneous behaviour was observed during cold storage of samples, regardless of the heat treatment and temperature of storage. Table 10 shows the percentage of survival in samples over the storage time as function of temperature of storage and duration of the treatment. Although counts of *S.* Enteritidis were below the quantification limit in all samples, some survival cells could be detected in some of them. Overall, any of the heat treatments applied were effective to obtain a complete reduction of S. Enteritidis. Due to external reasons, no data was obtained at day 5, 15 and day 30 of this experiment.

Table 10. Percentage of survival of *Salmonella* Enteritidis during cold storage at 4 and 8 ºC in pork loin samples cooked at 55 ºC for 30, 60 and 90 min.

Storage Temperature (ºC)	Time of heat Treatment (min)	Storage time (day)						
		0	5	10	15	20	25	30
4	30	100 [1]	nd [2]	33	nd	33	33	nd
	60	66	nd	33	nd	0	0	nd
	90	100	nd	33	nd	0	0	nd
8	30	100	nd	33	nd	0	0	nd
	60	66	nd	0	nd	33	66	nd
	90	100	nd	0	nd	33	0	nd

[1] Cells in green correspond to complete inactivation of the bacteria. Cells are red coloured if S. Enteritidis. was detected in all three replicates. Cells are in orange if S. Enteritidis was detected in one or two replicates
[2] nd: Not Determined

No significant differences were obtained in the survival probability due to the duration of heat treatment or the storage temperature (data not shown). *Sous-vide* cooking at 55 ºC during 30, 60 and 90 min cannot be considered a safe heat treatment regarding *Salmonella* spp., since even the less heat resistant strain included in this study was able to survive, with a mean probability of 0.29.

Salmonella Senftenberg

As seen in the previous section, the Mafart model estimated a δ value between 85.56 and 99.56 min. In accordance with this prediction, only the heat treatment of 90 min was able to reduce the initial count in one log. During the first five days of storage at 4 ºC of samples treated for 30 and 60 min, a significant reduction of heat-stressed *S.* Senftenberg cells was observed, probably caused by the cold environmental conditions. Thereafter, similar to samples treated for 90 min, counts of heat-resistant cells remained constant during cold storage (Figure 16). Although the growth of *Salmonella* spp. is believed to be controlled by low temperatures (refrigeration), and industry relies heavily on refrigerated storage of fresh foods to maintain their safety in relation to this pathogen outgrowth, *Salmonella* spp. can be resilient to adverse environmental conditions and some strains

can exhibit psychrotrophic properties (D'Aoust, 1991; ICMSF, 1996; Matthews et al., 2017).

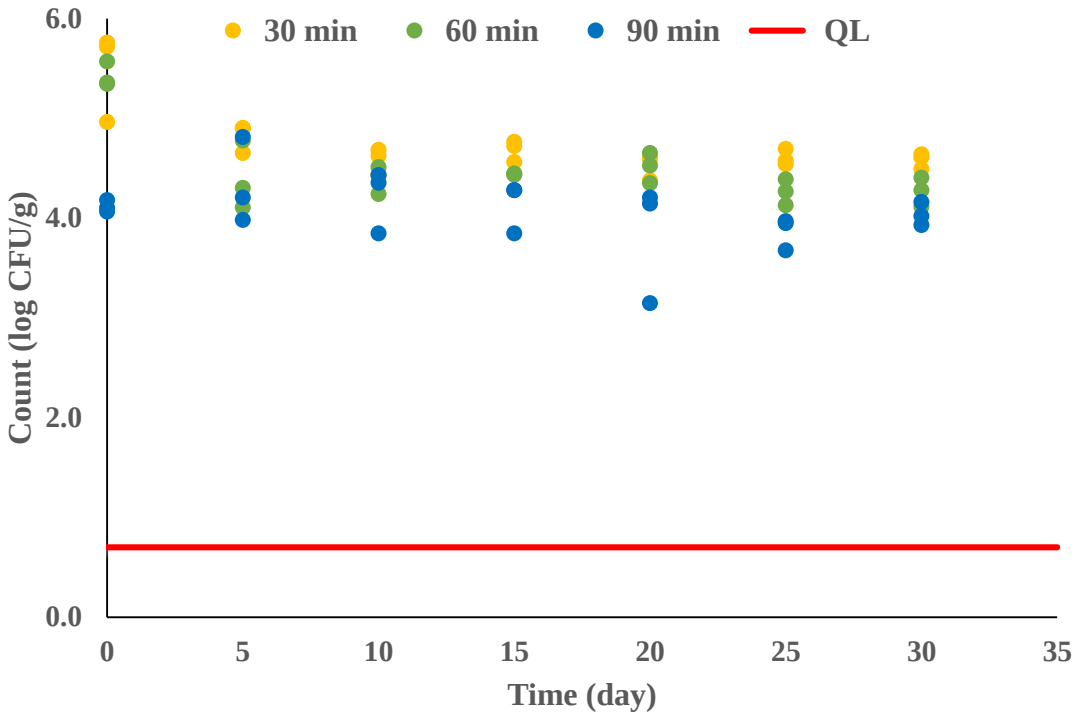


Figure 16. Counts of *Salmonella* Senftenberg in inoculated samples cooked at 55 ºC and stored at 4 ºC. Quantification limit (5 CFU/g) is shown with a red line.

Growth of *S.* Senftenberg was observed in samples stored at 8 ºC after a latency period of no more than 10 days. The raw growth data were fitted to the primary growth model of Baranyi and Roberts (1994) by the on-line tool DMFit of ComBase to estimate the lag time and the maximum growth rate (Grmax). The lag or latency phase is the initial stage in growth curves defining a certain period of time in which no increase of cell numbers is observed. Cells surviving the heat treatment undergo adaptation to the new environment and, once adapted, multiply showing an increase in the number of cells. This increase indicates the beginning of the exponential phase of the growth curve, that is mathematically described by means of the growth rate (i.e. increase of cells per time unit). Growth rate accelerates from a value of zero in the lag phase to reach a maximum value (Grmax). The maximum growth rate maintains constant during a certain period of time and then decelerates reaching a final value of zero, coinciding with the maximum population density achieved in the stationary phase (Pérez-Rodríguez, 2014).

In view of the high variability obtained between replicates, confidence intervals of the estimated kinetic parameters (Grmax and lag) as well as confidence bands for the fitted growth model were computed based on the Monte Carlo method using the standard error (SE) reported by DMFit. At each Monte Carlo iteration, randomly data-sets were generated at each sampling time and fitted with the model of Baranyi and Roberts. The Monte Carlo analysis results in a probability density distribution for each one of the model parameters and also in a probability distribution for the microbial load prediction at a certain time. The confidence intervals define the uncertainty range on the estimated regression parameters (Grmax and lag) while confidence bands define graphically the uncertainty range on the best-fit growth model (i.e. growth curve) (Posada-Izquierdo et al., 2013).

Figure 17 shows growth data of *S.* Senftenberg in inoculated samples cooked at 55 ºC and stored at 8 ºC. Quantification limit (5 CFU/g) is shown with a red line. In green, the best-fit prediction curves based on the Baranyi model and, indicated with blue lines, the confidence bands generated by Monte Carlo analysis. Table 11 present statistics for the estimated maximum growth rate (Grmax) and latency, generated by Monte Carlo analysis, for each heat treatment time.

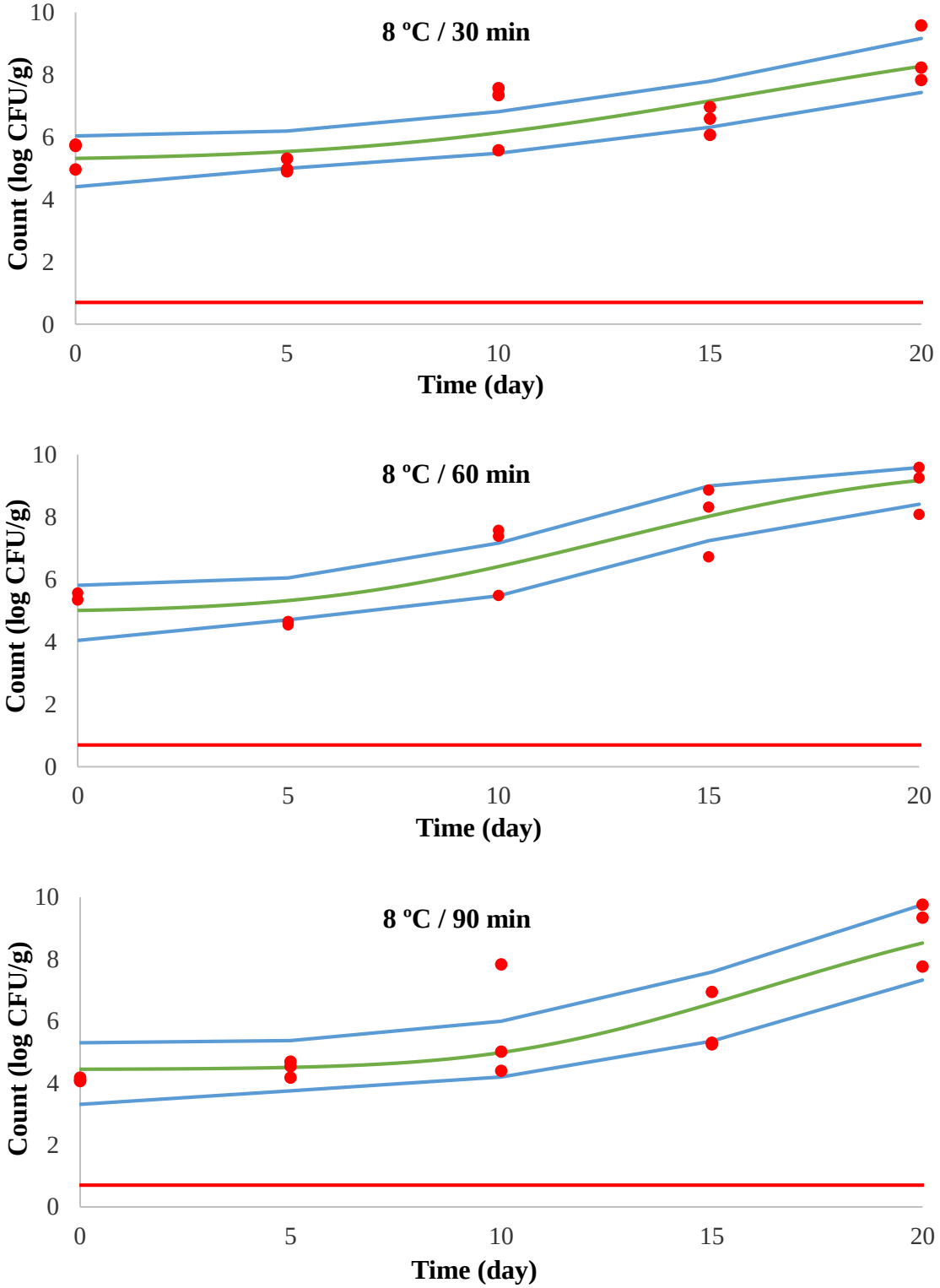


Figure 17. Counts of *Salmonella* Senftenberg in inoculated samples cocked at 55 ºC and prediction curves based on the Baranyi model, including best-fit line (green line) and confidence bands (blue lines) generated by the Monte Carlo analysis. The quantification limit (5 CFU/g) is shown with a red line.

Table 11. Statistics for the estimated maximum growth rate (Grmax) and latency of *Salmonella* Senftenberg, generated by the Monte Carlo analysis, for each treatment during the storage at 8 ºC.

