Investigating the effects of novel heat and high pressure processing on *Listeria* and *Bacillus* inactivation in a model food

Inaktiveringsstudie av *Listeria* and *Bacillus* i et modellprodukt prosessert ved en ny varmebehandlingsmetode og høytrykk

Philosophiae Doctor (PhD) Thesis

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1 PREFACE

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2 ABSTRACT

Thermal processing is a common method for controlling foodborne pathogens in various ready-to-eat (RTE) products. Traditional processes that involve static heating often result in undesirable quality changes in foods. These changes are due to slow heat penetration under conductive heating. Recent technologies such as agitating retorts or HPP can be an alternative for improving food quality through reduced processing time and overall heat load. Nevertheless, such novel technologies need to be thoroughly investigated with microbial inactivation studies to ensure that food safety requirements are met.

The overall aim of this PhD thesis was to investigate the effects of novel agitating retort and combined high pressure-temperature processing on elimination of *Listeria* and *Bacillus* in a model soup (pH 6.1). In this context, the effects of process parameters such as processing time, pressure and heating mode (agitated/static) on microbial survival were studied. Applicability of the classical thermal death model and response surface methodology (RSM) on agitating retort and combined high pressure-temperature processing was assessed, respectively. The same model soup (pH 6.1) was used for all experiments as the food substrate.

It was shown that novel agitating retort can give reductions of 86 to 89 % and 77 % in processing time compared to static heating for *Listeria* and *Bacillus* inactivation, respectively. Using agitation speeds up to 100 strokes per minute (spm) provided a homogenous heat load distribution and high heating rates (5.3 to 9.5 °C/min) in the soup. This led to correlated predicted and observed values for estimation of microbial inactivation.

Combined high pressure and temperature (*P*/*T*) showed synergistic effects for inactivation of vegetative cells of *Listeria* and spores of *Bacillus*. Pressurization at 600 MPa for 5min with initial temperatures above 45 °C prevented recovery of *Listeria* at 4 and 8 °C for three weeks when initial counts were 3 and 5 log CFU/ml. Combined *P*/*T* treatments provided up to 4 log increases in *Bacillus* spore inactivation when the initial temperature was increased from 20 °C to 65 °C at 650 MPa for 10 min.

Results from this work clearly indicated that the novel processes examined could offer a great potential for milder processing of foods. Results from this thesis can be used for designing

future studies on validation and process optimization on similar products. Combined *P*/*T* processing appears to be suitable for heat sensitive products, whereas agitating process could be desirable for less heat-sensitive liquid products without solid particles.

3 SAMMENDRAG

Prosessering ved bruk av varmebehandling er en vanlig metode for å kontrollere og inaktivere patogene mikroorganismer i ulike ferdigretter. Tradisjonelle prosesser som involverer statisk varme resulterer ofte i uønskede kvalitetsendringer i mat. Disse endringene er forårsaket av lav varmegjennomgang under konduktiv varmeoverføring. Relativt nye teknologier som autoklavering med risting og høytrykksprosessering (HP) kan benyttes som alternativer for å forbedre matkvaliteten som følge av redusert prosesseringstid og samlet varmebelastning. Denne nyere type teknologier må bli nøye undersøkt med hensyn på mikrobiell inaktivering for å sikre at matsikkerhetskrav blir ivaretatt.

Hovedmålsettingen for denne PhD oppgaven har vært å undersøke effekten av autoklavering med risting og høytrykksprosessering med varme (HP-varme), og deres effekt på eliminering av *Listeria* og *Bacillus* i en modellsuppe (pH 6.1). Mikrobiell overlevelse ble studert ved å variere ulike parametere som: temperatur, prosesseringstid, trykk og risting eller statisk varme. Anvendelse av klassisk varmeinaktivering og RSM modeller for autoklaver med risting og HP-varme har blitt undersøkt. Den samme modellsuppen ble benyttet som modellprodukt i alle eksperimentene.

Resultatene viste at autoklavering med risting kan gi en reduksjon i prosesseringstid på henholdsvis 86 til 89 % og 77 % for inaktivering av *Listeria* og *Bacillus* når man sammenligner med tradisjonell statisk varmebehandling. Ved å bruke ristehastigheter på opp til 100 slag per minutt oppnådde man en homogen varmefordeling og høy oppvarmingshastighet (5.3 til 9.5 °C/min) i suppen. Dette ga god korrelasjon mellom beregnede og observerte verdier for estimert mikrobiell inaktivering.

HP-varme ga synergistisk effekt for inaktivering av vegetative *Listeria*-celler og *Bacillus*-sporer. Prosessering ved 600 MPa i 5 min kombinert med starttemperaturer på over 45 °C hindret reparasjon og vekst av *Listeria* etter lagring ved 4 and 8 °C i tre uker etter prosessering. Startnivå av bakterier var da 3 og 5 log CFU/ml. Kombinasjonen HP-varme ga opp til 4 log inaktivering av *Bacillus* sporer da starttemperaturen ble økt fra 20 til 65 °C at 650 MPa for 10 min.

Resultatene fra PhD arbeidet har gitt tydelige indikasjoner på at disse nye prosesseringsmetodene kan ha stort potensiale for en midlere behandling av mat. Resultatene

kan videre bli benyttet som bakgrunnsmateriale for studier for validering og prosessoptimalisering av lignende produkter. HP-varme kan være en prosesseringsmetode som kan egne seg for varmesensitive produkter. Varme kombinert med risting kan også i flere tilfeller med fordel benyttes for mat som tåler mer varme.

4 LIST OF PAPERS

Paper I **Comparison of bacterial inactivation with novel agitating retort and static retort after mild heat treatments**

Ates, M. B., Skipnes, D., Rode, T. M., Lekang, O. 2014. *Food Control*, 43:150-154

Paper II **Effects of agitated and static heating on subsequent recovery of sublethally injured *Listeria***

Ates, M. B., Rode, T. M., Skipnes, D., Lekang, O.

Manuscript to be submitted to European Food Research & Technology

Paper III **Modelling of *Listeria monocytogenes* inactivation by combined high pressure and temperature treatments in model soup**

Ates, M. B., Rode, T. M., Skipnes, D., Lekang, O.

Submitted to European Food Research & Technology

Paper IV **Comparison of spore inactivation with novel agitating retort, static retort and combined high pressure-temperature treatments**

Ates, M. B., Skipnes, D., Rode, T. M., Lekang, O.

Submitted to Food Control

In the following chapters, papers are referred to with the roman letters.

5 INTRODUCTION

5.1 Objectives of the study

Milder processing technologies are desirable for the food industry and consumers since they can enable production of added-value products with improved nutritional and sensorial quality. New technologies can show different mechanisms for microbial inactivation, hence traditional heat inactivation kinetics may no longer apply for the new system. Conducting food safety studies are critical for a better understanding of new technologies. This PhD study contains 4 papers that investigate how novel agitating retort process and combined high pressure-temperature (*P*/*T*) treatments affected inactivation, injury and recovery of *Listeria* and destruction of *Bacillus* spores in a model soup (pH 6.1). Thesis outline is depicted in Fig. 1.

In compliance with FDA (2000) recommendations on the research needs for alternative food processing technologies, the objectives of the thesis were to:

i. Study the effects of key process variables on inactivation of selected organisms by novel heat and high pressure processing (Papers I, III and IV)
ii. Evaluate the applicability of different models for lethality estimation for novel heat and HP processes (I, III and IV)
iii. Investigate synergistic effects of combined *P*/*T* treatments on *Listeria* cells and *Bacillus* spores (III and IV).
iv. Investigate *Listeria* recovery after novel heat and high pressure treatments during refrigerated storage (II and III)

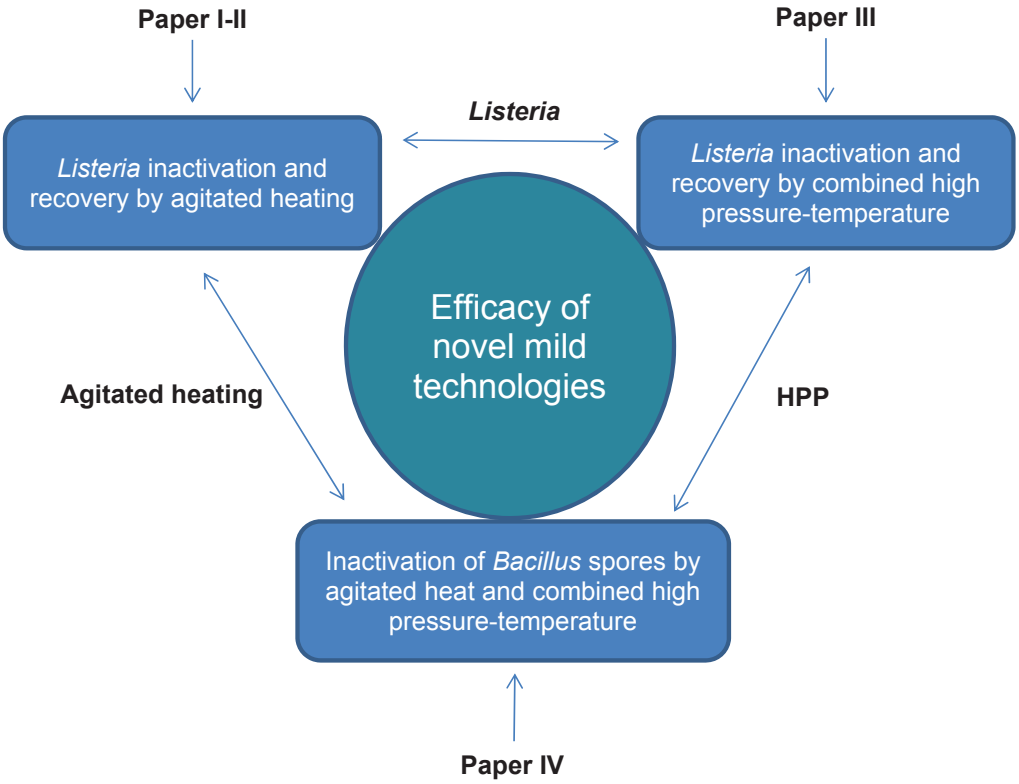

Fig. 1 PhD Thesis outline

5.2 Background

5.2.1 Novel mild technologies for microbial inactivation in foods

During the last three decades, consumer demand for mildly treated foods resulted in efforts for finding new processing methods for food preservation. New technologies such as HPP, high frequency agitating retorts, ionizing irradiation, pulsed electric fields, pulsed light, ultrasound, and magnetic fields were among the milder technologies of interest for microbial inactivation. These new technologies have been reviewed for interested readers (Lopez-Gomez et al., 2009; Rosnes, Skara & Skipnes, 2011).

5.2.1.1 Agitated heat processing

Thermal processing has been the most widely used method for microbial inactivation in foods. Improvement of food shelf life by heat processing was invented and patented without knowing microbiological principles lying under by Appert (1810). In the late 19th century, the process was named pasteurization that made inactivation of vegetative pathogens and spoilage organisms possible in foods. Heat processes were later developed using scientific methodology during early 20th century by the works of Richardson & Ball (1920), Bigelow (1921) and Esty & Meyer (1922). One drawback of these scientific developments was that they were based on wide microbiological stability margins. This problem is due to process control issues and temperature deviations during industrial heating processes. Therefore, safety and stability of the process have often been achieved at the expense of food quality degradation caused by overprocessing. Consequently, demand for higher quality foods has led to requirement of scientific and technological research on milder preservation methods without compromising on safety and stability (Smelt & Brul, 2014).

Heat transfer rate during a thermal process determines the speed of heating and cooling processes. Therefore, knowledge of thermal properties of foods becomes important for understanding thermal changes in foods during heat processing. Unsteady-state conditions may rule during heating (or cooling) of foods that mean changing heat transfer rate over time. When the rate of heat transfer stabilizes, steady-state heat transfer condition can be achieved. Knowledge on these thermal properties is vital for understanding basic mechanisms of heat

transfer. There are three types of heat transfer: (a) conduction, (b) convection and (c) radiation (Singh, 2007):

a. Conduction heat transfer takes place within a material from a high temperature zone to a low temperature zone through direct contact. There is no mass transfer involved. The rate of heat transfer (q/A) due to conduction is called Fourier's law: $q/A = -kdT/dx$. Thermal conductivity (k) is the steady-state heat transfer rate (q) through a unit cross-sectional area (A), when a unit temperature difference ($T_1 - T_2$) is maintained over a unit distance (L): $k = qL / [A(T_1-T_2)]$

b. Convection heat transfer takes place between a surface and the surrounding fluid. The rate of convective heat transfer depends on the properties of the fluid. Fluid flow properties determine the convective heat transfer coefficient (h). Equation for convective heat transfer rate is $q/A = h\ (T_s-T_\infty)$. There are two types of convective heat transfer which are forced convection and natural convection. Forced convection involves an externally provided fluid flow (i.e. agitation or fan), whereas natural convection is based on differences in fluid temperature and density.

c. Radiation heat transfer is based on three parameters of food materials: emissivity (ε), absorptivity (α), and transmittance (τ). Energy emission from a surface is based on the Stefan–Boltzmann law: $q = \sigma A \varepsilon T_A^4$

Industrial sterilization and pasteurization processes often involve unsteady-state heat transfer conditions. Thus, the time-temperature history of food products during heating and cooling periods must be measured in order to track the thermal process.

Agitating retorts can enhance heat flow rates through forced convection in liquid and semi-liquid foods. Through improved heat flow rates, agitated heating can minimize non-uniform temperature zones, over-processing and undesirable quality changes. First agitating retort with axial rotation mechanism came into use during 1920s. Axial rotation induced a process time reduction up to 50% in comparison to static mode (Eisner, 1988). End-over-end (EOE) agitation was later developed in 1950s (Clifcorn, Peterson, Boyd & Oneil, 1950). EOE agitation further improved heat transfer in canned foods and provided a reduction in process time extending to 65% compared to static heating. In 2006, a new agitating retort with high frequency reciprocal agitation was developed. Most recent agitating retort model is based on

high frequency longitudinal agitation mechanism which enables rotation speeds higher than 40 rpm (Walden, 2008). The new retort model is depicted in Fig. 2.

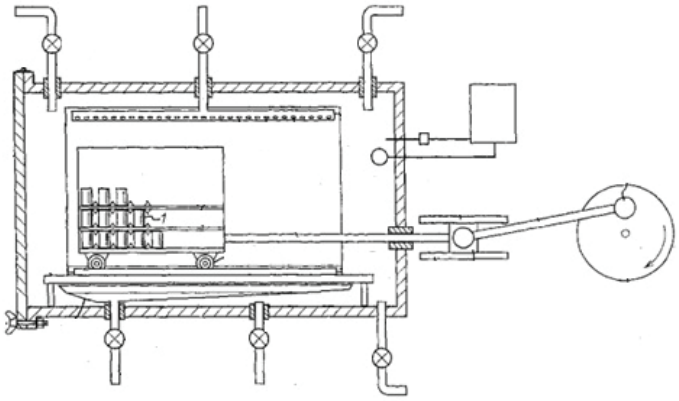

Fig. 2 Principle for the new retort system: products (1) within the basket are agitated in longitudinal direction (adopted from Paper I).

5.2.1.1.1 Modelling of microbial inactivation

Inactivation kinetics of the target microorganism(s) and the time-temperature history of in each spot in the container processed must be well understood in order to control thermal processes. Heat penetration curves obtained by thermocouples and data acquisition software can offer the required information for thermal process evaluations. On the other hand, some cases such as rotational sterilization and heating profile of food particles yet pose a challenge when the traditional tools are only used. Furthermore, process lethality estimations gets even more challenging when death rate of target microorganism(s) do not follow log-linear kinetics (Smelt & Brul, 2014).

Bigelow (1921) for the first time developed inactivation kinetics of bacterial spores using first-order reaction kinetics. Esty & Meyer (1922) then demonstrated that the heat resistance of *Clostridium botulinum* population was inoculum-size dependent and death rate of spores was exponential with time. Since then, inactivation kinetics has been described with decimal reduction time, ***D* value**, time to reduce the number of microbial cells by a factor of 10 and z

value which is the increase in temperature corresponding to a 10-fold reduction in *D* value. First-order microbial inactivation reactions are:

$$\log (N_t) = \log (N_0) - (t / D) \quad (1)$$

$$\log (D / D_{ref}) = - (1 / z) (T - T_{ref}) \quad (2)$$

where N_0 = the initial microbial counts, N_t = microbial counts at time t, D_{ref} = reference D value corresponding to the reference temperature (T_{ref})

Models based on first-order inactivation kinetics were developed for eliminating *Clostridium botulinum* hazard through "12*D* reduction" process. Excellent safety record of canned foods has validated the applicability of these models in industrial processes. For pathogenic vegetative cells, a thermal process design leading to "5*D* or 6*D* reduction" is mostly the target for pasteurized products (Smelt & Brul, 2014).

Nonlinearities can also be observed in microbial survival curves. These are often called "shoulder" and "tailing" effects (Peleg & Cole, 1998). If "shoulders" are present for microbial inactivation curves, log-linear approach is still applicable. However, in the case of having "tails", using a log-linear target may not be safe enough. Heating rate may also affect microbial resistance. For example, long exposure of vegetative cells to slow heating can lead to stress adaptation. As a result, vegetative cells may acquire higher heat resistance. Reasons causing deviation from linearity must be well understood for having more accurate inactivation models. Nonlinear models available for microbial inactivation kinetics have been reviewed (Smelt & Brul, 2014).

Response surface methodology (RSM) has been used in food research for studying the effects of process variables and their mutual interactions on the response. RSM can also take into account nonlinearities often shown with Weibull, Gompertz and log-logistic models. For more detailed descriptions on RSM, readers are referred to Paper III.

5.2.1.1.2 Bacterial heat resistance

Heating may cause injury in more than one target in vegetative cells. As a result of heating, some parts of the cell wall, cell membrane, ribosomes, ribosomal RNA and heat-sensitive enzymes involved in vital metabolic processes could be damaged (Wu, 2008). Differential scanning calorimetry (DSC) studies demonstrated that heat damage is apparently concentrated on the cell membrane at temperature range between 62 and 66 °C (Teixeira, Castro, Mohacsi, Farkas & Kirby, 1997). Heat-sensitive proteins such as α and β subunits of RNA polymerase denatures at higher temperatures, thereby causing cell death. Also, cell injury is promoted by leakage of nutrients (potassium, amino acids and proteins) through heat-damaged cell membrane (Smelt & Brul, 2014). Difference between heat damage mechanism at lower and higher temperatures is apparently due to the intrinsic stability of essential macromolecules within the cytoplasm, membrane and cell wall of vegetative cells (Smelt, Hellemons, Wouters & van Gerwen, 2002).

Stress responses are adaptive responses that permit bacteria to survive and grow even under undesirable conditions. Heat shock responses (HSPs) act as molecular chaperones when environmental stress factors are present. HSPs can repair or remove (through protease and peptidase activity) heat-damaged proteins essential for the survival of cells (Lindquist, 1992). HSPs are also reported to take role in DNA repair and replication, cell multiplication and concentrating osmolytes to improve protein stability against heat. The heat shock response system in cells is believed to modify and replace thermolabile proteins with heat-resistant (Wesche, Gurtler, Marks & Ryser, 2009).

Spore-forming microorganisms may switch to an irreversible sporulation process which takes many hours, when there is high scarcity of nutrients. Mature spores possess a spore core with a membrane which is the basis of the vegetative cell before the transformation. The spore core has 15 to 25% calcium dipicolinate (Ca-DPA) which may have a role in developing heat resistance in spores. It is known that DPA is released upon destruction of spores by heat (Coleman, De, Li, Cowan & Setlow, 2007). Unusual heat resistance of spores is attributed to very low water content and the physical state of the water in the spore core (Smelt & Brul, 2014).

There is an abundance of literature information on broth systems regarding heat inactivation but data with real foods is not available in equivalent amounts. A major difficulty in predictive microbiology is to generate knowledge in model systems that can be transferred to real food situations. An overview of inactivation data on pathogenic bacteria and spores in foods is shown in Table 1. It is possible to observe 10-fold difference in *D* values with regards to various food matrices. However, it is not yet clear if this is due to interlaboratory variation or the food matrix effect. Using adjusted parameters such as pH and water activity (a_w) in model buffer systems may provide comparable information with real food matrices. Nevertheless, it should be taken into account that microbial inactivation studies with buffers or broth media may not be extrapolated to real foods. Milk and cream is a case where underestimations can happen since microorganisms can be protected against temperature inactivation (Smelt et al., 2002).

A compilation of heat resistance data on important food pathogens in various food matrices is shown (Table 1 and 2). As it can be clearly seen, *D* values vary significantly on different food substrates, whereas the *z* value generally tends to be more comparable.

Factors that affect heat resistance

Cultivation, heating and recovery conditions have a significant impact on heat resistance. Cells at exponential growth phase are more sensitive to heat whereas stationary phase cells are more resistant. Higher incubation temperature enhances heat resistance due to formation of thicker cell membranes. Incubation at acidic and Ca^{+2}-rich environments improves heat resistance of spores. Acidic conditions enhances heat inactivation rate of microorganisms increasingly with higher pK_a values for the acid. On the other hand, it yet not fully understood whether heat inactivation of spores under acidic conditions is increased by the presence of undissociated organic acids. Reduced a_w is mostly known to be protective microorganisms against heat inactivation with maximal effect at a_w values between 0.2 – 0.4 for both spores and vegetative cells. On the contrary, a low a_w can hinder cell recovery following the heat treatment. Sugars are also more effective humectants than salt for giving protective effect (Smelt & Brul, 2014).

Table 1 Heat resistance of nonspore forming pathogens in foods with pH > 4.6 (Silva & Gibbs, 2010)

Bacteria	Substrate	*D* values (min)	*z* values (°C)	Temperature range (°C)
L. monocytogenes	Ground pork	47 – 0.085	5.9	55 - 70
	Chicken gravy	195 – 0.48	5.2 – 6.1	50 - 65
	Cooked lobster	97 – 1.1	5	51.6 – 62.7
	Rainbow trout roe	1.6 – 0.44	5.4	60 - 63
	Liquid egg yolk	1.3 – 0.58	6.1	60 – 62.2
	Liquid egg white	7.6 – 3.5	9.4	55.1 – 58.3
	Vacuum-packed minced beef	36 – 0.15	4.2	50 - 60
E. coli O157:H7	Ground pork	33 – 0.048	4.9	55 - 70
	Fully cooked frank	25 – 0.038	5.1	55 - 70
	Raw frank	21 – 0.031	-	55 - 70
	Ground beef	21 – 0.39	6	55 - 65
	Ground meat mix (lamb, chicken, turkey, pork)	12 – 0.29	6.5 – 6.8	55 - 65
	Ground morcilla sausage	5.5 – 0.6	7.4	54 - 62
	Ground meat mix (beef, pork sausage, chicken and turkey)	115 – 0.37	4.4 – 4.8	50 - 60
Salmonella spp.	Ground pork	46 – 0.083	5.9	55 - 70
	Green pea soup	10 - 1	5.7	60 – 71.1
	Thigh meat	12 – 3.2	6.9	55 – 62.5
	Chicken breast meat	6.1 – 0.66	8.1	55 – 62.5
	Liquid egg yolk	0.28 – 0.087	3.5 – 4.3	60 – 62.2
	Liquid egg white	8 - 1	-	55.1 – 58.3

Table 2 Heat resistance of spore forming pathogens in foods with pH > 4.6 (Silva & Gibbs, 2010)

Bacteria	Substrate	D-value (min)	z-value (°C)	Temperature (°C)
C. botulinum type II (non-proteolytic types B, E, F)	Crab meat	13 – 2.9	8.6	88.9 - 94.4
	Cod homogenate	59 – 0.79	8.6 – 8.3	75 - 90
	Turkey slurry	33 – 0.8	9.4	75 – 90
	Carrot homogenate	19 – 0.43	9.8	75 – 90
	Turkey slurry	52 – 1.2	9.9	70 - 85
	Whitefish paste	1.6 – 4.3	5.7 – 7.6	80
	Blue crab	13 – 0.49	7 – 8.4	73.9 - 85
	Oyster homogenate	9 – 0.08	4.2 – 7.1	73.9 – 82.2
C. perfringens	Ground turkey	23	-	99
B. cereus	Pork luncheon roll	31 – 1.9	8.6	85 - 95
	Distilled water	16 – 0.24	-	85 - 100

Heat injury and cell recovery

Sublethal injury can occur after exposure to a physical or chemical stress that will not kill but injure the microorganism (Hurst, 1977). Stress levels can vary from being minor to moderate and ultimately lethal. Minor stress level does not influence microbial growth rate due to full adaptation to the new situation. Low stress can lead to adaptive response coupled with a brief physiological shift ending with increased stress resistance. Moderate stress may cause a sequential injury that can be mild or severe leading to presence of both healthy and dead cells (Stephens et al., 1997). Lethal stress can ultimately cause the death of a part of bacterial population. Exposure to lethal stress by a subpopulation can lead to enhanced survival rate for overall population due to concomitant genetic responses and adaptive mutations (Wesche et al., 2009). Cell injury phenomenon poses a food safety risk, since food processes such as heating, refrigeration, freezing, drying, irradiation, use of preservatives, acidity, exposure to starvation and low a_w can result in presence of injured cells in foods. As a result, identification of sublethally injured microorganisms is important for the quality and safety of processed foods (Wu, 2008).

Metabolic and structural injury is distinguished by those cells becoming inapt to form visible colonies under selective conditions. While structural damages on cell wall or membrane

leakages can often occur, metabolic injury can also lead to reverse effects on functional components of the cell. For this purpose, growth/no growth on selective and nonselective media is compared for estimation of sublethal injury ratio. It should be noted that incubation temperature has a crucial impact on cellular repair processes. Surprisingly, incubation of injured cells at suboptimal growth temperatures may allow better recovery due to suppressing effect on cell division while allowing cells to concentrate on metabolic repair processes (Brashears, Amezquita & Stratton, 2001; Semanchek & Golden, 1998; Wesche et al., 2009).

Under favorable conditions, recovery of injured cells is expressed as 'resuscitation'. Repair process starts briefly after exposure to stress under optimum conditions. Initially, restoration of cellular functions happen before regular cell growth starts again. Important repair processes can be regeneration of ribosomes degraded during heating, synthesis of cell membrane, phospholipids, cell wall and essential proteins. Injured cells usually repair within 2 to 4 h if recovery conditions are convenient. Incubation temperature and non-selective medium are important parameters for recovery. Cells are often not equally exposed to cell injury. Consequently, lag times show variability among individual cells. Variation can increase when the initial injured cell concentration is low (Guillier, Pardon & Augustin, 2005; Wesche et al., 2009).

