Impact of Microbial Variability on Food Safety and Quality

Diah Chandra Aryani

Impact of Microbial Variability on Food Safety and Quality

Diah Chandra Aryani

INVITATION

You are cordially invited to attend the public defence of my PhD thesis entitled

Impact of Microbial Variability on Food Safety and Quality

The defence will take place on

Monday 6th June 2016 at 4 p.m.

in the Aula of
Wageningen University
Generaal Foulkesweg 1a 6703BG
Wageningen

After the defence ceremony you are also invited to join the reception at the same location

Diah Chandra Aryani
diah.chandraaryani@gmail.com

Paranymphs

Ioanna Stratakou
(ioanna.stratakou@wur.nl)

Irma van Rijswick
(irma.vanrijswick@wur.nl)

IMPACT OF MICROBIAL VARIABILITY ON FOOD SAFETY AND QUALITY

Diah Chandra Aryani

Thesis committee

Promotor
Prof. Dr M.H. Zwietering
Professor of Food Microbiology
Wageningen University

Co-promotor
Dr H.M.W. den Besten
Assistant professor, Laboratory of Food Microbiology
Wageningen University

Other members
Prof. Dr M.A.J.S. van Boekel, Wageningen University
Dr J.M. Membré, INRA, Nantes, France
Dr M.H.J. Wells-Bennik, NIZO Food Research, Ede
Prof. Dr F. Devlieghere, Ghent University, Belgium

This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences)

IMPACT OF MICROBIAL VARIABILITY ON FOOD SAFETY AND QUALITY

Diah Chandra Aryani

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 6 June 2016
at 4 p.m. in the Aula.

Diah Chandra Aryani
Impact of Microbial Variability on Food Safety and Quality,
198 pages.

PhD thesis, Wageningen University, Wageningen, NL (2016)
With references, with summary in English

ISBN 978-94-6257-738-1

TABLE OF CONTENTS

ABSTRACT

Microbial growth and inactivation kinetics in food can be predicted when the effects of intrinsic food properties and environmental conditions on microbial responses are known. However, the prediction result might not be accurate due to microbial variability. To ensure food safety and quality, knowledge on the sources of variability and the magnitude is needed to prioritize their importance. This thesis focused on various microbial variability aspects including between and within strain variabilities, the effect of growth history and physiological state of the cells, and the effect of food matrix on growth and thermal inactivation kinetics. *Listeria monocytogenes* and *Lactobacillus plantarum* were selected as model organisms to represent pathogenic and spoilage microorganisms. The result of this project underlines that many variability factors are important, but some are more important than others. Depending on the process characteristics, microbiological variability, especially strain variability, and in particular for thermal inactivation, will be the most determining factor affecting the final contamination level. This strain variability, however, is inherent to living organisms. Strain variability challenges food processors because strain variability cannot be well controlled unless complete inactivation is realized and no recontamination occurs within the food production chain. The integration of strain variability in prediction of microbial kinetics is, therefore, required in quantitative microbiology to obtain a more realistic prediction; and the most robust strains can be used in parallel or in cocktails to evaluate the efficacy of certain steps along the food production chain in controlling the growth.

CHAPTER 1

Introduction and outline of the thesis

INTRODUCTION

The world's population is estimated to reach 9 billion by 2050 (FAO, 2009; UN, 2015), which will also be accompanied by an increase in global food demand. Doubling the production by closing the yield gap, increasing the production limits, extension of arable land and aquaculture, reducing waste and changing the diets were some of the proposals given to meet the demand (Godfray et al., 2010). Implementing the proposals, amid the issue of climate change (Lobell et al., 2008; Singh, 2012), land degradation (Zdruli, 2014), depletion of natural resources (Childers et al., 2011; Neset and Cordell, 2012) and possible agricultural land conversion to biofuel production, would be a challenging task. However, failure to meet the demand will have a consequence on global food security. The world food summit of 1996 defined food security as the condition when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food which meets their dietary needs and food preferences for an active and healthy life. The keyword of an active and healthy life can be realized not only if food is available, easy to access and nutritious, but it should also be safe for consumption, which is shown in the interrelation of food safety and food security in figure 1.1. Food safety is a basis for a healthy life. Therefore, ensuring food safety will contribute to the achievement of food security, and meeting food security means that food safety measures are also implemented and controlled along food production chains.

Food safety becomes a serious concern in the growing world population due to internal and international migration, which increase the international trade of food products (Unnevehr and Roberts, 2002). The German outbreak of *Escherichia coli* O104:H4 in sprouts (Buchholz et al., 2011) is a good example on how the globalization of food supply had impact not only on food safety, but also on livelihood, economy and sustainable food supply (Luber and Hoorfar, 2014; Uyttendaele et al., 2014). The outbreak started when the German human health authorities detected several human EHEC cases caused by an infection with the Shiga-toxin-producing *E. coli* of the serotype O104:H4 (Luber and Hoorfar, 2014). The suspected vehicles were initially declared as cucumber, tomato and lettuce, but the outbreak was later found to be related to the sprouts of fenugreek seeds imported from Egypt when similar cases also occurred in France. In the first 2 weeks of the outbreak, the losses of the fruit and vegetable farmers were estimated at least 812 Million Euro. Also, a temporary export ban of vegetables to Russia occurred, constituting an annual value of 600 Million Euro. Furthermore, the European Commission also supported fruit and vegetable sectors on market intervention for a total value of 227 Million Euro, concerning the products most directly affected by the crisis, which were tomatoes, cucumbers, lettuces and certain endives, courgettes and sweet peppers

(DGSanco, 2012). The total number of reported cases of 3816 (including 54 deaths and 845 developed haemolytic–uremic syndrome) (Frank et al., 2011), massive market losses, authority intervention and the losses of consumer trust in fruit and vegetable products, underline the wide impact of certain food safety outbreaks and the importance of food safety measures on the global food supply.

Apart from these food safety crises, about one third of agricultural production is lost or wasted annually. The losses are caused among others by poor post-harvest technologies and facilities, which cause spoilage and damage of food products, food safety issues, inadequate market systems, and the appearance of high quality standards (FAO, 2011). To reduce the loss caused by food safety and quality issues, the implementation of good agricultural practices (GAP) and Good Manufacturing Practices (GMP), followed by the adoption of Good Hygienic Practices (GHP) and the compliance with quality standards are important and should be implemented along food production chain. Well implementation of those measures would be beneficial not only for food safety, but also food wastage reduction and the sustainable supply of food products.

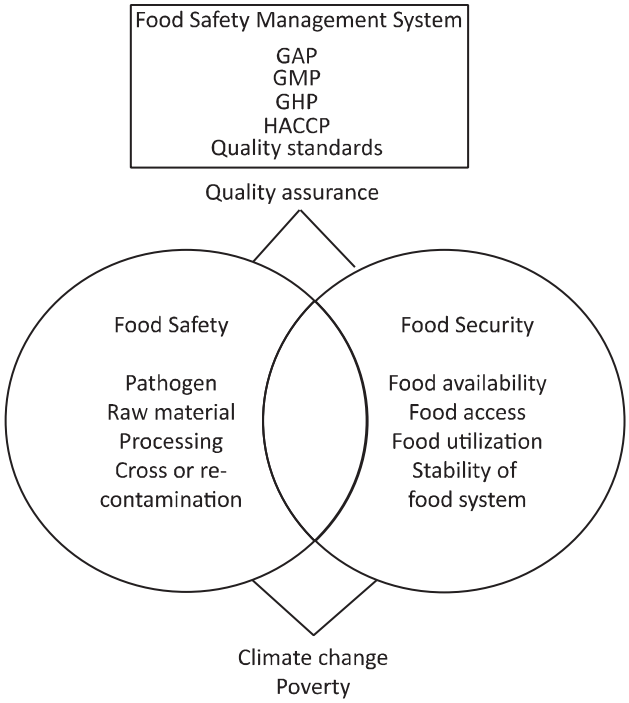

Figure 1.1. The interrelation between food security and food safety, adapted from Hanning et al. (2012).

FOOD SAFETY

"Food safety is an umbrella term that encompasses many facets of handling, preparation and storage of food to prevent illness and injury" (Hanning et al., 2012). Thus, food containing hazardous compounds, which might cause illness and injury, is considered unsafe and is not fit for consumption. A hazard according to the Codex Alimentarius Commission is a biological, chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect (CAC, 2003). These hazardous agents are including foreign objects such as broken glass, insects and other physical contaminants; pathogens; mycotoxins; pesticide residues; veterinary drugs; food additives; or genetically modified organisms which might contain toxins or allergens (Kuiper et al., 2001). Unlike the physical hazards, in which the relationship between source and the problem is straight forward, finding the correlation of chemical hazard and the disease is more complex. It is not only because of "the dose makes the poison", but also the disease manifestations, such as cancer might take years to develop. Although illnesses due to biological hazards (including pathogens, viruses, parasites etc.) appear quicker than that caused by chemical hazards and the link between the illness and the causative agents are more often found, effective control measures are difficult to put into practice due to biological evolution, strain variability or differences in susceptible groups among the population.

Ensuring food safety is not a one-time effort, but rather a long term activity involving continuous control and monitoring processes since food safety outbreaks and scandals occur on regular basis, including the incident of melamine in powdered infant formula in China, meat adulteration in the UK, the German outbreak of *Norovirus* in frozen strawberries imported from China in 2012 (Bernard et al., 2014), the 2012 multi states listeriosis outbreak associated with cantaloupe in the US (McCollum et al., 2013) and the listeriosis outbreak in caramel apple and Blue Bell creameries products in the US (CDC, 2015a; CDC, 2015b). To minimize the risk of a foodborne disease, a thorough knowledge on the process involving growth and survival as well as routes of contamination and the severity of the disease are of importance and are used as a basis in microbiological risk assessments.

Quantitative Microbial Risk Assessment (QMRA) is a scientifically based process, which is used as a tool to evaluate food production processes in relation to food safety and public health (Nauta, 2000). QMRA has four stages: hazard identification, hazard characterisation, exposure assessment and risk characterisation (CAC, 1999). In hazard identification, all possible microbiological hazards, which might be relevant for certain food product, are identified. Hazard identification might be conducted using three levels of identification: rough hazard identification, detailed hazard identification and

comprehensive hazard identification (Van Gerwen et al., 1997). The rough hazard identification identifies food pathogens that were involved in previously reported foodborne outbreaks in the selected product. The detailed hazard identification identifies food pathogens that have been reported or commonly associated with the ingredients of the selected product, while the comprehensive hazard identification identifies all food pathogens as hazardous, and therefore pathogens which might not be associated with the selected food product can also be included in the list (Van Gerwen et al., 1997). The combination of literature review, expert knowledge and the knowledge on the characteristics of food pathogens and food products are needed to select the most relevant hazards for specific food products.

In hazard characterisation, dose-response relationships and manifestation of disease are established. The dose-response will relate the amount of food pathogens or toxins to a probability of an adverse health effect. Exposure assessment describes all possible routes of contamination and exposure. The information on the nature of the food product and processing, which might eliminate or support the growth of microorganisms, as well as the possible re-contamination are also needed to estimate the level of the hazard present in food product at the point of consumption. Finally, the risk characterization needs all information from hazard characterization and exposure assessments, including the amount of food intake to estimate the probability of illnesses and the severity of the diseases. Up to date, several microbiological risk assessment documents, such as the ones for *Salmonella* in eggs and broiler chickens (FAO-WHO, 2002), *Listeria monocytogenes* in ready-to-eat foods (FAO-WHO, 2004) or viruses in foods (FAO-WHO, 2008), were produced and might be used as a basis to propose the measures for minimizing or preventing food contamination or foodborne illness for risk management.

PATHOGEN AND SPOILAGE MICROORGANISMS

Food products contaminated with pathogens often do not show any changes due to the low numbers sometimes involved, which make it difficult to observe unless people get ill after consumption. There are two types of foodborne diseases caused by pathogenic bacteria: intoxication and infection. In the intoxication case, bacteria, such as *Staphylococcus aureus*, *Bacillus cereus* or *Clostridium botulinum*, grow to a certain number, which allows them to produce relevant amounts of toxin (Argudín et al., 2010; Schelin et al., 2011; Taylor et al., 2005; Thompson and Tanner, 1925). Upon ingestion, the toxin can cause mild to severe health effects. In the infection case, vegetative cells or spores which survives the gastrointestinal tract might germinate (in case of spores), multiply and colonise the intestinal cells, and later produce toxin or invade other cells

causing illnesses ranging from nausea, (bloody) diarrhea, meningitis to haemolytic uremic syndrome or eventually in severe cases can lead to death. Groups of bacteria such as *Listeria monocytogenes* (Farber and Peterkin, 1991; Hamon et al., 2006), *Salmonella* spp. (D'Aoust, 1991). *Campylobacter* spp. (Dasti et al., 2010; Van Vliet and Ketley, 2001), *Vibrio* (Jones and Oliver, 2009; Su and Liu, 2007) and *E. coli* (Caprioli et al., 2014; Kaper et al., 2004), are example microorganisms able to cause infection in human through the consumption of contaminated food products.

Globally, 31 foodborne hazards, including bacteria, helminths, protozoa, virus and chemicals, were estimated to cause 600 (95% uncertainty interval [UI] 420–960) million foodborne illnesses and 420,000 (95% UI 310,000–600,000) deaths in 2010 (WHO, 2015). The agents responsible for most frequent foodborne illness were *Norovirus* and *Campylobacter* spp., while the agents caused 230,000 deaths (95% UI 160,000-320,000) were particularly non-typhoidal *Salmonella enterica*. The global burden of foodborne disease by those 31 foodborne hazards in 2010 was 33 (95% UI 25–46) million DALYs (Disability Adjusted Life Years), in which 40% of the foodborne disease burden was among children under 5 years old. Moreover, the highest disease burden was reported for the African region followed by South East Asia and the Eastern Mediterranean (WHO, 2015).

In the US, the center for disease control and prevention estimated that annually around 48 million people get ill from 31 known pathogens and unspecified agents, in which 128 thousands are hospitalised and 3 thousand die due to foodborne diseases (CDC, 2011). Similarly to the global report, *Norovirus* was listed as the most reported agent causing foodborne diseases in the US, while *Salmonella* (nonthypoidal) was the most reported pathogen to cause hospitalization and death (CDC, 2011; Scallan et al., 2011) (table 1.1).

Table 1.1. Top five pathogen contributing to foodborne illnesses, hospitalization and death in the US (CDC, 2011)

No.	Top five agents caused food borne disease	Top five agents caused hospitalisation due to foodborne diseases	Top five agents caused death due to foodborne diseases
1	*Norovirus*	*Salmonella* (nontyphoidal)	*Salmonella* (nontyphoidal)
2	*Salmonella* (nontyphoidal)	*Norovirus*	*Toxoplasma gondii*
3	*Clostridium perfringens*	*Campylobacter* spp.	*Listeria monocytogenes*
4	*Campylobacter* spp.	*Toxoplasma gondii*	*Norovirus*
5	*Staphylococcus aureus*	*E. coli* (STEC) O157	*Campylobacter* spp.

In Canada, about 4 million episodes of foodborne illness were estimated annually due to 30 known pathogens and unspecified agents, causing over 11,500 hospitalizations and 238 deaths. From 30 known pathogens, *Norovirus*, nontyphoidal *Salmonella* spp., *Campylobacter* spp., VTEC O157 and *L. monocytogenes* caused the highest number of hospitalizations, while *Norovirus*, nontyphoidal *Salmonella* spp., C*ampylobacter* spp., *C. perfringens* and *Bacillus cereus* were the cause of the highest number of illness (Thomas et al., 2015). In the European region, the most common pathogens reported for causing foodborne illnesses were *Salmonella* and *Campylobacter* (O'Brien and Motarjemi, 2014). Similar to this report, EFSA ranked *Campylobacter* as the most commonly reported causal of gastrointestinal diseases in 2014 by 236,851 confirmed campylobacteriosis cases, followed by salmonellosis with 88,715 cases, yersiniosis with 6,471 cases, VTEC infections with 5,955 cases and listeriosis 2,161 cases. Although the number of listeriosis cases was lower, the fatality rate of 15% was the highest compared to the other four foodborne diseases (EFSA and ECDC, 2015). The fatality rate of 17.8% was also reported in the age group over 65 years old. In the Netherlands, among 1.8 million cases of disease and 233 deaths caused by fourteen pathogens, approximately 680,000 cases and 78 deaths are attributable to foodborne transmission (Havelaar et al., 2012). From the total disease burden of 13,500 DALYs, approximately 45% is attributed to food, in which *Toxoplasma gondii*, *Campylobacter* spp., *Salmonella* spp. and *S. aureus* toxins were responsible for the majority of the burden.

Unlike food pathogens, spoilage organisms normally do not cause illness, but contamination of food with spoilage microorganisms affects quality, which eventually causes food loss due to deterioration of food products. In general, almost all groups of microorganisms can contribute to spoilage of foods. However, their ability to grow and to cause spoilage depend on nutrient composition and the chemical and physical parameters of food products (Gram et al., 2002), such as pH and water activity. Some lactic acid bacteria are found in different niches, and are able to cause spoilage in a diverse range of food products, such as vegetables and fermented products (Jespersen and Jakobsen, 1996; Tournas, 2005), meat (Borch et al., 1996; Hamasaki et al., 2003; Pothakos et al., 2015) and fisheries products (Dalgaard et al., 2003; Gram and Huss, 1996). Besides lactic acid bacteria, *Pseudomonas* spp. (Borch et al., 1996; Gram and Huss, 1996; Techer et al., 2014), *Enterobacteriaceae* (Borch et al., 1996; Gram and Huss, 1996; Techer et al., 2014), *Bacillus* spp. (Techer et al., 2014), moulds (Filtenborg et al., 1996; Tournas, 2005) and yeasts (Jakobsen and Narvhus, 1996) are also found as important agents causing spoilage in many food products.

The growth of pathogen and spoilage microorganisms in food if not controlled will have consequences on the safety and quality of food products, leading to food waste or

foodborne outbreaks, which threaten the sustainability of the food supply. Controlling the growth of pathogen and spoilage microorganisms can be done by modifying the intrinsic and extrinsic parameters of food products, such as pH, water activity, organic acid concentration and temperature. Certain growth limiting factors can be applied, which prevent the growth of pathogen and spoilage organisms, and therefore extending the shelf life of food products. Predictive modelling might be used as a tool to evaluate the effectiveness of certain processes and designs in controlling the growth of microorganisms. However, due to many variability factors the prediction result might not be realistic, which might underestimate the real level of the microorganisms. Therefore, any attempts, such as characterisation of variability factors, to improve the prediction results would contribute to the safety and quality of food products for sustainable food supply and food security.

PREDICTIVE MICROBIOLOGY

The concept of predictive microbiology, according to McMeekin et al. (2002), is that detailed knowledge of microbial responses to environmental conditions, including intrinsic food properties, enables objective evaluation of the effect of processing, distribution and storage operations on the microbiological safety and quality of foods. Therefore, predictive modelling is applicable for many activities along food production chains within the area of HACCP, risk assessment, microbial shelf life study, product research and development and experimental designs (McMeekin et al., 2007). Predictive modelling in microbiology was believed to be originated from the work on the thermal death curve of thermophilic bacterial spores (Bigelow and Esty, 1920; Bigelow, 1921; Esty and Meyer, 1922). But, the term “predictive modelling in microbiology” was introduced later by Roberts and Jarvis (1983).

The models used in predictive microbiology can be classified into three types: primary models, secondary models and tertiary models (Buchanan, 1993; Pérez-Rodríguez, 2014). Primary models are the models used for describing the changes in microbial concentration as function of time. The well-known primary models used in growth prediction are among others the logistic model (Gibson et al., 1987), modified Gompertz model (Gibson et al., 1987) or its re-parameterized form (Zwietering et al., 1990) and the model developed by Baranyi and Roberts (1994). The Bigelow model for inactivation was developed assuming that the log reduction of microbial load is linear with time (Bigelow and Esty, 1920; Bigelow, 1921). However, in reality many workers observed a non-linear relationship between time and log surviving cells. To accommodate the curvature of the inactivation curve, the Weibull model (Van Boekel, 2008), which is able to fit linear, concave

downward and concave upward inactivation curves might be used. Other approaches are the use of models such as the biphasic model and models accommodating shoulder and tail, etc. as described in a study of Geeraerd et al. (2005).

Secondary models are developed to explain the relationship between the kinetic parameters of the primary model, such as μ_{max} and *D*-value and environmental factors, such as temperature, pH or a_w. The secondary growth models commonly used for modelling purposes are the square root model (Ratkowsky et al., 1982), cardinal parameter model (Rosso et al., 1995), gamma model (Zwietering et al., 1993) and other secondary growth models as function of other environmental factors such as organic acid concentration. Other types of models such as probability models are also used when the minimum growth parameter is of concern due to the high virulence of a certain pathogen. The linear regression between log *D*-value and temperature to obtain the *z*-value is an example of the secondary model for inactivation. Finally, tertiary models are the implementation of both primary and secondary models implemented in certain user-friendly software. The widely used softwares amongst others are the Pathogen Modelling Program (PMP), Combase predictor or the Seafood Spoilage Safety Predictor (SSSP).

The vast number of primary and secondary models provided us with another challenge in selecting which model suits best for our purpose. The root mean squared error (RMSE) between observed and fitted data and the lack-of-fit values are commonly used to compare the goodness of fit of several models to the obtained data. Besides these statistical parameters, the number of model parameters and the number of parameters which have a biological meaning are among the criteria evaluated for selecting a best model. Following the principle of parsimony, if two models have similar goodness of fit, a model with fewer number of parameters is preferred. Similarly, the model having biologically relevant parameters would be preferred over another model containing mathematical parameters without biological meaning, when both models similarly fit to the data.

Once a predictive model is selected and set of parameters, such as cardinal growth parameter, maximum specific growth rate (μ_{max}) or *D*-value, are available, one might use them to predict the growth of microorganisms in certain conditions, and to estimate the reduction in log number of microorganisms when a certain inactivation process is applied. These predictions are commonly used in exposure assessment of QMRA or in estimating the shelf life of certain food product. However, as mentioned above, the prediction result might not be realistic due to variability factors. These variability factors among others are variation in initial contamination, variation of cells biology (between and within strains of the same species), variation in thermal process, food product characteristic and distribution or storage temperatures. These variability factors have influence on the

microbial kinetics along the food productions chain (figure 1.2), which might underestimate the true behaviour of the microorganisms. Finally, variability in the consumers' susceptibility toward certain foodborne pathogens would influence the dose response relationship, which might complicate risk characterization. Since using one point parameter estimate in predictive model might underestimate the behaviour of microorganisms, the effort to integrate variability into predictive models has been made for QMRA (Delignette-Muller and Rosso, 2000; Nauta, 2000). Furthermore, to have a more realistic kinetic prediction, knowledge on the source of the variability and its magnitude are, therefore, needed to prioritize their importance.

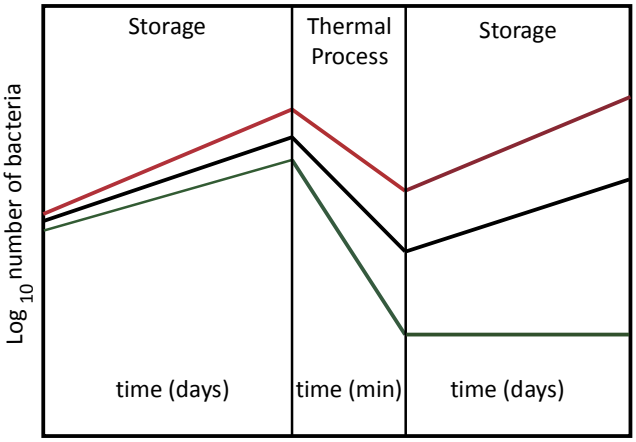

Figure 1.2. Illustration of the effect of variation in initial level, growth and thermal inactivation kinetics.

VARIATION: VARIABILITY AND UNCERTAINTY

The term variation can be divided into uncertainty and variability. Uncertainty represents the lack of perfect knowledge on the part of the analysis, which may be reduced by further measurement (Anderson and Hattis, 1999). Variability represents variability or heterogeneity in a population that is irreducible by additional measurements. It can be better characterised by additional measurements, however. In predictive microbiology, uncertainty might be related the technical and methodological uncertainties such as preciseness of measurements, lack of knowledge between interaction of the model parameters or lack of knowledge on the microbial strain present in the food products, while variability is the manifestation of biological heterogeneity such as genetic and

phenotypic differences between strains, or heterogeneity within a population due to the presence of sensitive and resistant sub-populations, which cannot be eliminated or reduced by additional measurements.

The level of variation might be reported using the standard deviation or coefficient of variation. The standard deviation quantifies the scatter of the data, and it has a similar unit as the unit of the data. The coefficient of variation also known as the relative variability equals to the standard deviation divided by the mean of the data, and can be expressed as fraction or percentage. Since it is a unitless measurement, the coefficient of variation can be used to compare the scatter of the data that have different units, such as rate (1/h) and time (h). Although both standard deviation and coefficient of variation are commonly used to express variability, the interpretation of the variability might be different from each other.

Quantification of variability factors related to food pathogens and spoilage microorganisms might provide knowledge, which improves prediction results, and is relevant for food safety and quality assurance. One of the aspects influencing the prediction is the growth and inactivation kinetics data used to generate the prediction. The data, which normally is generated from laboratory experiments, might vary as influenced by experimental variability, biological variability and strain variability. The experimental error can be obtained when two or more measurements of a parameter are performed using the same conditions and pre-culture. The difference between these measurements is considered as uncertainty due to measurement error. This type of error can be controlled and improved by performing good laboratory practices, and therefore we expect a low value of experimental error. The reproduction variation is obtained by taking duplicate or more measurements using independent cultures of a similar strain. Knowing that one can never obtain a similar culture due to biological variability and other varying conditions, one might expect larger reproduction variability than the experimental variability. The rational between the experimental and reproduction variability was pointed out in a study of Baranyi et al. (2014) on mould data. The environmental effect in the experimental error determination is minimized when cells are grown from the same batch/culture and the effect is mainly due to the experimental method used to generate the data, while the difference between repeats/reproduction is primarily the result of the environmental variability. Moreover, the strain variability is obtained by taking measurement using different strains from the same species or more widely known as between strain variability. Although belonging to one species, each strain might show different genotypic and phenotypic characteristics (De Jesús and Whiting, 2003; Donaldson et al., 2009; Nelson et al., 2004; Sanders et al., 2015; Siezen et al., 2010; Siezen and van Hylckama Vlieg, 2011; Whiting and Golden, 2002). Since one species consists of a

wide range of strains, one might expect that strain variability is higher than reproduction variability and experimental error.

VARIABILITY IN GROWTH AND THERMAL INACTIVATION KINETICS

Microbial growth and thermal inactivation parameters are an important aspect needed for predicting the growth and thermal inactivation of certain microorganisms in exposure assessment of QMRA or in estimation of shelf life of certain food products. However, these parameters, such as the maximum specific growth rate (μ_{max}) (Barbosa et al., 1994; Begot et al., 1997; Lianou and Koutsoumanis, 2011), lag phase (Aguirre et al., 2013), growth limit (Tienungoon et al., 2000; Van der Veen et al., 2008), *D*-value (De Jesús and Whiting, 2003; Doyle et al., 2001; Linton et al., 1990; Lou and Yousef, 1996), and *z*-values (Doyle et al., 2001; Van Asselt and Zwietering, 2006), vary depending on several factors amongst others within and between strain, cells history, physiological state of the cells and food matrices. Therefore, the prediction results might vary from one study to the others due to differences in the parameters used to generate the prediction.
Under similar condition, strain variability in μ_{max} was reported higher than within strain variability (Lianou and Koutsoumanis, 2011) and it was also higher than the experimental error in single and cocktail strains experiment (Whiting and Golden, 2002). The strain variability in lag phase duration, however, was reported higher than the variability in μ_{max} (Begot et al., 1997). Although lag phase duration is important for the prediction, yet it is laborious and difficult to obtain. Furthermore, lag phase is difficult to generically define and determine under conditions representative for practical contamination scenarios, and therefore, it is not included in this study. Besides strain variability, growth conditions, such as intrinsic and extrinsic parameters of the media, including pH, water activity, temperature, organic acid concentration and type of solute used to lower water activity, were also reported to affect the variability in microbial growth limit (Augustin and Carlier, 2000; Van der Veen et al., 2008). Similar to growth kinetics, the influence of strain variability was also reported for thermal inactivation kinetics of vegetative cells (Doyle et al., 2001; Lianou and Koutsoumanis, 2013; Mackey et al., 1990; Sörqvist, 1994) and spores (Berendsen et al., 2015; Luu-Thi et al., 2014). The effect of each reported influential factor on *D*-values was analysed in a previous study with a systematic approach to determine global thermal inactivation parameters for various food pathogens (Van Asselt and Zwietering, 2006). However, most factors reported to influence *D*-value are smaller than the variability of all published data, except in the presence of high salt concentration for *L. monocytogenes*, chocolate for *Salmonella*, the effect of strain variability and oily product for *Bacillus cereus* and the effect of different type of *Clostridium botulinum*. The analysis

was done using *D*-values collected from a diverse range of media and experimental set-up, and so the effect of each factor cannot be directly compared. Therefore, a study using the same set of strains under different conditions and media is needed to be able to quantitatively compare the effect of each variability factor on total variability of microbial kinetics.

OBJECTIVES AND OUTLINE OF THE THESIS

Since a realistic prediction is needed to improve the process and experimental designs, knowing the main sources of these variabilities and their magnitude are of importance. Two different microorganisms were used in the thesis for studying the variability factors in growth and thermal inactivation kinetics. *Listeria monocytogenes* is known as one of the important causative agents of foodborne disease. It has the highest fatality rate among foodborne pathogens, and is able to grow at relatively severe condition such as low temperature and high salt concentration. These characteristics make *L. monocytogenes* a good candidate to represent pathogenic bacteria. For spoilage bacteria, *Lactobacillus plantarum* was selected as a model since this member of the lactic acid bacteria group is often found as contaminant in different food products, such as ketchup, dressings and meat products.

The quantification of variability factors addressed in **Chapter 2** focuses on the growth kinetics of *L. monocytogenes*. The maximum specific growth rate (μ_{max}) was estimated as function of four different variables of pH, water activity (a_w), undissociated lactic acid concentration (HLa) and temperature. These μ_{max} values were used to quantify the experimental, reproduction and strain variabilities for each variable. Moreover, the secondary growth models were fitted to μ_{max} of each strain to estimate the cardinal growth parameters. These parameters represent strain variability and were used to predict the growth of *L. monocytogenes* in defined food products.

The variability in thermal inactivation kinetics of *L. monocytogenes* is discussed in **Chapter 3**. The experimental, reproduction and strain variabilities were calculated using *D*-values obtained at different temperature points. The effect of growth history on the variability of *D*-values was also quantified and compared to the effect of strain. The benchmarking of the experimental data to literature data provided information on the proportion of the variability that could be explained by these variability factors.

Chapter 4 describes the effect of variability factors on growth and thermal inactivation of a spoilage microorganism *L. plantarum*. The data obtained from growth and thermal inactivation experiments allowed us to calculate the experimental, biological and strain variabilities and obtain the growth and thermal inactivation parameters, such as cardinal

growth parameter of pH_{min}, $a_{w,min}$, T_{min}, $[HLa_{max}]$, *D*- and *z-values*. The effect of strain variability was also compared to the effect of growth history on the *D*-value variability, and both were benchmarked to the variability found in literature data.
Besides strain variability and the effect of growth history, the food matrix is also known to have an effect on the level and variability in growth and thermal inactivation kinetics. **Chapter 5** compares the predicted and observed growth kinetics in laboratory media, milk and ham for *L. monocytogenes* and *L. plantarum*. The effect of food matrix on growth kinetics, expressed as γ factor for milk and ham, was proposed. The effect of food matrix on thermal inactivation was also described and compared to the effect of strain variability. Finally in **Chapter 6** the findings of this study are discussed and perspectives for future research are provided.

REFERENCES

Aguirre, J.S., González, A., Özçelik, N., Rodríguez, M.R., García de Fernando, G.D. 2013. Modeling the *Listeria innocua* micropopulation lag phase and its variability. International Journal of Food Microbiology 164, 60-69.

Anderson, E.L., Hattis, D. 1999. Uncertainty and variability. Risk Analysis 19, 47-49.

Argudín, M.Á., Mendoza, M.C., Rodicio, M.R. 2010. Food poisoning and *Staphylococcus aureus* enterotoxins. Toxins 2, 1751-1773.

Augustin, J.-C., Carlier, V. 2000. Mathematical modelling of the growth rate and lag time for *Listeria monocytogenes*. International Journal of Food Microbiology 56, 29-51.

Baranyi, J., Roberts, T.A. 1994. A dynamic approach to predicting bacterial growth in food. International Journal of Food Microbiology 23, 277-294.

Baranyi, J., Csernus, O., Beczner, J. 2014. Error analysis in predictive modelling demonstrated on mould data. International Journal of Food Microbiology 170, 78-82.

Barbosa, W.B., Cabedo, L., Wederquist, H.J., Sofos, J.N., Schmidt, G.R. 1994. Growth variation among species and strains of *Listeria* in culture broth. Journal of Food Protection 57, 765-769.

Begot, C., Lebert, I., Lebert, A. 1997. Variability of the response of 66 *Listeria monocytogenes* and *Listeria innocua* strains to different growth conditions. Food Microbiology 14, 403-412.

Berendsen, E.M., Zwietering, M.H., Kuipers, O.P., Wells-Bennik, M.H.J. 2015. Two distinct groups within the *Bacillus subtilis* group display significantly different spore heat resistance properties. Food Microbiology 45, Part A, 18-25.

Bernard, H., Faber, M., Wilking, H., Haller, S., Höhle, M., Schielke, A., Ducomble, T., Siffczyk, C., Merbecks, S.S., Fricke, G., Hamouda, O., Stark, K., Werber, D. 2014. Large

multistate outbreak of *Norovirus* gastroenteritis associated with frozen strawberries, Germany, 2012. Euro Surveillance 19, 13-21.

Bigelow, W.D., Esty, J.R. 1920. The thermal death point in relation to time of typical thermophilic organisms. The Journal of Infectious Diseases 27, 602-617.

Bigelow, W.D. 1921. The logarithmic nature of thermal death time curves. The Journal of Infectious Diseases 29, 528-536.

Borch, E., Kant-Muermans, M.-L., Blixt, Y. 1996. Bacterial spoilage of meat and cured meat products. International Journal of Food Microbiology 33, 103-120.

Buchanan, R.L. 1993. Predictive food microbiology. Trends in Food Science & Technology 4, 6-11.

Buchholz, U., Bernard, H., Werber, D., Böhmer, M.M., Remschmidt, C., Wilking, H., Deleré, Y., an der Heiden, M., Adlhoch, C., Dreesman, J., Ehlers, J., Ethelberg, S., Faber, M., Frank, C., Fricke, G., Greiner, M., Höhle, M., Ivarsson, S., Jark, U., Kirchner, M., Koch, J., Krause, G., Luber, P., Rosner, B., Stark, K., Kühne, M. 2011. German outbreak of *Escherichia coli* O104:H4 associated with sprouts. New England Journal of Medicine 365, 1763-1770.

CAC. 1999. Principles and guidelines for the conduct of microbiological risk assessment. Document CAC/GL-30 [Online]. Available at http://www.fao.org/docs/eims/upload/215254/CAC_GL30.pdf (Accessed: 16 November 2015).

CAC. 2003. General principles of food hygiene [Online]. Available at http://www.codexalimentarius.org/standards/list-standards/en/?no_cache=1?provide=standards&orderField=title&sort=asc&num1= (Accessed: 26 October 2015).

Caprioli, A., Morabito, S., Scavia, G., Motarjemi, Y. 2014. Bacteria: shiga toxin-producing *Escherichia coli* and other pathogenic *Escherichia coli*, Encyclopedia of Food Safety. Academic Press, Waltham. 417-423.

CDC. 2011. Estimate of foodborne illness in the United States [Online]. Available at http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html (Accessed: 28 October 2015).

CDC. 2015a. Multistate outbreak of listeriosis linked to Blue Bell creameries products (Final Update) [Online]. Available at (Accessed: 11 December 2015).

CDC. 2015b. Multistate outbreak of listeriosis linked to commercially produced, prepackaged caramel apples made from bidart bros apples (final update) [Online]. Available at http://www.cdc.gov/listeria/outbreaks/caramel-apples-12-14/index.html (Accessed: 26 October 2015).

Childers, D.L., Corman, J., Edwards, M., Elser, J.J. 2011. Sustainability challenges of phosphorus and food: solutions from closing the human phosphorus cycle. BioScience 61, 117-124.

D'Aoust, J.-Y. 1991. Pathogenicity of foodborne *Salmonella*. International Journal of Food Microbiology 12, 17-40.

Dalgaard, P., Vancanneyt, M., Euras Vilalta, N., Swings, J., Fruekilde, P., Leisner, J.J. 2003. Identification of lactic acid bacteria from spoilage associations of cooked and brined shrimps stored under modified atmosphere between 0 degrees C and 25 degrees C. Journal of Applied Microbiology 94, 80-89.

Dasti, J.I., Tareen, A.M., Lugert, R., Zautner, A.E., Groß, U. 2010. *Campylobacter jejuni*: a brief overview on pathogenicity-associated factors and disease-mediating mechanisms. International Journal of Medical Microbiology 300, 205-211.

De Jesús, A.J., Whiting, R.C. 2003. Thermal inactivation, growth, and survival studies of *Listeria monocytogenes* strains belonging to three distinct genotypic lineages. Journal of Food Protection 66, 1611-1617.

Delignette-Muller, M.L., Rosso, L. 2000. Biological variability and exposure assessment. International Journal of Food Microbiology 58, 203-212.

DGSanco. 2012. Commission staff working document SANCO/13004/2011: lessons learned from the 2011 outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O104:H4 in sprouted seeds [Online]. Available at http://ec.europa.eu/food/food/biosafety/salmonella/docs/cswd_lessons_learned_en.pdf (Accessed: 24 November 2015).

Donaldson, J.R., Nanduri, B., Burgess, S.C., Lawrence, M.L. 2009. Comparative proteomic analysis of *Listeria monocytogenes* strains F2365 and EGD. Applied and Environmental Microbiology 75, 366-373.

Doyle, M.E., Mazzotta, A.S., Wang, T., Wiseman, D.W., Scott, V.N. 2001. Heat resistance of *Listeria monocytogenes*. Journal of Food Protection 64, 410-429.

EFSA, ECDC. 2015. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. EFSA Journal 13 (12):4329 (191 pp).

Esty, J.R., Meyer, K.F. 1922. The heat resistance of the spores of *B. botulinus* and allied anaerobes. XI. Journal of Infectious Diseases 31, 650-663.

FAO-WHO. 2002. Risk assessments of *Salmonella* in eggs and broiler chickens [Online]. Available at http://www.fao.org/food/food-safety-quality/scientific-advice/jemra/risk-assessments/salmonella0/en/ (Accessed: 28 October 2015).

FAO-WHO. 2004. Risk assessment of *Listeria monocytogenes* in ready-to-eat foods [Online]. Available at http://www.fao.org/food/food-safety-quality/scientific-advice/jemra/risk-assessments/listeria0/en/ (Accessed: 28 October 2015).

FAO-WHO. 2008. Viruses in food: scientific advice to support risk management activities [Online]. Available at http://www.fao.org/food/food-safety-quality/scientific-advice/jemra/risk-assessments/vibrio0/en/ (Accessed: 28 October 2015).

FAO. 2009. How to feed the world in 2050 [Online]. Available at http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf (Accessed: 24 October 2015).

FAO. 2011. Global food losses and food waste – extent, causes and prevention [Online]. Available at http://www.fao.org/docrep/014/mb060e/mb060e00.htm (Accessed: 28 October 2015).

Farber, J.M., Peterkin, P.I. 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiology Reviews 55, 476-511.

Filtenborg, O., Frisvad, J.C., Thrane, U. 1996. Moulds in food spoilage. International Journal of Food Microbiology 33, 85-102.

Frank, C., Werber, D., Cramer, J.P., Askar, M., Faber, M., an der Heiden, M., Bernard, H., Fruth, A., Prager, R., Spode, A., Wadl, M., Zoufaly, A., Jordan, S., Kemper, M.J., Follin, P., Müller, L., King, L.A., Rosner, B., Buchholz, U., Stark, K., Krause, G. 2011. Epidemic profile of shiga-toxin–producing *Escherichia coli* O104:H4 outbreak in Germany. New England Journal of Medicine 365, 1771-1780.

Geeraerd, A.H., Valdramidis, V.P., Van Impe, J.F. 2005. GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. International Journal of Food Microbiology 102, 95-105.

Gibson, A.M., Bratchell, N., Roberts, T.A. 1987. The effect of sodium chloride and temperature on the rate and extent of growth of *Clostridium botulinum* type A in pasteurized pork slurry. Journal of Applied Bacteriology 62, 479-490.

Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M., Toulmin, C. 2010. Food security: the challenge of feeding 9 billion people. Science 327, 812-818.

Gram, L., Huss, H.H. 1996. Microbiological spoilage of fish and fish products. International Journal of Food Microbiology 33, 121-137.

Gram, L., Ravn, L., Rasch, M., Bruhn, J.B., Christensen, A.B., Givskov, M. 2002. Food spoilage—interactions between food spoilage bacteria. International Journal of Food Microbiology 78, 79-97.

Hamasaki, Y., Ayaki, M., Fuchu, H., Sugiyama, M., Morita, H. 2003. Behavior of psychrotrophic lactic acid bacteria isolated from spoiling cooked meat products. Applied and Environmental Microbiology 69, 3668-3671.

Hamon, M., Bierne, H., Cossart, P. 2006. *Listeria monocytogenes*: a multifaceted model. Nature Reviews Microbiology 4, 423-434.

Hanning, I.B., O'Bryan, C.A., Crandall, P.G., Ricke, S.C. 2012. Food safety and food security. Nature Education Knowledge 3 (10), 9.

Havelaar, A.H., Haagsma, J.A., Mangen, M.-J.J., Kemmeren, J.M., Verhoef, L.P.B., Vijgen, S.M.C., Wilson, M., Friesema, I.H.M., Kortbeek, L.M., van Duynhoven, Y.T.H.P., van Pelt, W. 2012. Disease burden of foodborne pathogens in the Netherlands, 2009. International Journal of Food Microbiology 156, 231-238.

Jakobsen, M., Narvhus, J. 1996. Yeasts and their possible beneficial and negative effects on the quality of dairy products. International Dairy Journal 6, 755-768.

Jespersen, L., Jakobsen, M. 1996. Specific spoilage organisms in breweries and laboratory media for their detection. International Journal of Food Microbiology 33, 139-155.

Jones, M.K., Oliver, J.D. 2009. *Vibrio vulnificus*: disease and pathogenesis. Infection and Immunity 77, 1723-1733.

Kaper, J.B., Nataro, J.P., Mobley, H.L.T. 2004. Pathogenic *Escherichia coli*. Nature Reviews Microbiology 2, 123-140.

Kuiper, H.A., Kleter, G.A., Noteborn, H.P.J.M., Kok, E.J. 2001. Assessment of the food safety issues related to genetically modified foods. The Plant Journal 27, 503-528.

Lianou, A., Koutsoumanis, K.P. 2011. Effect of the growth environment on the strain variability of *Salmonella enterica* kinetic behavior. Food Microbiology 28, 828-837.

Lianou, A., Koutsoumanis, K.P. 2013. Evaluation of the strain variability of *Salmonella enterica* acid and heat resistance. Food Microbiology 34, 259-267.

Linton, R.H., Pierson, M.D., Bishop, J.R. 1990. Increase in heat resistance of *Listeria monocytogenes* Scott A by sublethal heat shock. Journal of Food Protection 53, 924-927.

Lobell, D.B., Burke, M.B., Tebaldi, C., Mastrandrea, M.D., Falcon, W.P., Naylor, R.L. 2008. Prioritizing climate change adaptation needs for food security in 2030. Science 319, 607-610.

Lou, Y., Yousef, A.E. 1996. Resistance of *Listeria monocytogenes* to heat after adaptation to environmental stresses. Journal of Food Protection 59, 465-471.

Luber, P., Hoorfar, J. 2014. 25 - The case of the European *Escherichia coli* outbreak from sprouts, Global Safety of Fresh Produce. Woodhead Publishing. 356-366.

Luu-Thi, H., Khadka, D.B., Michiels, C.W. 2014. Thermal inactivation parameters of spores from different phylogenetic groups of *Bacillus cereus*. International Journal of Food Microbiology 189, 183-188.

Mackey, B.M., Pritchet, C., Norris, A., Mead, G.C. 1990. Heat resistance of *Listeria*: strain differences and effects of meat type and curing salts. Letters in Applied Microbiology 10, 251-255.

McCollum, J.T., Cronquist, A.B., Silk, B.J., Jackson, K.A., O'Connor, K.A., Cosgrove, S., Gossack, J.P., Parachini, S.S., Jain, N.S., Ettestad, P., Ibraheem, M., Cantu, V., Joshi, M., DuVernoy, T., Fogg, N.W., Gorny, J.R., Mogen, K.M., Spires, C., Teitell, P., Joseph, L.A., Tarr, C.L., Imanishi, M., Neil, K.P., Tauxe, R.V., Mahon, B.E. 2013. Multistate outbreak of listeriosis associated with cantaloupe. New England Journal of Medicine 369, 944-953.

McMeekin, T.A., Olley, J., Ratkowsky, D.A., Ross, T. 2002. Predictive microbiology: towards the interface and beyond. International Journal of Food Microbiology 73, 395-407.

McMeekin, T.A., Mellefont, L.A., Ross, T. 2007. Predictive microbiology: past, present and future. In: Brull, S., Van Gerwen, S., Zwietering, M., (Eds.), Modelling microorganisms in food. Woodhead Publishing Limited and CRC Press LLC. 7-21.

Nauta, M.J. 2000. Separation of uncertainty and variability in quantitative microbial risk assessment models. International Journal of Food Microbiology 57, 9-18.

Nelson, K.E., Fouts, D.E., Mongodin, E.F., Ravel, J., DeBoy, R.T., Kolonay, J.F., Rasko, D.A., Angiuoli, S.V., Gill, S.R., Paulsen, I.T., Peterson, J., White, O., Nelson, W.C., Nierman, W., Beanan, M.J., Brinkac, L.M., Daugherty, S.C., Dodson, R.J., Durkin, A.S., Madupu, R., Haft, D.H., Selengut, J., Van Aken, S., Khouri, H., Fedorova, N., Forberger, H., Tran, B., Kathariou, S., Wonderling, L.D., Uhlich, G.A., Bayles, D.O., Luchansky, J.B., Fraser, C.M. 2004. Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. Nucleic Acids Research 32, 2386-2395.

Neset, T.-S.S., Cordell, D. 2012. Global phosphorus scarcity: identifying synergies for a sustainable future. Journal of the Science of Food and Agriculture 92, 2-6.

O'Brien, S.J., Motarjemi, Y. 2014. Foodborne diseases: prevalence of foodborne diseases in Europe, Encyclopedia of Food Safety. Academic Press, Waltham. 302-311.

Pérez-Rodríguez, F. 2014. Development and application of predictive microbiology models in foods, Mathematical and Statistical Methods in Food Science and Technology. John Wiley & Sons, Ltd. 321-362.

Pothakos, V., Devlieghere, F., Villani, F., Björkroth, J., Ercolini, D. 2015. Lactic acid bacteria and their controversial role in fresh meat spoilage. Meat Science 109, 66-74.

Ratkowsky, D.A., Olley, J., McMeekin, T.A., Ball, A. 1982. Relationship between temperature and growth rate of bacterial cultures. Journal of Bacteriology 149, 1-5.

Roberts, T.A., Jarvis, B. 1983. Predictive modelling of food safety with particular reference to *Clostridium botulinum* in model cured meat systems. In: Roberts, T.A., Skinner, F.A., (Eds.), Food microbiology: advances and prospects. Academic Press, New York. 85-95.

Rosso, L., Lobry, J.R., Bajard, S., Flandrois, J.P. 1995. Convenient model to describe the combined effects of temperature and pH on microbial growth. Applied and Environmental Microbiology 61, 610-616.

Sanders, J.W., Oomes, S.J.C.M., Membré, J.M., Wegkamp, A., Wels, M. 2015. Biodiversity of spoilage lactobacilli: phenotypic characterisation. Food Microbiology 45, Part A, 34-44.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M., Roy, S.L., Jones, J.L., Griffin, P.M. 2011. Foodborne illness acquired in the United States—major pathogens. Emerging Infectious Disease Journal 17, 7-15.

Schelin, J., Wallin-Carlquist, N., Thorup Cohn, M., Lindqvist, R., Barker, G.C., Rådström, P. 2011. The formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment. Virulence 2, 580-592.

Siezen, R.J., Tzeneva, V.A., Castioni, A., Wels, M., Phan, H.T.K., Rademaker, J.L.W., Starrenburg, M.J.C., Kleerebezem, M., van Hylckama Vlieg, J.E.T. 2010. Phenotypic and genomic diversity of *Lactobacillus plantarum* strains isolated from various environmental niches. Environmental Microbiology 12, 758-773.

Siezen, R.J., van Hylckama Vlieg, J.E.T. 2011. Genomic diversity and versatility of *Lactobacillus plantarum*, a natural metabolic engineer. Microbial Cell Factories 10, S3-S3.

Singh, R.B. 2012. Climate change and food security, Improving crop productivity in sustainable agriculture. Wiley-VCH Verlag GmbH & Co. KGaA. 1-22.

Sörqvist, S. 1994. Heat resistance of different serovars of *Listeria monocytogenes*. Journal of Applied Bacteriology 76, 383-388.

Su, Y.-C., Liu, C. 2007. *Vibrio parahaemolyticus*: A concern of seafood safety. Food Microbiology 24, 549-558.

Taylor, J.M.W., Sutherland, A.D., Aidoo, K.E., Logan, N.A. 2005. Heat-stable toxin production by strains of *Bacillus cereus*, *Bacillus firmus*, *Bacillus megaterium*, *Bacillus simplex* and *Bacillus licheniformis*. FEMS Microbiology Letters 242, 313-317.

Techer, C., Baron, F., Jan, S. 2014. Spoilage of animal products | microbial milk spoilage. Encyclopedia of Food Microbiology 2, 446-452.

Thomas, M.K., Murray, R., Flockhart, L., Pintar, K., Fazil, A., Nesbitt, A., Marshall, B., Tataryn, J., Pollari, F. 2015. Estimates of foodborne illness–related hospitalizations

and deaths in Canada for 30 specified pathogens and unspecified agents. Foodborne Pathogens and Disease 12, 820-827.

Thompson, L., Tanner, F.W. 1925. Toxin production by *Clostridium botulinum* in canned foods. The Journal of Infectious Diseases 37, 344-352.

Tienungoon, S., Ratkowsky, D.A., McMeekin, T.A., Ross, T. 2000. Growth limits of *Listeria monocytogenes* as a function of temperature, pH, NaCl, and lactic acid. Applied and Environmental Microbiology 66, 4979-4987.

Tournas, V.H. 2005. Spoilage of vegetable crops by bacteria and fungi and related health hazards. Critical Reviews in Microbiology 31, 33-44.

UN. 2015. World population prospects [Online]. Available at http://esa.un.org/unpd/wpp/Publications/Files/Key_Findings_WPP_2015.pdf (Accessed: 11 November 2015).

Unnevehr, L., Roberts, T. 2002. Food safety incentives in a changing world food system. Food Control 13, 73-76.

Uyttendaele, M., Jacxsens, L., Van Boxstael, S., Hoorfar, J. 2014. 4 - Issues surrounding the European fresh produce trade: a global perspective, Global Safety of Fresh Produce. Woodhead Publishing. 33-51.

Van Asselt, E.D., Zwietering, M.H. 2006. A systematic approach to determine global thermal inactivation parameters for various food pathogens. International Journal of Food Microbiology 107, 73-82.

Van Boekel, M.A.J.S. 2008. Kinetic modelling of reaction in foods CRC Press.

Van der Veen, S., Moezelaar, R., Abee, T., Wells-Bennik, M.H.J. 2008. The growth limits of a large number of *Listeria monocytogenes* strains at combinations of stresses show serotype- and niche-specific traits. Journal of Applied Microbiology 105, 1246-1258.

Van Gerwen, S.J.C., de Wit, J.C., Notermans, S., Zwietering, M.H. 1997. An identification procedure for foodborne microbial hazards. International Journal of Food Microbiology 38, 1-15.

Van Vliet, A.H.M., Ketley, J.M. 2001. Pathogenesis of enteric *Campylobacter* infection. Journal of Applied Microbiology 90, 45S-56S.

Whiting, R.C., Golden, M.H. 2002. Variation among *Escherichia coli* O157:H7 strains relative to their growth, survival, thermal inactivation, and toxin production in broth. International Journal of Food Microbiology 75, 127-133.

WHO. 2015. WHO estimates of the global burden of foodborne diseases [Online]. Available at http://apps.who.int/iris/bitstream/10665/199350/1/9789241565165_eng.pdf?ua=1 (Accessed: 11 December 2015).

Zdruli, P. 2014. Land resources of the mediterranean: Status, pressures, trends and impacts on future regional development. Land Degradation & Development 25, 373-384.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M., Van 't Riet, K. 1990. Modeling of the bacterial growth curve. Applied and Environmental Microbiology 56, 1875-1881.

Zwietering, M.H., Wijtzes, T., Rombouts, F.M., Van 't Riet, K. 1993. A decision support system for prediction of microbial spoilage in foods. Journal of Industrial Microbiology 12, 324-329.

CHAPTER 2

Quantifying strain variability in modelling growth of *Listeria monocytogenes*

D.C. Aryani, H.M.W. den Besten, W.C. Hazeleger and M.H. Zwietering

Published in International Journal of Food Microbiology 208, 19-29.

ABSTRACT

Prediction of microbial growth kinetics can differ from the actual behaviour of the target microorganisms. In the present study, the impact of strain variability on maximum specific growth rate (μ_{max}) (h^{-1}) was quantified using twenty *Listeria monocytogenes*. The μ_{max} was determined as function of four different variables, namely pH, water activity (a_w)/NaCl concentration [NaCl], undissociated lactic acid concentration ([HLa]), and temperature (T). The strain variability was compared to biological and experimental variabilities to determine their importance. The experiment was done in duplicate at the same time to quantify experimental variability and reproduced at least twice on different experimental days to quantify biological (reproduction) variability. For all variables, experimental variability was clearly lower than biological variability and strain variability; and remarkably, biological variability was similar to strain variability. Strain variability in cardinal growth parameters, namely pH_{min}, $[NaCl_{max}]$, $[HLa_{max}]$, and T_{min} was further investigated by fitting secondary growth models to the μ_{max} data, including a modified secondary pH model. The fitting results showed that *L. monocytogenes* had an average pH_{min} of 4.5 (5-95% prediction interval (PI) 4.4 – 4.7), $[NaCl_{max}]$ of 2.0 M (PI 1.8 M – 2.1 M), $[HLa_{max}]$ of 5.1 mM (PI 4.2 mM – 5.9 mM), and T_{min} of -2.2°C (PI (-3.3°C) – (-1.1°C)). The strain variability in cardinal growth parameters was benchmarked to available literature data, showing that the effect of strain variability explained around 1/3 or less of the variability found in literature. The cardinal growth parameters and their prediction intervals were used as input to illustrate the effect of strain variability on the growth of *L. monocytogenes* in food products with various characteristics, resulting in 2-4 $\log_{10}$ cfu/ml(g) difference in growth prediction between the most and least robust strains, depending on the type of food product. This underlined the importance to obtain quantitative knowledge on variability factors to realistically predict the microbial growth kinetics.