Heating time (min)	Grmax (log CFU/g/day)		Latency (day)	
	Mean ± SD	**95% CI**	**Mean ± SD**	**95% CI**
30	0.34 ± 0.26	0.10 – 0.77	6.73 ± 14.90	-11.07 – 15.48
60	0.52 ± 0.50	0.21 – 2.33	6.82 ± 3.68	-1.23 – 13.76
90	0.64 ± 0.75	0.20 – 2.92	10.04 ± 6.28	0.17 – 15.41

Although the high variability observed between replicates, an acceptable fitting of growth data was obtained with the Baranyi model. The standard error reported by DMFIT was 0.84, 0.88 and 1.13 log CFU/g for 30, 60 and 90 min treatments, respectively. The Monte Carlo analysis provides quantification of differences in growth kinetics caused by data variability. In fact, prediction intervals of the entire growth curves for 30 and 60 min treatments enclose a narrower zone (between 1.2 and 1.7 log CFU/g) than the prediction interval generated for the treatment lasting 90 min (between 1.6 and 2.4 log CFU/g). As stated in previous studies, the more severe the heat treatment was, the lower the average number of surviving bacteria and the greater the variability (Koutsoumanis, 2008; Aguirre et al., 2009).

A direct relationship can be outlined between Grmax or latency period and treatment duration. Thus, treatment of 30 min showed the minor value of Grmax and latency period, in contrast to higher values of both parameters in samples heated during 90 min. Surviving cells heated during 30 min at 55 ºC were barely affected and were able to begin growth immediately, but at low rates. Nevertheless, surviving cells of longer heat treatments were more stressed, needing a longer latency period to recover. Then, the exponential phase of growth began later at a major rate. Additionally, it can be seen that longer heat treatments had larger standard deviation of Grmax, resulting in higher uncertainty of predictions.

Other studies have established the relationship between stress and lag phase of *Salmonella* spp. Stephens et al. (1997) assessed the growth of heat-injured *S.* Typhimurium in microtiter plates by monitoring turbidity and then calculated the lag times by using a model that extrapolated the growth curve back to the initial inoculum level. While the lag times for individual healthy cells were narrowly distributed, the lag times for injured cells ranged from less than 12 h to more than 20 h, with some lag times longer than 30 h.

In their review about stress, sublethal injury, resuscitation and virulence of bacterial foodborne pathogens, Wesche et al. (2009) stated that incubation of injured bacteria at suboptimal growth temperatures reportedly suppresses cell division while permitting metabolic repair processes to continue, presumably reversing the effects of sublethal injuries prior to replication. Results of *S.* Senftenberg heat-treated at 55 ºC and stored at 8 ºC are in accordance with these authors, although growth took place on a food matrix (pork meat) and not in laboratory media.

Finally, it has to be noted that the non-linear regression algorithm for the fitting calculates negative values for the lower confidence interval of latency time of samples cooked during 30 and 60 min. These negative lower limits are artificial results related to the fitting algorithm, because it generates symmetrical confidence intervals for the growth parameters. Similar outputs are related by Garre et al. (2017). In these cases, the use of other fitting algorithms is suggested, but they are not provided by the tool used in this study.

Salmonella cocktail

As seen in the previous section, the Mafart model was used to characterise inactivation kinetics of the *S.* cocktail. The 95% confidence interval estimated for δref value was 50.30 - 77.70 min. At this moderate rate of inactivation, *Salmonella* was able to survive in all heat treatments. Survivor *Salmonella* cells of samples stored at 4 ºC could be quantified at each time point. Nevertheless, no growth tendency was detected during the storage period (Figure 18A). Although *S.* Senftenberg was able to recover and growth at 8 ºC, it could not growth when it was inoculated mixed in a cocktail (Figure 18B). This fact is probably due to biological (reproduction) variability. This is defined as the difference between independently reproduced experiments with the same strain, performed on different experimental days from new pre-cultures, newly prepared media and different competitive microbiota present in the food matrix that may inhibit growth of specific pathogens (NACMCF, 2010; Aryani et al., 2015a; b).

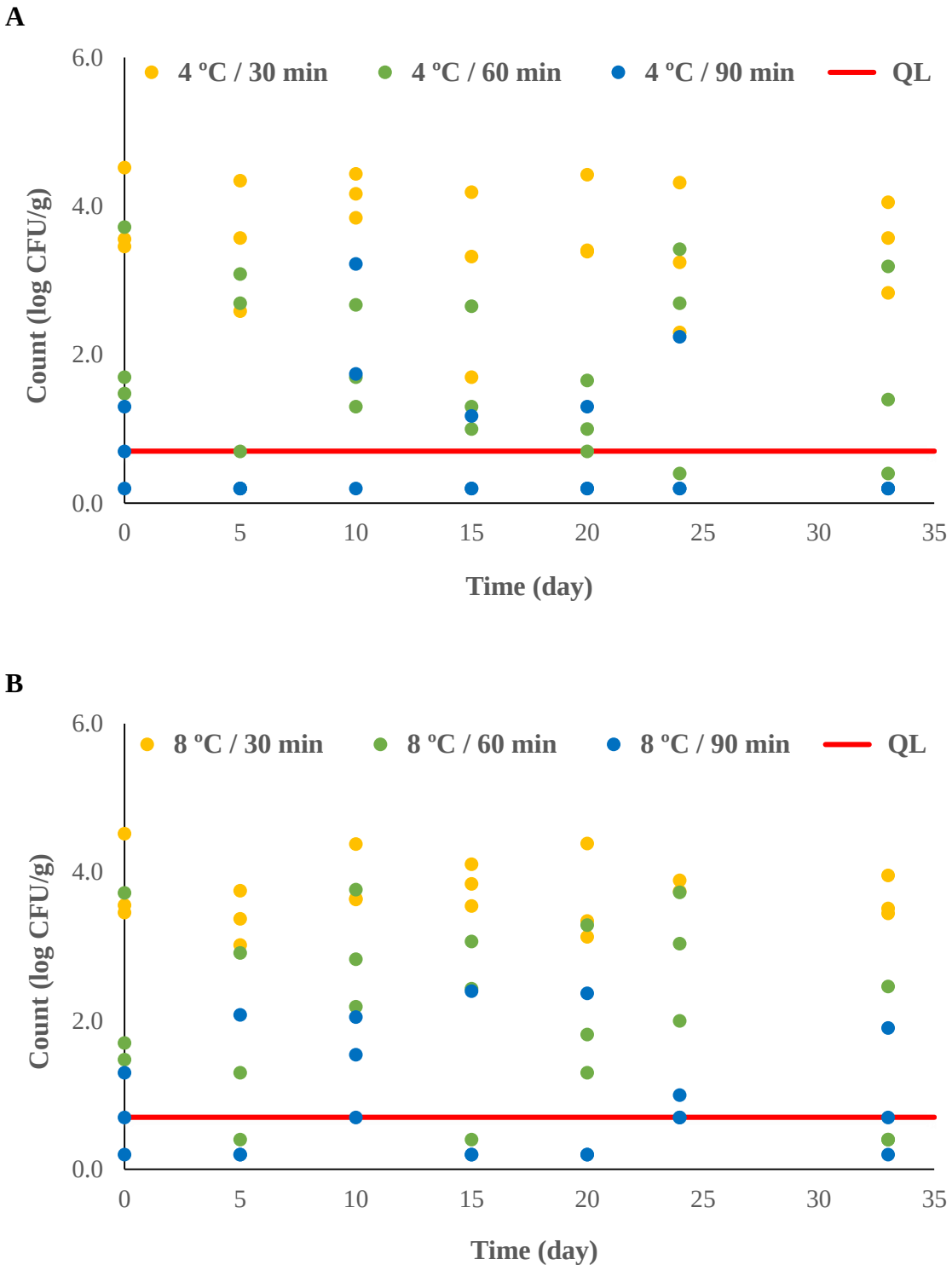


Figure 18. Counts of *Salmonella* cocktail in inoculated samples cocked at 55 ºC and stored at 4 ºC (A) and 8 ºC (B). The quantification limit (5 CFU/g) is shown with a red line.

When the probability of samples presenting counts higher than the QL as function of the duration of the treatment and the temperature of storage was evaluated, no differences were observed between 30 and 60 min treatments at any storage temperatures (Figure 19). In accordance with the δ value estimated by the Mafart model, significant differences

were observed in samples treated for 90 min. At this heat treatment time, storage at 4 ºC had a significant effect on bacterial counts, possibly caused by the fact that cold damage was added to heat damage in sublethal-stressed cells.

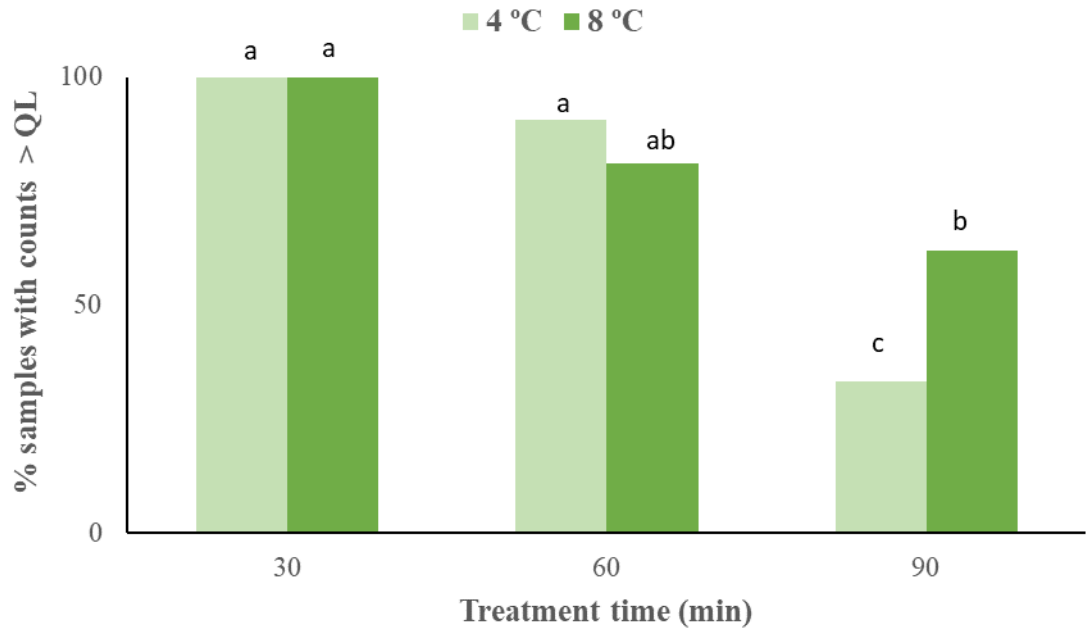


Figure 19. Proportion of samples inoculated with *Salmonella* cocktail and cooked at 55 ºC showing counts above the quantification limit (QL) during the storage at 4 and 8 ºC. Different letters indicate statistically significant differences ($p < 0.05$) between groups.

4.3 *Listeria monocytogenes*

4.3.1 Lethality

All heat treatments performed at 60 ºC achieved a lethality of *L. monocytogenes* strains higher than 5 log CFU/g, and all samples showed counts below the QL (5 CFU/g) at day 0 after the heat treatments (Table 12). However, statistical differences were observed at 55 ºC as function of the strain and the duration of the heat treatment (Table 12). Lethality caused was complete only for strain Lm 4031 while for strain Lm Scott A and for the Lm cocktail the lethality was significantly lower ($p < 0.05$). Besides, statistical differences were also observed between lethalities caused to Lm Scott A strain and the Lm cocktail ($p < 0.05$) when samples were cooked for 60 and 90 min (Table 12), being higher for Lm Scott A strain. This fact could be related to the biological variability, since the cooking

cycle of samples inoculated with Lm Scott A had a more intense heating phase than samples inoculated with Lm cocktail (Figure 11).

Table 12. Lethality of *Listeria monocytogenes* strains inoculated in pork meat samples *sous-vide* cooked at 55 and 60 ºC for 30, 60 and 90 min (mean ± standard deviation, log CFU/g).