Heat shock responses play an important role in food processes since it can cause microorganisms to gain higher heat resistance. Heat shock occurs if microorganisms are exposed to temperatures above their normal tolerance level. As a result, a subpopulation group can be heat inactivated. Typically, thermal processes with relatively long come-up phases such as slow roasting of meat, may promote sublethal heat injury to microorganisms. Therefore, genetic and physiological heat shock responses in microorganisms can become activated. Heating rate is a critical factor as under slow heating heat resistance may increase (Pagan, Condon & Sala, 1997; Quintavalla & Campanini, 1991).

5.2.1.2 High pressure processing (HPP)

Despite the fact that an opportunity was identified with high pressure inactivation of undesirable microorganisms as early as 1899 (Hite, 1899), scientific studies and industrial application of HPP intensified during the past three decades. HPP is employed in food

industry with the purpose of extending the shelf-life of foods while producing fresh, preservative-free and minimally-processed foods (Considine, Kelly, Fitzgerald, Hill & Sleator, 2008; Rendueles et al., 2011).

The efficiency of HPP mainly relies on the pressure applied, holding time and temperature of the pressure chamber. Since the process is isostatic, pressure transmission is unaffected by the physical shape of the food. Furthermore, the process has an adiabatic heating effect thereby causing product temperature to increase by 3 to 9 °C per 100 MPa based on the food composition. Higher fat content gives higher heating rates and higher water content leads to an opposite effect. Upon the release of pressure (decompression), temperature of the food goes back to the initial value, unless no heat losses/gains happen during the holding stage. Therefore, heat transfer from the walls of the pressure chamber should ideally be controlled to prevent having non-isothermal conditions. An overview of pressure effects and applications is also shown (Table 3). In Fig. 3, an illustration of the batch type HPP machine used in the current is shown.

Table 3 Overview of pressure effects on biological structures (Follonier, Panke & Zinn, 2012)

Pressure (MPa)	**Cellular effects**	**Applications**
0.1 – 1		Biotechnological processes
30 – 80	Inhibition of RNA transcription	
50 – 90	Inhibition of chromosome replication	
70 –100	Inhibition of protein synthesis (reversible)	
100 – 200	Loss of membrane integrity	
100 – 300		Protein refolding
200 – 300	Dissociation of multimeric proteins	
350 – 450	Protein denaturation	
300 – 700		Production of vaccines
450 – 600		Disinfection of biomaterials
500 – 1000		Dissociation of amyloid fibrils
300 – 1000 (or above)	Food processing	

In summary, HPP has key advantages such as allowing (1) pasteurization at ambient temperature or chilled temperatures; (2) uniform pressure transfer to the food product with any size/geometry; (3) minimally processed foods without preservatives/additives; and (4)

production of foods with novel functional properties (i.e. through protein modifications) (Rastogi, Raghavarao, Balasubramaniam, Niranjan & Knorr, 2007).

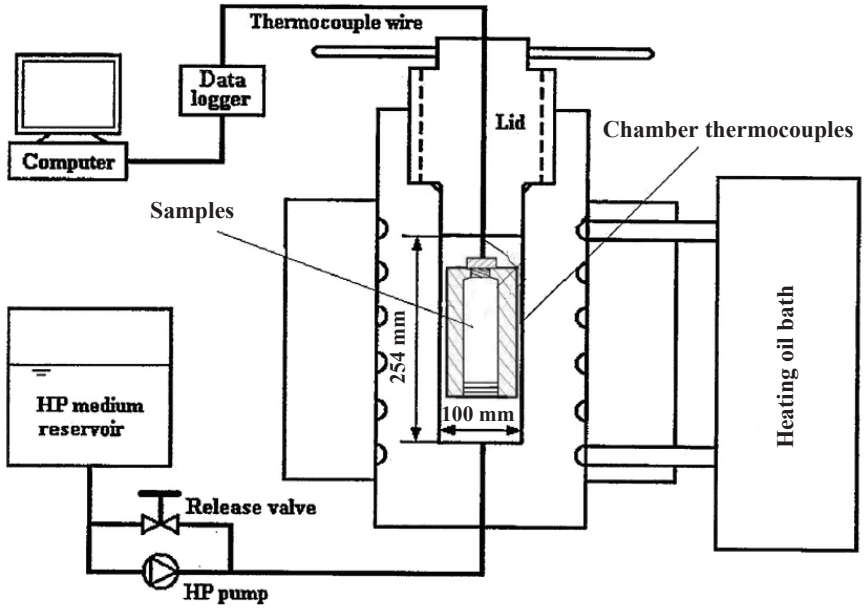

Fig. 3 Schematic diagram of the HPP machine used in this work (adopted from Ramaswamy, Shao, Bussey & Austin, 2013)

Effects of high pressure on biomolecules

Pascal defined pressure in 1648 which was then incorporated into scientific domains of physics, chemistry and geosciences before finding space in biology. Pressure is generated through heating a closed system or a mechanical volume reduction (Heinz & Buckow, 2010). Release of pressure always leads to a negative ΔV value. In other words, a decrease in volume is prompted by pressure, and vice-versa which is known as Le Chatelier principle. Furthermore, low energy levels requirement of HPP is believed to be the reason why covalent bonds of food components are almost unaffected (Rastogi et al., 2007). Therefore, basic research efforts in HPP focused on (1) the product volume changes (2) the impact of high pressure on weak chemical bonds. Proteins, other biomolecules and microbial inactivation studies were of particular attention (Rendueles et al., 2011).

High pressure does not change covalent bonds that are resistant against pressure ranges up to 1000 MPa (used in food industry). Eventually, primary food components such as peptides, lipids, vitamins, flavour compounds, and sugars remain unaffected (Considine et al., 2008). On the contrary, stabilization of hydrogen bonds and exposure of charged molecular groups towards water medium are enhanced by high pressure. Overall effect is the reduction in volume through better hydration. Since HPP affects weaker bonds (i.e. ionic, hydrophobic and hydrogen), structural changes may occur in secondary, tertiary and quaternary forms of proteins such as unfolding and volume contraction (Considine et al., 2008). Apart from that, large macromolecules such as starch may also be gelatinized (Rendueles et al., 2011).

Since pressure affects non-covalent bonds in molecular structures, various effects on foods are observed as a result of HPP. As previously mentioned, high pressure may alter proteins (especially tertiary structures). Water molecules may enter into the protein core as a result of weakened hydrophobic bonds under high pressure. These structural modifications in food proteins may lead to volume reduction, but also increase the digestibility or reduce the allergenicity (Demazeau & Rivalain, 2011). While protein denaturation is inevitable during HPP treatment of high-protein foods, resulting changes in organoleptic properties of such foods are less than those observed under thermal processing. Product shrinkage may also take place if air pockets are present in food products (Considine et al., 2008).

5.2.1.2.1 Microbial pressure resistance

High pressure apparently targets cell membranes resulting in leakages and molecular disruptions (Pagan & Mackey, 2000; Ritz, Tholozan, Federighi & Pilet, 2002). Cell death occurs when the accumulated injury imparted by high pressure extends beyond the capacity of microbial cells for self-repair. Due to differences in cell membrane structures, gram-positive bacteria are often more resistant against high pressure than gram-negative bacteria. Phospholipid bilayers (abundant in gram-negative cell membranes) are believed to be lost during compression/decompression cycles ultimately leading to pore formation and leakage of cellular material (Considine et al., 2008; Rendueles et al., 2011). Pressure can also affect membrane-bound enzymes such as the ATPase which takes role in acid-base physiology of the cell, thereby leading to pH sensitivity for the microbial cells (Hoover, Metrick, Papineau,

Farkas & Knorr, 1989). Above pressure ranges of 100-200 MPa, cell membrane collapse may occur (Follonier et al., 2012).

Morphological and physiological changes in cells may occur under high pressure. These are detachment of the cell membrane from the cell wall, elongation of the cell, compression of gas vacuoles, alterations in intracellular organelles (Manas & Mackey, 2004; Patterson, 2005). Other phenotypic effects of high pressure are suppression of cell division and loss of motility (Follonier et al., 2012). Additionally, ribosome, protein synthesis, cellular enzyme activity, DNA-enzyme complexes can be affected by pressure (Rendueles et al., 2011). However, nucleic acids (DNA and RNA) are even more stabilized by high pressure due to previously described effect of pressure on hydrogen bonds (Follonier et al., 2012).

Endospores are one of the most HPP resistant biological structures which can survive treatments above 1200 MPa. Therefore, a combination of temperature ranges from 90 to 121 °C and pressure ranges from 500 to 800 MPa are often used for inactivation of proteolytic *C. botulinum* spores (Rastogi et al., 2007; Smelt, 1998). It is hypothesized that spores are initially germinated by the applied pressure/temperature followed by the death of more pressure-sensitive germinated cells. At a pressure range of 50 – 300 MPa, nutrient-germinant receptors can be activated which later enhances the release of dipicolinic acid (DPA) and small acid-soluble spore proteins (SASPs). The later events are the hydrolysis of core and cortex, and the reduction of intracellular pH leading to a cascade of later germination events triggered by DPA (Ahn & Balasubramaniam, 2007). It is also possible to trigger rapid germination by direct release of Ca-DPA at higher pressure ranges above 500 MPa (Black, Setlow et al., 2007).

Bacterial inactivation by high pressure generally occurs in the range 200 – 600 MPa based on: (1) the pressure sensitivity (i.e. prokaryotic cells more resistant eukaryotes), (2) the growth phase (more pressure sensitivity during exponential growth phase), (3) food composition (presence of fat, proteins and carbohydrates), (4) presence of food additives, (5) combination of HPP with other treatments, (6) HPP settings. High pressure pasteurization (pascalization) process targets vegetative pathogens in acidic foods (pH < 4.6). Generally 5-6 log inactivation of target pathogen is desirable. While *L. monocytogenes* is the target organism for dairy and meat products, *Salmonella* is targeted in eggs and *E. coli* needs to be eliminated in fruit- and vegetable-based products (Demazeau & Rivalain, 2011). Inactivation of various food

pathogens and viruses in different substrates exposed to different pressure-time-temperature treatments with HPP is shown in Table 4.

Table 4 Inactivation of selected food pathogens and viruses by HPP (Rendueles et al., 2011)

Bacteria	Substrate	Pressure (MPa)	Time (min)	Temperature (°C)	Inactivation level (log)
L. monocytogenes	Broth	345	10	35	5
L. monocytogenes CA	Poultry meat	375	15	20	2
S. enteridis	Broth	345	10	35	8
S. senftenberg 775W	Baby food	340	10	23	< 2
E. coli O157:H7	Broth	345	10	35	8
E. coli O157:H7	Poultry meat	600	15	20	3
Campylobacter jejuni	Pork slurry	300	10	25	6
Vibrio parahaemolyticus O3:K6	Oysters	300	3	10	5
Staphylococcus aureus	Poultry meat	600	15	20	3
S. aureus 765	Broth	345	10	35	4
Hepatit A virus	Oysters	400	1	10	> 3
Norovirus	Oysters	400	5	5	4

High pressure sterilization aims to inactivate spores in foods with a pH value higher than 4.6. Since food sterilization often demands process conditions that are not “gentle”, maintaining organoleptic properties of foods treated with high pressure sterilization has been an important area of research. Three different strategies have been developed to kill spores which are: (1) direct destruction of spores, i.e. via combination of very high pressure and high temperature, (2) induction of the spore germination through manipulation of pressure and temperature followed by inactivation of resulting vegetative cells and (3) causing injuries in spores with high pressure with resulting spores becoming unable to germinate, when the food product has a low pH or a_w or intended to be stored under refrigerated temperature (Demazeau & Rivalain, 2011; Heinz & Buckow, 2010).

Target pathogenic spores in foods treated with HPP are *C. botulinum*, *C. perfringens* and *B. cereus*. Non-pathogenic surrogates such as *C. sporogenes* can be used in studies for research purposes (Ahn, Balasubramaniam & Yousef, 2007). Selected data with various foodborne pathogenic spores exposed to different pressure-time-temperature treatments with HPP is shown in Table 5.

Table 5 Inactivation of foodborne pathogenic spores by HPP (Rendueles et al., 2011)

Bacteria	Pressure (MPa)	Time (min)	Temperature (°C)	Inactivation (log reduction)
Clostridium botulinum proteolytic type A	600	6	80	5
C. botulinum proteolytic type B	600	70	80	5
C. botulinum proteolytic type B	800	4	80	2.3
C. botulinum nonproteolytic type B	827	20	75	> 6
C. botulinum type A	827	20	75	2-3
C. botulinum type E	827	5	55	~5
Clostridium perfringens	500	30	up to 65	0
C. perfringens type A	650	15	75	3.7
Bacillus cereus	400	25	30	0.5

Factors that affect pressure resistance

Cell membranes of barophiles and barotolerants have a lower degree of saturation in fatty acid (FA) composition of their membranes. Psychrophilic microorganisms possess high levels of polyunsaturated FA in their membranes and are usually more pressure-resistant. This is due to the increased fluidity in cell membranes being partially responsible for high pressure resistance (Smelt, 1998).

Research studies with model systems revealed that physiological condition of microorganisms exposed to HPP has an impact on pressure resistance. Increased pressure susceptibility during logarithmic growth phase, synthesis of stress response proteins, cell membrane composition, prior heat shock and cold shocks increases the resistance to HPP (Hayman, Anantheswaran & Knabel, 2008). In foods, microbial populations are diverse and in very different physiological states. On the other hand, sublethally injured cells (i.e. by a thermal treatment) are more susceptible to pressure (Rendueles et al., 2011).

Temperature can affect microbial survival after HPP treatment, with increased inactivation taking place below ambient temperatures (Alpas, Kalchayanand, Bozoglu & Ray, 2000; Bayindirli, Alpas, Bozoglu & Hizal, 2006). Lower pressure-resistance at temperatures below 20 °C is hypothesized to be due to modified membrane structure and fluidity as a result of weaker hydrophobic interactions as well as crystallization of phospholipids (Cheftel, 1995).

Furthermore, mild heating at 40 – 60 °C may also trigger enhanced microbial inactivation by pressure thereby providing an opportunity to operate at lower pressure levels.

Pressure-sensitivity is dramatically higher in either acidic or basic conditions (Alpas et al., 2000). Intracellular pH of microbial cells can temporarily decreases during pressurization. A pH reduction by 0.2 units per 100 MPa increase in pressure was observed in apple juice (Rastogi et al., 2007). Also, dissociation of organic acids is promoted under high pressure which can decrease their antimicrobial efficacy since the non-dissociated form has an antimicrobial effect (Rendueles et al., 2011). Nevertheless, upon decompression, pH value revert to its original but the impact of instant pH change on microbial survival during HPP treatment is unknown (Considine et al., 2008).

High pressure resistance can be possessed for more than 80 generations shown with some of *E. coli* strains (Vanlint, Rutten, Michiels & Aertsen, 2012). Various pressure resistant strains were obtained after HPP treatments. *E. coli* became resistant to pressures up to 800 MPa after cycles of exposure to mild pressure levels of 280 to 450 MPa (Hauben et al., 1997). Strains of *L. monocytogenes* that can persist 400 MPa for 20 min have also been shown (Karatzas & Bennik, 2002). The activation of genes such as those responsible for RpoS protein in *E. coli* and SigB in *L. monocytogenes* is proposed to play a role in pressure-resistance (Malone, Chung & Yousef, 2006).

Lastly, lower a_w values enhanced microbial resistance against HPP with model food studies. The effect is more visible when a_w is below 0.9. The same phenomenon also applies to spores as germination is restricted at lower a_w values (Hayman et al., 2008).

Pressure injury and cell recovery

HPP prolongs the shelf-life of food products. However, since HPP treated products are often nonsterile, refrigeration is used for keeping the product stable. Also, microorganisms subjected to HPP can get injuries, but may resuscitate if conditions are suitable for their growth following the treatment (Bozoglu, Alpas & Kaletunc, 2004). Psychrotrophic pathogens (such *L. monocytogenes*) that resist the treatment pose a significant health hazard. If provided with nutrient-rich food medium as well as high storage temperature and enough

time, sublethally injured cells can repair themselves following the HPP treatment and outgrow (Bozoglu et al., 2004). Shelf-life studies and challenge tests with HPP treated products should be assessed for the presence risk of sublethally injured pathogens. For this purpose, microbiological analysis procedure should not only include selective culture medium but also non-selective medium providing rich nutrients as well as sufficient incubation time and temperature to allow repair of damaged cells (Ritz, Pilet, Jugiau, Rama & Federighi, 2006). In real foods, presence of nutrients such as essential vitamins and amino acids permits sublethally damaged cells to repair themselves after processing more quickly (Black, Huppertz, Fitzgerald & Kelly, 2007).

5.2.1.2.2 Combined high pressure-mild temperature effects

High pressure and temperature often work synergistically for the inactivation of vegetative microorganisms and spores (Serment-Moreno, Barbosa-Canovas, Torres & Welti-Chanes, 2014). In case of vegetative microorganisms apparently highest resistance against inactivation is seen between 20 to 40 °C, whereas the stability is decreased at temperatures below 20 °C (Considine et al., 2008; Heinz & Buckow, 2010). Pressure resistance variability among food pathogens was highly reduced when temperature was increased from 25 to 50 °C during pressurization (Alpas et al., 1999). Combining high pressure and temperature is more vital regarding inactivation of spores (Wilson, Dabrowski, Stringer, Moezelaar & Brocklehurst, 2008). On the other hand, application of food sterilization by high pressure and temperature needs further knowledge on pressure-resistant pathogenic spores. Pressure resistance of spores is not often related to temperature resistance (Wilson et al., 2008).

Development of combined high pressure-temperature (*P*/*T*) processes can minimize the use of high temperatures that have been traditionally utilized in thermal processing (Huang, Lung, Yang & Wang 2014). In addition, reductions in pressure intensity and holding time can be achieved without comprising on food safety. This gives an economical advantage for a more widespread applicability of HPP (Scolari, Zacconi, Busconi & Lambri, 2015). Bacterial pressure resistance is often higher in foods compared to buffered suspensions or culture media (Patterson, 2005). Hence, further research is needed for investigating the effect of more complex food systems (Gao, Ju & Jiang, 2006).

Inactivation mechanisms for vegetative cells under high pressure and temperature have been related to structural changes in essential proteins/enzymes, cell membranes, ribosomes as well as intracellular pH drop. Physiological changes in bacteria and interactions between cells and food components under different *P*/*T* profiles need to be elucidated with respect to cell death and recovery (Georget et al., 2015). According to Hawley's theory, protein denaturation/unfolding follows an elliptical shape under various *P*/*T* combinations based on thermodynamic experiments (Hawley, 1971). Main limitation with this theory was the lack of any structural knowledge about the system (Smeller, 2002). Infrared and fluorescence spectroscopy studies revealed that *P*/*T* stability of studied model proteins were mainly affected by calcium ions, hydration and pH. Under *P*/*T* treatments, protein states were also shown to include partially unfolded, unfolded, molten globule and aggregated forms (Somkuti, Bublin, Breiteneder, & Smeller, 2012). Since this topic was beyond the scope of this study, readers are referred to related reviews (Marchal et al., 2009; Meersman, Smeller & Heremans, 2006).

A similarity was observed in elliptical shape of *Escherichia coli* inactivation diagram with those of model proteins under different *P*/*T* profiles (Smeller, 2002). The same relationship was not found for melting curves of membranes or nucleic acids, as they were both linear with respect to *P*/*T* profiles. Hence, it was concluded that specific proteins or enzymes may play a critical role in bacterial inactivation (Ludwig, Scigalla, & Sojka, 1996). There was a striking similarity between stability diagrams for Ribonuclease A at pH 2.0 and *L. monocytogenes* in smoothies at pH 3.5 (Fig. 4). This may be further evidence that high pressure and temperature may cause cell inactivation mainly through targeting essential proteins which requires further investigations.

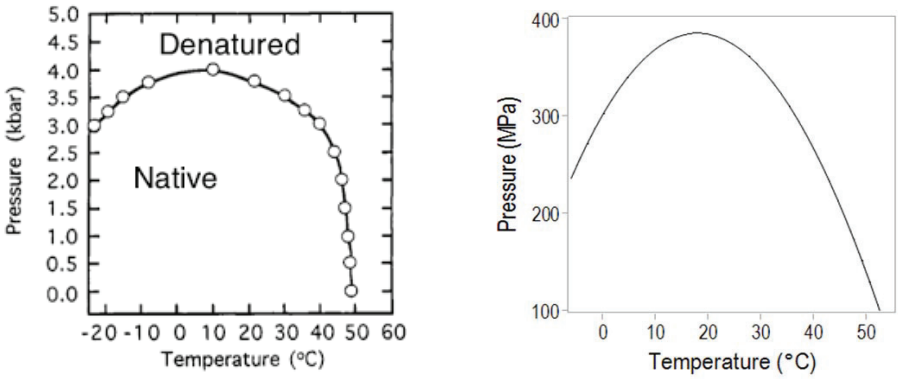

Fig. 4 Isorate diagrams for protein ribonuclease A denaturation (pH 2.0) (left) and 2-fold *L. monocytogenes* inactivation (pH 3.5) (right) (adopted from Scolari et al., 2015; Zhang, Peng, Jonas & Jonas, 1995)

Sizeable reductions (more than 8 logs) were shown with food pathogens at 300 MPa when combined with 50 °C heating during 5 min treatment (Alpas et al., 1999). However, differences were observed among pathogenic microorganisms (*L. monocytogenes*, *S. aureus*, *E. coli*, *S. typhimurium*) with reductions in the range of 0.5 to 8.5 log units. Furthermore, different strains of the same pathogen were also shown to have different pressure sensitivity (Alpas et al., 1999). Pressure-temperature combinations required for 5 log inactivation of selected foodborne vegetative pathogens and spores after 5 min isobaric/isothermal treatment are shown in Fig. 5.

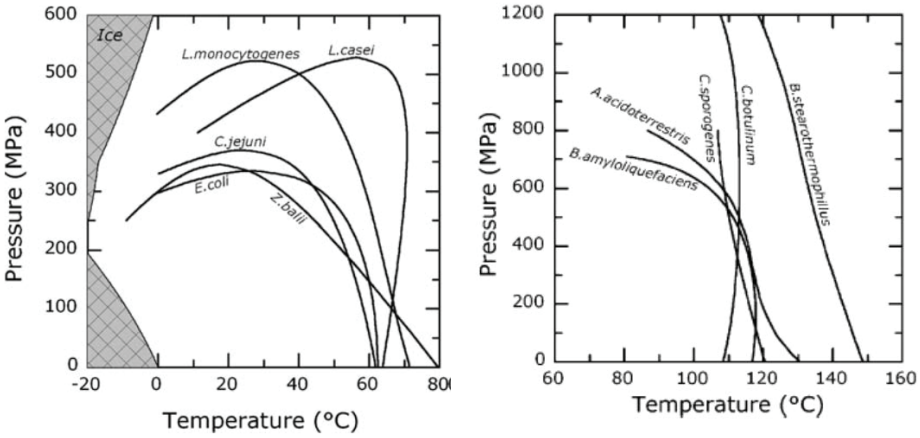

Fig. 5 Pressure-temperature isorate diagrams for 5 log inactivation of selected vegetative bacteria and spores after 5 min treatment (Heinz & Buckow, 2010)

5.2.2 Pathogens of concern for mild technologies

Microorganisms associated with foodborne ilnesses are *Aeromonas hydrophila*, *B. cereus*, *B. subtilis, Campylobacter jejuni*, *C. botulinum*, *C. perfringens*, pathogenic *E. coli*, *L. monocytogenes*, *Salmonella* serovars, *Shigella* spp, *S. aureus*, *Vibrio* spp. and *Yersinia enterocolitica*, Hepatitis A, *Cryptosporidium* and *Cyclospora* (FDA, 2000).

Since the coleslaw outbreak in 1981, *L. monocytogenes* has been identified as a ubiquitous organism responsible for very high hospitalization (90 %) and mortality (20-30 %) rates among other foodborne pathogens (Melo, Andrew, & Faleiro, 2015). According to Codex Alimentarius guidelines, *L. monocytogenes* growth in RTE foods can be controlled with pH ≤ 4.0, water activity ≤ 0.92, prevention of contamination after heat treatment, presence of antimicrobials and strict maintenance of cold chain. Under these conditions, *Listeria* presence is limited to 100 CFU/g at the end of shelf life. However, if favorable conditions exist in RTE products for *L. monocytogenes* growth, *Listeria* presence must be limited to < 0.04 CFU/g (or absence in 25 g product) (Luber, 2011).

B. cereus toxins can be produced when cell concentrations reach hazardous levels (IV). To control the growth of *B. cereus*, foods need to be stored at < 4 °C for less than a week. For longer durations, chilled products must be stored ≤ 3 °C to prevent nonproteolytic *C. botulinum* type E spores from producing toxins. Considering highly resistant strain types, heat treatments equivalent to 70 °C / 2 min, 90 °C / 10 min and 100 °C / 48 min are required for 6*D* reduction of *L. monocytogenes*, *C. botulinum* type E spores and *B. cereus* spores, respectively (Rosnes et al., 2011).

6 MAIN RESULTS AND DISCUSSION

Experiments performed for investigation of novel agitating retort and combined high pressure-temperature (*P*/*T*) effects on microbial inactivation are briefly summarized in Fig. 6. The whole work consists of four papers (I-IV). The same model soup (pH 6.1) was used for all experiments as the bacterial suspending medium. Process temperature, pressure intensity, heating/pressure holding time, type of microorganism (vegetative cell/spore) and heating mode (agitated/static) were used as experimental variables for assessment of microbial inactivation (I, III, IV) and injury (I, III). Microbial recovery after processing was assessed using storage temperature (II, III) and inoculum level (III) as additional factors. Methods used for prediction/modelling of microbial inactivation was based on classical thermal death model (I, IV) and response surface methodology (RSM) (III).