INTRODUCTION

Mathematical models can be used as a tool to simulate the behaviour of microorganisms in food within the product's shelf life. However, differences might occur when prediction results are compared to the actual behaviour of microorganisms in food, due to the variability caused by, amongst others, growth history, the effect of the food matrix and variability between strains. These differences between predictions and the actual behaviour of microorganisms affect stability prediction of food products and risk estimations of foodborne illnesses.

Strain variability is defined as an inherent characteristic of microorganisms that cannot be reduced when strains are identically treated under the same set of conditions (Whiting and Golden, 2002). Differences between strains showed to be an important source of variability in thermal inactivation kinetics (Aryani et al., 2015; Doyle et al., 2001), and explained between 1/2 and 2/3 of the variability found in literature (Aryani et al., 2015). In contrast, the contribution of strain variability to the total variability in growth kinetics is still unknown. Although strain variability in growth kinetics of different bacterial species was reported in previous studies (Barbosa et al., 1994; Lianou and Koutsoumanis, 2011a; Lindqvist, 2006; Nauta and Dufrenne, 1999; Whiting and Golden, 2002), only some reported the magnitude and these focused only on one or two experimental conditions or variables, except in the study performed by Nauta and Dufrenne (1999) who investigated the variability in growth parameters of *Escherichia coli* as a function of temperature, pH, and water activity. Since microbial growth in food is affected by different intrinsic and extrinsic factors, such as pH, a_w, undissociated lactic acid concentration (HLa) and temperature (T), it is of importance to quantify the impact of strain variability as function of those variability factors. In this work, strain variability in maximum specific growth rate μ_{max} was quantified and compared to biological and experimental variabilities to determine their importance. Experimental variability was defined as the difference between duplicate experiments carried out in parallel at the same time on the same experimental day. Reproduction (biological) 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, or the so-called intra-species variability.

Listeria monocytogenes was used as target organism since it is an important pathogen with a high mortality rate (Jones, 1990). It can be found in a wide range of environments and has been isolated from different sources including food processing plants (Cox et al., 1989; Farber and Peterkin, 1991; Weis and Seeliger, 1975). Moreover, its ability to survive

and grow in stress conditions such as refrigeration temperature and high concentration of salt makes it difficult to reduce during food processing (Azevedo et al., 2005; Bajard et al., 1996; Van der Veen et al., 2008; Walker et al., 1990). The *L. monocytogenes* strains used in this study were not only strains with a long history of culturing in the laboratory, but also strains isolated from food industries, which have a short laboratory history. The variability in growth kinetics of *L. monocytogenes* quantified in this study is of relevance for the food industry and food safety management to realistically predict microbial growth kinetics.

MATERIALS AND METHODS

Culture preparation

Twenty strains of *L. monocytogenes*, covering a wide range of origins, were used in this study (table 2.1). The strain stocks were kept frozen at -80°C in 70% Brain Heart Infusion (BHI) broth (Becton Dickinson, France) and 30% glycerol (v/v) (Sigma-Aldrich, Germany). From the stock culture, a streak was made onto a BHI agar plate (BHI broth with 15 gram/liter bacteriological agar, Oxoid, England) and incubated for 24 h at 30°C. A single colony was selected and grown in a 250 ml flask containing 100 ml of BHI broth. The culture was incubated for 16 h in a shaking incubator (Innova 4335, New Brunswick Scientific, Netherlands) at 200 rpm at 30°C. To prepare a working culture, a similar procedure was followed as previously described (Biesta-Peters et al., 2010b). Briefly, the overnight culture was divided in two 50 ml centrifuge tubes and centrifuged for 2 min at 15,557 x *g* (Centrifuge 5804 R, Eppendorf). The supernatant was discarded and the pellet was re-suspended in 2 ml of Peptone Physiological Salt (PPS, Tritium Microbiologie B.V., Netherlands). One ml of the pooled suspension of two pellets was diluted in PPS to obtain an OD_{600} of 0.3 (Spectrophotometer Novospec II, Pharmacia Biotech) or approximately 10^8 CFU/ml. This standardized culture was used further for the experiments.

Table 2.1. Twenty *Listeria monocytogenes* strains used during the study from different origins and their resistance profiles obtained from this study

Strains	Origins	Resistance Profiles	Serotype
ScottA	Human isolate from Massachusetts milk outbreak	Low pH_{min}	4b
F2365	Jalisco cheese	Low pH_{min}	4b
EGDe	Rabbit		1/2a
LO28	Healthy pregnant carrier		1/2c
AOPM3	Human isolate		4b
C5	Smoked meat	Low T_{min}	4b
H7764	Deli turkey		1/2a
H7962	Hotdog		4b
L6	Milk		1/2b
FBR12	Frozen vegetable mix		1/2a
FBR13	Frozen endive a la creme	High $[NaCl_{max}]$	1/2a
FBR14	Carrot piece		1/2a
FBR15	Ice cream packaging machine		1/2c
FBR16	Ham (after cutting machine)		1/2a
FBR17	Frozen fried rice	Low pH_{min}, High $[NaCl_{max}]$, High $[HLa_{max}]$	4d
FBR18	Ice cream		1/2a
FBR19	Frozen meat		1/2a
FBR20	Frozen vegetables for soup		1/2a
FBR21	Fresh yeast	Low T_{min}	4d
FBR33	Pancake		1/2c

Media preparation

Nine pH values between 7.3 - 4.2 were selected to test the effect of pH on the maximum specific growth rate (μ_{max}) of the *L. monocytogenes* strains. For each experiment, the BHI broth was pH adjusted using 0.5 M of sulphuric acid (Riedel-de Haën, Seelze, Germany) and filter sterilized (Steritop, Milipore Corporation, MA).

Eight different concentrations of sodium chloride (NaCl, VWR International, Leuven, Belgium) between 0.5 - 12.5% (w/v) were selected and NaCl was added to BHI broth and autoclaved. The NaCl concentration of the standard medium (BHI broth) was 0.5%. The corresponding a_w value of the NaCl adjusted BHI broth was measured using a Novasina water activity meter (Labmaster a_w, Novasina, Lachen, Switzerland) set at 30°C.

Five concentrations (0, 3, 4, 5, and 6 mM) of DL-lactic acid 85% (Sigma-Aldrich, Germany) in its undissociated form at pH 5.5 were used in this study. The preparation of the medium

adjusted with lactic acid was done as previously described (Biesta-Peters et al., 2010a). Briefly, the ratio between the dissociated and undissociated forms of lactic acid at set pH was calculated using the Henderson-Hasselbalch equation (equation (2.1)).

$$\mathrm{pH} = \mathrm{pK_a} + log\frac{[A^-]}{[HLa]} \quad [2.1]$$

Where pH is the pre-set pH (pH 5.5) of the medium using sulphuric acid as acidulant, pK_a is the acid dissociation constant (pK_a of lactic acid 3.86), *[A⁻]* is the concentration of lactate, and *[HLa]* is the concentration of the undissociated acid. The conjugated salt of lactic acid used in this study was potassium lactate (Corbion, Gorinchem, Netherlands).

For the effect of temperature, the growth experiments were conducted at four temperatures, namely 30°C, 20°C, 10°C, and 5°C using non-adjusted BHI medium (pH 7.3; a_w 0.997).

Estimating μ_{max} as function of pH, a_w/[NaCl], [HLa], and temperature.

In this study, each experiment was conducted in duplicate at the same time using the same standardized culture to quantify experimental variability. The experiment was also reproduced minimally two times on different days using new cultures to quantify biological variability. This procedure resulted in at least six μ_{max} estimates per value of pH, a_w/[NaCl], [HLa] and temperature, namely two μ_{max} estimates obtained per day (duplicate) times three independent reproductions. The standardized culture was diluted 10,000 fold in each pH, a_w/[NaCl], and [HLa] adjusted BHI broth and in non-adjusted BHI broth for the temperature experiment, aiming for an initial cell concentration of approximately 10^4 CFU/ml. The diluted suspension was spiral plated (Eddy Jet, IUL instruments) onto BHI agar plates in duplicate to determine the initial concentration (N_0) of each *L. monocytogenes* strain.

The μ_{max} was estimated using the 2-fold dilution method, which is based on time-to-detection (TTD) measurement of serially diluted concentration as described earlier by Biesta-Peters et al. (2010b). The TTD was defined as a time at which a well reaches a specific value of OD_{600} 0.2 (or 0.15 for the condition close to the growth boundary). Briefly, all wells in a 100-well honeycomb plate (Oy growth Curves AB ltd, Helsinki, Finland) were filled with 200 µl of optimum medium or adjusted medium (pH, a_w/[NaCl], and [HLa]). For each condition, every first well in the honeycomb plate was inoculated in duplicate with 200 µl of the diluted bacterial culture in the same medium. After mixing, a two-fold dilution was made from the first well to the fifth well. From the fifth well 200 µl was discarded to have the same volume in all wells. The honeycomb plates were incubated in the Bioscreen C (Oy growth Curves AB Ltd, Helsinki, Finland) at 30°C for pH, a_w/[NaCl], and [HLa] tests; or incubated at 20°C, 10°C, and 5°C for temperature tests. The

Bioscreen C was run with continuous and medium shaking for a certain period depending on the condition tested, which was up to 2 weeks, 10 days, 3 weeks, and 1 month for the lowest pH, a_w/[NaCl], [HLa] and temperature respectively. The OD_{600} data obtained from Bioscreen C were imported in Microsoft Excel for data processing to estimate the TTD. For practical reasons, which did not influence the results, the OD values in this study were not corrected for the background colour of BHI broth (~ 0.1 at OD_{600}) (Biesta-Peters et al., 2010b). The μ_{max} was calculated as the negative reciprocal slope of the linear regression between TTD and the natural logarithm of the initial bacterial concentration of the five wells. A manual check was performed to confirm that there were no systematically higher residuals for any of the five wells (especially the lowest inoculum) before performing the regression.

For the wells that did not show any changes in their OD values within the time frame of experiment, viability of the bacteria at the end of the experiment was verified by plating all the content of the well. When the plate counting showed an increase in number of bacteria, the term "growth" was put in the database, meaning that at that point growth occurred, but the μ_{max} could not be determined using the method used in this study. This typical growth situation was observed in 14 cases out of 360 cases at pH 4.6 - 4.3, 4 cases out of 120 cases when 11% of NaCl was added in the BHI medium, and 6 cases out of 120 cases in the presence of 5 mM [HLa] in BHI medium. When the plate counting showed reduction in number of bacteria in comparison to N_0 or total inactivation of bacteria, the μ_{max} was set to 0 h^{-1}.

In cases where no μ_{max} value was obtained using OD measurement, but the verification with plating showed some increase in number of bacteria, no μ_{max} value was included in the fitting procedures. When the verification with plating showed inactivation or no growth and the model fitting gave a negative fitted μ_{max} value, then the μ_{max} of 0 h^{-1} was excluded in the fitting procedures. In case a positive fitted μ_{max} value was observed and no growth or inactivation was confirmed by plating, the μ_{max} of 0 h^{-1} was included in the fitting procedures.

Quantifying experimental, biological/reproduction and strain variabilities

All the μ_{max} data were tabulated according to strain and the pH, a_w/[NaCl], [HLa], and temperature values. The experimental (E), biological/reproduction (R), and strain (S) variabilities were determined for each variable, namely pH, a_w/[NaCl], [HLa] and temperature based on the scheme in figure 2.1 using equations (2.2) – (2.4).

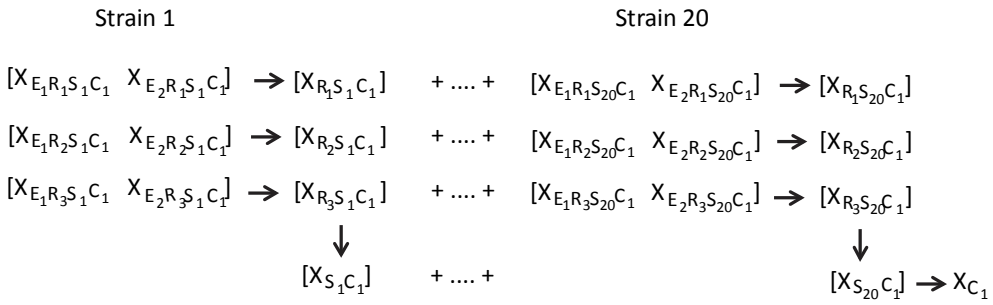

Figure 2.1. Schematic figure for calculating the experimental (E), biological/reproduction (R), and strain (S) variabilities. X_{ERSC} is the maximum specific growth rate (μ_{max}) obtained for experiment "E", reproduction "R", strain "S", and condition "C". X_{ERSC} is replicated (E_1 and E_2) on the same experimental day for strain "S" and condition "C". X_{RSC} is the average of two X_{ERSC} obtained on the same day for strain "S" and condition "C". X_{SC} is the average of X_{RSC} from three different experimental days for strain "S" and condition "C", and X_C is the average X_{SC} from all strains for condition "C".

Experimental variability:

$$MSE = \frac{RSS}{DF} = \frac{\sum_{C=1}^{i}\sum_{S=1}^{20}\sum_{R=1}^{3}\sum_{E=1}^{2}(X_{ERSC}-X_{RSC})^2}{n-p} \quad [2.2]$$

Where the mean square error (MSE) is calculated from the residual sum of squares (RSS) divided by the degrees of freedom (DF). The RSS is the sum of squared differences between X_{ERSC} and X_{RSC}. X_{ERSC} is the μ_{max} value (h^{-1}) of each replicate of duplicate experiments conducted at the same time for a certain strain per condition (C) (E=1, 2; R=1, 2 ,3; S=1, 2,…20; C=pH 7.3, 6.5, 6,…., etc.), as an example X_{1111} is the μ_{max} (h^{-1}) from the first replicate (E=1) conducted on day 1 (R=1) and for strain 1 (S=1) at condition 1 (C=1). X_{RSC} is the average μ_{max} (h^{-1}) obtained on the same experimental day for a certain strain per condition (R=1, 2, 3; S=1, 2,…20; C= pH 7.3, 6.5, 6,…., etc.), as an example X_{111} is the average μ_{max} (h^{-1}) from experimental day 1 for strain 1 and condition 1. The *DF* is the number of data points (n=6∗20) per condition minus the number of parameters (p=3∗20) times the number of conditions "C". The number of conditions per variable were 9, 8, 5, and 4 for pH, a_w/[NaCl], [HLa], and temperature respectively.

Biological/Reproduction variability:

$$MSE = \frac{RSS}{DF} = \frac{\sum_{C=1}^{i}\sum_{S=1}^{20}\sum_{R=1}^{3}(X_{RSC}-X_{SC})^2}{n-p} \quad [2.3]$$

Where X_{RSC} is the average μ_{max} (h^{-1}) obtained at the same experimental day for a certain strain per condition (R=1,2,3; S=1, 2,…,20; C=pH 7.3, 6.5, 6,…., etc.), X_{SC} is the average μ_{max} (h^{-1}) from three different experimental days for each strain per condition (S=1, 2,…,20; C=

pH 7.3, 6.5, 6,…., etc.). *DF* is the number of data points (*n*=3∗20) per condition minus the number of parameters (p=1∗20) times the number of conditions "C".

Strain variability:

$$MSE = \frac{RSS}{DF} = \frac{\sum_{C=1}^{i}\sum_{s=1}^{20}(X_{SC}-X_C)^2}{n-p} \quad [2.4]$$

Where X_{SC} is the average μ_{max} (h^{-1}) from three different experimental days for each strain per condition, X_C is the average μ_{max} (h^{-1}) of all 20 strains at condition *C*, *DF* is the number of data points (*n*=20) per condition minus the number of parameter (*p*=1) times the number of conditions "C".

Model selection and fitting performance

Secondary pH, a_w/[NaCl], [HLa] and temperature models were used to fit the μ_{max} data as function of pH, a_w/[NaCl], [HLa] and temperature for each strain. The model fitting performances were compared using four criteria: 1) having low mean square error of the model (MSE_{model}) (equation (2.5)); 2) having model parameters with biological meaning; 3) having low number of parameters; and 4) giving realistic model parameter estimates. The MSE_{model} was calculated as follows:

$$MSE_{model} = \frac{RSS}{DF} = \frac{\sum_{C=1}^{i}\sum_{R=1}^{3}\sum_{E=1}^{2}(X_{ERC}-\hat{X}_C)^2}{n-p} \quad [2.5]$$

Where X_{ERC} is the observed μ_{max} (h^{-1}) obtained from each condition (e.g. C=pH 7.3, 6.5, 6,…, etc.), $\hat{X}_C$ is the fitted μ_{max} (h^{-1}) (per strain) at each condition, *n* is the number of data points, and *p* is the number of parameters of the respective model.

The overall mean square error of the model was the sum of mean square errors of all 20 strains. An *F*-test was used to compare the overall mean square errors between the models.

When two models had similar fitting performances based on the overall mean square error and the *F*-test, then the lack-of-fit test (equation (2.6)) and the Akaike Information Criterion (AIC) (equation (2.8)) were further employed for each strain. The lack-of-fit test compares the MSE_{model} and the measuring error (MSE_{data}). The AIC test was used to compare models based on their number of parameters. The lack of fit test (Den Besten et al., 2006) was determined as follows:

$$\text{Lack-of-fit} = \frac{MSE_{model}}{MSE_{data}} \quad [2.6]$$

$$\text{Where } MSE_{data} = \frac{RSS}{DF} = \frac{\sum_{A=1}^{i}\sum_{R=1}^{3}\sum_{E=1}^{2}(X_{ERA}-X_A)^2}{n-m} \quad [2.7]$$

Where X_{ERA} is the observed μ_{max} (h^{-1}) obtained from each condition (e.g. A=pH 7.3, 6.5, 6,…, etc.), X_A is the average μ_{max} (h^{-1}) for each condition, *n* is the number of data points, and *m* equals to the number of conditions *i*.

The AIC (Van Boekel, 2008) was determined as follows:

$$AIC = n\ln(\hat{\sigma}^2) + 2(p+1) \quad [2.8]$$

Where *n* is the number of data points, $\hat{\sigma}^2$ is the maximum likelihood estimator for the variance (*RSS* model divided by *n*), and *p* is the number of model parameters.

When two models had comparable overall MSE based on the F-test, then the selected model was the model that fitted the data best for most of the strains based on the lack-of-fit test and AIC. All fitting was done using Excel Solver Add-in (Microsoft) and confirmed using TableCurve 2D v5.01.

Prediction of growth in food products

The gamma model (γ) (Zwietering et al., 1993) was used to predict the microbial growth in two food products, assuming that different variables have a multiplicative effect rather than an interaction effect (Biesta-Peters et al., 2010a). To integrate strain variability in the prediction, the cardinal growth parameters of each strain were used as inputs for the gamma model (equations (2.9) – (2.10)). The gamma model used the best secondary model selected for each condition (pH, a_w/[NaCl], [HLa], and T). Since only the a_w value was available for the food products and not the NaCl concentration, the a_w model was used in the prediction.

The reference condition then was set at pH 7.3, a_w 0.997, temperature 30°C, and [HLa] 0 mM, and each model was transformed to the reference condition (equations (2.11) – (2.14)). The model transformation, the resulting gamma factors and the obtained μ_{ref} are presented in supplement 2.1 and figure S2.1 respectively. The μ_{ref} of each strain used for calculation was the average μ_{ref} obtained from pH, a_w, temperature, and [HLa] models.

$$\gamma = \gamma(pH) * \gamma([NaCl]/a_w) * \gamma(T) * \gamma(HLa) \quad [2.9]$$

$$\mu_{max} = \mu_{ref} * \gamma \quad [2.10]$$

$$\gamma(pH) = \frac{1-2^{\frac{(pH-pH_{min})}{(pH_{min}-pH_{1/2})}}}{1-2^{\frac{(pH_{ref}-pH_{min})}{(pH_{min}-pH_{1/2})}}} \quad [2.11]$$

$$\gamma(a_w) = \frac{1-\left(\frac{1-a_w}{1-a_{w_{min}}}\right)^{\alpha_{a_w}}}{1-\left(\frac{1-a_{w_{ref}}}{1-a_{w_{min}}}\right)^{\alpha_{a_w}}} \quad [2.12]$$

$$\gamma(T) = \frac{(T-T_{min})^2}{(T_{ref}-T_{min})^2} \quad [2.13]$$

$$\gamma(HLa) = \left(1-\left(\frac{HLa}{HLa_{max}}\right)^{\alpha_{HLa}}\right) \quad [2.14]$$

Where μ_{ref} is μ_{max} at pH 7.3, a_w 0.997, [HLa] 0 mM, and temperature 30°C.

The μ_{max} estimated using the gamma model was then used in the logistic model (equation (2.15)), which is widely used in predictive growth modelling, to predict the growth of *L. monocytogenes* in specific food matrices.

$$ln(N_t) = ln(N_{max}) - ln\left\{1 + \left[\frac{N_{max}}{N_0}\right] exp(-\mu_{max} t)\right\} \quad [2.15]$$

Where N_t is the concentration of *L. monocytogenes* (CFU/ml or CFU/g) at time *t*, N_{max} is the maximum concentration (CFU/ml or CFU/g), N_0 is the initial concentration (CFU/ml or CFU/g), μ_{max} is the estimated μ_{max} using the gamma model (day^{-1}) and *t* is the incubation time (days).

Hundred simulations with 10,000 iterations were done in Microsoft Excel using @Risk add-in for Excel version 5.2 (Palisade Corporation, New York, USA) to estimate the 5 - 95% prediction intervals. In addition to the simulation, the 5 - 95% intervals of growth were calculated manually using the worst case scenario of each parameter.

The food products used for the *L. monocytogenes* growth prediction were milk and ham. The product specification of milk was described previously (Te Giffel and Zwietering, 1999), with pH was 6.6; a_w was 0.993; and temperature was 7°C. The product specification of ham was provided by Corbion, the Netherlands, in which pH was 6; a_w was 0.965; temperature was 7°C; and 3% (w/w) of 65% K-Lactate. The corresponding concentration of [HLa] was calculated using the Henderson-Hasselbalch equation (equation (2.1)), assuming that around 67% of ham is water (Fernández-Salguero et al., 1993) and 100% of [HLa] is in the water phase. Using the pH of ham and the provided concentration above, the [HLa] concentration was 1.58 mM.

RESULTS

The effect of pH, a_w/[NaCl], [HLa], and temperature on μ_{max} of *L. monocytogenes*

In figure 2.2A - C, the variability in μ_{max} of *L. monocytogenes* as function of pH is shown. The experimental variability was low as replicated μ_{max} estimates obtained on the same experimental day were rather similar (figure 2.2A). The variability increased when the data from three reproductions obtained on different days (figure 2.2B) or the data from all strains (figure 2.2C) were compared. Of all 20 strains, strain FBR15 consistently had the lowest μ_{max} over the whole pH range used in this study. In contrast to FBR15, strains F2365 and EGDe were among the strains that had the highest growth rate at all pH values.

Variability was also observed in pH values at which growth could still be detected. Strain F2365, which had the highest growth rate over the whole pH range, grew at pH 4.3 in one of three reproductions. Four strains, namely ScottA, EGDe and two industrial strains FBR17 and FBR21, were able to grow at pH 4.5, but failed to grow at pH 4.4. The other

strains grew at pH 4.6, except for L6, FBR12, and FBR14 that showed growth only at pH 5 and higher pH values, meaning that the pH to initiate growth was between pH 4.6 - pH 5.

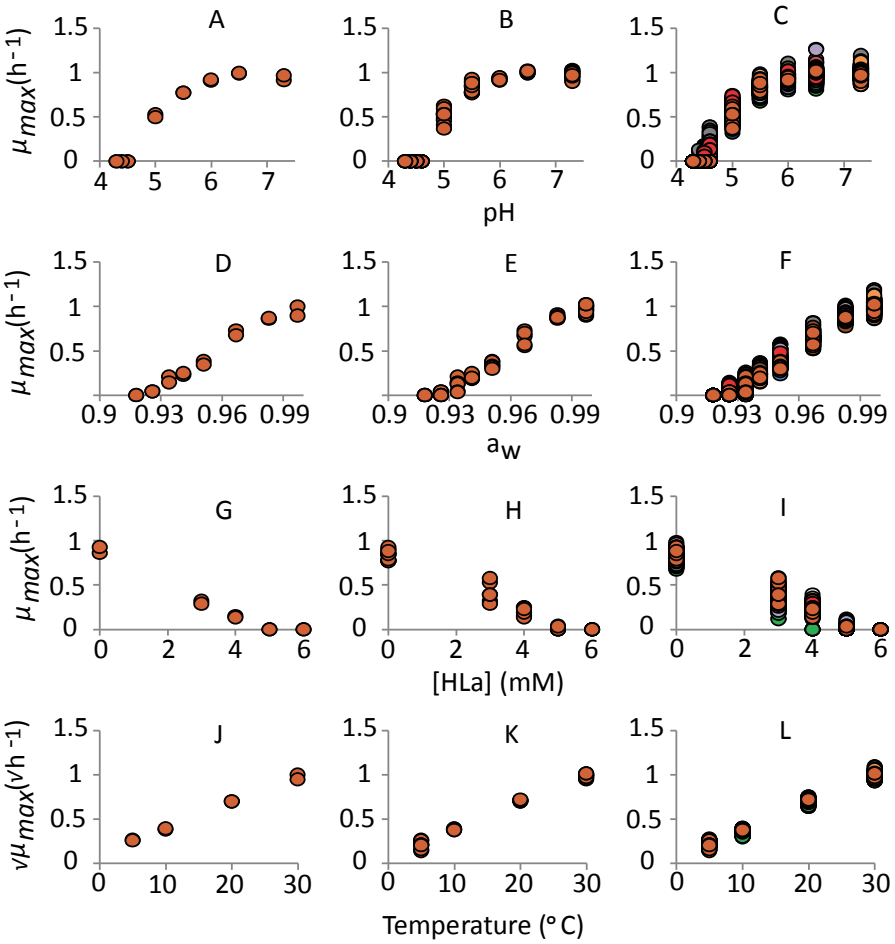

Figure 2.2. The effect of pH (A-C); a_w (D-F); undissociated lactic acid concentration ([HLa]) (G-I) on μ_{max} and temperature (J-L) on $\sqrt{\mu_{max}}$ of *L. monocytogenes*. Figure A, D, G, and J show the experimental data of FBR 33 obtained on the same experimental day to visualize the experimental variability; B, E, H, and K the experimental data of FBR 33 obtained on different experimental days to visualize the biological variability; and figure C, F, I, and L the experimental data of all 20 strains to visualize strain variability. ScottA, EGDe, F2365, LO28, AOPM3, C5, H7764, H7962, L6, FBR12, FBR13, FBR14, FBR15, FBR16, FBR17, FBR18, FBR19, FBR20, FBR21, and FBR33.

Similar to the effect of pH, combining the μ_{max} data from different experimental days (figure 2.2E) or different strains (figure 2.2F) increased the variability in μ_{max} as function of a_w or [NaCl]. The impact of variability caused by reproduction (biological variability) and strains, however, seemed to be comparable. Strain variability was observed at the a_w or [NaCl] values where growth could be measured. Strain ScottA, which had the highest growth rate over the whole a_w range, showed growth at a_w 0.926 (11.5% NaCl or 1.97 M). Besides ScottA, seven other strains were also able to grow at this a_w value. All strains, except H7962, grew at a_w of 0.934 (10.5%, 1.8 M).

The same trend was observed for the effect of [HLa]. Figures 2.2G – 2.2I show that the variability in μ_{max} increased when the μ_{max} data from different experiments were combined. Ten out of 20 strains did not grow at the concentration of 5 mM [HLa], while at 4 mM only strain FBR15 was not able to grow.

Although differences between replicates were much lower than differences between reproductions and strains for the variable temperature, the variance of μ_{max} as function of temperature varied across the temperature range (figure S2.2). Therefore, different transformations of μ_{max} were applied to stabilize the variance, namely square root, $\log_{10}$, and 1/square root transformations. The best transformation was the square root transformation, and this was then used to transform the μ_{max} data as can be seen in figures 2.2J – 2.2L.

Quantifying the impact of experimental, biological, strain variabilities on μ_{max} of *L. monocytogenes*

Experimental, reproduction and strain variabilities were quantified and are represented in figure 2.3. The same trend was observed for the variables pH, a_w/[NaCl] and [HLa]. For those variables, the strain and reproduction variabilities were in the same order of magnitude, and both were significantly higher than experimental variability (figures 2.3A – 2.3C). For the variable temperature, the strain variability was lower than reproduction variability when those were calculated using non transformed μ_{max} data (data not shown). A similar result was obtained when using a linear mixed model, in which the variance component of reproduction variability was higher than the variance component of strain variability. Since the variance of the temperature data varied across the temperature values (figure S2.2), the square root transformed μ_{max} data were used to calculate the variability. When the square root transformed μ_{max} data were used, the strain and biological variabilities were comparable and both were significantly higher than experimental variability (figure 2.3D).

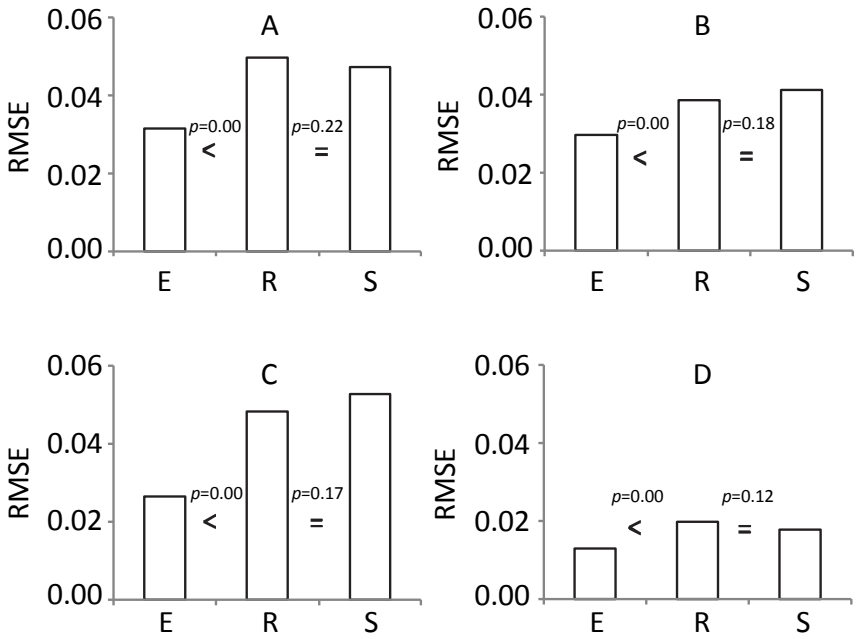

Figure 2.3. E (experimental), R (reproduction), and S (strain) variabilities, presented as the root mean square error (RMSE), calculated using the μ_{max} data of 20 *L. monocytogenes* strains: A) the variability as function of pH; B) the variability as function of a_w; C) the variability as function of undissociated lactic acid ([HLa]); and D) the variability as function of temperature for square root transformed μ_{max} data.

Model fitting

Modelling the μ_{max} as function of pH

Eleven secondary pH models, including a new proposed model, were used to fit the μ_{max} data of each *L. monocytogenes* strain (table 2.2). The new proposed model (model 11) was a modification of model 5, in which the value 10 of the model was changed into a parameter "α_{pH}" to increase curvature flexibility resulting in:

$$\mu_{max} = \mu_{opt}\left(1 - \alpha_{pH}^{(pH_{min} - pH)}\right) \quad [2.16]$$

The new model was further re-parameterized to replace the shape parameter "α_{pH}" with a parameter that has an interpretable meaning. The parameter $pH_{1/2}$ is defined as the pH at which the μ_{max} is half of the μ_{opt} (equation (2.17)).

$$\mu_{max} = \mu_{opt}\left(1 - 2^{\frac{(pH - pH_{min})}{(pH_{min} - pH_{1/2})}}\right) \quad [2.17]$$

From all secondary pH models used to fit the data, model 8 had the lowest overall mean square error (table 2.2). Model 11, however, was slightly better than model 8 based on the lowest mean square error for 10 out of 20 strains. Although the lack-of-fit test results of both models were almost similar, further comparison with the Akaike Information Criterion (AIC) showed that model 11 had lower AIC (13 out of 20 strains) than model 8 (7 out of 20 strains). Model 11 was also preferred since it has one parameter less than model 8, and in some cases the pH_{max} estimate of model 8 was unrealistic (due to unavailable data at higher pH values). Considering all those results, model 11 was selected as the best model for further estimating μ_{opt} (h^{-1}) (figure S2.3), $pH_{1/2}$ (figure S2.4), and pH_{min} (figure 2.4A) of each strain. The pH_{min} estimates ranged from 4.34 to 4.68, with an average of 4.51. The lowest pH_{min} (4.34) was estimated for strain F2365, while the three highest pH_{min} were estimated for strains L6 (4.60), FBR12 (4.64), and FBR33 (4.68).

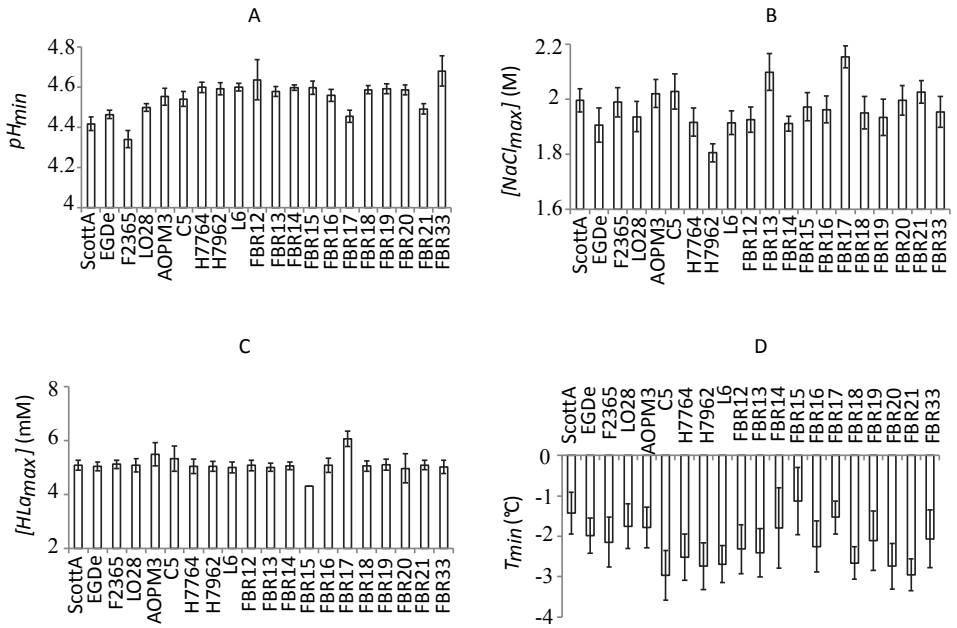

Figure 2.4. The pH_{min} (A), maximum sodium chloride concentration ($[NaCl_{max}]$) (B), maximum undissociated lactic acid concentration ($[HLa_{max}]$) (C), and T_{min} (D) estimated using secondary growth models for the 20 *L. monocytogenes* strains. The error bars show the confidence interval of each value. * the confidence interval cannot be estimated.

Table 2.2. Secondary pH models used for fitting the μ_{max} as function of pH, including their number of parameters and fitting performance

No	Model	No. of par.	Overall MSE*	Lowest MSE**	Ref.***)
1	$\mu_{max} = {\alpha_{pH}}^2(pH - pH_{min})$	2	0.775	0/20	1
2	$\mu_{max} = \alpha_{pH}\,(pH - pH_{min})(pH - pH_{max})$	3	0.122	0/20	2
3	$\mu_{max} = \alpha_{pH}(pH - pH_{min})\{1 - e^{b(pH - pH_{max})}\}$	4	0.125	0/20	2
4	$\mu_{max} = \left(\alpha_{pH}(pH - pH_{min})\left(1 - e^{b(pH - pH_{max})}\right)\right)^2$	4	0.216	0/20	3
5	$\mu_{max} = \mu_{opt}(1 - 10^{pH_{min} - pH})$	2	0.0899	1/20	4
6	$\mu_{max} = \mu_{opt}(1 - 10^{pH_{min} - pH})(1 - 10^{pH - pH_{max}})$	3	0.0917	0/20	4, 5
7	$\mu_{max} = \mu_{opt}\dfrac{(pH - pH_{max})(pH - pH_{min})^2}{(pH_{opt} - pH_{min})\left[(pH_{opt} - pH_{min})(pH - pH_{opt}) - (pH_{opt} - pH_{max})(pH_{opt} + pH_{min} - 2pH)\right]}$	4	0.123	0/20	6
8	$\mu_{max} = \mu_{opt}\dfrac{(pH - pH_{max})(pH - pH_{min})}{(pH - pH_{min})(pH - pH_{max}) - (pH - pH_{opt})^2}$	4	0.0628	9/20	7
9	$\mu_{max} = \mu_{opt}\dfrac{(pH - pH_{min})(pH_{max} - pH)}{(pH_{opt} - pH_{min})(pH_{max} - pH_{opt})}$	4	0.124	0/20	8

Table 2.2 continued

No	Model	No. of par.	Overall MSE*	Lowest MSE**	Ref.***
10	$\mu_{max} = \mu_{opt} \frac{(pH - pH_{min})(2pH_{opt} - pH_{min} - pH)}{(pH_{opt} - pH_{min})^2}$	3	0.122	0/20	8
11	$\mu_{max} = \mu_{opt}(1 - \alpha_{pH}{}^{(pH_{min} - pH)}) \equiv \mu_{max} = \mu_{opt}\left(1 - 2^{\frac{(pH - pH_{min})}{\left(pH_{min} - pH_{\frac{1}{2}}\right)}}\right)$	3	0.0630	10/20	9

* the sum of model's MSE of all 20 strains.

** shows how often the model had the lowest mean square error from all 20 strains. The value 10/20 means that the model had the lowest MSE for 10 out of 20 strains.

*** References

1) Adams et al., 1991; 2) Wijtzes et al., 1995; 3) Ratkowsky et al., 1983; 4) Presser et al., 1997; 5) Biesta-Peters et al., 2010a; 6) Rosso et al., 1993; 7) Rosso et al., 1995; 8) Zwietering et al., 1996; 9) This study.

Modelling the μ_{max} as function of a_w/[NaCl

In total 18 secondary models for the effect of a_w/[NaCl] (table 2.3) on μ_{max} were selected and tested based on four criteria described above. The transformation of a_w to [NaCl] model and vice versa was done by assuming that:

$$a_w = 1 - d[S] \quad [2.18]$$

In which a_w is the water activity value measured using Novasina a_w meter, *[S]* is NaCl concentration in mol/L, and *d* is 0.037 estimated from all a_w values and the corresponding salt concentrations in mol/L. The fitting of the secondary a_w model, however, was done using the measured a_w value instead of the a_w value calculated using the above equation, and therefore, slight differences were observed between MSE [S] and MSE a_w.

From all models used to fit the data, model 9b had the lowest overall mean square error, followed by models 3b and 3a. However, the overall mean square error of model 9b was not significantly lower than that of models 3a and 3b. Also, in some cases the μ_{opt} estimated using model 9b was considerably low (e.g. 0.2 h^{-1}). Considering these results, although model 9b had a lower AIC (17 out of 20 strains), models 3a and 3b were preferred since these had also fewer number of parameters. Although both models showed similar good fit for 18 out of 20 strains, model 3b had a lower AIC (13 out of 20 strains) than model 3a. Therefore model 3b was used for further estimating *[$NaCl_{max}$]*.

Using secondary [NaCl] model 3b three parameters, namely μ_{opt} (h^{-1}) (figure S2.3); maximum NaCl concentration *[$NaCl_{max}$]* in mol/L (figure 2.4B); and shape parameter "$\alpha_{[NaCl]}$" (figure S2.5A), were estimated. The μ_{opt} parameter of 20 strains estimated using this model was in agreement with the one obtained using the secondary pH model. Moreover, the shape parameter "$\alpha_{[NaCl]}$" was significantly different from one for 19 out of 20 strains, supporting the use of model 3b over a linear model. The *[$NaCl_{max}$]* estimates given by model 3b were in the range of 1.81-2.15 M, in which the highest concentration was estimated for strain FBR17.

Table 2.3. Secondary a_w and [NaCl] models and their fitting performance

No	Model	No. of par.	Overall MSE*	Lowest MSE**	Ref.[***)]
1a	$\mu_{max} = {\alpha_{a_w}}^2(a_w - a_{w_{min}})$	2	0.0631	0/20	1
1b	$\mu_{max} = {\alpha_{[NaCl]}}^2(d([s_{max}] - [s]))$	2	0.0779	0/20	1
2a	$\mu_{max} = {\alpha_{a_w}}^2\left(\frac{a_w - a_{w_{min}}}{1 - a_{w_{min}}}\right)$	2	0.0631	0/20	1, 2
2b	$\mu_{max} = {\alpha_{[NaCl]}}^2\left(\frac{[s_{max}] - [s]}{[s_{max}]}\right)$	2	0.0779	0/20	1, 2
3a	$\mu_{max} = \mu_{opt}\left(1 - \left(\frac{1 - a_w}{1 - a_{w_{min}}}\right)^{\alpha_{a_w}}\right)$	3	0.0451	3/20	3
3b	$\mu_{max} = \mu_{opt}\left(1 - \left(\frac{[s]}{[s_{max}]}\right)^{\alpha_{[NaCl]}}\right)$	3	0.0439	2/20	3
4a	$\mu_{max} = \alpha_{a_w}(1 - 10^{a_{w_{min}} - a_w})$	2	0.0579	0/20	4
4b	$\mu_{max} = \alpha_{[NaCl]}\left(1 - 10^{d([s] - [s_{max}])}\right)$	2	0.0679	0/20	4
5a	$\mu_{max} = \mu_{opt}\left(1 - 2^{\left(\frac{a_w - a_{w_{min}}}{a_{w_{min}} - a_{w_{1/2}}}\right)}\right)$	3	0.0513	0/20	5
5b	$\mu_{max} = \mu_{opt}\left(1 - 2^{\left(\frac{[s_{max}] - [s]}{[s_{1/2}] - [s_{max}]}\right)}\right)$	3	0.0490	0/20	5
6a	$\mu_{max} = \mu_{opt}\left(\frac{a_w - a_{w_{min}}}{1 - a_{w_{min}}}\right)$	2	0.0631	0/20	6
6b	$\mu_{max} = \mu_{opt}\left(\frac{[s_{max}] - [s]}{[s_{max}]}\right)$	2	0.0779	0/20	6

Table 2.3. continued

No	Model	No. of par.	Overall MSE*	Lowest MSE**	Ref.***
7a	$\mu_{max} = \mu_{opt}\left(1 - \sqrt{\frac{(1-a_w)}{(1-a_{w_{min}})}}\right)$	2	0.200	0/20	7
7b	$\mu_{max} = \mu_{opt}\left(1 - \sqrt{\frac{[s]}{[s_{max}]}}\right)$	2	0.246	0/20	7
8a	$\mu_{max} = \mu_{opt}\left(\frac{\alpha_{a_w}(a_w - a_{w_{min}})}{\alpha_{a_w}(1-a_{w_{min}}) - \frac{(1-a_{w_{min}})(1-a_w)}{d}}\right)$	3	0.0511	0/20	8
8b	$\mu_{max} = \mu_{opt}\left(\frac{\alpha_{[NaCl]}([s_{max}]-[s])}{[S_{max}](\alpha_{[NaCl]}-[s])}\right)$	3	0.0501	0/20	8
9a	$\mu_{max} = \mu_{opt}\left(1 + \frac{\alpha_{a_w}(1-a_w)/d}{b+(1-a_w)/d}\right)\left(1 - \frac{(1-a_w)}{(1-a_{w_{min}})}\right)$	4	0.0460	2/20	9
9b	$\mu_{max} = \mu_{opt}\left(1 + \frac{\alpha_{[NaCl]}[s]}{b+[s]}\right)\left(1 - \frac{[s]}{[s_{max}]}\right)$	4	0.0395	13/20	9

* the sum of model's MSE of all 20 strains.

** showed how often the model had the lowest mean square error from all 20 strains. The value 13/20 means that the model has 13 times the lowest mean square errors over the fitting with 20 strains.

*** References

1) Presser et al., 1998; 2) Zwietering et al., 1996; 3) Luong, 1985; 4) Presser et al., 1997; 5) This study; 6) Ghose and Tyagi, 1979; 7) Le Marc et al., 2002; 8) Houtsma et al., 1994; 9) Passos et al., 1993.

Modelling the μ_{max} as function of [HLa]

From five secondary models selected to fit the μ_{max} as function of [HLa], model 1 had the lowest overall mean square error (table 2.4). It also had the lowest mean square error for 16 out of 20 strains, and showed a good fit for all 20 strains. The closest alternative of this model was linear model 2 which had 0.018 point difference in overall MSE compared to model 1. However, model 2 had the lowest mean square error for only 4 out of 20 strains, and showed a good fit for 18 out of 20 strains. The AIC test also showed that model 1 (18 out of 20 strains) had a better performance than model 2 (2 out of 20 strains). Therefore, secondary [HLa] model 1 was selected to model μ_{max} as function of [HLa].

The μ_{opt} (h^{-1}) as function of [HLa] was the μ_{opt} (h^{-1}) at pH 5.5, because the experiments were done at pH 5.5. The fitting result showed that in 11 cases parameter "$\alpha_{[HLa]}$" was significantly different from one (figure S2.5B). For strain FBR 15, the confidence interval of parameters "$\alpha_{[HLa]}$" and $[HLa_{max}]$ could not be obtained because only two [HLa] values (0 and 3 mM) were available for fitting the secondary model with 3 parameters. In general, the estimated $[HLa_{max}]$ of each strain was around the average (5.1 mM), except for strains FBR17 (6.1 mM) and FBR 5 (4.3 mM) (figure 2.4C).

Table 2.4. Secondary models of [HLa] and their fitting performance

No.	Model	No. of par.	Overall MSE *	Lowest MSE**	Ref.***
1	$\mu_{max} = \mu_{opt}\left(1 - \left(\frac{[HLa]}{[HLa_{max}]}\right)^{\alpha_{[HLa]}}\right)$	3	0.0511	16/20	1
2	$\mu_{max} = \mu_{opt}\left(1 - \frac{[HLa]}{[HLa_{max}]}\right)$	2	0.0688	4/20	2
3	$\mu_{max} = \mu_{opt}\left(1 - \sqrt{\frac{[HLa]}{[HLa_{max}]}}\right)$	2	0.137	0/20	3
4	$\mu_{max} = \alpha_{[HA]}\left(1 - \frac{[HLa]}{[HLa_{max}](1 + 10^{pH-pKa})}\right)$	2	0.0701	0/20	4
5	$\mu_{max} = \mu_{opt} exp\left(-\alpha_{[HLa]}[HLa] - [HLa_{max}]\right)$	3	0.178	0/20	5

* the sum of model's MSE of all 20 strains.

** showed how often the model had the lowest mean square error from all 20 strains. The value 16/20 means that the model has 16 times the lowest mean square errors over the fitting with 20 strains.

*** References

1) Luong, 1985; 2) Ghose and Tyagi, 1979; 3) Le Marc et al., 2002; 4) Presser et al., 1997; 5) Yeh et al., 1991.

Modelling the μ_{max} as function of temperature

A linear model (equation (2.20)) (Ratkowsky et al., 1982) was used for estimating the parameter T_{min}.

$$\sqrt{\mu_{max}} = \alpha_T(T - T_{min}) \quad [2.20]$$

When fitting the secondary temperature model to μ_{max} data, the slope parameter "α_T" and parameter T_{min} (°C) had an average estimate of 0.031 (5-95% prediction interval (PI) between 0.029 and 0.033) and -2.2°C (PI between -3.3°C and -1.1°C). Strain C5 and FBR21 had the lowest T_{min}, which were about -3.0°C (figure 2.4D).

Predicting the growth of *L. monocytogenes* in food products

In this study the worst case scenario was assumed and no lag phase was taken into account in the growth prediction. This is a realistic scenario when the contaminant is adapted to the product and the environment. Low temperature (7°C) did not prevent the growth of *L. monocytogenes* when the other environmental factors were almost optimal as in the case of milk (table 2.5).

When *L. monocytogenes* is present in milk, it will be able to grow until it reaches maximum levels (figure 2.5). Strains C5 and FBR21, which were found to have the lowest T_{min}, were able to grow faster than the other strains when temperature is the only growth limiting factor. In the case of ham, the combination of low temperature, mild concentration of NaCl, and the use of lactic acid as preservative slightly inhibited the growth of *L. monocytogenes*. In this case, strain FBR21 grew slightly faster than the other strains. In both products strain FBR15 grew slower than the other strains because it had the lowest growth rate. To compare these predictions to available literature data, the growth parameter estimates of the cardinal model without interaction from Coroller et al. (2012) (figure 2.6) were used. This latter study estimated the growth parameters from different data sources including literature, unpublished sources and databases. These growth parameter estimates were obtained from various products and media. Therefore these estimates were lower or higher than our parameter estimates because in our study defined broth conditions were used. So, as expected, the growth kinetics predicted using the mean parameter values were higher than ours, both in ham and milk. But, when the 95% prediction intervals of these parameters were used, the intervals of predicted growth kinetics in both products overlapped with our intervals.

Table 2.5. Average value of μ_{ref} and $\gamma^{*)}$ factors calculated for each food product

Food Products	Average μ_{ref} and γ factors
Milk pH 6.6, a_w 0.993, T=7°C	μ_{ref} = 0.99 (h^{-1}); γ(pH) = 0.98; γ(T) = 0.082; $\gamma(a_w)$ = 0.96; γ (total)= 0.077
Ham pH 6, a_w 0.965, T=7°C, [HLa] 1.58 mM	μ_{ref} = 0.99 (h^{-1}); γ(pH) = 0.93; γ(T) = 0.082; $\gamma(a_w)$ = 0.62; γ(HLa) = 0.74; γ (total)= 0.035

*) γ was defined as being relative to the reference condition at pH 7.3, a_w 0.997, [HLa] 0 mM, and T 30°C.

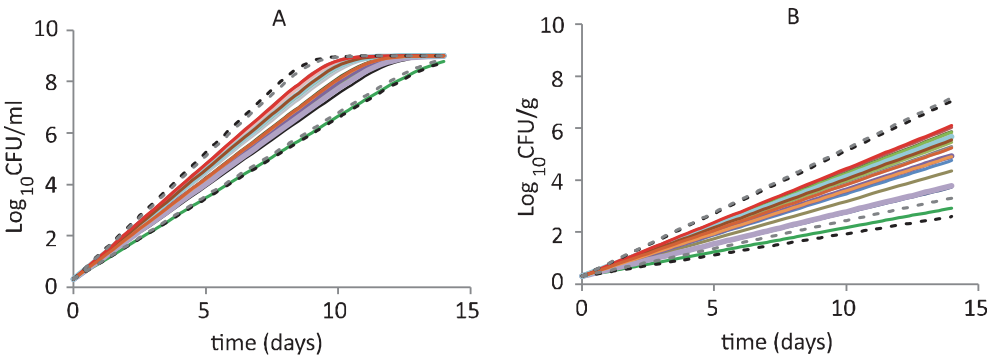

Figure 2.5. The predicted growth of 20 strains of *Listeria monocytogenes* in milk (A) and ham (B) with initial concentration (N_0) 2 CFU/ml (or g), N_{max} 10^9 CFU/ml and T 7°C. Black dashed line is the worst case with all input values (pH_{min}, a_{wmin}, [HLa_{max}], T_{min}, and μ_{ref}) as being upper level (95%) or lower level (5%) of prediction interval. Grey dashed line is the simulation result with @Risk using 5-95% prediction interval of each parameter as the input value. ScottA, EGDe, F2365, LO28, AOPM3, C5, H7764, H7962, L6, FBR12, FBR13, FBR14, FBR15, FBR16, FBR17, FBR18, FBR19, FBR20, FBR21 and FBR33.

DISCUSSION

Variability in microbial growth kinetics have been observed in studies using *Staphylococcus aureus* (Lindqvist, 2006), *Salmonella* (Lianou and Koutsoumanis, 2011a; Oscar, 2000) and *Listeria* (Barbosa et al., 1994; Begot et al., 1997) species. The variability of μ_{max} among *Salmonella enterica* strains was reported to be greater than within strain

variability (Lianou and Koutsoumanis, 2011a). Strain variability was described to be larger than the error calculated from experimental procedures using single strains or cocktails of *Escherichia coli* O157:H7 (Whiting and Golden, 2002). It was also suggested to be four to six times higher than the inherent variability of the method and experimental protocol for a single strain of *S. aureus* (Lindqvist, 2006). In contrast to what was already reported, our finding showed that biological variability and strain variability were in the same order of magnitude, and both were around two to four times higher than experimental variability. A previous study (Lianou and Koutsoumanis, 2011a) suggested that strain variability amongst *S. enterica* strains was larger when the growth conditions became unfavourable. It should be noted, however, that the degree of variability reported by Lianou and Koutsoumanis (2011a) was based on the coefficient of variation (CV) ratio value. When the standard deviations are similar for all growth conditions such as in our study, the CV ratio will increase when the growth conditions become unfavourable. We also observed larger variability in μ_{max} near the growth boundary for pH than for NaCl conditions, which corresponded to the previous result from Lianou and Koutsoumanis (2011a) study.

The estimated cardinal parameters were used to screen the presence of robust strains in all growth conditions tested. However, lack of correlation was observed between cardinal parameters (supplement 2.2). From all 20 strains, only strain FBR17 was observed to have low pH_{min}, high $[NaCl_{max}]$ and high $[HLa_{max}]$. Among all 20 strains, strain FBR15, which is an industrial environmental isolate (equipment), consistently had lower μ_{max} over the pH and [HA] ranges. In contrast, Begot et al. (1997) showed that strains originating from industrial environments were among the faster growing strains. It was suggested that those strains were accustomed to growing in a wide range of harsh conditions allowing them to adapt to unfavourable conditions. Our study showed that also industrial isolates can be slow growers, underlining the wide strain variability.

Many factors influence variability in cardinal growth parameters. The fitted cardinal parameters were benchmarked to the available literature data (figure 2.6). Figure 2.6 shows that strain variability in cardinal growth parameter explained around 1/3 or less of the variability found in literature. Since the effect of strain alone did not explain all variability found in literature, other factors such as growth history and possible interactions between growth limiting factors might also contribute to the observed differences in cardinal growth parameter estimates. Van der Veen et al. (2008) reported the pH_{min} of 138 strains *L. monocytogenes* in BHI at 30°C using HCl as acidulant, and these pH_{min} values were in the range of our fitted pH_{min}. In the latter study when the growth temperature was changed to 7°C, the pH_{min} values were reported higher than the ones at 30°C. The pH_{min} was also reported higher when organic acids, such as lactic acid and citric acid, were used as acidulant (Cole et al., 1990; Conner et al., 1986). In this case, besides

pH, the undissociated form of organic acid also influences the pH_{min}. When only the effect of strain was taken into account, our fitted $a_{w,min}/[NaCl_{max}]$ had a wider range than the $a_{w,min}$ reported by Van der Veen et al. (2008) in BHI at 30°C and pH 7.4. Besides growth conditions, the type of solutes used to lower water activity also influenced the variability in the cardinal growth parameters (Augustin and Carlier, 2000; Farber et al., 1992; Nolan et al., 1992; Tapia De Daza et al., 1991).