Temperature (º C)	***L. monocytogenes* strains**	**Lethality (log CFU/g)**		
		Heating time (min)		
		30	**60**	**90**
60	**Lm 4031**	5.69 ± 0.00^{a}	5.69 ± 0.00^{a}	5.69 ± 0.00^{a}
	Lm Scott A	5.58 ± 0.00^{a}	5.58 ± 0.00^{a}	5.58 ± 0.00^{a}
	Lm cocktail	5.52 ± 0.00^{a}	5.52 ± 0.00^{a}	5.52 ± 0.00^{a}
55	**Lm 4031**	5.58 ± 0.00^{a}	5.58 ± 0.00^{a}	5.58 ± 0.00^{a}
	Lm Scott A	0.98 ± 0.39^{bx}	2.20 ± 0.38^{by}	2.60 ± 0.45^{by}
	Lm cocktail	0.84 ± 0.25^{bx}	1.39 ± 0.64^{cxy}	1.38 ± 0.16^{cy}

[a-c] Different letters on the same column indicate statistically significant differences ($p < 0.05$) between groups.
[x-y] Different letters on the same row indicate statistically significant differences ($p < 0.05$) between groups.

4.3.2 Modelling of thermal inactivation

Dynamic heating conditions were modelled with the non-isothermal fitting module of the Bioinactivation FE software. Heat inactivation was characterized only for meat samples inoculated with Lm Scott A and Lm cocktail and cooked at 55 ºC. All three replicates of these two experiments had counts above the QL immediately after the heat treatment. The best fit to experimental data were obtained for the Bigelow and the Geeraerd models (Figures 20 and 21and Table 13). The summary of the statistical data of the model fitting is shown in Table 13.

Table 13. Statistical indexes and parameter estimation (mean, standard deviations and their 95% confidence intervals) of the inactivation model used for *Listeria monocytogenes* strains.

	Units	**Lm Scott A at 55 ºC**		**Lm cocktail at 55 ºC**	
Model	-	Bigelow	Geeraerd	Bigelow	Geeraerd
RMSE	-	1.90	1.03	1.47	1.01
B_f	-	0.82	0.99	1.22	0.98
z ± SD	(ºC/log min)	7.43 ± 0.27	7.47 ± 0.28	7.98 ± 0.56	6.77 ± 0.28
z CI	(ºC/log min)	6.54 – 7.46	6.52 – 7.48	6.08 – 7.95	6.53 – 7.48
log N_0 ± SD	log CFU/g	5.40 ± 0.14	5.22 ± 0.14	5.41 ± 0.09	5.64 ± 0.09
log N_0 CI	log CFU/g	5.01 – 5.49	5.01 – 5.48	5.41 – 5.69	5.41 – 5.69
D_{ref} ± SD	min	16.76 ± 4.94	7.05 ± 0.43	54.80 ± 7.89	8.29 ± 1.99
D_{ref} CI	min	14.69 – 32.80	6.03 – 7.48	29.07 – 56.82	8.18 – 14.86
log N_{res} ± SD	log CFU/g	-	2.54 ± 2.32	-	4.22 ± 3.98
log N_{res} CI	log CFU/g	-	2.23 – 2.97	-	3.59 – 4.54
C_c ± SD	-	-	920.39 ± 59.67	-	59.91 ± 2.72
C_c CI	-	-	804.80 – 993.86	-	50.34 – 59.48

As can be seen in Table 13, RMSE and B_f indicate that fitting for the Geeraerd model has better goodness and reliability than for the Bigelow model. Parameter estimations of z values with both models (Bigelow and Geeraerd) were similar to those reported by Van Asselt and Zwietering (2006). In particular, D values generated by the Geeraerd model were similar to the mean D value obtained at 55 ºC (12.51 min), while D values estimated by the Bigelow model were higher, but below the 95% CI at 55 ºC (72.25 min) reported by these authors.

Different values of D at 55 ºC and z have been documented for *L. monocytogenes*. Murphy et al. (2004) studied the heat inactivation of some pathogens inoculated in samples of 5 g of ground pork placed in bags of less than 1 mm thick and cooked between 55 and 70 ºC in a water bath, obtaining a D value of 47.17 ± 1.99 min at 55 ºC and a z value of 5.92 for *L. monocytogenes*. In a similar study carried out with ground pork meat obtained from breaded pork patties, Osaili et al. (2007) obtained a similar z value but a higher D value for *L. monocytogenes* at 55 ºC. Karyotis et al. (2017) compared thermal inactivation of *L. monocytogenes* inoculated in marinated and non-marinated (control)

chicken breast in pieces weighing 5 g each (approximately 1.5 cm thickness), that were heated in a water bath, obtaining z values of 6.32 and 6.92 ºC, respectively. D values obtained at 55 ºC were 45.05 ± 0.86 min in marinated samples and 54.81 ± 1.49 min in control samples. In another study about strain variability on thermal resistance of *L. monocytogenes* Aryani et al. (2015b) estimated that z values of all strains ranged from 4.4 to 5.7 °C, and D values ranged from 9 to 30 min at 55 °C. This experiment was done using flasks pre-filled with brain heart infusion broth and heated in a water bath at the desired temperature prior to the inoculation. In this way, the observed temperature drop at the moment of inoculation was negligible and inactivation started immediately. In conclusion, the z and D values from different sources are difficult to compare due to differences in the experimental design, bacterial type, substrate or environmental factors (Murphy et al., 2004). As stated previously for *Salmonella* spp., outcomes from models based on bibliography must be validated.

The shoulder effect taken into account by the Geeraerd model implies lower D values in relation to the Bigelow model, as thermal inactivation is delayed, i.e. counts are maintained at the inoculation level for some time. Therefore, time to get a ten-fold decrease in count is lower with respect to the Bigelow model, which computes inactivation since the beginning of the thermal process. This fact is especially evident in Lm Scott A (Figure 20). Considering the singular long heating phase of Lm Scott A heat treatment, both the fitted curve and the 90% prediction intervals generated by Bioinactivation FE manifest a smooth initiation of thermal inactivation in contrast to the gradient predicted by the Bigelow model since the meat temperature is higher than 50 ºC.

The Geeraerd model describes the shoulder effect by the Cc parameter, that is attributed to the presence of cells with sublethal injuries of increasing severity during the first instants of the treatment. The microbial counts does not start to drop until the amount and severity of the damages surpass a given threshold (Geeraerd et al., 2000; Cebrián et al., 2017). Comparing Geeraerd model predictions for Lm Scott A and for the Lm cocktail, Cc was 15 folds higher for Lm Scott A, indicating a higher thermotolerance when a single inoculum was used. Actually, in samples inoculated with Lm Scott A and cooked for 30 minutes, the heat treatment was applied at a mean temperature of 50 ºC during 63 min, while in samples inoculated with Lm cocktail the mean temperature was 47 ºC applied during 54 min. Consequently, Lm Scott A was affected by a higher level of heat stress than the Lm cocktail during the heating phase. The higher Cc value obtained for Lm Scott

A could explain why there were no differences between the lethality of both strains after 30 min of treatment, although significant different lethalities were obtained after 60 and 90 min. It should be noted that, as the Bigelow model, the Geeraerd model is an empirical one, as it is constructed in order to obey a set of predefined requirements as well as to be consistent with literature arguments (Geeraerd et al., 2005).

The survival curve displayed tailing (Figures 20A and 21A). This type of kinetic has been previously described for *Listeria* spp. at similar temperatures (Noriega et al., 2013). A survivor subpopulation (log N_{res}) around 4.22 log CFU/g was predicted in samples inoculated with the Lm cocktail and cooked for 60 and 90 min at 55 ºC. In samples inoculated with Lm Scott A, the tail effect began in the last minutes of the heat treatment at a level of 2.54 log CFU/g (**Table 13**). In the next section, cell recovery and growth of these cells will be discussed.

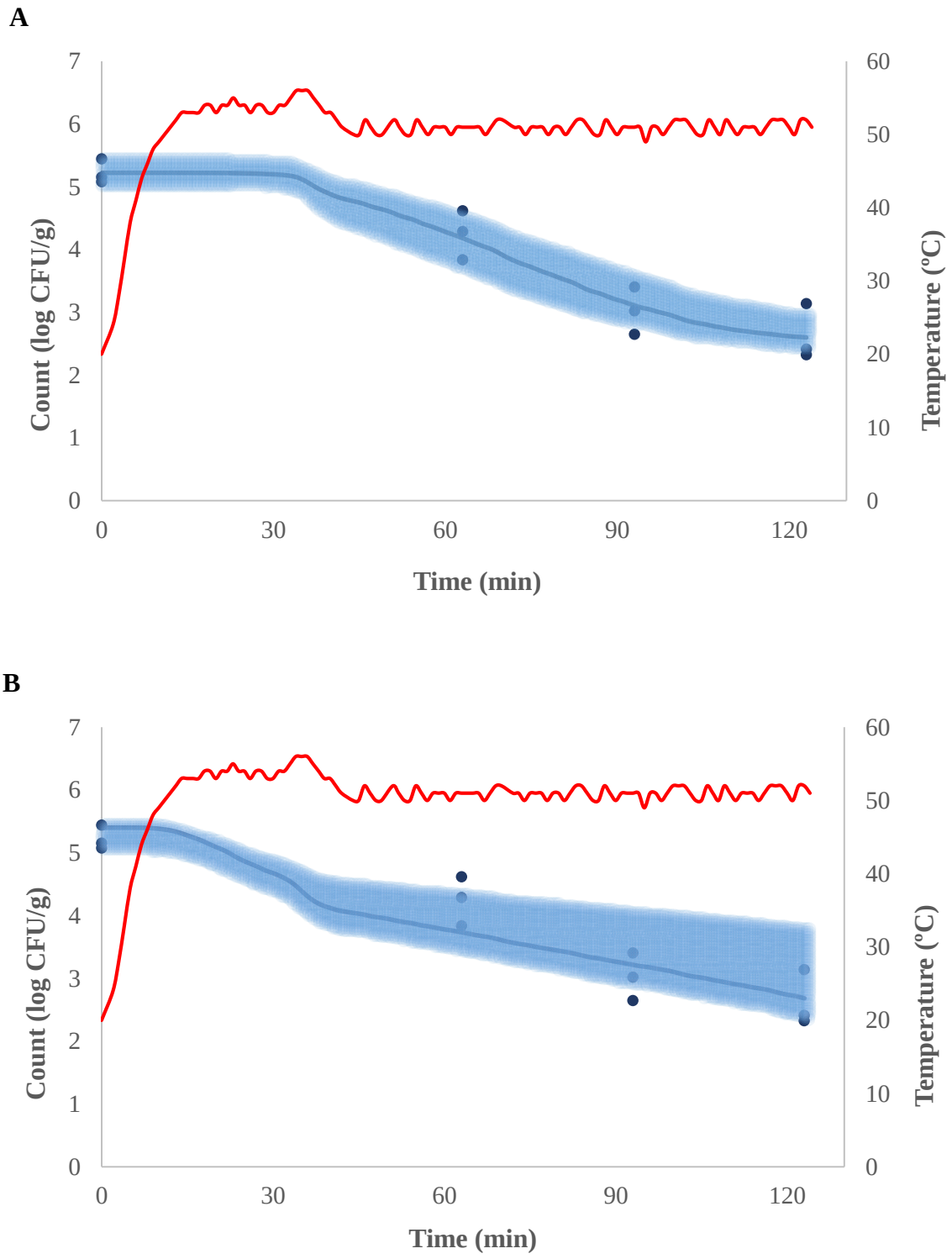


Figure 20. *Listeria monocytogenes* Scott A inactivation data in inoculated samples cooked for 30, 60 and 90 min at 55 ºC. Experimental counts (blue dots), temperature profile recorded during the experiment (red line) and the survivor curve generated by Bioinactivation FE by fitting the Geeraerd model (A) and the Bigelow model (B) (blue line), including the 90 % prediction interval of the microbial reduction (blue band).