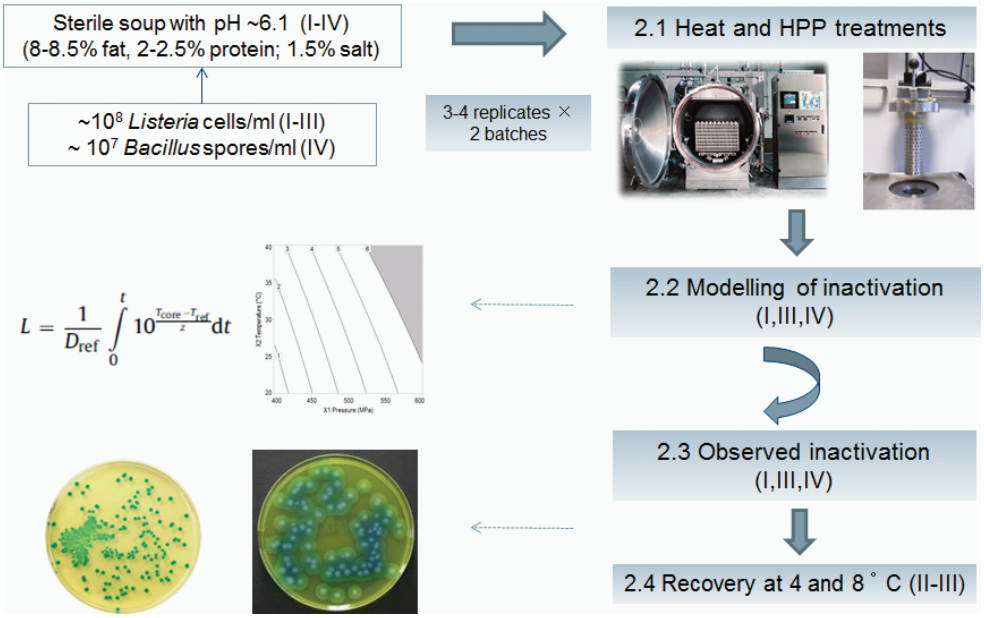

Fig. 6 Experimental outline of the thesis

6.1 Effects of novel heat and high pressure processing on *Listeria* inactivation (I, III)

The main purpose of this chapter (7.1) was to identify optimal process variables for achieving > 6 log reduction of *Listeria* in the model soup in compliance with (FDA, 2008) guidance on RTE foods. The secondary aim was to model/predict microbial inactivation using log-linear (I) and RSM models (III). The model soup inoculated with *L. innocua* (I) and *L. monocytogenes* (III) to a level of 10^8 CFU/ml was used for all experiments in this chapter. Samples were exposed to heat treatments in agitating/static mode (I) and combined *P*/*T* treatments (III). Process intensity is the critical factor for the overall thermal load on the product. Hence, various time-temperature (I) and *P*/*T* combinations were tested for *Listeria* inactivation. Investigation of these parameters can help the food industry to determine test conditions for safety studies in similar products as well as to identify possibilities for achieving a milder process.

Heating in agitating mode showed a remarkable reduction in processing time required for thorough inactivation of *Listeria* inoculated into 350 g soup (in trays). At retort temperatures of 62, 65 and 68 °C, no colonies were detected after 13.5, 8.8 and 7.5 min processing in agitating mode compared to 95, 85 and 70 min in static mode (based on 2 and 18 min cooling times). Model organism *L. innocua* and the soup were used as means for comparison of two retort processes under same conditions. Using a test product with different viscosity, volume, package, or target organism would alter the results. Minimally heated foods need to be exposed to a heat load equivalent to 70 °C for 2 min at the coldest point to ensure a 6 log kill effect on *L. monocytogenes* (FAO/WHO, 2004) based on unusually high heat resistance of *L. monocytogenes* on challenging food matrices ($D_{60°C}$ range: 1.6 to 16.7 min) (Beuchat et al., 2013; Mackey & Bratchell, 1989). Nevertheless, these results from Paper I clearly imply that novel agitating retort dramatically reduces processing time required for *Listeria* inactivation without compromising on food safety concerning liquid or semi-liquid products without solid particles.

In agitated process, integration of isothermal heat resistance parameters for *Listeria* into dynamic conditions gave accurate predictions for microbial inactivation (Fig. 7). For this purpose, *F*-value concept was used where $D_{60°C}$ and *z* values were chosen as 2.40 min and

4.71 °C (I). Accuracy (A_f) and bias (B_f) factors introduced by (Ross, 1996) were used as prediction accuracy indexes. Paper III can be referred to for descriptions on A_f and B_f. Comparison of predicted and observed lethality with agitating treatments gave A_f = 1.42 and B_f = 0.81 (n = 120) (calculated from Paper I). These values were in the acceptable ranges, as A_f was below 1.60 and B_f was in the range of 0.75 to 1.25 (Dalgaard, 2000; Ross, 1996). As shown in Fig. 7, regression analysis of predicted and mean observed inactivation values for *Listeria* gave satisfactory correlation (R^2_{adj} = 0.94; P < 0.0001; n = 20). Based on the results, adding a safety margin of at least 2 log units on predicted values would be a valid approach. This is based on the variation commonly observed with respect to microbial detection and processing.

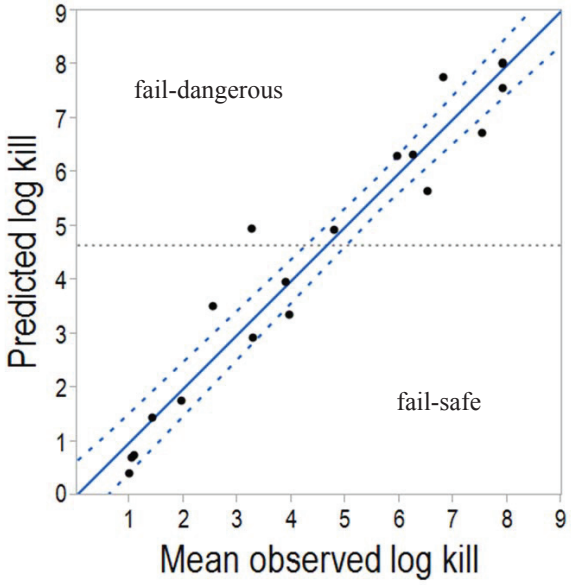

Fig. 7 Regression analysis of predicted and observed mean log inactivation after agitated heating (α = 0.05 for blue dotted lines; gray line: mean of response)

Agitating treatments provided relatively high heating rates at the coldest point (5.3 to 9.5 °C/min) (II). Most likely, this was the reason for obtaining similar estimated and observed lethality values with *Listeria* using the log-linear model (I). Earlier studies with *Listeria* showed that nonisothermal inactivation can be predicted accurately from isothermal log-linear kinetics. This was possible when the heating rates were more than 5 °C/min. In contrast, heating rates lower than 5.0 °C/min were shown to cause overestimated predictions due to heat shock effect (Hassani, Manas, Raso, Condon, & Pagan, 2005; Stephens, Cole, & Jones,

1994). With heating rates less than 5.0 °C/min, the magnitude of heat shock on *Listeria* was also enhanced at higher pH (5.5 and 7.4) (Hassani, Condon, & Pagan, 2007; Jorgensen, Hansen, & Knochel, 1999). Relatively high pH (6.1) of the soup and the slow heating rates during static heating (≤ 1.1 °C/min) possibly increased the heat-tolerance of *Listeria*. This could be the reason for almost 10-fold increase in processing times required for the same level of *Listeria* inactivation, when heating was in static mode (I).

An orthogonal central composite design (CCD) with two variables (pressure and initial temperature) was used for studying the effects of combined *P*/*T* treatments on inactivation of *Listeria* inoculated into 40 ml soup (III). Agitated heating experiments provided a homogenous distribution of heat which enabled accurate estimation of process lethality. However, prediction of lethality during a combined process that would be expected to give a synergistic effect may require a polynomial model. Furthermore, RSM can include nonlinearities often studied with Gompertz, Weibull and log-logistic type models (III). Therefore, RSM was selected for modeling of *Listeria* inactivation in the same soup used in heating experiments. Results showed that higher temperatures (≥ 30 °C) can enhance bacterial inactivation by 1.5 to 2 log units under pressurization at 400 MPa or above (based on the raw data). This result was in agreement with earlier studies with microscopy and flow cytometry that showed cellular structures of gram-positive bacteria can withstand pressure levels up to 400 MPa (Ananta, Heinz, & Knorr, 2004; Ritz, Tholozan, Federighi, & Pilet, 2001). Consequently, a contour plot was obtained from the regression model showing *P*/*T* combinations that would give a 1 to 6 log inactivation range for *Listeria* (III).

The deduced model in the soup (pH 6.1) and the one obtained by (Gao et al., 2006) in buffered milk (pH 7.0) showed comparable results for *Listeria* inactivation (III). Compatibility of two models could be due to presence of milk-based ingredients (36.1 % whole milk and 12 % cream) in the soup. Predicted values from each model were also compared and A_f =1.45 and B_f = 0.79 were found to be within the acceptable ranges. Based on the results, apparently cell death occurs at a higher rate under suboptimal temperatures for microbial growth in accordance with earlier studies (III). If this phenomenon is validated with further structural studies, food industry can largely benefit from it through operating at milder process conditions (lower pressure levels and holding times). This is valuable for reduction of processing costs and improving product quality (III).

Findings in this chapter can be beneficial for food processors for evaluating the possibilities with three processes presented. If product quality requirements outweigh processing costs, combined *P*/*T* treatments seems to be the most attractive option. The deduced RSM model showed that a range of *P*/*T* combinations (such as 525 MPa / 40°C and 600 MPa / 25 °C) could give 6 log kill effect on *Listeria* in 5 min (III). This can provide wide a processing window for optimization of product quality. If higher temperatures (> 60 °C) does not affect the quality negatively, the new agitating process would be very effective for increased throughputs and energy savings. However, a number of products (with solid particles) might not be able to preserve its intact form after agitation. In case of using lower agitation frequencies (< 80 strokes per min) for such products, new safety studies need to be performed. Especially, when heating rates are below 5 °C/min, log-linear inactivation kinetics may no longer be able to apply. Lastly, static processing is the most suitable option for products with solid particles that need to remain intact. Clearly, sensory studies are needed for a complete assessment of the technologies studied with respect to food safety in this work.

6.2 Effects of novel heat and high pressure processing on *Listeria* injury and recovery (II, III)

It is well-known that pressure or temperature may sublethally stress bacteria (Metrick, Hoover, & Farkas, 1989). Psychrotrophic organisms such as *Listeria* pose a challenge in mildly treated RTE foods with pH > 4.6 (FDA, 2000). Even when the product is frozen, *Listeria* can survive in the product and resume growth when the product is thawed. Furthermore, when studying novel processing effects on microbial inactivation in foods, selection of recovery media that permits detection of both injured and noninjured cells is important (FDA, 2000) These conditions apply to the product and processes studied (I-III). Hence, the aim of this chapter (7.2) was to study *Listeria* injury following heat/pressure treatments and the subsequent recovery during cold storage.

Sublethal injury was detected at some of agitating and static heat treatments targeted to leave survivors. In those observations, only 0.01 to 0.001 % of the survivors managed to avoid injury (I). Injury takes place when cells no longer persist beyond a stressor level. Cells lose cultivability under more severe stress which results in cell death (Smelt & Brul, 2014). Subsequent cell repair is largely affected by the acidity, as low pH can suppress the recovery

of damaged cell proteins (II). Main objective of Paper II was to assess survival and recovery of *Listeria* in the soup after exposure to mild temperatures relevant for "sous vide" production. The heat treatments were performed with agitation and static modes, and the samples were stored for three weeks at 4 and 8 °C. Overall results indicated that regardless of initial number of survivors (after heating), cells were predominantly in the lag phase during three weeks at 4 °C after exposure to agitated heating. However, static heating left a survivor population that showed a higher variability among samples than agitated heating did. In the case of very mild treatments in static mode, cells apparently switched to growth phase within 3 weeks at 4 °C. Storage at 8 °C did not show significant differences for *Listeria* recovery, as cells were mostly recovered and they showed further growth at 8 °C (II).

The results from Paper II could be relevant for a product intended to be frozen or with a short shelf-life (< 7 days) at < 3.0 °C. Otherwise, there is a risk of toxin formation from the spores of nonproteolytic *C. botulinum* type E. Since *Listeria* may survive freezing and regrow after thawing, carrying out a storage test would be beneficial. Experimental conditions in Paper II were selected to study *Listeria* recovery under favorable conditions. Besides heat stress level and storage temperature, microflora, food composition and temperature history of a food product are critical factors for *Listeria* survival (I). Eliminating the microflora by using a sterile fish soup exposed *Listeria* to reduced microbial competition for the nutrients in soup. Furthermore, presence of lactic acid bacteria (i.e. bacteriocins), could suppress *Listeria* survival during storage (Arques, Rodriguez, Langa, Landete, & Medina, 2015). Salt and pH levels in the soup were not also expected to create a harsh environment for *Listeria* growth.

For studying HPP effects on cell injury, surviving colonies after treatments chosen for CCD (III) were also counted in selective medium that restrains growth of injured *Listeria*. The objective was to determine sublethal injury ratio with respect to different *P*/*T* combinations. Most treatments resulted in a range of 72.5 to 99.5 % injured cell population. Lowest sublethal injury rate was observed with 600 MPa / 40 °C treatment. This indicated that using elevated temperatures during pressurization were effective in suppressing survival of injured *Listeria* population. On the other hand, it was previously shown that even though initially inactivated, *Listeria* can regrow in milk, buffer and broth media within hours to weeks (Bozoglu et al., 2004; Bull, Hayman, Stewart, Szabo & Knabel, 2005; Jofre, Aymerich, Bover-Cid & Garriga, 2010; Koseki, Mizuno & Yamamoto, 2008).

The recovery problem during storage after HPP poses a significant food safety risk (Koseki et al., 2008). This can be very problematic for *Listeria*, since it can survive in frozen conditions and grow at temperatures as low as -1.5 °C. In this frame, *Listeria* recovery at 4 °C (normal condition) and 8 °C (abuse) following combined *P*/*T* treatments (III) was investigated. Results (III) indicated that 600 MPa treatments at 45 and 50 °C could be effective in controlling *Listeria* recovery in the model soup at 4 and 8 °C during three weeks. This was possible when samples were initially inoculated with 10^3 and 10^5 cells/ml. (Koseki et al., 2008) showed that heat treatments ranging from 37 °C / 240 min to 50 °C / 10 min following a 5 min HPP treatment at 550 MPa was able to prevent *Listeria* recovery in milk during 70 days at 25 °C. Results demonstrated that combining *P*/*T* treatments is an option for suppressing *Listeria* recovery in the model soup without an additional thermal process. According to Hawley's theory, proteins often undergo irreversible changes under pressurization above 45 °C (Hawley, 1971). Based on the empirical results, repair of critical proteins apparently plays an essential role in bacterial recovery at milder temperatures (< 45 °C). This phenomenon requires further understanding through structural studies. Nevertheless, an opportunity was shown for controlling *Listeria* recovery in soup when pressurization (600 MPa) was assisted with mild temperatures above 45 °C.

Similar to heat, pressure recovery is affected by various factors listed in Paper III. All these factors were considered for design of experiments in this work. *Listeria* recovery after HPP apparently could be included in the inactivation model as a parameter with further studies. Although cells were initially fully inactivated, there was subsequent recovery after some treatments (III). This phenomenon could be related to cellular repair mechanisms when one or more critical protein is reversibly denatured after combined *P*/*T* processing.

6.3 Effects of novel heat and high pressure processing on *Bacillus* spores (IV)

As a final step, model soup inoculated with *B. subtilis* spores was treated with static, agitating retort and combined *P*/*T* treatments. The objective of this chapter (7.3) was to compare spore inactivation efficacy of these processes in a similar way to *Listeria* (I and III). The mildly treated soup in Paper I would have to be stored below 3 °C for a very limited time or frozen. Otherwise, nonproteolytic *C. botulinum* type E spores could outgrow and produce toxins,

Type E spores pose a risk to mildly treated RTE foods (Lindstrom et al., 2003). Hence, it would be critical to study the effects of novel treatments (IV) on spore inactivation. Various RTE soups and ingredients (such as milk, flour) have been associated with *Bacillus* outbreaks (Evelyn & Silva, 2015). Some *B. cereus* spores from outbreaks showed very high heat resistance ($D_{100°C}$ = 27 min) (EFSA, 2005). Hence, *B. subtilis* spores may not be suitable as a surrogate organism for *B. cereus*. However, *Bacillus* spp. spores were generally reported to have a higher heat tolerance than type E spores (Silva & Gibbs, 2010).

Processing times required with agitating and static heat treatments for through inactivation of spores in the soup were 12 and 53 min at 110 °C (based on 3 and 18 min cooling times), respectively. Retort processing times included come-up, holding and cooling phases. $D_{95°C}$ and z values were found 4.67 min and 8.65 °C for *Bacillus* spores in soup. These values successfully predicted the inactivation of spores with agitating retort treatments. The same effect was also observed in Paper I. Therefore, there is strong evidence that the new agitation mechanism during heating provided a homogenous heat load distribution within the soup at selected agitation frequencies (I, IV). In practice, this finding can be very useful for thermal processing calculations. There are various challenges with long come-up times and temperature irregularities in static retorts. This often results in significant overprocessing for obtaining safety margins. After further validation studies in liquid or semi-liquid products, the new agitating retort can offer a great opportunity with process optimization. This could result in improved food quality and nutrition as well as lower energy usage.

Food composition and sporulation conditions can influence spore inactivation kinetics. Relatively low amounts of acids (pH 6.1) and salt (1.5 %) but high level of fat (8 – 8.5 %) in the soup may potentially provide favorable conditions for survival of *B. subtilis* spores during heating (Esteban, Huertas, Fernandez, & Palop, 2013). For a comparison with other scientific studies, a well-established protocol was used as the sporulation method (Nicholson & Setlow, 1990). For similar investigations in food industry, mimicking the food structure (a_w, pH, mineral ions) and process conditions (such as temperature, oxygen availability) would be the ideal approach for preparation of target spores.

Assisting HPP with moderate temperatures showed a synergistic effect for inactivation of *B. subtilis* spores in soup. After 10 min pressurization at 650 MPa / 20 °C, less than 0.5 log spore reduction was observed. When initial temperatures from 55 to 65 °C were combined with

HPP, this resulted in up to 4.5 log spore reduction in the soup. Depending on determined initial spore contamination levels, processors can utilize combined *P*/*T* processing for product quality improvements. For a higher log-kill effect, antimicrobials and elevated pH could also be used for this purpose. If the product of interest is more suitable for processing at higher temperatures (> 95 °C), agitating retort process would be a better option.

Using a sterile soup eliminated competitive flora as well as heat-resistant spores that could be introduced especially with dry ingredients (Beuchat et al., 2013). In addition, products with higher fat content may provide protection to spores under heat and pressure (Georget et al., 2015). Such conditions would need to be taken into account in future safety studies. Survival of nonproteolytic *C. botulinum* type E spores should also be investigated with combined *P*/*T*, since limited literature data is available in this respect. (Skinner et al., 2014) observed that a 600 MPa / 80 °C treatment gave full inactivation (6.7 log) for all type E spores in ACES buffer (pH 7.0) in 5 min. However, authors did not study temperature effects < 80 °C. Treatments used in this study would be expected to provide a high kill effect on type E spores which should nevertheless be investigated.

A representative graph showing pressure medium temperature change during pressurization at 650 MPa combined with an initial temperature of 60 °C is depicted in Fig. 8. The graph is shown as a reference for the *P*/*T* combinations investigated in Paper III and IV.

For a visualization of combined *P*/*T* effects on *B. subtilis* spores, Fig. 9 shows transmission electron microscopy (TEM) images of spores exposed to 400 MPa for 30 min at 25 to 55 °C. Apparently, combining high pressure with increasing temperatures resulted in ruptures at spore surfaces leading to partial leakage of intracellular compounds.

Results evidently indicated that utilizing novel mechanisms in food processing enabled new possibilities for milder food processing.

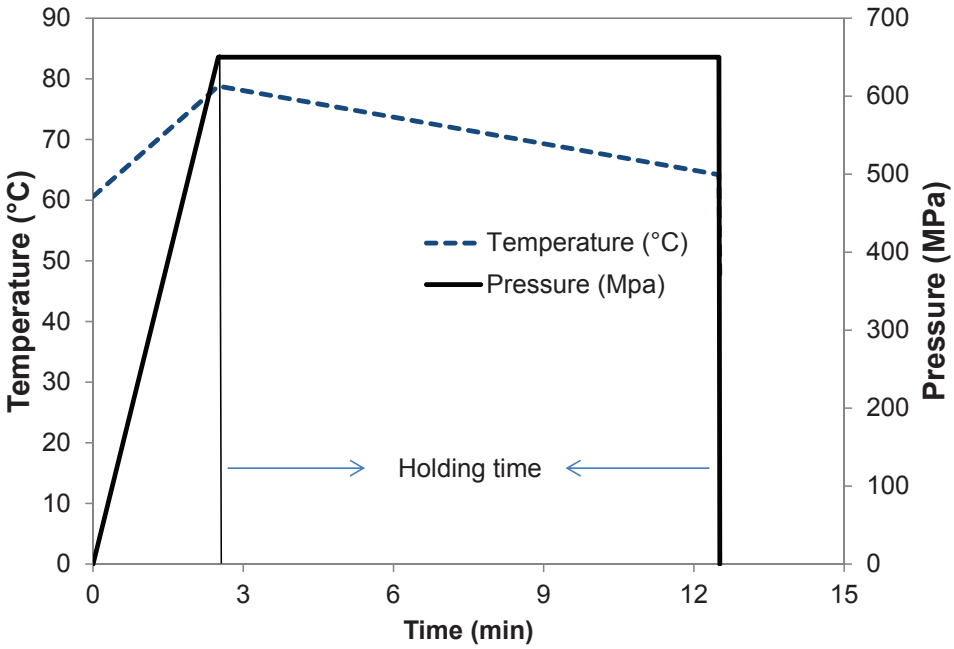

Fig. 8 Representative graph showing *P*/*T* profile during 650 MPa/60 °C treatment for 10 min

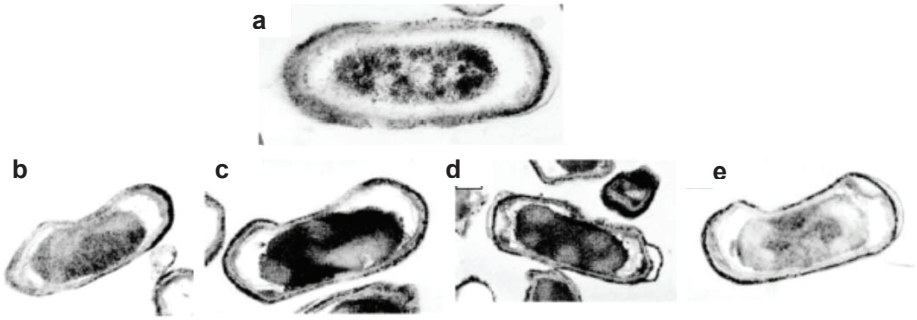

Fig. 9 TEM images of *B. subtilis* spores pressurized at 400 MPa for 30 min: (a) untreated spores; HPP combined with (b) 25 °C, (c) 35 °C, (d) 45 °C, (e) 55 °C (adopted from Huang et al., 2014)

7 CONCLUSIONS AND FURTHER RESEARCH

This PhD project investigated heat and high pressure processing effects on microbial survival when assisted with high frequency agitation and mild heat, respectively. The effects were compared using a model soup inoculated with *Listeria* cells and *Bacillus* spores. Overall results indicated that the novel mechanisms examined in this study could offer an opportunity for achieving milder processing compared to traditional static heat processing. Results from this thesis can be beneficial for designing validation studies on similar products and optimization of retort and high pressure processing.

Results drawn from investigations with the new agitating retort are:

- High frequency agitation mode could enable 86 – 89 % processing time reductions at 62 to 68 °C compared to static heating for *Listeria* inactivation.
- Agitating mode at 80 to 100 strokes per min (spm) could provide a homogenous heat load distribution and high heating rates (5.3 to 9.5 °C/min) in the soup.
- Inactivation of *Listeria* could be estimated with good precision from time-temperature profiles during agitating process using isothermal log-linear kinetics ($R^2_{adj} = 0.94$).
- Higher heating rates with agitated processing could extend lag times of *Listeria* survivors at 4 °C for storage periods less than three weeks.
- Agitated heating could enable a 77 % processing time reduction at 110 °C compared to static mode for inactivation of *Bacillus* spores.
- Similar to *Listeria* results, using the classical *F*-value concept could give accurate predictions for inactivation of *Bacillus* spores from time-temperature data with agitating process.

Results from combined high pressure-temperature (*P*/*T*) processing are:

- A 6 log kill effect on *Listeria* was possible within combined *P*/*T* ranges of 525 MPa / 40 °C to 600 MPa / 25 °C for 5 min.
- The deduced model showing the effects of *P*/*T* on *Listeria* in the soup was statistically significant ($P < 0.0001$, $R^2_{adj} = 0.95$).

- Combining 600 MPa with initial temperatures ≥ 45 °C was effective in controlling *Listeria* recovery at 4 and 8 °C for three weeks when initial counts were 3 to 5 log CFU/ml.
- Combined *P*/*T* processing was highly synergistic for inactivation of *Listeria* cells and *Bacillus* spores.
- Up to 4 log increases in *Bacillus* spore inactivation was possible when initial temperature was elevated from 20 to 65 °C at 650 MPa for 10 min.
- Combined *P*/*T* processing could enable rapid processing within lower temperature ranges than heat processing alone.

The processing of chilled RTE foods should be designed to inactivate nonproteolytic *C. botulinum* type E spores, when the product is not acidified (pH > 4.6) and stored at ≥ 3.0 °C. Similar to heat, combined *P*/*T* processing could be expected to give a higher reduction on type E spores than *Bacillus* spores. Furthermore, *Bacillus* spp. spores are often more heat resistant than type E spores. Hence, the results shown in Paper IV would possibly eliminate type E spore risk. Results from Paper I would be suitable for a RTE product intended to be stored for less than a week at ≤ 3.0 °C or at frozen conditions.

For heat sensitive products aimed to for chilled distribution, combined *P*/*T* processing would be an attractive option. If the product does not contain solid particles and relatively less heat-sensitive, agitating process could be used for targeting spores. For products with large solid particles, lower agitation speeds or static heating mode can be suitable for aesthetic or sensorial reasons.

Future studies may look into:

- Effects of different products considering pH, viscosity, composition, presence of solid particles, package type, volume and target organisms/strain cocktails on processing time requirements and heat load distribution with the new retort process.
- Applicability of the *F*-value concept with different products and slower agitation speeds (lower than 80 spm).
- Comparison of agitated and static processing on food matrices that provides very high heat resistance for *Listeria* (equivalent to 70 °C / 2 min process).