The reported T_{min} of *L. monocytogenes* from literature data was between - 6.1°C and 9.1°C (Augustin and Carlier, 2000). This wide range of reported T_{min} in comparison to our data was influenced by different factors, such as the presence of organic acid and nitrite in the medium, or the modification of the atmospheric conditions. Because each reported experiment was done using a different set of strains and conditions, it is difficult to clearly distinguish the effect of each factor on T_{min} variability. The T_{min} in our study was estimated using four temperature points using the square root model with two parameters, assuming that there is a linear correlation between temperature and the square root of μ_{max}. Notably, some studies reported a nonlinear behaviour of *L. monocytogenes* at suboptimal temperatures (Bajard et al., 1996; Le Marc et al., 2002). One then could argue that with only four temperature points and assuming a linear correlation between temperature and square root of μ_{max} we might overestimate the T_{min} of the corresponding strains used in this study. However, the MSE_{model} was comparable to the MSE_{data} for 11 out of 20 strains. When the MSE_{data} is much lower than the MSE_{model} then the use of a more complex model, such as a temperature model with inflection point, might be justified. But, when this model is used to estimate parameter T_{min} more data points should be available at different suboptimal temperatures.

The quantitative knowledge on the impact of strain variability on the growth parameters can be integrated into a mathematical model describing growth kinetics (Delignette-Muller and Rosso, 2000; Lianou and Koutsoumanis, 2011b; Nauta and Dufrenne, 1999). Although strain variability was similar to biological variability in our study and explained only a part of the variability found in the literature, the attempt to integrate strain variability into mathematical models will result in a more realistic prediction of the growth of *L. monocytogenes*, such as in the examples of milk and ham. This integration resulted in 2-4 $\log_{10}$ cfu/ml(g) difference in the growth prediction between the most and least robust strains, depending on the food product type. In cases where growth is limited, higher differences in prediction between strains might be expected. Then the negligence on this difference will result in an underestimation of the true microbial growth behaviour.

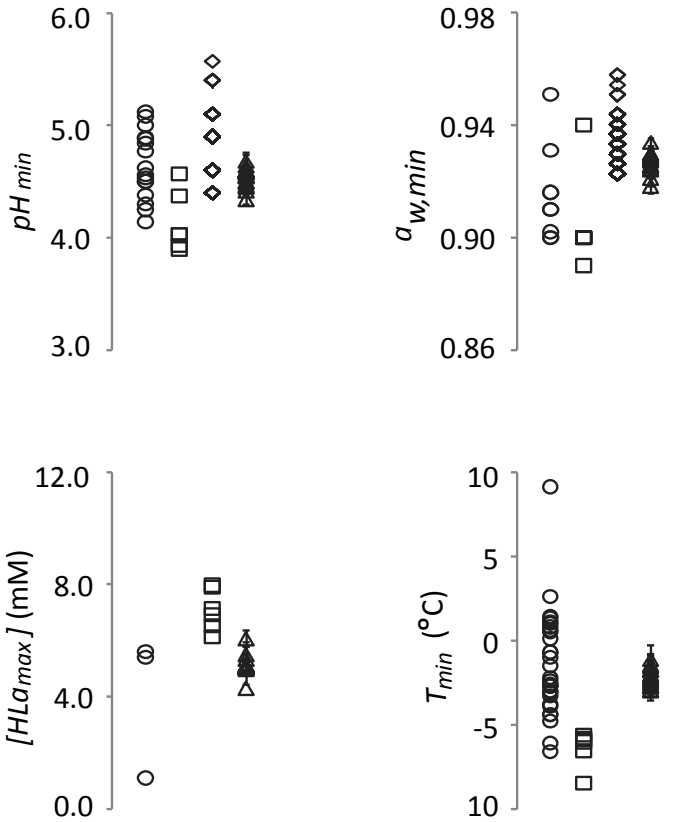

Figure 2.6. The fitted cardinal growth parameters of 20 *L. monocytogenes* strains from this study (△ the error bars show the confidence interval of each parameter) plotted together with the available literature data: A) pH_{min}, B) $a_{w,min}$, C) $[HLa_{max}]$, D) T_{min} to visualize the impact of variability on cardinal growth parameters found from literature (○ Augustin and Carlier, 2000; ◇ Van der Veen et al., 2008; □ Coroller et al., 2012).

ACKNOWLEDGMENTS

We would like to thank the Nederlandse Voedsel- en Warenautoriteit (*NVWA*) for serotyping our *L. monocytogenes* strains and Gerard Weenk of Top Institute for Food and Nutrition (TIFN) and Danone Schiphol, The Netherlands for his managerial role in the TIFN modelling project. The research is funded by TI Food and Nutrition, a public-private

partnership on precompetitive research in food and nutrition. The funders had no role in study design, data collection and analysis, or preparation of the manuscript.

REFERENCES

Adams, M.R., Little, C.L., Easter, M.C. 1991. Modelling the effect of pH, acidulant and temperature on the growth rate of *Yersinia enterocolitica*. Journal of Applied Microbiology 71, 65-71.

Aryani, D.C., Den Besten, H.M.W., Hazeleger, W.C., Zwietering, M.H. 2015. Quantifying variability on thermal resistance of *Listeria monocytogenes*. International Journal of Food Microbiology 193, 130-138.

Augustin, J.-C., Carlier, V. 2000. Mathematical modelling of the growth rate and lag time for *Listeria monocytogenes*. International Journal of Food Microbiology 56, 29-51.

Azevedo, I., Regalo, M., Mena, C., Almeida, G., Carneiro, L., Teixeira, P., Hogg, T., Gibbs, P.A. 2005. Incidence of *Listeri*a spp. in domestic refrigerators in Portugal. Food Control 16, 121-124.

Bajard, S., Rosso, L., Fardel, G., Flandrois, J.P. 1996. The particular behaviour of *Listeria monocytogenes* under sub-optimal conditions. International Journal of Food Microbiology 29, 201-211.

Barbosa, W.B., Cabedo, L., Wederquist, H.J., Sofos, J.N., Schmidt, G.R. 1994. Growth variation among species and strains of *Listeria* in culture broth. Journal of Food Protection 57, 765-769.

Begot, C., Lebert, I., Lebert, A. 1997. Variability of the response of 66 *Listeria monocytogenes* and *Listeria innocua* strains to different growth conditions. Food Microbiology 14, 403-412.

Biesta-Peters, E.G., Reij, M.W., Gorris, L.G.M., Zwietering, M.H. 2010a. Comparing nonsynergistic gamma models with interaction models to predict growth of emetic *Bacillus cereus* when using combinations of pH and individual undissociated acids as growth-limiting factors. Applied and Environmental Microbiology 76, 5791-5801.

Biesta-Peters, E.G., Reij, M.W., Joosten, H., Gorris, L.G.M., Zwietering, M.H. 2010b. Comparison of two optical-density-based methods and a plate count method for estimation of growth parameters of *Bacillus cereus*. Applied and Environmental Microbiology 76, 1399-1405.

Cole, M.B., Jones, M.V., Holyoak, C. 1990. The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. Journal of Applied Bacteriology 69, 63-72.

Conner, D.E., Brackett, R.E., Beuchat, L.R. 1986. Effect of temperature, sodium chloride, and pH on growth of *Listeria monocytogenes* in cabbage juice. Applied and Environmental Microbiology 52, 59-63.

Coroller, L., Kan-King-Yu, D., Leguerinel, I., Mafart, P., Membré, J.-M. 2012. Modelling of growth, growth/no-growth interface and nonthermal inactivation areas of *Listeria* in foods. International Journal of Food Microbiology 152, 139-152.

Cox, L.J., Kleiss, T., Cordier, J.L., Cordellana, C., Konkel, P., Pedrazzini, C., Beumer, R., Siebenga, A. 1989. *Listeria* spp. in food processing, non-food and domestic environments. Food Microbiology 6, 49-61.

Delignette-Muller, M.L., Rosso, L. 2000. Biological variability and exposure assessment. International Journal of Food Microbiology 58, 203-212.

Den Besten, H.M.W., Mataragas, M., Moezelaar, R., Abee, T., Zwietering, M.H. 2006. Quantification of the effects of salt stress and physiological state on thermotolerance of *Bacillus cereus* ATCC 10987 and ATCC 14579. Applied and Environmental Microbiology 72, 5884-5894.

Doyle, M.E., Mazzotta, A.S., Wang, T., Wiseman, D.W., Scott, V.N. 2001. Heat resistance of *Listeria monocytogenes*. Journal of Food Protection 64, 410-429.

Farber, J.M., Coates, F., Daley, E. 1992. Minimum water activity requirements for the growth of *Listeria monocytogenes*. Letters in Applied Microbiology 15, 103-105.

Farber, J.M., Peterkin, P.I. 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiology Reviews 55, 476-511.

Fernández-Salguero, J., Gómez, R., Carmona, M.A. 1993. Water activity in selected high-moisture foods. Journal of Food Composition and Analysis 6, 364-369.

Ghose, T.K., Tyagi, R.D. 1979. Rapid ethanol fermentation of cellulose hydrolysate. II. Product and substrate inhibition and optimization of fermentor design. Biotechnology and Bioengineering 21, 1401-1420.

Houtsma, P.C., Kusters, B.J.M., de Wit, J.C., Rombouts, F.M., Zwietering, M.H. 1994. Modelling growth rates of *Listeria innocua* as a function of lactate concentration. International Journal of Food Microbiology 24, 113-123.

Jones, D. 1990. Foodborne listeriosis. The Lancet 336, 1171-1174.

Le Marc, Y., Huchet, V., Bourgeois, C.M., Guyonnet, J.P., Mafart, P., Thuault, D. 2002. Modelling the growth kinetics of *Listeria* as a function of temperature, pH and organic acid concentration. International Journal of Food Microbiology 73, 219-237.

Lianou, A., Koutsoumanis, K.P. 2011a. Effect of the growth environment on the strain variability of *Salmonella enterica* kinetic behavior. Food Microbiology 28, 828-837.

Lianou, A., Koutsoumanis, K.P. 2011b. A stochastic approach for integrating strain variability in modeling *Salmonella enterica* growth as a function of pH and water activity. International Journal of Food Microbiology 149, 254-261.

Lindqvist, R. 2006. Estimation of *Staphylococcus aureus* growth parameters from turbidity data: characterization of strain variation and comparison of methods. Applied Environmental Microbiology 72, 4862-4870.

Luong, J.H.T. 1985. Kinetics of ethanol inhibition in alcohol fermentation. Biotechnology and Bioengineering 27, 280-285.

Nauta, M., Dufrenne, J. 1999. Variability in growth characteristics of different *E. coli* O157:H7 isolates, and its implications for predictive microbiology. Quantitative Microbiology 1, 137-155.

Nolan, D.A., Chamblin, D.C., Troller, J.A. 1992. Minimal water activity levels for growth and survival of *Listeria monocytogenes* and *Listeria innocua*. International Journal of Food Microbiology 16, 323-335.

Oscar, T.P. 2000. Variation of lag time and specific growth rate among 11 strains of *Salmonella* inoculated onto sterile ground chicken breast burgers and incubated at 25^{o}C. Journal of Food Safety 20, 225-236.

Passos, F.V., Fleming, H.P., Ollis, D.F., Hassan, H.M., Felder, R.M. 1993. Modeling the specific growth rate of *Lactobacillus plantarum* in cucumber extract. Applied Microbiology and Bacteriology 40, 143-150.

Presser, K.A., Ratkowsky, D.A., Ross, T. 1997. Modelling the growth rate of *Escherichia coli* as a function of pH and lactic acid concentration. Applied and Environmental Microbiology 63, 2355-2360.

Presser, K.A., Ross, T., Ratkowsky, D.A. 1998. Modelling the growth limits (growth/no growth interface) of *Escherichia coli* as a function of temperature, pH, lactic acid concentration, and water activity. Applied and Environmental Microbiology 64, 1773-1779.

Ratkowsky, D.A., Lowry, R.K., McMeekin, T.A., Stokes, A.N., Chandler, R.E. 1983. Model for bacterial culture growth rate throughout the entire biokinetic temperature range. Journal of Bacteriology 154, 1222-1226.

Ratkowsky, D.A., Olley, J., McMeekin, T.A., Ball, A. 1982. Relationship between temperature and growth rate of bacterial cultures. Journal of Bacteriology 149, 1-5.

Rosso, L., Lobry, J.R., Bajard, S., Flandrois, J.P. 1995. Convenient model to describe the combined effects of temperature and pH on microbial growth. Applied and Environmental Microbiology 61, 610-616.

Rosso, L., Lobry, J.R., Flandrois, J.P. 1993. An unexpected correlation between cardinal temperatures of microbial growth highlighted by a new model. Journal of Theoretical Biology 162, 447-463.

Tapia De Daza, M.S., Villegas, Y., Martinez, A. 1991. Minimal water activity for growth of *Listeria monocytogenes* as affected by solute and temperature. International Journal of Food Microbiology 14, 333-337.

Te Giffel, M.C., Zwietering, M.H. 1999. Validation of predictive models describing the growth of *Listeria monocytogenes*. International Journal of Food Microbiology 46, 135-149.

Van Boekel, M.A.J.S. 2008. Kinetic modelling of reaction in foods. CRC Press.

Van der Veen, S., Moezelaar, R., Abee, T., Wells-Bennik, M.H.J. 2008. The growth limits of a large number of *Listeria monocytogenes* strains at combinations of stresses show serotype- and niche-specific traits. Journal of Applied Microbiology 105, 1246-1258.

Walker, S.J., Archer, P., Banks, J.G. 1990. Growth of *Listeria monocytogenes* at refrigeration temperatures. Journal of Applied Bacteriology 68, 157-162.

Weis, J., Seeliger, H. 1975. Incidence of *Listeria monocytogenes* in nature. Applied Microbiology 30, 29-32.

Whiting, R.C., Golden, M.H. 2002. Variation among *Escherichia coli* O157:H7 strains relative to their growth, survival, thermal inactivation, and toxin production in broth. International Journal of Food Microbiology 75, 127-133.

Wijtzes, T., de Wit, J.C., Huis in 't Veld, J.H.J., Van 't Riet, K., Zwietering, M.H. 1995. Modelling bacterial growth of *Lactobacillus curvatus* as a function of acidity and temperature. Applied and Environmental Microbiology 61, 2533-2539.

Yeh, P.L.-H., Bajpai, R.K., Iannotti, E.L. 1991. An improved kinetic model for lactic acid fermentation. Journal of Fermentation and Bioengineering 71, 75-77.

Zwietering, M.H., de Wit, J.C., Notermans, S. 1996. Application of predictive microbiology to estimate the number of *Bacillus cereus* in pasteurised milk at the point of consumption. International Journal of Food Microbiology 30, 55-70.

Zwietering, M.H., Wijtzes, T., Rombouts, F.M., Van 't Riet, K. 1993. A decision support system for prediction of microbial spoilage in foods. Journal of Industrial Microbiology 12, 324-329.

SUPPLEMENTARY MATERIALS

Supplement 2.1. Secondary growth models transformation

pH model

$$\mu_{max} = \mu_{opt}\left(1 - 2^{\frac{(pH - pH_{min})}{(pH_{min} - pH_{1/2})}}\right)$$

$$\mu_{ref} = \mu_{opt}\left(1 - 2^{\frac{(pH_{ref} - pH_{min})}{(pH_{min} - pH_{1/2})}}\right)$$

$$\mu_{opt} = \frac{\mu_{ref}}{\left(1 - 2^{\frac{(pH_{ref} - pH_{min})}{(pH_{min} - pH_{1/2})}}\right)}$$

$$\mu_{max} = \mu_{ref}\frac{\left(1 - 2^{\frac{(pH - pH_{min})}{(pH_{min} - pH_{1/2})}}\right)}{\left(1 - 2^{\frac{(pH_{ref} - pH_{min})}{(pH_{min} - pH_{1/2})}}\right)}$$

a_w model

$$\mu_{max} = \mu_{opt}\left(1 - \left(\frac{1 - a_w}{1 - a_{a_{w_{min}}}}\right)^{\alpha_{a_w}}\right)$$

$$\mu_{ref} = \mu_{opt}\left(1 - \left(\frac{1 - a_{a_{w_{ref}}}}{1 - a_{a_{w_{min}}}}\right)^{\alpha_{a_w}}\right)$$

$$\mu_{opt} = \frac{\mu_{ref}}{\left(1 - \left(\frac{1 - a_{w_{ref}}}{1 - a_{w_{min}}}\right)^{\alpha_{a_w}}\right)}$$

$$\mu_{max} = \mu_{ref}\frac{\left(1 - \left(\frac{1 - a_w}{1 - a_{w_{min}}}\right)^{\alpha_{a_w}}\right)}{\left(1 - \left(\frac{1 - a_{w_{ref}}}{1 - a_{w_{min}}}\right)^{\alpha_{a_w}}\right)}$$

[HLa] model (experiment at pH 5.5)

$$\mu_{max} = \mu_{pH\ 5.5}\left(1 - \left(\frac{[HLa]}{[HLa_{max}]}\right)^{\alpha_{[HLa]}}\right)$$

$$\mu_{pH\ 5.5} = \mu_{ref}\frac{\left(\frac{(pH5.5 - pH_{min})}{1-2^{(pH_{min}-pH_{1/2})}}\right)}{\left(\frac{(pH_{ref}-pH_{min})}{1-2^{(pH_{min}-pH_{1/2})}}\right)}$$

$$\mu_{max} = \mu_{ref}\frac{\left(\frac{(pH5.5 - pH_{min})}{1-2^{(pH_{min}-pH_{1/2})}}\right)}{\left(\frac{(pH_{ref}-pH_{min})}{1-2^{(pH_{min}-pH_{1/2})}}\right)}\left(1 - \left(\frac{[HLa]}{[HLa_{max}]}\right)^{\alpha_{[HLa]}}\right)$$

Temperature model

$$\mu_{max} = \left(\alpha_T(T - T_{min})\right)^2$$
$$\mu_{max} = {\alpha_T}^2(T - T_{min})^2$$
$$\mu_{ref} = {\alpha_T}^2\left(T_{ref} - T_{min}\right)^2$$
$${\alpha_T}^2 = \frac{\mu_{ref}}{\left(T_{ref} - T_{min}\right)^2}$$
$$\mu_{max} = \mu_{ref}\frac{(T - T_{min})^2}{\left(T_{ref} - T_{min}\right)^2}$$

Supplement 2.2. The Pearson correlation coefficient between cardinal growth parameters

Correlation between cardinal growth parameters	Pearson correlation coefficient (r)
pH_{min} and $[NaCl_{max}]$	-0.35
pH_{min} and $[HLa_{max}]$	-0.33
$[NaCl_{max}]$ and $[HLa_{max}]$	0.049
T_{min} and pH_{min}	-0.24
T_{min} and $[NaCl_{max}]$	0.18
T_{min} and $[HLa_{max}]$	0.024

SUPPLEMENTARY FIGURES

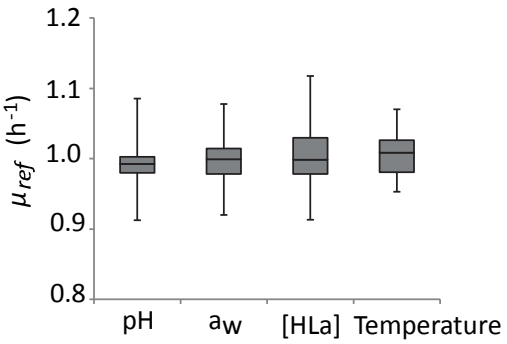

Figure S2.1. μ_{ref} values calculated from the μ_{opt} of each strain using the transformed secondary models.

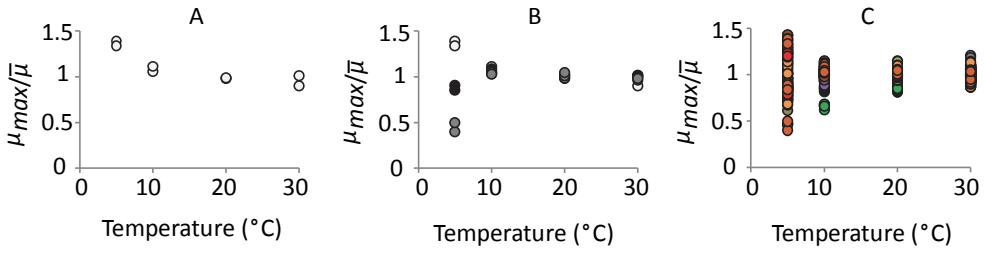

Figure S2.2. The effect of temperature (A-C) on $\mu_{max}/\bar{\mu}$ (μ_{max} is the maximum specific growth rate of each *L. monocytogenes* strain and $\bar{\mu}$ is the average μ_{max} of all 20 strains at the respective temperature). Figure A shows the experimental data of FBR33 obtained on the same experimental day to visualize the experimental variability; B the experimental data of FBR33 obtained on different experimental days to visualize the biological variability; and C the experimental data of all 20 strains to visualize strain variability. ScottA, EGDe, F2365, LO28, AOPM3, C5, H7764, H7962, L6, FBR12, FBR13, FBR14, FBR15, FBR16, FBR17, FBR18, FBR19, FBR20, FBR21, and FBR33.

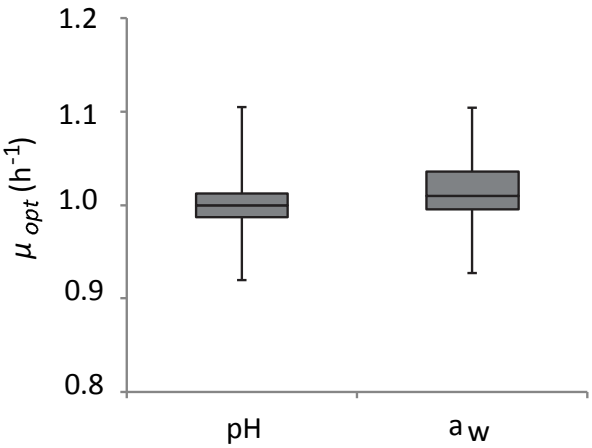

Figure S2.3. Parameter μ_{opt} (h^{-1}) estimated using secondary pH and secondary a_w models.

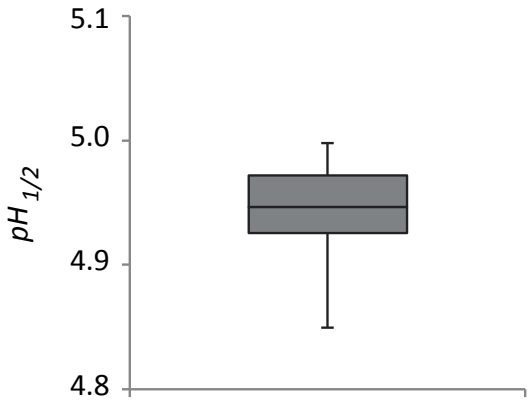

Figure S2.4. Parameter $pH_{1/2}$ estimated using secondary pH model 11.

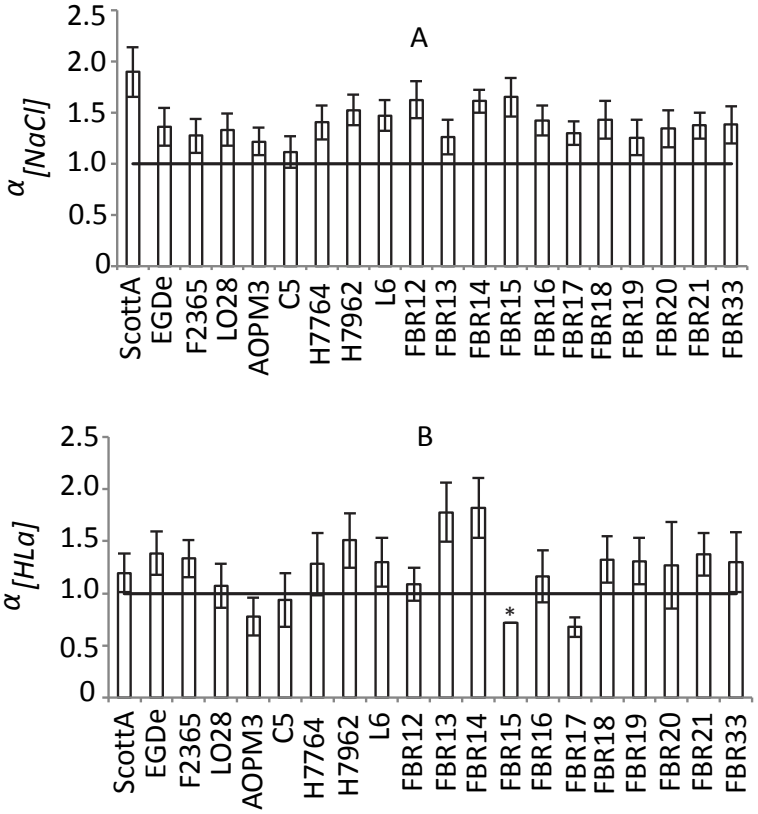

Figure S2.5. Parameter "α" estimated using (A) secondary [NaCl] model 3b, (B) secondary [HLa] model 1. * the confidence interval cannot be estimated.

CHAPTER 3

Quantifying variability on thermal resistance of *Listeria monocytogenes*

D.C. Aryani, H.M.W. den Besten, W.C. Hazeleger and M.H. Zwietering

Published in International Journal of Food Microbiology 193, 130-138

ABSTRACT

Knowledge on the impact of strain variability and growth history on thermal resistance is needed to provide a realistic prediction and adequate design of thermal treatments. In the present study, apart from quantifying strain variability on thermal resistance of *Listeria monocytogenes*, also biological (reproduction) and experimental variabilities were determined to prioritize their importance. Experimental variability was defined as the repeatability of parallel experimental replicates and biological (reproduction) variability was defined as the reproducibility of biologically independent reproductions. Furthermore, the effect of growth history was quantified. The thermal inactivation curves of 20 *L. monocytogenes* strains were fitted using the modified Weibull model, resulting in total 360 *D*-value estimates. The *D*-value ranged from 9 to 30 minutes at 55°C; from 0.6 to 4 minutes at 60°C; and from 0.08 to 0.6 minutes at 65°C. The estimated *z*-values of all strains ranged from 4.4°C to 5.8°C. The strain variability was ten times higher than experimental variability and four times higher than biological variability. Furthermore, the effect of growth history on thermal resistance variability was not significantly different from strain variability, and was mainly determined by the growth phase.

INTRODUCTION

Thermal treatment is widely used in food processing, and aims to eliminate spoilage microorganisms and pathogens in food products. The treatment is generally determined by the thermal resistance profiles of the most resistant target microorganism. The *D*-value is known as the time needed to reduce the bacterial concentration by one log cycle; while the *z*-value is the temperature increase needed to reduce the *D*-value by a factor of ten. The thermal resistance of bacterial cells varies and product formulation, such as salt concentration, pH and fat content can alter the thermal resistance profiles of bacteria. Therefore, it is important to know the factors that influence the variability of thermal resistance and their magnitude in order to design an adequate thermal treatment for a specific food product.

Many factors have been considered to contribute to the variability in thermal resistance of microorganisms, amongst others, strain differences, physiological state of the cell, growth condition, and test condition (Bayles et al., 2000; Doyle et al., 2001; Jørgensen et al., 1996; Kilstrup et al., 1997; Martínez et al., 2003; Miller et al., 2000). Considerable variability of *Listeria monocytogenes* thermal resistance was observed in a comprehensive meta-analysis study on thermal inactivation parameters (Van Asselt and Zwietering, 2006). Following this meta-analysis study, a multiple regression model for thermal inactivation of *L. monocytogenes* in liquid food products was constructed (Van Lieverloo et al., 2013) as an alternative for the single regression model. In both studies however, the factors reported to influence the *D*-value were smaller than the variability of all published data, except for the effect of high salt concentration.

To date, several studies have focused on investigating the effect of strain variability on thermal resistance of vegetative cells (Benito et al., 1999; De Jesús and Whiting, 2003; Lianou and Koutsoumanis, 2013; Mackey et al., 1990; Ng et al., 1969; Rodríguez-Calleja et al., 2006; Sörqvist, 1994) and spores (Berendsen et al., 2014; Luu-Thi et al., 2014). However, the results between studies, even those using the same species, were different. An older study on *Salmonella* species, as an example, reported that one strain (D_{57} 31 min*)* was 30 times more thermal resistant than the reference strain (D_{57} 1.2 min*)* used in the study (Ng et al., 1969), while a more recent *Salmonella* thermal resistant study reported about a two-fold difference in D_{57}-values between the least and the most thermal resistant strains (Lianou and Koutsoumanis, 2013). Another study on *L. monocytogenes* reported D_{57}-value between 6.5 to 26 min in 27 *L. monocytogenes* strains (Mackey et al., 1990).

While previous studies focused on strain variability, none compared or prioritized the importance of strain, biological, and experimental variability. In the present study, strain

variability is defined as the variability between strains from the same species. Biological (reproduction) variability is defined as the variability between biologically independent reproductions, and experimental variability is defined as the variability between parallel experimental replicates. Moreover, from the literature strain variability and other variability factors, such as growth history and the effect of physiological state of the cells, cannot be easily compared, because available data were generated using different sets of strains. To quantify different variability factors and to compare their magnitudes, a study is needed which uses the same set of strains from the same species. Therefore, our main objectives were to quantify the impact of strain variability on thermal resistance in perspective to biological and experimental variability and variability caused by growth history. The effect of growth history in the present study includes the effect of pre-culturing condition and the physiological state of the cells. Quantification of these variability factors allows to compare their magnitudes and to include them in prediction of thermal inactivation kinetics or in the exposure assessment of risk assessment studies. *L. monocytogenes* was selected as a model organism due to its ability to grow at harsh conditions, such as low temperature and relatively high concentration of salt (Bajard et al., 1996; Van der Veen et al., 2008).

MATERIALS AND METHODS

Culture preparation

Twenty *L. monocytogenes* strains, covering a wide range of origins, were used in this study (table 2.1). The stock cultures were kept frozen at -80°C in 70% brain heart infusion (BHI) broth (Becton Dickinson, France) and 30% glycerol (v/v) (Sigma Aldrich). From the stock culture, a streak was made onto a BHI agar plate (BHI broth with 1.5% (w/v) of bacteriological agar, Oxoid, England) and incubated for 24 h at 30°C. A single colony was inoculated in a 250 ml Erlenmeyer flask pre-filled with 100 ml of BHI broth and incubated until stationary phase for 17 h at 30°C, 200 rpm (Forma Orbital Shakers, Thermo Electron Corporation, USA).

Thermal inactivation experiments

The thermal inactivation experiment was done using a water bath (Julabo SW23, Julabo Labortechnik GmbH, Germany) set at 55°C (the duration was between 84 min for the most thermal sensitive strains to 280 min for the most thermal resistant strain), 60°C (between 6 min to 24 min), and 65°C (between 45 s to 210 s). Three sterile 250 ml flasks were pre-filled with 40 ml of BHI broth and pre-heated in the water bath at the desired temperature. One flask was used to measure the temperature using a thermocouple

(PeakTech 3150, Thermocouple K-type), while the other two were used for the experiment. The stationary phase culture was inoculated 1:100 (v/v, final concentration approximately 10^7 CFU/ml) in the pre-heated BHI to immediately start the inactivation, because in this way the observed temperature drop was negligible (±0.3°C). The same dilution (1:100) in non-heated BHI was also done for time point t=0. At each time point, one ml of sample was diluted in 9 ml of peptone physiological salt (PPS, Tritium Microbiologie), after which further decimal dilutions were made and the appropriate dilution was plated in duplicate onto BHI agar plates using a spiral plater (Eddy Jet, IUL instrument). For the time points with expected low concentrations of viable cells, one ml of sample was transferred into a sterile cup, rapidly cooled down on ice to room temperature, and spread plated onto three BHI agar plates. This method allowed us to have a detection limit of 1 CFU/ml. All plates were incubated for 5 days at 30°C, and the colonies were counted and reported in $\log_{10}$ CFU/ml. Each experiment was conducted in duplicate on the same day and reproduced at least two times on different days to quantify the experimental and the biological variability. The inactivation experiments were conducted using on average eight sampling time points for 55°C, six sampling points for 60°C and five samplings points for 65°C.

Growth history experiments: the effect of pre-culturing conditions and physiological state of the cells

Three strains were selected based on their thermal resistance at 60°C to quantify the effect of growth history on thermal resistance, namely the least thermal resistant strain (ScottA), the most thermal resistant strain (L6), and an intermediate thermal resistant strain (C5). To quantify the effect of pre-culturing condition, we cultured the cells until stationary phase in BHI medium adjusted to certain suboptimal conditions, namely, pH (5 and 6); % NaCl (2.5 and 5% (w/v)); and temperature (7°C and 15°C). For pH experiments, the BHI broth was adjusted to pH 6 and pH 5 using 0.5 M H_2SO_4 (Riedel-de Haën; Seelze, Germany) and then filter sterilized using Steritop 500 ml (Millipore Corporation, USA). For NaCl adjusted medium, 2.5% and 5% of NaCl (AnalaR NORMAPUR, VWR International, Belgium) was added to the medium and sterilized for 15 minutes at 121°C; and for temperature experiments plain BHI broth was used. A single colony from a BHI agar plate was inoculated into 100 ml of BHI medium in a 250 ml flask and incubated for 24 h (pH 6), 42 h (pH 5), 22 h (2.5% NaCl), and 28 h (5% NaCl) at 30°C, 200 rpm. For temperature experiments, the culture was incubated at 200 rpm for 3 days at 15°C, and for 8 days at 7°C. For the control condition cells were grown in BHI medium until the stationary phase. Furthermore, to evaluate the effect of physiological state on thermal resistance of *L. monocytogenes*, cells were also sub cultured in BHI broth at 30°C until the exponential

phase (OD_{600}=0.5). All cultures were inactivated at 60°C as described in previous section of thermal inactivation experiments.

Estimation of *D*- and *z*-value

The $\log_{10}$ surviving counts were plotted against the inactivation time to obtain the thermal inactivation curve of each inactivation experiment. The modified Weibull model (Metselaar et al., 2013) was used to fit each thermal inactivation curve and to estimate the 6 decimal reduction time, which is the time to reach 6 $\log_{10}$ reduction (equation (3.1)). The *6D* is selected because the reduction was within the experimental range and is of relevance for pathogen inactivation (FDA, 2004). The modified Weibull model allows to fit linear, concave, and convex inactivation curves and is able to fit the different thermal inactivation curves of the strains. Points below the detection limit were not included in the fitting procedures.

$$\mathrm{Log}_{10}\, N_t = \mathrm{Log}_{10}\, N_0 - 6\left(\frac{t}{6D}\right)^{\beta} \qquad [3.1]$$

Where $\mathrm{Log}_{10}\, N_t$ is $\log_{10}$ number of surviving organism ($\log_{10}$ CFU/mL) at time *t*; $\mathrm{Log}_{10}\, N_0$ is $\log_{10}$ initial number ($\log_{10}$ CFU/mL); t is time (min/s); 6*D* is the time to reach 6 $\log_{10}$ reduction (min/s); and β is the shape parameter. The fitting procedure was done using Microsoft Solver Add-in and was confirmed using TableCurve 2Dv5.1. The *D*-value was calculated as 6*D*/6. The *D*-values among strains for each temperature were compared using Anova, followed by a post hoc (Tukey) test to classify strains based on their heat resistance.

The *D*-values were used for calculating the *z*-value per strain as being the negative reciprocal of the linear regression slope between the $\log_{10}$ *D*-values (six values per temperature) and temperatures. For *z*-value calculation, additional thermal inactivation experiments were performed for strain L6 (i.e the most heat resistant strain) at 62°C and 70°C.

Quantifying variability

For quantifying experimental variability, biological (reproduction) variability, and strain variability, the *D*-values were tabulated according to strain. Experimental, reproduction, and strain variability were determined for each temperature and for all temperatures combined using equations (3.2)-(3.4) following the scheme in figure 3.1.

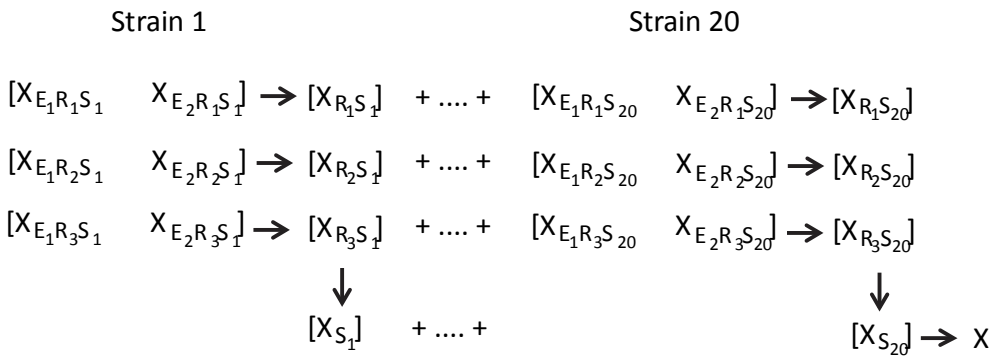

Figure 3.1. Schematic figure to quantify Experimental (E), Reproduction (R), and Strain (S) variability. X_{ERS} is the $\log_{10}$ *D*-value obtained for experiment "E", biological reproduction "R", and strain "S". X_{RS} is the average of two X_{ERS} obtained on the same day for strain "S", X_S is the average of X_{BS} from three different experimental days for strain "S", and *X* is the average $\log_{10}$ *D*-value of all strains.

Experimental variability:

$$MSE = \frac{RSS}{DF} = \frac{\sum_{S=1}^{20}\sum_{R=1}^{3}\sum_{E=1}^{2}(X_{ERS}-X_{RS})^2}{n-p} \quad [3.2]$$

Where MSE is mean square error, X_{ERS} is the $\log_{10}$ *D*-value (min) of each experiment "E", biological reproduction "R" and strain "S", X_{RS} is the average $\log_{10}$ *D*-value (min) of duplicate experiments obtained on the same day for strain "S", and *DF* is the number of data points (*n*=6*20) minus the number of parameters (*p*=3*20), which is 60 per temperature or 180 for three temperatures.

Reproduction variability:

$$MSE = \frac{RSS}{DF} = \frac{\sum_{S=1}^{20}\sum_{R=1}^{3}(X_{RS}-X_S)^2}{n-p} \quad [3.3]$$

Where X_{RS} is the average $\log_{10}$ *D*-value (min) of duplicate experiments obtained on the same day for strain "S", X_S is the average of X_{RS} from three different experimental days for strain "S", and *DF* is the number of data points (*n*=3*20) minus the number of parameters (*p*=1*20), which is 40 per temperature or 120 for three temperatures.

Strain variability:

$$MSE = \frac{RSS}{DF} = \frac{\sum_{S=1}^{20}(X_S-X)^2}{n-p} \quad [3.4]$$

Where X_S is the average of X_{BS} from three different experimental days for strain "S", *X* is the average X_S of all 20 strains, and *DF* is the number of data points (*n*=20) minus the number of parameters (*p*=1), which is 19 per temperature or 57 for three temperatures.

The *F*-test was used to compare strain variability, reproduction variability, and experimental variability (equation (3.5))

$$F = \frac{MSE_1}{MSE_2} \quad [3.5]$$

Where MSE_1 is the mean square error of the first variability factor and MSE_2 is the mean square error of the second variability factor. The *F*-test result was compared to the *F*-critical value according to an alpha of 0.05. To quantify the effect of strain variability and growth history on heat resistance at 60°C using three strains of *Listeria monocytogenes*, the data were tabulated according to strain and growth history condition. The variability factors were calculated using equations (3.6)-(3.7) following the scheme in figure 3.2.

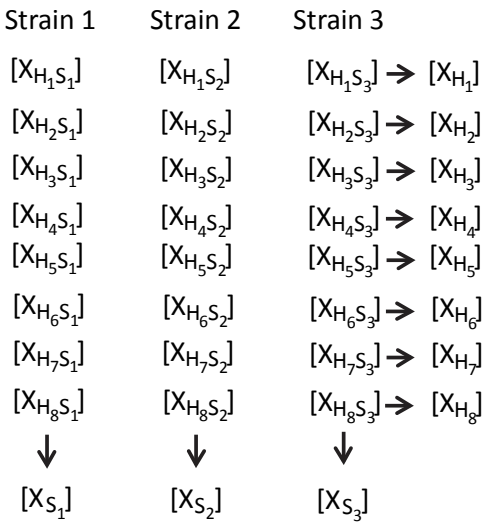

Figure 3.2. Schematic figure to quantify the effect of strain and growth history for thermal inactivation at 60°C. X_{HS} is the average $\log_{10}$ D_{60}-value for growth history "H" for strain "S", X_H is the average $\log_{10}$ D_{60}-value for growth history "H" for all three strains, and X_S is the average $\log_{10}$ D_{60}-value for strain "S" for all eight growth history factors. The eight growth history factors are stationary phase, exponential phase, pH 6 and 5, 2.5% and 5% NaCl, and temperature 7°C and 15°C.

Strain variability at 60°C using 3 strains:

$$MSE = \frac{RSS}{DF} = \frac{\sum_{S=1}^{3}\sum_{H=1}^{8}(X_{HS}-X_H)^2}{n-p} \quad [3.6]$$

Where X_{HS} is the average of $\log_{10}$ D_{60}-value for growth history "H" for strain "S"; X_H is the average of $\log_{10}$ D_{60}-value for growth history "H" for all three strains; and *DF* is the degrees of freedom from *n* number of data points minus *p* number of parameters (*DF*=24-8).

Growth history effect at 60°C using 3 strains:

$$MSE = \frac{RSS}{DF} = \frac{\sum_{H=1}^{8}\sum_{S=1}^{3}(X_{HS}-X_S)^2}{n-p} \quad [3.7]$$

Where X_S is the average of $\log_{10}$ D_{60}-value for strain "S" for all growth history factors; X_{HS} is the average of $\log_{10}$ D_{60}-value for growth history "H" for strain "S"; and *DF* is the degrees of freedom from *n* number of data points minus *p* number of parameters (*DF*=24-3).

To investigate which growth history factor mostly influenced the variability per strain, a multiple linear regression was used (equation (3.8)).

$$Y = \check{Y} + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_6 X_6 + \beta_7 X_7 \quad [3.8]$$

Where Y is the obtained $\log_{10}$ *D*-values for all growth history factors, $\check{Y}$ is the average $\log_{10}$ *D*-value at optimum condition (stationary phase cells), β is the coefficient of each independent variable *X*, and *X* is each growth history factor (either 0 or 1). The results of the multiple linear regression were also compared to the results of student *t*-test to confirm the influence of a specific growth history factor on the *D*-value.

RESULTS

Thermal inactivation kinetics of *L. monocytogenes*

The thermal inactivation curves of 20 *L. monocytogenes* strains at 55°C, 60°C, and 65°C are presented in figure 3.3. The difference between two replicates obtained at the same experimental day (experimental variability) was relatively small at each temperature (figures 3.3A, 3.3D, and 3.3G). The difference between three reproductions (each had two replicates) carried out on different experimental days (biological variability) was higher than differences between replicates (figures 3.3B, 3.3E, and 3.3H). But differences between strains (figures 3.3C, 3.3F, and 3.3I) were much higher than differences between replicates or between reproductions. Moreover, at all temperatures, we observed that strains L6, FBR14, and FBR16 had lower inactivation rates than the other strains, and strains ScottA and LO28, which are often used as model strains (Casadei et al., 1998; Chen and Hoover, 2003; Gaze et al., 1989; Stephens et al., 1994; Sumner et al., 1991; Van Boeijen et al., 2011), were amongst the most heat sensitive strains at 60°C and 65°C.

To estimate the *D*-value, the inactivation curves were fitted using a modified Weibull model. The fitting was done for each data set, giving a total of 360 *D*-value estimates. The *D*-values of the twenty *L. monocytogenes* strains ranged from 9 to 30 minutes at 55°C, from 0.6 to 4 minutes at 60°C, and from 0.08 to 0.6 minutes at 65°C. There was a relatively good correlation between the *D*-values obtained at the different temperatures (R^2=0.93 (60°C and 65°C), 0.87 (65°C and 55°C), 0.87 (65°C and 55°C)) meaning that the strain having a high *D*-value at 65°C was also found to have a high *D*-value at 60°C or 55°C. Further analysis using Anova and post hoc test showed that strain L6 had the highest *D*-

values from all strains at all temperatures, and it was followed by strains FBR14 and FBR16.

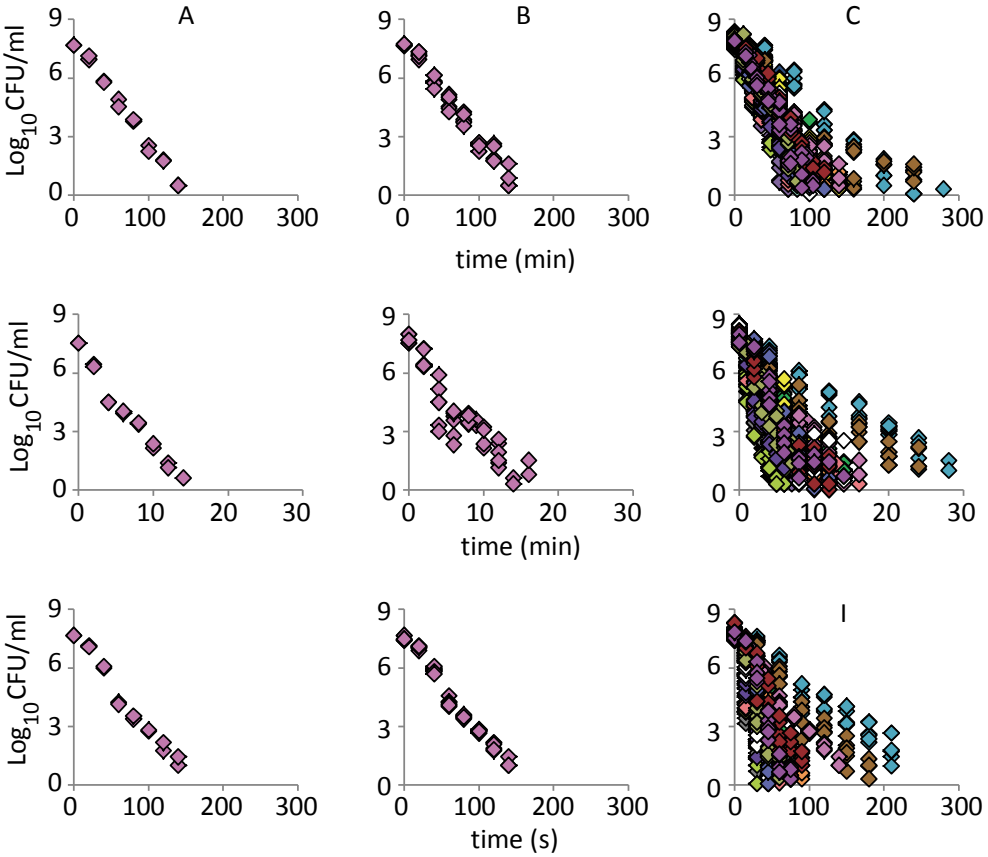

Figure 3.3. Thermal inactivation curves of twenty *L. monocytogenes* strains at 55°C (A-C), 60°C (D-F), 65°C (G-I). Figure A, D, G are the thermal inactivation curves of FBR16 from the same experimental day; figure B, E, H are the thermal inactivation curves of FBR16 from three different experimental days. In Figure C, F, and I: Scott A, LO28, EGDe, F2365, AOPM3, C5, H7764, H7962, L6, FBR12, FBR13, FBR14; FBR15; FBR16; FBR17; FBR18; FBR19; FBR20; FBR21; FBR33.

The *D*-values per strain were used to estimate the *z*-value by taking the negative reciprocal of the slope of the linear regression between $\log_{10}$ *D*-values and temperature (figure 3.4). The linear regression for strain L6, the most heat resistant strain, was based

on five temperatures of 55°C, 60°C, 62°C, 65°C, and 70°C, while the linear regression of the other strains was based on three temperatures. The estimated *z*-values (table 3.1) ranged from 4.4°C to 5.8°C.

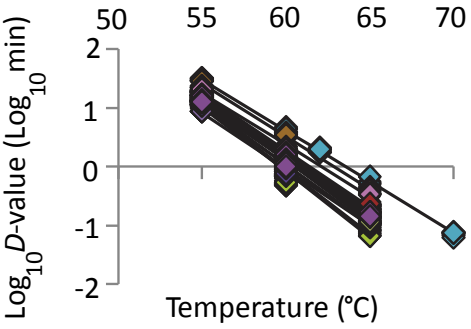

Figure 3.4. The linear regression between $\log_{10}$ *D*-value and temperature used to estimate the *z*-value of each *Listeria monocytogenes* strain: Scott A, LO28, EGDe, F2365, AOPM3, C5, H7764, H7962, L6, FBR12, FBR13, FBR14, FBR15, FBR16, FBR17; FBR18, FBR19, FBR20, FBR21 and FBR33.

Table 3.1. The average *D*-values of twenty *Listeria monocytogenes* strains at 55°C, 60°C, and 65°C and the estimated *z*-values

Strain	*D*-value (min)*			*z*-value (°C)**
	55°C	60°C	65°C	
ScottA	13.1 (1.2) ^{c,d,e***}	0.58 (0.094)^{a}	0.084 (0.011)^{a}	4.5 (4.2-4.9)
LO28	13.3 (0.68)^{h,i}	0.59 (0.13)^{a,b}	0.075 (0.0067)^{a}	4.4 (4.1-4.8)
EGDe	18.5 (0.36)^{h,i}	1.6 (0.30)^{f,g,h}	0.17 (0.022)^{e,f,g}	4.9 (4.7-5.1)
F2365	18.7 (1.1)^{d,e}	1.7 (0.18)^{g,h}	0.20 (0.032)^{f,g}	5.1 (4.9-5.2)
AOPM3	16.4 (0.20)^{f,g,h}	1.4 (0.041)^{e,f,g}	0.16 (0.029)^{d,e,f}	5.0 (4.8-5.1)
C5	18.4 (0.46)^{g,h,i}	1.6 (0.27)^{f,g,h}	0.22 (0.0095)^{g}	5.2 (5.0-5.4)
H7764	9.18 (0.79)^{a}	0.76 (0.080)^{a,b,c}	0.11 (0.0048)^{b,c}	5.2 (5.0-5.4)
H7962	13.4 (0.50)^{d,e}	1.2 (0.21)^{d,e}	0.17 (0.013)^{e,f,g}	5.3 (5.0-5.5)
L6	30.2 (1.2)^{k}	4.1 (0.41)^{i}	0.57 (0.080)^{i}	5.7 (5.6-5.8)
FBR12	13.3 (0.32)^{d,e}	0.78 (0.093)^{b,c}	0.12 (0.014)^{c}	4.9 (4.5-5.2)
FBR13	13.4 (0.90)^{d,e}	0.62 (0.11)^{a,b}	0.086 (0.022)^{a,b}	4.5 (4.2-4.9)
FBR14	24.5 (3.2)^{j}	3.4 (0.23)^{i}	0.41 (0.027)^{h}	5.6 (5.5-5.8)
FBR15	11.4 (0.37)^{b}	1.5 (0.10)^{e,f,g}	0.14 (0.028)^{c,d,e}	5.2 (5.0-5.5)
FBR16	19.8 (0.98)^{i}	2.0 (0.15)^{h}	0.34 (0.0088)^{h}	5.7 (5.4-5.9)
FBR17	11.5 (0.48)^{b,c}	0.92 (0.17)^{c,d}	0.11 (0.021)^{c}	5.0 (4.7-5.2)

Strain	*D*-value (min)*			*z*-value (°C)**
	55°C	60°C	65°C	
FBR18	14.7 (1.4)[e,f]	1.2 (0.10)[e,f]	0.12 (0.015)[c]	4.8 (4.6-4.9)
FBR19	13.8 (1.3)[d,e]	1.3 (0.10)[e,f,g]	0.12 (0.011)[c,d]	4.9 (4.8-5.0)
FBR20	12.8 (0.36)[b,c,d]	1.1 (0.15)[d,e]	0.14 (0.013)[c,d,e]	5.1 (4.9-5.2)
FBR21	16.1 (0.53)[f,g]	1.2 (0.16)[d,e]	0.19 (0.028)[f,g]	5.2 (4.8-5.5)
FBR33	14.9 (1.6)[e,f]	1.2 (0.19)[e,f]	0.16 (0.013)[d,e,f]	5.1 (4.9-5.3)
Average ****	15.9	1.4	0.18	5.1
Standard deviation*****	4.9	0.88	0.12	0.35

* value within bracket is the standard deviation.

** value within bracket is 95% confidence interval.

*** similar code shows comparable thermal resistance.

**** value is the average of 20 strains.

***** value is the standard deviation of 20 strains.

Variability in thermal inactivation profile of twenty *L. monocytogenes* strains

Effect of experimental, biological (reproduction) and strain variability

As expected, when the *D*-value data were plotted against temperature, the variability increased with the decrease in temperature. Thus, the assumption for statistical analysis that the variance was equal over the temperature range was not met. Therefore, before using the *D*-value data for variability calculation, transformation to $\log_{10}$ *D*-value was done to normalize the data. Figures 3.5A, 3.5B, and 3.5C show the calculated variability using the $\log_{10}$ *D*-value at 55°C, 60°C, and 65°C and figure 3.5D shows the calculated variability when the $\log_{10}$ *D*-value from all temperatures were combined. In general strain variability was much larger than experimental and biological variability. Strain variability at all conditions was ten times higher than experimental variability and four times higher than reproduction variability.

Effect of growth history: pre-culturing conditions and physiological state of the cells

The results showed that except for the exponential-phase cells, the effect of growth history on thermal inactivation kinetics was strain dependent (figure 3.6 and supplement table 3.1). Strain ScottA pre-cultured until the stationary phase in low pH medium, high NaCl medium or at low temperatures was significantly more resistant than when pre-cultured in optimum medium (figures 3.6A and 3.6D). In contrast, strains C5 and L6 pre-cultured at low temperatures were significantly less resistant than cells pre-cultured at 30°C (figures 3.6B, 3.6C, 3.6E and 3.6F). However, strains C5 and L6 pre-cultured in 2.5% and 5% NaCl had similar thermal resistance to cells pre-cultured in optimum conditions (figures 3.6B, 3.6C, 3.6E and 3.6F). The effect of pre-culturing at low pH, however, was

different for both strains. Strain C5 was significantly less resistant when it was pre-cultured at pH 5, while strain L6 was significantly less resistant when it was pre-cultured at pH 6. While the effect of pre-incubation condition was strain dependent, the results showed that for all three strains, exponential phase cells had a much lower thermal resistance than that of stationary phase cells. Furthermore, both multiple linear regression (supplement table 3.2) and *t*-test analyses showed that exponential-phase cells had the highest effect on *D*-value for strains L6 and C5. Although exponential-phase cells also had a significant effect on *D*-value for ScottA, this effect was less than the effect of pH.