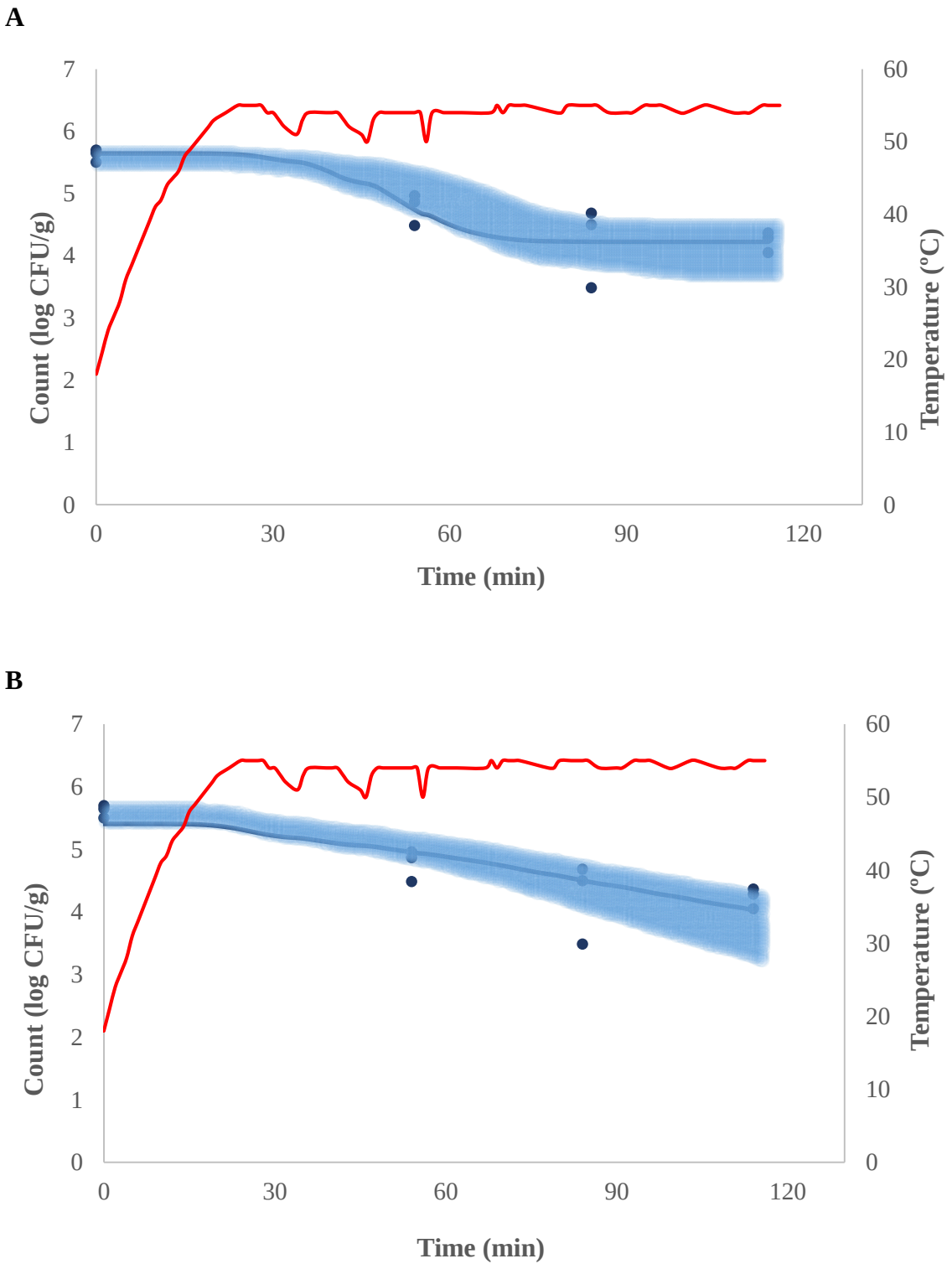


Figure 21. *Listeria monocytogenes* cocktail inactivation data in inoculated samples cooked for 30, 60 and 90 min at 55 ºC. Experimental counts (blue dots), temperature profile recorded during the experiment (red line) and the survivor curve generated by Bioinactivation FE by fitting the Geeraerd model (A) and the Bigelow model (B) (blue line), including the 90 % prediction interval of the microbial reduction (blue band).

4.3.3 Effect of the cooking treatment on the survival and recovery of *L. monocytogenes*

As for *Salmonella* spp., effect of cooking treatments on degree of damage of *L. monocytogenes* was evaluated during cold storage at 4 and 8 ºC. Results showed a highly heterogeneous behaviour. Because of that, data were processed considering the individual values of triplicates. Results are shown in tables indicating survival % or figures showing counts trough time of storage depending on how cooking treatment or cold storage affected the behaviour of different *Listeria* strains.

4.3.3.1 *L. monocytogenes* behaviour in pork loin samples cooked at 60 ºC

The effect of the heat treatment was intense, as the obtained counts for all samples were below the quantification limit at any temperature of storage. Complete inactivation was observed in samples inoculated with single strains (Lm 4031 and Lm Scott A). However, surviving cells from the Lm cocktail were detected in some samples just after being cooked for 30 and 60 min, and during the storage period just in some samples treated for 30 min (Table 14). Due to the fact that no significant differences were obtained between samples stored at 4 ºC and 8ºC, the overall results were combined. As expected, the longer the time of heat treatment, the lower the probability of survival. Significant differences ($p < 0.05$) in survival behaviour were observed between the percentage of survival in treatments of 30 min (0.25) and treatments of 60 min (0.03). On the other hand, complete inactivation was obtained in 90 min treatments. In view of these results, heat treatment of 90 min at 60 ºC can be considered safe.

Table 14. Percentage of survival of the *Listeria monocytogenes* cocktail in pork loin samples cooked at 60 ºC for 30, 60 and 90 min during cold storage at 4 and 8 ºC.

Storage Temperature (º C)	Time of heat Treatment (min)	Storage time (day)						
		0	5	10	15	20	24	33
4	30	66[1]	0	0	0	66	0	33
	60	33	0	0	0	0	0	0
	90	0	0	0	0	0	0	0
8	30	66	0	33	0	66	33	nd[2]
	60	33	0	0	0	0	0	nd
	90	0	0	0	0	0	0	nd

[1] Cells in green correspond to complete inactivation of the bacteria. Cells are in orange if *L monocytogenes* was detected in one or two replicates.
[2] nd: Not determined

4.3.3.2 *L. monocytogenes* behaviour in pork loin samples cooked at 55 ºC

L. monocytogenes CECT 4031

The effect of the heat treatment on Lm 4031 was intense because it was not detected in any sample immediately after the heat treatment. Moreover, during the storage period at 4 and 8 ºC, Lm 4031 was no detected in the 60% of samples. However, in some samples (around 9%) Lm 4031 could be recovered during the cold storage achieving counts above 2 log CFU/g, that is the limit established in the EU regulation on microbiological criteria for foodstuffs (European Parliament and Council, 2005).

A high experimental variability was observed between replicates of Lm 4031. Independently of the heat treatment, the time applied and the storage temperature, simultaneous growth (> QL), survival (< QL) and death (not detected, ND) of Lm 4031 was observed (Figure 22). According to Aspridou et al. (2018), the heterogeneity of single cells behaviour in stressing conditions is more intense and can be manifested as the coexistence of three different behavioural responses. Moreover, these findings are in accordance with published data referred to longer times and spread distribution of the lag phase of a microbial population after sublethal stress (Métris et al., 2008; Pin et al., 2013; Aguirre and Koutsoumanis, 2016; Valdramidis and Koutsoumanis, 2016; Ding et al., 2018).

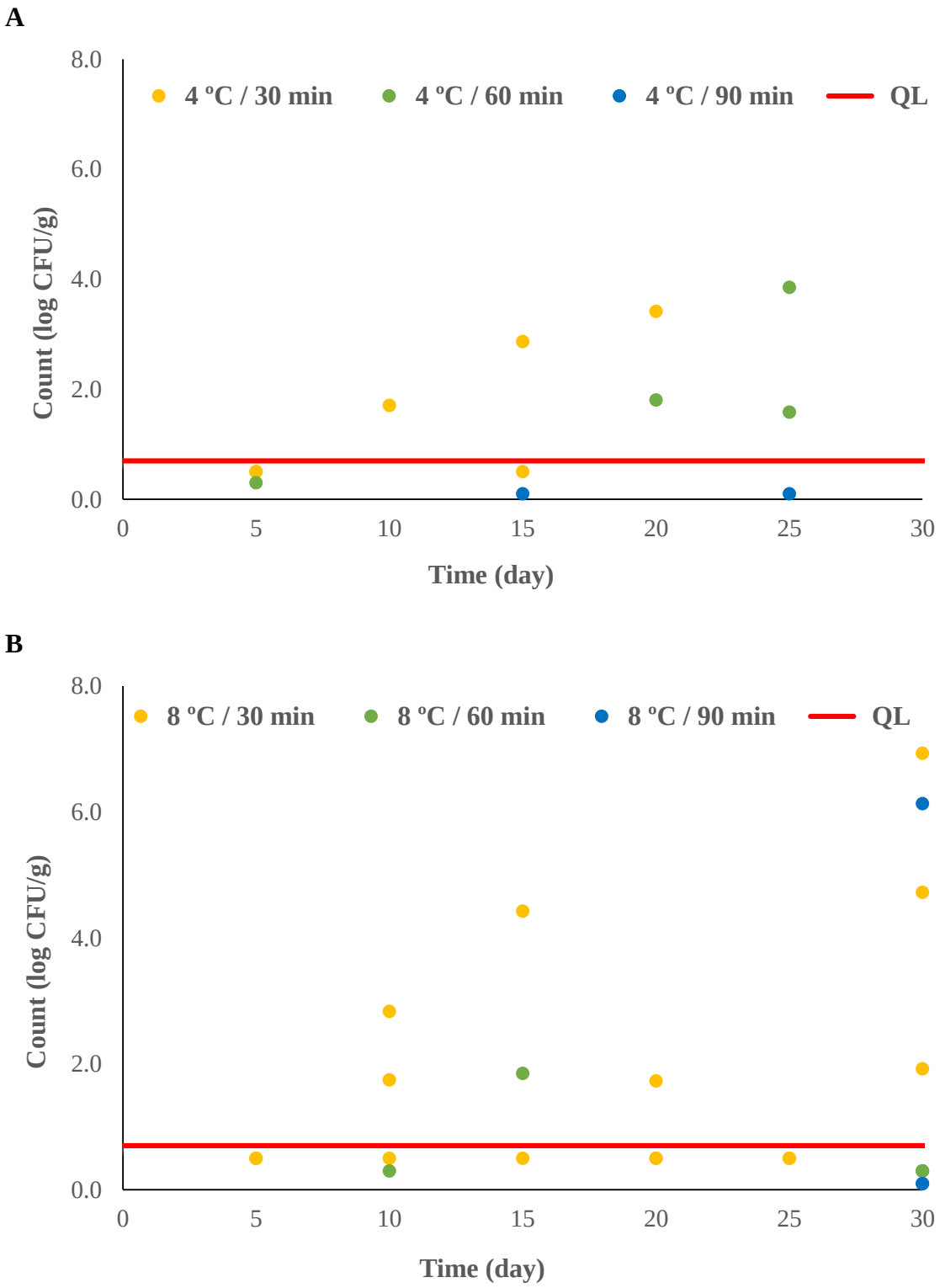


Figure 22. Counts of *Listeria monocytogenes* 4031 in inoculated samples cooked at 55 ºC and stored at 4 ºC (A) and 8 ºC (B). The quantification limit (5 CFU/g) is shown with a red line.