- Comparison of agitated and static heating on nonproteolytic *C. botulinum* type E spores (equivalent to 90 °C / 10 min) and *C. sporogenes* spores (121 °C / 3 min).
- Influence of different heating rates (1.1 to 5.3 °C/min) with agitating retort on bacterial recovery.
- Improving the value of the RSM model by studying the effects of product composition within an extended *P*/*T* range.
- Comparison of RSM model predictions to a larger dataset including alternative modeling approaches that have been used in published/unpublished studies.
- Effects of mild *P*/*T* combinations on some strains of *S. aureus* and *E. coli* that exhibit higher tolerance to pressure than *Listeria*.
- Inclusion of *Listeria* recovery as a parameter in future combined *P*/*T* inactivation models.
- Effects of mild *P*/*T* combinations (650 MPa / 45 to 65 °C) on nonproteolytic *C. botulinum* type E spores.

Finally, the impact of studied process parameters on key product quality attributes determined by food processors could be evaluated with sensory analyses.

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PAPER I

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Comparison of bacterial inactivation with novel agitating retort and static retort after mild heat treatments

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ABSTRACT

Lower thermal load on foods is desirable for food producers and consumers as the food gets higher quality. With reduced thermal load, the investigation of food safety is of importance. In this study, microbial inactivation efficacy of a new retort process with high frequency longitudinal agitation was compared to static retort process which was used as a benchmark. As a model, fish soup samples, inoculated with approximately 10^8 cells/ml *Listeria innocua*, was exposed to mild heat treatments at 62, 65 and 68 °C. Results clearly demonstrated that agitating mode can provide equivalent lethality to the model organism *L. innocua* within significantly shorter heating times compared to static mode. Bacteria were not detected on TSA-YE plates after 11.5, 6.8 and 5.5 min processing in agitating mode; 77, 67 and 52 min processing in static mode at 62, 65 and 68 °C respectively. Bacterial inactivation in agitating mode was generally correlated with estimated inactivation based on product core temperature, D- and z-values for *L. innocua*. This may indicate that distribution of the heat load over the soup was enhanced through agitation. Results showed that utilization of high frequency longitudinal agitation mechanism in retorts is promising for reducing the thermal load on food products without compromising on food safety related to non-spore forming pathogens.

1. Introduction

Thermal processing in hermetically sealed containers is a common method to produce foods with extended shelf-life utilized historically since the Napoleonic era. Static retorts have been widely used for industrial applications of thermal processing. Early versions of these retorts used steam as the heating medium but later on water, steam/air, raining and spray water systems were also developed as heating media. In 1920s, agitating retorts were introduced for the first time where agitation mechanism was based on axial rotation (rolling of cans) (Eisner, 1988). Rotary retort was later developed with end-over-end rotation principle (Clifcorn, Peterson, Boyd, & O'Neil, 1950).

Development of agitating retorts was the outcome of a need to overcome some weaknesses of conventional static retorts. The weaknesses included differing temperature zones within heated product, over-cooking, lack of consistency in texture and flavor in processed products and slow heat penetration (Eisner, 1988). On the other hand, agitation of packed foods has enabled more uniform distribution of heat and process time reduction. These benefits often give higher quality food products while being only applicable for liquid and semi-liquid foods (Rosnes, Skara, & Skipnes, 2011). Furthermore, product consistency, headspace, fill-in weight and rotation speed has to be strictly controlled to prevent under-processing as these parameters influence the heat transfer effectiveness of agitating process (Awuah, Ramaswamy, & Economides, 2007). Recently, an agitating retort with high frequency longitudinal agitation mechanism was developed in 2006 (Fig. 1). The new process allows rotation speeds beyond the 20–40 rpm range with the help of reciprocating agitation mechanism (Rosnes et al., 2011). Although critical factors for the new retort process are believed to be same as rotary retorts, investigation of food safety through microbial inactivation studies is of importance with such novel processes (Walden, 2008).

Microbial inactivation studies with artificially inoculated foods are typically used for food safety studies with novel processes. Lower number of survivors than the hazardous level in the food product over a determined shelf-life period after thermal processing is desirable (NACMF, 2010). Traditionally, process lethality calculations have been based on thermal resistance data for

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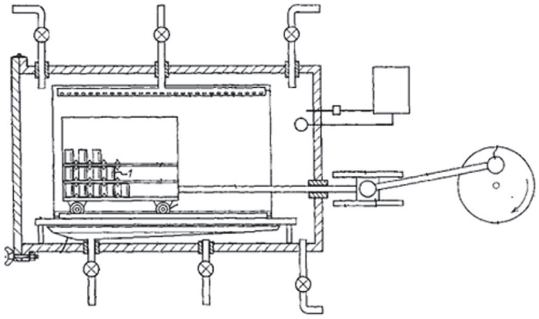

Fig. 1. Principle for the new retort system: Products (1) within the basket are agitated in longitudinal direction. The corresponding patent can be referred to for descriptions on each number shown in the figure modified from (Walden & Ferguson, 2007).

bacteria and spores. Thermal resistance has been mathematically expressed by decimal reduction time (*D*-value) and *z*-value. *D*- and *z*-values are based on the assumption that thermal inactivation of bacteria follows log-linear kinetics. *D* value is the duration of heat treatment at a specific temperature necessary to kill 90% of the microbial population and *z* value is the temperature change required to shift *D* value by 1 log unit (Stumbo, 1973). Other available thermal inactivation models for microorganisms have been reviewed by (Smelt & Brul, 2014) for interested readers.

Milder heat treatment is generally applied on foods designed to have short shelf-life under refrigeration temperatures. For such foods, *Listeria monocytogenes* contamination is a large problem as the bacterium can cause lethal diseases. Compared to other non-spore forming food-borne pathogens, *Listeria* is generally reported to have higher heat resistance as well as being able to grow at temperatures from −1.5 °C up to 44 °C (Hudson & Mott, 1993). Furthermore, *L. monocytogenes* has been reported to grow in foods from a_w of 0.91–0.93 and pH value of 4.2 (FAO/WHO, 2004). It is generally agreed that sufficient pasteurization can eliminate *L. monocytogenes*. Mild heated products are required to be heated for at least 2 min at 70 °C (at the coldest point) in order to achieve 6-log kill effect on *L. monocytogenes* (FAO, 1999, p. 34; Rosnes et al., 2011).

In research studies, *Listeria innocua* is proposed as a potential surrogate microorganism for *L. monocytogenes*. This is because *L. innocua* is safer to work with as well as having major phenotypic similarity with *L. monocytogenes* (Kamat & Nair, 1996; Lorentzen, Ytterstad, Olsen, & Skjerdal, 2010; Miller, Gil, Brandão, Teixeira, & Silva, 2009). In a recent review, Milillo et al. (2012) recommended more precise selection of surrogate microorganisms for *L. monocytogenes* based on experimental conditions. Although several strains of *L. innocua* have been found to be more heat tolerant than *L. monocytogenes* (Friedly et al., 2008; O'Bryan, Crandall, Martin, Griffis, & Johnson, 2006; Sorqvist, 2003), these results may sometimes vary with process conditions and product matrix (Murphy, Duncan, Beard, & Driscoll, 2003). Lorentzen et al. (2010) compared the survival of *L. innocua* ATCC 33090, *L. monocytogenes* NCTC 11994 and No. 4006 and found ATCC 33090 to be the most heat tolerant one. *L. innocua* is apparently a useful model organism for inactivation studies but should not be directly used for thermal validation purposes.

To the best of our knowledge, there are no studies published regarding microbial inactivation efficacy of the retort process with high frequency longitudinal agitation mechanism. Therefore, objective of this study was to investigate bacterial inactivation over the whole food product with agitating retort process in comparison to static process which was used as a benchmark. This was done by conducting bacterial inactivation experiments through a large set of heat treatments with fish soup samples inoculated with *L. innocua* model organism. The product was intended to have limited shelf life at refrigerated temperatures lower than 3.3 °C.

2. Materials and methods

2.1. Fish soup preparation

A common recipe for making fish soup was used. Ingredients consisted of approximately 2% fish bouillon, 8–8.5% fat (from butter, milk and cream), 0.66% salt, and the rest being mainly water. The soup was cooked, packed and treated with a sterilization program of $F_{121C}^{10C} \geq 3$ min based on core temperature. As a next step, the soup was packed in polypropylene plastic trays (Promens, Kristiansand, Norway) with dimensions 9 × 4 × 13.2 cm and sealed with a plastic film. Samples were then stored at 1 °C until the day of experiments.

2.2. Culture preparation and inoculation into fish soup samples

L. innocua ATCC 33090 (Oxoid, Hampshire, U.K) was stored in Microbank (Pro-Lab Diagnostics, Canada) at −80 °C. *L. innocua* was initially grown in Tryptic Soy Broth (Oxoid) supplemented with 0.6% w/w Yeast Extract (Merck, Darmstadt, Germany) (TSB-YE) at 37 °C for 20 h at 150 rpm. The overnight culture was then sub-cultured in TSB-YE with 20 h incubation at 30 °C, 150 rpm. Resulting cell density was 10^8–10^9 cells/ml. In order to concentrate the cells further, bacteria were centrifuged at 3500 × *g* for 4 min and cell pellets were collected and re-suspended in peptone water (Merck) to obtain 10^{10}–10^{11} cells/ml. Bacteria were then added to fish soup samples to obtain an initial concentration of approximately 10^8 cells per ml fish soup. Finally, inoculated fish soup samples, each having 350 g weight, were separately packed and shaken thoroughly to distribute the bacteria evenly before subsequent heat treatments.

2.3. Heat treatments

A batch retort (Steriflow, Roanne, France) was used for all heat treatments since it was possible to run the retort both in agitating and static heating modes. The retort was previously calibrated and checked for even heat distribution and the heat transfer medium was steam and raining water. The range used in agitating mode was 80–100 strokes per minute (spm). There were two process batches where three replicates of inoculated fish soup were heated in each ($n = 6$). Core temperature histories of three additional soup samples were recorded during treatments. A negative control (without bacteria) was also processed and analyzed in each process batch.

For a detailed assessment of bacterial inactivation pattern with longitudinal agitating heating mode in comparison to static mode, three different mild temperatures (62, 65 and 68 °C) were selected for investigation. Retort program phases were (1) come-up period for retort water temperature; (2) heating; (3–4) cooling (Fig. 2). Phases 1, 3 and 4 were always kept constant for each thermal treatment. Only the effect of selected time–temperature combinations for heating phase (2) on bacterial inactivation was studied. For this purpose, time intervals analyzed in agitating mode were 4–9.5 min (at 62 °C), 3–4.8 min (at 65 °C) and 1.5–3.5 min (at 68 °C). In static mode, time intervals 40–70 min (at 62 °C), and 30–65 min (at 65 °C) and 25–50 min (at 68 °C) were investigated. Additionally,

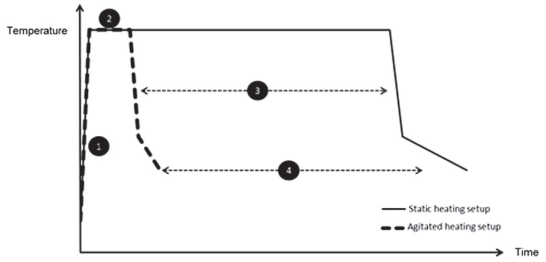

Fig. 2. Retort programs for thermal treatments in agitating and static modes (1: come-up period for retort water temperature, 2: heating period at constant retort temperature, 3–4: cooling periods).

pre-heating of retort water temperature was 2 min for agitating and static heating modes for all treatments.

Detailed settings of phases in agitating and static heating setups were as follows:

- (1) Retort water heated up to set temperature (62, 65 or 68 °C). Process time kept constant as 2 min with 80 strokes per minute (spm) shaking frequency in agitating mode whereas process time was 2 min in static mode without shaking,
- (2) various temperature-time combinations at 100 spm shaking frequency in agitating mode and without agitation in static mode,
- (3) retort water cooled down to 30 °C in 2 min at 100 spm shaking frequency in agitating mode and in 3 min without shaking in static mode,
- (4) retort water further cooled down to 20 °C within 5 min at 80 spm shaking frequency in agitating mode and within 15 min without shaking in static heating mode

The reason for choosing a longer cooling time for static process was to ensure fish soup samples were below ca. 45 °C since cooling was slower in static process compared agitated cooling. The retort was otherwise too warm to touch before the samples could immediately be placed on ice-water.

2.4. Calculation of process lethality and core temperature data acquisition

Process lethality was estimated only on agitating process samples based on coldest spot. Lethality was not calculated on static process samples since different temperature zones within the product causes misleading results. Classical thermal death model was used for process lethality calculations. Heat resistance data for the *L. innocua* culture was previously obtained as $D_{60°C} = 2.40$ min with a standard deviation (SD) of 0.04 and $z = 4.71$ °C with an SD of 0.08 after experiments made in TSB-YE medium. As a comparison, in previous studies $D_{60°C}$ was found 2.43 ± 0.17 min for *L. innocua* ATCC 33090 in peptone water medium (Ahn & Balasubramaniam, 2007). Based on experiments with various food media, *z*-values for *L. innocua* were obtained 5.8 ± 0.8 °C (Sorqvist, 2003).

Heat resistance data available for *L. innocua* culture was used for estimating microbial inactivation (in log units) based on lowest core temperature values (±0.05 °C accuracy). The temperature data was obtained via E-Val Flex thermocouple system (Ellab A/S, Hilleroed, Denmark) integrated to PC software (Valsuite, Ellab A/S). Threshold temperature value was set to 50 °C where *L. innocua* inactivation was assumed to begin. Since dynamic heating condition was the case for all treatments, time–temperature history data was incorporated to isothermal heat inactivation kinetics to calculate the lethality (L) of each thermal treatment based on Equation (1) where $D_{ref} = 2.4$ min; $T_{ref} = 60$ °C; $z = 4.71$ °C; t and T_{core} were treatment time (min) and core temperature (°C) respectively (Monfort, Sagarzazu, Gayán, Raso, & Álvarez, 2012):

$$L = \frac{1}{D_{\mathrm{ref}}} \int_0^t 10^{\frac{T_{\mathrm{core}} - T_{\mathrm{ref}}}{z}} \mathrm{d}t \tag{1}$$

2.5. Enumeration of survivors

Along with positive and negative controls, triplicate samples of 2 ml processed fish soup (taken from 350 g aseptically opened fish soup packs) were diluted in 18 ml peptone water and vortexed. Further ten-fold serial dilutions were prepared for subsequent surface plating on Tryptic Soya Agar (Oxoid) supplemented with 0.6% yeast extract (TSA-YE). Additionally, dilutions were plated on Brilliance Listeria Agar added with selective and differential supplements (Oxoid). The latter medium, that is selective for *Listeria*, can give information about cell injury, as injured cells have difficulties to grow on this medium (Hansen & Knochel, 2001; Miller, Brandão, Teixeira, & Silva, 2006). Eddy Jet spiral plater instrument (IUL Instruments, Barcelona, Spain) was used for surface plating procedure unless manual plating was necessary to count lower amounts of bacteria. All plates were incubated at 30 °C for 2–5 days.

2.6. Statistical analysis of data

L. innocua inactivation data was calculated as $\log_{10}$ (N_0/N), where N_0 is the initial bacteria concentration and N number of survivors on TSA-YE and Brilliance plates after heat treatment. Detection limit was 10^2 cells/ml. If there were no survivors on plates with lowest dilution, all bacteria were considered killed after the heat treatment. Results for each data point were obtained by calculating the mean-value and standard deviation from six replicates. The general linear modeling (GLM) and Tukey's HSD test were used to compare means at significance level $p < 0.05$ using Minitab Statistical Software v15 (Minitab Ltd., Coventry, UK).

3. Results and discussion

3.1. Heat penetration data

Agitating mode caused faster product heating than the static mode for all experiments. As a representative for other heat penetration curves, selected heat penetration values and simulated core temperature data are shown (Fig. 3).

3.2. Microbial inactivation data

Results showed agitating heating mode shortens process time needed to inactivate the model organism *L. innocua* compared to static mode. No colonies were detected after 11.5, 6.8 and 5.5 min processing in agitating mode compared to 77, 67 and 52 min processing in static mode at 62, 65 and 68 °C respectively (Figs. 4 and 5). Another study concluded that the heat resistance of *L. innocua* in liquid medium decreases at higher heating rates shown with non-isothermal heating experiments at 1.5, 1.8 and 2.6 °C/min from 20 to 65 °C (Miller et al., 2011). Similar cases were also reported for *L. monocytogenes* (Quintavalla & Campanini, 1991) and *Salmonella*

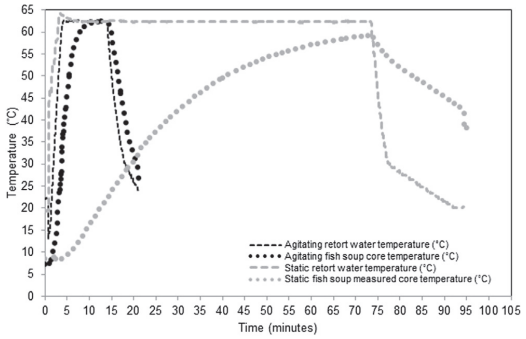

Fig. 3. Time–temperature history of fish soup and retort water temperature during agitating heating mode (62 °C, 11.5 min process) and static heating mode (62 °C, 72 min process).

typhimurium (Mackey & Derrick, 1987). In the present study, the phenomenon is likely a synergistic factor for microbial inactivation since agitating heating mode provides faster heating rates in comparison to static mode.

Bacterial inactivation data from TSA-YE plates and calculated log kill (based on product coldest point and first order model) after agitated processing was similar (Fig. 4). This may indicate the thermal load was rather homogenously distributed over the product in agitated process. Nevertheless, using traditional methods for thermal process validation is more convenient since the heat resistance among different bacterial strains may vary.

L. innocua cells were sublethally injured after agitated processing for 8, 9, 9.5 and 10 min at 62 °C; 5.5 min at 65 °C; 4.5 min at 68 °C and after static processing for 47, 32 and 27 min at 62, 65 and 68 °C respectively (Figs. 4 and 5). Occurrence of cell injury was determined by the difference between colony counts on TSA-YE and Brilliance plates ($p < 0.05$). Highest difference observed was in the range of 4–5 log units suggesting that only 0.01–0.001% of the survivors were not sublethally injured in those cases. The possible explanation is that under insufficient heat treatment, injured cells appear to be able to repair themselves and grow on TSAYE medium but not on selective medium as similar cases were reported in previous studies (Crawford, Beliveau, Peeler, Donnelly, & Bunning, 1989; Mackey, Boogard, Hayes, & Baranyi, 1994).

Competitive microflora, composition and temperature history of a model food product should be considered for inactivation studies. In the present study, potential effects from the competitive microflora were knocked out by using sterile fish soup. However, since *Listeria* has higher heat-resistance than other non-sporulating bacteria, survival risk of other pathogens such as *Salmonella* spp. and pathogenic *Escherichia coli* would be eliminated along with *Listeria* under sufficient heat treatment (Farber, 1989). Furthermore, cells may develop modified heat resistance based on food composition (high concentration of salt, acids, sugar and inhibitors) and prior heat shocks which have been reviewed in the literature (Doyle, Mazzotta, Wang, Wiseman, & Scott, 2001; Farber & Brown, 1990). This factor should be taken into account for such inactivation studies. Finally, attention should be given when processing liquid products with large solid particles. Underestimation of *L. monocytogenes* inactivation in ground beef with log-linear and Weibull model during dynamic heating was reported (Huang, 2009). In the present study, the product had neutral pH as well as being absent of solid particles and excessive amounts of salt.

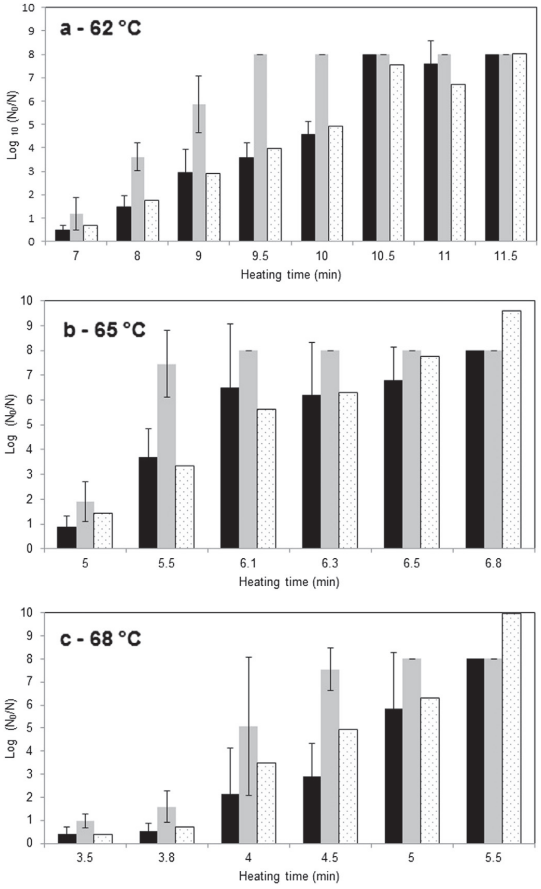

Fig. 4. Inactivation of *L. innocua* exposed to agitating heating at 62 °C (a), 65 °C (b) and 68 °C (c). Black bars: inactivation data ($\log_{10}$) obtained from TSA-YE plates; gray bars: data from Brilliance plates; and white bars with dots show mathematically calculated log inactivation based on lowest core temperature data. Data are means from six replicate samples. Error bars represent standard deviation of each mean value. Retort cooling times were 7 and 18 min for agitating and static modes (not shown).

4. Conclusions

Results clearly demonstrated that agitating retort process could deliver equivalent lethality to the model organism *L. innocua* within significantly shorter times compared to static process. No colonies were observed on TSA-YE plates after heat treatments of 11.5, 6.8 and 5.5 min in agitating mode; 77, 67 and 52 min in static mode at 62, 65 and 68 °C respectively. Results could be relevant for food producers interested in minimal food processing. Furthermore, estimated bacterial inactivation was generally correlated with real inactivation with samples treated in agitating mode. The result was supportive of the assumption that the heat load through the soup was homogenously distributed with the help of agitation. Apparently, further inactivation studies using more heat-resistant *L. monocytogenes* strains (with 70 °C/2 min process) and spores are also needed for understanding the efficacy of the agitating process at higher temperature ranges. Last but not the least,

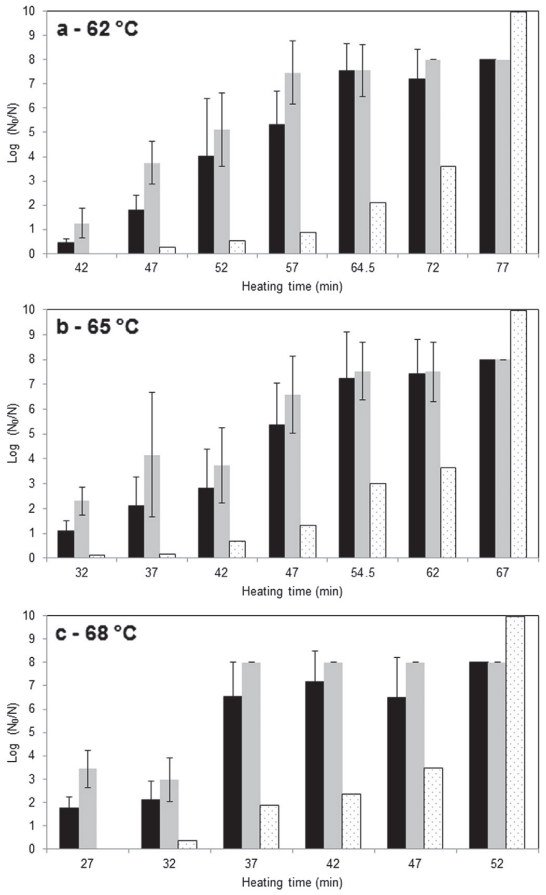

Fig. 5. Inactivation of *L. innocua* exposed to static heating mode at 62 °C (a), 65 °C (b) and 68 °C (c). Black bars: inactivation data (log_{10}) obtained from TSA-YE plates, gray bars show data from Brilliance plates; and white bars with dots show mathematically calculated log inactivation based on lowest core temperature data. Data are means from six replicate samples. Error bars represent standard deviation of each mean value. Retort cooling times were 7 and 18 min for agitating and static modes (not shown).

samples should be checked for recovery risk of *Listeria* during a refrigerated storage test.

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PAPER II

Ates, M. B., Rode, T. M., Skipnes, D., Lekang, O. Effects of agitated and static heating on subsequent recovery of sublethally injured *Listeria*. *To be submitted for publication in European Food Research & Technology*

Effects of agitated and static heating on survival and recovery of sublethally injured *Listeria*

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Abstract Investigations on *Listeria* recovery in ready-to-eat (RTE) foods after mild treatments could be useful. In this work, survival and recovery of *Listeria innocua* after mild heat exposure in a model food matrix were investigated. The heat treatments were performed with or without agitation in a temperature range that can be used for "sous vide" production. Survival and the recovery of *Listeria* were detected after three weeks at two different storage temperatures (4 and 8 °C). Cell injury and recovery were determined with growth differences of heat-stressed *Listeria* on nonselective TSAYE and selective Brilliance media before and after refrigerated storage. Overall results indicated that cells heated with agitation were predominantly in the lag phase during three weeks at 4 °C, irrespective of the average concentration of survivors left after heating. This indicated that those cells were still repairing injuries. However, static heating left a survivor population that showed a higher variability after three weeks at 4 °C. Cells heated in static mode for short durations mostly recovered and switched to growth phase within three weeks at 4 °C. Storage at 8 °C did not show significant differences for *Listeria* recovery with agitated/static heating, as cells mostly resuscitated and grew further during three weeks. Differences in cell recovery after mild treatments in agitated and static modes could be due to temperature irregularities in the product triggered by static heating mode. This apparently results in a cell population possessing varying degrees of sublethal injury.

Keywords *Listeria*, recovery, sublethal injury, agitating retort

Introduction

Sublethal injury can occur when cells no longer persist beyond a stressor level [1]. More severe stress can ultimately cause cell death [2]. Identification of sublethally injured microorganisms is required for improving the safety of processed products. Hurdles such as heating, refrigeration, freezing, acidity, use of preservatives, low water activity, and exposure to starvation may result in presence of injured cells in foods [3]. Recovery of injured cells is denoted as 'resuscitation' which only takes place under favorable conditions. Regeneration processes for cellular functions and structures are initially triggered before cells can resume growth. Injured cells can repair within very short times (2 – 4 h), when recovery conditions are suitable [4].

