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Power Ultrasound and High Pressure Processing Inactivation of Specific Microbial Spores in Foods

by
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A thesis submitted in fulfilment of the requirement for the degree of Doctor of
Philosophy in Chemical and Materials Engineering

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Under the supervision of

Dr. Filipa Silva



DEDICATION

Dedicated to my incredibly wonderful husband Muhamad Yusa, our beautiful and precious daughter Shavina Alifia, my beloved mother and parents in law.

Thank you for your patience, support and love throughout my PhD study.

In loving memory of my father

(1949 – 2004)

Abstract

Bacterial and fungal spores are a great concern in food industries due to their extreme resistance to physical and chemical treatments. Thermal processing at high temperatures often diminishes food quality. Therefore in this study, high pressure processing (HPP) and power ultrasound alone and in combination with heat (HPP-thermal) and thermosonication (TS) were investigated for their abilities to inactivate the spores of *Clostridium perfringens* and psychrotrophic *Bacillus cereus* in low-acid (pH>4.6) foods, and *Alicyclobacillus acidoterrestris*, *Neosartorya fischeri*, and *Byssochlamys nivea* spores in high-acid (pH<4.6) foods. The spore inactivation was compared with thermal processing alone and the inactivation kinetics was modeled.

The 600 MPa HPP-thermal and TS treatments were better than thermal processing alone for the microbial spore inactivation, requiring between 8–30°C lower temperatures to obtain the same lethality. The 600 MPa HPP-75°C was the best technique to inactivate *C. perfringens* in beef slurry, and *N. fischeri*, and *B. nivea* moulds in juice/puree. With respect to *C. perfringens*, spore reductions of ≥ 2 log after 20 min were obtained for HPP-thermal vs. almost no inactivation for TS and thermal processes. Regarding the moulds, the 600 MPa HPP-75°C inactivated ≥ 4 log after 20 min, while the TS and thermal treatments increased the spore numbers by up to 2.5 log.

Regarding *B. cereus* spores, TS was the most effective method to inactivate them in skim milk and beef slurry. Over 15 min, TS caused ≥ 5 log in milk vs 3 log after HPP-thermal and 2 log with thermal process. In beef slurry, TS was actually able to increase the thermal spore inactivation in beef slurry by more than 6 fold.

TS and thermal processing alone at 78°C had no effect on *A. acidoterrestris* spores. However, TS treatment (78°C) of HPP pretreated spores suspended in orange juice increased the spore

inactivation by ≥ 1.6 fold. Lower D -values were obtained at higher acoustic power densities. In addition, heat shock (HS) and ultrasonication pretreatment of the spores doubled the spore thermal inactivation of *C. perfringens* and *A. acidoterrestris*: pretreated *C. perfringens* spores $D_{95^\circ\text{C}} = 9.8$ min vs 22 min in beef slurry; pretreated *A. acidoterrestris* spores $D_{95^\circ\text{C}} = 0.8$ min vs 1.5 min in orange juice.

With respect to overall spore resistance to different technologies at 70-75°C, psychrotrophic *B. cereus* spores were the least resistant. The spores of *B. nivea* and *N. fischeri* showed the highest resistance to thermal treatment over 30 min. *A. acidoterrestris* and *C. perfringens* were more difficult to inactivate with TS processing and *C. perfringens* was more difficult to inactivate with HPP-thermal treatment.

The mould ascospore resistance to HPP-thermal and TS processes increased with increasing spore age. Regarding 4 week old *N. fischeri* spores at 75°C TS or HPP-thermal, 27 min were required for 1 log reduction, whereas 74 min was required to obtain the same spore inactivation for 12 week old spores. With respect to 4 week old *B. nivea* spores, the results were closer. While 13 min were required for 1 log reduction, 29 min were required to obtain the same spore inactivation for 12 week old spores.