Considering the % of samples with counts higher than the quantification limit (> QL), samples with counts below the quantification limit (< QL) and samples where Lm 4031 was inactivated (ND), significant differences were observed in the behaviour of Lm 4031 between samples cooked for 30 min and stored at 8 ºC and all the other samples (Figure

23). In samples cooked for 30 min, storage at 4 ºC had a restrictive effect on the recovery and growth of *Listeria* cells, possibly caused by the fact that cold damage was added to heat damage in sublethal-stressed cells. Heat treatment for 60 and 90 minutes were intense enough to limit the recovery of heat-injured cells, despite the psychotrophic nature of *Listeria*.

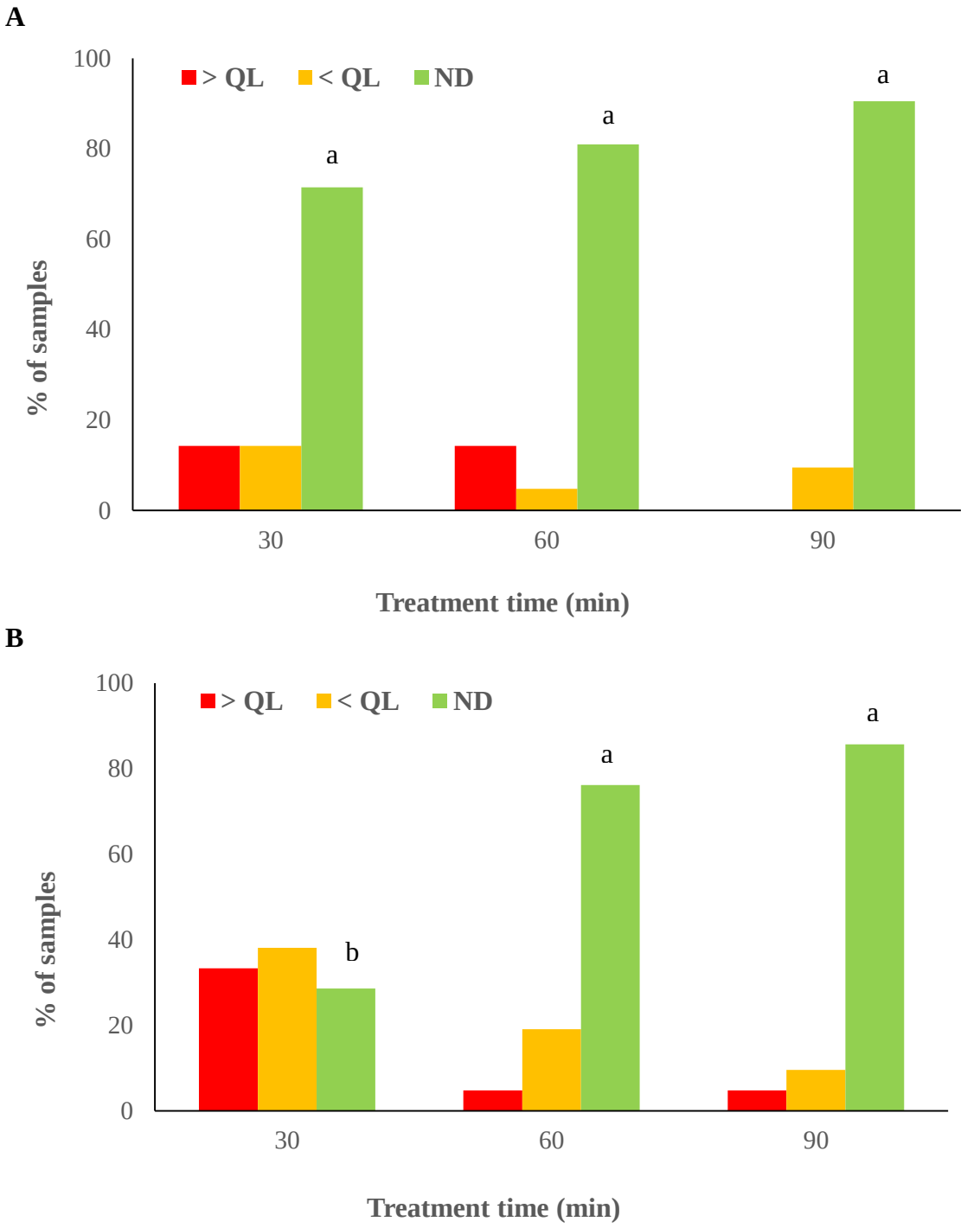


Figure 23. Proportion of samples inoculated with *Listeria monocytogenes* 4031 and cooked at 55 ºC with counts above the quantification limit (QL) (in red), counts below the QL (in orange) and samples where inactivation was complete (in green) during the storage at 4 ºC (A) and 8 ºC (B). a-b. Different letters indicate statistically significant differences ($p < 0.05$) between counts of samples cooked during 30, 60 and 90 min and stored at 4 and 8 ºC.

L. monocytogenes Scott A

As seen in the previous section, lethalities obtained for Lm Scott A were 0.98 ± 0.39, 2.20 ± 0.38 and 2.60 ± 0.45 log CFU/g after 30, 60 and 90 min heat treatments, respectively. In samples cooked for 30 min these lethalities were significantly different ($p < 0.05$) than those cooked for 60 and 90 min. A similar trend was observed in counts of Lm Scott A during cold storage at 4 ºC. In fact, after heat treatments no differences were found in counts during all the storage periods, although variability between replicates was higher as the duration of the treatment increased (Figure 24). Absence of growth during cold storage was not expected since *L. monocytogenes* is a psycrotrophic bacteria (Bover and Garriga, 2014; Matthews et al., 2017), so that the absence of growing of this microorganism was probably due to a high degree of sublethal injury caused in heat-stressed cells.

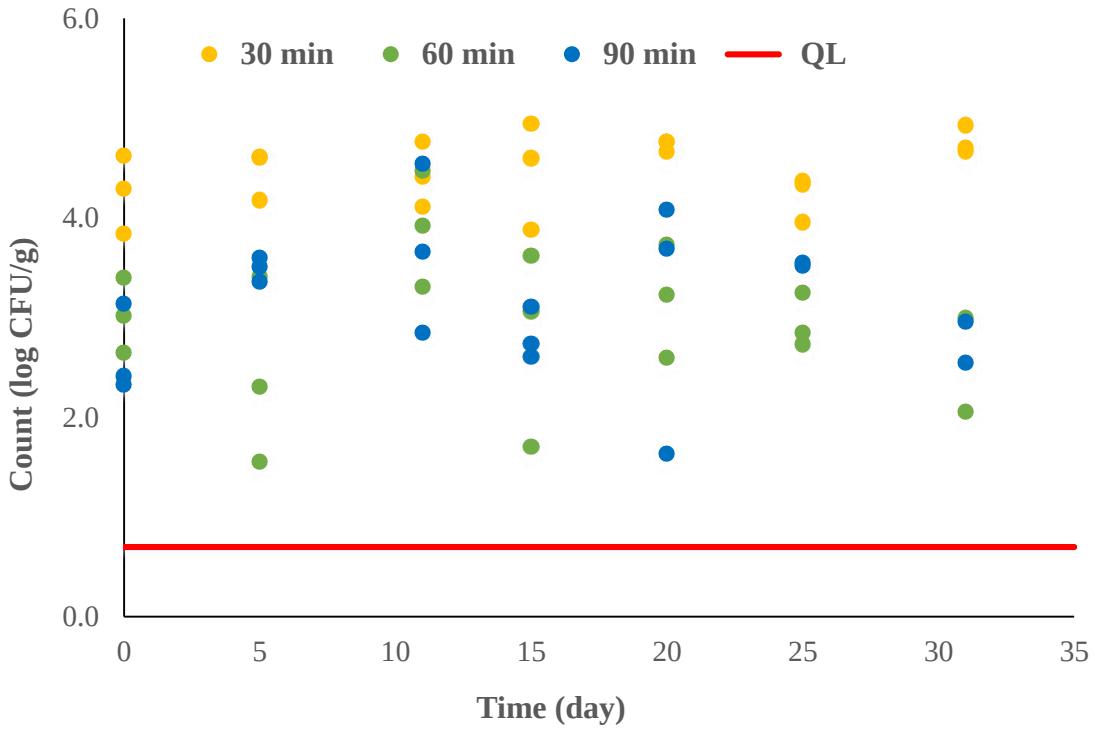


Figure 24. Counts of *Listeria monocytogenes* Scott A in inoculated samples cocked at 55 ºC and stored at 4 ºC. The quantification limit (5 CFU/g) is shown with a red line.

Figure 25 shows the growth data of Lm Scott A in the inoculated samples cooked at 55 ºC and stored at 8 ºC. Table 15 present the statistics for the estimated maximum growth rate (Grmax) and latency, generated by Monte Carlo analysis, for each treatment time. Growth of Lm Scott A was observed after a latency period between 10 and 15 days for

all heat treatments. About this result, it is necessary to comment that with respect to other strains, Lm Scott A appears to have an intermediate thermal resistance (Doyle et al., 2001) and has a lower growth rate than the average (Begot et al., 1997; Lianou et al., 2006). However, it was selected because this strain has been widely used in research to set up models (Doyle et al., 2001).

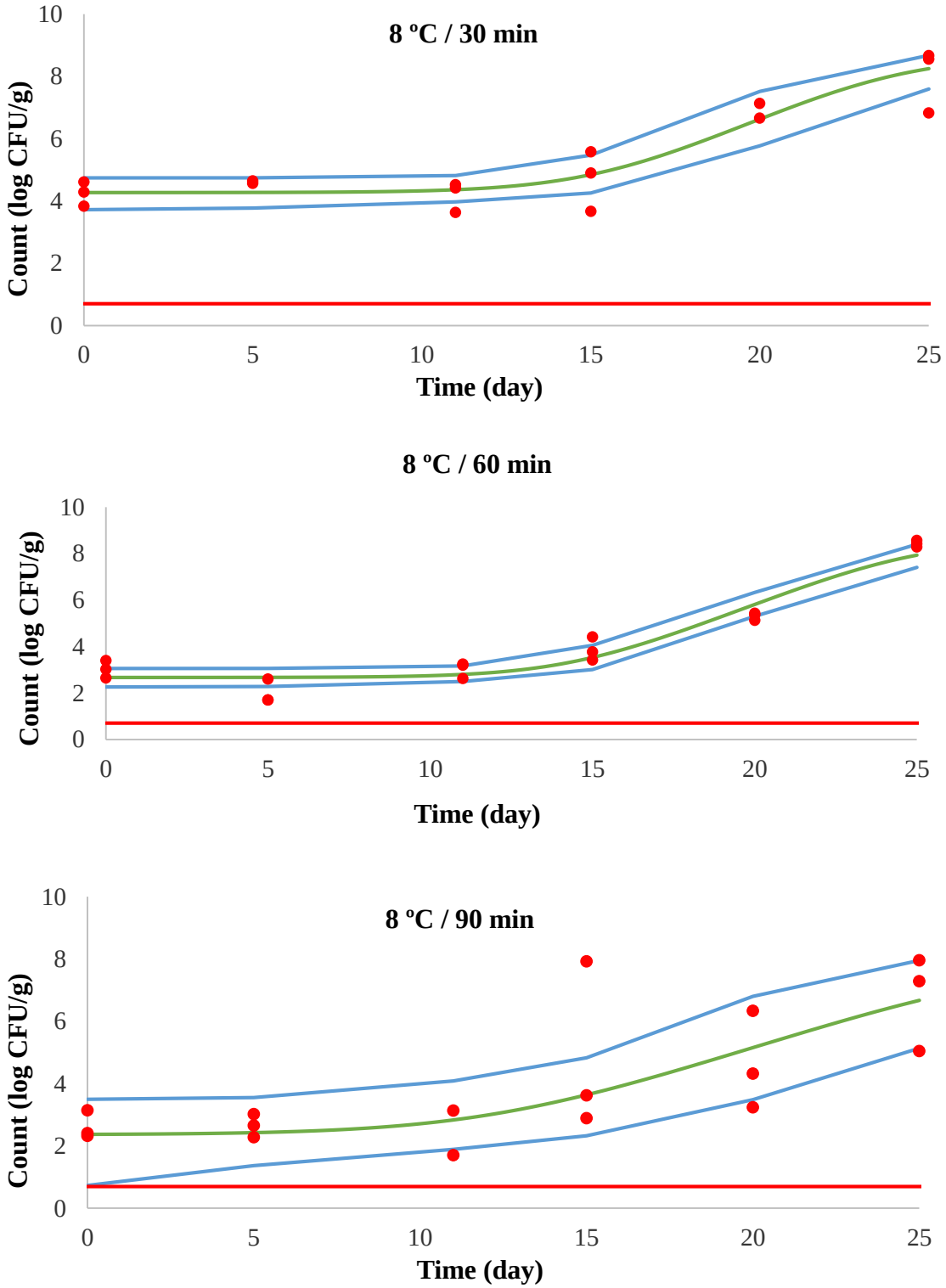


Figure 25. Growth data of *Listeria monocytogenes* Scott A in inoculated samples cocked at 55 ºC and prediction curves based on the Baranyi model, including best-fit line (green line) and confidence bands (blue lines) generated by the Monte Carlo analysis. The quantification limit (5 CFU/g) is shown with a red line.