Listeria monocytogenes has been identified as a ubiquitous organism associated with high hospitalization (90 %) and mortality (20 – 30 %) rates [5]. Codex Alimentarius recommended that presence of *Listeria* should be limited to 100 CFU/g at the end of shelf life, if *L. monocytogenes* growth can be controlled. This is only possible with pH ≤ 4.0, water activity ≤ 0.92, use of antimicrobials and strict maintenance of the cold chain. However, if favorable conditions exist in RTE products for *L. monocytogenes* growth, its presence must be limited to < 0.04 CFU/g (or absence in 25 g product) [6]. *Listeria* can grow at very low temperatures (-1.5 °C) [7]. Furthermore, it is among the most heat tolerant non-spore forming food pathogens [8]. *Listeria innocua* is usually more heat-tolerant than *L. monocytogenes* and accepted as a model surrogate for thermal inactivation studies [9-13].

Listeria recovery after heat treatments has been reported with respect to incubation temperature, salt level, cell growth phase, storage temperature, recovery medium, treatment temperature and pH [10, 14-16]. Agitation of product contents during heating can result in a homogenous heat load distribution compared to varying temperature zones often seen with static processes. Temperature irregularities may expose the cell population to varying intensities of heat stress, which may in return affect cell survival during subsequent storage. Suboptimal temperatures may permit higher recovery rates based on the suppressing effects on cell division, thereby activating metabolic repair processes. Cells are usually not homogenously exposed to cell injury which may lead to variable lag times among individual cells [4,2].

Refrigerated RTE products with pH > 4.6 require a heat treatment equivalent to at least 90 °C / 10 min and storage at < 3.0 °C in order to prevent the risk of toxin formation from nonproteolytic *Clostridium botulinum* type E spores [17]. Otherwise, such products may

require a heat load equivalent to at least 70 °C / 2 min followed by frozen storage [18]. If survivors of *Listeria* are present after processing, they can survive freezing and resume growth upon thawing. This risk needs to be considered for mildly heated products intended to be distributed in frozen conditions. Furthermore, effects of product heating rate, lethality distribution and microbial survival during storage are necessary for thermal process design and risk assessment [19,20].

Therefore, the objective of the present study was to investigate the effects of mild heat treatments in agitated/static mode on survival and recovery of the model organism *L. innocua* in a model soup matrix after three weeks storage. Heat treatments were performed within a temperature range relevant for "sous vide" production and subsequent storage temperatures were 4 and 8 °C.

Materials and Methods

Preparation of inoculated model soup

Two successive cultures of *L. innocua* ATCC 33090 (Oxoid, Hampshire, U.K) were grown in tryptic soy broth (Oxoid) supplemented with 0.6 % w/w yeast extract (Merck, Darmstadt, Germany) (TSB-YE) for two consecutive days at 37 and 30 °C for 20 h under 150 rpm shaking. Resulting late stationary phase cell concentration was 10^8 – 10^9 cells/ml. Cell pellets were then collected after a 3500 g centrifugation process for 4 min. Concentrated cell culture was added to sterilized model soup ($F_0 > 3$) at 1:1000, giving a population density about 10^8 cells per ml soup.

The soup (pH 6.1) used in the current study composed of water (38.8 %), whole milk (36.1 %), cream (12 %), flour (4.5 %), fish bouillon (2.4 %), salt (0.6 %), lemon juice (0.6 %), garlic powder (0.6 %), sugar (0.4 %), white pepper and dill (0.02 %) (w/w). Inoculated packs (containing 350 g soup) were PP trays (Promens, Kristiansand, Norway) with dimensions 9 × 4 × 13.2 cm. Trays were thermosealed with a 90 µm PA/PP film layer (Dynopack, Kristiansand, Norway) and kept at 1 °C before experiments.

Heat treatments

Heat treatments were carried out using a pilot 1-basket water cascading retort (Steriflow, Roanne, France). For detailed retort descriptions, readers are referred to Ates *et al.* [21]. Retort settings are shown in Table 1.

Coldspot temperature of three samples was measured with thermocouples (± 0.05) (Ellab A/S, Hilleroed, Denmark). Dynamic temperature data was logged with E-Val Flex data acquisition system (Valsuite, Ellab A/S). Average heating rate ($\Delta T/\Delta t$) at the coldest point of soup was calculated with the slope of the soup heating curve (Fig. 1). For this purpose, Eq. 1 was formulated where T', T_0, t' were heated core temperature, initial core temperature and time to reach the highest core temperature, respectively:

$$\Delta T/\Delta t = \frac{T' - T_0}{t'} \quad (1)$$

Overall, three sample replicates were heated in two different batches (n = 6). Following heat treatments, soup samples were repacked and stored aerobically at 4 ± 0.5 °C and 8 ± 0.5 °C for three weeks in a cooling cabinet (Porkka CM 710F, Huurre Group OY, Finland). A temperature logger (Tracksense® Pro, Ellab A/S) was used for tracking the temperature in the storage chamber.

Microbial and statistical analyses

Cells were analyzed after heat treatments and storage test for three weeks. For microbial analysis, two ml soup from each sample was serially diluted in 0.1 % peptone water (Merck, Darmstadt, Germany). Surface plating method was used for counting procedure. Both injured and non-injured cells were counted on tryptic soy agar (Oxoid) with 0.6% yeast extract (TSA-YE). Non-injured cells were enumerated on Brilliance agar (Oxoid) which is a selective medium for *Listeria*. Brilliance agar contained selective and differential supplements for *Listeria* (Oxoid). Eddy Jet spiral plater (IUL Instruments, Barcelona, Spain) was mainly used for counting procedure. Manual plating with 0.1 and 0.5 ml of undiluted cell suspensions was also performed in order to count lower dilutions and increase sensitivity. Detection limit was 1.3 log CFU/ml. All plates were incubated at 30 °C for 2 – 5 days. Results were reported as colony forming units/ml (CFU/ml). Mean values from six samples were calculated as log (N),

where N was the number of cells after heat treatments and storage period. The General Linear Model (GLM) and Tukey's HSD test were applied to compare means at 95 % significance level. JMP Pro 11 (SAS, USA) was used for the statistical analysis procedure.

Results and discussion

During retort treatments, time-temperature histories at the coldest points showed that soup heating rates ($\Delta T/\Delta t$) were 5.3, 6.6 and 9.5 °C/min in agitated mode, compared to 0.7, 0.8 and 1.1 °C/min in static mode at 62, 65 and 68 °C, respectively. A representative time-temperature graph is shown in Fig. 2.

Results generally indicated that cells heated under agitation were predominantly in the lag phase during three weeks at 4 °C, irrespective of initial mean cell concentration in the soup. This implied that those cells were generally at the repair stage. After agitated heating for 9, 9.5 and 10 at 62 °C; 5.5, 6.3 and 6.5 min at 65 °C; 4.5 and 5 min at 68 °C, the repair of injuries was incomplete (Figs. 3A, 4A and 5A). In those cases, 0.5 to 2 log differences in mean bacterial counts on TSAYE and Brilliance were observed after three weeks at 4 °C, when compared to initial levels ($P < 0.05$). This finding indicated that a 30 to 99 % of bacterial population was still sublethally injured.

Furthermore, agitated heating for 52 – 72 min at 62 °C, 5.5, 6.3 and 6.5 min at 65 °C gave decreased mean cell counts on TSAYE (by 0.6 to 1.8 log) after three weeks at 4 °C, when compared initial number of survivors ($P < 0.05$) (Figs. 3A and 4A). Similarly, only 42 min static heating at 68 °C resulted in reduction of cells below the detection limit after 4 °C storage (Fig. 5B). The findings could be interpreted as a part of the sublethally injured population was unable to resuscitate and died during the storage period at 4 °C.

Static heating left a survivor population that showed a higher variability than agitated heating after three weeks at 4 °C. Applying static heating for 52, 57, 64.5 min at 62 °C; 42, 47 and 62 min at 65 °C; 32 min at 68 °C resulted in cell counts on both TSAYE and Brilliance being increased by 0.5 to 4 log after three weeks at 4 °C (Figs. 3B, 4B and 5B). With agitated heating, 0.8 and 2 log increases were observed after 6.1 min at 65 °C and 4 min at 68 °C, respectively (only on TSAYE) (Figs. 4A and 5A). These results indicated that heating in static mode provided more favorable conditions for cell recovery and a quicker shift to growth phase within three weeks at 4 °C, when shorter heating times were applied.

Differential scanning calorimetry (DSC) studies demonstrated that heat damage is apparently concentrated on the cell membrane at temperature ranges between 62 and 66 °C [22]. This may play a role in observing a clear difference in *Listeria* recovery between agitated and static heating modes at 62 and 65 °C but not at 68 °C. Temperature irregularities within the product after static heating could end up with bacterial membranes being exposed to varying levels of heat damage at 62 and 65 °C. Hence, cells located at colder points of soup packs could be exposed to reversible changes in their cellular structures. On the contrary, a more homogenous heat load over the whole soup with agitated heating could be expected to provide equal heat damage on cells located on various regions of soup packs. This effect could be the reason for extended lag times for *Listeria* after agitated heating.

Microbial growth rate is not affected under minor stress levels since full adaptation to the new situation takes place rapidly. Low stress (i.e. 48 °C for *Listeria*) may lead to an adaptive response resulting in increased stress resistance. Moderate stress such as the treatments used in this work may cause varying degrees of injury that could give live, injured and dead cells [23]. In previous studies, slow heating rates were shown to cause heat shock induced heat resistance development for *L. monocytogenes* and *L. innocua* [24,25]. This effect was attributed to heat-shock protein synthesis during extended exposure times (such as 30 min) to sublethal temperatures (< 55 °C) [26]. It was also shown that *L. monocytogenes* became more heat resistant with heating rates ≤ 0.7 °C/min compared to 5 °C/min [27]. Furthermore, *L. monocytogenes* was found more thermotolerant at pH values of 5.5 and 7.4 along with heating rates ≤ 2 °C/min [24]. Effects from the soup pH (6.1) coupled with slower heating rates in static mode may additionally play a role in observed differences for *Listeria* recovery with agitated and static heating.

Overall findings from the current study could be relevant for a product intended to be frozen or for having a short shelf-life (< 7 days) under refrigeration (< 3 °C). Otherwise, there is a risk of toxin formation from the spores of nonproteolytic *C. botulinum* type E. Subsequent cell repair is largely affected by the recovery medium. Especially, lower pH can suppress the recovery of damaged cell proteins. In the current work, eliminating the microflora by using a sterile soup exposed *Listeria* to reduced microbial competition for the nutrients. Presence of lactic acid bacteria (bacteriocins) could also suppress *Listeria* survival during storage [28]. Salt and pH levels in the soup were expected to provide favorable conditions for *Listeria* growth at 4 °C. This was validated with generating hypothetical growth curves for *L. monocytogenes* and *L. innocua* using ComBase growth predictor (not shown) (www.combase.cc/).

Results obtained in this work could be useful for evaluating the effects of varying heat intensities, heat load distribution and heating rates on physiological state of *Listeria* on a realistic food substrate. The physiological state of cells after heat treatments apparently influenced the lag times for *Listeria* during subsequent recovery. Effects of different temperatures (50 – 65 °C) on repair of heat-treated *L. innocua* on a single cell level were previously modelled [29]. The authors found that lag times increased linearly at sublethal temperatures from 50 to 55 °C. In future investigations, effects of the food matrix, heat load distribution over the product and heating rate can be included in predictive models related to lag time and growth rate predictions for heat-treated *Listeria*. Such models can be based on the Baranyi equation, the modified Gompertz and logistic functions [30].

Conclusions

Overall findings demonstrated that agitated heating could result in longer lag times for injured *L. innocua* in the model soup matrix during three weeks at 4 °C. This observation was apparently due to a more even heat damage on cells at various locations of soup packs as a result of agitation. Milder heat treatments in static mode enabled more favorable conditions for cell recovery and a quicker shift to growth phase within three weeks at 4 °C, compared to agitated heating. Under temperature abuse conditions (8 °C), there were generally no significant differences in bacterial recovery. Extended lag times for *Listeria* would be beneficial for improved food safety. Results indicated that optimization of thermal processing with novel agitation mechanisms may add an additional safety factor with respect to survival of food pathogens. Findings could be relevant for adding a further safety margin to 70 °C / 2 min processing for elimination of *Listeria*. Existing models on *Listeria* recovery could be improved with future studies on foods considering the effects of varying temperature profiles on the physiological state of cells after exposure to dynamic heat stress.

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Conflict of Interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal objects.

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Table 1 Design of heat treatments

	Agitating process			Static process	
Phase	*Temperature (°C)*	*Time (min)*	*Agitation (spm[1])*	*Temperature (°C)*	*Time (min)*
Come-up	20 to 62/65/68	2	80	20 to 62/65/68	2
Holding	62	6 - 9.5	100	62	50 - 75
	65	3.5 - 4.8	100	65	40 - 65
	68	2 - 3.5	100	68	30 - 50
Cooling	62/65/68 to 30	2	100	62/65/68 to 30	3
Cooling	30 to 20	5	80	30 to 20	15

[1] Strokes per minute

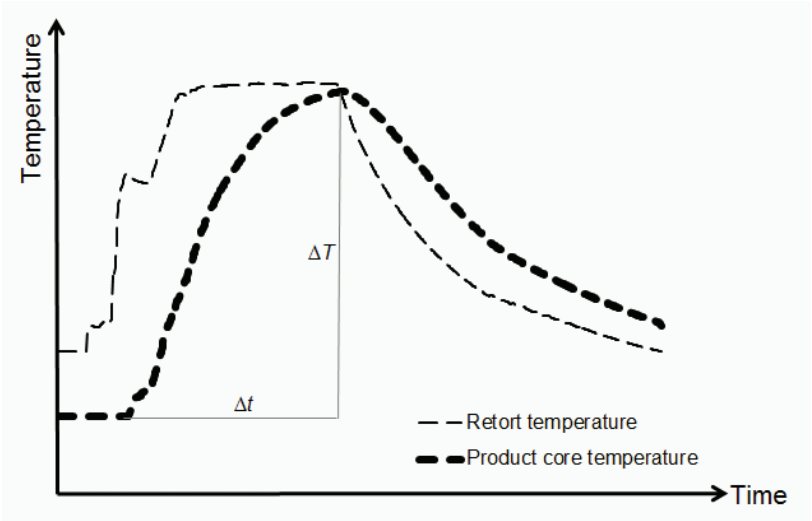

Fig. 1 Calculation method for average soup heating rate.

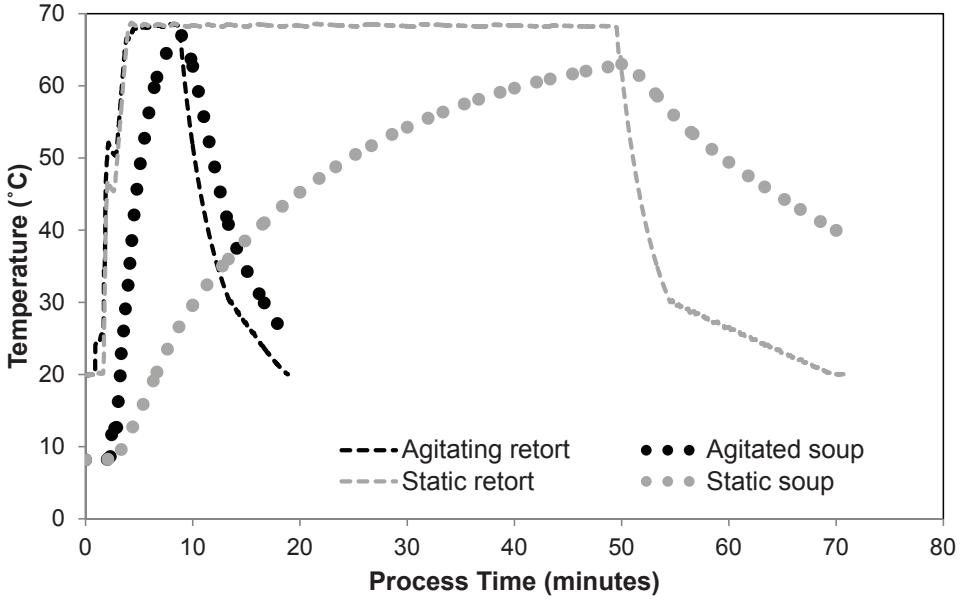

Fig. 2 Representative curves for soup core and retort temperature during 68 °C treatments.

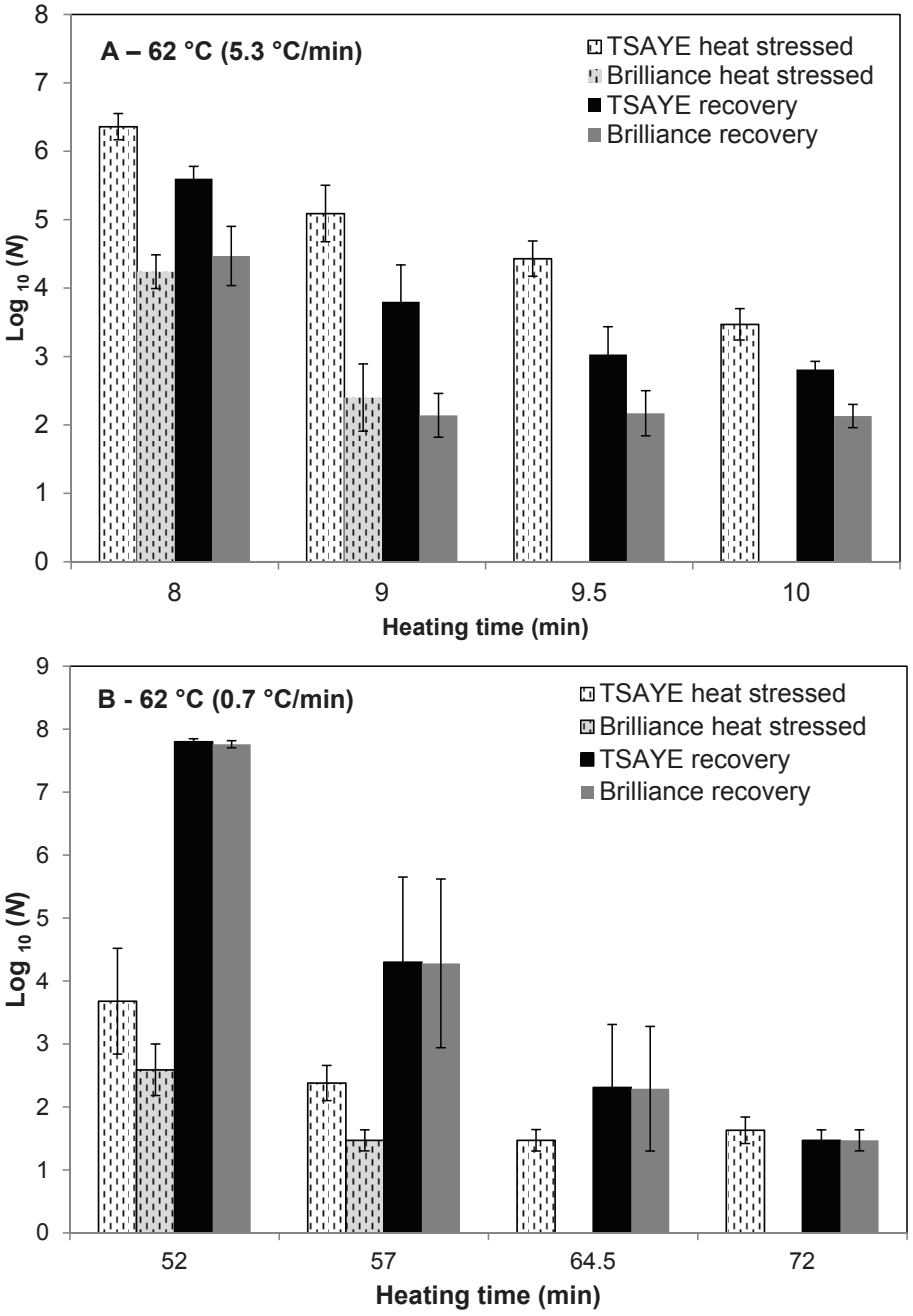

Fig. 3 Mean *Listeria* survivors/ml in soup packs after agitating (A) and static (B) heat stress at 62 °C and after three weeks at 4 °C. Survivors were counted on TSAYE (white and black) and Brilliance (gray). Initial counts before heat stress were 8 ± 0.2 log CFU/ml (n = 6).

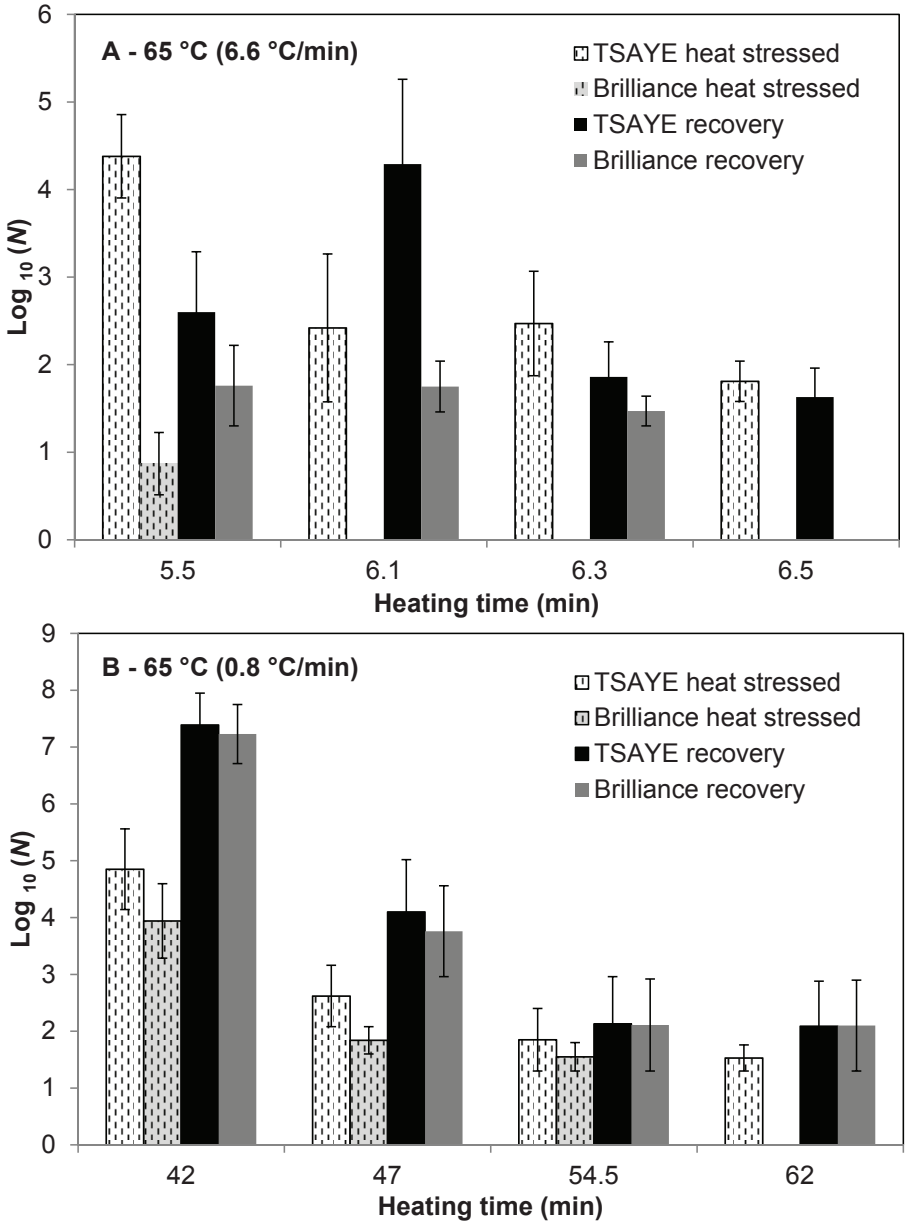

Fig. 4 Mean *Listeria* survivors/ml in soup packs after agitating (A) and static (B) heat stress at 65 °C and after three weeks at 4 °C. Survivors were counted on TSAYE (white and black) and Brilliance (gray). Initial counts before heat stress were 8 ± 0.2 log CFU/ml (n = 6).

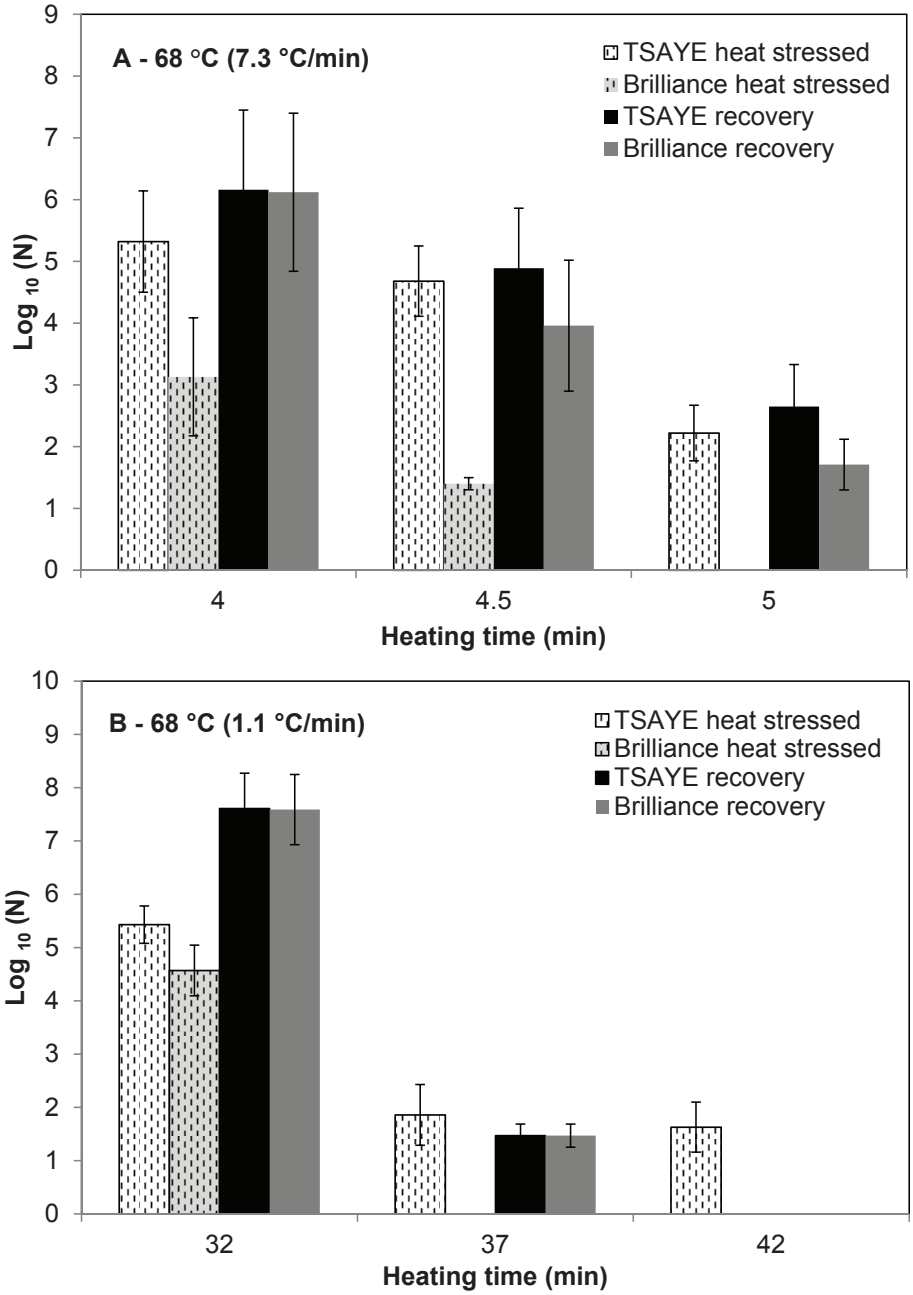

Fig. 5 Mean *Listeria* survivors in soup packs after agitated (A) and static (B) heat stress at 68 °C and after three weeks at 4 °C. Survivors were counted on TSAYE (white and black) and Brilliance (gray). Initial counts before heat stress were 8 ± 0.2 log CFU/ml (n = 6).