The HPP-thermal inactivation for all the microbial spores was well described with the Weibull model, whereas the inactivation kinetics for TS treatment was species/strain/food dependent. The TS inactivation of psychrotrophic *B. cereus* spores in skim milk and *A. acidoterrestris* spores in orange juice followed simple first order kinetics, whereas log logistic and Weibull models described the TS inactivation of *B. cereus* and *C. perfringens* spores in beef slurry, respectively. Lorentzian distribution modeled the 4-10 week old mould spore inactivation with TS treatment. With the exception of thermal inactivation of mould spores at $T \leq 85^\circ\text{C}$, all the spore thermal inactivations followed the simple first order kinetic model.

Abstrak

Spora bakteri and fungi merupakan hal yang sangat mengkhawatirkan bagi industri pengolahan makanan karena resistensi spora yang tinggi terhadap perlakuan fisika dan kimia. Proses thermal pada temperatur yang tinggi sering berdampak buruk bagi kualitas makanan. Oleh sebab itu dalam studi ini, pengolahan tekanan tinggi atau High pressure processing (HPP) and teknik power ultrasound secara tersendiri serta kombinasi dengan panas (HPP-thermal and thermosonication, TS) diinvestigasi untuk menginaktivasi spora dari *Clostridium perfringens* dan psychrotrophic *Bacillus cereus* dalam makanan pH rendah ($\text{pH} > 4.6$), dan *Alicyclobacillus acidoterrestris*, *Neosartorya fischeri*, dan *Byssoschlamys nivea* spores dalam makanan pH tinggi ($\text{pH} < 4.6$). Selain itu, jumlah spora yang terinaktivasi juga dibandingkan dengan proses thermal dan kinetika proses dimodelkan.

HPP (600 MPa)-thermal and TS lebih baik jika dibandingkan dengan hanya menggunakan proses thermal untuk inaktivasi spora, menunjukkan temperatur 8–30°C lebih rendah untuk mendapatkan lethalitas yang sama. HPP pada 600 MPa dan 75°C adalah teknik yang paling baik untuk menginaktivasi *C. perfringens* dalam slurry daging, dan kapang *N. fischeri*, dan *B. nivea* dalam jus/puree buah. Reduksi spora *C. perfringens* ≥ 2 log setelah 20 min dapat diperoleh dengan HPP-thermal vs. hampir tidak ada inaktivasi dengan TS dan proses thermal. HPP pada 600 MPa dan 75°C dapat menginaktivasi kapang ≥ 4 log setelah 20 min, sementara TS dan thermal hanya meningkatkan jumlah spora sampai 2.5 log.

TS merupakan metode paling efektif untuk menginaktivasi spora *B. cereus* dalam susu skim and slurry daging. TS dapat menghasilkan ≥ 5 log dalam susu vs. 3 log dengan HPP-thermal dan 2 log dengan proses thermal setelah 15 min. TS bahkan dapat meningkatkan inaktivasi proses thermal dalam slurry daging 6 kali lebih besar.

TS dan proses thermal pada 78°C tidak berpengaruh terhadap spora *A. acidoterrestris*. Tetapi, TS pada 78°C dengan didahului oleh HPP spora dalam jus jeruk dapat menaikkan inaktivasi spora ≥ 1.6 kali. Nilai *D* (decimal reduction time) lebih rendah dapat diperoleh pada tingkat power densiti akustik yang lebih tinggi. Perlakuan shok panas (heat shock, HS) dan pretreatment dengan ultrasound pada spora dapat meningkatkan dua kali lipat inaktivasi thermal spora *C. perfringens* dan *A. acidoterrestris*: dengan pretreatment $D_{95^\circ\text{C}} = 9.8$ min vs 22 min untuk *C. perfringens* dalam slurry daging; dengan pretreatment $D_{95^\circ\text{C}} = 0.8$ min vs 1.5 min untuk *A. acidoterrestris* dalam jus jeruk.

Spora psychrotrophic *B. cereus* paling rendah tingkat resistensinya terhadap HPP-thermal, TS, dan thermal pada 70-75°C. Spora *B. nivea* dan *N. fischeri* menunjukkan resistan paling tinggi terhadap thermal selama 30 min proses. Spora dari *A. acidoterrestris* dan *C. perfringens* lebih sulit untuk diinaktivasi dengan TS dan *C. perfringens* lebih sulit untuk diinaktivasi dengan HPP-thermal.