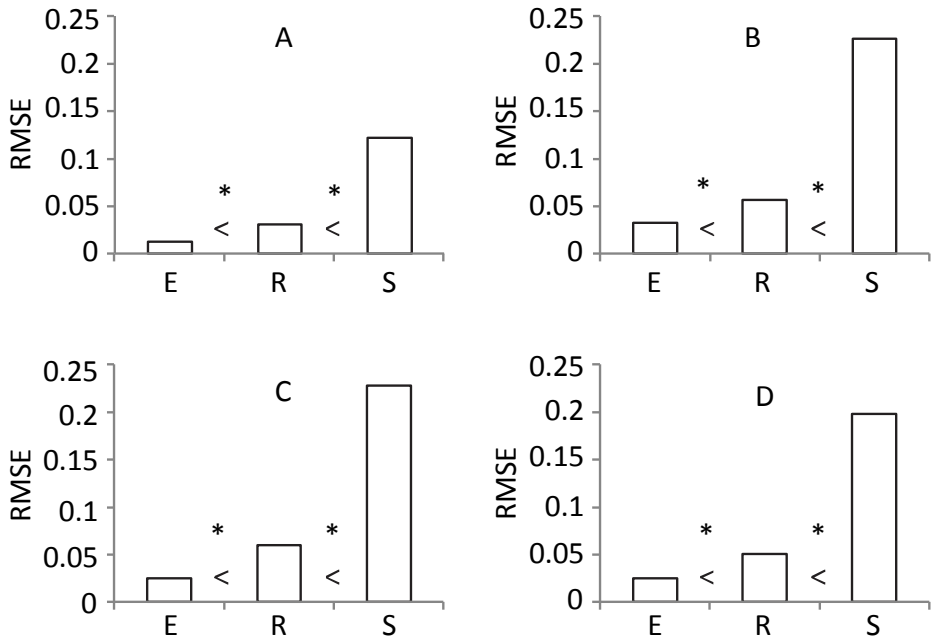

Figure 3.5. Experimental (E), Reproduction (R), and Strain (S) variability expressed in RMSE (Root Mean Square Error) which was calculated using $\log_{10}$ *D*-value at (A) 55°C, (B) 60°C, (C) 65°C, and (D) all three temperatures. The RMSE is the root mean squared differences between each experiment "E", or between biological reproductions "R", or between strains "S". * *p* value is less than 0.0001.

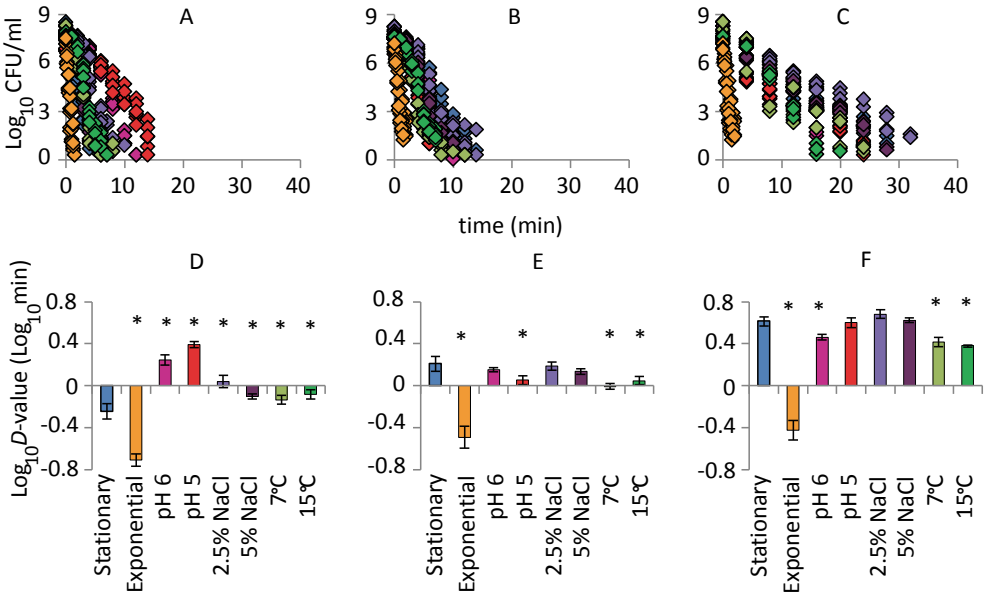

Figure 3.6. Effect of growth history on thermal resistance of ScottA (A, D), C5 (B, E), and L6 (C, F) at 60°C. The bottom figures are the $\log_{10}$ *D*-values fitted using modified Weibull model from the respective curves of ScottA (D), C5 (E), and L6 (F). ◇ stationary-phase cells, ◇ exponential-phase cells, ◇ pH 6, ◇ pH 5, ◇ NaCl 2.5%, ◇ NaCl 5%, ◇ 7°C, ◇ 15°C. * the value was significantly different from the cells pre-cultured until stationary phase in optimum condition (30°C).

The $\log_{10}$ transformed D_{60}-values of the three strains from different growth conditions were used to compare variability caused by strain and by growth history. In general, the effect of strain on the variability of thermal resistance was similar to the effect of growth history (figure 3.7). However, when the effect of the exponential cells was not taken into account, the effect of strain on the variability of thermal resistance was significantly higher than the effect of growth history.

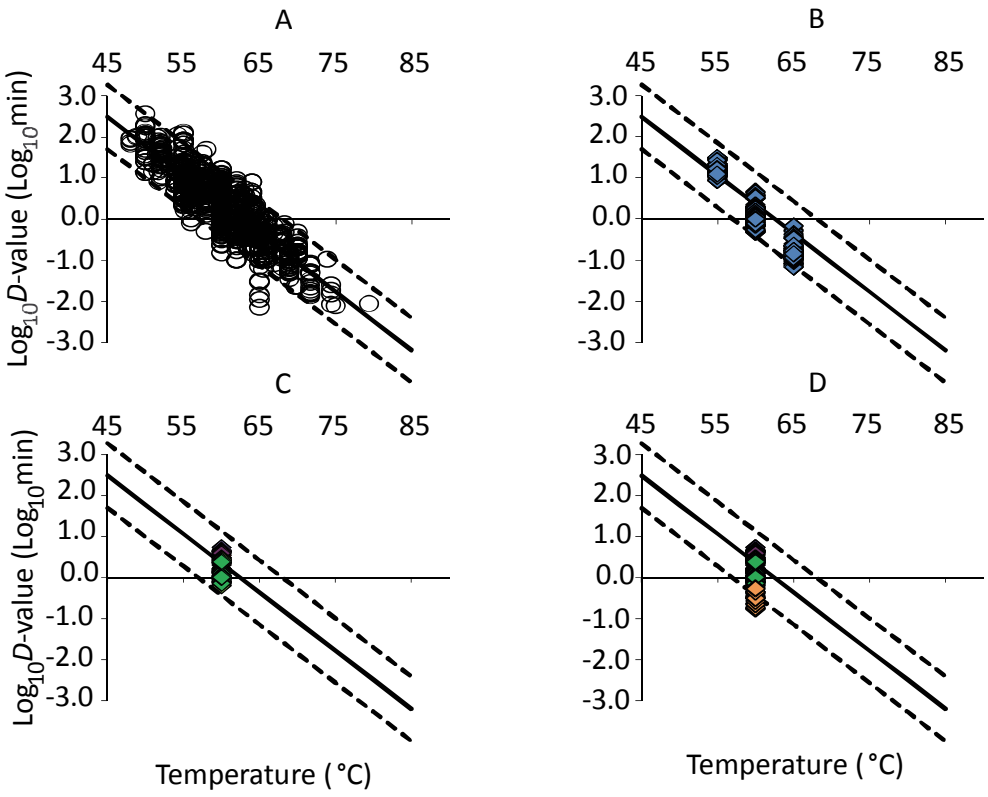

Figure 3.7. Variability in thermal resistance of *Listeria monocytogenes*: A) thermal resistance data collected in the meta-analysis study of Van Asselt and Zwietering (2006); B) the effect of experimental, biological and strain variability based on 20 strains; C) the effect of growth history (pH, NaCl, and temperature) based on 3 strains; and D) the effect of growth history and physiological state based on 3 strains. The solid and the dotted lines in figures A-D are the average and 95% prediction interval of $\log_{10}$ *D*-value from the meta-analysis study of Van Asselt and Zwietering (2006) (thermal resistance data in the presence of high concentration of salt were not included). O literature data, ◇ Stationary-phase cells, ◇ exponential-phase cells, ◇ pH 6, ◇ pH 5, ◇ NaCl 2.5%, ◇ NaCl 5%, ◇ 7°C, ◇ 15°C.

DISCUSSION

Knowledge on the sources of variability is needed to generate realistic estimates of thermal inactivation parameters. The replications and reproductions made and the use of twenty strains allowed us to quantify experimental, biological, and strain variability as sources of variability. With a broad selection of *L. monocytogenes* strains we observed strain to strain variation with respect to their thermal resistance. Interestingly, the origin of the strains seemed to have no relation with the thermal resistance profiles of the strains. For example the most thermal resistant strains L6, FBR14, and FBR16 were isolated from different food products, and not all human isolates were in the group of the least thermal resistant strains. The strain variability found in our study at 60°C was higher than previously reported using twelve strains of *L. monocytogenes* (Sörqvist, 1994). However, in the latter study no discrimination was made between strain, biological, and experimental variability, although replication of each experiment was reported. Using the temperature range of 55°C, 60°C, and 65°C, we estimated the *z*-value to be in the range of 4.4°C - 5.8°C. These ranges were smaller than the results of previous studies, which reported *z*-values between 5°C - 7°C (Doyle et al., 2001; Van Asselt and Zwietering, 2006), and between 4.8°C - 7.1°C based on narrow temperature ranges, namely 58°C, 60°C and 62°C or 60°C, 62°C and 64°C (Sörqvist, 1994). Wide temperature ranges might assure the accuracy of the estimated *z*-values and this is especially relevant for the most heat resistant strains.

Although many studies are available on thermal inactivation kinetics of *L. monocytogenes*, little is known about the magnitude of strain variability in perspective to biological and experimental variability. Strain variability, calculated on $\log_{10}$ scale, in the present study was found to be four times higher than biological variability and ten times higher than experimental variability. This result was in agreement with the result of an *Escherichia coli* study where the magnitude of strain variation was greater than the uncertainties calculated from experimental procedures using single strains or cocktails (Whiting and Golden, 2002). In contrast, strain variability was reported to be similar to the biological variability in another study using *L. monocytogenes* (De Jesús and Whiting, 2003). Similarity between strain variability and biological variability was also reported from a recent publication using *Salmonella enterica* strains, where the coefficient of variation (CV) of strain variability on specific inactivation rate (k_{heat}) was 18.3% compared to the CV among replicates of 10.2±6.5% (Lianou and Koutsoumanis, 2013).

In the current study, experimental, biological, and strain variability were calculated using estimated $\log_{10}$ *D*-values, which were obtained from fitting the modified Weibull model. The goodness of fit of the model was evaluated using the mean square residual (MSE)

between the observed and the fitted data (Den Besten et al., 2006). The average MSE value was between 0.17 - 0.27 (supplement table 3.3). The MSE value obtained from the model fitting cannot be directly compared to the variability calculated using the estimated $\log_{10}$ *D*-value. However, the MSE obtained from the model fitting was not only influenced by the model, but also by the accuracy of the method used to obtain the thermal inactivation data, namely plate counting error and pipetting error.

Besides strain differences, the physiological state of the cells and growth history are known to influence the thermal resistance of *L. monocytogenes*. However, no study compared structurally the magnitude of various growth history effects on thermal resistance variability. It is generally known that exponential phase cells are more sensitive than stationary phase cells (Doyle et al., 2001; Martínez et al., 2003; Ng et al., 1969). In the present study, the thermal resistance of the stationary-phase cells were four to eleven times higher than that of exponential phase cells (figure 3.6). Moreover, the enhanced thermal resistance of cells when pre-adapted to certain stresses has been reported (Den Besten et al., 2006; Den Besten et al., 2010; Farber and Pagotto, 1992), and was explained by stress-induced adaptation (Lou and Yousef, 1996), which might provide (cross) protection to other stresses (Lou and Yousef, 1996). In the present study the effect of acid adaptation on thermal resistance showed to be strain dependent. Although some studies reported that exposure to acid by means of acid adaptation and acid shock prior to heating increased the thermal resistant of *L. monocytogenes*, *Salmonella*, or *E. coli* (Álvarez-Ordóñez et al., 2008; Farber and Pagotto, 1992; Leyer and Johnson, 1993; Mazzotta, 2001), these results were not considered as conclusive since decreased thermal resistance was also observed for *L. monocytogenes* which were acid shocked for longer than one hour before heating at 58°C (Farber and Pagotto, 1992).

Similar to acid, the effect of sodium chloride was strain dependent. Enhanced thermal resistance was observed only for ScottA grown in the medium supplemented with 2.5% and 5% of sodium chloride. The effect of sodium chloride, however, was not as high as the effect of pH, because the adaptation only resulted in between 1.3 to 1.9 times increase in D_{60}-value. The increase in heat resistance after adaptation to salt was also reported for *Bacillus cereus* by Den Besten et al. (2006). As for acid stress, salt stress exposure was found to be able to induce heat shock proteins, amongst others DnaK (Kilstrup et al., 1997; Meury and Kohiyama, 1991), which was found to be responsible for the increased thermo tolerance of *E. coli* (Delaney, 1990) and was also induced during heat shock experiments in *L. monocytogenes* EGDe (Van der Veen et al., 2007). The degree of thermal resistance of salt adapted cells, however, was influenced by the physiological state of the cells as shown in the study of Den Besten et al. (2006). In their study, the increase in heat resistance was less pronounced for transition and stationary-phase cells than for exponential-phase cells.

In our study, the stress adapted cells were all in stationary-phase, which can be the explanation for the less pronounced effect of mild stress adaptation on thermal resistance.

Pre-culturing of strains C5 and L6 at low temperature (15°C and 7°C) decreased the D_{60}-value. This result was in agreement with the study of Álvarez-Ordóñez et al. (2008) who showed that the thermal resistance of *Salmonella* Typhimurium was maximal in cells grown at 45°C and decreased with the decrease in pre-culturing growth temperature to 10°C. The reduction in D_{60}-value by 13%-37% was also reported when *L. monocytogenes* was cold shocked at 0°C for 3 h (Miller et al., 2000). In contrast to C5 and L6, pre-culturing ScottA at low temperatures significantly enhanced its thermal resistance at 60°C. This result might provide the indication that pre-incubation at low temperature increases the thermal resistance of thermal sensitive strains. Interestingly, no study reported the increase in thermal resistance when the cells were pre-cultured at low temperature until stationary phase. The increase in thermal resistance was reported for young cultures (lag phase) of *Streptococcus faecalis* (White, 1953), but the thermal resistance decreased upon the cells entering the exponential phase. Another study reported two-fold increase in thermal resistance of *Aeromonas hydrophila* when 48 h cells incubated at 30°C were further incubated at 7°C for 72 h (Condon et al., 1992). Therefore, investigating the effect of low temperature pre-culturing for the other thermal sensitive strains might be of interest.

In general, our study showed that strain differences and growth history had similar influence on the variability of thermal resistance of *L. monocytogenes*. However, the high differences in *D*-values between stationary and exponential cells influenced the overall effect of growth history on variability. Without the effect of exponential cells, the effect of growth history on variability was significantly less than the effect of strain variability.

To extend our knowledge on these sources of variability, we benchmarked our results with the available thermal resistance data of *L. monocytogenes* found in literature (Van Asselt and Zwietering, 2006) (figure 3.7A). The variability of the thermal resistance data was presented as the average and its prediction interval (the solid and dotted lines in figures 3.7A - 3.7D). The average and its prediction interval were calculated based on *D*-values from various data sets, with different strains, conditions, and experimental methods, excluding those coming from the treatments using high percentage of salt (figure 3.7A). When all our data collected in this study were plotted into the meta-analysis data of *L. monocytogenes* (Van Asselt and Zwietering, 2006) (figure 3.7A), the effect of strain alone was able to explain between 1/2 (55°C) to 2/3 (60°C and 65°C) of the variability found in literature (figure 3.7B). When the effect of growth history excluding exponential cells at 60°C was added, almost no difference with the effect of strain alone was seen (figure

3.7C). When also the effect of exponential cells was included at 60°C, the variability in our set of data had an almost equal band width compared to the literature data, although there was a slight bias (figure 3.7D).

CONCLUSIONS

The thermal resistance of *L. monocytogenes* was influenced by strain differences and growth history. However, except for the exponential-growth phase that resulted in the most thermal sensitive cells for all tested strains, the effect of different growth history was strain dependent. Strain to strain variability was found to be four times higher than biological variability and ten times higher than experimental variability. The effect of strain variability on thermal resistance at 60°C was similar to the effect of growth history, which was mainly determined by the effect of growth state (stationary and exponential cells). Further study on the effect of other factors such as the synergistic effect of pre-culturing conditions, heating menstruum and food matrix may be a valuable complement to explain the remaining variability reported in literature. Additionally, the investigation of different pre-culturing conditions for exponential cells is also of interest to provide a better comparison to those evaluated in the stationary phase.

ACKNOWLEDGMENTS

We would like to thank Sylvia Sari Barkey, Chenxi Zhang, and Vasiliki Akridopoulou for their assistance during experimental work, and Gerard Weenk of Top Institute for Food and Nutrition (TIFN) and Danone Schiphol, The Netherlands for his managerial role in the TIFN modelling project.
The research is funded by TI Food and Nutrition, a public-private partnership on precompetitive research in food and nutrition. The funders had no role in study design, data collection and analysis, or preparation of the manuscript.

REFERENCES

Álvarez-Ordóñez, A., Fernández, A., López, M., Arenas, R., Bernardo, A. 2008. Modifications in membrane fatty acid composition of *Salmonella* Typhimurium in response to growth conditions and their effect on heat resistance. International Journal of Food Microbiology 123, 212-219.

Bajard, S., Rosso, L., Fardel, G., Flandrois, J.P. 1996. The particular behaviour of *Listeria monocytogenes* under sub-optimal conditions. International Journal of Food Microbiology 29, 201-211.

Bayles, D.O., Tunick, M.H., Foglia, T.A., Miller, A.J. 2000. Cold shock and its effect on ribosomes and thermal tolerance in *Listeria monocytogenes*. Applied and Environmental Microbiology 66, 4351-4355.

Benito, A., Ventoura, G., Casadei, M., Robinson, T., Mackey, B. 1999. Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat, and other stresses. Applied and Environmental Microbiology 65, 1564-1569.

Berendsen, E.M., Zwietering, M.H., Kuipers, O.P., Wells-Bennik, M.H.J. 2014. Two distinct groups within the *Bacillus subtilis* group display significantly different spore heat resistance properties. Food Microbiology 45, Part A, 18-25.

Casadei, M.A., Esteves de Matos, R., Harrison, S.T., Gaze, J.E. 1998. Heat resistance of *Listeria monocytogenes* in dairy products as affected by the growth medium. Journal of Applied Microbiology 84, 234-239.

Chen, H., Hoover, D.G. 2003. Modeling the combined effect of high hydrostatic pressure and mild heat on the inactivation kinetics of *Listeria monocytogenes* Scott A in whole milk. Innovative Food Science & Emerging Technologies 4, 25-34.

Condon, S., Garcia, M.L., Otero, A., Sala, F.J. 1992. Effect of culture age, pre-incubation at low temperature and pH on the thermal resistance of *Aeromonas hydrophila*. Journal of Applied Bacteriology 72, 322-326.

De Jesús, A.J., Whiting, R.C. 2003. Thermal inactivation, growth, and survival studies of *Listeria monocytogenes* strains belonging to three distinct genotypic lineages. Journal of Food Protection 66, 1611-1617.

Delaney, J.M. 1990. Requirement of the *Escherichia coli dnaK* gene for thermotolerance and protection against H_2O_2. Journal of General Microbiology 136, 2113-2118.

Den Besten, H.M.W., Mataragas, M., Moezelaar, R., Abee, T., Zwietering, M.H. 2006. Quantification of the effects of salt stress and physiological state on thermotolerance of *Bacillus cereus* ATCC 10987 and ATCC 14579. Applied and Environmental Microbiology 72, 5884-5894.

Den Besten, H.M.W., van der Mark, E.-J., Hensen, L., Abee, T., Zwietering, M.H. 2010. Quantification of the effect of culturing temperature on salt-induced heat resistance of *Bacillus* species. Applied and Environmental Microbiology 76, 4286-4292.

Doyle, M.E., Mazzotta, A.S., Wang, T., Wiseman, D.W., Scott, V.N. 2001. Heat resistance of *Listeria monocytogenes*. Journal of Food Protection 64, 410-429.

Farber, J.M., Pagotto, F. 1992. The effect of acid shock on the heat resistance of *Listeria monocytogenes*. Letters in Applied Microbiology 15, 197-201.

FDA. 2004. Guidance for industry: recommendations to processors of apple juice or cider on the use of ozone for pathogen reduction purposes [Online]. Available at http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/ucm072524.htm (Accessed: 29 September 2014).

Gaze, J.E., Brown, G.D., Gaskell, D.E., Banks, J.G. 1989. Heat resistance of *Listeria monocytogenes* in homogenates of chicken, beef steak and carrot. Food Microbiology 6, 251-259.

Jørgensen, F., Panaretou, B., Stephens, P.J., Knøchel, S. 1996. Effect of pre- and post-heat shock temperature on the persistence of thermotolerance and heat shock-induced proteins in *Listeria monocytogenes*. Journal of Applied Bacteriology 80, 216-224.

Kilstrup, M., Jacobsen, S., Hammer, K., Vogensen, F.K. 1997. Induction of heat shock proteins DnaK, GroEL, and GroES by salt stress in *Lactococcus lactis*. Applied and Environmental Microbiology 63, 1826-1837.

Leyer, G.J., Johnson, E.A. 1993. Acid adaptation induces cross-protection against environmental stresses in *Salmonella* Typhimurium. Applied and Environmental Microbiology 59, 1842-1847.

Lianou, A., Koutsoumanis, K.P. 2013. Evaluation of the strain variability of *Salmonella enterica* acid and heat resistance. Food Microbiology 34, 259-267.

Lou, Y., Yousef, A.E. 1996. Resistance of *Listeria monocytogenes* to heat after adaptation to environmental stresses. Journal of Food Protection 59, 465-471.

Luu-Thi, H., Khadka, D.B., Michiels, C.W. 2014. Thermal inactivation parameters of spores from different phylogenetic groups of *Bacillus cereus*. International Journal of Food Microbiology 189, 183-188.

Mackey, B.M., Pritchet, C., Norris, A., Mead, G.C. 1990. Heat resistance of *Listeria*: strain differences and effects of meat type and curing salts. Letters in Applied Microbiology 10, 251-255.

Martínez, S., López, M., Bernardo, A. 2003. Thermal inactivation of *Enterococcus faecium*: effect of growth temperature and physiological state of microbial cells. Letters in Applied Microbiology 37, 475-481.

Mazzotta, A.S. 2001. Thermal inactivation of stationary-phase and acid-adapted *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in fruit juices. Journal of Food Protection 64, 315-320.

Metselaar, K.I., Den Besten, H.M.W., Abee, T., Moezelaar, R., Zwietering, M.H. 2013. Isolation and quantification of highly acid resistant variants of *Listeria monocytogenes*. International Journal of Food Microbiology 166, 508-514.

Meury, J., Kohiyama, M. 1991. Role of heat shock protein *DnaK* in osmotic adaptation of *Escherichia coli*. Journal of Bacteriology 173, 4404-4410.

Miller, A.J., Bayles, D.O., Eblen, B.S. 2000. Cold shock induction of thermal sensitivity in *Listeria monocytogenes*. Applied and Environmental Microbiology 66, 4345-4350.

Ng, H., Bayne, H.G., Garibaldi, J.A. 1969. Heat resistance of *Salmonella*: the uniqueness of *Salmonella* Senftenberg 775W. Applied Microbiology 17, 78-82.

Rodríguez-Calleja, J.M., Cebrián, G., Condón, S., Mañas, P. 2006. Variation in resistance of natural isolates of *Staphylococcus aureus* to heat, pulsed electric field and ultrasound under pressure. Journal of Applied Microbiology 100, 1054-1062.

Sörqvist, S. 1994. Heat resistance of different serovars of *Listeria monocytogenes*. Journal of Applied Bacteriology 76, 383-388.

Stephens, P.J., Cole, M.B., Jones, M.V. 1994. Effect of heating rate on the thermal inactivation of *Listeria monocytogenes*. Journal of Applied Bacteriology 77, 702-708.

Sumner, S.S., Sandros, T.M., Harmon, M.C., Scott, V.N., Bernard, D.T. 1991. Heat resistance of *Salmonella* Typhimurium and *Listeria monocytogenes* in sucrose solutions of various water activities. Journal of Food Science 56, 1741-1743.

Van Asselt, E.D., Zwietering, M.H. 2006. A systematic approach to determine global thermal inactivation parameters for various food pathogens. International Journal of Food Microbiology 107, 73-82.

Van Boeijen, I.K.H., Francke, C., Moezelaar, R., Abee, T., Zwietering, M.H. 2011. Isolation of highly heat-resistant *Listeria monocytogenes* variants by use of a kinetic modeling-based sampling scheme. Applied and Environmental Microbiology 77, 2617-2624.

Van der Veen, S., Hain, T., Wouters, J.A., Hossain, H., de Vos, W.M., Abee, T., Chakraborty, T., Wells-Bennik, M.H.J. 2007. The heat-shock response of *Listeria monocytogenes* comprises genes involved in heat shock, cell division, cell wall synthesis, and the SOS response. Microbiology 153, 3593-3607.

Van der Veen, S., Moezelaar, R., Abee, T., Wells-Bennik, M.H.J. 2008. The growth limits of a large number of *Listeria monocytogenes* strains at combinations of stresses show serotype- and niche-specific traits. Journal of Applied Microbiology 105, 1246-1258.

Van Lieverloo, J.H.M., de Roode, M., Fox, M.B., Zwietering, M.H., Wells-Bennik, M.H.J. 2013. Multiple regression model for thermal inactivation of *Listeria monocytogenes* in liquid food products. Food Control 29, 394-400.

White, H.R. 1953. The heat resistance of *Streptococcus faecalis*. Journal of General Microbiology 8, 27-37.

Whiting, R.C., Golden, M.H. 2002. Variation among *Escherichia coli* O157:H7 strains relative to their growth, survival, thermal inactivation, and toxin production in broth. International Journal of Food Microbiology 75, 127-133.

SUPPLEMENTARY MATERIALS

Supplement table 3.1. The thermal resistance of three *Listeria monocytogenes* strains following different pre-culturing conditions and physiological state of the cells

Growth history	*D*-value (min)*		
	Scott A (SD)	C5	L6
1. Pre-culturing condition			
pH 5	2.5 (0.18)	1.1 (0.11)	4.0 (0.43)
pH 6	1.8 (0.21)	1.4 (0.065)	2.9 (0.18)
NaCl 2.5%	1.1 (0.15)	1.5 (0.14)	4.8 (0.46)
NaCl 5%	0.79 (0.046)	1.4 (0.088)	4.2 (0.21)
T 7°C	0.74 (0.071)	0.98 (0.061)	2.6 (0.27)
T 15°C	0.83 (0.081)	1.1 (0.10)	2.4 (0.061)**
2. Physiological state			
Stationary cells	0.58 (0.094)	1.6 (0.27)	4.1 (0.41)
Exponential cells	0.20 (0.028)	0.33 (0.078)	0.38 (0.087)

*Standard deviation of six replicates

**Standard deviation of four replicates

Supplement Table 3.2. Multiple linear regression results for the effect of growth history of strain L6, strain C5 and strain ScottA

Strain L6

	Coefficients	Standard Error	*t* stat	*p*-value	Lower 95%	Upper 95%
Intercept	0.614	0.0197	31.1	$1.16*10^{-28}$	0.574	0.654
Exponential	-1.04	0.0279	-37.2	$1.62*10^{-31}$	-1.090	-0.982
pH 6	-0.152	0.0279	-5.46	$3.14*10^{-6}$	-0.209	-0.0958
pH 5	-0.0139	0.0279	-0.499	0.620	-0.0703	0.0425
NaCl 2.5%	0.0685	0.0279	2.46	0.0187	0.0120	0.125
NaCl 5%	0.00990	0.0279	0.355	0.724	-0.0465	0.0663
7°C	-0.198	0.0279	-7.12	$1.7*10^{-8}$	-0.255	-0.142
15°C	-0.235	0.0312	-7.54	$4.69*10^{-9}$	-0.298	-0.172

Strain C5

	Coefficients	Standard Error	*t* stat	*p*-value	Lower 95%	Upper 95%
Intercept	0.208	0.0220	9.45	$9.67*10^{-12}$	0.164	0.253
Exponential	-0.698	0.0311	-22.4	$2.88*10^{-24}$	-0.761	-0.635
pH 6	-0.0541	0.0311	-1.74	0.0900	-0.117	0.00884
pH 5	-0.155	0.0311	-4.97	$1.32*10^{-5}$	-0.218	-0.0917
NaCl 2.5%	-0.0226	0.0311	-0.725	0.473	-0.0855	0.0404
NaCl 5%	-0.0722	0.0311	-2.32	0.0257	-0.135	-0.00924
7°C	-0.216	0.0311	-6.93	$2.39*10^{-8}$	-0.279	-0.153
15°C	-0.161	0.0311	-5.17	$6.79*10^{-6}$	-0.224	-0.0982

Strain ScottA

	Coefficients	Standard Error	*t* stat	*p*-value	Lower 95%	Upper 95%
Intercept	-0.244	0.0203	-12.0	$7.17*10^{-15}$	-0.285	-0.203
Exponential	-0.464	0.0287	-16.2	$3.61*10^{-19}$	-0.522	-0.407
pH 6	0.490	0.0287	17.1	$5.43*10^{-20}$	0.432	0.548
pH 5	0.635	0.0287	22.2	$4.49*10^{-24}$	0.577	0.693
NaCl 2.5%	0.284	0.0287	9.92	$2.45*10^{-12}$	0.226	0.342
NaCl 5%	0.142	0.0287	4.95	0.0000137	0.0841	0.200
7°C	0.120	0.0287	3.82	0.000451	0.0517	0.168
15°C	0.160	0.0287	5.58	$1.81*10^{-6}$	0.102	0.218

Supplement Table 3.3. Summary of model fitting error

Temperature	MSE			
	Mean	Median	Min	Max
55°C	0.173	0.127	0.00954	0.723
60°C	0.207	0.165	0.00751	0.676
65°C	0.271	0.193	$7.47*10^{-5}$	0.917

CHAPTER 4

Quantifying variability factors: effect of strain variability on growth and thermal inactivation kinetics of *Lactobacillus plantarum*

D.C. Aryani, H.M.W den Besten, M.H. Zwietering

Submitted for publication

ABSTRACT

The presence and growth of spoilage organisms in food affect the shelf life, and thereby contribute to decrease of food quality and to food loss. In this study, the effects of experimental variability, reproduction variability and strain variability were quantified with respect to growth and thermal inactivation using 20 *Lactobacillus plantarum* strains. Also the effect of growth history on thermal resistance was quantified using different pre-culturing conditions. The strain variability in μ_{max} was similar to reproduction variability as function of pH, a_w and temperature, while slightly lower as function of [HLa]. The cardinal growth parameters were estimated for the 20 *L. plantarum* strains and pH_{min} was between 3.2 and 3.5; $a_{w,min}$ between 0.936 and 0.953; [HLa_{max}] at pH 4.5 between 29 and 38 mM and T_{min} between 3.4°C and 8.3°C. The *D*-values ranged from 0.80 min to 19 min at 55°C, 0.22 min to 3.9 min at 58°C, 3.1 s to 45 s at 60°C, and 1.8 s to 19 s at 63°C. In general, the impact of strain variability on thermal resistance was much higher than on growth. Also, unlike in growth, strain variability in thermal resistance was much higher than reproduction and experimental variabilities. Strain variability was also slightly higher than the effect of growth history, which was mostly determined by the physiological state of the cells and the effect of pre-culturing temperature of 12°C. The combined effects of strain variability and growth history on *D*-value explained all variability as found in literature, although with bias.

INTRODUCTION

Spoilage microorganisms are of interest for the food industry since their presence and growth in food products causes decay, and when not well controlled contribute to undesired food product loss. Lactic acid bacteria are an important group of spoilage microorganisms and are abundant in the environment. They can be found in diverse niches (Siezen et al., 2010) and are commonly isolated from plant and plant material (Hammes and Vogel, 1995), soil (Chen et al., 2005), silage (Yang et al., 2010), meat (Hamasaki et al., 2003), vegetables (Tournas, 2005), and milk (Khedid et al., 2009). Since they are natural contaminants of raw food ingredients, controlling their presence and growth are critical steps to control shelf life of food products.

Accurate control and realistic prediction of shelf life is, however, complicated by the natural diversity among strains. Recently, strain variability in growth and thermal resistance of *Listeria monocytogenes* was quantified (Aryani et al., 2015a; Aryani et al., 2015b). The impact of strain variability on growth and thermal resistance was also reported for other pathogens such as, *Salmonella enterica* (Juneja et al., 2003; Lianou and Koutsoumanis, 2011; Lianou and Koutsoumanis, 2013), *Staphylococcus aureus* (Lindqvist, 2006; Rodríguez-Calleja et al., 2006), *Bacillus cereus* (Väisänen et al., 1991), and *Escherichia coli* (Benito et al., 1999; Nauta and Dufrenne, 1999; Whiting and Golden, 2002). While strain variability in growth and thermal inactivation kinetics has been reported for pathogens, limited information is available for spoilage microorganisms. To extend the knowledge of variability in growth and thermal resistance of spoilage microorganisms, *Lactobacillus plantarum* was chosen as a model species. Although high diversity in genomic and phenotypic levels of *L. plantarum* have been reported (Sanders et al., 2015; Siezen et al., 2010; Siezen and van Hylckama Vlieg, 2011), none of the studies focused on quantification of strain variability in growth and thermal resistance. Therefore, the objectives of the present study were to quantify strain variability, reproduction (biological) variability and experimental variability with respect to growth and thermal inactivation kinetics of *L. plantarum* and to quantify the variability in thermal resistance attributed to growth history. Strain variability was defined as the difference between strains from the same species; reproduction variability was defined as the difference between independent reproductions of the same strain (within strain variability); and experimental variability was defined as the difference between duplicate measurements conducted using the same culture. Quantitative knowledge on variability in growth and thermal resistance will contribute to better characterize this species, and to better predict the behaviour of this spoilage organism in food products.

MATERIALS AND METHODS

Growth experiments

Culture preparation

Twenty strains of *L. plantarum* were used in this study (table 4.1). Stock cultures were stored at -80°C in 70% De Man, Rogosa and Sharpe (MRS) broth (Merck Millipore) supplemented with 30% glycerol (v/v) (Sigma-Aldrich, Germany). From the stock, *L. plantarum* strains were grown on an MRS agar plate made from MRS broth and bacteriological agar (1.5% w/w, Oxoid, England) and incubated in microaerobic conditions for 48h at 30°C. Then, one colony was inoculated in a 30 ml tube containing 10 ml MRS broth, which was incubated for 24h at 30°C. A second culture was then made by transferring 0.5% (v/v) of the first overnight culture to 100 ml of MRS broth followed by incubation for 24h at 30°C. The working culture was prepared from this second overnight culture following the same procedure as previously described (Aryani et al., 2015a; Biesta-Peters et al., 2010b). Briefly, the culture was transferred to 50 ml centrifuge tubes and centrifuged for 15 min for strains FBR02, FBR06, FBR30, and SF2A35B and 2 min for the other strains at 15,557 x *g* (Centrifuge 5804 R, Eppendorf). Longer centrifugation times were needed for the former four strains because they produced slimy extracellular polymeric substances, which influenced sedimentation. The supernatant was discarded and the pellet was re-suspended in 2 ml of Peptone Physiological Salt (PPS, Tritium Microbiologie B.V., Netherlands). One ml of the pooled suspension of two pellets was diluted in PPS to obtain an OD_{600} of 0.5 (Spectrophotometer Novospec II, Pharmacia Biotech) or approximately 10^8 CFU/ml. This standardized working culture was used for further experiments.

Table 4.1. Twenty *L. plantarum* strains and their source of isolation

Strains	Isolation source
FBR01	Dressing
FBR02	Dressing
FBR03	Salad dressing
FBR04	Cheese with garlic
FBR05	Dressing
FBR06	Onion ketchup
FBR22	Sausage
FBR23	Potato salad
FBR24	Luncheon meat

Strains	Isolation source
FBR25	Sliced salami
FBR26	Frankfurter
FBR27	Sliced cooked ham
FBR28	Spoiled tomato ketchup
FBR29	Lettuce
FBR30	Raw vegetable salad
WCFS1	Human saliva
LMG18035	Milk
LMG23454	Healthy adult faeces
LMG6907	Pickled cabbage
SF2A35B	Sour cassava

Media preparation

Eight pH values, namely 7.0, 6.0, 5.0, 4.0, 3.6, 3.5, 3.4, and 3.3, were selected to test the effect of pH on the maximum specific growth rate (μ_{max}) of *L. plantarum*. For each experiment, the MRS broth was buffered using 100 mM sodium phosphate buffer (1 M Na_2HPO_4, Merck KGaA, Darmstadt Germany; 1 M NaH_2PO_4, Merck KGaA, Darmstadt Germany). The pH of the broth was adjusted to the appropriate pH using 1 M of sulphuric acid (Riedel-de Haën, Seelze, Germany) and the broth was filter sterilized (Steritop, Milipore Corporation, MA) before use.

Six different concentrations of sodium chloride (NaCl, AnalaR NORMAPUR, VWR International, Leuven, Belgium), namely 2%, 4%, 6%, 8%, 9%, and 10% (w/v), were added to MRS broth and the broth was autoclaved. The corresponding a_w values of the NaCl adjusted MRS broth and plain MRS broth (0% added NaCl) were measured using a Novasina water activity meter (Labmaster a_w, Novasina, Lachen, Switzerland) set at 30°C.

Five concentrations (2.5, 5, 10, 20 and 30 mM) of DL-lactic acid 85% (Sigma-Aldrich, Germany) in its undissociated form at pH 4.5 were used in this study. The preparation of the medium adjusted with lactic acid was done as previously described (Aryani et al., 2015a; Biesta-Peters et al., 2010a). Briefly, the ratio between the dissociated and undissociated forms of lactic acid at set pH was calculated using the Henderson-Hasselbalch equation (equation (4.1)).

$$pH = pK_a + log\frac{[A^-]}{[HLa]} \quad [4.1]$$

Where pH is the pre-set pH (pH 4.5) of the medium using sulfuric acid as acidulant, pK_a is the acid dissociation constant (pK_a of lactic acid 3.86), $[A^-]$ is the concentration of anions, and $[HLa]$ is the concentration of the undissociated acid. The conjugated salt of lactic acid used in this study was potassium lactate (Corbion, Gorinchem, the Netherlands).

For the effect of temperature, the growth experiments were conducted at seven temperature points, namely 30°C, 25°C, 20°C, 18°C, 12°C, 10°C and 7°C in plain MRS medium (pH 5.7, a_w of 0.993).

Estimating μ_{max} as function of pH, a_w, undissociated lactic acid concentration ([HLa]), and temperature

The growth experiments were performed following the procedure described previously (Aryani et al., 2015a). Briefly, the standardized culture was diluted 10,000 fold in each pH, a_w, and HLa adjusted MRS broth and in plain MRS broth for the temperature experiment, aiming for an initial cell concentration of approximately 10^4 CFU/ml for the highest concentration of the four sequential 2-fold dilutions. The diluted suspension was spiral plated (Eddy Jet, IUL instruments) onto MRS agar plates in duplicate to determine the initial concentration (N_0) of each *L. plantarum* strain.

The μ_{max} was estimated by monitoring the OD_{600} of four sequential 2-fold dilutions in five neighbouring wells using the Bioscreen C (Aryani et al., 2015a; Biesta-Peters et al., 2010b). The TTD was defined as the time at which a certain well reaches a specific value of OD_{600} 0.2 (or 0.15 for the condition close to the growth boundary). The Bioscreen C was run for a certain period depending on the condition tested, which was up to 2 weeks for pH, a_w, and [HLa], and 30 days for the lower temperature experiments. The Bioscreen C was set at medium shaking for 15 s before each measurement. The μ_{max} was calculated as the negative reciprocal slope of the linear regression between TTD and the natural logarithm of the initial bacterial concentration of the five wells. If the wells showed no changes in the OD values, the viability of the bacteria at the end of the experiment was determined by plating all the content of the well. When the plate counting showed a reduction in number of bacteria in comparison to N_0 or total inactivation of bacteria, the μ_{max} was set to 0 h^{-1}. The maximal decrease in pH of the medium when the OD_{600} value reached 0.2 was 0.1-0.2 from the initial pH of 5.7 depending on the strain.

Quantification of variability factors and model fitting

The experimental, reproduction, and strain variability factors were quantified using the previously described method (Aryani et al., 2015a) (figure 2.1 and equations (4.2)-(4.4)).

Experimental variability:

$$MSE = \frac{RSS}{DF} = \frac{\sum_{C=1}^{i}\sum_{S=1}^{20}\sum_{R=1}^{3}\sum_{E=1}^{2}(X_{ERSC}-X_{RSC})^2}{n-p} \quad [4.2]$$

The mean square error (MSE) is calculated from the residual sum of squares (RSS) divided by the degrees of freedom (DF). The RSS is the sum of squared differences between X_{ERSC} and X_{RSC}. X_{ERSC} is the μ_{max} value (h^{-1}) of each replicate of duplicate experiments conducted

at the same time using the same culture of a certain strain per condition (C) (E=1, 2; R=1, 2 ,3; S=1, 2,…20; C=pH 7, 6, 5,…., etc.). X_{RSC} is the average μ_{max} (h^{-1}) obtained using the same culture of a certain strain per condition (R=1, 2, 3; S=1, 2,…20; C= pH 7, 6, 5,…., etc.). The *DF* is the number of data points (*n*=6∗20) per condition minus the number of parameters (*p*=3∗20) times the number of conditions "C".

Reproduction variability:

$$MSE = \frac{RSS}{DF} = \frac{\sum_{C=1}^{i}\sum_{S=1}^{20}\sum_{R=1}^{3}(X_{RSC}-X_{SC})^2}{n-p} \quad [4.3]$$

Where X_{RSC} is the average μ_{max} (h^{-1}) obtained using the same culture of a certain strain per condition (R=1,2,3; S=1, 2,…,20; C=pH 7, 6, 5,…., etc.), X_{SC} is the average μ_{max} (h^{1}) from three independent reproductions performed on different experimental days for each strain per condition (S=1, 2,…,20; C= pH 7, 6, 5,…., etc.). *DF* is the number of data points (*n*=3∗20) per condition minus the number of parameters (p=1∗20) times the number of conditions "C".

Strain variability:

$$MSE = \frac{RSS}{DF} = \frac{\sum_{C=1}^{i}\sum_{S=1}^{20}(X_{SC}-X_{C})^2}{n-p} \quad [4.4]$$

Where X_{SC} is the average μ_{max} (h^{-1}) from three independent reproductions for each strain per condition, X_C is the average μ_{max} (h^{-1}) of all 20 strains at condition *C*, *DF* is the number of data points (*n*=20) per condition minus the number of parameters (*p*=1) times the number of conditions "C".

The secondary growth models used to fit the μ_{max} data as function of pH, temperature, a_w and [HLa] were the selected best models from our previous study (Aryani et al., 2015a) (equations (4.5)-(4.8)). The data obtained from temperature experiments (pH 5.7, T 30°C) and [HLa] experiment (pH 4.5, T 30°C, 0 mM [HLa]) was included in the fitting process of pH data. The obtained cardinal growth parameters were used to calculate the specific growth rate at the reference condition (μ_{ref}) (pH 5.7, a_w 0.993, temperature of 30°C, and 0 mM [HLa]) using the transformed growth models provided in supplement 4.1.

pH model

$$\mu_{max} = \mu_{opt}\left(1 - 2^{\frac{(pH-pH_{min})}{\left(pH_{min}-pH_{\frac{1}{2}}\right)}}\right) \quad [4.5]$$

Temperature model

$$\sqrt{\mu_{max}} = \alpha_T(T - T_{min}) \quad [4.6]$$

a_w model

$$\mu_{max} = \mu_{opt}\left(1 - \left(\frac{1-a_w}{1-a_{w_{min}}}\right)^{\alpha_{a_w}}\right) \quad [4.7]$$

[HLa] model

$$\mu_{max} = \mu_{opt}\left(1 - \left(\frac{[HLa]}{[HLa]_{max}}\right)^{\alpha_{[HLa]}}\right) \quad [4.8]$$

All fitting was done using Excel solver add-in (Microsoft) and confirmed using TableCurve 2D v5.01. Moreover, the lack-of-fit test (Den Besten et al., 2006) (equations (4.9a) and (4.9b)) was used to observe the goodness of fit of the selected secondary growth models for each strain.

$$\text{Lack-of-fit} = \frac{MSE_{model}}{MSE_{data}} \quad [4.9a]$$

$$\text{Where } MSE_{data} = \frac{RSS}{DF} = \frac{\sum_{A=1}^{i}\sum_{R=1}^{3}\sum_{E=1}^{2}(X_{ERA}-X_A)^2}{n-m} \quad [4.9b]$$

Where X_{ERA} is the observed μ_{max} (h^{-1}) obtained from each condition (e.g. A=pH 7, 6, 5,…, etc.) for specific strain, X_A is the average μ_{max} (h^{-1}) for each condition for a specific strain, *n* is the number of data points, and *m* equals to the number of conditions *i*.

Thermal inactivation experiments

Culture preparation

From the stock culture, a streak was made onto an MRS agar plate and incubated in microaerobic condition for 48h at 30°C. A single colony was selected and inoculated in a 30 ml tube containing 10 ml of MRS broth and incubated for 24h at 30°C. From the first culture, 0.5% (v/v) was inoculated in a 100 ml flask containing 50 ml of MRS broth and incubated for 24h at 30°C.

Thermal inactivation experiments

The thermal inactivation experiments were done in a water bath at 80 rpm (Julabo SW23, Julabo Labortechnik GmbH, Germany) set at 55°C (the duration was between 6 min for the most thermal sensitive strains to 120 min for the most thermal resistant strain), 58°C (between 1.67 min to 30 min), 60°C (between 0.75 min to 6 min) and 63°C (between 24 s to 2 min); following the method previously described (Aryani et al., 2015b). Briefly, three sterile 250 ml flasks were pre-filled with 40 ml of MRS broth and pre-heated in the water bath at the desired temperature. One flask was used to measure the temperature using a thermocouple (PeakTech 3150, Thermocouple K-type), while the other two flasks were used to obtain duplicate inactivation experiments using the same culture. The stationary phase culture was inoculated 1:100 (v/v, final concentration approximately 10^7 CFU/ml) in

the pre-heated MRS to immediately start the inactivation, because in this way the observed temperature drop was negligible (±0.3°C). The same dilution (1:100) in non-heated MRS was also done for time point t=0. At each time point, one ml of sample was diluted in 9 ml of PPS at room temperature, after which further decimal dilutions were made, and the appropriate dilution was plated in duplicate onto MRS agar plates using a spiral plater. For the time points with expected low concentrations of viable cells, one ml of sample was transferred into a sterile cup, rapidly cooled down on ice to room temperature, and spread plated onto three MRS agar plates. This method allowed us to have a detection limit of 1 CFU/ml. All plates were incubated for 5 days in microaerobic condition at 30°C, and the colonies were counted and reported in $\log_{10}$ CFU/ml. Each inactivation experiment was conducted in duplicate on the same day using the same culture to quantify experimental variability. Also the inactivation experiments were reproduced at least two times on different days using freshly prepared cultures to quantify the biological or reproduction variability.

Growth history: the effect of pre-culturing conditions and physiological state of the cells on thermal resistance

Three strains were selected based on their thermal resistance at 58°C to quantify the effect of growth history on thermal resistance, namely the least thermal resistant strain (SF2A35B), the most thermal resistant strain (FBR05), and an intermediate thermal resistant strain (LMG18035). To determine the effect of pre-culturing condition, the cells were cultured until the stationary phase in MRS medium adjusted to certain suboptimal conditions, namely, pH 4; 2.5% NaCl (w/v); and 12°C. For pH experiments, the MRS broth was buffered using 100 mM of sodium phosphate buffer and the pH was adjusted to pH 4 using 1 M of sulphuric acid and filter sterilized. For NaCl adjusted medium, 2.5% of NaCl was added to the medium and sterilized for 15 minutes at 121°C; and for temperature experiments plain MRS broth was used. From the stock culture, a streak was made onto an MRS agar plate and incubated in microaerobic condition for 48h at 30°C. A single colony was selected and incubated in 10 ml MRS broth for 24h at 30°C. From this culture, 0.1% (v/v) was transferred into a 100 ml flask containing 50 ml MRS broth and incubated at 30°C for 40 h (pH 4), 24 h (2.5% NaCl), and 7- 8 days (12°C) to obtain a stationary phase culture. For the control condition cells were grown in plain MRS medium at 30°C for 24 h until the stationary phase. To evaluate the effect of physiological state, cells were also sub cultured in MRS broth at 30°C until the OD_{600}=0.5 was reached. All cultures were then inactivated at 58°C as described in section thermal inactivation experiments.

Estimation of *D*- and *z*-values and quantification of variability factors and statistical analysis

The $\log_{10}$ surviving counts were plotted against the inactivation time to obtain the thermal inactivation curve of each data set. The modified Weibull model (equation (4.10)) (Metselaar et al., 2013) was used to fit each thermal inactivation data set and to estimate the 5 or 6 decimal reduction time, which is the time to reach 5 or 6 $\log_{10}$ reduction. The model allows to fit linear, concave, and convex inactivation curves and is able to fit the different thermal inactivation curves of the strains. Points below the detection limit were not included in the fitting procedures.

$$\mathrm{Log}_{10}\, N_t = \mathrm{Log}_{10} N_0 - \Delta\left(\frac{t}{\Delta D}\right)^{\beta} \qquad [4.10]$$

Where $\mathrm{Log}_{10}\, N_t$ is $\log_{10}$ number of surviving organism ($\log_{10}$ CFU/mL) at time t; $\mathrm{Log}_{10}\, N_0$ is $\log_{10}$ initial number ($\log_{10}$ CFU/mL); t is time (min or s); ΔD is the time to reach Δ $\log_{10}$ reduction (min or s); and β is the shape parameter. The fitting procedure was done using Microsoft Solver Add-in and was confirmed using TableCurve 2Dv5.1. The *D*-value was calculated as $\Delta D/\Delta$.

The $\log_{10}$ *D*-values were used to quantify the experimental, reproduction, and strain variability following the above described method (equations (4.2)-(4.4)). The *D*-values among strains for each temperature were compared using Anova, followed by a post hoc (Tukey HSD) test to classify strains based on their thermal resistance. The *D*-values were also used for calculating the *z*-value per strain as being the negative reciprocal of the linear regression slope between $\log_{10}$ *D*-values (six values per temperature) and temperature. Finally, the effect of growth history condition was compared to the control condition using a *t*-test and multiple linear regression (Aryani et al., 2015b). The comparison between the effect of growth history and strain variability on thermal resistance was performed following the scheme presented in figure 3.2.

Comparison of strain variability in growth and thermal inactivation

The cardinal growth parameters, the average μ_{ref} and the *D*-values were used to predict the growth and inactivation of *L. plantarum* in a model process (growth in milk: pH 6.6 ($\pm$0.1), a_w 0.997 ($\pm$ 0.003), T 7°C; thermal inactivation at 60°C for 2 minutes). The gamma modelling approach without interaction (Zwietering et al., 1993) (equations (4.11)-(4.12)) was used to predict the μ_{max}, and the logistic growth model (equation (4.13)) and the linear inactivation model (equation (4.14)) were used to predict the final level of *L. plantarum*. Since the gamma approach is based on a reference condition, each secondary growth model used to calculate the gamma factor was transformed using the reference condition of pH 5.7, a_w 0.993, temperature 30°C and [HLa] 0 mM (supplement 4.1).

$$\gamma(total) = \gamma(pH) * \gamma(a_w) * \gamma(T) * \gamma(HLa) \quad [4.11]$$

$$\mu_{max} = \mu_{ref} * \gamma(total) \quad [4.12]$$

$$Log\ N_t = Log\ N_0 - Log\left(1 + \left(\frac{N_{max}}{N_0} - 1\right) exp(-\mu_{max} t_d)\right) \quad [4.13]$$

$$Log\ N_t = Log\ N_0 - \frac{t_m}{D-value} \quad [4.14]$$

Where μ_{max} is the maximum specific growth rate (h^{-1}), μ_{ref} is the specific growth rate at the reference condition (pH 5.7, a_w 0.993, temperature 30°C and [HLa] 0 mM) calculated using the transformed secondary growth models (h^{-1}) (supplement 4.1), N_t is the bacterial concentration at time *t* (CFU/ml), N_0 is the initial bacterial concentration (CFU/ml), N_{max} is the maximum bacterial concentration (CFU/ml), t_d is the storage time (days), *D*-value is the time needed to kill 1 log of bacterial concentration (min) and t_m is the thermal process time.

RESULTS

Growth kinetics of *L. plantarum*

The growth kinetics of 20 *L. plantarum* strains as function of pH, temperature (T), water activity (a_w) and undissociated lactic acid ([HLa]) are presented in figure 4.1. As expected, the differences between duplicate measurements using the same culture (figures 4.1A, 4.1D, 4.1G, 4.1J) were lower than the differences from independent reproductions carried on different experimental days (figures 4.1B, 4.1E, 4.1H, 4.1K) and the differences between strains (figures 4.1C, 4.1F, 4.1I, 4.1L).

For strain LMG18035, growth/no growth was already observed at pH 3.4, while at pH 3.3 most of strains grew at least in one of the three reproductions, but no μ_{max} could be obtained. The experiment at a lower pH value of pH 3.2 could not be performed since precipitation of the media was observed at this pH value. FBR26 showed growth/no growth behaviour at pH 5 and below, and therefore the result of FBR26 was excluded from the variability calculation as function of pH and the secondary model fitting for pH.

For variable of water activity (a_w), most strains grew at least in one reproduction when 8% NaCl was added in the medium (a_w 0.948). Less strains grew when concentration of NaCl was increased to 9% (a_w 0.943), and only three strains (FBR01, FBR22, and LMG6907) showed growth in one of the three reproductions.

When the effect of undissociated lactic acid ([HLa]) on growth kinetics was tested, strain FBR26 already showed inconsistent growth at control condition of pH 4.5 and was therefore excluded also from the variability calculation as function of [HLa] and the secondary model fitting for [HLa]. When 30 mM of [HLa] was added to MRS broth, most strains were able to grow in one, two or in all reproductions.