PAPER III

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Modelling of *Listeria monocytogenes* inactivation by combined high pressure and temperature treatments in model soup

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Abstract High pressure processing (HPP) in combination with mild heat is known to have a synergistic effect on bacterial inactivation in model substrates such as milk. However, this synergistic effect also needs to be assessed in more complex food matrices. In this study, *Listeria monocytogenes* inactivation in a model soup under combined high pressure and temperature treatments was modelled according to a central composite design. The model was significant ($P < 0.0001$) with a satisfactory predictability ($R^2_{adj} = 0.95$). Pressure and temperature effects on *L. monocytogenes* inactivation were assessed by solving the deduced quadratic equation and analyzing its contour plot. More than 6 log inactivation of *Listeria* was possible at the combined pressure-temperature ranges of 525 MPa / 40 °C to 600 MPa / 25 °C for 5 min in accordance with FDA guidance on refrigerated ready-to-eat foods. Moreover, 600 MPa treatments at ≥ 45 °C resulted in no *L. monocytogenes* recovery in the model soup during three weeks at 4 and 8 °C when the inoculum size was 10^3 or 10^5 CFU/ml. Results clearly indicated that use of mild temperatures in combination with HPP can induce a more complete inactivation, hence reducing the microbial recovery after HPP in foods. Experimental results and the fitted model in this study may be utilized as a comparison to other inactivation models and for determining test conditions for process safety assessments on similar refrigerated products in food industry.

Keywords HPP, mild heat, *Listeria monocytogenes,* inactivation model, injury, recovery

Introduction

More than a century ago, it was discovered that high pressure can improve the shelf-life of milk [1]. Later scientific studies and technological developments on high pressure processing (HPP) led to its application in food industry during the last two decades [2]. HPP is advantageous for retention of vitamins, phytochemicals and aroma compounds when compared to traditional thermal processing meanwhile being lethal to microorganisms [3]. A major favorable aspect of HPP is providing a rapid and uniform effect over foods in contrast to especially slowly penetrating conductive heating processes [4].

HPP with lower pressure intensity and holding time is desirable for treatment of HPP sensitive products, achieving higher product throughput levels and lower capital equipment costs without compromising on food safety [5]. Recent studies have shown that microbial inactivation can be enhanced when HPP is used in combination with other hurdles, such as thermal processing [5], natural antimicrobials [6] and modified atmosphere packaging [7]. In a previous study, a combined treatment of 400 MPa and 50 °C resulted in five log bacterial reduction in milk, whereas either treatment alone gave less than one log reduction [8].

Mathematical modelling is a useful tool for assessment of microbial inactivation in food processes. Response surface methodology (RSM) is an empirical modelling approach commonly used in food research, as it can demonstrate the effects of process variables and their mutual interactions on the response [9,10]. RSM can also include nonlinearities that have been often studied with Weibull, Gompertz and log-logistic models to describe microbial inactivation kinetics by HPP [11,12]. RSM has been used for modelling high pressure inactivation of *Listeria innocua* in peptone water [9] and *L. monocytogenes* in smoothies [5], milk buffer [10] and dry-cured ham [13].

L. monocytogenes is a major concern as lethal outbreaks and product recalls related to this organism are frequently reported [14]. *L. monocytogenes* is the target organism in ready-to-eat foods as well as being one of the most baroresistant and heat tolerant non-spore forming food pathogens [2,15]. FDA requires a process that can achieve min. 6 log reduction of this organism in refrigerated ready-to-eat foods [16]. Due to its psychrotrophic nature, controlling *Listeria* growth during chilled storage can be notoriously difficult if some bacteria are sublethally injured after processing. This also applies to HPP as a bacterial subpopulation is proposed to have higher pressure resistance, thus surviving the HPP treatment [17]. Recovery of pressure injured *L. monocytogenes* during subsequent storage has been reported in phosphate and citrate buffers [18], phosphate-buffered saline [19], milk [20] and brain-heart

infusion (BHI) [21]. Heat injury and subsequent resuscitation of *Listeria* has also been reported previously [22-24]. Hence, investigation of *L. monocytogenes* recovery after combined pressure and temperature treatments is vital for food safety.

In addition to technological and microbial variables, the suspending medium is known to influence microbial inactivation by HPP. Therefore, it is suggested to validate HPP effects using food products since bacterial pressure resistance has been reported as higher in foods than in buffered suspensions or culture media [25-27]. Development of new predictive models in different foods describing *L. monocytogenes* inactivation by HPP can be valuable for comparison with existing models and the food industry [2,10,13].

The objectives of this study were i) to build a regression model for *L. monocytogenes* inactivation in a model soup under combined high pressure and temperature treatments and ii) to investigate whether HPP assisted with mild temperatures can reduce/prevent microbial recovery in the soup during chilled storage for three weeks.

Materials and methods

Study design

Firstly, *L. monocytogenes* inactivation in a model soup was assessed using a regression model based on RSM. The deduced model was then statistically compared with a previous model in milk buffer [10]. Sublethal injury of *L. monocytogenes* was determined by the difference of cultivable bacteria on selective (Brilliance) and nonselective (TSAYE) medium after HPP treatments used in regression modelling. Finally, cell recovery during three weeks storage test at 4 and 8 °C with a separate HPP experiment was examined. All HPP treatments were performed in duplicate with four technical repeats (n=8).

Bacterial strain and culture preparation

L. monocytogenes ATCC35152 was identified as the most barotolerant strain among six *L. monocytogenes* strains (from our culture collection, ATCC35152, ATCC15313, ATCC19112, ATCC7644, 1BR5 and 2BR14) after a screening experiment at 400 MPa / 20 °C for 5 min (not shown). The latter two strains isolated from salmon were obtained from the National Institute of Nutrition and Seafood Research (Norway). Koseki & Yamamoto [19] observed

that *L. monocytogenes* ATCC19117 was slightly more barotolerant than ATCC35152. However, another study detected recovery of ATCC35152 within two days at 22 °C after 600 and 900 MPa treatments whereas ATCC19117 did not recover [21]. Therefore, we decided to use *L. monocytogenes* ATCC35152 as a test strain in the current study. All strains were maintained in Microbank (Pro-Lab Diagnostics, Richmond Hill, Canada) at -80 °C. Prior to use in all experiments, cultures were initially grown overnight in tryptic soy broth (Oxoid, Hampshire, UK) supplemented with 0.6 % yeast extract (Merck, Darmstadt, Germany) (TSBYE) at 37 °C. Bacteria were then re-cultured and grown in TSBYE at 30 °C, 150 rpm for 20 h to obtain ca. $10^9 – 10^{10}$ colony forming units (CFU)/ml.

Sample inoculation and packaging

L. monocytogenes was suspended in sterilized model soup (1:100) to obtain initial counts of ca. 10^8 CFU/ml for regression modelling study. For the storage test, soup samples were added 10^3, 10^5 and 10^8 CFU/ml using calculated dilutions. Main ingredients in the model soup (pH 6.1) were water (38.8 %), whole milk (36.1 %), cream (12 %), flour (4.5 %), fish bouillon (2.4 %), salt (0.6 %), lemon juice (0.6 %), garlic powder (0.6 %) and sugar (0.4 %) (w/w). All samples were vacuum packed (Webomatic vacuum chamber, Bochum, Germany) in heat-sealed polyethylene bags each containing 40 ml inoculated soup. Samples were kept at 1 °C shortly before HPP treatments.

HPP treatments

A high hydrostatic machine QFP 2L-700 (Avure Technologies Inc., Columbus, USA) with a pressure vessel (10 cm diameter and 25.4 cm high, 2 L capacity and 690 MPa limit) was used for carrying out treatments. Water acted as the pressure transmitting medium in the vessel. Water temperature was adjustable between 10 to 90 °C as the vessel could be heated with a recirculating jacket connected to an oil bath. Process temperature (on external surface of samples) was tracked using a K-type thermocouple. Pressure level and holding time were controlled automatically. Pressure come-up rate was approximately 250 MPa/min whereas holding and release times were 5 min and < 1 sec for all treatments. Holding time did not include come-up and release times. Before treatments, soup samples were kept in the pressure chamber for 5 min in order to heat samples to the desired initial temperature. Adiabatic temperature increase in the pressure medium was less than 2.5°C/100 MPa during treatments.

Immediately after HPP treatments, samples were cooled on ice slurries and analyzed on the same day.

Microbial analysis

Immediately after treatments, aliquots of 2 ml soup from each sample replicate were diluted (1:10) in buffered peptone water (Merck) and vortexed. Quantification of viable *L. monocytogenes* colonies (CFU/ml) was performed with surface plating method using non-selective tryptic soy agar (Oxoid) supplemented with 0.6 % yeast extract (TSAYE). Additionally, surface plating on selective Brilliance Listeria agar added with Listeria selective and differential supplements (both Oxoid) was done. Injured bacteria can grow on selective medium only after full recovery, whereas they may repair themselves on nonselective medium [30]. Untreated and non-inoculated soup samples were also surface plated as positive and negative controls. TSAYE and Brilliance agar plates were incubated for 2 – 5 days at 30 °C. A mechanical spiral plater (Eddy Jet, IUL Instruments, Barcelona, Spain) was mainly used for counting procedure. Manual plating was also performed to count lower dilutions and to improve sensitivity. Detection limits were 1 log CFU/ml for samples used in regression study and 0.3 log CFU/ml for storage test samples.

Regression modelling for L. monocytogenes inactivation in model soup

Modelling the effects of combined pressure and temperature treatments were based on a two variable orthogonal central composite design (CCD, 16 runs) (Table 1). CCD is the most frequently used technique in RSM, as it can provide optimal fitting of polynomial models and equal estimations in all directions from the center from a minimal number of experiments [10]. Experimental variables were predetermined with initial trials with the aim of covering the whole inactivation range as well as considering practical relevance. Pressure holding time was chosen as 5 min for all treatments, since a lower process time is desirable for optimal HPP [28]. Pressure (X_1) and temperature (X_2) were selected as variables in the CCD. The lower, middle, and upper levels of each variable were set as −1, 0, and +1, respectively (Table 2). Coding of variables was according to the Eq. 1:

$$x_i = (X_i - X_c)/\Delta X \quad (1)$$

where x_i is the dimensionless coded value of X_i (such as x_1: -1 for X_1: 400 MPa); X_0 is the value of X_i at the center point (500 MPa and 30 °C), and ΔX is the step change (100 MPa and 10 °C).

A regression model was built with a second-order polynomial equation (Eq. 2):

$$Y = B_0 + B_1 x_1 + B_2 x_2 + B_{12} x_1 x_2 + B_{11} x_1^2 + B_{22} x_2^2 \quad (2)$$

where Y is the predicted response ($\log_{10}$ (N_0/N) with N_0 as the inoculum level and N as the number of surviving bacteria after HPP treatments. Regression coefficients in Eq.2 included a constant (B_0), linear terms (B_1 and B_2), interaction term (B_{12}) and quadratic terms (B_{11} and B_{22}).

Model analysis

The deduced model was assessed by a comparison with an earlier model [10] obtained under similar process conditions for *L. monocytogenes* inactivation in milk buffer. In order to do so, the modified validation indexes for accuracy (Eq. 3) and bias (Eq. 4) of the models relative to each other were calculated based on Baranyi et al. [29]:

$$\text{Accuracy factor } (A_f') = 10^{\frac{\sum|\log(Y_1/Y_2)|}{n}} \quad (3)$$

$$\text{Bias factor } (B_f') = 10^{\frac{\sum\log(Y_1/Y_2)}{n}} \quad (4)$$

where Y_1 and Y_2 are the predicted inactivation values from the current and the earlier [10] model, respectively. Prediction points were selected among the treatments in this study since pressure holding time was included in the latter model but not vice versa. An ideal predictive model is expected to have $A_f = B_f = 1$, showing a precise correlation [13].

Storage test for L. monocytogenes recovery in model soup

Samples were pressurized in a separate experiment than regression study using the same sample inoculation and packaging protocols. For this purpose, eight soup replicates with three inoculum levels of *L. monocytogenes* (10^3, 10^5 and 10^8 CFU/ml) were selected. Pressure-temperature combinations of 500 MPa – 50 °C, 550 MPa – 50 °C, 600 MPa –40 °C, 600 MPa – 45 °C and 600 MPa – 50 °C were applied in order to have minimal microbial survival after treatments. Following HPP treatments, samples were repacked and stored aerobically at 4.0 ± 0.5 and 8 ± 0.5 °C in a cooling cabinet (Porkka CM 710 F, Huurre Group OY, Finland) without exposure to light. A temperature logger (Tracksense ®Pro, Ellab AS, Hillerød, Denmark) was used for tracking temperature in the storage chamber during the whole experiment. Microbial recovery in the model soup was determined immediately after pressurization (I), and after 7, 14 and 21 days of storage according to the presence of *L. monocytogenes* colonies (growth/no-growth) in samples.

Statistical analysis

The General Linear Model (GLM) and Tukey's HSD test were used for comparing the results of different treatments at a 95 % significance level using JMP Pro 11 (SAS Institute, Cary, USA). As for the storage test, cell recovery was quantified by the number of sample replicates with colony presence (growth/no-growth). Estimation of regression equation coefficients for *L. monocytogenes* inactivation according to the CCD was carried out using JMP Pro 11 (SAS Institute). The goodness-of-fit for the regression model was assessed by the adjusted coefficient of determination (R^2_{adj}). The model performance to explain variability in observations was determined by mean square error (*MSE*). The statistical significance of the model variables and the fitted model were determined by the *P*-values derived from the Fisher's *F*-test. For this purpose, backward stepwise linear regression was performed to find significant model variables ($P < 0.05$). Consequently, a response surface contour plot was obtained from the deduced model. Visualization of combined pressure and temperature effects on bacterial inactivation in the model soup was realized thereof.

Results and discussion

Bacterial inactivation data

Experimental results clearly indicated that combining high pressure and elevated temperatures gave an additional lethality effect on *L. monocytogenes* compared to high pressure alone. Selected process variables accomplished a reduction range of 0.40 – 7.44 log with a mean of 4.18 for *L. monocytogenes* in the model soup. Initial cell count before treatments was approx. 10^8 CFU/ml. Mean values of observed inactivation data were obtained from eight sample replicates (n=8) from two independent trials. Experimental variability was generally low. Summary of trial results at selected pressure and temperature combinations according to the CCD is shown in Table 2.

Experimental data indicated that *L. monocytogenes* in the model soup was sensitive to varying levels of pressure and temperature. Pressure and temperature showed a clear synergistic effect when used in combination as reported previously [9,10,31-33]. Chen [34] observed that pressure sensitivity of *L. monocytogenes* on turkey breast was increased at initial sample temperatures ≥ 30 °C. In this study, comparable results were obtained with the exception of 358.6 MPa – 30 ° C treatment. Studies performed with microscopy and flow cytometry suggested that up to 400 MPa, various Gram-positive bacteria such as *L. monocytogenes* can maintain cell integrity and metabolic activity, consequently a limited inactivation takes place [17,18,35].

Regression modelling

Independent effects of varying high pressure and temperature levels on *L. monocytogenes* inactivation were evaluated by a stepwise backwards regression procedure. Hence, a quadratic equation quantifying the effects of high pressure and temperature on bacterial inactivation was deduced at a fixed level of 5 min pressure holding time. Experimental values were generally in agreement with predicted values (Table 2). The quadratic term for temperature and the interaction term did not show significant effects for the model ($P > 0.1$), therefore they were excluded from the final regression equation. Summary of analysis of variance (ANOVA) for the selected model is shown in Table 3. A high goodness-of-fit (R^2_{adj}=0.95) and a low *MSE* value (0.20) indicated that the model explained the variability in observations relatively well. Previous similar models on *L. monocytogenes* inactivation in milk [10] and in

0.1 % peptone water [9] had R^2_{adj} of 0.98 and 0.85; and *MSE* of 0.039 and 0.30, respectively. The model was significant (*F*-value = 101.6, $P < 0.0001$). Consequently, an empirical equation for the regression was obtained (Eq. 5):

$$Y = 4.339 + 2.626x_1 + 0.993x_2 - 0.356x_1^2 \quad (5)$$

Based on the regression model, a response surface contour plot was obtained showing the predicted bacterial inactivation (as log reductions) in Fig. 1. The highlighted area in the figure visualizes pressure-temperature combinations that are expected to give > 6 log reduction of *L. monocytogenes*. This was achievable in the pressure-temperature ranges of 525 MPa / 40 °C to 600 MPa / 25 °C in the model soup. According to the deduced model (Fig. 1), approx. 1.5 – 2.0 log increase in *L. monocytogenes* inactivation was attained from 20 °C to 40 °C (at the same pressure level) in the model soup after a 5 min holding time. Ritz et al. [32] reported that a temperature rise from 20 °C to 40 °C corresponded to a 1.2 log increase in *L. monocytogenes* inactivation by HPP in buffer based on a fractional experimental design. RSM model by Gao et al. [10] observed 1.0 – 1.5 log inactivation increase for *L. monocytogenes* in milk, when temperature was increased from 30 to 50 °C at the same pressure level with a 11 min holding time. With a thermal process, 2.2 – 4.8 min heating at 58 °C would be needed to achieve 1 log reduction of *L. monocytogenes* in milk [36,37]. Hence, a clear positive relationship between high pressure and elevated temperatures for *L. monocytogenes* inactivation is seen. It should be noted that a thermal process at 70 °C for 2 min is recommended for 6 log reduction of *L. monocytogenes*, considering food matrixes that provide unusually high bacterial heat resistance [38]. Our product also contained high concentrations of milk and fat that are known to increase pressure and heat resistance of microorganisms.

Model analysis

The relative accuracy factor (A_f') and the bias factor (B_f') obtained for the model in the current study are shown in Table 4. Calculation of these parameters was based on the predicted values from Eq.5 (Table 2) and the model by Gao et al. [10]. Predicted values for our CCD variables showed low uncertainty when applied to the earlier model [10] (Table 4). Finding the uncertainty in regression models is recommended to evaluate whether data interpolation or extrapolation causes high deviations in prediction [39]. A_f and B_f were calculated as 1.45 and

0.79 according to Eq. 3 and 4, respectively. Ross et al. [40] proposed an upper limit of 1.60 for A_f but ideally being ≤ 1.40. Dalgaard [41] recommended B_f to be in the range of 0.75 – 1.25 in seafood spoilage models. It is suggested that B_f values in the range of 0.70 – 0.90 can be considered acceptable; and 0.90-1.05 good [42]. According to our analysis (Table 5), A_f was below the upper limit (1.45 < 1.60) and B_f was in the acceptable range (0.70 < 0.79 < 0.90). It is important to note that certain amount of bias could also be generated by the error in viable counts [42]. In this study, the uncertainty in predicted and experimental values was generally low.

Sublethal injury of L. monocytogenes

Testing *Listeria* growth on a selective medium after HPP treatments has previously been used as a method for detection of injured cells [7,33,43]. Following treatments used in regression modelling, surviving colonies of *L. monocytogenes* were also counted on selective Brilliance medium in order to investigate cell injury with respect to pressure and temperature. Sublethal injury was determined with the difference between viable counts on TSAYE and Brilliance (P < 0.05). Most treatments resulted in 0.8 – 2.2 log differences, indicating that approx. 85 to 99.5 % of the cell population was sublethally injured (Fig. 2). We previously observed that only 0.01 to 0.001 % of *L. innocua* population was not sublethally injured in the same model soup (used in this study) after mild thermal treatments at 62, 65 and 68 °C [24]. Another investigation observed that mild heating at 60 °C for 20 min resulted in less than 60 % sublethal injury of *L. monocytogenes* in TSBYE [44]. It seems that bacterial suspension medium is influential since higher levels of sublethal injury were detected in our observations in soup. Both cell inactivation and injury after 400 MPa – 20 °C treatment was minimal apparently due to a mild process effect. On the other hand, 600 MPa – 40 °C was the most intensive treatment that gave the highest mean log reduction and lowest sublethal injury, as the results on TSAYE and Brilliance agar were not significantly different (P > 0.05). Results in this study indicated a lower level of injury was achievable under combined high pressure and temperature treatments.

In previous studies, comparable results on the effects on sublethal injury of *L. monocytogenes* were reported after exposure to combined high pressure and temperature treatments. In a recent study, lower inactivation of *L. monocytogenes* in milk was seen on both selective and nonselective media under milder treatments (for instance 400 MPa – 27 °C), but vice versa with more severe treatments (such as 600 MPa – 43 °C) [33]. Similarly

also in peptone water, *L. monocytogenes* inactivation was dramatically increased after pressurization at 45 °C both on selective and nonselective media in contrast to treatments at 25 and 35 °C under 207 MPa for 5 min [31]. The mechanism for the effect of temperature on cell injury during pressurization was proposed as the membrane fluidity of *L. monocytogenes* is more easily disrupted beyond its optimal growth temperatures (30 – 37 °C) [45].

Higher temperatures during pressurization showed a favorable effect for enhancing bacterial inactivation and minimizing sublethal injury in the model soup in accordance with previous studies. On the other hand, recovery of *L. monocytogenes* in milk, buffer or broth media within hours to several weeks after exposure to HPP has been documented previously [12,19,21,30,46]. Bozoglu et al. [30] coined the terms structural injury (*I1*) for HPP-treated cells that can grow on non-selective but not on selective agar, and metabolic injury (*I2*) for those that cannot initially form visible colonies both on non-selective and selective agar. Presence of *I2* type injury needs to be evaluated with shelf life studies, as it may pose a safety risk with the product of interest (especially with pH ≥ 4.4) and storage conditions (if product is not frozen) [16].

L. monocytogenes recovery in the model soup

The model soup used in this study had a relatively neutral pH (6.1) which makes it challenging to control regrowth of surviving or injured bacteria after processing. It is also known that, *L. monocytogenes* is able grow at refrigerated temperatures. Therefore, a new set of experiments shown in Table 5 were carried out in order to investigate bacterial recovery in the model soup during chilled storage. Based on the results, it was shown that 600 MPa treatments at 45 and 50 °C kept *L. monocytogenes* level below the detection limit in the model soup stored at 4 or 8 °C during three weeks. This was when initial counts were 10^3 and 10^5 CFU/ml (Table 5). When samples were inoculated with 10^8 CFU/ml, lesser number of replicates recovered with more severe treatments but complete inhibition of bacterial recovery was not possible (Table 5). A previous study demonstrated that higher pressure levels and holding times were required to prevent *L. monocytogenes* recovery in phosphate-buffered saline when samples were spiked with higher initial counts [19]. In the current study, all recovered samples increased by approx. 9 log CFU/ml both on selective and nonselective media after 7, 14 and 21 days. Bozoglu et al. [30] proposed that repair of metabolic injury (*I2*) may take longer time (up to 6 days based on their experiments). However, once repair is completed, cells can rapidly grow both on selective and nonselective media. Hence, detection

of structural injury (*I1*) can become difficult on agar media. We also observed that recovered cells grew by the same level (~9 log units) both on selective and nonselective agar after 7 days which may indicate that *I2* type injury could be present in our system.

Koseki et al. [20] observed that although *L. monocytogenes* was initially completely inactivated in milk after pressurization at 550 MPa for 5 min, cells increased by > 8 log units following 28 days storage at 4 °C regardless of the inoculum size (3, 5 and 7 log CFU/ml). In the same study, mild heat treatments at 37 – 50 °C for 10 – 240 min after the HPP treatment prevented bacterial recovery during 70 day storage at 25 °C. Furthermore, Scolari et al. [5] obtained a synergistic effect with combined high pressure and temperature treatments below 15 °C. Based on these observations, bacterial survival during pressurization seems to be largely influenced at suboptimal conditions, both below and above optimal bacterial growth temperatures.

A mechanism for the synergistic effect of temperature (> 37 °C) and high pressure was speculated as membrane damage caused by the latter process might become more difficult to repair as a result of weakened intermolecular forces among membrane molecules [47]. Through a different approach, Smeller [48] proposed a potential link between elliptic nature of pressure-temperature phase diagrams for protein denaturation and inactivation of *Escherichia coli*. Both protein denaturation and bacterial inactivation was shown to increase at a lower or higher temperature than a threshold level. Hawley [49] demonstrated that reversible and irreversible denaturation of proteins were indifferent at temperatures above 45 °C. Apparently, identification of essential proteins affected by combined pressure-temperature through molecular studies may elucidate inactivation mechanisms.

Prior bacterial growth conditions, strain type, treatment severity, recovery medium and storage temperature are among the most significant factors on pressure inactivation kinetics and the recovery time [7,50]. In the current study, late stationary phase cultures grown at 37 °C were used which is expected to add a safety margin since higher baroresistance has been reported for them [51]. The strain used in this study (*L. monocytogenes* ATCC35152) was a relatively barotolerant type, as it was previously shown to recover in BHI within 2 days at 22 °C after up to 900 MPa treatments [21]. Moreover, test conditions in this study covered an industrially relevant range of pressure-temperature combinations. A HPP model soup with a relatively neutral pH (6.1) and high milk content (known to have a baroprotective effect [52]) would be a challenging product. Hence, its effects as a recovery medium would require investigation. Last but not least, previous studies observed different optimal recovery temperatures for *L. monocytogenes* such as 10 °C [53], 15 °C [46,50] and 15 to 20 °C [19].