Resistensi spore kapang terhadap HPP-thermal dan TS naik dengan kenaikan umur spora. Waktu proses 27 min dibutuhkan untuk memperoleh reduksi sebanyak 1 log pada 4 minggu spora *N. fischeri* dengan metoda TS atau HPP-thermal pada 75°C, sementara 74 min dibutuhkan untuk memperoleh inaktivasi spora yang berumur 12 minggu. Sementara untuk spora *B. nivea*, jarak waktunya lebih dekat yaitu 13 min untuk reduksi 1 log spora berumur 4 minggu dan 29 min untuk spora yang berumur 12 minggu.

Inaktivasi semua spora dengan HPP-thermal dapat dimodelkan dengan baik oleh model Weibull, sementara kinetika inaktivasi untuk TS tergantung dari species/strain/food yang digunakan. Inaktivasi psychrotrophic *B. cereus* spora dalam susu skim dan *A. acidoterrestris* spora dalam jus jeruk mengikuti laju reaksi orde pertama, sementara log logistic dapat mendeskripsikan dengan baik inaktivasi spora *B. cereus* dan Weibull untuk *C. perfringens*

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dengan proses TS, keduanya dalam slurry daging. Distribusi Lorentzian dapat memodelkan inaktivasi spora kapang yang berumur 4-10 minggu. Kecuali untuk spora kapang proses thermal pada temperature $\leq 85^{\circ}\text{C}$, semua inaktivasi proses thermal umumnya mengikuti model laju reaksi orde pertama.

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CHAPTER 2: Modeling the HPP-thermal inactivation of Clostridium perfringens spores in beef slurry.

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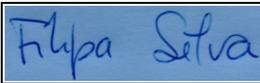
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CHAPTER 3: Use of power ultrasound to enhance the thermal inactivation of Clostridium perfringens spores in beef slurry.

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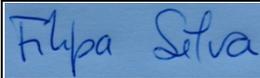
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CHAPTER 4: High pressure processing of milk: Modeling the inactivation of psychrotrophic *Bacillus cereus* spores at 38–70°C.

Evelyn, & Silva, F.V.M. (2015). High pressure processing of milk: Modeling the inactivation of psychrotrophic *Bacillus cereus* spores at 38–70°C. *Journal of Food Engineering*, 165, 141-148.

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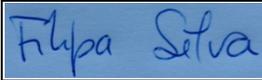
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CHAPTER 5: High pressure processing combined with 38-70°C to inactivate psychrotrophic Bacillus cereus spores in beef slurry: Modeling the HPP and thermal inactivation

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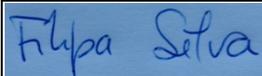
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CHAPTER 6: Thermo-sonication versus thermal processing of skim milk and beef slurry: Modeling the inactivation kinetics of psychrotrophic *Bacillus cereus* spores.

Evelyn, & Silva, F.V.M. (2015). Thermo-sonication versus thermal processing of skim milk and beef slurry: Modeling the inactivation kinetics of psychrotrophic *Bacillus cereus* spores. Food Research International, 67, 67-74.

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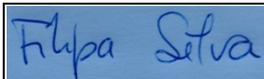
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CHAPTER 7: Efficacy of thermosonication vs HPP-thermal for Clostridium perfringens and psychrotrophic Bacillus cereus spores inactivation. To be submitted.

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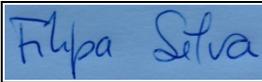
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CHAPTER 8: Ultrasound assisted thermal inactivation of Alicyclobacillus acidoterrestris spores in orange juice

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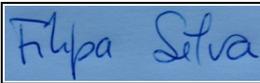
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CHAPTER 9: Inactivation of *Byssochlamys nivea* ascospores in strawberry puree by high pressure, power ultrasound and thermal processing.

Evelyn, & Silva, F.V.M. (2015). Inactivation of *Byssochlamys nivea* ascospores in strawberry puree by high pressure, power ultrasound and thermal processing. *International Journal of Food Microbiology*, 214: 129-136.