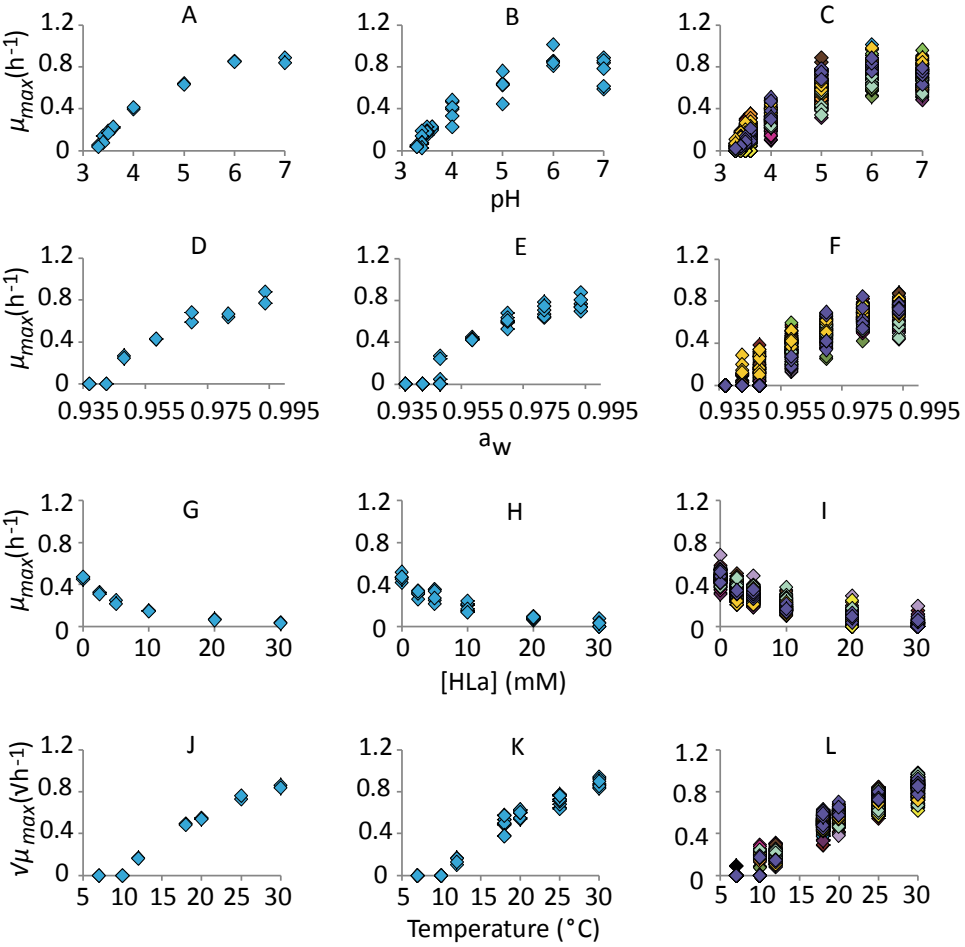

Figure 4.1. The growth kinetics of 20 *L. plantarum* strains as function of pH (A-C), a_w (D-F), undissociated lactic acid concentration ([HLa]) at pH 4.5 (G-I) and temperature (J-L). Panels A, D, G and J are the duplicate growth kinetics curves of FBR05 using the same culture; panels B, E, H, and K are the growth kinetics curves of FBR05 from three independent reproductions. In panels C, F, I, and L: ◆ FBR01; ◆ FBR02; ◆ FBR03; ◆ FBR04; ◆ FBR05; ◆ FBR06; ◆ FBR22; ◆ FBR23; ◆ FBR24; ◆ FBR25; ◆ FBR26; ◆ FBR27; ◆ FBR28; ◆ FBR29; ◆ FBR30; ◆ WCFS1; ◆ LMG18035; ◆ LMG23454; ◆ LMG6907 and ◆ SF2A35B.

For the variable of temperature, the μ_{max} was square root transformed since the variance was not distributed evenly over the temperature range. In general the transformed data looked linear (figure 4.1F), although this was not always the case for an individual set of μ_{max} data of all strain (figures 4.1D - 4.1E).

Variability in growth kinetics

The quantified experimental (E), reproduction (R) and strain (S) variabilities for each variable are presented in figure 4.2. The variability as function of pH (figure 4.2A), a_w (figure 4.2B) and temperature (figure 4.2D) followed the same trend, in which strain variability was comparable to reproduction variability; and both variabilities were significantly higher than experimental variability. A different trend was observed for variables [HLa], for which the strain variability was significantly lower than the reproduction variability (figure 4.2C), but both variabilities were also significantly higher than experimental variability.

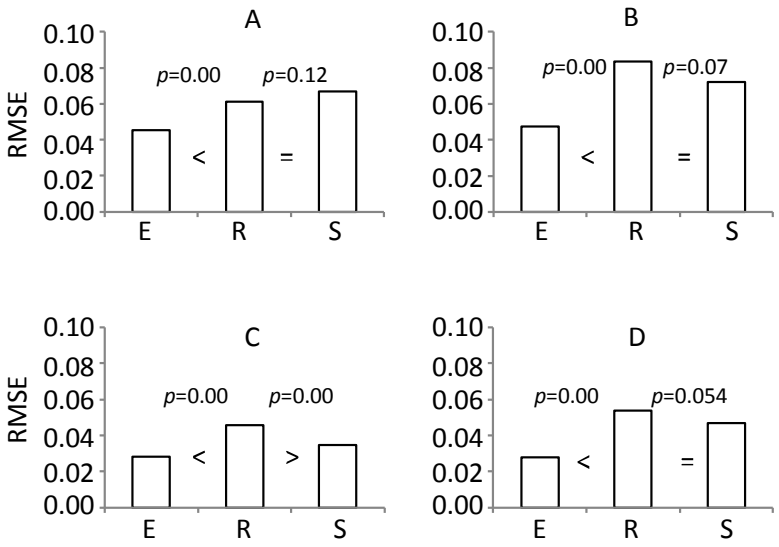

Figure 4.2. The calculated variability factors (E, experimental; R, reproduction, S, strain) presented as the root mean square error (RMSE) based on the μ_{max} as function of pH (A); the μ_{max} as function of a_w (B); the μ_{max} as function of undissociated lactic acid concentration ([HLa]) (C); and square root μ_{max} as function of Temperature (D).

Variability in cardinal growth parameters

The secondary growth models were used to fit all μ_{max} data per strain as function of pH, temperature, a_w and [HLa], resulting in the cardinal growth parameter estimates per strain (figure 4.3). The minimum pH estimated for the 19 strains ranged from pH 3.2 and pH 3.5, and LMG6907, which was isolated from pickled cabbage, was estimated to have the lowest pH_{min} of 3.17 (CI 3.08 - 3.27). The estimated $a_{w,min}$ for the 20 *L. plantarum* strains was between 0.936 and 0.953 and strain LMG6907 was also estimated to have the lowest $a_{w,min}$ of all 20 strains. The [HLa_{max}] at pH 4.5 estimated for *L. plantarum* was between 29 and 38 mM. The confidence intervals of most of the [HLa_{max}] estimates were high since at the highest concentration used in this study most of the strains were still able to grow, resulting in extrapolation beyond the [HLa] concentration which was available. The T_{min} estimated using the square root model was between 3.4°C and 8.3°C and strain FBR29 and WCFS1 were estimated to have the lowest T_{min} of all 20 strains.

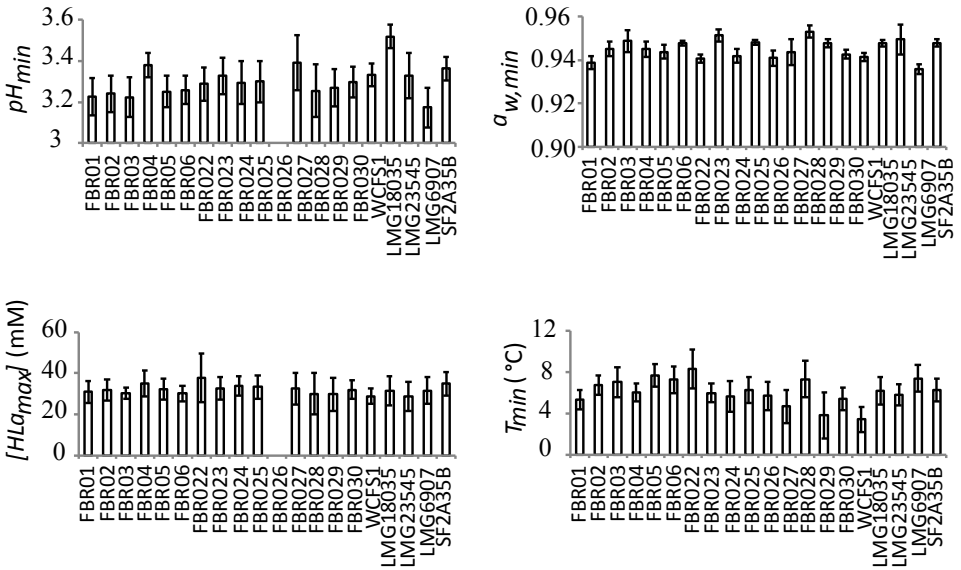

Figure 4.3. The cardinal growth parameters pH_{min}, $a_{w,min}$, $[HLa_{max}]$ and T_{min} estimated using secondary growth models of Equations (4.5) – (4.8). The error bars indicate the 95% confidence intervals of the estimated parameters.

The specific growth rate at the reference condition (μ_{ref}) was calculated from the cardinal parameters obtained in present study using the transformed growth models (figure S4.1). The μ_{ref} of the variables pH and [HLa] was lower than the μ_{ref} obtained for variables T and a_w, most probably due to the characteristic of the selected secondary pH model. The μ_{opt} of the secondary pH model was the μ_{opt} at pH infinite. If the μ_{max} value of the higher pH point decreased, such as in the case of FBR01 and FBR05 (figure S4.2), these values would influence the μ_{opt} estimate, resulting in underestimation of μ_{opt} by the pH model. Since the μ_{ref} was calculated from the μ_{opt}, the lower value of μ_{opt} would result in the lower estimate of μ_{ref}. For predicting the growth of *L. plantarum* strains the average μ_{ref} obtained from four variables was used.

Thermal inactivation kinetics of *L. plantarum*

Thermal inactivation kinetics of 20 strains *L. plantarum* and the effect of variability factors on thermal inactivation kinetics.

The thermal inactivation kinetics of 20 *L. plantarum* strains are presented in figure 4.4. Small differences were observed from duplicate inactivation experiments using the same bacterial culture (figures 4.4A, 4.4D, 4.4G, 4.4J). The differences were larger when the data from different independent reproductions were compared (figures 4.4B, 4.4E, 4.4H, 4.4K) and much larger when the data from all strains were combined (figures 4.4C, 4.4F, 4.4I, 4.4L). From all 20 strains, strain SF2A35B, which was isolated from sour cassava, had the lowest *D*-value at all temperatures.

In contrast, FBR05, which was isolated from dressing, had the highest *D*-value at 55°C and 58°C. However, its *D*-value was less than WCFS1 and FBR06 at 60°C and 65°C.

The *D*-value and the shape parameter (β) of each thermal inactivation curve for each strain was estimated using the modified Weibull model. On average, the *D*-value of 20 *L. plantarum* strains ranged from 0.8 min to 19 min at 55°C, 0.22 min to 3.9 min at 58°C, 3.1 s to 45 s at 60°C, and 1.8 s to 19.2 s at 63°C. These *D*-values were then used to estimate the *z*-value of each strain. The *z*-values obtained from this study were between 3.9°C and 6.0°C (table 4.2). The value of the β parameter also varied per strain, but for most strains the β values was larger than 1 (supplement table S4.1).

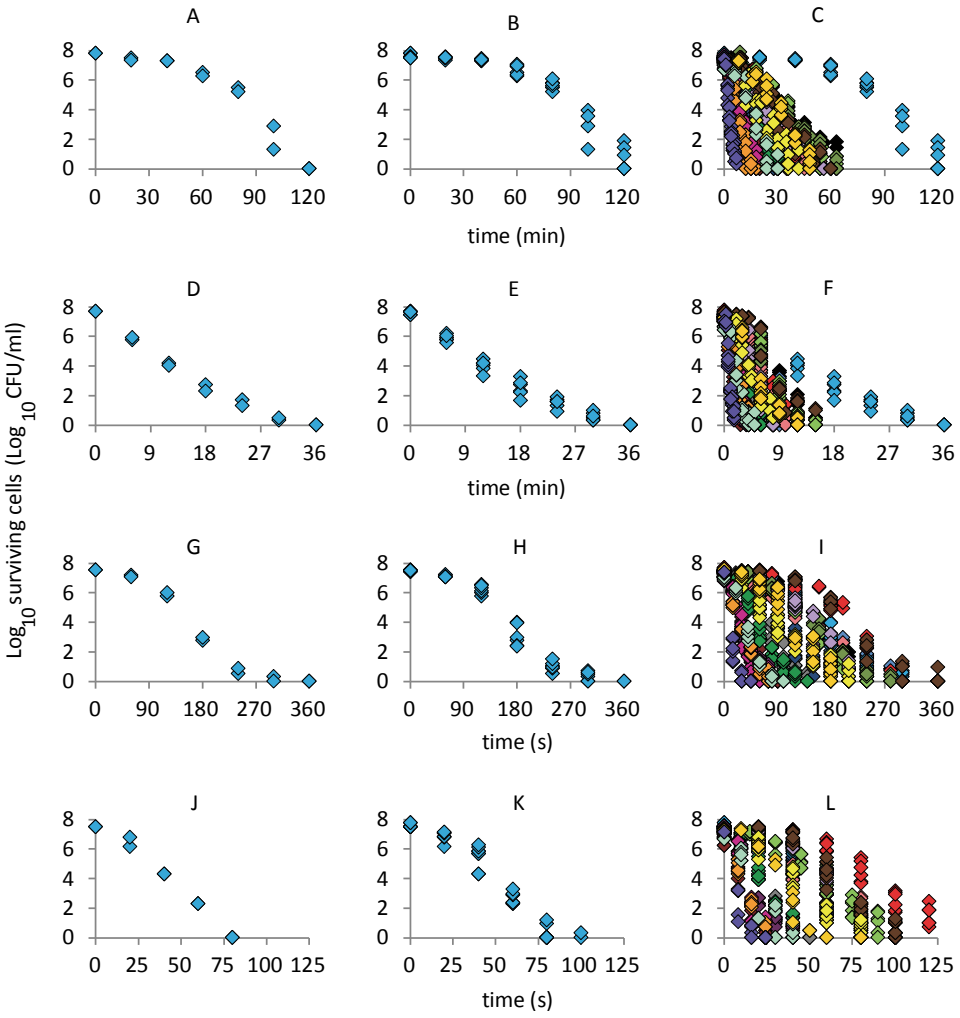

Figure 4.4. The thermal inactivation kinetics of 20 *L. plantarum* strains at 55°C (A-C), 58°C (D-F), 60°C (G-I), and 63°C (J-L). Panels A, D, G and J are the thermal inactivation curves of FBR05 using the same culture; panels B, E, H, and K are the thermal inactivation curves of FBR05 from three independent reproductions. In panels C, F, I, and L : FBR01; FBR02; FBR03; FBR04; FBR05; FBR06; FBR22; FBR23; FBR24; FBR25; FBR26; FBR27; FBR28; FBR29; FBR30; WCFS1; LMG18035; LMG23454; LMG6907 and SF2A35B.

Table 4.2. The *D*- and *z*-values of 20 *L. plantarum* strains

Strain	*D*-value (min)[1)]				*z*-value (°C)[2)]
	55°C	58°C	60°C	63°C	
FBR01	8.47 (1.21)$^{k\ 3)}$	1.94 (0.198)^{m}	0.591 (0.0189)^{e,f,g}	0.180 (0.0132)^{g,h}	4.73 (4.53-4.93)
FBR02	4.20 (0.567)^{e,f}	0.723 (0.0780)^{d,e}	0.285 (0.0325)^{c}	0.0980 (0.00500)^{d,e}	4.91 (4.60-5.23)
FBR03	3.29 (0.0762)^{d}	0.942 (0.0443)^{f,g}	0.420 (0.0303)^{d}	0.163 (0.00445)^{f,g,}	6.01 (5.75-6.26)
FBR04	4.64 (0.209)^{f,g}	1.53 (0.208)^{j,k}	0.611 (0.0570)^{f,g,}	0.187(0.0134)^{g,h,i}	5.50 (5.29-5.70)
FBR05	18.6 (1.14)^{l}	3.91 (0.223)^{n}	0.609 (0.0414)^{f,g}	0.190 (0.0112)^{g,h,i,j}	3.88 (3.61-414)
FBR06	6.05 (0.515)^{h,i}	1.56 (0.166)^{j,k,l}	0.652 (0.0709)^{f,g}	0.318(0.0228)^{l}	6.19 (5.72-6.67)
FBR22	3.67 (0.372)^{d,e}	0.416 (0.0766)^{b}	0.149 (0.0269)^{b}	0.0565 (0.00899)^{b}	4.41 (3.98-4.85)
FBR23	7.55 (0.814)^{k}	1.34 (0.0766)^{i,j}	0.575 (0.0346)^{e,f}	0.215 (0.00619)^{h,i,j,k}	5.15 (4.78-5.52)
FBR24	4.20 (0.250)^{e,f}	0.867 (0.0866)^{e,f}	0.255 (0.0186)^{c}	0.0987 (0.00358)^{e}	4.83 (4.52-5.13)
FBR25	8.20 (0.523)^{k}	1.78 (0.0545)^{k,l,m}	0.550 (0.0138)^{e,f}	0.232 (0.0281)^{i,j,k}	5.07 (4.79-5.36)
FBR26	8.65 (0.228)^{k}	1.58 (0.0743)^{j,k,l}	0.559 (0.0169)^{e,f}	0.237(0.0150)^{j,k}	5.25 (4.89-5.61)
FBR27	1.99 (0.219)^{b}	0.449 (0.0388)^{b}	0.159(0.0398)^{b}	0.0785 (0.00365)^{c,d}	5.77 (5.22-6.33)
FBR28	6.60 (0.615)^{i,j}	1.21 (0.150)^{h,i}	0.513 (0.0413)^{d,e,f}	0.224 (0.0177)^{h,i,j,k}	5.59 (5.18-6.01)
FBR29	2.64 (0.192)^{c}	0.570 (0.0482)^{c}	0.144 (0.0263)^{b}	0.0535 (0.00787)^{b}	4.60 (4.28-4.92)
FBR30	1.89 (0.267)^{b}	0.615 (0.0753)^{c,d}	0.151 (0.00745)^{b}	0.0533 (0.00474)^{b}	5.00 (4.67-5.33)
WCFS1	7.23 (0.603)^{i,j,k}	1.88 (0.0576)^{l,m}	0.754 (0.0640)^{g}	0.243 (0.0179)^{k}	5.48 (5.33-5.64)
LMG18035	5.34 (1.01)^{g,h}	1.07(0.0781)^{g,h}	0.471 (0.0517)^{d,e}	0.196 (0.00815)^{g,h,i,j,k}	5.60 (5.17-6.03)
LMG23454	3.90 (0.446)^{d,e,f}	0.588 (0.0622)^{c}	0. 166 (0.00280)^{b}	0.0801 (0.0222)^{c}	4.63 (4.16-5.10)
LMG6907	7.60 (0.575)^{j,k}	1.32 (0.0613)^{i,j}	0.427 (0.0316)^{d}	0.140 (0.0137)^{f}	4.57 (4.33-4.82)
SF2A35B	0.796 (0.0393)^{a}	0.219 (0.0126)^{a}	0.0521 (0.0134)^{a}	0.0297 (0.00468)^{a}	5.34 (4.74-5.94)
Average	5.77 (3.79)[4)]	1.23 (0.809)	0.405 (0.212)	0.154 (0.0801)	5.13 (0.576)

1) the value within brackets is the standard deviation from 6 *D*-values.

2) the value within brackets is the 95% confidence intervals obtained from linear regression of log *D*-value against temperature using Table curve 2D v5.01.

3) similar codes in a column show comparable thermal resistance (post hoc Tukey HSD test p 0.05).

4) the value within brackets is the standard deviation from the *D* or *z*-values of 20 *L. plantarum* strains.

The experimental, reproduction and strain variability of the $\log_{10}$ *D*-values are expressed in root mean square error (RMSE) (figure 4.5). Strain variability was much higher than reproduction and experimental variabilities at all temperatures. Strain variability was about 6 times higher than reproduction variability and more than 10 times higher than experimental variability.

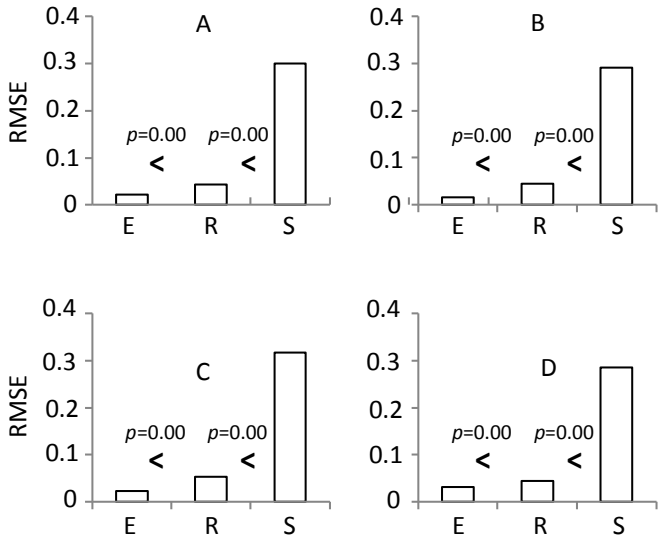

Figure 4.5. The variability factors (E, experimental; R, reproduction; S, strain) presented as the root mean square error (RMSE) calculated from the $\log_{10}$ *D*-values obtained at A) 55°C; B) 58°C; C) 60°C; D) 63°C.

The effect of growth history and physiological state of the cells on thermal resistance.

The thermal inactivation kinetics as influenced by growth history and physiological state of the cells are presented in figure 4.6. The cells grown in MRS medium containing 2.5% NaCl had similar kinetics as the cells grown until stationary phase in plain MRS for strain SF2A35B, as the representative of the least heat sensitive strains, and strain LMG18035, as the representative of the intermediate heat resistant strains. Slight differences were observed for strain FBR05, in which the cells grown in the medium with higher NaCl concentration unexpectedly had a lower *D*-value than those grown in plain medium.

The thermal resistance of cells grown in the media with lower pH (pH 4) was not different from the cells grown in plain MRS media (pH 5.7) for strain FBR05 and strain SF2A35B (figures 4.6D - 4.6F). However, growing the cells in the MRS pH 4 significantly decreased

the *D*-value of strain LMG18035. Growing the cells until stationary phase at 12°C or until exponential phase greatly reduced the *D*-value as compared to the stationary cells grown in plain media at 30°C for all three strains.

The effect of growth history on thermal resistance was also analysed using multiple linear regression to determine the factors influencing the *D*-value. This analysis confirmed that both pre-culturing temperature and exponential phase grown cells significantly influenced the *D*-value (figure 4.6). When the effect of growth history on thermal resistance was compared to the effect of strain variability using the previously described scheme (Aryani et al., 2015b) (figure 3.2), the strain variability was slightly higher than the variability caused by growth history (p=0.03).

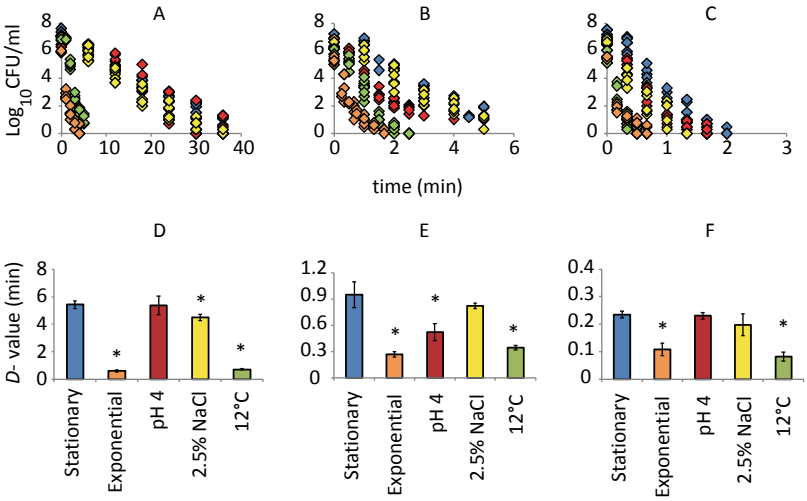

Figure 4.6. The effect of growth history and physiological state of the cells of strain FBR05 (A and D); LMG18035 (B and E); and SF2A35B (C and F) on thermal inactivation kinetics at 58°C. Cells grown until stationary phase in: ◇ MRS at 30°C; ◆ MRS pH 4 at 30°C; ◆ MRS at 12°C; ◇ MRS+2.5% NaCl at 30°C; and ◆ cells grown until exponential phase in MRS at 30°C. * significantly different than the stationary phase cells grown in MRS at 30°C ($p < 0.05$).

Comparison of strain variability in growth and thermal inactivation of *L. plantarum*

Direct comparison on the effect of variability observed in growth and thermal inactivation kinetics could not be made due to differences in the data format/unit. Therefore, the cardinal parameters and *D*-values data of 19 strains were used to visualise the growth and inactivation kinetics in a model process, namely growth in milk (pH 6.6, a_w 0.997 ($\pm$ 0.003)

using gamma approach, followed by thermal inactivation at 60°C for 2 min. The illustration (figure 4.7) shows that the impact of strain variability in thermal inactivation was much higher than in growth kinetics.

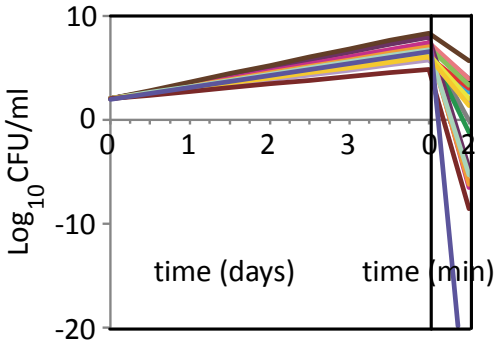

Figure 4.7. Illustration on the effect of strain variability on growth and thermal inactivation of 19 *L. plantarum* strains. FBR01, FBR02, FBR03, FBR04, FBR05, FBR06, FBR22, FBR23, FBR24, FBR25, FBR27, FBR28, FBR29, FBR30, WCFS1, LMG18035, LMG23454, LMG6907 and SF2A35B.

DISCUSSION

The strain variability in growth kinetics and thermal inactivation kinetics of *L. plantarum*, except for growth as function of [HLa], were between 1.3-2.6 times higher than previously reported for the foodborne pathogen *L. monocytogenes* (Aryani et al., 2015a). The strain variability observed in *L. plantarum* might be related to the high genetic diversity reported for this species (Sanders et al., 2015; Siezen et al., 2010; Siezen and van Hylckama Vlieg, 2011), which corresponded to the observed phenotypic diversity, including the strain diversity in stress tolerance. A clear example for the observed strain variability in growth kinetics is given for strain FBR26. The result of FBR26 was excluded from the analysis of pH and HLa since growth/no growth was already observed at pH 5 and below. Using the μ_{max} as function of pH available for FBR26, the pH_{min} was estimated 3.86 (CI 3.64 - 4.08). This value was higher than the estimated pH_{min} of the other 19 strains in our study (pH 3.2 - pH 3.5), and the latter range corresponded to the pH_{min} reported in literature (G-Alegría et al., 2004; Giraud et al., 1991; Lambert, 2011). Interestingly, growth of FBR26 was observed in one of experiments performed at pH 3.4, but no μ_{max} at this pH level could be obtained for

FBR26. Additional investigation on the effect of aerobic, microaerobic, and static incubation with different volume of headspace using 10 *L*. *plantarum* strains showed that the growth of strain FBR26 was temporarily disrupted during exponential phase in aerobic growth. This disruption led to a much lower growth rate estimated for aerobic growth than for microaerobic and static growth (data not shown). This lower growth rate in aerobic growth was also reported in a previous study with *L*. *plantarum* ATCC 8014 and *L. plantarum* P5 (Gupta et al., 2011; Murphy and Condon, 1984). In our study, the growth disruption during exponential phase was only observed for strain FBR26, because no difference in growth kinetics was observed from the other nine *L*. *plantarum* strains, including strain WCFS1 in the three different incubation conditions. The phenomenon observed for FBR26 was not new since a previous study also reported a temporary growth stagnation in early exponential phase of strain WCFS1 grown aerobically in MRS medium (Stevens et al., 2008), which was correlated to the limited CO_2 concentration in the medium. This reported stagnation for WCFS1 during aerobic growth, however, was not observed in our study and in the study of Watanabe et al. (Watanabe et al., 2012). Whether the growth disruption during aerobic growth correlated to the growth/no growth behaviour of strain FBR26 at pH below 5, when tested using Bioscreen C, is not yet clear. Moreover, less strain variability was observed when strains were incubated in microaerobic than in static and aerobic conditions. This can be an indication that the microaerobic condition might be more suitable to study the growth kinetics of *L. plantarum* strains (especially strain FBR26) than the method using Bioscreen C. However, FBR26 was able to grow to the same extent as the other 19 *L*. *plantarum* strains when the other experiments as function of a_w and temperature were performed using Bioscreen C. Further investigation on correlation between pH and aerobic growth might be needed to explain the behaviour of strain FBR26 at low pH values. However, since this phenomenon provided disadvantage rather than the advantage over growth kinetics, this might not be of interest compared to the effect of others conditions, which support growth or protect cells from being inactivated by certain processes.

The higher confidence intervals of [HLa] obtained in this study could be explained by the fact that most of the strains were still able to grow even at the highest [HLa] used. When data in the growth/no growth boundary are available, then the confidence intervals of the growth limit might be smaller. However, in our study higher concentrations than 30 mM [HLa] were not used since it might influence the water activity of the medium. At the concentration of 20 - 30 mM, the measured a_w medium was 0.991 ($\pm$ 0.002), which was close to the a_w of the plain MRS medium. Comparing the estimated [HLa_{max}] obtained in our study with the one reported in literature was rather complicated since [HLa_{max}] might be influenced by pH media and incubation temperature used in the studies. However, a

previous study reported that the influence of pH on the MIC of sodium lactate depended on the type of microorganisms, and that temperature did not have a specific influence on the MIC of sodium lactate (Houtsma et al., 1996). The pH had a minor influence on the MICs of sodium lactate for lactic acid bacteria M18, M52, M75, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* SK3 (Houtsma et al., 1996). The MICs of sodium lactate of those strains were between 268 mM and 714 mM at pH 5.7 and incubation temperature of 20°C or corresponded to 3.82 - 10.17 mM of [HLa_{max}], which was lower than what was estimated in our study. When we considered only the effect of initial pH medium, in which the growth at pH 4.5 would be slower than the growth at pH 5.7, one might expect lower [HLa] would be needed at pH 4.5 than at pH 5.7 to inhibit the growth of *L. plantarum*. However, as also described by Houtsma et al. (1996), three factors played a role in determining the growth of microorganism when lactate was added in the medium: pH, [HLa], and a_w. At higher pH value of pH 7, the inhibitory effect of sodium lactate was mostly due to lowering effect of a_w, while at the lower pH, the effect of undissociated form of the organic acid became more important (Houtsma et al., 1996). The addition of 268 mM - 714 mM sodium lactate in peptone-yeast extract broth (a_w =0.998) used in Houtsma et al. (1993) study resulted in the medium a_w between 0.992 and 0.977. The influence of a_w and the type of microorganism might then be the reason for the difference between the [HLa_{max}] obtained in our studies and those reported from literature.

Most of the *L. plantarum* strains in our study had an estimated T_{min} between 4.7°C - 8.3°C, except for two strains FBR29 and WCFS1. These two strains had T_{min} estimates between 3°C - 4°C which was similar to the reported T_{min} estimate for *L. plantarum* (Zwietering et al., 1994). Notably, the square root μ_{max} data as function of temperature for some *L. plantarum* strains in this study seemed to slightly deviate from linearity, as indicated by the lack-of-fit of the square root model for 10 out of 20 strains. However, no structural deviation of the model was observed for those 10 strains since the deviation was not always found at the same location/temperature point. This phenomenon is different compared to *L. monocytogenes* and *L. innocua* where in certain cases a deviation was found at the same temperature point (Bajard et al., 1996; Le Marc et al., 2002), and therefore a two phase temperature model was used in these studies. So far, no deviations in linear relationship between square root μ_{max} and temperature have been reported for *L. plantarum*.

Heating temperature seemed not to have an influence on strain variability of *L. plantarum* since the strain variability in $\log_{10}$ *D*-value was remarkably similar at all temperatures. Moreover, while the average *D*-value of all strains was temperature dependent, the β parameter was independent of temperature. Similar result was also observed in a previous study on the application of the Weibull model for thermal inactivation of

vegetative cells (Van Boekel, 2002), although linear dependency of the β parameter with temperature was also reported.

As for *L. monocytogenes*, the physiological state of the cells had the biggest effect on the thermal resistance, but the magnitude was similar to the effect of pre-culturing at low temperature before thermal treatment. Culturing the cells at low pH seemed to have a limited impact on the thermal resistance of strain FBR05 and SF2A3B as compared to the cells cultured in plain medium. This can be explained by the fact that when the cells were grown until stationary phase for 24 h in plain medium, the pH of the medium dropped to between 3.9 and 3.8. This reference culturing condition therefore also exposed cells to a lowered pH before the thermal process. Although the medium for low pH growth (pH 4) was buffered, the pH decreased to 3.2 and 3.4 when the cells reached stationary phase. This limited difference in end pH of both culturing conditions might explain why both conditions showed no difference in the thermal resistance result. However, the effect of pre-culturing at low pH was observed for strain LMG18035. No study reported the effect of pre-culturing in low pH medium on the thermal resistance of *L. plantarum*. But, a decrease in thermal resistance of the cells pre-cultured in low pH medium was also observed for *L. monocytogenes* (Aryani et al., 2015b).

Lower thermal resistance was observed for FBR05 when it was pre-cultured in higher concentration of NaCl. In contrast to our result, enhanced thermal resistance was reported for *L. monocytogenes* (Aryani et al., 2015b) and *B. cereus* (Den Besten et al., 2006) pre-cultured in the presence of higher NaCl concentration. The decrease in thermal resistance of strain FBR05 could not be related to its sensitivity to NaCl since it was able to grow up to the concentration of 8% NaCl in the growth experiment. No report about the decreasing effect of NaCl on thermal resistance was found for lactic acid bacteria and for other mesophilic bacteria. Testing the effect of NaCl on thermal resistance for other *L. plantarum* strains could confirm whether the decreased effect of NaCl was strain specific.

To benchmark the strain variability and variability introduced by growth history, these variability factors were compared to the variability found in *D*-values reported in literature. In total, 121 *D*-values data were collected from different studies. These studies used different methods, strains, treatments and different types of heating media. Following a similar approach as used in the study of Van Asselt and Zwietering (2006), the 95% prediction intervals of the data were obtained (figure 4.8A) to quantify the overall variability found in literature.

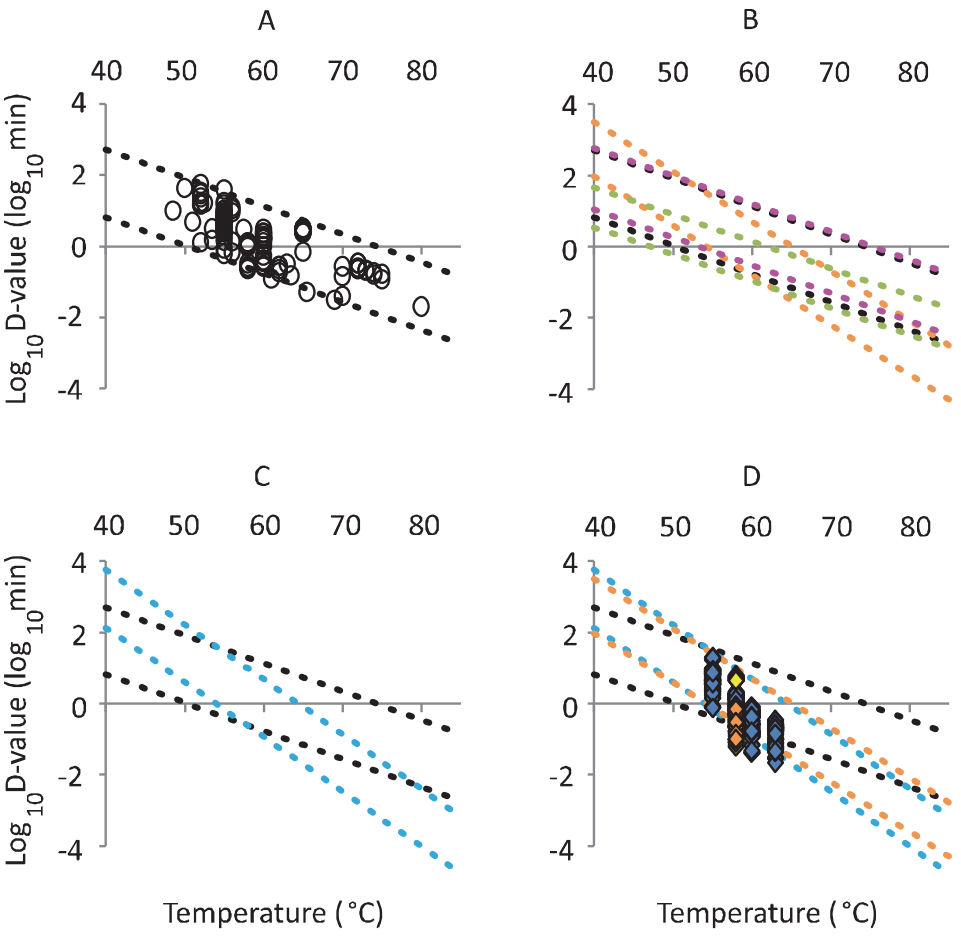

Figure 4.8. The meta-analysis of thermal inactivation data: A) Thermal inactivation data from literature and its 95% prediction intervals (PI, all data); B) 95% PI --- all literature data, --- laboratory media, --- milk, --- juices; C) 95% PI --- all literature data, --- literature data of 50-63°C and D) 95% PI --- all literature data, --- laboratory media, --- literature data of 50-63°C. ○ Literature data[1)] in panel A; Cells grown until stationary phase in: ◆ MRS at 30°C; ◆ MRS pH 4 at 30°C; ◆ MRS+2.5% NaCl at 30°C; ◆ MRS at 12°C; and ◆ cells grown until exponential phase in MRS.

1) References: (Alwazeer et al., 2002; Augusto et al., 2011; Cole and Jones, 1990; De Angelis et al., 2004; Franchi et al., 2003 ; Golowczyc et al., 2010; Jordan and Cogan, 1999; Liu et al., 2011; Milly et al., 2007; Minervini et al., 2012; Paéz et al., 2012; Parente et al., 2010; Tajchakavit et al., 1998; Turner et al., 1986)

When all data from the literature was used (48.5°C - 80°C) (figure 4.8A), the slope of the linear regression between $\log_{10}$ *D*-value and temperature was rather low, so the estimated *z*-value of *L. plantarum* from literature data was rather high (12°C), in comparison to the *z*-value of our experimental data that was between 3.9°C and 6.0°C. The higher *z*-value of the literature data was due to the effect of different matrices included in the meta-analysis. When the literature data of *L. plantarum* was split into different categories, namely laboratory media, milk, and juices, the estimated *z*-values were 7.2°C, 12.8°C, and 13.3°C respectively (figure 4.8B). The effect of food matrix on thermal resistance was also reported for *Salmonella* spp. in chocolate (*z*-value 20.4°C) (Van Asselt and Zwietering, 2006) and *L. plantarum* in milk (20°C) (De Angelis et al., 2004) and in apple juice (15°C) (Tajchakavit et al., 1998). This highlighted the effect of heating menstruum on thermal inactivation. Moreover, higher *D*-values between 63°C and 80°C, which were mostly obtained from experiments using milk and were available only in fewer numbers than those at lower temperature, might also contribute to the lower slope of the regression. When only *D*-values at the temperature range of our study (50°C and 63°C) (figure 4.8C) was used for the regression analysis, the estimated *z*-value was 6.5°C, which corresponded more or less to the reported *z*-values of vegetative cells which was between 5°C - 7°C (Doyle et al., 2001; Jordan and Cogan, 1999; Van Asselt and Zwietering, 2006).
When our data was benchmarked to the intervals of all literature data, strain variability explained most of the variability in *D*-values found in literature (figure 4.8D). When our data was benchmarked to the intervals of literature data at truncated temperature range or to the intervals of literature data obtained from laboratory media, strain variability explained rather all of the variability found in literature, but with bias at higher temperature of 60°C and 63°C (figure 4.8D). As in figure 4.6, strain variability in log *D*-value was consistently observed over temperature range used in this study. When the data of growth history effect was added, our data explained all variability found in literature, although with bias (figure 4.8D).

CONCLUSSIONS

Phenotypic diversity in growth and thermal resistance of *L. plantarum* was quantified. In general the impact of strain variability was much higher for thermal resistance than for growth. Strain variability in thermal resistance was also higher than the effect of growth history, but the combined effects of strain and growth history were able to explain all of variability found in literature, although with bias. The quantitative knowledge obtained on experimental, reproduction and strain variabilities can be used to improve experimental designs and to adequately select strains for challenge growth and inactivation tests.

Moreover, integration of strain variability in prediction of microbial kinetics will result in more realistic predictions of *L*. *plantarum* growth and inactivation dynamics.

ACKNOWLEDGMENTS

The research is funded by TI Food and Nutrition, a public-private partnership on precompetitive research in food and nutrition. The funders had no role in study design, data collection and analysis, or preparation of the manuscript.
We also would like to thank Jia Zhang, Esther Pothuis, Bertine Smit, and Su Man for their assistance during experimental work.

REFERENCES

Alwazeer, D., Cachon, R., Divies, C. 2002. Behavior of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* in fresh and thermally processed orange juice. Journal of Food Protection 65, 1586-1589.

Aryani, D.C., Den Besten, H.M.W., Hazeleger, W.C., Zwietering, M.H. 2015a. Quantifying strain variability in modeling growth of *Listeria monocytogenes*. International Journal of Food Microbiology 208, 19-29.

Aryani, D.C., Den Besten, H.M.W., Hazeleger, W.C., Zwietering, M.H. 2015b. Quantifying variability on thermal resistance of *Listeria monocytogenes*. International Journal of Food Microbiology 193, 130-138.

Augusto, P.E.D., Tribst, A.A.L., Cristianini, M. 2011. Thermal inactivation of *Lactobacillus plantarum* in a model liquid food. Journal of Food Process Engineering 34, 1013-1027.

Bajard, S., Rosso, L., Fardel, G., Flandrois, J.P. 1996. The particular behaviour of *Listeria monocytogenes* under sub-optimal conditions. International Journal of Food Microbiology 29, 201-211.

Benito, A., Ventoura, G., Casadei, M., Robinson, T., Mackey, B. 1999. Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat, and other stresses. Applied and Environmental Microbiology 65, 1564-1569.

Biesta-Peters, E.G., Reij, M.W., Gorris, L.G.M., Zwietering, M.H. 2010a. Comparing nonsynergistic gamma models with interaction models to predict growth of emetic *Bacillus cereus* when using combinations of pH and individual undissociated acids as growth-limiting factors. Applied and Environmental Microbiology 76, 5791-5801.

Biesta-Peters, E.G., Reij, M.W., Joosten, H., Gorris, L.G.M., Zwietering, M.H. 2010b. Comparison of two optical-density-based methods and a plate count method for

estimation of growth parameters of *Bacillus cereus*. Applied and Environmental Microbiology 76, 1399-1405.

Chen, Y.S., Yanagida, F., Shinohara, T. 2005. Isolation and identification of lactic acid bacteria from soil using an enrichment procedure. Letters in Applied Microbiology 40, 195-200.

Cole, M.B., Jones, M.V. 1990. A submerged-coil heating apparatus for investigating thermal inactivation of micro-organisms. Letters in Applied Microbiology 11, 233-235.

De Angelis, M., Di Cagno, R., Huet, C., Crecchio, C., Fox, P.F., Gobbetti, M. 2004. Heat shock response in *Lactobacillus plantarum*. Applied and Environmental Microbiology 70, 1336-1346.

Den Besten, H.M.W., Mataragas, M., Moezelaar, R., Abee, T., Zwietering, M.H. 2006. Quantification of the effects of salt stress and physiological state on thermotolerance of *Bacillus cereus* ATCC 10987 and ATCC 14579. Applied and Environmental Microbiology 72, 5884-5894.

Doyle, M.E., Mazzotta, A.S., Wang, T., Wiseman, D.W., Scott, V.N. 2001. Heat resistance of *Listeria monocytogenes*. Journal of Food Protection 64, 410-429.

Franchi, M.A., Serra, G.E., Svilosen, J., Cristianini, M. 2003 Thermal death kinetics of bacterial contaminants during cane sugar and alcohol production. International Sugar Journal 105, 527-530

G-Alegría, E., López, I., Ruiz, J.I., Sáenz, J., Fernández, E., Zarazaga, M., Dizy, M., Torres, C., Ruiz-Larrea, F. 2004. High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol. FEMS Microbiology Letters 230, 53-61.

Giraud, E., Lelong, B., Raimbault, M. 1991. Influence of pH and initial lactate concentration on the growth of *Lactobacillus plantarum*. Applied Microbiology and Biotechnology 36, 96-99.

Golowczyc, M.A., Silva, J., Abraham, A.G., De Antoni, G.L., Teixeira, P. 2010. Preservation of probiotic strains isolated from kefir by spray drying. Letters in Applied Microbiology 50, 7-12.

Gupta, S., Abu-Ghannam, N., Scannell, A.G.M. 2011. Growth and kinetics of *Lactobacillus plantarum* in the fermentation of edible Irish brown seaweeds. Food and Bioproducts Processing 89, 346-355.

Hamasaki, Y., Ayaki, M., Fuchu, H., Sugiyama, M., Morita, H. 2003. Behavior of psychrotrophic lactic acid bacteria isolated from spoiling cooked meat products. Applied and Environmental Microbiology 69, 3668-3671.

Hammes, W.P., Vogel, R.F. 1995. The genus *Lactobacillus*, p. 19-54. *In* Wood BJB, Holzapfel WH (ed.), The genera of lactic acid bacteria, vol. 2. Springer US, Boston, MA.

Houtsma, P.C., de Wit, J.C., Rombouts, F.M. 1993. Minimum inhibitory concentration (MIC) of sodium lactate for pathogens and spoilage organisms occurring in meat products. International Journal of Food Microbiology 20, 247-257.

Houtsma, P.C., de Wit, J.C., Rombouts, F.M. 1996. Minimum inhibitory concentration (MIC) of sodium lactate and sodium chloride for spoilage organisms and pathogens at different pH values and temperatures. Journal of Food Protection 59, 1300-1304.

Jordan, K.N., Cogan, T.M. 1999. Heat resistance of *Lactobacillus* spp. isolated from Cheddar cheese. Letters in Applied Microbiology 29, 136-140.

Juneja, V.K., Marks, H.M., Huang, L. 2003. Growth and heat resistance kinetic variation among various isolates of *Salmonella* and its application to risk assessment. Risk Analysis 23, 199-213.

Khedid, K., Faid, M., Mokhtari, A., Soulaymani, A., Zinedine, A. 2009. Characterization of lactic acid bacteria isolated from the one humped camel milk produced in Morocco. Microbiological Research 164, 81-91.

Lambert, R.J. 2011. A new model for the effect of pH on microbial growth: an extension of the Gamma hypothesis. Journal of Applied Microbiology 110, 61-68.

Le Marc, Y., Huchet, V., Bourgeois, C.M., Guyonnet, J.P., Mafart, P., Thuault, D. 2002. Modelling the growth kinetics of *Listeria* as a function of temperature, pH and organic acid concentration. International Journal of Food Microbiology 73, 219-237.

Lianou, A., Koutsoumanis, K.P. 2011. Effect of the growth environment on the strain variability of *Salmonella enterica* kinetic behavior. Food Microbiology 28, 828-837.

Lianou, A., Koutsoumanis, K.P. 2013. Evaluation of the strain variability of *Salmonella enterica* acid and heat resistance. Food Microbiology 34, 259-267.

Lindqvist, R. 2006. Estimation of *Staphylococcus aureus* growth parameters from turbidity data: characterization of strain variation and comparison of methods. Applied Environmental Microbiology 72, 4862-4870.

Liu, H., Xu, W., Luo, Y., Tian, H., Wang, H., Guo, X., Yuan, Y., Huang, K. 2011. Assessment of tolerance to multistresses and in vitro cell adhesion in genetically modified *Lactobacillus plantarum* 590. Antonie van Leeuwenhoek 99, 579-589.

Metselaar, K.I., Den Besten, H.M.W., Abee, T., Moezelaar, R., Zwietering, M.H. 2013. Isolation and quantification of highly acid resistant variants of *Listeria monocytogenes*. International Journal of Food Microbiology 166, 508-514.

Milly, P.J., Toledo, R.T., Harrison, M.A., Armstead, D. 2007. Inactivation of food spoilage microorganisms by hydrodynamic cavitation to achieve pasteurization and sterilization of fluid foods. Journal of Food Science 72, M414-422.

Minervini, F., Siragusa, S., Faccia, M., Dal Bello, F., Gobbetti, M., De Angelis, M. 2012. Manufacture of Fior di Latte cheese by incorporation of probiotic lactobacilli. Journal Dairy Science 95, 508-520.

Murphy, M.G., Condon, S. 1984. Comparison of aerobic and anaerobic growth of *Lactobacillus plantarum* in a glucose medium. Archives of Microbiology 138, 49-53.

Nauta, M., Dufrenne, J. 1999. Variability in growth characteristics of different *E. coli* O157:H7 isolates, and its implications for predictive microbiology. Quantitative Microbiology 1, 137-155.

Paéz, R., Lavari, L., Vinderola, G., Audero, G., Cuatrin, A., Zaritzky, N., Reinheimer, J. 2012. Effect of heat treatment and spray drying on lactobacilli viability and resistance to simulated gastrointestinal digestion. Food Research International 48, 748-754.

Parente, E., Ciocia, F., Ricciardi, A., Zotta, T., Felis, G.E., Torriani, S. 2010. Diversity of stress tolerance in *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum*: A multivariate screening study. International Journal of Food Microbiology 144, 270-279.

Rodríguez-Calleja, J.M., Cebrián, G., Condón, S., Mañas, P. 2006. Variation in resistance of natural isolates of *Staphylococcus aureus* to heat, pulsed electric field and ultrasound under pressure. Journal of Applied Microbiology 100, 1054-1062.

Sanders, J.W., Oomes, S.J.C.M., Membré, J.M., Wegkamp, A., Wels, M. 2015. Biodiversity of spoilage lactobacilli: phenotypic characterisation. Food Microbiology 45, Part A, 34-44.

Siezen, R.J., Tzeneva, V.A., Castioni, A., Wels, M., Phan, H.T.K., Rademaker, J.L.W., Starrenburg, M.J.C., Kleerebezem, M., van Hylckama Vlieg, J.E.T. 2010. Phenotypic and genomic diversity of *Lactobacillus plantarum* strains isolated from various environmental niches. Environmental Microbiology 12, 758-773.

Siezen, R.J., van Hylckama Vlieg, J.E.T. 2011. Genomic diversity and versatility of *Lactobacillus plantarum*, a natural metabolic engineer. Microbial Cell Factories 10, S3-S3.

Stevens, M.J.A., Wiersma, A., de Vos, W.M., Kuipers, O.P., Smid, E.J., Molenaar, D., Kleerebezem, M. 2008. Improvement of *Lactobacillus plantarum* aerobic growth as directed by comprehensive transcriptome analysis. Applied and Environmental Microbiology 74, 4776-4778.

Tajchakavit, S., Ramaswamy, H.S., Fustier, P. 1998. Enhanced destruction of spoilage microorganisms in apple juice during continuous flow microwave heating. Food Research International 31, 713-722.

Tournas, V.H. 2005. Spoilage of vegetable crops by bacteria and fungi and related health hazards. Critical Reviews in Microbiology 31, 33-44.

Turner, K.W., Lawrence, R.C., Lelievre, J. 1986. A microbiological specification for milk for aseptic cheesemaking. New Zealand Journal of Dairy Science and Technology 21, 249-254.

Väisänen, O.M., Mwaisumo, N.J., Salkinoja-Salonen, M.S. 1991. Differentiation of dairy strains of the *Bacillus cereus* group by phage typing, minimum growth temperature, and fatty acid analysis. Journal of Applied Bacteriology 70, 315-324.

Van Asselt, E.D., Zwietering, M.H. 2006. A systematic approach to determine global thermal inactivation parameters for various food pathogens. International Journal of Food Microbiology 107, 73-82.

Van Boekel, M.A.J.S. 2002. On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. International Journal of Food Microbiology 74, 139-159.

Watanabe, M., van der Veen, S., Nakajima, H., Abee, T. 2012. Effect of respiration and manganese on oxidative stress resistance of *Lactobacillus plantarum* WCFS1. Microbiology 158, 293-300.

Whiting, R.C., Golden, M.H. 2002. Variation among *Escherichia coli* O157:H7 strains relative to their growth, survival, thermal inactivation, and toxin production in broth. International Journal of Food Microbiology 75, 127-133.

Yang, J., Cao, Y., Cai, Y., Terada, F. 2010. Natural populations of lactic acid bacteria isolated from vegetable residues and silage fermentation. Journal Dairy Science 93, 3136-3145.

Zwietering, M.H., Wijtzes, T., Rombouts, F.M., Van 't Riet, K. 1993. A decision support system for prediction of microbial spoilage in foods. Journal of Industrial Microbiology 12, 324-329.

Zwietering, M.H., Cuppers, H.G.A.M., de Wit, J.C., Van 't Riet, K. 1994. Evaluation of data transformations and validation of a model for the effect of temperature on bacterial growth. Applied and Environmental Microbiology 60, 195-203.