Therefore, it would be of interest whether lower storage temperatures can restrain cell recovery during the storage. In this study, no effect on cell repair time was seen with respect to results obtained at 4 or 8 °C.

Conclusions

L. monocytogenes inactivation under combined high pressure and temperature treatments was modelled by RSM using a model soup. Statistical parameters indicated that the model was significant (R^2_{adj} = 0.95, $P < 0.0001$). High pressure and temperature showed a synergistic effect and > 6 log inactivation was realizable at pressure-temperature ranges of 525 MPa / 40 °C to 600 MPa / 25 °C. Moreover, pressurization at 600 MPa above 45 °C resulted in no bacterial regrowth in the soup during three weeks at 4 °C and 8 °C, when samples had initial counts of 10^3 or 10^5 CFU/ml. It is necessary to perform a specific inactivation study when developing an industrial process for a refrigerated product. The regression model for the inactivation of *L. monocytogenes* presented in this study may be used for determining the test conditions for soups and sauces with a comparable base recipe. Using HPP in combination with mild heat offers an opportunity to meet consumer demands for minimally processed foods without compromising on FDA requirements (6 log-kill effect on *L. monocytogenes*) [16]. Further inactivation studies on different food matrixes under different combinations of high pressure-temperature treatments can provide valuable data using RSM approach. With those studies, *L. monocytogenes* recovery after treatments should also be investigated. Cellular mechanisms for temperature influence on bacterial inactivation and recovery under high pressure also need to be elucidated.

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Conflict of Interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal objects.

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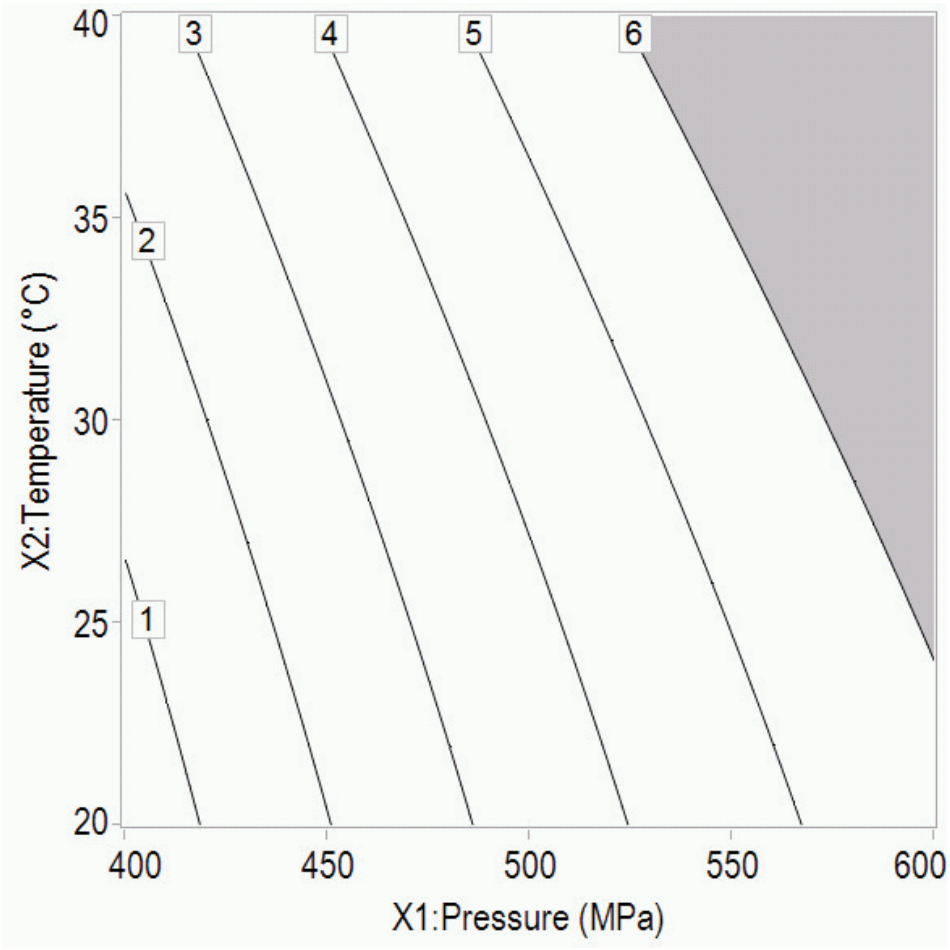

Fig. 1 Contour plot of regression showing combined high pressure and temperature effects on *L. monocytogenes* inactivation (1 – 6 log units) in the model soup by 5 min treatments

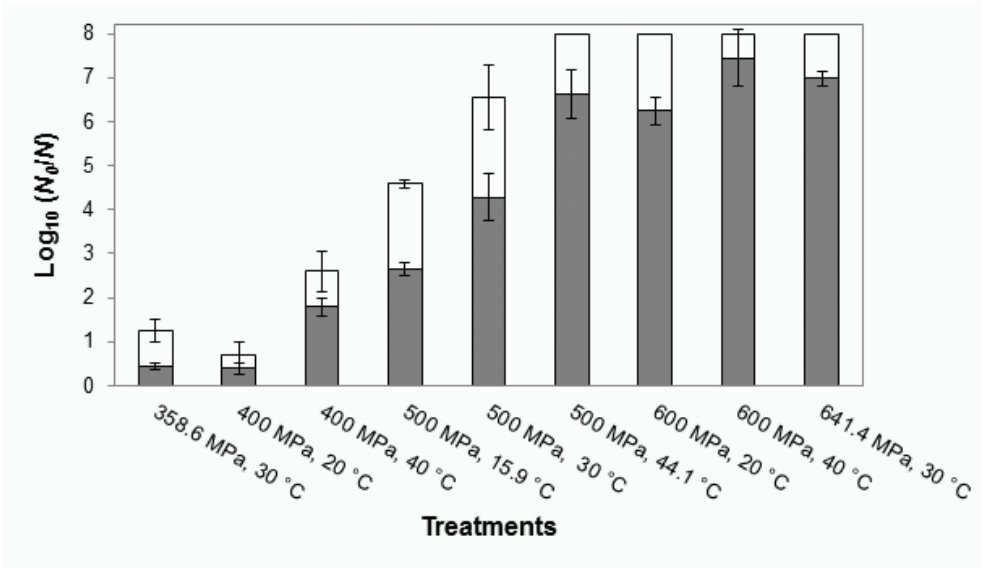

Fig. 2 *L. monocytogenes* inactivation detected on nonselective (TSAYE, gray colored column area) and selective (Brilliance, the whole column) media after treatments according to the regression study (initial count in the soup approx. 10^8 CFU/ml). Error bars show ±1 standard deviation (n=8 for all treatments besides 500 MPa, 30 °C where n=32).

Table 1 Variable codes and levels selected for the Central Composite Design (CCD)

Factor	Symbols		Level[a]				
	Coded	Uncoded	-1.414	-1	0	+1	+1.414
Pressure (MPa)	x_1	X_1	358.6	400.0	500.0	600.0	641.4
Temperature (°C)	x_2	X_2	15.9	20.0	30.0	40.0	44.1

[a] $x_1 = (X_1 - 500)/100$; $x_2 = (X_2 - 30)/10$

Table 2 Observed and fitted responses for *L. monocytogenes* inactivation in model soup after 5 min treatments according to the CCD arrangement

x_1: Pressure (MPa)	x_2: Temperature (°C)	Y: Log_{10} (N_0/N)	
		Observed (sd)	Fitted (sd)
400	20	0.40 (0.13)^{A}	0.39 (0.27)
400	40	1.79 (0.19)^{B}	2.44 (0.27)
600	20	6.24 (0.32)^{C}	5.57 (0.27)
600	40	7.44 (0.64)^{D}	7.63 (0.27)
358.6	30	0.44 (0.07)^{A}	0.00 (0.35)
641.4	30	6.98 (0.16)^{DF}	7.33 (0.35)
500	15.9	2.64 (0.15)^{E}	2.89 (0.27)
500	44.1	6.62 (0.55)^{F}	5.80 (0.27)
500	30	4.34 (0.24)^{G}	4.35 (0.14)
500	30	4.31 (0.25)^{G}	4.35 (0.14)
500	30	4.56 (0.16)^{G}	4.35 (0.14)
500	30	4.34 (0.09)^{G}	4.35 (0.14)
500	30	4.61 (0.35)^{G}	4.35 (0.14)
500	30	4.25 (0.44)^{GH}	4.35 (0.14)
500	30	4.14 (1.32)^{GH}	4.35 (0.14)
500	30	3.75 (0.23)^{H}	4.35 (0.14)

[1]Two independent trials were conducted (n=8)
[2]Values with the same superscript letters did not show significant differences according to Tukey's HSD test ($P > 0.05$).
[3]Colonies were counted on TSAYE agar.

Table 3 ANOVA table for the regression model

Source	Degrees of freedom	Sum of squared errors	Mean squared error	*F* value	*P*
Model	3	63.13	21.04	101.58	< 0.0001
Residual error	12	2.48	0.21		
Corrected total	15	65.61			

$R^2_{adj} = 0.95$, $MSE = 0.20$

Table 4 Predicted values based on the earlier model [10] and calculated relative accuracy and bias factors for the current model

x1 : Pressure (MPa)	x2 : Temperature (°C)	Predicted based on earlier model [10][a]	A_f'	B_f'
400	20	2.43 (0.22)	1.45	0.79
400	40	3.29 (0.09)		
500	15.9	3.81 (0.33)		
500	30	4.15 (0.11)		
500	44.1	5.03 (0.10)		
600	20	5.50 (0.32)		
600	40	6.37 (0.22)		
641.4	30	6.55 (0.33)		

[a] Predicted log reductions of *L. monocytogenes* (standard error)

Table 5 Number of positive replicates immediately after selected treatments (I) and following 7, 14 and 21 days of storage for *L. monocytogenes* in the model soup (n=8)

Pressure (MPa)	Temperature (°C)	Inoculum: 10^8 CFU/ml				Inoculum: 10^3 or 10^5 CFU/ml			
		I	7d	14d	21d	I	7d	14d	21d
500	50	4	8	8	8				
550	50	2	6	6	6				
600	40	2	6	6	6				
600	45	0	4	4	4	0	0	0	0
600	50	0	4	4	4	0	0	0	0

[1] Number of recovered samples was same at 4 and 8 °C.

PAPER IV

Ates, M. B., Skipnes, D., Rode, T. M., Lekang, O. Comparison of spore inactivation with novel agitating retort, static retort and combined high pressure-temperature treatments. *Submitted for publication in Food Control*

Comparison of spore inactivation with novel agitating retort, static retort and combined high pressure-temperature treatments

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Abstract

Optimization of food preservation technologies is necessary for improved product quality and nutrition as well as energy and environmental sustainability. In the current study, inactivation of *Bacillus subtilis* spores in a model soup (pH 6.1) with agitating retort, static retort and combined high pressure-temperature treatments was investigated. With isothermal experiments, $D_{95°C}$ and z values for *B. subtilis* spores in the soup were obtained as 4.67 min and 8.65 °C. Log-linear model performed well for describing isothermal spore inactivation kinetics with satisfactory R^2_{adj} values (0.94 – 0.97). Agitating retort treatments caused a dramatic reduction in processing times, as 17 min processing in agitating mode was required for 7-log inactivation of *B. subtilis* spores, compared to 53 min in static mode at 110 °C. For agitating process, observed and predicted lethality values were similar. This implied a homogenous heat load distribution within the soup with the help of high frequency agitation. Combined HPP and mild temperature treatments were highly synergistic for elimination of *B. subtilis* spores in the same model soup used in heat treatments. HPP treatments combining 650 MPa and 55 – 65 °C for 10 min resulted in up to 4.5 log kill effect on spores. The findings from the current study can be utilized in selection of test conditions for similar products in future safety studies. Results clearly showed that using novel mechanisms in food processing provide an opportunity for milder processing which can lead to better food quality and sustainability.

Keywords:
Agitating retort;
HPP;
Bacillus spores;
Inactivation kinetics

1. Introduction

Thermal processing and high pressure processing (HPP) were introduced as food preservation technologies long time ago by Appert (1810) and Hite (1899). Traditional static heating has some limitations due to slow heat penetration in foods. This often results in significant overprocessing to ensure that a safe product is obtained. Furthermore, inactivation of some bacterial spores requires extremely high pressure levels (>1,000 MPa) which renders this application unfeasible in food industry (H. Zhang & Mittal, 2008). During the last few decades, research efforts have focused on developing milder food preservation technologies (Gao & Ju, 2008). Agitating retort and combined high pressure-temperature (*P*/*T*) processing are among promising next generation technologies as an alternative to the slowly penetrating conductive heating processes (Knorr, 1993; Rosnes, Skara, & Skipnes, 2011).

Survival of spores can be a risk factor in several ready-to-eat (RTE) foods, since this type of products is often exposed to mild treatments. One of major concerns in refrigerated RTE foods with low acidity is the control of *Bacillus* spp. spores due to spore germination at pH >4.6 (Evelyn & Silva, 2015). *B. cereus* can grow at suboptimal temperatures (4–55 °C), pH (4.9–9.3) and a_w (0.92–1.0) and produce heat-stable emetic toxins at >10 °C (EFSA, 2005). Absence of *B. cereus* toxins in foods is recommended as the safety criterion. Initial levels of *B. cereus* in foods is mostly up to 10^3 CFU/ml. However, hazardous toxin levels are produced when bacterial concentration grows to concentrations above 10^6 CFU/g (FDA, 2012). Other strains such as *B. subtilis* and *B. licheniformis* were also reported in foodborne poisoning cases (WHO, 2000). Outbreaks related to *Bacillus* spp. have been associated with starchy foods, milk, cheese, meat and various chilled products such as soups, sauces and salads (FDA, 2012; Evelyn & Silva, 2015)).

Multiple mechanisms generally conserved across *Bacillus* spp. play a role in spore resistance. Among these species, *B. subtilis* has been the most characterized strain with respect to resistance against heat, radiation and chemicals. Critical factors for *Bacillus* heat resistance are mainly based on water and mineral ion content of the spore core, strain type, sporulation conditions and suspending medium (Setlow, 2006). *Bacillus* spores were generally found to be more heat tolerant than non-proteolytic *Clostridium botulinum* type E which is another major risk factor in pasteurized and refrigerated RTE foods.

Inactivation of spores is generally assumed to follow log-linear kinetics (Eq. 1) (Stumbo, 1973; Teixeira, 1992). According to the classical thermal death model (Bigelow and Esty, 1920), temperature-dependent decimal reduction time (D) and z value are used as microbial heat resistance parameters:

$$\log \frac{N}{N_0} = \frac{-t}{D\ (T)} \tag{1}$$

$$z = \frac{T_2 - T_1}{\log D_1 - \log D_2} \tag{2}$$

where log (N_0/N): logarithmic spore inactivation, t : isothermal heating time, and T_1 and T_2 are two different heating temperatures that give D_1 and D_2 values according to Eq. 1.

Deviations from linearity have been reported with survival curves of vegetative pathogens due to mechanisms that are not fully elucidated (Silva & Gibbs, 2010). Inactivation curves may show a slowly accelerating gradient (shoulder) at the initial phase of heating, followed by a linear slope and a braking phase (tail) after a specific heating time point (Cerf, 1977). Presence of shoulders was linked with the time needed for reaching a threshold level of heat damage accumulation on one or more critical spore proteins (Coleman & Setlow, 2009). On the other hand, tailing is attributed to effects from the test method and spore clumping (Furukawa et al., 2005).

Agitating retorts can enhance the heat flow rates within liquid or semi-liquid foods through forced convection. Hence, production of higher quality products can be achieved within shorter processing times (van Loey et al., 1994). Earlier agitation methods (axial and end-over-end rotation) provided a rotation speed range of 20-40 rpm (Knap & Durance, 1998), whereas the most recent reciprocating model enables greater frequencies up to 320 rpm (Rosnes et al., 2011). The recent retort model with longitudinal reciprocating agitation caused a significant processing time reduction for *Listeria* inactivation when compared to static retort (Ates, Skipnes, Rode, & Lekang, 2014). However, inactivation studies with spores are required for evaluating the efficacy of the new agitating retort at higher temperature ranges.

HPP can deliver a uniform effect on foods within rapid processing times, thereby overcoming challenges related to non-stationary conditions observed in conductive processes (Patterson, Quinn, Simpson, & Gilmour, 1995). Although spores are often barotolerant, combining HPP with heat, antimicrobials, ultrasound, pulsed electric fields and irradiation has a synergistic effect on elimination of spores (Evelyn & Silva, 2015; Olivier, Smith, Bull, Chapman, & Knoerzer, 2015; Siemer, Toepfl, & Heinz, 2014; H. Zhang & Mittal, 2008). Inactivation of vegetative organisms in foods with HPP has already found several applications in food industry (Georget et al., 2015). Further spore inactivation studies in foods under combined *P*/*T* would be useful for establishing a database similar to that of thermal inactivation (Serment-Moreno, Barbosa-Canovas, Torres, & Welti-Chanes, 2014).

Survival of *Bacillus* spp. spores in foods after processing poses a challenge to the food industry. In low-acid foods (pH > 4.6), spores can germinate and grow to hazardous concentrations to produce toxins even under chilled conditions (Ju, Gao, Yao, & Qian, 2008; Silva & Gibbs, 2010). Studying microbial inactivation in inoculated products is a useful tool for assessment of food safety with novel processes (NACMF, 2010). It is also known that spore resistance can be influenced from food composition both under thermal and high pressure processing (Georget et al., 2015; Smelt & Brul, 2014). Therefore, the objective of this study was to investigate inactivation of *B. subtilis* spores in a model soup under isothermal conditions, dynamic heating (static and agitating) and combined *P*/*T* processing.

2. Materials and methods

2.1 Study design

Firstly, log-linear and modified Gompertz models were fitted for inactivation of *B. subtilis* spores under isothermal heating at 95 – 110 °C. Then, spore inactivation with retort treatments was compared to estimated lethality based on isothermal inactivation kinetics. Lastly, effect of selected *P*/*T* treatments on spores was studied. All treatments were performed in duplicate (using two spore batches) with three technical repeats ($n = 6$).

A sterile soup (pH 6.1) was used as a model product for all treatments. The model soup contained water (38.8 %), whole milk (36.1 %), cream (12 %), flour (4.5 %), fish bouillon

(2.4 %), salt (0.6 %), lemon juice (0.6 %), garlic powder (0.6 %) and sugar (0.4 %) (w/w). Free amino acid (FAA) content of the soup was analyzed in order to detect if any potential spore germinants were present at a significant concentration (Table 1).

2.2 Spore preparation

As a well characterized prototrophic derivative of *B. subtilis 168* strain, *B. subtilis* PS533 spores (kindly donated by Dr. P. Setlow, University of Connecticut) were used in all inactivation experiments. Prior to their use, spores were maintained in Microbank (Pro-Lab Diagnostics, Canada) at -80 °C. Initial pure culture was grown overnight in lactose broth (LB) medium (Oxoid, Hampshire, UK) at 37 °C, 150 rpm. Sporulation was made according to the method described by Nicholson and Setlow (1990) in 1 L flasks filled with 200 ml liquid 2 × SG medium at 37 °C, 225 rpm within 5-7 days. Harvested spores were centrifuged and washed multiple times until the resulting suspension had > 98 % purity. Presence of germinated spores or cell debris was determined with phase contrast microscopy. Spores were suspended in sterile MiliQ water (10^{10}-10^{11} spores/ml) and stored in the refrigerator without exposure to light for a limited time.

2.3 Sample preparation

Preparation of inoculated samples is summarized in Table 2. The model soup was too dense for injection into capillary tubes used in isothermal treatments. Hence, it was diluted (1:10). Dimensions of the trays used in retort treatments were 9 × 4 × 13.2 cm (Promens, Kristiansand, Norway). Trays were then thermosealed with a 90 µm PA/PP film layer (Dynopack, Kristiansand, Norway). All HPP samples were vacuum packed (Webomatic, Bochum, Germany) and heat-sealed after filling. All samples were vortexed, shaken or mixed in a stomacher machine in order to have even spore distribution and kept at 1 °C shortly before treatments.

2.4 Isothermal heat treatments

The capillary tube method was used for determination of spore inactivation kinetics (Stern & Proctor, 1954). Inoculated samples (100 µl) were aseptically injected into autoclaved 200 µl micropipettes (Blaubrand, Wertheim, Germany). Each glass tube had 0.025 mm diameter and

125 mm length. The tubes were then heat sealed about 20 mm from the sample at open ends. Samples for each time-temperature point were exposed to isothermal heating by fully immersing them in a circulating oil bath (±0.05 °C). Samples were removed after 5–35 min at 95 °C; 1–10 min at 100 °C; 0.5–2.5 min at 105 °C; and 15–60 s at 110 °C. The come-up time for the samples (< 5 s) was excluded from the total heating time. At the end of heating, capillary tubes were cooled on ice slurries and washed with ethanol. Before microbial analysis, both ends of the capillary tubes were aseptically cut and each sample was serially diluted in 0.9 ml 0.1% peptone water (Merck, Darmstadt, Germany). Each treatment was repeated with two spore batches (n = 6).

2.5 Nonisothermal heat treatments

For nonisothermal heat treatments, a pilot-scale single basket water cascading retort operable in static/agitating modes (Steriflow, Roanne, France) was used. Retort temperature increase was supplied by direct steam injection and preheated water. For detailed descriptions on the retort process, see Ates et al., 2014. Three samples were processed in two process batches (n = 6). In each batch, time-temperature history at the coldspot of three additional samples was logged with E-Val Flex data acquisition system (Ellab A/S, Hilleroed, Denmark). A negative control (non-inoculated sample) was also processed along with inoculated samples. Summary of agitating and static heating setups are shown in Table 3. Retort pressure was 1.8, 2.0, 0.3, 0.2 bars during come-up, holding, cooling 1 and cooling 2 phases. Following treatments, samples were immediately placed on ice slurries and analyzed on the same day.

2.6 Modeling thermal inactivation of spores

2.6.1 Inactivation kinetics under isothermal conditions

D values for *Bacillus* spores at 95 – 110 °C were calculated with Eq. 1. Fitting procedures were carried out using data points above the detection limit. Combining Eq. 1 and Eq. 2 enabled calculation of the z value with a nonlinear regression (Eq. 3):

$$Log\frac{N}{N_0} = \frac{-t}{D_{ref}10^{\frac{T_{ref}-T}{z}}} \quad (3)$$

where D_{ref} was the D value at the selected reference temperature (T_{ref}) of 95 °C.

Gompertz model was previously recommended for assaying sigmoidal inactivation kinetics (Gil, Brandao, & Silva, 2006). It also performed better for fitting *B. coagulans* spore inactivation curves with capillary method (Zimmermann, Miorelli, Schaffner, & Aragao, 2013). Hence, modified Gompertz-inspired model based on Huang (2009) was selected for a comparison with log-linear model:

$$Log\frac{N}{N_0} = logN_0(-e^{-e^{-\mu(t-M)}}) \quad (4)$$

where μ : relative inactivation rate (s^{-1} or min^{-1}) and M : time constant (s or min). Other thermal inactivation models have been reviewed by Smelt & Brul (2014) for interested readers.

2.6.2 Estimation of process lethality under nonisothermal conditions

Ball (1927) introduced the *F*-value concept for sterilization of cans, which has been applied in the food industry with great success. *D* and *z* values obtained with Eqs. 1 and 3 were integrated to dynamic conditions for predicting process lethality with agitating and static retort treatments using Ball method (Eqs. 5 and 6). Dynamic time–temperature data at the product coldspot was obtained with E-Val Flex data acquisition system (Ellab). Threshold value where inactivation of *B. subtilis* spores assumed to initiate was set to 90 °C.

$$F = \int_0^t L(T)\ dt \quad (5)$$

$$L(T) = \frac{1}{D_{ref}}\int_0^t 10^{\frac{T_c - T_{ref}}{z}}\ dt \quad (6)$$

where $L(T)$: process lethality as a function of time-temperature data, t : total treatment time (min), T_c : temperature at the product coldspot (°C) and T_{ref} : 95 °C.

2.7 Combined high pressure-temperature treatments

HPP was carried out using the QFP 2L-700 (Avure Technologies Inc., Columbus, USA). The cylindrical pressure vessel had 10 × 25.4 cm dimensions, 2 L capacity and 690 MPa upper pressure limit. Mild temperatures above 45 °C during pressurization previously showed a synergistic effect for preventing *Listeria* survival in the same model soup (unpublished results). Hence, selected treatments were 650 MPa / 20 °C, 650 MPa / 55 °C, 650 MPa / 60 °C, 650 MPa / 65 °C. A holding time of 10 min was used. Temperature of the pressure medium (water) was tracked with a K-type thermocouple located on the external surface of samples. Pressure come-up rate and release times were 250 MPa/min and < 1 sec. Samples were preheated to the selected initial temperature within 5 min before treatments. Samples were kept in ice slurries before and after all treatments before microbial analysis.

2.8 Microbial analysis

Immediately after treatments, aliquots of 1 ml soup from each sample were serially diluted (1:10) in 0.1 % peptone water and vortexed. Quantification of *B. subtilis* colonies (CFU/ml) was performed with surface plating method using polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) medium selective for *Bacillus* (Oxoid, UK) (Chon, Song, Kim, & Seo, 2014; van Netten & Kramer, 1992). Untreated and non-inoculated soup samples were also surface plated as positive and negative controls. Agar plates were incubated at 37 °C for 2 to 5 days. This was done to give enough time for repair and germination of surviving spores. A mechanical spiral plater (Eddy Jet, IUL Instruments, Barcelona, Spain) was mainly used for counting procedure. Manual plating was also performed to count lower dilutions and to improve sensitivity. The detection limit for spore counts was 1 log CFU/ml.

2.9 Statistical analysis

Inactivation of *B. subtilis* spores was calculated as $\log_{10}(N/N_0)$, where N_0 was the initial counts in samples and N was the number of survivors on PEMBA plates after treatments. Spores were assumed fully inactivated if no colonies were detected on all sample replicates with the lowest dilution. Tukey's HSD test was used for comparing means at a 95 % significance level. The goodness-of-fit for each linear regression model was evaluated with the adjusted coefficient of determination (R^2_{adj}) and root mean squared error (*RMSE)* along

with *P*-values derived from the Fisher's *F*-test. Performance of nonlinear inactivation models was assessed by root mean square error (*RMSE*). Evaluation of R^2 as a measure for nonlinear models in biological studies is not recommended due to overestimated values (Spiess & Neumeyer, 2010). JMP Pro 11 (SAS Institute, Cary, USA) was used for modelling and statistical analysis procedures.