Table 15. Statistics for the estimated maximum growth rate (Grmax) and latency period of *Listeria monocytogenes* Scott A, generated by the Monte Carlo analysis for each treatment during the storage at 8 ºC

Heating time (min)	Grmax (log CFU/g)		Latency (day)	
	Mean ± SD	95% CI	Mean ± SD	95% CI
30	0.75 ± 0.69	0.33 – 3.11	15.59 ± 2.06	11.41 – 19.44
60	0.59 ± 0.15	0.43 – 0.85	14.45 ± 1.21	11.95 – 16.78
90	0.64 ± 0.95	0.11 – 3.41	10.09 ± 21.33	-10.90 – 20.49

The high variability observed between replicates during the storage time in samples cooked during 90 min implied worse goodness-of-fit indexes, showing discrepancies between the best-fit line and the observed data. The standard error of fit reported by DMFIT for this treatment was 1.63 log CFU/g compared to the values of 0.67 and 0.55 log CFU/g observed for the 30 and 60 min treatments, respectively. Besides, the Monte Carlo analysis provides quantification of differences in growth kinetics caused by data variability by means of the confidence bands. As seen in **Figure 25**, confidence bands of the 90 min treatment comprised a wider range (between 2.19 and 3.32 log CFU/g) than the confidence bands of the 30 and 60 min treatments (between 0.67 and 1.75 log CFU/g). As stated in previous studies, the more severe was the heat treatment, the lower the average number of surviving bacteria and the greater the variability observed (Koutsoumanis, 2008; Aguirre et al., 2009)

Similar values for Grmax with different CI were generated for the three different heat treatments. However, variability was high for the latency period, even for the 60 min treatment, that obtained a considerable repeatability among replicates. Although lag phase duration is important for the prediction, yet it is laborious and difficult to obtain. Furthermore, lag phase is difficult to define and determine generically under representative conditions for practical contamination scenarios, and therefore it is not included in many challenge studies (Beaufort et al., 2014; Aryani, 2016).

As in growth predictions for *S. Senftenberg*, negative values were generated for the lower confidence interval of the latency time of samples cooked during 90 min. This negative lower limits are artificial results related to the fitting algorithm.

L. monocytogenes cocktail

As seen in the previous section, lethality obtained for Lm cocktail (mean ± SD) was of 0.84 ± 0.25, 1.39 ± 0.64 and 1.38 ± 0.16 log CFU/g for 30, 60 and 90 min heat treatments, respectively. This lethality was significantly different ($p < 0.05$) between samples cooked for 30 min and those cooked for 60 and 90 min. However, after heat treatment no differences were found in counts during all storage period at 4 ºC except for the heat treatment of 90 min, that showed significantly lower counts at day 25 and 30 of storage. Sublethal heat damage of these cells added to cold stress were probably the cause of a mean inactivation at the end of the storage period of 1.91 ± 0.37 log CFU/g. Moreover, variability between replicates was higher as time of treatment increased (Figure 26). As mentioned previously for Lm Scott A, no growth at 4 ºC was not expected, since *L. monocytogenes* is a psycrotrophic bacteria. This fact was probably due to a high sublethal injury in heat-stressed cells.

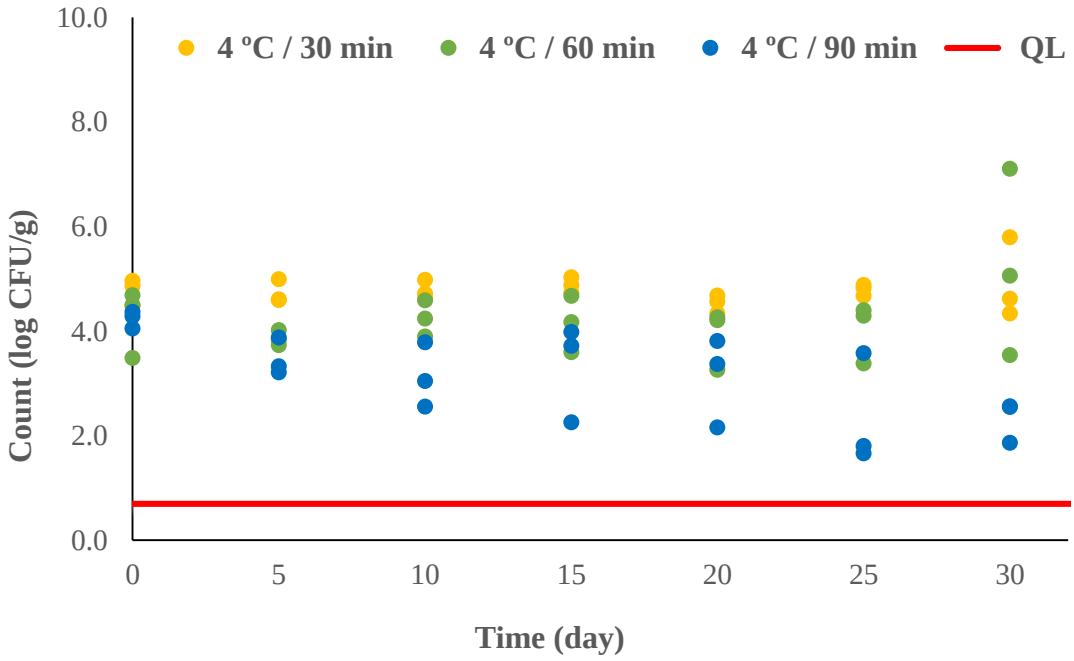


Figure 26. Counts of *Listeria monocytogenes* cocktail.in inoculated samples cocked at 55 ºC and stored at 4 ºC. The quantification limit (5 CFU/g) is shown with a red line.

On the other hand, surviving cells after 60 and 90 min heat treatments did not show significant differences during the storage at 8ºC, Cell counts remained at a constant level during cold storage until day 25, in which a significant growth took place in two up to three replicates (Figure 27). As seen previously, samples inoculated with Lm Scott A as a single strain and stored at 8 ºC after a heat treatment of 30, 60 and 90 min at 55 ºC were

able to growth from day 20. The reason why this strain could no growth as part of a mixed inoculum at that time could be explained by the biological (reproduction) variability. Considering that the cooking cycle of samples inoculated with Lm Scott A had a more intense heating phase, competitive microbiota could have been reduced as well. So, growth of Lm Scott A cells could be facilitated (NACMCF, 2010).

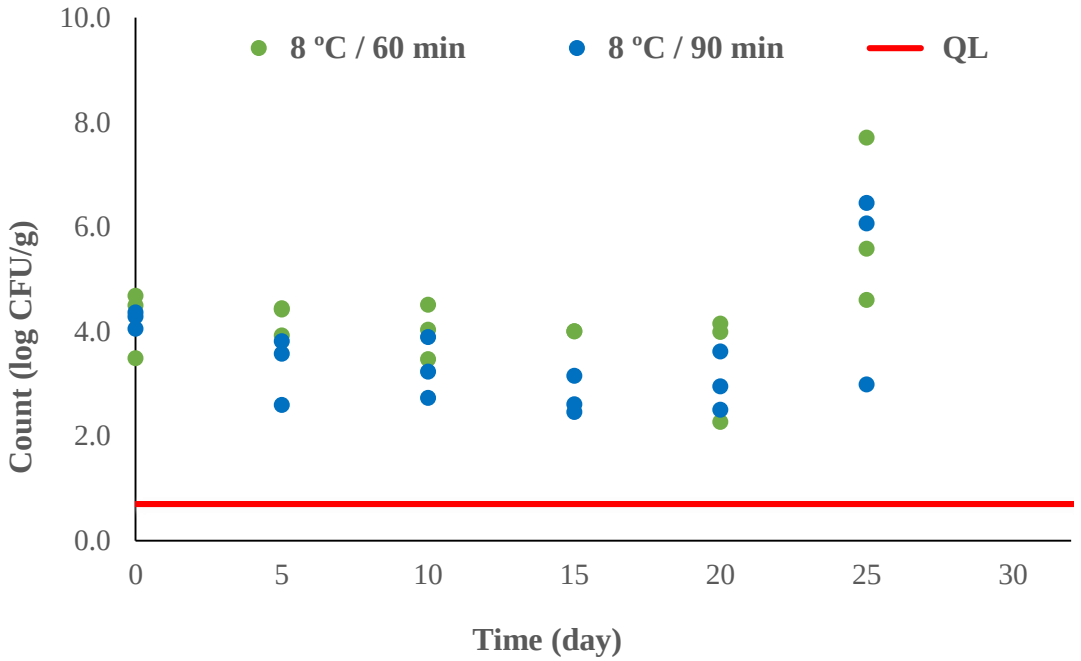


Figure 27. Counts of the *Listeria monocytogenes* cocktail in samples cocked at 55 ºC and stored at 8 ºC. The quantification limit (5 CFU/g) is shown with a red line.

On the other hand, growth was observed in samples cooked for 30 min after a latency period of 20 days in two up to three replicates. Then, the primary growth model of Baranyi and Roberts (1994) could be fitted to the raw growth data. Figure 28 shows the growth data of the Lm cocktail in inoculated samples cooked at 55 ºC during 30 min and stored at 8 ºC.

The estimated maximum growth rate (Grmax) was 0.53 ± 0.78 log CFU/g/day, with a confidence interval of 0.02 – 2.85 log CFU/g/day. The estimated latency period was 8.25 ± 52.32 days with a confidence interval of -103.61 – 25.32 days. As mentioned previously, the estimation of the latency period is inaccurate because the great variability of the adaptation time, that is related to the cellular history (Swinnen et al., 2004; Posada-Izquierdo, 2013; Aryani, 2016). A standard error of 1.00 log CFU/g generated by DMFIT for this treatment accounted for the variability observed between replicates after the lag phase. Besides, Monte Carlo analysis provides quantification of differences in the growth

kinetics caused by data variability by means of the confidence bands, which comprised a range between 0.67 and 1.05 log CFU/g.

The abovementioned variability observed at the end of the storage period at 8 ºC in samples heat treated for 30, 60 and 90 min could be caused by the presence of a non-growing fraction of the population. As stated by Aguirre and Koutsoumanis (2016), growth-limiting conditions divide population as function of its ability to growth. As the environment becomes more stressful an increasing number of cells of the population is not able to grow. Then, the population lag is divided into a physiological lag (estimated by fitting only the growth data of the growing fraction) and the pseudo-lag, that is caused by the presence of the non-growing fraction of the population. In view of these results, heat treatments of 60 and 90 min resulted in a higher non-growing fraction of survivors and, consequently, a longer latency period.