SUPPLEMENTARY MATERIALS

Supplement 4.1. Secondary growth models transformation

pH model

$$\mu_{max} = \mu_{opt}\left(1 - 2^{\frac{(pH - pH_{min})}{(pH_{min} - pH_{1/2})}}\right)$$

$$\mu_{ref} = \mu_{opt}\left(1 - 2^{\frac{(pH_{ref} - pH_{min})}{(pH_{min} - pH_{1/2})}}\right)$$

$$\mu_{opt} = \frac{\mu_{ref}}{\left(1 - 2^{\frac{(pH_{ref} - pH_{min})}{(pH_{min} - pH_{1/2})}}\right)}$$

$$\mu_{max} = \mu_{ref}\frac{\left(1 - 2^{\frac{(pH - pH_{min})}{(pH_{min} - pH_{1/2})}}\right)}{\left(1 - 2^{\frac{(pH_{ref} - pH_{min})}{(pH_{min} - pH_{1/2})}}\right)}$$

$$\gamma(pH) = \frac{\left(1 - 2^{\frac{(pH - pH_{min})}{(pH_{min} - pH_{1/2})}}\right)}{\left(1 - 2^{\frac{(pH_{ref} - pH_{min})}{(pH_{min} - pH_{1/2})}}\right)}$$

a_w model

$$\mu_{max} = \mu_{opt}\left(1 - \left(\frac{1 - a_w}{1 - a_{a_{w_{min}}}}\right)^{\alpha_{a_w}}\right)$$

$$\mu_{ref} = \mu_{opt}\left(1 - \left(\frac{1 - a_{a_{w_{ref}}}}{1 - a_{a_{w_{min}}}}\right)^{\alpha_{aw}}\right)$$

$$\mu_{opt} = \frac{\mu_{ref}}{\left(1 - \left(\frac{1 - a_{w_{ref}}}{1 - a_{w_{min}}}\right)^{\alpha_{a_w}}\right)}$$

$$\mu_{max} = \mu_{ref}\frac{\left(1 - \left(\frac{1 - a_w}{1 - a_{w_{min}}}\right)^{\alpha_{a_w}}\right)}{\left(1 - \left(\frac{1 - a_{w_{ref}}}{1 - a_{w_{min}}}\right)^{\alpha_{a_w}}\right)}$$

$$\gamma(a_w) = \frac{\left(1 - \left(\frac{1 - a_w}{1 - a_{w_{min}}}\right)^{\alpha_{a_w}}\right)}{\left(1 - \left(\frac{1 - a_{w_{ref}}}{1 - a_{w_{min}}}\right)^{\alpha_{a_w}}\right)}$$

[HLa] model (experiment at pH 4.5)

$$\mu_{max} = \mu_{pH\ 4.5}\left(1 - \left(\frac{[HLa]}{[HLa_{max}]}\right)^{\alpha_{[HLa]}}\right)$$

$$\mu_{pH\ 4.5} = \mu_{ref}\frac{\left(\frac{1}{1-2^{(pH_{min}-pH_{1/2})}}\right)^{(pH\ 4.5-pH_{min})}}{\left(\frac{1}{1-2^{(pH_{min}-pH_{1/2})}}\right)^{(pH_{ref}-pH_{min})}}$$

$$\mu_{max} = \mu_{ref}\frac{\left(\frac{1}{1-2^{(pH_{min}-pH_{1/2})}}\right)^{(pH4.5-pH_{min})}}{\left(\frac{1}{1-2^{(pH_{min}-pH_{1/2})}}\right)^{(pH_{ref}-pH_{min})}}\left(1 - \left(\frac{[HLa]}{[HLa_{max}]}\right)^{\alpha_{[HLa]}}\right)$$

$$\gamma(HLa) = \left(1 - \left(\frac{[HLa]}{[HLa_{max}]}\right)^{\alpha_{[HLa]}}\right)$$

Temperature model

$$\mu_{max} = \left(\alpha_T(T - T_{min})\right)^2$$
$$\mu_{max} = {\alpha_T}^2(T - T_{min})^2$$
$$\mu_{ref} = {\alpha_T}^2\left(T_{ref} - T_{min}\right)^2$$
$${\alpha_T}^2 = \frac{\mu_{ref}}{\left(T_{ref} - T_{min}\right)^2}$$
$$\mu_{max} = \mu_{ref}\frac{(T - T_{min})^2}{\left(T_{ref} - T_{min}\right)^2}$$
$$\gamma(T) = \frac{(T - T_{min})^2}{\left(T_{ref} - T_{min}\right)^2}$$

Supplement Table S4.1. The shape parameter (β) of thermal inactivation curve of *L. plantarum*

Strains	Shape parameter (β)[1)]			
	55°C	58°C	60°C	63°C
FBR01	1.41 (0.134)	1.94 (0.198)	1.59 (0.465)	1.91 (0.247)
FBR02	1.10 (0.0797)	1.20 (0.131)	1.57 (0.149)	1.36 (0.241)
FBR03	1.19 (0.163)	2.63 (0.485)	1.40 (0.202)	2.11 (0.392)
FBR04	1.31 (0.155)	2.03 (0.44)	1.47 (0.124)	1.29 (0.201)
FBR05	3.43 (0.340)	0.89 (0.209)	1.89 (0.270)	1.80 (0.455)
FBR06	1.27 (0.0856)	1.79 (0.147)	3.19 (0.369)	1.92 (0.214)
FBR22	0.828 (0.0622)	0.859 (0.0465)	1.01 (0.228)	1.15 (0.144)
FBR23	1.67 (0.401)	1.70 (0.243)	1.65 (0.235)	1.70 (0.538)
FBR24	1.88 (0.220)	1.37 (0.135)	1.16 (0.0643)	1.17 (0.0911)
FBR25	1.53 (0.166)	1.56 (0.142)	1.68 (0.140)	1.54 (0.315)
FBR26	1.15 (0.0904)	1.70 (0.393)	1.64 (0.141)	2.00 (0.590)
FBR27	1.66 (0.192)	0.896 (0.0672)	0.886 (0.158)	0.947 (0.174)
FBR28	1.18 (0.129)	2.17 (0.179)	2.05 (0.366)	2.17 (0.496)
FBR29	1.22 (0.126)	1.52 (0.332)	1.06 (0.0197)	1.32 (0.329)
FBR30	1.36 (0.307)	1.12 (0.0936)	0.726 (0.0451)	1.06 (0.284)
WCFS1	1.25 (0.214)	1.70 (0.0462)	1.88 (0.221)	1.82 (0.158)
LMG18035	1.11 (0.134)	1.34 (0.394)	1.02 (0.0959)	1.16 (0.0691)
LMG23454	1.67 (0.259)	1.32 (0.301)	1.32 (0.119)	1.24 (0.0889)
LMG6907	1.48 (0.261)	1.18 (0.126)	2.16 (0.333)	1.70 (0.377)
SF2A35B	1.04 (0.0697)	1.11 (0.216)	0.525 (0.0542)	0.538 (0.241)

1) The value between brackets is the standard deviation of six β estimates.

SUPPLEMENTARY FIGURES

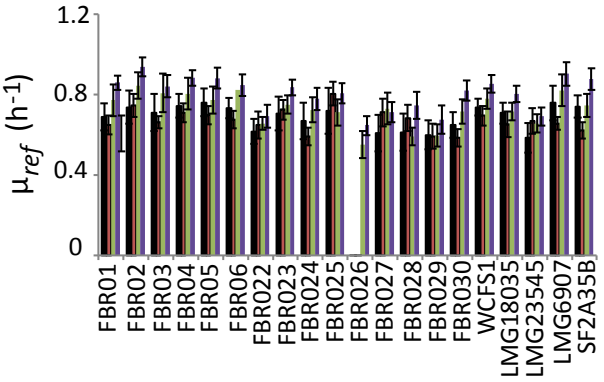

Figure S4.1. The calculated μ_{ref} as function of pH, HLa, a_w and temperature.

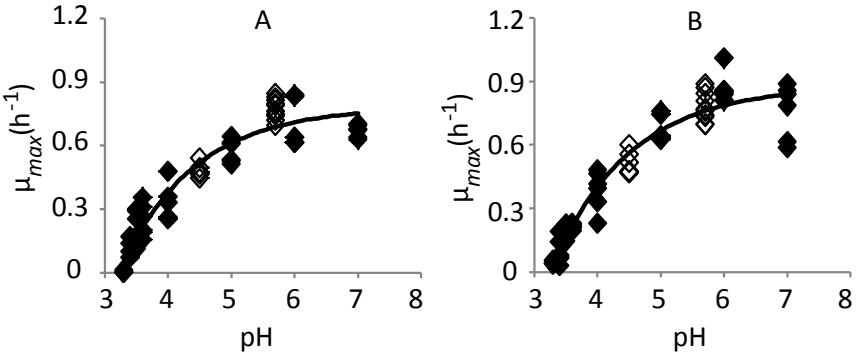

Figure S4.2. the μ_{max} data as function of pH: A) strain FBR01 and B) strain FBR05. Closed symbols: the data obtained from the pH experiment. Open symbols: the data obtained from temperature (30°C, pH 5.7), and HLa experiment (30°C, pH 4.5, 0 mM [HLa]). The solid line is the average prediction if μ_{max} data of pH 7 was used in the fitting process.

CHAPTER 5

The effect of different matrices on the growth and thermal inactivation of pathogenic and spoilage microorganisms

D.C. Aryani, M.H. Zwietering, H.M.W den Besten

Submitted for publication

ABSTRACT

Microbial growth and inactivation kinetics in food can be predicted when the effects of food properties and environmental conditions on microbial responses are available. However the effects of these intrinsic and extrinsic variables on microbial kinetics are often obtained using laboratory media, and deviations between predictions and true behaviour might occur if the specific effect of a food product is not known or considered in the prediction. Therefore, knowing the food specific effect on microbial kinetics might not only result in a more realistic growth and inactivation prediction, but also extend the knowledge on factors influencing growth and heat resistance. In this study, growth predictions of *Listeria monocytogenes* and *Lactobacillus plantarum* were validated in laboratory media and in milk and ham as model food products. A good agreement between the predicted and observed growth kinetics in laboratory media highlighted the possibility to predict μ_{max} based on cardinal growth parameters obtained from OD-based measurement in laboratory media. Only in two conditions a possible interaction between growth limiting factors was observed, yet existing interaction models were not better in predicting growth. Growth validation in the two model foods showed that the food specific effects were strain dependent, which might further complicate accurate prediction. For both species the effect of strain variability on thermal inactivation was similar to the food specific effects, and the latter was mainly determined by the effect of ham as heating medium. The combination of both effects explained (almost) all variability found in literature, however, with some bias.

INTRODUCTION

Quantitative microbiology relies on the concept that the responses of microorganisms to intrinsic food properties and environmental conditions are reproducible (Ross and McMeekin, 1994), allowing the use of past knowledge to predict the behaviour of microorganisms. Quantitative microbiology is used, amongst others, as a tool to evaluate the efficacy of certain product designs and thermal processes in controlling the presence and growth of microorganisms to ensure an acceptable level at the end of the shelf life period. The mathematical models and parameters used for prediction are generally established from experimental data obtained in well-defined experimental conditions using laboratory media (Adams et al., 1991; Buchanan and Phillips, 1990; Duh and Schaffner, 1993; Le Marc et al., 2002; Presser et al., 1997; Ross et al., 2003). When growth or inactivation kinetics in an actual food product are investigated, then the prediction is sometimes found to be unrealistic (Pin et al., 1999). The reason is that the model is based on data generated in laboratory media and only focuses on the intrinsic and extrinsic parameters available for that media, and does not take into account additional food specific factors (Xanthiakos et al., 2006). These food factors are also specific since the growth model developed specially for a certain food product type might not be realistic for other food products (Schvartzman et al., 2010). To quantify these food specific factors, the model established using laboratory media based data must be validated in food products, such as done by Murphy et al. (1996), or by using available literature data (te Giffel and Zwietering, 1999).

In previous studies, the growth and thermal inactivation of *Listeria monocytogenes* and *Lactobacillus plantarum* in laboratory media were characterized (Aryani et al., 2015a; Aryani et al., 2015b; Aryani et al., submitted for publication), in which strain variability in thermal inactivation was found to be much larger than strain variability in growth kinetics for both species. The obtained cardinal growth parameters were initially used to predict the microbial maximum specific growth rate (μ_{max}) in a defined food product (Aryani et al., 2015b; Aryani et al., submitted for publication). This initial prediction was made using a gamma modelling approach (Zwietering et al., 1996) to demonstrate the effect of strain variability on the growth behaviour of *L. monocytogenes* and *L. plantarum*. No product factors, however, were included in our previous studies, so the initial prediction results might not be sufficient for predicting the actual microbial behaviour in real food products. Similarly, food factors, such as fat, are also known to influence the heat resistance of microorganisms (Doyle et al., 2001; Van Asselt and Zwietering, 2006), and might provide additional challenges to model the inactivation rate of microorganisms.

The knowledge on food product specific factors is not only needed to improve the prediction result, but is also needed to prioritize the importance in comparison to other variability factors during experimental or challenge study designs. Therefore, the objectives of the present study were: 1) to validate the established growth model using experimental and literature growth data; 2) to examine the food product specific effects on microbial growth and heat resistance; and 3) to compare the food product specific effects with other variability factors to prioritize their importance. The model food products used in our study were milk and ham, representing liquid and solid food products.

MATERIALS AND METHODS

Predicting the growth of *L. monocytogenes* and *L. plantarum*

Growth kinetics of *L. monocytogenes* and *L. plantarum* was predicted in laboratory media, skimmed milk and ham. The properties of skimmed milk and ham are listed in table 5.1. The pH and water activity were measured using a pH meter and a_w meter (Novasina water activity machine), respectively. The concentration of total lactic acid in ham was determined using HPLC (Ultimate 3000 system, Dionex, equiped with a Shodex RI detector and Aminex HPX 87H column). Briefly, the ham sample was homogenized in demineralized water using an ultraturrax (IKA® ultraturrax® tube dispenser). The ham suspension was then centrifuged at 12,000 x *g* for 2 min (Biofuge Pico, Heraeus instruments). Ten microliter of the supernatant was injected in the column and elution was performed using 0.005M H_2SO_4 at a flow rate of 0.6 ml/min. The obtained total lactic acid concentration was then corrected and converted into the total undissociated lactic acid ([HLa]) knowing also the pH of the ham.

Table 5.1. Food matrices characteristics

Variables	Milk	Commercial ham	In-house produced ham
pH	6.6-6.7	6.5	6.0
a_w	0.997 (±0.003)	0.968 (±0.003)	0.967 (±0.003)
[HLa] (mM)	0	0.21 (σ= 0.060)	1.24 (σ =0.47)

The growth of *L. monocytogenes* and *L. plantarum* was predicted using the three-phase linear model (Equations (5.1a) and (5.1b)) adapted from Buchanan et al. (1997).

$$Log_{10}(N_t) = Log_{10}N_0 \;\; for\; t \leq \lambda \qquad [5.1a]$$

$$Log_{10}(N_t) = \min\left(Log_{10}N_0 + \frac{\mu_{max}}{\ln(10)}(t-\lambda), Log_{10}N_{max}\right) for\; t > \lambda \qquad [5.1b]$$

Where N_t is the concentration (CFU/ml or CFU/g) at time t, N_0 is the initial concentration (CFU/ml or CFU/g), μ_{max} is the maximum specific growth rate at a certain condition (h^{-1}), N_{max} is the maximum concentration (CFU/ml or CFU/g), t and λ are the time and lag time (h), respectively.

The maximum specific growth rate (μ_{max}) used in the three-phase linear model was estimated using the gamma model without interaction (equations (5.2a) and (5.2b)), which included the best fitting secondary growth models from our previous study (Aryani et al., 2015b). These secondary growth models were transformed following the reference conditions of pH 7.3, a_w 0.997, T 30°C, 0 mM [HLa] for *L. monocytogenes* and pH 5.7, a_w 0.993, T 30°C, [HLa] 0 mM for *L. plantarum* (equations (5.3)-(5.6)).

$$\gamma(total) = \gamma(pH) * \gamma(a_w) * \gamma(T) * \gamma(HLa) \quad [5.2a]$$

$$\mu_{max} = \mu_{ref} * \gamma(total) \quad [5.2b]$$

$$\gamma(pH) = \frac{1-2^{\frac{(pH-pH_{min})}{(pH_{min}-pH_{1/2})}}}{1-2^{\frac{(pH_{ref}-pH_{min})}{(pH_{min}-pH_{1/2})}}} \quad [5.3]$$

$$\gamma(a_w) = \frac{1-\left(\frac{1-a_w}{1-a_{w_{min}}}\right)^{\alpha_{a_w}}}{1-\left(\frac{1-a_{w_{ref}}}{1-a_{w_{min}}}\right)^{\alpha_{a_w}}} \quad [5.4]$$

$$\gamma(T) = \frac{(T-T_{min})^2}{(T_{ref}-T_{min})^2} \quad [5.5]$$

$$\gamma([HLa]) = 1 - \left(\frac{[HLa]}{[HLa_{max}]}\right)^{\alpha_{[HLa]}} \quad [5.6]$$

Where γ is the gamma factor for each variable; μ_{max} is the maximum specific growth rate (h^{-1}): μ_{ref}, pH_{ref}, $a_{w,ref}$ and T_{ref} are the specific growth rate (h^{-1}), pH, a_w, and temperature (°C) at the reference conditions; pH, a_w, T, $[HLa]$ are the actual pH, a_w, temperature (°C) and undissociated lactic acid concentration (mM). The cardinal growth parameters pH_{min}, $pH_{1/2}$, $a_{w,min}$ T_{min} and $[HLa_{max}]$ and the shape parameters α_{aw} and $\alpha_{[HLa]}$ were obtained from the previous studies (Aryani et al., 2015b, Aryani et al., submitted for publication) (supplement table 5.1).

The lag time was estimated following the same approach as Koutsoumanis et al. (2006) and Zwietering et al. (1994), assuming $\lambda * \mu_{max}$ between 0-4. Using this assumption, the λ duration of cells grown in BHI and milk was between 0 and 5 h when cells were grown in laboratory media at 30°C or 0-5 days when cells were grown in laboratory media and milk at 7°C for *L. monocytogenes* and at 15°C for *L. plantarum*. For the cells grown in ham at 7°C for *L. monocytogenes* and at 15°C for *L. plantarum* the λ duration was between 0 and 12 days. Moreover, since the initial culture used in the validation study was between 2 and 3 $\log_{10}$ CFU/ml or $\log_{10}$ CFU/g, those values were used for the lowest and highest

initial concentration in the prediction. The maximum concentration (N_{max}) obtained from the validation study varied according to the type of media. Therefore, for the cells grown in laboratory media, the maximum concentration used for prediction was between 9 and 10 $\log_{10}$ CFU/ml. For the cells grown in milk and ham, in which less nutrients are available, the maximum concentration was between 7 and 8 $\log_{10}$ CFU/ml or $\log_{10}$ CFU/g; and for the cells grown in laboratory media in the presence of severe growth limiting factors, the maximum concentration was between 7 and 8 $\log_{10}$ CFU/ml.

The prediction was presented with two different intervals: i) 95% confidence intervals (95% CI) of the respective strain and ii) 95% prediction intervals (95% PI) of all strains. In the first interval, the lowest or highest estimates (95% CI) of the cardinal parameters and μ_{ref} were used to calculate the 95% CI of μ_{max} for each strain. These 95% CI μ_{max} values represent within strain (reproduction) variability. In the second interval, the mean estimate of each cardinal parameter and μ_{ref} was used to calculate the μ_{max} of each strain. The 95% prediction interval was then calculated from the average μ_{max} of all 20 strains. Since the 95% PI was calculated from the μ_{max} of the 20 strains, this interval represents strain variability.

The prediction result was then compared to the obtained growth kinetics in laboratory media to validate the gamma model. If a deviation between the observed and predicted kinetics was observed, the gamma model with interaction of Le Marc et al. (2002) (equation (5.7)) and Augustin and Carlier (2000) (equations (5.8a) and (5.8b)) were also tested.

$$\mu_{max} = \mu_{ref} * \gamma(pH) * \gamma(a_w) * \gamma(T) * \gamma([HLa]) * \xi(pH, a_w, T, [HLa]) \quad [5.7]$$

The $\xi(pH, a_w, T, [HLa])$ is the interaction term between parameters proposed by Le Marc et al. (2002).

The model of Augustin and Carlier does not include a specific interaction factor, but the cardinal growth parameters are adapted independently, integrating the effect of the other growth parameter factors. The adapted cardinal growth parameters were then used in the gamma model without interaction.

$$X_{min,new} = X_{opt} - (X_{opt} - X_{min}) * \left\{ \left(1 - \frac{[HLa]}{[HLa_{max}]}\right) - \left(\frac{Y_{opt} - Y}{Y_{opt} - Y_{min}}\right)^3 - \left(\frac{Z_{opt} - Z}{Z_{opt} - Z_{min}}\right)^3 \right\}^{1/3} \quad [5.8a]$$

$$[HLa_{max,new}] = [HLa_{max}] * \left(1 - \left\{ \left[\frac{pH_{opt} - pH}{pH_{opt} - pH_{min}}\right]^3 + \left[\frac{T_{opt} - T}{T_{opt} - T_{min}}\right]^3 + \left[\frac{a_{w_{opt}} - a_w}{a_{w_{opt}} - a_{w_{min}}}\right]^3 \right\}\right) \quad [5.8b]$$

Where *X*, *Y*, and *Z* are pH, a_w, or T. The value of T_{opt} and pH_{opt} for *L. monocytogenes* used were 37°C and pH 7 as found from literature (ICMSF, 1996) and the $a_{w,opt}$ was set at 1.

Additionally, the bias and accuracy factors (Ross, 1996) (equations (5.9)-(5.10)) were used to assess the performance of the gamma model in predicting growth kinetics in laboratory

media. For this purpose, only the mean predicted μ_{max} of the Gamma model was compared to the mean observed μ_{max} obtained from fitting Gompertz model to the experimental data. The μ_{max} was converted to generation time (GT) following equation (5.11).

$$B_f = 10^{(\sum \log(GT_{predicted}/GT_{observed})/n)} \quad [5.9]$$

$$A_f = 10^{(\sum |\log(GT_{predicted}/GT_{observed})|/n)} \quad [5.10]$$

$$GT = \frac{LN(2)}{\mu_{max}} \quad [5.11]$$

Where *GT* is the generation time (h), $GT_{predicted}$ is the predicted generation time; $GT_{observed}$ is the observed generation time, *n* is the number of $GT_{observed}$ and μ_{max} is the maximum specific growth rate (h^{-1}).

Culture preparation

Three strains of *L. monocytogenes* (strain L6, FBR17 and FBR15) and two strains of *L. plantarum* (strain WCFS1 and FBR05) were selected for this study. Strains L6, FBR17, and FBR15 were selected based on their characteristic as heat resistance strain, robust strain at low pH, low a_w and high concentration of HLa, and slow growing strain, respectively, while FBR05 and WCFS1 were selected for their characteristic as heat resistant strains and also robust strain at low temperature for WCFS1.

From frozen cultures kept at -20°C, a streak was made on Brain Heart Infusion (BHI) agar (BHI broth, Becton Dickinson, France, supplemented with 1.5% of bacterial agar (w/v), Oxoid, England) for *L. monocytogenes* and on De Man, Rogosa and Sharpe (MRS, Merck Millipore) agar for *L. plantarum* and incubated for 48h (at microaerobic condition for *L. plantarum*) at 30°C. A single colony from the plate was selected and transferred to 100 ml of BHI broth for *L. monocytogenes* and MRS broth for *L. plantarum*. The cultures were incubated at 200 rpm for 8-10 days at 7°C and 16h at 30°C for *L. monocytogenes* and were incubated statically for 7 days at 15°C and 24h at 30°C for *L. plantarum*. Then, all cultures were diluted to the concentration of approximately 10^4 $\log_{10}$ CFU/ml using Peptone Physiological Salt (PPS, Tritium Microbiologie B.V., Netherlands).

Growth Kinetics

Validation in laboratory media

Validation in laboratory media was conducted in three types of experiments:

1) Validation of the model at optimal conditions (30°C) in plain medium using *L. monocytogenes* L6, FBR17 and FBR15 and *L. plantarum* WCFS1 and FBR05. For this purpose, one milliliter of the diluted culture (pre-cultured at 30°C) was transferred into a 250 ml flask containing 100 ml of BHI broth or MRS broth in duplicate. The

flasks were then incubated at 30°C at 200 rpm for *L*. *monocytogenes* and 30°C at static condition for *L*. *plantarum*. Sampling was performed every 2 h until the samples reached stationary phase. Each sample was diluted in PPS and the appropriate dilution was plated on BHI/MRS agar plates in duplicate using a spiral plater (Eddy Jet, IUL instruments). The plates were incubated at 30°C for *L*. *monocytogenes* and in microaerobic condition at 30°C for *L*. *plantarum* for 24-48 h. The colonies found on the plate were counted and reported in $\log_{10}$ CFU/ml.

2) Validation of the model at low incubation temperature (7°C and 15°C) in plain medium using *L. monocytogenes* L6, FBR17 and FBR15 and *L. plantarum* WCFS1 and FBR05. One milliliter of the diluted culture (pre-cultured at 7°C, 15°C or 30°C) was transferred into a 250 ml flask containing 100 ml of BHI broth or MRS broth in duplicate. The flasks were incubated statically at 7°C and 15°C for *L*. *monocytogenes* and *L*. *plantarum* respectively until reaching stationary phase. Also, flasks containing 100 ml of BHI broth were incubated at 7°C at 200 rpm to examine the effect of shaking on the growth of *L*. *monocytogenes*. Sampling was performed every 2 days until the samples reached stationary phase. The samples were plated and incubated following a similar procedure as described earlier in 1).
3) Validation of the model when the multiple growth limiting factors were present in the media. For these experiments, only *L. monocytogenes* strain L6 was selected as a representative strain. The effect of growth limiting factors was tested using different conditions: a) BHI +2.5% NaCl (AnalaR NORMAPUR, VWR International, Leuven Belgium), b) BHI+ DL-lactic acid 85% (pH 6.1, 0.13 [HLa]) (Sigma-Aldrich, Germany), c) BHI adjusted to pH 5.5 using H_2SO_4 (Riedel-de Haen, Seelze Germany) and 1 mM [HLa] (calculated using the Henderson-Hasselbalch equation described in a previous study (Aryani et al., 2015b)), and d) BHI adjusted to pH 5.5, 1 mM [HLa] and 2.5% NaCl.

One milliliter of the diluted culture (pre-cultured at 30°C or 7°C) was transferred into a 250 ml flask containing 100 ml of each adjusted BHI broth. The flasks then were incubated at 30°C and 7°C, 200 rpm for almost all experiments, except for the experiment using BHI adjusted to pH 5.5, 1 mM [HLa] and 2.5% NaCl, which was only done at 7°C and 200 rpm. Sampling was performed every 2 h for the samples incubated at 30°C and every 2 days for the samples incubated at 7°C until the samples reached stationary phase. The samples were plated and incubated following a similar procedure as used for the validation at optimum condition.

Validation in milk

One milliliter of the diluted culture (incubated at 30°C, 7°C or 15°C) was transferred into a 250 ml flask containing 100 ml of milk (UHT milk 0% fat, a_w 0.997 ($\pm$0.003), pH 6.6 ($\pm$0.1)).

The flasks were incubated statically at 7°C and 15°C for *L. monocytogenes* and *L. plantarum* respectively until reaching stationary phase. Sampling was performed every 2 days until the samples reached stationary phase. At each sampling time, 1 ml of milk samples was taken, diluted in PPS and plated in duplicate using a spiral plater on BHI and MRS agar plates. The plates were incubated following the method described earlier for laboratory media. Additionally, experiments were also conducted in milk at 7°C and 15°C containing 1.5% and 3% of fat (semi-skimmed milk and full milk) to study the effect of fat concentration and also to validate the gamma factor obtained from skimmed milk.

Growth validation on commercial and in-house produced hams

Two different hams were used for the experiments, commercial ham (pH 6.7, a_w 0.968, [HLa] 0.21 mM (standard deviation of 0.060, ±12-13 g per ham slice) and in-house produced ham provided by Corbion, the Netherlands (pH 6, a_w 0.967, [HLa] 1.24 mM (standard deviation of 0.47, 5 - 7 g per ham slice). Before the ham slices were used for the experiment, each slice was put in the stomacher bag and sent for irradiation (10 kGy). The slices were then stored at 4°C prior to use.

Hundred microliter of diluted culture was spread on both sides of each ham slice to obtain approximately 2-3 $\log_{10}$ CFU/g of *L. monocytogenes* and *L. plantarum* respectively. The ham slice in the stomacher bag was then incubated at 7°C and 15°C until stationary phase. At each sampling time point a bag was removed and diluted 10 times in PPS and homogenized using a stomacher (30 s, 160 rpm). The homogenized suspension was then further diluted in PPS and plated in duplicate on BHI and MRS agar. The agar was incubated at 30°C for *L. monocytogenes* and at 30°C in microaerobic conditions for *L. plantarum* for 24 to 48 h. The colonies obtained on the plate were counted and reported as $\log_{10}$ CFU/g.

Validation using literature data

Besides using experimental data, the validation can be performed using data obtained from literature. For this purpose, the *L. monocytogenes* growth data in BHI at 30°C, BHI at 3°C, milk at 7°C, milk at 10°C and ham at 8°C were extracted from Combase. The Gompertz model was then fitted to the log counts data extracted from Combase to estimate the μ_{max}. This literature μ_{max} was used to validate the predicted μ_{max} obtained using gamma model. Since no information was available on the type of the strain used in literature, the predicted 95% PI of μ_{max} from all strains were used for comparison.

Model fitting and gamma food product calculation

The re-parameterized modified Gompertz model (Zwietering et al., 1990) (equation (5.12)) was used to fit the $\log_{10}$ CFU/ml or $\log_{10}$ CFU/g data as function of time.

$$Log_{10}N_t = Log_{10}N_0 + (Log_{10}N_{max} - Log_{10}N_0)exp\left(-exp\left[\frac{\frac{\mu_{max}}{LN_{10}}exp(1)}{(Log_{10}N_{max}-Log_{10}N_0)}\right](\lambda - t) + 1\right) \quad [5.12]$$

Where N_0 is the initial bacterial concentration (CFU/ml or CFU/g), N_{max} is the maximum bacterial concentration (CFU/ml or CFU/g), μ_{max} is the maximum specific growth rate at a certain temperature (30°C, 7°C, or 15°C) (h^{-1}), λ is the lag time (h) and t is the time (h).

The gamma factor for the food product specific effect was calculated following equation (5.13), assuming that the observed μ_{max} is the product of predicted μ_{max} and food product factor (γ(product))

$$\mu_{max,obs} = \mu_{max,predict} * \gamma(product) \quad [5.13]$$

Where $\mu_{max,obs}$ is the observed μ_{max} obtained from fitting the modified Gompertz model to the experimental data; $\mu_{max,predicted}$ is the predicted μ_{max} obtained using the gamma model; and γ(product) is food product factor.

The fitting was done using excel solver Add-In and was confirmed using TableCurve 2D v5.01.

Heat resistance

The effect of heating menstruum on heat resistance

To test the effect of heating menstruum, one ml of diluted overnight culture incubated at 30°C (see section culture preparation) was transferred to the flask containing 100 ml of BHI for *L. monocytogenes* and MRS for *L. plantarum* and incubated until stationary phase at 7°C for *L. monocytogenes* (14 days) and at 15°C for *L. plantarum* (10 days). At the end of the incubation period in those laboratory media, the obtained culture was used for testing the effect of heating menstruum following the procedure described previously (Aryani et al., 2015a). Briefly, a flask containing 40 ml of heating menstruum (BHI or milk for *L. monocytogenes* and MRS or milk for *L. plantarum*) was preheated in a water bath until reaching the set temperature (65°C for *L. monocytogenes* and 60°C for *L. plantarum*). The diluted culture was then transferred to the preheated heating menstruum (1:100 v/v) to start the thermal inactivation treatment. One ml of aliquot was removed from the flask at certain time intervals and diluted directly in 9 ml of PPS. Serial dilutions were made and plated on BHI/MRS agar in duplicate using a spiral plater. For the time point at which no dilution was needed, one ml aliquot was put in a cup standing on ice for a few seconds to stop the inactivation process. The 1 ml aliquot was then distributed and spread plated on three different plates. This allowed us to have the detection limit of 0 $\log_{10}$ CFU/ml for the

cells inactivated in liquid media. All the plates were incubated at 30°C for *L. monocytogenes* and at 30°C in microaerobic conditions for *L. plantarum* for 4-5 days.
When ham was used as heating menstruum, 100 µl of the diluted culture was spread on each side of a ham slice. The bag containing ham was then heat sealed and heated in a water bath at 65°C for *L. monocytogenes* and at 60°C for *L. plantarum*. The time needed to reach 99% of those waterbath temperatures was 20-25 s for control cooked ham (0.5 mm thickness) and 35-40 s for commercial ham (1.0 mm thickness), measured using a thermocouple probe (PeakTech 3150, Thermocouple K-type) inserted into the ham before each experiment. At each sampling point a bag containing a ham slice was removed from the water bath and the bag was aseptically opened. The ham was diluted 10 times in PPS and homogenized using a stomacher (30 s, 160 rpm). From this stage, serial dilutions were made and the appropriate dilution was plated in duplicate on BHI or MRS plates. The plates were incubated at 30°C (in microaerobic condition for *L. plantarum*). The colonies found on the plate were reported in $\log_{10}$ CFU/g. The detection limit for ham was 1 $\log_{10}$ CFU/g.

The effect of incubation media on heat resistance

One ml of diluted overnight culture incubated at 30°C (see section culture preparation) was transferred to the flask containing 100 ml of BHI or milk for *L. monocytogenes* and MRS or milk for *L. plantarum* and incubated until stationary phase at 7°C for *L. monocytogenes* (14 days) and at 15°C for *L. plantarum* (10 days). At the end of the incubation period, the culture was used for thermal inactivation using similar media as used for incubation. For example, the culture obtained from incubation in laboratory media was transferred to the preheated laboratory media and the culture obtained from incubation in milk was transferred to the preheated milk to start the thermal inactivation treatment following the procedure described in the effect of heating menstruum on heat resistance section. The heat resistance data of *L. monocytogenes* and *L. plantarum* pre-cultured in laboratory media at 30°C obtained from our previous studies (Aryani et al., 2015a; Aryani et al., submitted for publication) were used as a control.
For ham, 100 µl of diluted overnight culture incubated at 30°C (from section culture preparation) was spread on each side of the ham slice. The ham slice was then incubated at 7°C for *L. monocytogenes* and 15°C for *L. plantarum* until reaching the stationary phase. At the end of the incubation time, the bag containing ham was heat sealed using a heat sealer, and then heated in the water bath following the procedure described in the effect of heating menstruum on heat resistance.

Data fitting and statistical analysis

The time points of the ham data were corrected (using equations (5.14a) and (5.14b)) with the time needed to heat the ham to 65°C for *L. monocytogenes* and 60°C for *L. plantarum*.

$$L = 10^{\left(\frac{T-T_{ref}}{z}\right)} \quad [5.14a]$$

$$F_{ref} = \int_0^t L\, dt \quad [5.14b]$$

Where *L* is ratio of inactivation rate in comparison to reference temperature (T_{ref}); T_{ref} is the reference temperature (65°C and 60°C for *L. monocytogenes* and *L. plantarum*), *T* is the actual temperature at certain heating time (°C), *z* is the temperature needed for one $\log_{10}$ reduction in the *D*-value (°C), F_{ref} is the amount of time which give an equivalent heating process as T_{ref} does, and *t* is the total time needed to reach the temperature T (s). The obtained F_{ref} value was used to correct the heating up time.

The log number of surviving cells was then plotted against the corrected time points to obtain the thermal inactivation curve for each experiment, and the modified Weibull model (Metselaar et al., 2013) (equation (5.15)) was used to fit this thermal inactivation curve.

$$\mathrm{Log}_{10}N_t = \mathrm{Log}_{10}N_0 - \Delta\left(\frac{t_F}{\Delta D}\right)^{\beta} \quad [5.15]$$

Where Log N_t is the $\log_{10}$ number of surviving organism ($\log_{10}$ CFU/ml/g) at time t_F; Log N_0 is the $\log_{10}$ initial number ($\log_{10}$ CFU/ml/g); t_F is the corrected time (min or s); ΔD is the time needed to reach Δ $\log_{10}$ reduction (min/s); and β is the shape parameter.

To characterize the factors influencing the *D*-value, multiple linear regression (equation (5.16)) was used and the effect of strain and food matrix on the variability was also compared using a previously described method (figure 3.2) (Aryani et al., 2015a).

$$\mathrm{Y} = \widehat{\mathrm{Y}} + \beta_1\mathrm{Heating\ Media} + \beta_2\mathrm{Growth\ Media} + \beta_3\mathrm{Strain} \quad [5.16]$$

After the dummy variables were created for temperature, heating media, growth media and strain, the regression analysis was done in Excel (Microsoft Office 2010) using the data analysis tools.

RESULTS

Validation of growth kinetics of *L. monocytogenes* and *L. plantarum*

Validation in laboratory media

Validation in laboratory media is needed to investigate if the prediction made using previously obtained data match with the observed kinetics of microorganisms. The validation in laboratory media is also important since the cardinal growth parameters used for predicting μ_{max} were obtained from OD_{600} based measurement of a series of

experiments in laboratory media using the 2-folds dilution method with Bioscreen C, while the current growth data was obtained from plate count experiments. The validation in laboratory media with different growth limiting factors for *L. monocytogenes* strain L6 is presented in figure 5.1, and the validations in plain media at 30°C and lower temperature for strains FBR17, FBR1, WCFS1 and FBR05 are presented in figure S5.1. The maximum specific growth rate (μ_{max}) was predicted using the gamma model and the cardinal growth parameters obtained from a previous study on *L. monocytogenes* (Aryani et al., 2015b) and *L. plantarum* (Aryani et al., submitted for publication).

The growth of *L. monocytogenes* strain L6 at 30°C, when all growth parameters were in optimal condition, was in agreement with the 95% confidence intervals of predicted growth (95% CI) for strain L6 and the 95% prediction intervals (95% PI) of 20 *L. monocytogenes* strains (figure 5.1A). The difference between these two intervals is small because biological variability (represented by 95% CI) and strain variability (represented by 95% PI) were in the same order of magnitude (Aryani et al., 2015b).

When one or two parameters were adjusted to sub-optimal condition, such as with the addition of 2.5% NaCl (figure 5.1B), the addition of DL-lactic acid (pH 6.11, [HLa] 0.13 mM) (figure 5.1C), the addition of H_2SO_4 to pH 5.5 (figure 5.1D) and the addition of H_2SO_4 to pH 5.5 and 1 mM [HLa] (figure 5.1E), but the temperature was kept at 30°C, the observed data was also between the two intervals of growth prediction. Similar result was also obtained when the temperature was decreased to 7°C in: plain media (figure 5.1F), media with 2.5% NaCl (figure 5.1G) and media with the addition of DL-lactic acid (pH 6.1 and [HLa] 0.13 mM) (figure 5.1H). However, when temperature was decreased to 7°C in combination with pH 5.5 and in combination with pH 5.5 and 1 mM [HLa] (figures 5.1I and 5.1J), the observed data slightly deviated from the 95% CI of L6, although they were still within the 95% PI of *L. monocytogenes* strains. Different result was observed when 2.5% NaCl was added to the media of pH 5.5 containing 1 mM [HLa] incubated at 7°C (figure 5.1K). Although the observed data was outside the 95%CI of strain L6, the deviation seems to be affected more by the lag phase than the growth rate. When the lag phase used in the prediction process was adjusted from 0-5 days to 8-12 days, the predicted models were in agreement to the observed data (figure 5.1L).

Since we observed a slight deviation between the 95% CI and the observations of the cells incubated in BHI pH 5.5 and BHI pH 5.5, 1 mM [HLa] at 7°C, we considered the possibility of interaction effect between temperature and pH and between temperature, pH and [HLa]. Therefore, the performances of the gamma models with interaction of Le Marc et al. (2002) and Augustin and Carlier (2000) were evaluated. The calculated interaction factor of pH and temperature, or pH, temperature, and [HLa] following the Le Marc model was 1, meaning that there was no interaction predicted yet between those above

variables. From the Augustin and Carlier model, a set of new cardinal growth parameters was estimated following the equations (5.8a) - (5.8b) (data not shown). The new cardinal growth parameters were then used to calculate each of the gamma factors, and subsequently for calculating the μ_{max}. However, the resulting μ_{max} was much lower than what was obtained from the observed data, indicating that the model with new cardinal growth parameters predicted a larger interaction effect than what was observed from the experimental data.

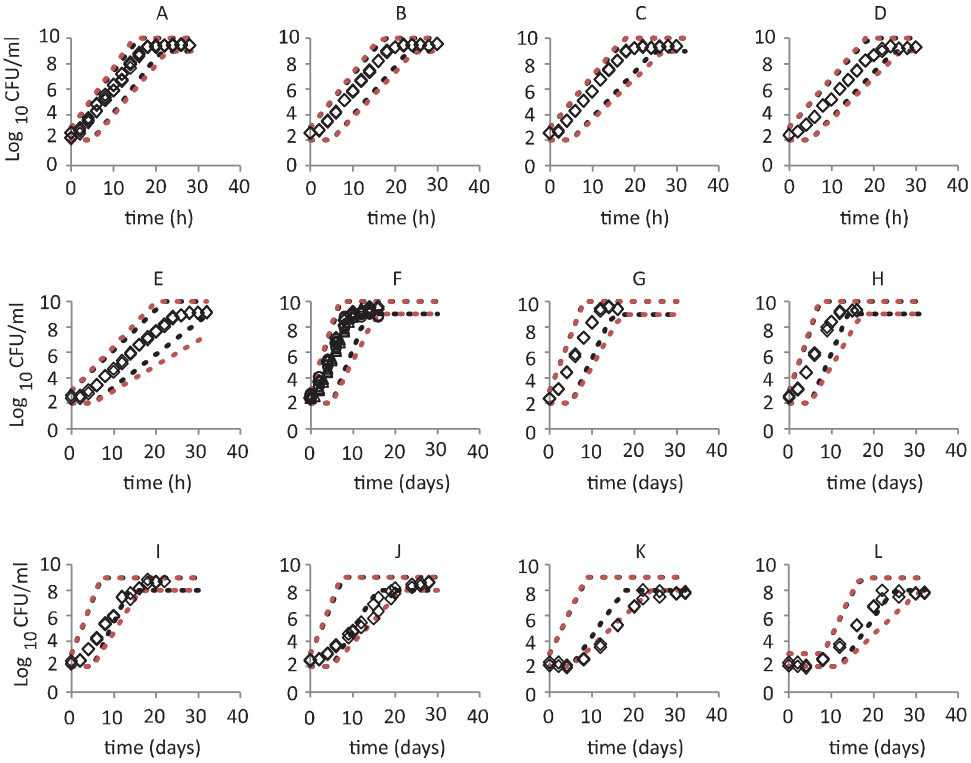

Figure 5.1. growth kinetics of *L. monocytogenes* strain L6 in BHI media: A) BHI (pH 7.3; a_w 0.997) at 30°C; B) BHI + 2.5% NaCl (pH 7.3; a_w 0.983) at 30°C; C) BHI pH 6.11 HLa 0.13 mM at 30°C; D) BHI pH 5.5 (a_w 0.997) at 30C; E) BHI pH 5.5 HLa 1 mM at 30°C; F) condition as in A at 7°C; G) condition as in B at 7°C; H) condition as in C at 7°C; I) D at 7°C; J) E at 7°C; K) BHI pH 5.5, 2.5% NaCl, 1 mM HLa at 7°C; L) condition as in K with lag phase of 8-12 days. - - - 5-95% confidence intervals of predicted growth kinetics of L6, - - - 5-95% prediction intervals of predicted growth kinetics of *L. monocytogenes* strains. ◇ Experiment data with similar temperature for pre-culturing and growth conditions; △ pre-cultured at 30°C followed with growth at 7°C 200rpm; ○ pre-cultured at 30°C followed with static growth at 7°C.

As for strain L6, the observed growth kinetics of four other strains cultured in plain media at 30°C, 7°C and 15°C were within the two intervals of the predicted kinetics (figure S5.1). Also, no difference in the fitted μ_{max} could be observed between the *L. monocytogenes* cells pre-cultured at 30°C or 7°C prior to growth at 7°C (figures 5.1F and S5.1D). However, pre-culturing at 7°C prior to growth at 7°C significantly reduced the lag phase of the slow grower strain FBR15. Although, the lag phase of strain L6 decreased upon pre-cultured at 7°C, it was not significantly different from the lag phase of the cells pre-cultured at 30°C. Moreover, the use of shaking (200 rpm), which introduced aerobic condition, did not have any effect on the observed μ_{max} and lag phase for both strains L6 and FBR15. The bias (B_f) and accuracy (A_f) factors of the gamma model are presented in table 5.2. The B_f values of the growth model in comparison to the measured growth in laboratory media ranged between 0.545 and 1.91 and the A_f between 1.00 and 1.91. Unlike in the comparison of the measured growth data to the predicted 95% CI and 95% PI, the B_f and A_f factors used the mean value of the prediction. The highest B_f of 1.97 was observed for the growth of FBR05 at pH 7°C, underlining that longer generation time was predicted using the gamma model (fail dangerous prediction). The lowest B_f was observed for the growth of L6 at pH 5.5, 7°C and at pH 5.5, 1 mM [HLa] at 7°C, underlining that the predicted growth was faster than the observed growth (fail safe prediction).

Table 5.2. Bias factor (B_f) and accuracy factor (A_f) of the gamma model estimates based on generation time in laboratory media

Strain	Variable				B_f	A_f
	pH	a_w	Temperature (°C)	[HLa] mM		
L6	7.3	0.997	30	0.0	1.19	1.19
L6	7.3	0.983	30	0.0	1.29	1.29
L6	6.11	0.997	30	0.13	1.20	1.20
L6	5.5	0.997	30	0.0	1.17	1.17
L6	5.5	0.997	30	1.0	1.10	1.10
L6	7.3	0.997	7	0.0	0.987[1)]	1.01[1)]
L6	7.3	0.997	7	0.0	1.04[2)]	1.04[2)]
L6	7.3	0.997	7	0.0	1.03[3)]	1.04[3)]
L6	7.3	0.983	7	0.0	1.00	1.00

Strain	Variable				B_f	A_f
	pH	a_w	Temperature (°C)	[HLa] mM		
L6	6.11	0.997	7	0.13	0.961	1.04
L6	5.5	0.997	7	0.0	0.703	1.42
L6	5.5	0.997	7	1.0	0.545	1.83
L6	5.5	0.983	7	1.0	0.847	1.18
FBR17	7.3	0.997	30	0.0	1.10	1.10
FBR17	7.3	0.997	7	0.0	1.20[2)]	1.20[2)]
FBR17	7.3	0.997	7	0.0	1.13[3)]	1.13[3)]
FBR15	7.3	0.997	30	0.0	1.20	1.20
FBR15	7.3	0.997	7	0.0	0.821[1)]	1.22[1)]
FBR15	7.3	0.997	7	0.0	0.768[2)]	1.30[2)]
FBR15	7.3	0.997	7	0.0	0.808[3)]	1.24[3)]
FBR05	5.7	0.993	30	0.0	1.19	1.19
FBR05	5.7	0.993	15	0.0	1.91	1.91
WCFS1	5.7	0.993	30	0.0	1.10	1.10
WCFS1	5.7	0.993	15	0.0	1.17	1.17

1) Pre-cultured at 30°C followed by growth at 7°C,200 rpm
2) Pre-cultured at 30°C followed by static growth at 7°C.
3) Pre-cultured at 7°C followed by static growth at 7°C.

Validation in milk

A good agreement between the predicted and observed growth kinetics in laboratory media was expected since the cardinal growth parameters used for prediction were also obtained from experiments using laboratory media. However, when those parameters are used to predict the growth kinetics in more complex media, such as food, deviation between the predicted and observed kinetics might be expected. Milk has almost similar characteristic, such as pH and water activity, as laboratory media. Therefore, when deviation is observed between prediction and growth kinetics in milk, it is due to the product specific effect of milk.

The observed and predicted growth kinetics of *L. monocytogenes* and *L. plantarum* in skimmed milk are presented in figure 5.2. Although the observed data were inside the 95% CI of the respective strain and the 95% PI of *L. monocytogenes* and *L. plantarum* strains, the exponential phase of the curve was less steep than that from our prediction

(figures 5.2A-E), except for strain FBR05. Consequently, the μ_{max} estimate of both species in milk (supplement table 5.2), except for strain FBR05, was lower than the prediction, underlining the presence of a product specific effect (γ(milk)). The γ(milk) calculated using equation 10 is presented in figure S5.2. Additionally the γ(milk) was also calculated using the μ_{max} estimates of the logistic and Baranyi models to compare if growth model selection influences the γ(milk) estimates. The γ(milk) of strains L6, FBR17, FBR15 and WCFS was between 0.6 and 0.8, while for strain FBR05 the value was above 1. Little differences were observed between the γ(milk) estimates calculated from the μ_{max} estimate of all models, except for strain FBR05 when the μ_{max} estimate of the Gompertz model gave a significantly higher γ(milk) estimate than that of the logistic and Baranyi models. The difference in model fitting might be caused by the limited growth data points available in the exponential phase *L. plantarum* at 15°C.

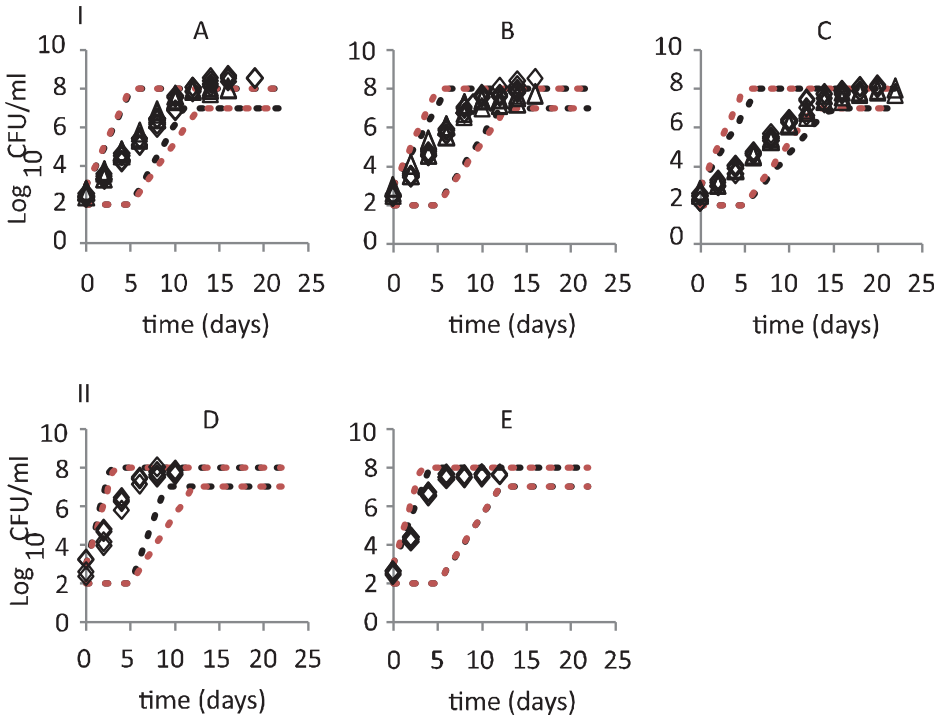

Figure 5.2. The growth kinetics in skimmed milk of I) *L. monocytogenes* at 7°C: A) L6; B) FBR17; C) FBR15; and II) *L. plantarum* at 15°C: D) WCFS1); E) FBR05. - - - 5-95% confidence intervals of predicted growth kinetics of each strain; - - - 5-95% prediction intervals of predicted growth kinetics of *L. monocytogenes* strains; ◇ pre-cultured at 7/15°C; △ pre-cultured at 30°C.

To study the effect of fat concentration on growth kinetics, *L. monocytogenes* strains L6 and FBR17 and *L. plantarum* strains WCFS1 and FBR05 were grown in semi skimmed milk (1.5% fat) and full milk (3% fat), which has similar characteristic as the skimmed milk used in this study (pH and a_w) except for the fat content. No difference could be observed from the growth kinetics of L6, FBR17, WCFS1 and FBR05 grown in skimmed milk, semi-skimmed milk, and full milk (figure S5.3), and therefore the $\gamma(milk)$ estimate obtained from skimmed milk can also be used for semi-skimmed milk and full milk.

Validation in Ham

Two different hams, commercial ham bought in the supermarket and in-house produced ham, were used in this study. Commercial ham contained, besides lactic acid, also sodium nitrite (E250) as preservative agent. No sodium nitrite, however, was added in in-house produced ham. The growth kinetics of *L. monocytogenes* and *L. plantarum* are presented in figures 5.3 and 5.4, respectively. The growth of *L. monocytogenes* in ham was dependent on the strain and the characteristics of the ham. The observed kinetics of *L. monocytogenes* strain L6, FBR17 and FBR15 grown in commercial ham (figures 5.3-IA – 5.3-IC) were inside the 95% CI of the respective strain and the 95% PI of the *L. monocytogenes* strains, but the slope of the growth curve of FBR15 was less steep than those of both predictions. The slope of the growth curve of the three *L. monocytogenes* strains in in-house produced ham was similar to the lower 95% PI of the *L. monocytogenes* strains, but the slope again was smaller than that of the 95% CI for strain L6 and FBR15 (figures 5.3-IIA, 5.3-IIB, and 5.3-IIC). Strain dependency was also observed from the growth kinetics of *L. plantarum* strains (figure 5.4). Although the observed kinetics of *L. plantarum* strains was in the range of both prediction intervals, the slope of the observed kinetics was smaller than that of the predicted kinetics, indicating the presence of a ham specific effect.

Just as with milk, a similar approach was used for ham to estimate the effect of γ(ham) on the growth kinetics of *L. monocytogenes* and *L. plantarum*. The average γ(ham) for the commercial ham was calculated from the observed μ_{max} divided by the predicted μ_{max} and was around 1 for *L. monocytogenes* L6 and FBR17 and between 0.2 and 0.5 for *L. monocytogenes* FBR15, and *L. plantarum* WCFS1 and FBR05. Moreover, the average γ(ham) for the in-house produced ham was between 0.2 and 0.7 (figure S5.2). Of all strains, *L. plantarum* WCFS1 had the lowest γ(ham) in both ham types.

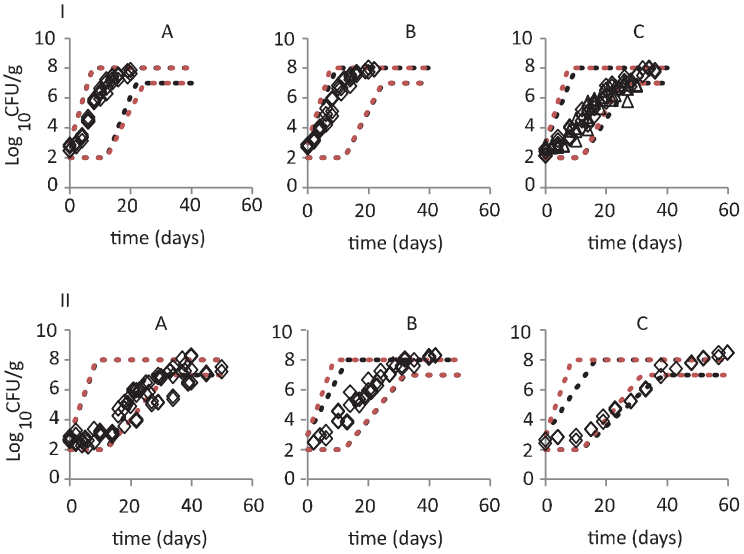

Figure 5.3. The growth kinetics of *L. monocytogenes* in I) Commercial ham and II) in-house produced ham at 7°C: A) L6; B) FBR17; C) FBR15. - - - 5-95% confidence intervals of predicted growth kinetics of each strain; - - - 5-95% prediction intervals of predicted growth kinetics of *L. monocytogenes* strains; ◇ pre-cultured at 7/15°C; △ pre-cultured at 30°C.

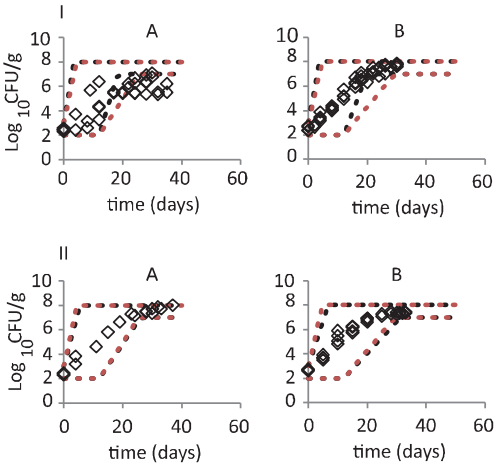

Figure 5.4. The growth kinetics of *L. plantarum* in I) commercial ham and II) in-house produced ham at 15°C: A) WCFS1 Ham; B) FBR05. - - - 5-95% confidence intervals of predicted growth kinetics of each strain; - - - 5-95% prediction intervals of predicted growth kinetics of *L. plantarum* strains; ◇ experimental data.

Validation using literature data

The comparison between literature and predicted μ_{max} is presented figure 5.5. The μ_{max} transformation was not only needed to better visualize the μ_{max} prediction (95% PI) at low temperature, but was also needed to stabilize the variance (95% PI) over the whole temperature range. Difference transformation of μ_{max}, such as log (Den Besten and Zwietering, 2012) and square root transformations (Zwietering et al., 1994) have been reported to stabilize the variance. Similar to the results of Zwietering et al. (1994), the variance in the predicted μ_{max} was best stabilized with the square root transformation (figures 5.5A-C). In general, the predicted μ_{max} was in agreement with the μ_{max} obtained from literature data (figure 5.5). Although the γ(milk/ham) was not included in the prediction, no deviation could be seen between the predicted and observed μ_{max} in milk at 7°C. Only slight deviation was observed between the predicted and observed μ_{max} in milk at 10°C and ham at 8°C, in which the observed was a bit higher than the predicted μ_{max} for milk at 10°C and vice versa for ham at 8°C.