3. Results and discussion.

3.1 Spore inactivation kinetics under isothermal conditions

Fitted survival curves for *B. subtilis* spores in the model soup under isothermal heating with capillary method are shown in Fig. 1. Representative data in the figure exhibits spore inactivation at 95, 100 and 105 °C. Inactivation rate was close to log-linear at 95 – 105 °C (Fig. 1a). This was confirmed with satisfactory linear regression coefficients (R^2_{adj}) that ranged from 0.94 to 0.97. When *RMSE* values were compared, Gompertz model performed better than the linear model at 100 and 105 °C, but vice versa at 95 °C (Fig. 1). *RMSE* values were 0.45, 0.35, 0.39 for the Gompertz model; and 0.38, 0.48, 0.60 for the linear model at 95, 100 and 105 °C, respectively. Overall, linear model predicted *B. subtilis* inactivation with a slightly lower uncertainty at 95 °C compared to 100 and 105 °C. This effect is possibly due to longer heating time at 95 °C which can reduce the variation among time-temperature points.

Using a higher heating temperature provided increased inactivation rate (Fig. 1). *D*-values were obtained as 4.67 (0.23), 1.02 (0.06), 0.23 (0.02) and 0.15 (0.03) at 95, 100, 105 and 110 °C, respectively. The values in parentheses are standard deviations at a 95 % confidence level. The *z* value was calculated as 8.65 °C with a standard error of 0.07. In previous studies, $D_{95\,°C}$ and *z* values for *B. cereus* and *B. subtilis* spores were generally found in the ranges of 1.2 – 36.2 and 2.7 – 54 min; 6.7 – 10.1 °C and 6.1 – 9.3 °C on various media (Condon, Palop, Raso, & Sala, 1996; Conesa, Periago, Esnoz, Lopez, & Palop, 2003; ICMSF, 1996; Kort et al., 2005; Nakayama, Yano, Kobayashi, Ishikawa, & Sakai, 1996; Serp, von Stockar, & Marison, 2002). It should be noted that some *B. cereus* spores from outbreaks were found to have very high heat resistance ($D_{100°C}$ = 27 min) (EFSA, 2005). For the same *B. subtilis* strain used in this study, $D_{95°C}$ was found 4.35 – 5.76 min milk (Jagannath, Tsuchido, & Membre, 2005).

Heat resistance of the strain used in the current work was generally within the range of previously reported results.

Survival curves at 100 and 105 °C were slightly sigmoidal shaped as illustrated in Fig. 1b. This was the reason for lower *RMSE* values obtained with Gompertz model in those cases. Various studies with vegetative cells and some studies with spores reported survival curves with shoulders and tails (Gil, Miller, Brandao, & Silva, 2011; van Zuijlen et al., 2010; Zimmermann et al., 2013). However, long shoulders and tails were not observed in the current work. This could be due to effects from the bacterial strain, absence of spore clumps, and rapid come-up times (< 5 s) in tubes. Shoulders are most apparent under conductive heating processes with viscous products in larger containers due to longer come-up times (Juneja & Huang, 2004).

There was a linear relationship between temperature and Gompertz model heat resistance parameters (μ, M) as shown in Fig 2. The same linear correlation was previously observed between temperature and modified Gompertz and Weibull models parameters (Huang, 2009; Zimmermann et al., 2013). When D value was replaced with μ, M in Eq. 2, inverse of the slope gave 8.8 and 8.6 °C. These values were similar to the z-value obtained in the current study (8.65 °C). It would be useful to investigate whether μ shows a similar trend with different vegetative cells and spores. Hence, μ can be incorporated to nonisothermal inactivation models more conveniently. This can be useful for implementation of such nonlinear models in thermal processing.

3.2 Spore inactivation under nonisothermal heating with retort treatments

Agitating mode provided a faster heating at the coldest point of soup compared to static mode for all experiments. As a representative for other heat penetration curves, time-temperature history of retort water and soup core temperatures are shown (Fig. 3). It was apparent that static process required a longer cooling time than agitating process to reach lower temperatures than the used levels (8 and 18 min for agitating and static modes, respectively). Risk of spore germination was minimized by immediately submerging the samples in ice slurries following heat treatments.

Spore inactivation data clearly indicated that agitating mode dramatically reduced heating times required for equivalent level of *B. subtilis* spore inactivation in the soup when compared to static mode (Fig. 3). When come-up and holding times were taken into account, 7-log inactivation of spores took place after 9 and 35 min treatments in agitating and static modes at 110 °C. Reduction of *Listeria* by 8-fold took place after 11.5, 6.8, 5.5 heating in agitating mode compared to 77, 67 and 52 min heating in static mode at 62, 65 and 68 °C, respectively (Ates et al., 2014). These findings indicated that utilizing high frequency agitation in retorts is an effective method for inactivation of vegetative cells and spores in liquid products. Attempts on optimization of heat treatments can be a valuable option for energy and environmental sustainability without compromising on food safety (van Zuijlen et al., 2010).

Results illustrated in Fig. 4 showed that traditional *F*-value concept was applicable for estimation of spore inactivation with agitating retort treatments. This was based on similar predicted and observed lethality values with agitated heating. In our previous study, predicted lethality and observed inactivation for *Listeria* in the same model soup was also correlated (Ates et al., 2014). Static retort treatments underestimated the process lethality after 20 and 25 min treatments. As expected, varying temperature zones within the product in static heating may lead to underestimations. Furthermore, slow heating rates (1 °C/min) caused underestimated predictions for *B. subtilis* and *B. sporothermodurans* at pH 7.0 (Conesa et al., 2003; Esteban, Huertas, Fernandez, & Palop, 2013). This may also be an additional factor for longer heating times required in static retort treatments. Overall, results implied that high frequency reciprocal agitation enabled a uniform lethality effect over the whole soup. However, liquid products with solid particles must be evaluated for safety in future studies.

Food composition effects should be taken into account when interpreting results from spore inactivation studies. The model soup used in this work was low in salt content (0.6 %) and acids (pH 6.1) and it contained relatively high amount of fat (8 – 8.5 %). Heat resistance of *Bacillus* spores can be higher in this type of product. This is based on lack of suppressing effects from acidity and salt and protective effect of lipids (Esteban et al., 2013). Heat inactivation kinetics of *B. subtilis* spores greatly differed in milk and soy sauce (Jagannath et al., 2005). Authors detected that inactivation kinetics in buffer was able to predict inactivation in milk well but not soy sauce and kayu (a traditional food). In the current study, isothermal inactivation kinetics of *B. subtilis* in diluted soup was successful in predicting process lethality in the soup with agitating retort treatments.

Spore preparation protocol can also influence the spore heat resistance. It was shown that low aeration, low calcium content, higher incubation temperature and pH can increase the heat resistance of *B. subtilis* spores (Minh, Durand, Loison, Perrier-Cornet, & Gervais, 2011). We used a well-established sporulation protocol developed by Nicholson and Setlow (1990) in order to facilitate comparison with other published studies. An ideal approach for similar inactivation studies in the food industry would be to mimic the food structure (such as a_w, pH, mineral and salt content) and processing effects (such as temperature, oxygen availability) during spore preparation. Samples were kept at 1 °C before treatments to prevent germination of spores. Presence of free amino acids such as valine and asparagine was much lower in the soup (Table 1) than the levels used for initiating germination of *B. subtilis* spores (10 mM) (J. Q. Zhang, Griffiths, Cowan, Setlow, & Yu, 2013).

It was proposed that *F*-value can lose its additive pattern if the survival curve of the target microorganism is not log-linear (Mafart, Leguerinel, Couvert, & Coroller, 2010). For instance, presence of a shoulder and an upward concavity in isothermal curves could cause overestimations. Estimation of process lethality based on nonlinear inactivation models often poses a challenge due to presence of multiple heat resistance parameters and time dependence of the inactivation model. When the derivative of Eq. 4 was incorporated into Eq. 5, an ordinary differential equation was obtained (Eq. 7) based on Huang (2009). Hence, time variable was eliminated and a single heat resistance parameter (μ) was obtained. As shown in Fig.2, μ showed a linear relationship with temperature. Consequently, a dynamic time-temperature data can be used for lethality estimation with Eq. 7 for *B. subtilis* spores. A numerical method such as Runge-Kutta would be required for solving the equation (van Impe, Nicolai, Martens, Debaerdemaeker, & Vandewalle, 1992). In the future, development of user-friendly computer-aided tools can enable the food industry to use such nonlinear lethality prediction models. This could be used for optimization of heat treatments when microbial inactivation follows nonlinear kinetics on challenging food matrices.

$$L(T) = \int_0^t \mu(T)(Y)\ln(\frac{Y}{Y_0})\, dt \tag{7}$$

where Y : predicted lethality as $\log_{10}(N_0/N)$ and Y_0 : initial log spore counts.

3.3 Spore inactivation under combined high pressure-temperature treatments

Inactivation of *B. subtilis* spores in the model soup under combined *P*/*T* treatments are shown in Fig 4. Log reductions of *Bacillus* spores were 0.48 (0.25), 3.81 (1.02), 4.02 (0.79) and 4.62 (0.25) after 650 MPa treatments at 20, 55, 60 and 65 °C, respectively. Values in parentheses are standard deviations at a 95 % confidence interval. There was no statistically significant difference between the values obtained at 55 to 65 °C. There was a clear synergistic effect between high pressure and mild temperatures for spore inactivation when compared to high pressure or temperature alone. In order to achieve equivalent spore inactivation (4 – 5 log), 1, 4 and 20 min isothermal heating would be required at 95, 100 and 105 °C, respectively. Similarly, a total processing of 16 and 48 min at 110 °C would be needed for the equivalent kill effect with agitating and static retort treatments. A strong synergistic effect between high pressure and temperature was reported for spores of various *Bacillus* strains in Bolognese sauce compared to thermal processing alone (Olivier et al., 2011).

Results shown in Fig. 5 indicated that higher temperatures would be needed for obtaining > 6 log inactivation of *B. subtilis* spores. Since there was no significant difference at initial temperatures between 55 to 65 °C, it would be useful to assay the effect of temperatures below 55 °C and above 65 °C. A 6-log kill effect on *B. cereus* spores was shown within combined *P*/*T* ranges of 500 MPa / 80 °C to 600 MPa / 60 °C in milk buffer (Ju et al., 2008). It was found that optimum process parameters for having 5-log reduction of *B. sporothermodurans* spores were 472 MPa at 53°C for 5 min in presence of 121 IU/ml nisin (Aouadhi, Simonin, Mejri, & Maaroufi, 2013). Synergistic effects from such antimicrobials, germinants and elevated pH can be used for enhancing inactivation of *Bacillus* spores in heat sensitive food products treated with HPP.

Using a sterile soup eliminated any potential spore contamination that could potentially be introduced by the soup ingredients. Especially, components with low a_w such as flours and herbs can carry along dehydrated spores with increased heat resistance. Furthermore, fats and oils can increase barotolerance of spores in a similar way they act in increasing heat resistance (Georget et al., 2015). These effects need to be carefully assessed in future studies. Sporulation conditions were also found to influence high pressure resistance of *Bacillus* spores, albeit at a lower degree than heat resistance (Minh et al., 2011). This is apparently due to possible germination of spores above 350 MPa followed by inactivation of cells under mild

heat and pressure (Black et al., 2007). Olivier, Bull, & Chapman (2012) detected a barotolerance increase when sporulation temperature was increased from 30 °C to 37 °C for *Bacillus* spores. In this work, 37 °C was also used.

4. Conclusions

B. subtilis spore inactivation under isothermal treatments, dynamic heating with agitating/static retort processes and combined high pressure-temperature treatments was investigated. Isothermal heat resistance parameters for *B. subtilis* spores in the model soup were obtained as $D_{95°C}$ = 4.67 min and z = 8.65 °C. These values performed well for estimation of agitating process lethality on *B. subtilis* spores in the soup. This also indicated a homogenous heat load distribution within the product, when agitation was used. Agitated processing at 110 °C gave 7-log reduction of spores in the soup after 17 min processing, whereas the same effect was possible after 53 min processing in static mode. When the same model organism and product were exposed to combined high pressure-mild temperature (*P*/*T*) treatments, a dramatic synergistic effect was observed. Pressurization at 650 MPa and 55 – 65 °C for 10 min provided up to 4.5 log kill effect on spores. The results presented in this work can be used for determination of test conditions on similar products for evaluation of novel agitating retort and combined high pressure-temperature (*P*/*T*) effects. Further studies using more heat-resistant spores (12*D* concept) and different liquid or semi-liquid products (with solid particles) would be required for assessing the applicability of the new agitating retort for sterilization processes. Further investigations on combined *P*/*T* effects when assisted with other germinant systems could also enable processing at milder temperatures.

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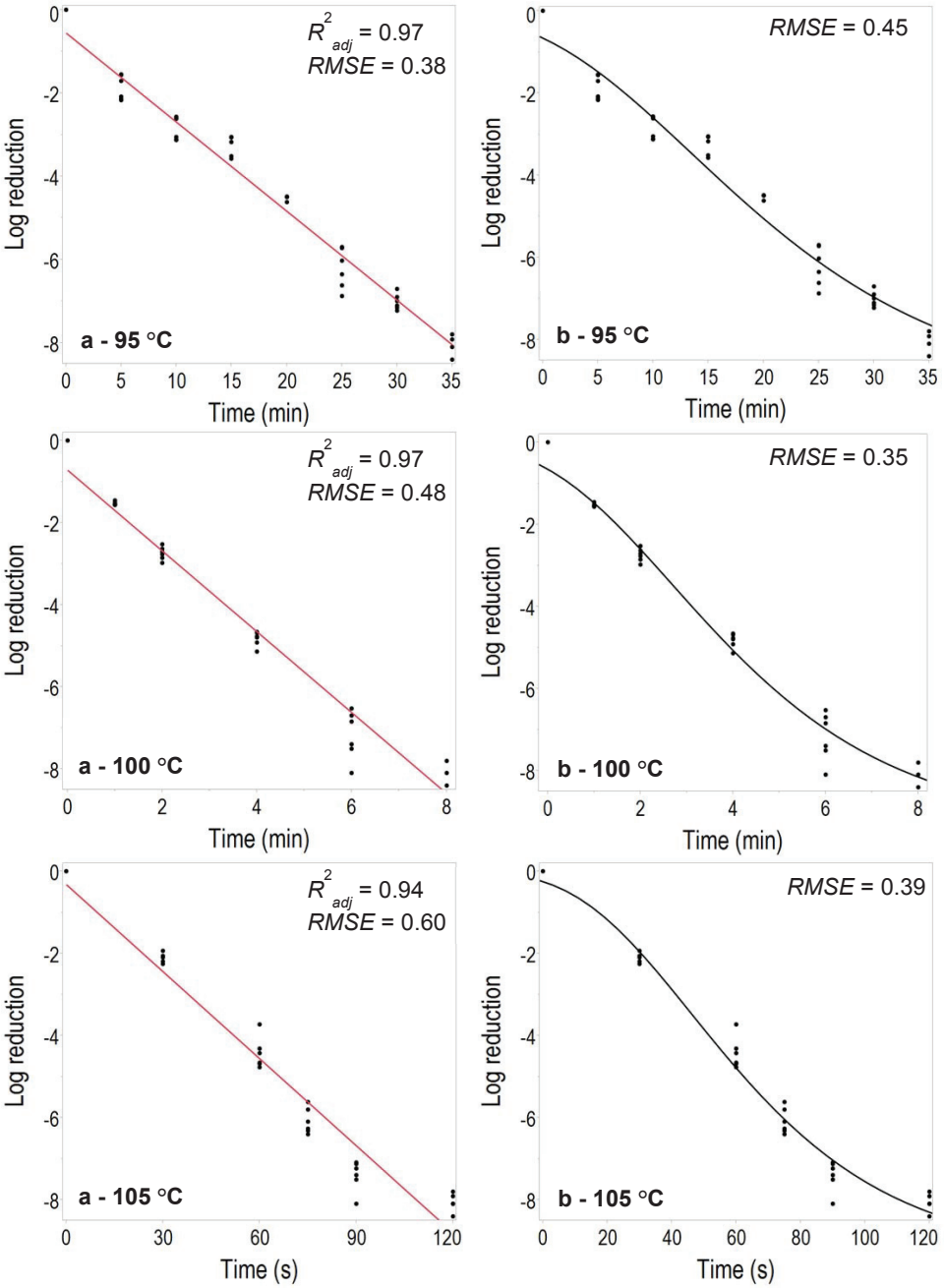

Fig. 1 Isothermal survival curves for *B. subtilis* spores in soup at 95, 100 and 105 °C. Dots are raw data (n = 6). The same data set was fitted to log-linear model (a) and modified Gompertz model (b). Initial count in the soup was 9.5 log spores/ml.

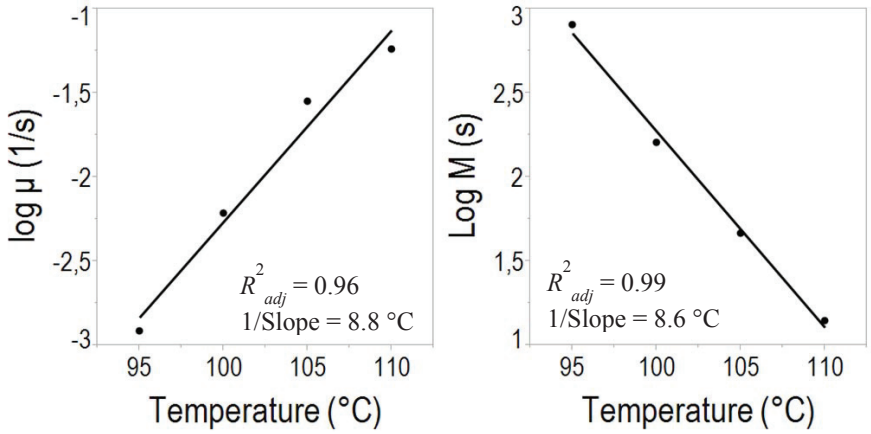

Fig. 2 Temperature effects on μ, M and the corresponding regression lines.

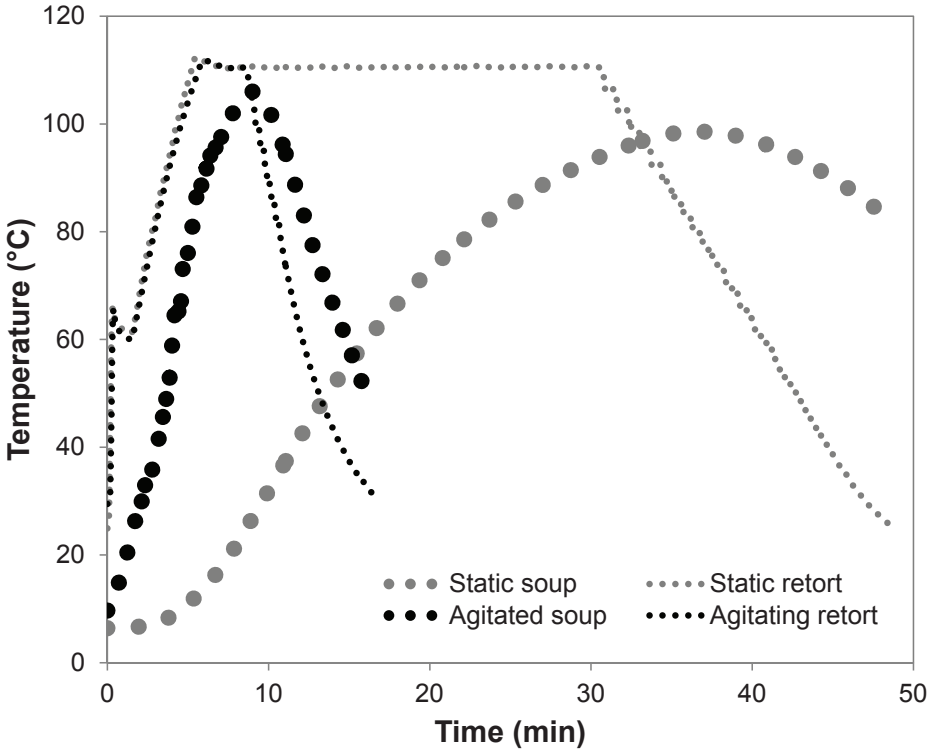

Fig. 3 Time–temperature curves during agitating heating (16 min) and static heating (48 min) at 110 °C

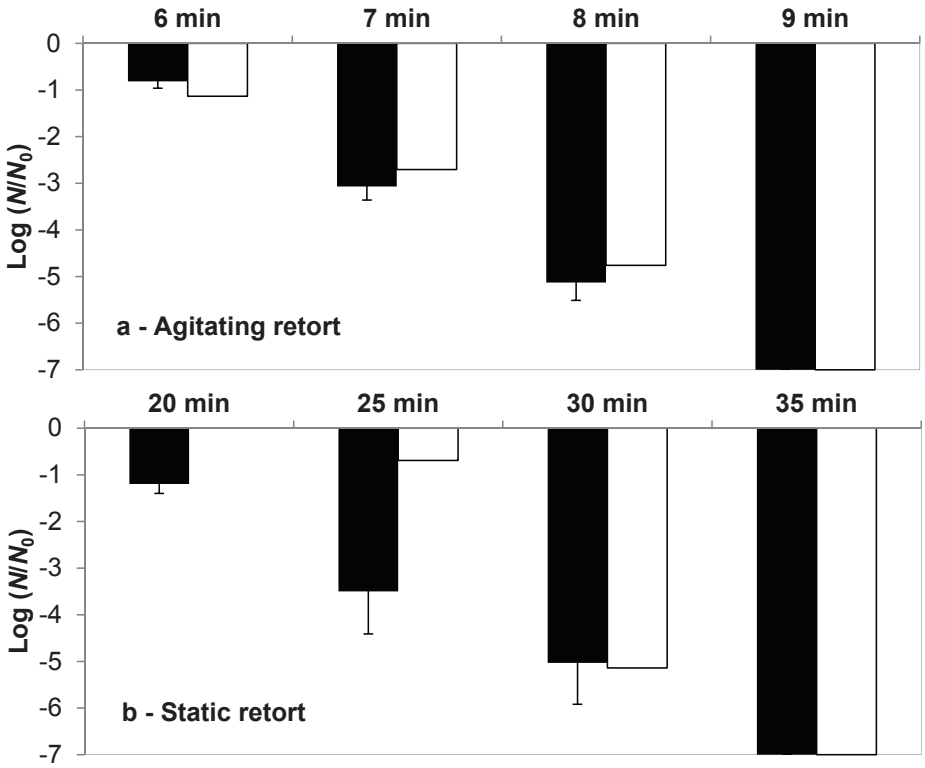

Fig. 4. Inactivation of *B. subtilis* spores in the model soup at 110 °C after (a) agitating (b) static retort treatments. Black bars: observed log-kill and white bars: predicted lethality based on log-linear model (initial count in the soup was 7 log spores/ml). Error bars show ±1 standard deviation (n = 6). Total come-up and holding times are shown. Cooling times were 8 and 18 min for agitating and static treatments.

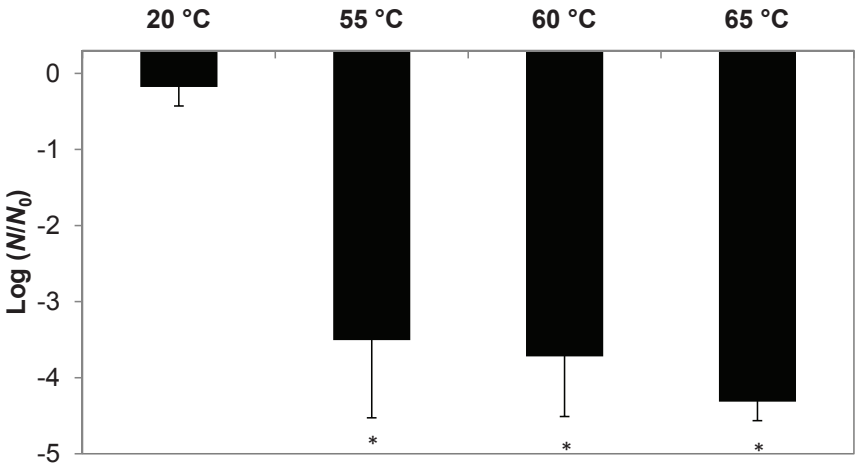

Fig. 5. Inactivation of *B. subtilis* spores in the model soup after combined *P*/*T* treatments of 650 MPa at 20, 55, 60 and 65 °C with 10 min holding time. Initial spore count in the soup was 7 log spores/ml). Error bars show ±1 standard deviation (n=6). Means with an asterisk did not show significant differences according to Tukey's HSD test ($P > 0.05$).

Table 1. Content of free amino acids in the soup (grams per 100g soup)

Type	Level	Type	Level	Type	Level
Creatinine	<0.001	Aspartic acid	0.010	Glutamic acid	0.250
Hydroxyproline	<0.001	Serine	0.009	Asparagine	0.006
Glycine	0.007	Glutamine	0.009	β-Alanine	<0.001
Taurine	0.008	Histidine	0.004	γ-Aminobutyric acid	<0.001
Citrulline	<0.001	Threonine	0.023	Alanine	0.014
Carnosine	<0.001	Arginine	0.036	Proline	0.003
Anserine	0.012	Tyrosine	0.011	Valine	0.009
Methionine	0.005	Cystine	<0.001	Isoleucine	0.005
Phenylalanine	0.007	Tryptophan	0.004	Ornitine	0.002
Lysine	0.016				

Table 2. Summary of sample preparation

Treatment	Initial counts (spores/ml)	Sample size	Sample dilution	Container type
Isothermal	10^9-10^{10}	100 µl	1:10	Capillary tube
Nonisothermal	~10^7	350 g	-	PP Trays
HPP	~10^7	40 ml	-	PE bags

Table 3. Retort treatments setup

	Agitating process			Static process	
Phase	*Temperature (°C)*	*Time (min)*	*Agitation (spm[1])*	*Temperature (°C)*	*Time (min)*
Come-up	20–110	5	80	20–110	5
Holding	110	1/2/3/4	100	110	15/20/25/30
Cooling 1	110–35	5	100	110–35	15
Cooling 2	35–20	3	80	35–20	3

[1] Strokes per minute