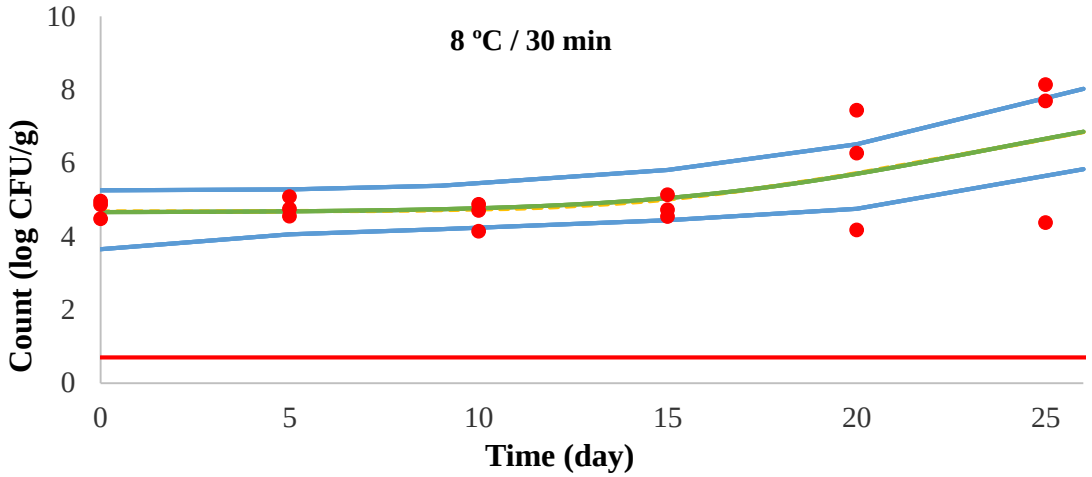


Figure 28. Counts of *Listeria monocytogenes* cocktail in inoculated samples cocked at 55 ºC and prediction curves based on the Baranyi model, including best-fit line (green line) and confidence bands (blue lines) generated by the Monte Carlo analysis. The quantification limit (5 CFU/g) is shown with a red line.

4.4. General discussion

4.4.1 Published vs experimental D values

Many different researchers have developed models for specific microorganisms that are available in the public domain through peer-reviewed publications (McKellar and Lu, 2004; Carrasco et al., 2012; Pérez-Rodríguez and Valero, 2013; Pin et al., 2013; Tenenhaus-Aziza and Ellouze, 2015; Ding et al., 2018). From an industry perspective, the utility of these public-domain models is often somewhat limited. In many cases,

models have been developed under laboratory conditions. These models are based on specific combinations of parameters that might not be appropriate for the particular food products. Moreover, they have not always been validated, or even used, in real food systems. Despite that, such models can be useful as long as their limitations are recognized and considered in their application (Membré and Lambert, 2008).

For almost one century, the food industry assumed that thermal inactivation followed first-order kinetics during the estimation of the outcome of a thermal treatment on the survival of microorganisms. However, there is growing evidence to support that the inactivation of microbial cells does not always follow the traditional first-order kinetics, especially during a mild thermal treatment (Augustin et al., 1998; Valdramidis et al., 2006). In the present study, shoulder and tail phenomena were observed on three survival curves: *S.* Senftenberg cooked at 60 ºC and *L. monocytogenes* (either cocktail of strains and Scott A strain) cooked at 55 ºC. It has been a consensus that D values should be used with care when the isothermal survival curves are not really log-linear (Peleg, 2004). However, many published articles do not show inactivation curves, but just D values. It is not clear if the original data were indeed log-linear, so that the derived D values can have a clear meaning. Therefore, it is recommended that the 'D values', including the ones reported in literature, were critically assessed (Wang et al., 2015).

Salmonella

Table 16 shows the lethality estimated from published D-values and from the thermal inactivation tool of Combase. Van Asselt and Zwietering (2006) collected from literature a large quantity of D values (n =4066) for various pathogens and linear regression was applied to obtain mean D values together with the 95% upper prediction level. A wide range of D values was reported. Lethality estimated from published mean D values was higher than obtained in our experimental conditions, while lethality estimated from the 95% upper prediction levels was lower (Table 7). As stated by these authors, the 95% upper prediction levels of D values can be used as a conservative estimate of inactivation. Combase tool was applied to calculate lethality using non-restrictive factors (a_w=1, physiological state=1 and a pH of 7). Combase lethality values were higher than those observed in our study at 60 ºC (between 3.74 and 4.89 log CFU/g) and similar to lethalities obtained at 55 ºC, especially for *S.* Senftenberg and the S. cocktail (between 0.16 and

3.80 logCFU/g). This fact is relevant because a wrong selection of the D-value as critical limit could have significant consequences on food safety.

Table 16. Lethality of *Salmonella* spp. in samples cooked at 55 and 60 ºC for 30, 60 and 90 min according to Van Asselt and Zwietering (2006) and the thermal inactivation tool of Combase.

Temperature (º C)	Prediction model	D-value prediction	Lethality (log CFU/g)		
			Heating time (min)		
			30	60	90
60	Van Asselt and Zwietering (2006)	95%	0.61	1.23	1.84
		mean	16.15	32.30	48.46
	Combase	mean	16.69	33.39	50.08
55	Van Asselt and Zwietering (2006)	95%	0.17	0.35	0.52
		mean	4.56	9.12	13.67
	Combase	mean	0.71	1.43	2.14

Listeria monocytogenes

Table 17 shows lethality estimated from published D-values, (Van Asselt and Zwitering (2006) and the thermal inactivation tool of Combase. Lethality data is not characterised for *L. monocytogenes* in the predictor tool of Combase at 55 ºC since it covers just the range between 60 ºC and 68 ºC. As it was previously described for *Salmonella* spp., at 60 ºC lethality estimated from published mean D values and Combase was higher than the obtained in our experimental conditions (between 5.52 and 5.69 log CFU/g), while lethality estimated from the 95% upper prediction levels was lower. At 55 ºC, lethalities calculated with the published mean D-values were higher than those obtained in our study (between 0.84 and 2.60 log CFU/g), except for Lm 4031 heat treated during 30 and 60 min (5.58 log CFU/g). The 95% upper prediction values can be seen as the worst-case estimation of lethality as they were very close to the most heat-resistant inoculum (Lm cocktail). As stated previously, a decision-making process related to food safety in real conditions have to validate published lethalities.

Table 17. Lethality of *Listeria monocytogenes* in samples cooked at 55 and 60 ºC for 30, 60 and 90 min according to Van Asselt and Zwietering (2006) and the thermal inactivation tool of Combase.

Temperature (º C)	Prediction model	D-value prediction	Lethality (log CFU/g)		
			Heating time (min)		
			30	60	90
60	Van Asselt and Zwietering (2006)	95%	2.15	4.30	6.45
		mean	12.43	24.85	37.28
	Combase	mean	28.01	56.02	84.03
55	Van Asselt and Zwietering (2006)	95%	0.42	0.83	1.25
		mean	2.40	4.80	7.20
	Combase	mean	Temperature out of the model range		

4.4.2 Effect of stress and sublethal injury

Current literature related to microbial injuries accounts for different levels of stress, which progress in severity from minor to moderate, severe, extreme and, eventually, lethal. With minor stress, bacterial cells adapt completely to the changed conditions, and growth rate is not affected. Low levels of stress may cause a transient adaptation (adaptive response) accompanied by a temporary physiological change that often results in increased stress tolerance. Lethal stress, however, can cause the death of some, but not necessarily all, bacterial cells. When lethality is experienced by only a fraction of the population, accompanying gene responses and adaptive mutations may actually improve survival of the overall population. In conclusion, moderate stress may result in a continuum of injury, ranging from mild to severe, including healthy and dead cells (Wesche et al., 2009; Abee et al., 2016).

In accordance with the above mentioned, results obtained for each inoculum and process condition (heat treatment and further storage) outline three different scenarios shown in Figure 29: complete inactivation (green), viability (red) and growth/no growth status (orange). In case of complete inactivation, *Salmonella* or *Listeria* were not detected in any of the three replicates. Scenarios of viability were those with all replicates showing counts above the QL. In some cases, counts described a growth or a decreasing tendency

that could be modelled. Finally, some experimental conditions lead to a "growth, no growth" scenario with different proportions of death, viable and injured cells, probably due to different degrees of sublethal heat injury at single-cell level. Figure x show these three scenarios, including outcomes immediately after the heat treatment and during the storage period at 4 and 8 ºC. Models fitted to experimental data are indicated with capital letters.

Table 18. Behaviour scenarios in inoculated samples with *Salmonella* and *Listeria monocytogenes* immediately after heat treatments and during storage.

Heating temperature (ºC)	Microorganism	Immediately after heat treatment			During storage at 4 ºC			During storage at 8ºC		
		t30[a]	t60	t90	t30	t60	t90	t30	t60	t90
60	*S.* Enteritidis									
	S. Senftenberg		G							
	S. cocktail									
55	*S.* Enteritidis									
	S. Senftenberg		M					B	B	B
	S. cocktail		M							
60	Lm 4031									
	Lm Scott A									
	Lm cocktail									
55	Lm 4031									
	Lm Scott A		G					B	B	B
	Lm cocktail		G					B		

Complete inactivation — G Geeraerd model
Growth / no growth — M Mafart model
Viability — B Baranyi model

[a] t30, t60 and t90: *sous-vide* cooking treatments for 30, 60 and 90 min

"Growth / no growth" scenarios have in common a low, or a very low, level of contamination due to damages produced by heat treatments. Koutsoumanis and Aspridou (2017) stated that as a result of the heterogeneity in the cell division time, the growth of

single cells of small microbial populations shows high variability and can be considered as a pool of events each one having its own probability to occur. The apparent variability in population growth gradually decreases with increasing initial population size (N_0). For bacterial populations with N_0 over 100 cells, the variability is almost eliminated and the system seems to behave deterministically even though the underlying law is stochastic. The "growth / no growth" behaviour of single cells has been intensively studied for various pathogens (Carrasco et al., 2012; Coroller et al., 2012). Although a deterministic approach is possible, probability models have been widely adopted for "growth/no growth" modelling. These models deal with data which can be measured as "positive" or "negative", "detectable" or "not detectable". Unfortunately, stochastic modelling is out of the scope of the present study due to the reduced number of samples. Nevertheless, plain data exposed in this chapter can be useful in future research, and respond to the call for data to expand "growth/no growth modelling" (Carrasco et al., 2012; Stringer et al., 2012).

4.4.3 Heterogeneity of results

Biological or reproduction variability was defined as the difference between independently reproduced experiments of the same strain performed on different experimental days from new pre-cultures and newly prepared media, and strain variability was defined as the difference between strains of the same species

Performing experiments using technical replicates is a common practice that has to be clearly reported to differentiate between experimental and biological or reproduction variability (den Besten et al., 2018). Differences in heat penetration curves obtained in different experiments of our study were a relevant source of reproduction variability. Other authors reported different sources of biological or experimental variability. Mackey et al. (1994) reported biological variability in the decrease of heat-injured *L. monocytogenes* counts during incubation at 5°C, probably related to differences between experiments or between batches of prepared media. Aryani et al. (2015a) quantified the differences among experimental, biological and strain variabilities on μmax of *L. monocytogenes*. The same trend was observed for some variables like pH or a_w. In particular, strain and biological variabilities were in the same order of magnitude, and both were significantly higher than experimental variability. In another study of Aryani et al. (2015b), the differences among these three sources of variability on D values of *L.*

monocytogenes at different temperatures were quantified. In this case, strain variability was much larger than experimental and biological variabilities. Strain variability at all conditions was ten times higher than experimental variability and four times higher than biological variability.