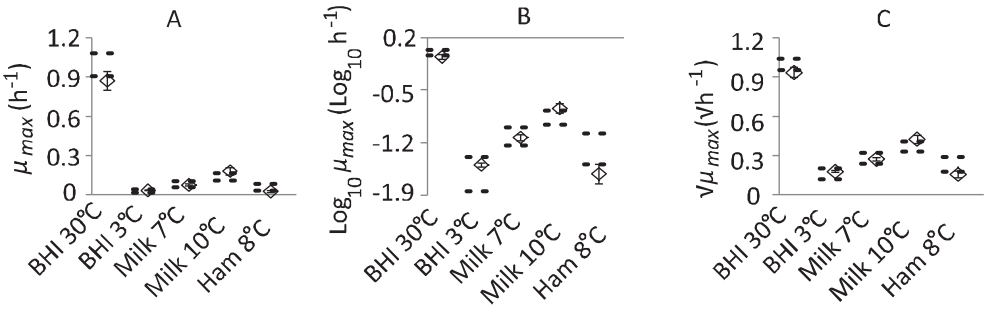

Figure 5.5. Validation using growth data extracted from Combase. ◇ Observed μ_{max} (A), log μ_{max} (B) and $\sqrt{\mu_{max}}$ (C) obtained from fitting with Gompertz model; **----** 95% the μ_{max}, log μ_{max} and $\sqrt{\mu_{max}}$ prediction intervals of *L. monocytogenes* strains. The error bars represent the 95% confidence interval of the μ_{max}, log μ_{max} and $\sqrt{\mu_{max}}$ estimates.

The effect of heating and incubation media on heat inactivation of *L. monocytogenes* and *L. plantarum*

The heat inactivation results of *L. monocytogenes* and *L. plantarum* as influenced by heating and incubation matrices are presented in figure 5.6. The effect of heating medium on heat resistance of low-temperature grown culture was strain and matrix dependent. Cells of *L. monocytogenes* FBR17 and FBR15 heated in laboratory media had similar heat resistance as those heated in milk. However, a contrast effect was observed for the other strains. A slight decrease in heat resistance was observed for *L. monocytogenes* L6 and *L.*

plantarum FBR05, while a slight increase in heat resistance was observed for strain *L. plantarum* WCFS1 when they were heated in milk compared to in laboratory media. The heat resistance of *L. monocytogenes* (figures 5.6A, B, C) and *L. plantarum* (figures 5.6D and E) heated in ham were much higher than that of in laboratory media and milk. In addition, the cells of strain L6, FBR17, WCFS1 and FBR05 inactivated in commercial ham had slightly but significantly higher heat resistance compared to the cells inactivated in control ham, while the cells of strain FBR15 inactivated in both hams had similar heat resistance.

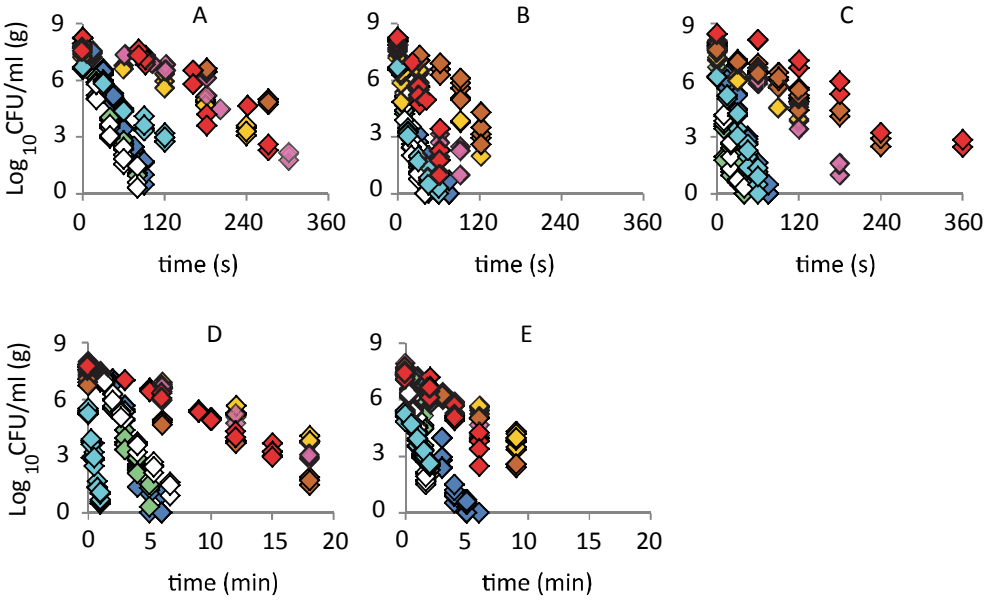

Figure 5.6. The thermal inactivation of I) *L. monocytogenes* at 65°C: A) L6; B) FBR17; C) FBR15; and II) *L. plantarum* at 60°C: D) WCFS1; E) FBR05 as influenced by heating and incubation media. ◆ Grown in BHI/MRS at 30°C inactivated in BHI; ◆ grown in BHI/MRS at 7°C/15°C inactivated in BHI/MRS; ◇ grown in BHI/MRS at 7°C/15°C inactivated in milk; ◆ grown in BHI/MRS at 7°C/15°C inactivated in commercial ham; ◆ grown in BHI/MRS at 7°C/15°C inactivated in in-house produced ham; ◆ grown in milk at 7°C/15°C inactivated in milk; ◆ grown in commercial ham at 7°C/15°C inactivated in commercial ham; ◆ grown in in-house produced ham at 7°C/15°C inactivated in in-house produced ham.

The heat resistance also depended on the type of incubation media used to grow microbial cells at low temperature before thermal treatment. In general, the cells grown in ham had similar heat resistance with those grown in laboratory media when they were inactivated in ham. The cells grown in milk had significantly higher heat resistance than

those grown in laboratory media prior to heat treatment in milk, except for strain WCFS1. Strain WCFS1 grown in milk had significantly lower heat resistance than that grown in laboratory media.

The multiple linear regression results (supplement table 5.3) showed that the effects of strain and heating medium were significant for both *L. monocytogenes* and *L. plantarum*, while the effect of incubation media was only significant for *L. plantarum*. To explain whether the product effect on heat resistance was mainly given by the effect of food as heating or incubation matrices, the *D*-values of the cells grown and inactivated in food product and those grown in laboratory media and inactivated in food product were compared to the *D*-values of the control condition (the cells were grown in laboratory media at 7°C and inactivated in laboratory media). The effect of ham on heat resistance was mostly determined by its effect as heating medium since the heat resistance of cells grown in ham and inactivated in ham was similar to that of cells grown in BHI and inactivated in ham. So, no additional effect on heat resistance due to incubation in ham medium was found. In contrast, the effect of milk on heat resistance of *L. monocytogenes* and *L. plantarum* was mostly influenced by its function as incubation medium. Further analysis using the scheme in figure 3.2 showed that the effect of food product in general was similar to the effect of strain on heat resistance. Also, the benchmarking of *D*-value data obtained from the thermal inactivation experiments in food products to literature data showed that the combination of strain and food product effects explained (almost) all of variability found in literature, however, with some bias (figure 5.7).

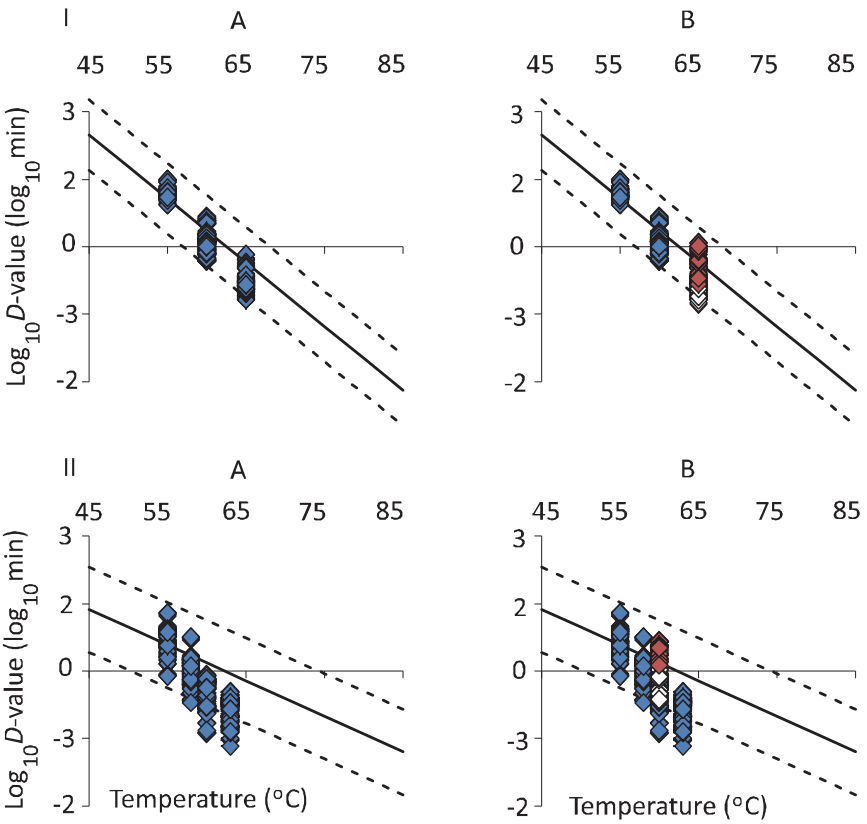

Figure 5.7. The benchmarking of *D*-value of *L. monocytogenes* (I) and *L. plantarum* (II) to literature data. Panel A the effect strain; B) the effect of strain and food products. ◆ $\log_{10}$ *D*-values as function of strains; ◇ $\log_{10}$ *D*-values as function of milk; ◆ $\log_{10}$ *D*-values as function of ham; the mean prediction (solid lines) and the 95% prediction intervals calculated from all literature data of *L. monocytogenes* (dashed lines) (Aryani et al., 2015a) and *L. plantarum* (Aryani et al., submitted for publication).

DISCUSSION

A validation study was carried out to evaluate the performance of the gamma model and cardinal growth parameters obtained from laboratory media in predicting microbial kinetics in food products. The prediction of microbial growth kinetics was made using the gamma model approach, assuming that no interaction occurs between different intrinsic and extrinsic variables. Note that the predicted μ_{max} data were obtained from OD based

experiments, and the new data set we used to validate our model prediction was obtained from plate count experiments and over a large range of logs increase. In most conditions the B_f values were above 1, indicating the average predicted generation time is longer than the observed value. Although these can be considered as a "fail dangerous" prediction, most of B_f values were within the range (0.75 - 1.25) considered as the successful prediction range used in seafood spoilage models (Dalgaard, 2000). The higher failed dangerous prediction was observed for the growth of *L. plantarum* FBR05 at 15°C, but not at 30°C. This might indicate that the prediction at low temperature for this strain was inaccurate. However, the difference between the prediction and the observed μ_{max} of FBR05 at 15°C, which caused high $B_f$value, was within the biological variability observed from the original data set as function of temperature (Aryani et al., submitted for publication) used for the gamma model (Figure S5.4). This suggests that the B_f factor interpretation should also account for the presence of biological variability in microbial growth behaviour. Moreover, the agreement between our prediction intervals and the observation highlighted the possibility of using the μ_{max} predicted using the cardinal growth parameters derived from OD based experiments.

A slight deviation was found in only two conditions of pH 5.5 at 7°C and pH 5.5, 1 mM [HLa] at 7°C. However, no interaction between variables was concluded when the formula proposed by Le Marc was used for calculating the interaction term, while new cardinal growth parameters obtained from Augustin and Carlier interaction model were underestimating the observed data when they were used to calculate the μ_{max}. The interactive effect between growth limiting factors was suggested to happen at the point where growth ceases (McMeekin et al., 2000). Therefore, if a synergistic interaction only occurs when one or more growth limiting variables become severe, this might explain why we observed deviation only when the pH was 5.5, but not when the pH was 6.1 or when the 2.5% NaCl was added to the medium at 7°C. Interestingly, when the 2.5% NaCl was added to the medium with pH 5.5 and 1 mM HLa, the deviation seemed to be affected more by the lag phase rather than the growth rate. In theory, growth should be further limited when another growth limiting factor is added to the media. But, strain L6 grown in the media with added 2.5% NaCl at 7°C contrarily had slightly higher growth rate compared to that without 2.5% NaCl at 7°C. To have a solid conclusion on the possible interaction between pH and low temperature, a further investigation is needed in future studies.

We assumed based on the growth results in laboratory media that our model was able to predict the growth of *L. monocytogenes* and *L. plantarum* when mild growth limiting factors are present in the matrix. Since mild growth limiting factors were present in milk and ham, we assumed that the difference between prediction and the observed growth in

milk and ham was mainly due to the food product specific effect represented as γ(milk/ham). Although a food product specific effect was found in the current study, the effect was in general still smaller than the effect given by low temperature of 7°C and 15°C (data not shown). The temperature is a variable that can be controlled without having a negative effect on the taste and other aspects, such as given by pH, a_w, [HLa] or the other preservative agents. Those latter variables, when added in higher amount, might provide similar prevention of bacterial growth, but when doing so, the higher amount will influence the sensory aspects and acceptability of the food product. When the intrinsic parameters of certain food products, such as milk, are almost similar to optimum conditions, then the temperature is the most important aspect to consider for preventing the growth of microorganisms. In other cases when also others hurdles are presents, such as in ham, both temperature and the other hurdles play an important role in preventing the growth of microorganisms.

Moreover, there were also differences in the growth response of *L. monocytogenes* and *L. plantarum* in the two types of hams. Commercial ham had more effect on the growth of FBR15 than on FBR17 or L6. All three *L. monocytogenes* strains also grew better in commercial ham than in in-house produced ham because of the higher pH and lower [HLa] of the commercial ham. It was different in the case of *L. plantarum*, since the growth in in-house produced ham was better than the growth in commercial ham. For *L. monocytogenes*, the effect of lower pH and higher concentration of [HLa] might be the reason for the lower growth observed in in-house produced ham than in commercial ham. However, this effect was less for *L. plantarum* since it grows at lower pH and higher concentration of [HLa] than *L. monocytogenes* does. The concentration of sodium nitrite permitted in meat product is up to 150 ppm in the EU (EU, 1995). Nitrite was reported to have little effect in delaying the growth of *L. monocytogenes* at pH 6 and above, at concentrations up to 400 μg/ml (McClure et al., 1991). Therefore, in combination with the pH of the commercial ham of 6.5, the nitrite presence in low level might have only a little effect on the growth of *L. monocytogenes*. In contrast, sodium nitrite might be the reason for the lower growth of *L. plantarum* in commercial ham, although a limited effect of 50 and 100 mg/L sodium nitrite on growth was also reported for lactic acid bacteria (Korkeala et al., 1992). Due to the strain dependency of the specific effect of food product, all relevant strains need to be inhibited for spoilage and risk prevention.

Certain components present in food product are able to protect bacterial cells during thermal inactivation (Doyle et al., 2001). Also, growing bacterial cells in food products might adapt to different components in food, which might influence the heat resistance. A large and consistent enhanced effect in heat resistance was seen when ham was used as heating or incubation matrices. However, the protective effect of ham on heat resistance

was mainly given by its effect as heating medium, rather than its effect as growth media. The heat resistance of the cells as influenced by ham was even significantly larger than that of the stationary cells grown at 30°C as shown in figure 5.7 (Aryani et al., 2015a; Aryani et al., submitted for publication).
The effect of milk on heat resistance, on the contrary, was determined by its effect as incubation medium. Though, in general, only little enhanced effect in heat resistance was observed when milk was used as incubation media. This might be due to the characteristics of milk, which was quite close to the characteristics of the laboratory media in terms of pH and a_w. Unlike in ham, the enhanced effect was inconsistent since one strain showed a decrease heat resistance when it was incubated in milk prior to thermal inactivation. However, the milk used was skimmed milk with 0% fat concentration. Fat is reported to have influence on the *D*-value (Schultze et al., 2007) due to its protective effect to the cells (Doyle et al., 2001). Further tests using milk with different fat content provided preliminary indication that the fat content seemed to have no or little effect on the heat resistance of strain L6 and strain FBR05 (data not shown).

CONCLUSIONS

The efficacy of an established gamma model used to predict microbial growth kinetics was validated using laboratory media. Slight deviations between the observed and predicted growth kinetics were seen only when both pH and temperature were decreased to sub-optimal conditions, indicating possible interaction between variables. Yet, existing interaction models were not better in predicting growth. Furthermore, the food product specific effect on growth kinetics was strain dependent, which might complicate accurate growth prediction. The effect of food product as heating medium on heat resistance was comparable to the effect introduced by strain diversity, while both were more important that the effect of growth media. The combination of strain and food product effect on heat resistance also explained (almost) all of variability found in literature with bias.

ACKNOWLEDGMENTS

The research is funded by TI Food and Nutrition, a public-private partnership on precompetitive research in food and nutrition. The funders had no role in study design, data collection and analysis, or preparation of the manuscript.
We would like to thank Lin Daokun and Su Yufei for their assistance during experimental work.

REFERENCES

Adams, M.R., Little, C.L., Easter, M.C. 1991. Modelling the effect of pH, acidulant and temperature on the growth rate of *Yersinia enterocolitica*. Journal of Applied Microbiology 71, 65-71.

Aryani, D.C., Den Besten, H.M.W., Hazeleger, W.C., Zwietering, M.H. 2015a. Quantifying variability on thermal resistance of *Listeria monocytogenes*. International Journal of Food Microbiology 193, 130-138.

Aryani, D.C., Den Besten, H.M.W., Hazeleger, W.C., Zwietering, M.H. 2015b. Quantifying strain variability in modeling growth of *Listeria monocytogenes*. International Journal of Food Microbiology 208, 19-29.

Aryani, D.C., Den Besten, H.M.W., Zwietering, M.H. submitted for publication. Quantifying variability factors: effect of strain variability on growth and thermal inactivation kinetics of *Lactobacillus plantarum*.

Augustin, J.-C., Carlier, V. 2000. Modelling the growth rate of *Listeria monocytogenes* with a multiplicative type model including interactions between environmental factors. International Journal of Food Microbiology 56, 53-70.

Buchanan, R.L., Phillips, J.G. 1990. Response surface model for predicting the effects of temperature pH, sodium chloride content, sodium nitrite concentration and atmosphere on the growth of *Listeria monocytogenes*. Journal of Food Protection 53, 370-381.

Buchanan, R.L., Whiting, R.C., Damert, W.C. 1997. When is simple good enough: a comparison of the Gompertz, Baranyi, and three-phase linear models for fitting bacterial growth curves. Food Microbiology 14, 313-326.

Dalgaard, P. 2000. Fresh and lightly preserved seafoods. In: Man, C.M.D., Jones, A.A., (Eds.), Shelf-life evaluation of food. Aspen Publishing, Maryland, USA. 110-139.

Den Besten, H.M.W., Zwietering, M.H. 2012. Meta-analysis for quantitative microbiological risk assessments and benchmarking data. Trends in Food Science & Technology 25, 34-39.

Doyle, M.E., Mazzotta, A.S., Wang, T., Wiseman, D.W., Scott, V.N. 2001. Heat resistance of *Listeria monocytogenes*. Journal of Food Protection 64, 410-429.

Duh, Y.-H., Schaffner, D.W. 1993. Modeling the effect of temperature on the growth rate and lag time of *Listeria innocua* and *Listeria monocytogenes*. Journal of Food Protection 56, 205-210.

EU. 1995. European parliament and council directive no 95/2/EC on food additives other than colours and sweeteners [Online]. Available at

http://ec.europa.eu/food/fs/sfp/addit_flavor/flav11_en.pdf (Accessed: 15 October 2015).

ICMSF. 1996. Microorganisms in Foods 5 Springer US.

Korkeala, H., Alanko, T., Tiusanen, T. 1992. Effect of sodium nitrite and sodium chloride on growth of lactic acid bacteria. Acta Vet Scand 33, 27-32.

Le Marc, Y., Huchet, V., Bourgeois, C.M., Guyonnet, J.P., Mafart, P., Thuault, D. 2002. Modelling the growth kinetics of *Listeria* as a function of temperature, pH and organic acid concentration. International Journal of Food Microbiology 73, 219-237.

McClure, P.J., Kelly, T.M., Roberts, T.A. 1991. The effects of temperature, pH, sodium chloride and sodium nitrite on the growth of *Listeria monocytogenes*. International Journal of Food Microbiology 14, 77-91.

McMeekin, T.A., Presser, K., Ratkowsky, D., Ross, T., Salter, M., Tienungoon, S. 2000. Quantifying the hurdle concept by modelling the bacterial growth/no growth interface. International Journal of Food Microbiology 55, 93-98.

Metselaar, K.I., Den Besten, H.M.W., Abee, T., Moezelaar, R., Zwietering, M.H. 2013. Isolation and quantification of highly acid resistant variants of *Listeria monocytogenes*. International Journal of Food Microbiology 166, 508-514.

Murphy, P.M., Rea, M.C., Harrington, O. 1996. Development of a predictive model for growth of *Listeria monocytogenes* in a skim milk medium and validation studies in a range of dairy products. Journal of Applied Bacteriology 80, 557-564.

Pin, C., Sutherland, J.P., Baranyi, J. 1999. Validating predictive models of food spoilage organisms. Journal of Applied Microbiology 87, 491-499.

Presser, K.A., Ratkowsky, D.A., Ross, T. 1997. Modelling the growth rate of *Escherichia coli* as a function of pH and lactic acid concentration. Applied and Environmental Microbiology 63, 2355-2360.

Ross, T., McMeekin, T.A. 1994. Predictive microbiology. International Journal of Food Microbiology 23, 241-264.

Ross, T. 1996. Indices for performance evaluation of predictive models in food microbiology. Journal of Applied Bacteriology 81, 501-508.

Ross, T., Ratkowsky, D.A., Mellefont, L.A., McMeekin, T.A. 2003. Modelling the effects of temperature, water activity, pH and lactic acid concentration on the growth rate of *Escherichia coli*. International Journal of Food Microbiology 82, 33-43.

Schultze, K.K., Linton, R.H., Cousin, M.A., Luchansky, J.B., Tamplin, M.L. 2007. Effect of preinoculation growth media and fat levels on thermal inactivation of a serotype 4b strain of *Listeria monocytogenes* in frankfurter slurries. Food Microbiology 24, 352-361.

Schvartzman, M.S., Belessi, X., Butler, F., Skandamis, P., Jordan, K. 2010. Comparison of growth limits of *Listeria monocytogenes* in milk, broth and cheese. Journal of Applied Microbiology 109, 1790-1799.

Te Giffel, M.C., Zwietering, M.H. 1999. Validation of predictive models describing the growth of *Listeria monocytogenes*. International Journal of Food Microbiology 46, 135-149.

Van Asselt, E.D., Zwietering, M.H. 2006. A systematic approach to determine global thermal inactivation parameters for various food pathogens. International Journal of Food Microbiology 107, 73-82.

Xanthiakos, K., Simos, D., Angelidis, A.S., Nychas, G.J.E., Koutsoumanis, K. 2006. Dynamic modeling of *Listeria monocytogenes* growth in pasteurized milk. Journal of Applied Microbiology 100, 1289-1298.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M., Van 't Riet, K. 1990. Modeling of the bacterial growth curve. Applied and Environmental Microbiology 56, 1875-1881.

Zwietering, M.H., Cuppers, H.G.A.M., de Wit, J.C., Van 't Riet, K. 1994. Evaluation of data transformations and validation of a model for the effect of temperature on bacterial gtrowth. Applied and Environmental Microbiology 60, 195-203.

Zwietering, M.H., de Wit, J.C., Notermans, S. 1996. Application of predictive microbiology to estimate the number of *Bacillus cereus* in pasteurised milk at the point of consumption. International Journal of Food Microbiology 30, 55-70.

SUPPLEMENTARY TABLES

Supplement Table 5.1. The growth parameters of *L. monocytogenes* and *L. plantarum* (Aryani et al., 2015b; Aryani et al., submitted for publication)

Growth parameters	*L. monocytogenes*			*L. plantarum*	
	L6	FBR17	FBR15	WCFS1	FBR05
μ_{ref} (h^{-1})	1.01 (0.994-1.02)	1.04 (1.05-1.04)	0.931 (0.963-0.899)	0.766 (0.698-0.833)	0.777 (0.853-0.702)
pH_{min}	4.60 (4.58-4.62)	4.45 (4.43-4.48)	4.60 (4.57-4.63)	3.34 (3.29-3.39)	3.24 (3.15-3.34)
$pH_{1/2}$	4.95 (4.92-4.98)	4.94 (4.89-4.98)	4.99 (4.96-5.02)	3.90 (3.80-3.99)	4.10 (3.91-4.29)
$a_{w,min}$	0.930 (0.928-0.931)	0.918 (0.916-0.921)	0.927 (0.925-0.929)	0.941 (0.939-0.943)	0.945 (0.941-0.948)
T_{min} (°C)	-2.69 (-2.23/-3.15)	-1.53 (-1.12/-1.94)	-1.13 (-0.295/-1.96)	3.40 (2.15-4.64)	7.69 (6.56-8.82)
HLa_{max} (mM)	5.01 (4.81-5.21)	6.06 (5.77-6.35)	3.99*	29.3 (25.5-33.1)	30.3 (26.5-34.1)
α_{aw}	1.36 (1.22-1.50)	1.14 (1.01-1.27)	1.53 (1.36-1.70)	2.47 (1.90-3.04)	2.62 (1.72-3.54)
α_{HLa}	1.30 (1.06-1.53)	0.678 (0.583-0.772)	0.723*	0.56 (0.452-0.669)	0.386 (0.310-0.462)

*) No 95% Confidence Intervals could be estimated due to limited number of available data.

Supplement Table 5.2. Growth rate of *L. monocytogenes* and *L. plantarum* in Milk at 7°C and 15°C

Conditions	μ_{max} (h^{-1})				
	7°C			15°C	
	L6	FBR17	FBR15	WCFS1	FBR05
Preculture 30°C	0.0517-0.0616	0.0519-0.0695	0.0410-0.0465	NA	NA
Preculture 7°C/15°C	0.0483-0.0573	0.0587-0.0682	0.0413-0.0480	0.0811-0.123	0.134-0.158
95% Confidence Intervals	0.0802-0.0940	0.0683-0.0802	0.0510-0.0741	0.123-0.181	0.0676-0.116
95% Prediction Intervals of 20 strains		0.0623-0.0979		0.0685-0.154	

Supplement Table 5.3. The result of multiple linear regression between $\log_{10}$ *D*-value ($\log_{10}$ min) of *L. monocytogenes* at 65°C and *L. plantarum* 60°C and the effect of different factors influencing heat resistance

1. *Listeria monocytogenes*

	Coefficients	Standard Error	*t* stat	*p*-value	Lower 95%	Upper 95%
Intercept	1.09	0.03459	30.9	$3.47*10^{-45}$	1.00	1.14
Heating media	0.369	0.0442	8.34	$2.22*10^{-12}$	0.281	0.457
strains	0.3749	0.0581	6.44	$9.54*10^{-9}$	0.258	0.490

2. *Lactobacillus plantarum*

	Coefficients	Standard Error	*t* stat	*p*-value	Lower 95%	Upper 95%
Intercept	1.69	0.0262	64.5	$1.55*10^{-58}$	1.64	1.74
Heating media	0.389	0.0315	12.3	$2.81*10^{-18}$	0.325	0.451
growth media	-0.196	0.0545	-3.59	0.000655	-0.304	-0.0866
strains	0.266	0.0476	5.59	$5.52*10^{-7}$	0.171	0.361

SUPPLEMENTARY FIGURES

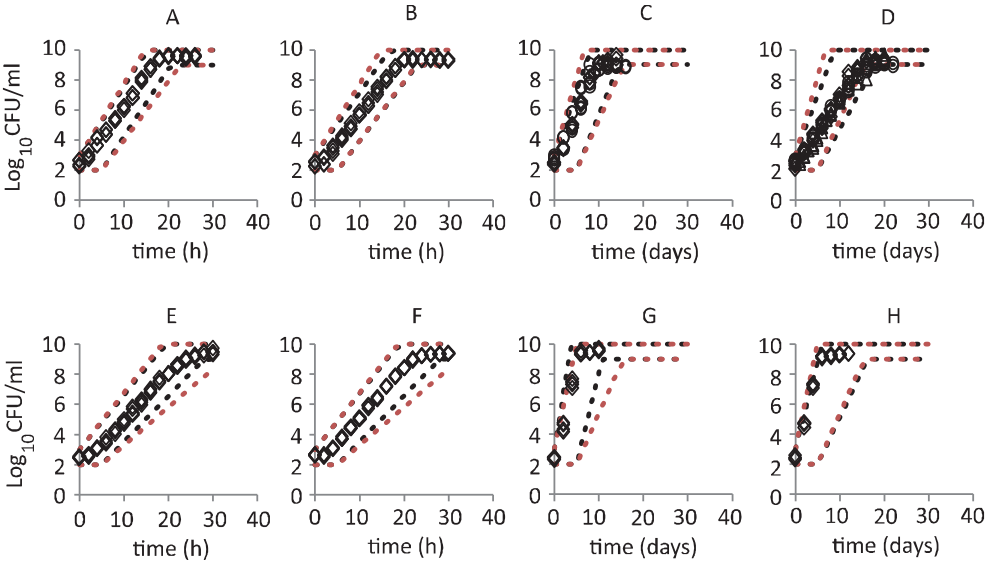

Figure S5.1. Growth kinetics of *L. monocytogenes* A) FBR17 in BHI at 30°C; B) FBR15 in BHI at 30°C; C) FBR17 in BHI at 7°C; D) FBR15 in BHI at 7°C; and *Lactobacillus plantarum* E) WCFS1 in MRS at 30°C; F) FBR05 in MRS at 30°C; G) WCFS1 in MRS at 15°C; H) FBR05 in MRS at 15°C. - - - 5-95% confidence intervals of predicted growth kinetics of each strain; - - - 5-95% prediction intervals of predicted growth kinetics of *L. monocytogenes* strains; ◇ cells were precultured at 7°C or 15°C; For B and D: △ cells were precultured at 30°C, grown at 7°C 200 rpm; ○ cells were precultured at 30°C, grown in static condition at 7°C.

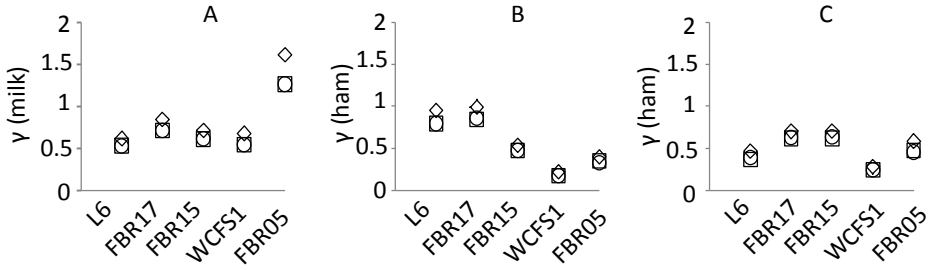

Figure S5.2. The gamma food product: A) milk; B) Supermarket Ham; C) Control Ham. The gamma value is calculated using μ_{max} of : ◇ Gompertz model; □ logistic model; ○ Baranyi model.

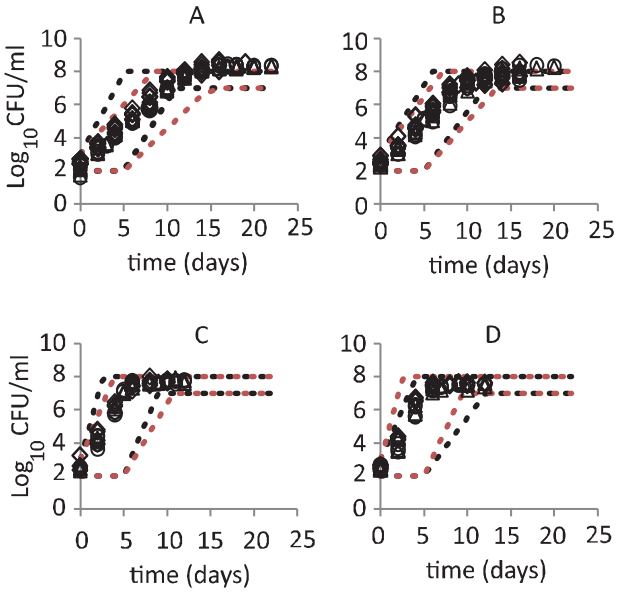

Figure S5.3. The growth kinetics of A) L6 at 7°C; B) FBR17 at 7°C; C) WCFS1 at 15°C; D) FBR05 at 15°C in milk: ◇ skimmed milk; △ semi- skimmed milk, ○ full milk. ---- the 95% confidence intervals of the respective strain; ---- the 95% confidence intervals of the respective strain with the integration of γ *(milk)* for the specific strain calculated using the μ_{max} of Gompertz model.

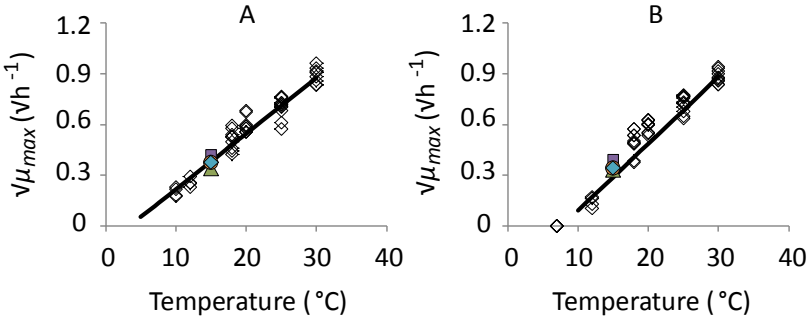

Figure S5.4. Comparison between the growth data of A) *L. plantarum* WCFS1 and B) *L. plantarum* FBR05 as function of temperature obtained from previous experiment using Bioscreen C (Aryani et al., submitted for publication (◇); the prediction using Gamma model (solid line); the observed μ_{max} at 15°C obtained from: ▲ the slope of the linear phase of the growth curve, ■ fitting with Gompertz model, ● fitting with logistic model and ◆ fitting with Baranyi model.

CHAPTER 6

Discussion and future perspectives

Some parts of this chapter were used for the publication: Microbial variability in growth and heat resistance of a pathogen and a spoiler: all variabilities are equal but some are more equal than others. Den Besten, H.M.W., D.C. Aryani, K.I. Metselaar and M.H. Zwietering. Accepted in International Journal of Food Microbiology.

INTRODUCTION

Most food products are perishable, and therefore specific formulation or processes are needed to preserve their quality. Heavy processing such as sterilization eliminates rather all microorganisms present in raw material, thus the shelf life of these food products is long. However, the change in lifestyle and the increase in awareness toward tastier and healthier food affect the consumer demand toward mildly processed food. Mildly processed foods generally have shorter shelf life and depend on the combination of different growth limiting factors to control the growth of microorganisms. The principle of the so-called hurdle technology is that each hurdle has its own effect on microorganisms and the total effect is the multiplicative effect with or without synergy between the hurdles. Though the healthier image, mildly processed foods have a risk of the presence of robust microorganisms, which may grow in mildly processed foods and causing spoilage and food poisoning.

Since zero risk does not exist, minimizing the risk is the ultimate goal to reduce the disease burden caused by microorganisms. To minimize the risk, effective control measures should be implemented along food production chains. An effective control measure can be designed when information on the behaviour of microorganisms is known. The past knowledge on the responses of microorganisms toward environmental factors can be used to predict the behaviour of the microorganisms in new situations using quantitative microbiology. The concept of quantitative microbiology, according to McMeekin et al. (2002), is that detailed knowledge of microbial responses to environmental conditions and intrinsic food properties, enables objective evaluation of the effect of processing, distribution and storage operations on the microbiological safety and quality of foods. Therefore, quantitative microbiology is often used as a tool to evaluate certain food processes and designs on their effectiveness in eliminating or controlling the growth of microorganisms (Augustin et al., 2011; Coroller et al., 2012; Koutsoumanis et al., 2010; Van Lieverloo et al., 2013; Xanthiakos et al., 2006). However, many variability factors are known to influence prediction, such as strain variability (Benito et al., 1999; Lianou and Koutsoumanis, 2011), process variability, or variability in the initial contamination (Awaisheh, 2010; Lambertz et al., 2012). Although, integration of these variability factors in predictive modelling, such as in stochastic or probabilistic modelling, has been applied (Koutsoumanis et al., 2010; Membré et al., 2006), quantitative knowledge on the various specific variability factors and their magnitude are also needed to prioritize their importance.

Cardinal growth and heat resistance parameters are amongst the inputs required in quantitative microbiology to predict the microbial growth and inactivation behaviour in a given condition and process. These parameters vary due to strain variability or the effect

of growth history, and may influence the accuracy of the prediction results. In this thesis, sources of variability factors affecting the growth and heat resistance were quantified and compared to prioritize their importance. Since the data used in predictive modelling are generally derived from studies performed using laboratory media, in this thesis the effects given by food product specific factors on microbial kinetics were also investigated and compared to the effect attributed to strain variability. In addition, in this chapter the microbiological variability will be compared to other variability factors often encountered in a model food chain to evaluate the impact of such factors in the variability of the final microbial concentration.

VARIABILITY IN GROWTH KINETICS: THE EFFECT OF STRAIN VARIABILITY ON GROWTH RATE AND CARDINAL GROWTH PARAMETERS

Reproduction variability (within strain variability) and between strain variability in microbial growth kinetics has been reported in studies using *Staphylococcus aureus* (Lindqvist, 2006), *Salmonella* (Lianou and Koutsoumanis, 2011; Oscar, 2000), *Escherichia coli* (Fernández-Escudero et al., 2014), *Listeria* spp. and *Listeria monocytogenes* (chapter 3) (Barbosa et al., 1994; Begot et al., 1997) and *Lactobacillus plantarum* (chapter 4). Different results, however, were reported from these studies. No significant difference between strains in maximum specific growth rate (μ_{max}) was reported for *E. coli*, while only minor difference was observed for *Salmonella* (Oscar, 2000). In other studies, variability between strains in μ_{max} was reported to be greater than within strain variability (Lianou and Koutsoumanis, 2011) and the variability attributed to the method and experimental protocol (Lindqvist, 2006; Whiting and Golden, 2002). On the contrary as shown in chapter 2 and 4, the strain variability in μ_{max} of *L. monocytogenes* was in general similar to reproduction variability for all variables tested, while for *L. plantarum* strain variability in μ_{max} was similar to reproduction variability for pH, a_w and temperature variables, but even slightly lower than reproduction variability for undissociated lactic acid concentration (figures 6.1A – 6.1D).

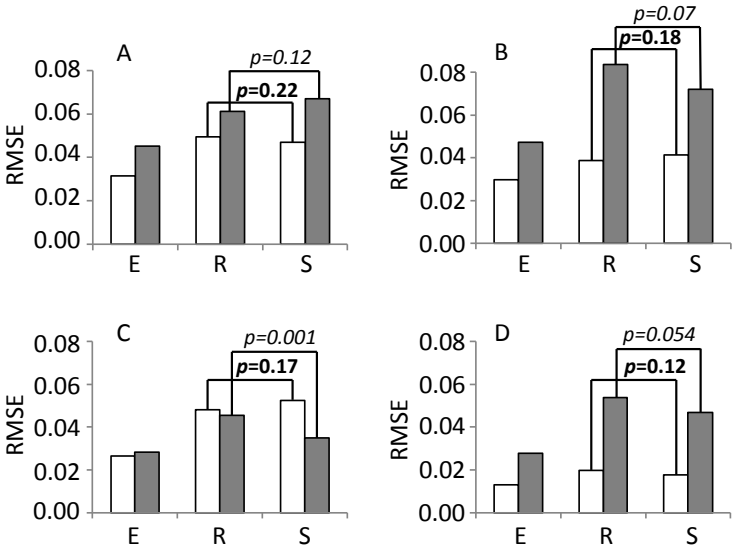

Figure 6.1. Experimental (E), Reproduction (R), and Strain (S) variabilities represented as Root Mean Square Error (RMSE) of *L. monocytogenes* (open bars, *p* value to indicate significant differences between the reproduction and strain variabilities presented in bold case) and *L. plantarum* (filled bars, *p* value presented in italic case) in: A) μ_{max} as function of pH, B) μ_{max} as function of a_w, C) μ_{max} as function of [HLa], and D) square root of μ_{max} as function of temperature.

The strain variability was also reported to be larger when the growth conditions became unfavourable (Lianou and Koutsoumanis, 2011). In contrast, strain variability was rather comparable in favourable and unfavourable conditions for *L. monocytogenes* (Figure 6.2A and 6.2B). The variability might be expressed as the coefficient of variation (CV), such as done for *Salmonella enterica* (Lianou and Koutsoumanis, 2011), or in the root mean square error (RMSE≈standard deviation) as we used in our studies. The coefficient of variation is the ratio of the standard deviation to the mean, which is used to describe the dispersion in a variable (relative error). If the standard deviations are comparable across the concentration range tested, but the mean value decreases linearly with the increase in concentration, such as in our case for the a_w variable (Figures 6.2A and 6.2B) for example, then the CV ratio will be higher at unfavourable growth conditions (lower a_w value) (Figure 6.2C). Both RMSE and CV ratio can be used to present variability, but as also discussed in chapter 1, care should be taken when comparing variabilities reported using different measurements.

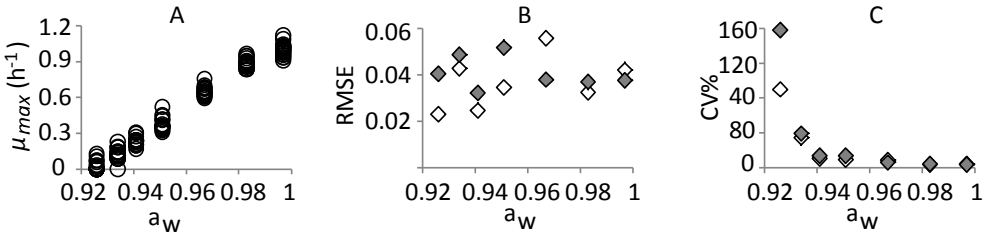

Figure 6.2. The maximum specific growth rate (μ_{max}) of *L. monocytogenes* and the variability as function of a_w expressed in RMSE and %CV. A) Mean value of μ_{max} for a_w (○); B) the variability expressed is RMSE (standard deviation) and C the variability expressed in % Coefficient of variation (%CV). ◇ Reproduction variability; ◆ Strain variability.

In general, the strain variability of *L. plantarum* in μ_{max} was significantly larger than the strain variability of *L. monocytogenes*, except for [HLa] (figure 6.1) (supplementary table 6.1). The average μ_{max} of *L. monocytogenes* grown in laboratory media at 30°C for example was between 0.93 and 1.03 h^{-1}, while at the same temperature the average μ_{max} of *L. plantarum* was between 0.72 and 0.92 h^{-1}. The higher strain variability in μ_{max} of *L. plantarum* might be caused by the differences in optimum growth requirements among strains of this species. Further study on the growth of *L. plantarum* under static, microaerobic and aerobic conditions showed that the strain and reproduction variabilities of 10 *L. plantarum* strains in microaerobic condition were lower than that in static and aerobic conditions (chapter 4). To reduce the variability in growth kinetics of *L. plantarum*, the experiment using Bioscreen C might be done in microaerobic condition. However, in an actual food environment, even when modified atmosphere packaging (MAP) is used for the food product, the variability among *L. plantarum* strains cannot be expected as low as the one obtained in well controlled microaerobic condition.

As for μ_{max}, the effect of strain variability was also quantified with respect to the minimum cardinal growth parameters (chapter 2 and chapter 4). While *L. monocytogenes* is known for its ability to grow in severe conditions such as low temperature of -2°C (Bajard et al., 1996) and relatively low water activity of 0.92 (Nolan et al., 1992), *L. plantarum*, as a member of the lactic acid bacteria, is known for its capability to grow at pH levels as low as pH 3 (Ingham et al., 2008) and at high concentrations of undissociated lactic acid (Houtsma et al., 1993; Houtsma et al., 1996). However, these growth limits depend on the strains used and growth conditions, including the type of acidulants or solutes used for lowering the pH or water activity and the presence of multiple hurdles. When the effect of multiple hurdles is excluded, strain variability in cardinal growth parameters of 20 *L. monocytogenes* strains used in our study explained around 50% or less of variability found in literature for pH, a_w, temperature and [HLa] growth limits (figures 6.3A – 6.3B). Strain

variability in pH_{min} and $a_{w,min}$ among 20 *L. monocytogenes* strains was also similar to those obtained from 138 *L. monocytogenes* strains (Van der Veen et al., unpublished data). Besides strain variability, the number of data points and the type of secondary growth model used to fit the growth data also influenced the variability in cardinal growth parameters. When the model from Augustin and Carlier (2000) was used to fit the few μ_{max} points as function of pH or water activity for *L. monocytogenes* obtained from the study of Brocklehurst et al. (1995) and Vasseur et al. (1999), the resulting pH_{min} and $a_{w,min}$ estimates were 4.06 and 0.864, respectively. Different pH_{min} estimates of 4.15, 4.03 and 3.84 were also reported by Wijtzes et al. (2001) when different secondary growth models were used to fit the growth data. In other cases, some workers (Bajard et al., 1996; Le Marc et al., 2002) reported a non-linear behaviour of *L. monocytogenes* at sub-optimal condition. Thus, a two-phase-secondary growth model was used to fit the temperature data, resulting in the lower T_{min} estimate than that obtained using a linear model.

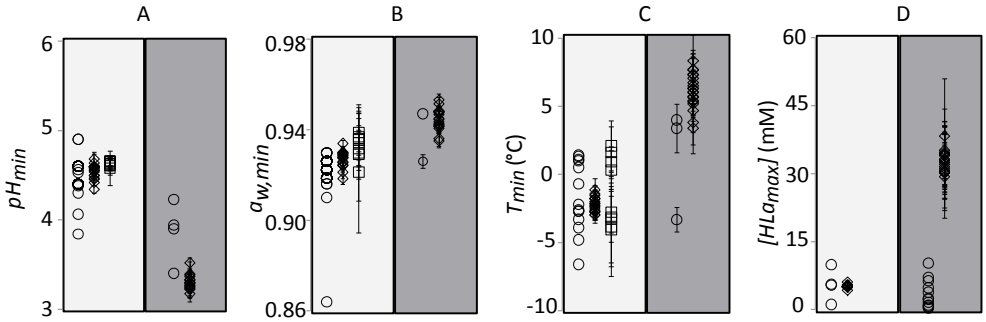

Figure 6.3. Benchmarking of growth limits of *L. monocytogenes* (light grey panel) and *L. plantarum* (dark grey panel) to literature data. ○ Literature data: Augustin and Carlier (2000), Gänzle et al. (1998), Giraud et al. (1991), Houtsma et al. (1996), Van der Veen et al. (2008); Wijtzes et al. (1995; 2001), Zwietering et al. (1991). ◇ Our study of strain variability; □ population heterogeneity of Metselaar et al. (submitted for publication). The error bars represent the 95% confidence interval of the estimate.

The effect of multiple hurdles on the cardinal growth parameter estimates was described in Van der Veen et al. (2008) in which the growth limit of *L. monocytogenes* strains increased when one or two hurdles were added or altered to sub-optimum conditions. With a fix experimental time, however, this result can be logical, but might also be an artifact of the experimental design when incubation times are too short to observe growth. Moreover, the use of organic acid or different solutes, such as sucrose and glycerol to lower pH and a_w changed the microbial growth limit of pH_{min} and $a_{w,min}$ (Cole et

al., 1990; Conner et al., 1986; Farber et al., 1989; Farber et al., 1992; Nolan et al., 1992; Tapia De Daza et al., 1991). The minimum pH at which growth of *L. monocytogenes* was observed at 30°C, when HCL or H_2SO_4 was used as acidulant, was between 4.2 and 4.3 (George et al., 1988; Tienungoon et al., 2000) (chapter 2). This pH limit increased when organic acid, such as propionic, acetic, or lactic acids, were used as acidulant (Augustin and Carlier, 2000; Conner et al., 1990) due to the effect of both pH and undissociated lactic acid presence in the media. The reported growth limit of a_w 0.92 for *L. monocytogenes* was based on the use of sodium chloride as the solute lowering agent. When other solutes, such as sucrose and glycerol, were used in the media, the growth limit was reported to be 0.93 and 0.90, respectively (Farber et al., 1992; Miller, 1992). Additionally, the effect of population heterogeneity in growth limits was reported in the study of Metselaar et al. (submitted for publication). The acid resistant variants isolated in their study (Metselaar et al., 2013) had a wide range of T_{min} (figure 6.3C). Interestingly, although isolated after exposure to low pH, these acid resistant variants did not show increased acid tolerance as indicated by the small range of pH_{min}.

The amount of undissociated acid concentration depends not only on the strain, but also on the pH of the media. The lower the pH of the media, the more acid is present in its undissociated form, causing complexity in comparing the limit of undissociated lactic acid ([HLA_{max}]) obtained from experiments using different initial pH values (figure 6.3D). When only the effect of pH is considered, the growth of microorganism in the media is slower when pH is lowered. The growth will be further limited when other growth limiting factors are also present. Therefore, an assumption can be made that at lower pH, the amount of other growth limiting factors needed to inhibit growth of microorganism will be less than at higher pH value. This assumption can be true for other growth limiting factors, such as a_w or temperature, but not for undissociated acid concentration. Although Houtsma et al. (1996) reported a decrease in the total sodium lactate required to inhibit the growth of lactic acid bacteria and *L. monocytogenes* with a decrease in pH value, confirming the above assumption, the amount of undissociated lactic acid ([HLa]] is contrarily higher at lower pH value. At higher pH value of 7, the total lactate needed to inhibit the growth of lactic acid bacteria was between 446 mM and 1339 mM, corresponding to a [HLa_{max}] value of 0.32 mM - 0.95 mM, while the total lactate at pH 5.7 was between 268 mM and 714 mM, corresponding to a [HLa_{max}] value of 3.7 mM - 9.9 mM. Unlike strong acid, weak organic acid when added in the media will not only affect the pH, but also the amount of undissociated acid and the water activity of the medium. Houtsma et al. (1993) reported the a_w of the media without sodium lactate was 0.998, while the measured a_w of the media with 268 mM and 714 mM sodium lactate was 0.992 and 0.977, respectively. The effect of sodium lactate at higher pH value then was also affected by the change of the a_w

of the media (Houtsma et al., 1993) rather than only by the undissociated acid concentration. In chapter 2 and 4, the estimated limit of undissociated lactic acid [HLa_{max}] for *L. monocytogenes* at pH 5.5 and *L. plantarum* at pH 4.5 were between 4.2 mM - 5.9 mM and 29 mM - 38 mM (corresponding to 178 mM - 268 mM and 129 mM - 170 mM potassium lactate). At this concentration, the effect of the weak organic acid on microbial growth is mostly determined by the effect of the undissociated lactic acid concentration since the a_w of the [HLa] adjusted medium was not largely different from the a_w of the plain media.

Strain variability in cardinal growth parameters of *L. plantarum* explained more than 50% of the variability from literature, although with bias (figure 6.3). However, only few data points were found in literature for the growth limits of *L. plantarum*, and therefore the data obtained from other lactic acid bacteria was also included. Thus, the literature data also represented species variability among the group of lactic acid bacteria. The pH_{min} reported in chapter 4 was lower than what was reported from *L. curvatus* and *L. sanfranciscensis* (Gänzle et al., 1998; Wijtzes et al., 2001), but was similar to the reported pH_{min} of *L. plantarum* from the Giraud study (1991). Although the variability in literature data looked similar to the strain variability for $a_{w,min}$ and T_{min} (figures 6.3A and 6.3B), the $a_{w,min}$ and T_{min} estimates reported for *L. curvatus* and *L. plantarum* (Wijtzes et al., 2001; Zwietering et al., 1991; Zwietering et al., 1994) were lower than what was estimated from our study. When more growth limit data will be available for *L. plantarum*, the effect of strain variability on the growth limit of *L. plantarum* can be better compared to the effect of other variability factors.

The information obtained from variability in maximum specific growth rate and growth limits might be used for selection of representative strains for a challenge study. However, no single robust strain, which has high tolerance to all four variables (pH, a_w, temperature and [HLa]) was observed from both species. For *L. monocytogenes*, strain FBR17 was the only strain which grew better at low pH, low a_w and high concentration of HLa, and therefore might be a good model strain for the challenge study. Apart for those three parameters, this strain is not robust at lower temperature, making it less suitable for the study performed at low temperature. When a challenge study is performed in food where more hurdles are often present, a cocktail of strains might be used. For example, the cocktail might consist of FBR17, which is robust at lower pH, a_w and higher [HLa], and FBR21 or C5, which are robust at lower temperature. When the knowledge on the effect of certain conditions on microbial kinetics is of interest, those strains might be tested in parallel, so the effect of certain growth conditions in food on each strain can be better monitored and explained.

The integration of strain variability in the growth prediction, such as done for milk in chapter 2, showed at least 2 log difference between the least and the most robust strain. Integrating strain variability then will result in more realistic prediction of growth kinetics. Moreover, since the parameters used for the prediction were established using the data obtained in laboratory media, a validation is needed to also take into account a potential product specific factor. Validation of the model in laboratory media when the effect of different hurdles in growth kinetics was combined using a multiplicative approach showed a good agreement between the prediction result and observed data in laboratory media (chapter 5). Only in two conditions slight deviation was observed, indicating the possible interactive effect between growth limiting factors. The existing interaction models of Le Marc et al. (2002) and Augustin and Carlier (2000), however, were also not better in predicting the growth in those two conditions. The specific effect of food product was observed when milk and ham were used as representative food products for validation. However, no similar factor can be used for all products (Schvartzman et al., 2010) or strains (chapter 5), complicating the accurate prediction of microbial growth kinetics in food matrices.

The variability factors quantified in this thesis and from literature data might also be used to compare variability factors to prioritize their importance. In general, strain variability and population heterogeneity are the important factors determining the variability in cardinal growth parameters (figure 6.3). Although the variability in T_{min} between variants is larger than strain variability, this does not mean that the population diversity is more important than strain variability. The μ_{max} of most variants, especially when they were grown at 7°C, was lower than that of the wild type (Metselaar et al., 2015). These slower growing variants, if present in a population, might be easily overgrown by the wild type, and therefore have a limited impact on the final concentration of the microorganism in food products.

VARIABILITY IN THERMAL INACTIVATION KINETICS: THE EFFECT OF STRAIN VARIABILITY AND GROWTH HISTORY ON *D*-VALUE

Similarly to growth kinetics, strain variability in thermal inactivation kinetics has also been studied for vegetative cells (Benito et al., 1999; De Jesús and Whiting, 2003; Lianou and Koutsoumanis, 2013; Mackey et al., 1990; Ng et al., 1969; Rodríguez-Calleja et al., 2006; Sörqvist, 1994; Whiting and Golden, 2002) and spores (Berendsen et al., 2015; Luu-Thi et al., 2014). Although differences between strains were reported, strain variability was suggested to be more or less similar to within strain (reproduction) variability in previous studies using *L. monocytogenes* and *Salmonella* (De Jesús and Whiting, 2003; Lianou and Koutsoumanis, 2013). In contrast, strain variability was found to be four and six times

higher than within strain variability for *L. monocytogenes* and *L. plantarum* (chapter 3 and chapter 4; figures 6.4A-B). The strain variability in thermal inactivation kinetics was also found to be at least 10 times higher than experimental variability, which was far higher than what was found for growth kinetics. As for growth kinetics, strain variability in *L. plantarum* was higher than that of *L. monocytogenes*.