"Growth/no growth" scenarios showed in Table 18 correspond to results presenting heterogeneity, both during heat treatment and during storage. For example, although *S.* Senftenberg inoculated in meat samples and heat treated at 60 ºC displayed tailing, one up to three replicates at 30, 60 and 90 minutes had counts below the QL. The most significant case of heterogeneity was observed in Lm 4031 inoculated samples cooked at 55 ºC. Although counts were not detected in any sample immediately after heat treatment nor in the 60% of samples throughout the cold storage period, Lm 4031 could recover and growth at over 2 log CFU/g in about 9% of the samples.

The impact of microbial variability at population level on food safety and quality has been extensively reported by using deterministic models for *Staphylococcus aureus* (Lindqvist, 2006), *Salmonella* (Oscar, 2000; Lianou and Koutsoumanis, 2011) and *Listeria* (Barbosa et al., 1994; Begot et al., 1997). Posada-Izquierdo et al. (2013) described variability in growth of *Escherichia coli* O157:H7 in fresh-cut lettuce subjected to chlorine washing and MAP and stored at different temperatures. At 8 ºC they found considerable variation between replicates in the experiments with differences of up to 2 log CFU/g between replicates. Stress-induced variability may be important in determining the reliability of predictive microbiological models.

Variability at single-cell level by using a stochastic approach has also been investigated. Influence of stress on individual lag time distributions of *L. monocytogenes* was studied by Guiller et al. (2005). It was established that the main source of the variability on the "times to turbidity" for stressed cells was the variability of individual lag times. Metris et al. (2008), also stated that lag times can vary widely between individual cells in a population, and the inherent variability in the lag time of single cells increases with severity of heat treatment. Then, knowing how heat treatments affect the variability of single-cell lag times is extremely important in assessing the risk of cell recovery and growth in processed foods, where low numbers of stressed cells of pathogenic bacteria may be distributed among different packs of food. Variability in the number of viable bacteria after mild heat treatments in relation to water activity (a_w) has been analysed by

Aguirre et al. (2009) for different bacterial species. They found that the more severe the heat treatment was, the lower the average number of surviving bacteria, but the greater the variability; at growth-limiting conditions, variability in the growth limits of individual cells has an important role to the lag phase of microbial populations. In case of inactivation kinetics, confidence intervals get broader at larger cooking times due to the presence of subpopulations of cells in the surviving population, with different distributions for the heat resistance parameters. This conclusion totally agrees with the 95% CI of parameters related to bacterial growth (Gr max of Lm Scott A cooked at 55 ºC during 90 min) or inactivation (δ ref of *S*. cocktail cooked at 55 ºC) obtained in this study.

In conclusion, when applying outcomes from models based on laboratory media and condition to foods it is thus important to validate these models carefully and take into account differences that might occur due to other composition, texture and physico-chemical characteristics of the food matrix and indigenous competing microbiota. If different bibliographical sources are compared, then substantial differences can be obtained (Zwietering, 2002; Murphy et al., 2004; Van Asselt and Zwietering, 2006; Wang et al., 2015). Furthermore, the variability between individual members of a bacterial population can render preservation treatments ineffective. Negligence on this difference will result in an underestimation of the true microbial growth behaviour (Nauta, 2007; Aguirre et al., 2009; Aryani et al., 2015a).

4.4.4 Food safety perspective

Lethalities obtained in meat samples cooked at 55 and 60 ºC showed that *L. monocytogenes* was less heat resistant than *Salmonella* spp. These results are in accordance with thermal destruction curves using the 95% prediction upper level of D values reported by Van Asselt and Zwietering (2006). However, the safety of a mild heat treatment should not be measured only by the log reduction of a concrete food pathogen, but also by the possibility of acclimation to the thermal stress, degree of injury at cell level and recovery capability of surviving microbiota (Métris et al., 2008). This fact was clearly observed during cold storage of meat samples inoculated with Lm 4031 cooked at 55 ºC. In general, the cellular structures or targets commonly affected by heat are the outer and inner membrane, the peptidoglycan cell wall, the nucleoid, the cell's RNA, the ribosomes and diverse enzymes. Heat affects diverse cellular structures and functions to a different degree, and those structures are interlinked. Moreover, the intrinsic resistance

of each particular cell target may vary depending on the type of microorganism and the environmental conditions, i.e., the composition of the treatment medium. Thus, the possibilities (and its level) of alteration of a given cell are diverse. This directly links to another important phenomenon: cellular sublethal injury and recovery. Sublethally injured cells present damages in cellular structures and functions that can only be repaired by the cellular machinery if environmental conditions are appropriate (Mackey, 2000). The practical implications of this phenomenon for the food safety are of tremendous importance (Cebrián et al., 2017). Injured cells respond to stress by entering a physiological state that requires specific reparative processes. Accordingly, damaged survivors may show a longer lag phase, compared to healthy cells, due to time needed to repair, not reaching often the detection threshold of analytical methods (Agranovski et al., 2003; Janssen et al., 2008; Noriega et al., 2013). Sublethal injuries emphasises the need for traditional and rapid methodologies to include preenrichment and repair steps, particularly when competing microbiota may preclude recovery of injured cells (Wesche et al., 2009).

Sous-vide cooking at 55 ºC of all pork loin samples inoculated with *S.* Senftenberg and Lm Scott A were able to survive to all treatments and growth at 8 ºC. Besides, a surviving fraction of the initial microbial load of *S.* Enteritidis and Lm 4031 was present after heat treatments and cold storage at 4 and 8 ºC although lethality obtained for these pathogens were 4.20 and 5.58 log CFU/g, respectively. Moreover, around 9% of samples inoculated with Lm 4031 showed growth during cold storage over 2 log CFU/g. In gastronomical and safety terms, pork loin cooked at 55 ºC during 30, 60 and 90 min would only be appropriate for direct consumption, i.e. to be served just after cooking.

On the other hand, some treatments at 60 ºC presented complete inactivation during all the storage period. However, *S.* Senftenberg, the S. cocktail and Lm cocktail were detected in some samples, presenting a growth/no growth scenario. Considering the results of all treatments at 60 ºC (30, 60 and 90 min), the probability of survival in samples stored at 4 and 8 ºC ranged from 0.13 to 0.38 for *S.* Senftenberg, from 0.03 to 0.36 for the *S.* cocktail, from 0.03 to 0.08 for *S.* Enteritidis, and less than 0.25 for the Lm cocktail. Complete inactivation was obtained in heat treatments of 90 min at 60 ºC for all samples inoculated with *L. monocytogenes* and all samples inoculated with *S.* Enteritidis. Therefore, this time/temperature combination could be considered reasonably safe and

suitable for cooking and chilling systems, provided that any termoresistant pathogen, like *S*. Senftenberg, are not present in the product prior to the *sous-vide* cooking.

Although this study is not able to provide more data to further asses the risk of cell recovery and growth of the pathogens under study, it confirms the presence of sublethally injured cells. This fact poses major public health concerns and is an essential aspect when assessing the microbial responses to food preservation strategies (Métris et al., 2008; Noriega et al., 2013). Then, a conservative perspective could suggest do not extend for more than five days the shelf-life for meat samples cooked at 55 and 60 ºC (1), in accordance with Regulation (EC) No. 2073/2005 for *L. monocytogenes* in ready-to-eat food or (2) preparing these products only for direct consumption, i.e. to be served just after cooking.

The Good Hygiene Practices Guide on Vacuum Cooking (Agència de Salut Pública de Catalunya, 2012), proposed a period of 10 days of conservation at ≤ 4 ºC for vacuum cooked products at 65 ºC during 15 minutes (or equivalent combinations at higher temperatures). Direct service (with no storage) should be required for products heat treated below this time/temperature combination. Therefore, advice rendered by the Guide with respect to shelf-life of *sous-vide* products was confirmed. However, further research carried out on this technique can provide new data that could allow the adjustment of those cold storage requirements.

Finally, we have some doubts in relation to recommendations established in the EURL Lm technical guidance document for conducting shelf-life studies on *L. monocytogenes* in ready-to-eat (RTE) foods. This guidance recommends to inoculate the product in its final packaged format. So, this challenge tests respond to reality if cross-contamination prior to packaging takes place, not in case of products heat treated in their final package. Adaptation of the strain to cold storage is mentioned, but no more indications to reproduce process stress or adaptations are given. Moreover, only deterministic growth is expected. In view of the expansion of preserving technologies consisting in a combination of mild stresses in RTE foods, it seems reasonable to expect a review of this procedure to include heterogeneity in the behaviour of the inoculum as a response to sublethal stresses or adaptation mechanisms (NACMCF, 2010). The National Advisory Committee on Microbiological Criteria for Foods mention several types of challenge studies that deal with validation of food safety processing procedures, product storage conditions and

shelf-life. Combined growth and inactivation studies may be used to evaluate the ability of a particular food or process to inactivate certain bacterial pathogens and to inhibit their growth when held under specific storage conditions.

5. Conclusions

1. Convection steam ovens do not heat food products uniformly in the range of 55-60 ºC during *sous-vide* cooking processes. The duration and the temperature rate of the heating phase and the temperature oscillations in the core of the product during the holding phase of each heat treatment are important sources of experimental variability. The Monte Carlo analysis has proven to be a useful tool to incorporate the variability found between replicates in the predictive models of thermal inactivation and growth.

2. Depending on the outcomes of the heat treatment, four scenarios were observed: (i) limited inactivation (*S.* Senftenberg cooked at 55 ºC); (ii) inactivation with the presence of a tailing phenomenon (*S.* Senftenberg at 60 ºC, *L. monocytogenes* Scott A and cocktail of *L. monocytogenes* at 55ºC); (iii) inactivation without the presence of a tailing phenomenon (cocktail of *Salmonella* spp. at 55 ºC) and (iv) severe inactivation, if low levels of contamination remained (*S.* Enteritidis, cocktail of *Salmonella* spp. and individual or combined strains of *L. monocytogenes* at 60 ºC, *S.* Enteritidis and *L. monocytogenes* CECT 4031 at 55 ºC). Non-lineal models could be fitted to survivor curves of scenarios (i), (ii) and (iii).

3. Depending on the results obtained during the storage period, three different scenarios were observed: (i) total inactivation (*S.* Enteritidis and *L. monocytogenes Scott A* and CECT 4031, in samples cooked at 60 ºC); (ii) presence of viable cells, with all replicates showing counts above the quantification limit, whether growth was observed or not (*S.* Senftenberg, *L. monocytogenes* Scott A, cocktail of *L. monocytogenes* strains cooked at 55 ºC, and cocktail of *Salmonella* spp. strains cooked at 55ºC during 30 min), and (iii) growth/no growth interface, with different proportions of death, viable and injured cells, probably due to different degrees of sublethal heat and cold injuries at a single-cell level. This situation took place in inoculated samples cooked at 60 ºC (S. Senftenberg, cocktail of *L. monocytogenes*) and 55 ºC (cocktail of *Salmonella* spp. and *L. monocytogenes* CECT 4031).

4. Experimental conditions with limited inactivation or with a tailing effect in the survivor curve after heat treatments at 55 ºC showed a growth trend during the storage period at 8 ºC. A primary growth model was fitted to experimental data. During storage at 4 ºC cold stress was added to the sublethal injuries caused by the heat treatments and interfered in the repair and growth mechanisms of survivor cells, even in samples inoculated with the psychrotrophic strains.

5. Heat treatments at 60 ºC for 90 min applied to pork loin could be considered reasonably safe and suitable for "cook-chill" systems in relation to *Salmonella* spp. and *L. monocytogenes* strains included in the essay, as long as no temperature abuse occurs during shelf-life. Treatments at 60 ºC during 30 and 60 minutes must be used only for "cook-serve" systems

6. *Sous-vide* cooking of pork loin at 55 ºC during 30, 60 and 90 minutes cannot be considered safe in relation to *S.* Senftenberg and *L. monocytogenes* Scott A due to the presence of survivor cells that can growth at 8 ºC. This treatment must be used only for "cook-serve" systems.

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