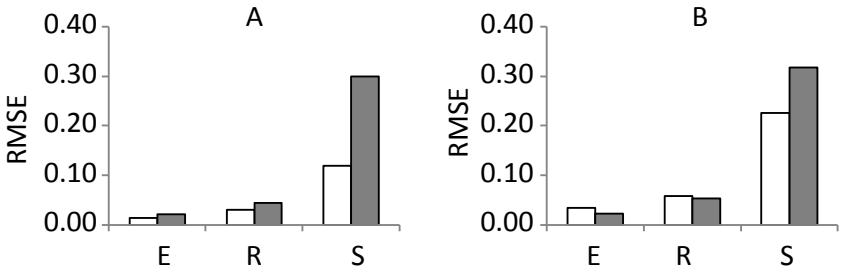

Figure 6.4. Experimental (E), Reproduction (R) and Strain (S) variability on log *D*-value at A) 55°C and B) 60°C. Open bars are *L. monocytogenes* and grey filled bars are *L. plantarum.*

The variability in *D*-values of bacterial cells was influenced among others by strain variability, previous growth conditions, the effect of growth history and the composition of heating menstruum (De Jesús and Whiting, 2003; Doyle et al., 2001; Farber and Pagotto, 1992). A previous study used a systematic approach to determine global thermal inactivation parameters for various food pathogens (Van Asselt and Zwietering, 2006) and the corresponding variabilities. Although the variability of all data obtained from Van Asselt and Zwietering can be used as a conservative estimate of inactivation variability as suggested by the authors, it is useful to know the contribution of each variability factor to the total variability to prioritize their importance. The overall variability obtained from literature for *L. monocytogenes* and *L. plantarum* was, therefore, used as a benchmark to evaluate the effect of each variability factor. Remarkably, the effect of strain alone explained more than 50% of the variability in reported *D*-values from literature for both species (chapter 3 and 4, figure 6.5). This was different from the overview of the growth kinetics, since less variability could be attributed to the effect of strain variability, especially when the effect of growth conditions including multiple hurdles on variability in cardinal growth parameters was considered (chapter 2). The combined effects of strain variability and growth history on heat resistance explained almost all variability, although with bias for *L. monocytogenes*. The effect given by the growth history was in general similar to the effect of strain variability for *L. monocytogenes* and slightly lower than strain variability for *L. plantarum* (chapter 2, chapter 4, figure 6.5). The effect of growth history

in figure 6.5 was presented using heat resistance data of strain C5 and LMG18035, as representative of intermediate heat resistant strains. However, the effect of growth history was not generic, since different strains behave differently toward similar stresses as described in chapter 3, chapter 4, and chapter 5. The only generic effect was given by the physiological state of the cells, confirming the "rule of thumb" that stationary phase cells have much higher heat resistance than exponential phase cells. Moreover, the effect given by population heterogeneity (Metselaar et al., submitted for publication) was more or less similar to the effect of strain variability, but remarkably, some stress resistant variants had higher *D*-value than the most heat resistant strain of *L. monocytogenes*.

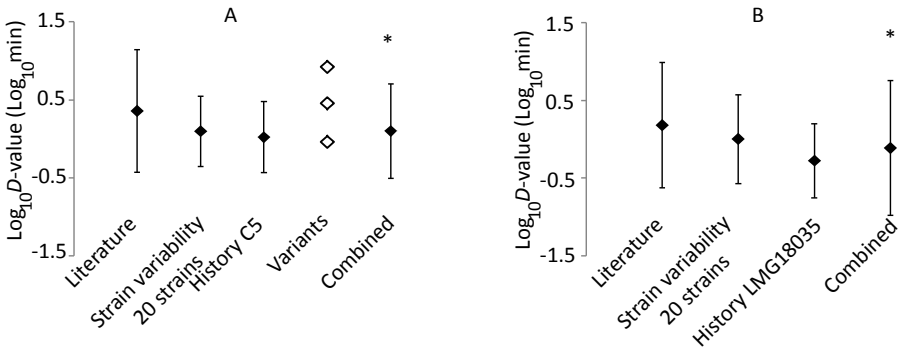

Figure 6.5. The benchmarking of $\log_{10}$ *D*-value data to literature data: A) *L. monocytogene*s and B) *L. plantarum*. C5 and LMG18035 were the representative of intermediate heat resistant strains used in chapter 2 and 4.

* Combination of strain variability from 20 strains, growth history from 3 strains and variants for *L. monocytogenes* and combination of strain variability from 20 strains and growth history from 3 strains for *L. plantarum*.

Food components, such as fat are known to have protective effect on cell robustness (Doyle et al., 2001). However, the magnitude of the effect of food product on heat resistance in comparison to the effect of strain variability was not yet known. Therefore, the effect of a model food matrix on heat resistance in this thesis was investigated in chapter 5, and showed that the total effect given by the food matrix was similar to the effect given by strain variability. The total effect of food product was mainly determined by the product ham. A consistent heat protective effect was observed for cells grown and/or inactivated in ham, and the protective effect was mainly determined by its function as heating menstruum. While the strong protective effect of ham was consistent,

a smaller and inconsistent effect was introduced by milk. The strain dependency effect of milk was mostly apparent when milk was used as incubation medium.

Knowledge on the strain variability in *D*-value can be used to assess the capability of current pasteurization processes in eliminating *L. monocytogenes* strains. When *L. monocytogenes* strains were grown at optimal conditions and inactivated in laboratory media, the D_{72}-values of *L. monocytogenes* were between 0.14 s and 1.41 s based on a *z*-value of between 4.4°C and 5.7°C (chapter 3). The pasteurization process at 72°C for 15 second then can eliminate this microorganism by more than 10 $\log_{10}$ CFU/ml. Since only small enhanced effect on heat resistance was observed when cells were incubated in milk at low temperature, almost similar reduction can be expected when cells are present in milk. This reduction is more than what is used as the performance criterion for *L. monocytogenes* in pasteurized milk, which is at least 6 $\log_{10}$ reduction (ILSI Europe, 2012). Although a higher protective effect was given by ham, the D_{72}-value of 3 *L. monocytogenes* strains grown and/or inactivated in ham were calculated to range from 0.5 s to 2 s. When a similar thermal processing for 15 s is applied for ham, the heat treatment will eliminate more than 7 $\log_{10}$ CFU/g of the bacterial cells. However, Van Boeijen et al. (2011) also reported the occurrence of resistant variants with average D_{72} value of 3.02 ± 0.21 s, indicating that the current pasteurization process might not be sufficient to inactivate all heat stress robust variants or to meet the 6 $\log_{10}$ reduction of the performance criterion as described by ILSI Europe.

In general, the most important factors to be considered for thermal inactivation, in which each having about equal impact, are strain variability, the presence of resistant fractions in microbial populations, the effect of food matrix and effect of growth history. Although the effect of growth history on heat resistance was similar to the effect of strain variability, the effect was mostly determined by the physiological state of the cells (exponential versus stationary). Certain pre-culturing conditions were known to also increase the heat resistance of exponentially grown cells (Den Besten et al., 2006; Linton et al., 1990), but their heat resistance was still lower than that of stationary grown cells. On the other hand, exposing cells to severe conditions might select for stable resistant variants that have higher heat resistance than stationary grown cells (Metselaar et al., 2013; Van Boeijen et al., 2011). Moreover, the strong and consistent protective effect of ham as heating matrix increased the heat resistance of microorganisms, and this effect should also be considered during thermal inactivation. To summarize, the effect of strain variability on thermal inactivation is similar to population heterogeneity, the effect of food matrix, especially ham, and the effect of growth history, especially exponential/stationary. Integrating those variability factors might widen the prediction intervals, and therefore influences the time or temperature needed for the thermal process. Our findings

demonstrate that the overall variability found in literature could be explained by the combination of strain variability, growth history, effect of food composition, and stable resistant variants, indicating that the conservative estimates suggested earlier by Van Asselt and Zwietering (2006) can be easily encountered in reality, and thus justifying the use of the conservative estimate (for example the upper limit *D*-value of the 95% prediction intervals) in the design of thermal process.

THE IMPACT OF VARIABILITY FACTORS IN THE PREDICTION AND FUTURE PERSPECTIVES

The effect of strain variability on growth and thermal inactivation kinetics was compared in chapter 4 and in this chapter, which demonstrated that strain variability in thermal inactivation was much higher than strain variability in growth. In a food chain, however, many variability factors contribute to the variability in microbial concentration at the end of the product shelf life. Therefore, knowledge on the most important factors/steps needed to control food pathogens and spoilage organisms along the food production chain is needed to prioritize the importance of control measures. To elaborate on this, an illustrative process was taken as an example to visualize the effect of different variability factors, such as strain variability, variability in thermal process, storage time and temperature, on variability in microbial concentration at the end of storage time. For this purpose, a milk process chain model combining the growth and thermal inactivation kinetics was simulated using the exponential growth model (equation (6.1)) and linear thermal inactivation model (equation (6.2)) to estimate the impact of variability in inputs (table 6.1) on the variability of *L. monocytogenes* concentration in the final product. The simulation was done using @Risk add-in for Excel version 6 (Palisade Corporation, New York, USA).

Table 6.1. Pasteurized milk chain variables used as model inputs for Monte Carlo simulation

Parameters	Description	Source
Raw material		
Initial contamination (log_{10} cfu/ml)	RiskNormal (2,0.5)	Assumed
Storage at farm		
Temperature (°C)	RiskPert(0,2,4)	Assumed
time (h)	RiskPert(2,36,72)	Dairy farmer (personal communication)
Heating process at factory		
Temperature (°C)	RiskNormal(65,0.1)[1)]; Risk Normal (70,0.1)[2,4)]; Risk Normal(70,2)[3)]	Scenarios

Parameters	Description	Source
time (s)	RiskNormal(15,0.1)	
Domestic storage		
Temperature (°C)	RiskNormal(6.3,2.7)[1,2,3]; RiskNormal(8.4,3.0)[4]	Koutsoumanis et al. (2010)
time (h)	RiskCumulative(0,120,0\24\48\72\96\120,0\ 0.23\0.74\0.96\0.978\1)	Koutsoumanis et al. (2010)
Growth and thermal inactivation characteristics		
μ_{max} (h^{-1})	RiskNormal(0.99,0.037)	Chapter 2
pH_{min}	RiskNormal(4.55, 0.0809)	Chapter 2
$pH_{1/2}$	RiskNormal(4.94,0.0433)	Chapter 2
pH_{ref}	7.3	Chapter 2
T_{ref} (°C)	30°C for growth; 65°C for thermal process	
T_{min} (°C)	RiskNormal(-2.20,0.52)	Chapter 2
Log_{10} *D*-ref (Log_{10} s)	RiskNormal(0.980,0.228)	Chapter 3
z-value (°C)	RiskNormal(4.94,0.34)	Chapter 3

1), 2), 3) and 4) are scenario 1, 2, 3 and 4.

$$Log_{10}\, N_t = Log_{10}\, N_0 + \frac{\mu_{max}}{ln\,(10)}\, t_s \quad [6.1]$$

$$Log_{10}\, N_t = Log_{10}\, N_0 - \frac{t_h}{D_T} \quad [6.2]$$

Where N_t is the maximum bacterial concentration (CFU/ml); N_0 is the initial bacterial concentration (CFU/ml); μ_{max} is the maximum specific growth rate (h^{-1}), t_h is the heating process time (s), t_s is the storage time (h) and D_T (s) is the *D*-value of *L. monocytogenes* at temperature T (°C).

The μ_{max} was calculated using the gamma model without interaction (equations (6.3)-(6.6)). The effect of a_w was not considered since the a_w of milk was similar to $a_{w,ref}$ (0.997±0.003) and therefore the $\gamma(a_w)$ is 1.

$$\mu_{max} = \mu_{ref} * \gamma(total) \quad [6.3]$$

$$\gamma(total) = \gamma(pH) * \gamma(a_w) \quad [6.4]$$

$$\gamma(pH) = \frac{\left(1-2^{\frac{(pH-pH_{min})}{(pH_{min}-pH_{1/2})}}\right)}{\left(1-2^{\frac{(pH_{ref}-pH_{min})}{(pH_{min}-pH_{1/2})}}\right)} \quad [6.5]$$

$$\gamma(T) = \frac{(T-T_{min})^2}{(T_{ref}-T_{min})^2} \quad [6.6]$$

Where μ_{ref} is the reference growth rate (h^{-1}); pH is the actual pH; pH_{min} is the pH limit, $pH_{1/2}$ is the pH at which the μ_{max} is half of the optimum specific growth rate; T is the actual temperature (°C); T_{min} is the temperature limit (°C) and T_{ref} is the reference temperature (°C).

The contribution of each variability factor in table 6.1 on final levels of *L. monocytogenes* at the end of domestic storage time was evaluated using four different scenarios presented in table 6.2.

Table 6.2. Scenarios used to evaluate the impact of different variability factors on the variability in final microbial levels

Scenarios	Pre-storage	Heating process at Factory*)	Domestic storage*)
Scenario 1	Temperature 0°C-4°C time 2h-72h	Temperature 65°C (0.1°C) time 15 s (0.1 s)	Temperature 6.3°C (2.7°C) time 0h-120h
Scenario 2	Temperature 0°C-4°C time 2h-72h	Temperature 70°C (0.1°C) time 15 s (0.1 s)	Temperature 6.3°C (2.7°C) time 0h-120h
Scenario 3	Temperature 0°C-4°C time 2h-72h	Temperature 70°C (2°C) time 15 s (0.1 s)	Temperature 6.3°C (2.7°C) time 0h-120h
Scenario 4	Temperature 0°C-4°C time 2h-72h	Temperature 70°C (0.1°C) time 15 s (0.1 s)	Temperature 8.4°C (3.0°C) time 0h-120h

*) value within brackets is the standard deviation.

For each scenario hundred simulations and 10,000 iterations were conducted using Latin Hypercube sampling in combination with a Mersenne twister random number generator, and a fixed seed value of 1 was selected to allow for the reproduction if the same

spreadsheet is used. The Spearman rank correlation coefficient was used to illustrate the impact of each variability factor on the variability in final levels of *L. monocytogenes*.
The milk process chain of the first scenario is illustrated in figure 6.6A. When the heating temperature was relative low and well controlled (65°C, σ=0.1°C), the most influential factor was the $\log_{10}D_{ref}$, representing strain variability in heat resistance, followed by domestic storage temperature, domestic storage time, and initial contamination (figure 6.7A). In the second scenario where a higher and well controlled heating temperature (70°C; σ=0.1°C) was used, higher degree of microbial reduction was realised as visualized in figure 6.6B. The contribution of $\log_{10}D_{ref}$ and *z*-value on the variability in final levels of *L. monocytogenes* in this scenario was much more pronounced than that of the other variables (figure 6.7B). In scenario 3, the heating temperature was poorly controlled (70°C; σ=2°C), resulted in a wider 95% prediction interval of microbial reduction during the heating process (figure 6.6C). Heating temperature followed by $\log_{10}D_{ref}$ became the most dominant factors influencing variability of the final microbial level (figure 6.7C). When well controlled heating temperature (70°C; σ=0.1°C) was combined with higher domestic storage temperature, a slight higher increase of the surviving *L. monocytogenes* was observed (figure 6.6D). In this scenario, besides the $\log_{10}D_{ref}$ and *z*-value as the most influencing factors, also storage temperature and time became slightly more important (figure 6.7D). The scenarios assumed that no post process contamination with *L. monocytogenes* occurred in the milk production chain. When post contamination is considered, domestic storage temperature, domestic storage time and strain variability in growth also become more important affecting the final microbial concentration at the end of storage time.

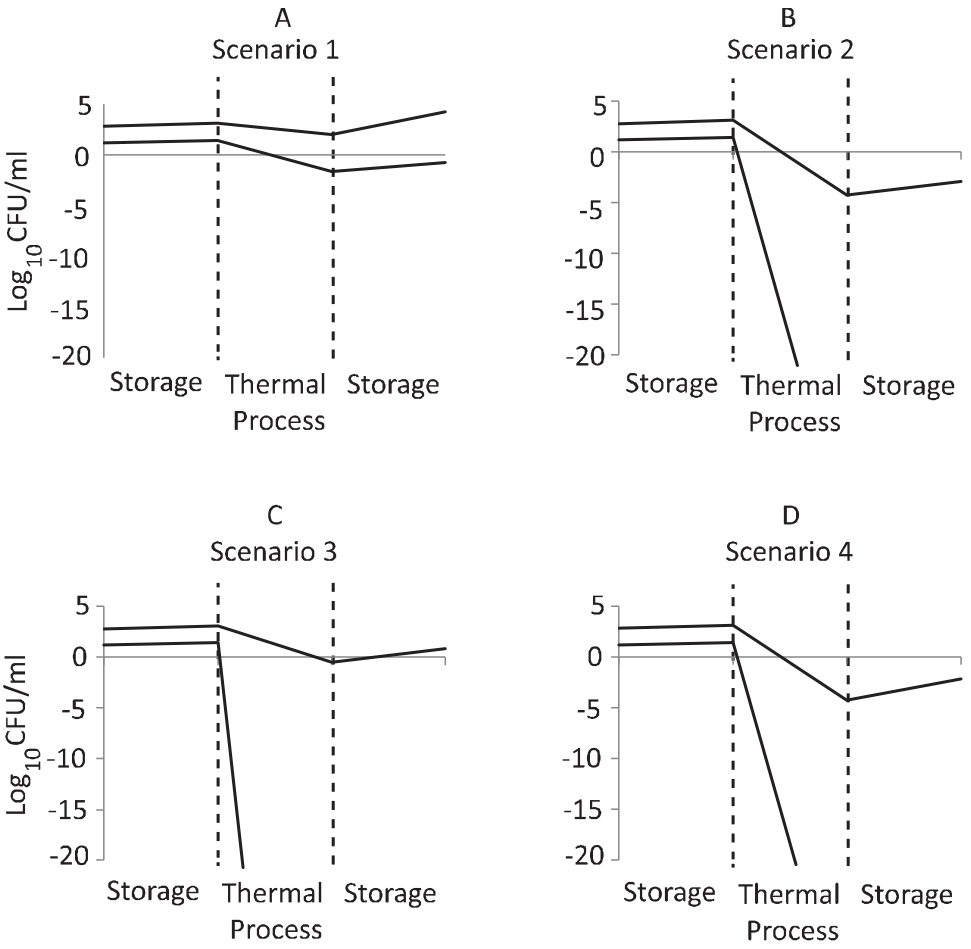

Figure 6.6. The simulated milk processing chain and concentrations of *L. monocytogenes* at different steps. The lines represent the 95% prediction interval. See Table 6.2 for the scenario details.

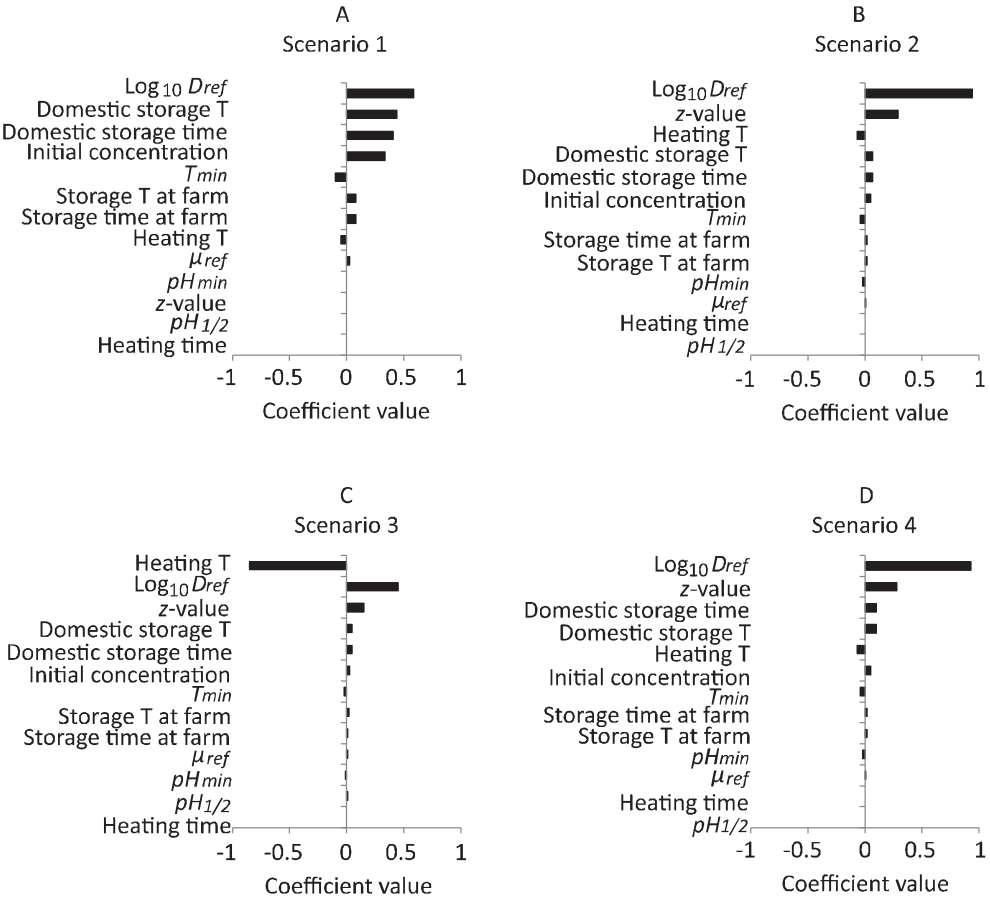

Figure 6.7. Spearman rank correlation between variability in final levels of *L. monocytogenes* at the end of domestic storage and variability in the input variables. See Table 6.1 for details on the input variables.

While strain variability in thermal inactivation kinetics was found to be one of the most important factors determining the variability in microbial concentrations at the end of domestic storage, the underlying mechanisms behind this natural variability still need to be explained. Gene-trait matching can be performed to better understand the diverse phenotypic characteristics seen in the growth and thermal inactivation kinetics of *L. monocytogenes* and *L. plantarum*. For the 20 strains of *L. monocytogenes* gene-trait matching did show some potential directions for further research (data not shown). Genetic markers for robustness might also be used for early detection of robust organisms in raw materials. The correlation between growth and thermal inactivation features and

other phenotypic characteristics, such as biofilm forming capability and virulence capacity, might also be relevant for understanding the fate of these microorganisms along the food production chain. Since variants from a heat sensitive strain LO28 showed highly increased heat resistance (Van Boeijen et al., 2011), further research on the occurrence of heat resistance fractions for the most heat resistant strains used in our study, such as L6 for *L. monocytogenes*, might also be of interest.

The sensitivity analysis underlines that many variability factors are important, but some are more important than others. Depending on the process characteristics, microbiological variability, especially strain variability in thermal resistance, is the most determining factor affecting the final contamination level. This strain variability, however, is inherent to living organisms. Strain variability challenges food processors because strain variability cannot be well controlled unless complete inactivation is realized and no recontamination occurs during food production chain. The integration of strain variability in prediction of microbial kinetics is, therefore, required in quantitative microbiology to obtain a more realistic prediction; and the most robust strains can be used in parallel or in cocktails to evaluate the efficacy of certain steps along the food production chain in controlling the growth of microorganisms.

REFERENCES

Augustin, J.-C., Carlier, V. 2000. Mathematical modelling of the growth rate and lag time for *Listeria monocytogenes*. International Journal of Food Microbiology 56, 29-51.

Augustin, J.C., Bergis, H., Midelet-Bourdin, G., Cornu, M., Couvert, O., Denis, C., Huchet, V., Lemonnier, S., Pinon, A., Vialette, M., Zuliani, V., Stahl, V. 2011. Design of challenge testing experiments to assess the variability of *Listeria monocytogenes* growth in foods. Food Microbiology 28, 746-754.

Awaisheh, S.S. 2010. Incidence and contamination level of *Listeria monocytogenes* and other *Listeria* spp. in ready-to-eat meat products in Jordan. Journal of Food Protection 73, 535-540.

Bajard, S., Rosso, L., Fardel, G., Flandrois, J.P. 1996. The particular behaviour of *Listeria monocytogenes* under sub-optimal conditions. International Journal of Food Microbiology 29, 201-211.

Barbosa, W.B., Cabedo, L., Wederquist, H.J., Sofos, J.N., Schmidt, G.R. 1994. Growth variation among species and strains of *Listeria* in culture broth. Journal of Food Protection 57, 765-769.

Begot, C., Lebert, I., Lebert, A. 1997. Variability of the response of 66 *Listeria monocytogenes* and *Listeria innocua* strains to different growth conditions. Food Microbiology 14, 403-412.

Benito, A., Ventoura, G., Casadei, M., Robinson, T., Mackey, B. 1999. Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat, and other stresses. Applied and Environmental Microbiology 65, 1564-1569.

Berendsen, E.M., Zwietering, M.H., Kuipers, O.P., Wells-Bennik, M.H.J. 2015. Two distinct groups within the *Bacillus subtilis* group display significantly different spore heat resistance properties. Food Microbiology 45, Part A, 18-25.

Brocklehurst, T.F., Parker, M.L., Gunning, P.A., Coleman, H.P., Robins, M.M. 1995. Growth of food-borne pathogenic bacteria in oil-in-water emulsions: II—effect of emulsion structure on growth parameters and form of growth. Journal of Applied Bacteriology 78, 609-615.

Cole, M.B., Jones, M.V., Holyoak, C. 1990. The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. Journal of Applied Microbiology 69, 63-72.

Conner, D.E., Brackett, R.E., Beuchat, L.R. 1986. Effect of temperature, sodium chloride, and pH on growth of *Listeria monocytogenes* in cabbage juice. Applied and Environmental Microbiology 52, 59-63.

Conner, D.E., Scott, V.N., Bernard, D.T. 1990. Growth, inhibition, and survival of *Listeria monocytogenes* as affected by acidic conditions. Journal of Food Protection 53, 652-655.

Coroller, L., Kan-King-Yu, D., Leguerinel, I., Mafart, P., Membré, J.-M. 2012. Modelling of growth, growth/no-growth interface and nonthermal inactivation areas of *Listeria* in foods. International Journal of Food Microbiology 152, 139-152.

De Jesús, A.J., Whiting, R.C. 2003. Thermal inactivation, growth, and survival studies of *Listeria monocytogenes* strains belonging to three distinct genotypic lineages. Journal of Food Protection 66, 1611-1617.

Den Besten, H.M.W., Mataragas, M., Moezelaar, R., Abee, T., Zwietering, M.H. 2006. Quantification of the effects of salt stress and physiological state on thermotolerance of *Bacillus cereus* ATCC 10987 and ATCC 14579. Applied and Environmental Microbiology 72, 5884-5894.

Doyle, M.E., Mazzotta, A.S., Wang, T., Wiseman, D.W., Scott, V.N. 2001. Heat resistance of *Listeria monocytogenes*. Journal of Food Protection 64, 410-429.

Farber, J.M., Sanders, G.W., Dunfield, S., Prescott, R. 1989. The effect of various acidulants on the growth of *Listeria monocytogenes*. Letters in Applied Microbiology 9, 181-183.

Farber, J.M., Coates, F., Daley, E. 1992. Minimum water activity requirements for the growth of *Listeria monocytogenes*. Letters in Applied Microbiology 15, 103-105.

Farber, J.M., Pagotto, F. 1992. The effect of acid shock on the heat resistance of *Listeria monocytogenes*. Letters in Applied Microbiology 15, 197-201.

Fernández-Escudero, I., Caro, I., Mateo, J., Tejero, J., Quinto, E.J. 2014. Low variability of growth parameters among six O157:H7 and non-O157:H7 *Escherichia coli* strains. Journal of Food Protection 77, 1988-1991.

Gänzle, M.G., Ehmann, M., Hammes, W.P. 1998. Modeling of growth of *Lactobacillus sanfranciscensis* and *Candida milleri* in response to process parameters of sourdough fermentation. Applied and Environmental Microbiology 64, 2616-2623.

George, S.M., Lund, B.M., Brocklehurst, T.F. 1988. The effect of pH and temperature on initiation of growth of *Listeria monocytogenes*. Letters in Applied Microbiology 6, 153-156.

Giraud, E., Lelong, B., Raimbault, M. 1991. Influence of pH and initial lactate concentration on the growth of *Lactobacillus plantarum*. Applied Microbiology and Biotechnology 36, 96-99.

Houtsma, P.C., de Wit, J.C., Rombouts, F.M. 1993. Minimum inhibitory concentration (MIC) of sodium lactate for pathogens and spoilage organisms occurring in meat products. International Journal of Food Microbiology 20, 247-257.

Houtsma, P.C., de Wit, J.C., Rombouts, F.M. 1996. Minimum inhibitory concentration (MIC) of sodium lactate and sodium chloride for spoilage organisms and pathogens at different pH values and temperatures. Journal of Food Protection 59, 1300-1304.

ILSI Europe. 2012. Risk assessment approaches to setting thermal processes in food manufacture. Report

Ingham, C.J., Beerthuyzen, M., van Hylckama Vlieg, J. 2008. Population heterogeneity of *Lactobacillus plantarum* WCFS1 microcolonies in response to and recovery from acid stress. Applied and Environmental Microbiology 74, 7750-7758.

Koutsoumanis, K., Pavlis, A., Nychas, G.-J.E., Xanthiakos, K. 2010. Probabilistic model for *Listeria monocytogenes* growth during distribution, retail storage, and domestic storage of pasteurized milk. Applied and Environmental Microbiology 76, 2181-2191.

Lambertz, S.T., Nilsson, C., Brådenmark, A., Sylvén, S., Johansson, A., Jansson, L.-M., Lindblad, M. 2012. Prevalence and level of *Listeria monocytogenes* in ready-to-eat foods in Sweden 2010. International Journal of Food Microbiology 160, 24-31.

Le Marc, Y., Huchet, V., Bourgeois, C.M., Guyonnet, J.P., Mafart, P., Thuault, D. 2002. Modelling the growth kinetics of *Listeria* as a function of temperature, pH and organic acid concentration. International Journal of Food Microbiology 73, 219-237.

Lianou, A., Koutsoumanis, K.P. 2011. Effect of the growth environment on the strain variability of *Salmonella enterica* kinetic behavior. Food Microbiology 28, 828-837.

Lianou, A., Koutsoumanis, K.P. 2013. Evaluation of the strain variability of *Salmonella enterica* acid and heat resistance. Food Microbiology 34, 259-267.

Lindqvist, R. 2006. Estimation of *Staphylococcus aureus* growth parameters from turbidity data: characterization of strain variation and comparison of methods. Applied Environmental Microbiology 72, 4862-4870.

Linton, R.H., Pierson, M.D., Bishop, J.R. 1990. Increase in heat resistance of *Listeria monocytogenes* Scott A by sublethal heat shock. Journal of Food Protection 53, 924-927.

Luu-Thi, H., Khadka, D.B., Michiels, C.W. 2014. Thermal inactivation parameters of spores from different phylogenetic groups of *Bacillus cereus*. International Journal of Food Microbiology 189, 183-188.

Mackey, B.M., Pritchet, C., Norris, A., Mead, G.C. 1990. Heat resistance of *Listeria*: strain differences and effects of meat type and curing salts. Letters in Applied Microbiology 10, 251-255.

McMeekin, T.A., Olley, J., Ratkowsky, D.A., Ross, T. 2002. Predictive microbiology: towards the interface and beyond. International Journal of Food Microbiology 73, 395-407.

Membré, J.M., Amézquita, A., Bassett, J., Giavedoni, P., de W. Blackburn, C., Gorris, L.G.M. 2006. A probabilistic modeling approach in thermal inactivation: Estimation of postprocess *Bacillus cereus* spore prevalence and concentration. Journal of Food Protection 69, 118-129.

Metselaar, K.I., Den Besten, H.M.W., Abee, T., Moezelaar, R., Zwietering, M.H. 2013. Isolation and quantification of highly acid resistant variants of *Listeria monocytogenes*. International Journal of Food Microbiology 166, 508-514.

Metselaar, K.I., Den Besten, H.M.W., Boekhorst, J., van Hijum, S.A.F.T., Zwietering, M.H., Abee, T. 2015. Diversity of acid stress resistant variants of *Listeria monocytogenes* and the potential role of ribosomal protein S21 encoded by rpsU. Frontiers in Microbiology 6.

Metselaar, K.I., Zwietering, M.H., Abee, T., Den Besten, H.M.W. Submitted for publication. Modeling and validation of ecological behaviour of Listeria monocytogenes wild type and stress resistant variants.

Miller, A.J. 1992. Combined water activity and solute effects on growth and survival of *Listeria monocytogenes* Scott A. Journal of Food Protection 55, 414-418.

Ng, H., Bayne, H.G., Garibaldi, J.A. 1969. Heat resistance of *Salmonella*: the uniqueness of *Salmonella* Senftenberg 775W. Applied Microbiology 17, 78-82.

Nolan, D.A., Chamblin, D.C., Troller, J.A. 1992. Minimal water activity levels for growth and survival of *Listeria monocytogenes* and *Listeria innocua*. International Journal of Food Microbiology 16, 323-335.

Oscar, T.P. 2000. Variation of lag time and specific growth rate among 11 strains of *Salmonella* inoculated onto sterile ground chicken breast burgers and incubated at 25^{o}C. Journal of Food Safety 20, 225-236.

Rodríguez-Calleja, J.M., Cebrián, G., Condón, S., Mañas, P. 2006. Variation in resistance of natural isolates of *Staphylococcus aureus* to heat, pulsed electric field and ultrasound under pressure. Journal of Applied Microbiology 100, 1054-1062.

Schvartzman, M.S., Belessi, X., Butler, F., Skandamis, P., Jordan, K. 2010. Comparison of growth limits of *Listeria monocytogenes* in milk, broth and cheese. Journal of Applied Microbiology 109, 1790-1799.

Sörqvist, S. 1994. Heat resistance of different serovars of *Listeria monocytogenes*. Journal of Applied Bacteriology 76, 383-388.

Tapia De Daza, M.S., Villegas, Y., Martinez, A. 1991. Minimal water activity for growth of *Listeria monocytogenes* as affected by solute and temperature. International Journal of Food Microbiology 14, 333-337.

Tienungoon, S., Ratkowsky, D.A., McMeekin, T.A., Ross, T. 2000. Growth limits of *Listeria monocytogenes* as a function of temperature, pH, NaCl, and lactic acid. Applied and Environmental Microbiology 66, 4979-4987.

Van Asselt, E.D., Zwietering, M.H. 2006. A systematic approach to determine global thermal inactivation parameters for various food pathogens. International Journal of Food Microbiology 107, 73-82.

Van Boeijen, I.K.H., Francke, C., Moezelaar, R., Abee, T., Zwietering, M.H. 2011. Isolation of highly heat-Resistant *Listeria monocytogenes* variants by use of a kinetic modeling-based sampling scheme. Applied and Environmental Microbiology 77, 2617-2624.

Van der Veen, S., Moezelaar, R., Abee, T., Wells-Bennik, M.H.J. 2008. The growth limits of a large number of *Listeria monocytogenes* strains at combinations of stresses show serotype- and niche-specific traits. Journal of Applied Microbiology 105, 1246-1258.

Van Lieverloo, J.H.M., de Roode, M., Fox, M.B., Zwietering, M.H., Wells-Bennik, M.H.J. 2013. Multiple regression model for thermal inactivation of *Listeria monocytogenes* in liquid food products. Food Control 29, 394-400.

Vasseur, C., Baverel, L., Hébraud, M., Labadie, J. 1999. Effect of osmotic, alkaline, acid or thermal stresses on the growth and inhibition of *Listeria monocytogenes*. Journal of Applied Microbiology 86, 469-476.

Whiting, R.C., Golden, M.H. 2002. Variation among *Escherichia coli* O157:H7 strains relative to their growth, survival, thermal inactivation, and toxin production in broth. International Journal of Food Microbiology 75, 127-133.

Wijtzes, T., de Wit, J.C., Huis in 't Veld, J.H.J., Van 't Riet, K., Zwietering, M.H. 1995. Modelling bacterial growth of *Lactobacillus curvatus* as a function of acidity and temperature. Applied and Environmental Microbiology 61, 2533-2539.

Wijtzes, T., Rombouts, F.M., Kant-Muermans, M.L.T., Van ’t Riet, K., Zwietering, M.H. 2001. Development and validation of a combined temperature, water activity, pH model for bacterial growth rate of *Lactobacillus curvatus*. International Journal of Food Microbiology 63, 57-64.

Xanthiakos, K., Simos, D., Angelidis, A.S., Nychas, G.J.E., Koutsoumanis, K. 2006. Dynamic modeling of *Listeria monocytogenes* growth in pasteurized milk. Journal of Applied Microbiology 100, 1289-1298.

Zwietering, M.H., de Koos, J.T., Hasenack, B.E., de Wit, J.C., Van 't Riet, K. 1991. Modeling of bacterial growth as a function of temperature. Applied and Environmental Microbiology 57, 1094-1101.

Zwietering, M.H., Cuppers, H.G.A.M., de Wit, J.C., Van 't Riet, K. 1994. Evaluation of data transformations and validation of a model for the effect of temperature on bacterial growth. Applied and Environmental Microbiology 60, 195-203.

Supplementary Table 6.1A. Comparison of Experimental (E), Reproduction (R) and Strain (S) variabilities between *L. monocytogenes* and *L. plantarum* as function of pH, a_w, T and [HLa]

Variables	Variability (RMSE)	Species		F-test[1)]	*p*-value
		L. monocytogenes	*L. plantarum*		
pH	Experimental (E)	0.0315	0.0452	2.06	**0.00**[2)]
	Reproduction (R)	0.0497	0.0612	1.52	**0.00**
	Strain (S)	0.0470	0.0670	2.03	**0.00**
a_w	Experimental (E)	0.0297	0.0472	2.52	**0.00**
	Reproduction (R)	0.0387	0.0836	4.67	**0.00**
	Strain (S)	0.0412	0.0720	3.05	**0.00**
T[3)]	Experimental (E)	0.0130	0.0278	4.62	**0.00**
	Reproduction (R)	0.0199	0.0537	7.24	**0.00**
	Strain (S)	0.0177	0.0470	7.00	**0.00**
[HLa]	Experimental (E)	0.0265	0.0282	1.13	0.14
	Reproduction (R)	0.0483	0.0456	1.12	0.20
	Strain (S)	0.0526	0.0350	2.26	**0.00**[4)]

1) F-test between variability of *L. monocytogenes* and *L. plantarum*.

2) Bold case indicates significant different.

3) RMSE for T was based on square root value.

4) Strain variability was significantly lower than reproduction variability for *L. plantarum*.

Supplementary Table 6.1B. Comparison between Experimental (E), Reproduction (R) and Strain (S) variabilities per species for pH, a_w, T and [HLa]

Variables	*p*-value	*L. monocytogenes* RMSE			*p*-value	*L. plantarum* RMSE		
		E	R	S		E	R	S
pH		0.0315	0.0497	0.0470		0.0452	0.0612	0.0670
E and R	**0.00**[1)]				**0.00**			
E and S	**0.00**				**0.00**			
R and S	0.22				0.12			
a_w		0.0297	0.0387	0.0412		0.0472	0.0836	0.0720
E and R	**0.00**				**0.00**			
E and S	**0.00**				**0.00**			
R and S	0.18				0.070			
T[2)]		0.0130	0.0199	0.0177		0.0278	0.0537	0.0470
E and R	**0.00**				**0.00**			
E and S	**0.00**				**0.00**			
R and S	0.12				0.054			
[HLa]		0.0265	0.0483	0.0526		0.0282	0.0456	0.0350
E and R	**0.00**				**0.00**			
E and S	**0.00**				**0.00**			
R and S	0.17				**0.001**			

1) Bold case indicates significant different.
2) RMSE T was based on square root value.

SUMMARY

The increase in the world's population is accompanied by an increase in global food demand. Most food products are perishable and therefore specific formulation and processes are needed to preserve their quality. The change in lifestyle and the increase in awareness toward tastier and healthier food affect the consumer demand toward mildly processed food. Mildly processed foods generally have shorter shelf life and thus depend on the combination of different growth limiting factors to prevent the growth of microorganisms during the shelf life of the product. Though its healthier image, mildly processed foods have a risk of the presence of robust microorganisms, which might grow and cause spoilage or foodborne illnesses. Since zero risk does not exist, minimizing the risk is the ultimate goal to reduce the disease burden caused by microorganisms.

Quantitative microbiology can be used as a tool to simulate the behaviour of microorganisms in food within the product's shelf life. Microbial growth and thermal resistance parameters are important inputs needed for predicting the growth and thermal resistance of microorganisms in the exposure assessment of a quantitative microbial risk assessment or in estimating the shelf life of food products. However, these parameters, such as the maximum specific growth rate (μ_{max}) vary depending on several factors amongst others within and between strain variabilities, cells history, physiological state of the cells and food matrix characteristics. Therefore, the prediction results might vary from one study to others due to differences in the parameters used to generate the prediction.

Since a realistic prediction is needed to improve the process and experimental designs, knowing the main sources of these variabilities and its magnitude are of importance. Two different microorganisms were used in the thesis for studying the variability factors in growth and thermal inactivation kinetics. *Listeria monocytogenes* is known as one of the important causative agents of foodborne disease. It has the highest case-fatality rate among foodborne pathogens, and is able to grow at relatively severe conditions, such as low temperature and high salt concentration. These characteristics make *L. monocytogenes* a good candidate to represent pathogenic bacteria. For spoilage bacteria, *Lactobacillus plantarum* was selected as a model species since this member of the lactic acid bacteria group is often found as contaminant in different food products, such as ketchup, dressings, and meat products.

The experimental (E), reproduction (R) and strain (S) variabilities in maximum specific growth rate (μ_{max}) (h^{-1}) were determined in chapter 2 and chapter 4 as function of four different variables, namely pH, water activity (a_w)/NaCl concentration ([NaCl]),

undissociated lactic acid concentration ([HLa]) and temperature (T). All experiments were done in duplicate at the same time using the same culture to quantify experimental variability and independently reproduced at least twice on different experimental days to quantify reproduction variability, and the use of 20 strains for both species accommodated the quantification of strain variability. For all four variables, experimental variability was clearly lower than reproduction variability and strain variability; and remarkably, reproduction variability was similar to strain variability for all variables for *L. monocytogenes* and for the variables pH, a_w and temperature for *L. plantarum*. Reproduction variability was even slightly higher than strain variability for the variable [HLa] for *L. plantarum*. The strain variability in cardinal growth parameters, namely pH_{min}, $[NaCl]_{max,}$ $[HLa_{max}]$, and T_{min} was further investigated by fitting secondary growth models to the μ_{max} data, including a modified secondary pH model. The fitting results showed that *L. monocytogenes* had an average pH_{min} of 4.5 (5-95% prediction interval (PI) 4.4 – 4.7), $[NaCl]_{max}$ of 2.0 M (PI 1.8 M – 2.1 M), $[HLa_{max}]$ of 5.1 mM (PI 4.2 mM – 5.9 mM), and T_{min} of -2.2°C (PI (-3.3°C) – (-1.1°C)), with the given prediction intervals of the means representing the variation in behaviour that can be encountered and needs to be controlled. Using similar secondary growth models as for *L. monocytogenes*, the average pH_{min} was 3.3 (PI 3.1 - 3.5); $a_{w,min}$ was 0.94 (PI 0.93 - 0.96); $[HLa_{max}]$ at pH 4.5 was 32.5 mM (PI 27.8 mM - 37.2 mM) and T_{min} was 6.1°C (PI 3.6°C - 8.6°C).

The strain variability in cardinal growth parameters was benchmarked to available literature data, showing that the effect of strain variability explained around 1/3 or less of all cardinal growth parameters found in literature, and since the literature data was rather limited for lactic acid bacteria, strain variability in cardinal growth parameters of *L. plantarum* explained more than 50% of the variability from literature, although with bias.

The quantification of variability in thermal resistance in chapter 3 and chapter 4 was conducted following the same method as used for growth. Besides the effect of temperature, the effect of growth history in the variability of *D*-values was also determined for *L. monocytogenes* and *L. plantarum* strains. Each thermal inactivation curve obtained in this study was fitted using the modified Weibull model, resulting in total of 360 *D*-value estimates for *L. monocytogenes* and 480 *D*-value estimates for *L. plantarum*. The *D*-value of *L. monocytogenes* ranged from 9 to 30 minutes at 55°C; from 0.6 to 4 minutes at 60°C; and from 0.08 to 0.6 minutes at 65°C. The *D*-value estimates of the 20 *L. plantarum* strains ranged from 0.80 min to 18.6 min at 55°C, 0.22 min to 3.91 min at 58°C, 3.1 s to 0.75 min at 60°C, and 1.8 s to 19.2 s at 63°C. Unlike in growth, strain variability in thermal resistance was much higher than reproduction and experimental variabilities. Strain variability was similar to the effect of growth history for *L. monocytogenes* and slightly higher than the effect of growth history for *L. plantarum*. The

effect of growth history was mostly determined by the physiological state of the cells (i.e. exponential phase versus stationary phase), and additionally also by the effect of pre-culturing temperature of 12°C for *L. plantarum*. Although the magnitude of strain variability in growth and thermal resistance could not be directly compared due to differences in measurement unit, the simulations using cardinal growth parameters and *D*-values showed that the effect of strain variability in thermal resistance was much larger than the effect of strain variability in growth.

Prediction of growth of microorganisms in food is often based on the effects of intrinsic and extrinsic variables as obtained in laboratory media. To combine the effect of different variables, those variables can be integrated in a gamma model with or without interaction to predict the maximum specific growth rate (μ_{max}), which can be used to predict microbial numbers in a defined food product. Deviations might occur because the effect of food product specific characteristics is not considered when laboratory media is used. Therefore a validation in actual food products is needed to quantify a potential product specific effect. Knowledge on food product specific effects on microbial kinetics will not only result in a more realistic growth prediction, but also extend the knowledge on factors influencing growth and heat resistance. Growth validation using two food models in chapter 5 showed that the effect of food product was strain dependent, which might further complicate the prediction. The lower value of the γ(T) at 7°C and 15°C compared to γ(milk/ham) observed in the present study highlighted the importance of the effect of temperature in controlling the growth of microorganisms. Moreover, the effect of food product, which was mainly determined by ham, and strain variability in thermal inactivation was similar for both species, and in general the combination of strain variability, the effect of growth history and specific effect of food products explained variability found in literature with bias.

In a food chain, other variability factors, such as variability in thermal process and storage time and temperature, also affect the microbial concentration at the end of the product shelf life. An illustrative process was taken as an example to visualize the impact of different variability factors, such as strain variability, variability in thermal process time and temperature, variability in storage time and temperature, on the variability in microbial concentration at the end of storage time. The simulation result showed that the importance of certain factors depended on the process condition. When well controlled high heating temperature was used, the strain variability in *D*-values was the most determining factors in the final level of microorganisms. This conclusion did change when the control in heating temperature was poor or when the lower heating temperature was used. However, even at those conditions, strain variability in *D*-value remains one of the important determinants for the final level of the microorganisms. Unlike other variability

factors of temperature and time, this strain variability is inherent to living organisms and challenges food processors because it cannot be well controlled unless complete inactivation is realized and no recontamination occurs during food production chain. Inclusion of strain variability in prediction of microbial kinetics is, therefore, required in quantitative microbiology to obtain a more realistic prediction; and the most robust strains can be used in parallel or in cocktails to evaluate the efficacy of certain steps along the food production chain in controlling the growth of microorganisms.

ACKNOWLEDGMENTS

Doing a PhD is like being in a journey. As in every journey, excitement sparks the first time the journey starts, getting overwhelmed in the middle of the journey and being exhausted toward the end of the journey. Despite the challenges, the happy moment comes when the journey is completed and the goal is accomplished. However, a successful journey is not only the work of a single person, but rather a mutual work since a traveller also receives assistance and support from the fellow travellers, local people, family and friends and perhaps certain authorities. Therefore, upon the completion of my PhD journey, I, as many other travellers would also do, would like to convey my gratitude to supervisors, colleagues, friends, and family, from whom I received encouragements and never ending supports during my PhD journey.

First, I would like to pass my gratitude to my promotor Marcel Zwietering and my co-promotor Heidy den Besten for their excellent supervisions during my PhD. Marcel, I thank you a million for your support, encouragement, in depth knowledge and positive feedback, which helped me to go through this journey and finalize it. Thanks for giving me a chance to start this journey and having confidence that I will make it to the end. Heidy, your critical thinking, high standard and encouragement helped to make me who I am now, and I am very thankful for that. To my other supervisor, Wilma Hazeleger thank you for your enthusiasm, support and encouragement to me and also to my students from the beginning till the end of my journey.

Second, to the former and successor project managers of the modelling project, Gerard Weenk, Masja Nierop Groot and Marjon Wells-Bennik, and all other members of food safety and preservation project for their enthusiasm, support and encouragement to all PhD students involved in the project. To Janneke Wijman, thanks for the help to arrange in-house produced ham, which I used for experiments described in chapter 5 of this thesis.

I would like also to thank Gerda van Laar-Engelen, Ingrid Maas, Judith Wolkers-Rooijackers and the other food microbiology staff. Gerda many thanks for helping me to sort out the administrative issues. Ingrid, thanks for the help in the kitchen, in the lab, for the chat and for the Germany trip. It really helps to relieve stress when you deserve to have one. Judith, thanks for your help in the molecular work. Although the work in the end did not result in a publication, but the result is useful for future research. The other food microbiology staff: Tjakko, Eddy, Martine, Marcel T, Gerrieke, Svetlana, Ida, Oscar and Jeroen thanks for the warmth and support during my stay in the department.

The girls Hasmik, Monica, Karin and Alicja, thanks so much guys for the talks we shared in and outside the office, flex room and during TIFN expert meetings. Those are the oases we sometimes need in the middle of our journey. Wish you all success in every path you choose after completing your own journey. Irma, Maciek and Natalia, thanks a lot for being great office mates. Maciek wish you all the best for your future career and life. Irma and Natalia, wish you both all the best for the remaining part of your own journey. Ioanna, thanks for providing me a place whenever I needed to stay away a moment from the world and together with Irma for being my paranymphs during my promotion.

My students Sylvia, Vasiliki, Jia, Tianyang, Su Man, Esther, Bertine, Daokun and Yufei, thanks a lot for your hard work. The results of your work are described in chapter 3, 4 and 5 of this thesis.

To Jimmy, Nanik and mbak Yuni, I was inspired by you the first time I accepted this project. Thanks for being there and giving me valuable suggestions when I started my journey. This four years journey was not always smooth, and sometimes we need friends to share when we badly miss home. Therefore, to the former house mates of Kortestraat 6G Yessie and Depi, Troelstraweg 33 mbak Vitri and Esti, Kolkakkerweg 27 mbak Eva and Rani and Lawickse Allee 16 mbak Vivi, mbak Hikmah, Ika, Titis, Tika and Aziza; also to Pak Dikky, Tika, Yuda, Indraningrat, Pak Fajar, Eda, Atin, Uma and the other Indonesian master students and PhD-ers, many thanks for the friendship and fun we shared during my stay in Wageningen.

I would like also to thank the former DG of the Agency for Food Security Pak Achmad Suryana and the Director of the Centre for Food Availability and Vulnerability Pak Tjuk Eko Hari Basuki for giving me support and permission to start my PhD journey. To colleagues and HRD thanks for taking care of the administration issues regarding to my leave.

To my mother, sister and brother, my other parents: uncle and aunts, also beloved cousins Reni, Ima and Widya, it never has been easy to leave all of you back home, but your supports gave me spirits to continue this journey till the end.

Finally, to my husband Widi Aribowo, this four years long distance marriage would not work out without your unconditional support and understanding. Thanks for your confidence when others doubted that this would work just fine. Although the next journey might not be easy, but I believe duo travellers are much better and stronger than solo traveller.

Diah

LIST OF PUBLICATIONS

Aryani, D.C., H.M.W. den Besten, W.C. Hazeleger, M.H. Zwietering (2015). Quantifying variability on thermal resistance of *Listeria monocytogenes*. International Journal of Food Microbiology 193, 130-138.

Aryani, D.C., H.M.W. den Besten, W.C. Hazeleger, M.H. Zwietering (2015). Quantifying strain variability in modelling growth of *Listeria monocytogenes*. International Journal of Food Microbiology 208, 19-29.

Aryani D.C., H.M.W. den Besten, M.H. Zwietering. Quantifying variability factors: effect of strain variability on growth and thermal inactivation kinetics of *Lactobacillus plantarum*. Submitted for publication.

Aryani, D.C., M.H. Zwietering, H.M.W. den Besten. The effect of different matrices on the growth and thermal inactivation of pathogenic and spoilage microorganisms. Submitted for publication.

Den Besten, H.M.W., **D.C. Aryani,** K.I. Metselaar, M.H. Zwietering. Microbial variability in growth and heat resistance of a pathogen and a spoiler: all variabilities are equal but some are more equal than others. Accepted in International Journal of Food Microbiology.

Perdana, J., H.M.W. den Besten, **D.C. Aryani**, O. Kutahya, M.B. Fox, M. Kleerebezem, R.M. Boom, M.A.I Schutyser (2014). Inactivation of *Lactobacillus plantarum* WCFS1 during spray drying and storage assessed with complementary viability determination methods. Food Research International 64, 212-217.

Overview of Completed Training Activities

Discipline Specific Activities

Courses

Management of Microbiological Hazards in Foods (MMFH)
MMHF - distance learning modules (preservation, HACCP, food related hazards)
Reaction Kinetics in Food Science
Food Fermentation
Basic Statistics
Advanced Course in Predictive Modelling and Risk Assessment
Genetic and Physiology of Food-associated Microorganisms

Meetings

International Conference on Predictive Modeling in Food 8, Paris, France
FoodMicro 2013, Nantes, France
IAFP2015, Cardiff, UK

General Courses

VLAG PhD Week
Scientific Writing
Workshop Presentation Skills
Technique for Writing and Presenting a Scientific Paper
Project and Time Management
Advanced Course Guide to Scientific Artwork

Optionals

Preparation of PhD Project Proposal
TIFN Project Meeting (2011-2015)
Seminars Laboratory of Food Microbiology (2011-2015)
PhD Trip Japan (2012)
PhD Trip Ireland (2014, organization)

ABOUT THE AUTHOR

Diah Chandra Aryani was born on 2 March 1980 in Rembang, Central Java, Indonesia. She moved to Bogor in 1998 to continue her education in Food Technology at Bogor Agricultural Institute. Following her graduation, she worked for the Agency for Food Security, Ministry of Agriculture in Indonesia. In 2009 she received STUNED scholarship to follow the master program of Food Safety at Wageningen University. In September 2011 she started her PhD project at the laboratory of Food Microbiology, Wageningen University. The result of her project “vegetative cells chain model” is described in this thesis. Diah will continue working at the Agency for Food Security, Ministry of Agriculture after completing her PhD.

The research described in this thesis was financially supported by the Top Institute Food and Nutrition (TIFN), Wageningen, The Netherlands.

Cover design and lay out by Diah Chandra Aryani and Asep Bayu Ekawijaya
Printed by Ridderprint BV, Ridderkerk, The Netherlands