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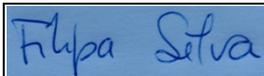
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CHAPTER 10: Modeling the inactivation of Neosartorya fischeri ascospores in apple juice by high pressure, power ultrasound and thermal processing

Evelyn, Kim, H.J., & Silva, F.V.M. (2016). Modeling the inactivation of Neosartorya fischeri ascospores in apple juice by high pressure, power ultrasound and thermal processing. Food Control, 59, 530-537

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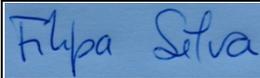
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Dr Filipa Silva	Idea, main supervision of the work and editing of the paper
Hyun Jae Kim	Minor experimental work

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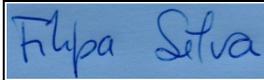
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Introduction and thesis outline

Introduction

Food safety and preservation relates to the inactivation of microorganisms including their vegetative and spore forms (bacterial and fungal). The spores can be very difficult to inactivate with heat processing due to their high thermal resistance (Silva & Gibbs, 2009). Generally if spores of a microbial species are inactivated, one can assume all vegetative forms are also destroyed. Thermal processing is the most common technology used to kill microorganisms in foods (pasteurization and sterilization). However, non-thermal technologies have been increasingly investigated since there is less impact on the food quality. The study of these emerging technologies with respect to microbial inactivation and food preservation is an area of great scientific and industrial interest. In this work, power ultrasound and high pressure processing have been used to inactivate spores in foods.

High Pressure Processing (HPP) is a commercial non-thermal technology used for microbial and enzyme inactivation. Pressure applications between 100 to 600 MPa combined with mild heat (HPP-thermal) is generally performed on the food/beverage to inactivate spoilage/pathogenic microorganisms and to extend food shelf-life. Results with *Bacillus stearothermophilus*, *Bacillus subtilis*, *Bacillus coagulans*, *Bacillus amyloliquefaction*, *Clostridium sporogenes*, *Clostridium botulinum* spores inactivation were reported (Ardia, 2004; Daryaei & Balasubramaniam, 2013; Margosch et al., 2006; Nguyen Thi Minh et al., 2010; Ramaswamy et al., 2010; Reineke, 2012).

Power ultrasound is an innovative technology that can be used for microbial and enzyme inactivation and was also used in this research. It relies on the application of ultrasonic waves at intensities higher than 1 W/cm^2 (typically in the range $10\text{--}1000 \text{ W/cm}^2$) and frequencies between 18 and 100 kHz (McClements et al., 2001). Sometimes the ultrasound is used in combination with moderate temperature (TS, thermosonication) for microbial

inactivation in foods. The thermosonication and ultrasound assisted thermal inactivation of bacterial spores, including the kinetics, is not yet well understood and documented, although a few works were found in the literature on *Bacillus subtilis*, *Bacillus cereus* and *Bacillus licheniformis* in glycerol, milk, and model solutions, respectively (Broda, 2007; Burgos et al., 1972; Garcia et al., 1989; Goodenough & Solberg, 1972; Ordonez & Burgos, 1976).

Since not many of the past works model the kinetics of microbial destruction of important microbial spores (e.g. psychrotrophic *Bacillus cereus*, *Clostridium perfringens*, *Neosartorya fischeri*, *Byssochlamys nivea*, *Alicyclobacillus acidoterrestris*) in foods and there is also an increasing consumer demand for fresh and minimally processed food products, there is an interest in studying non-thermal food preservation technologies such as HPP and ultrasound processing. The inactivation of microbial spores by high pressure or ultrasound alone is not feasible, so a combination with a mild thermal process is required. A detailed review on this topic has been carried out and is presented in Chapter 1. Given the variability of HPP and ultrasound resistance among the spores, a variety of spores representing low acid cold-stored and high acid ambient-stored foods was chosen to be studied in this research, which focuses on spore inactivation in these foods by HPP-thermal, TS, ultrasound enhanced thermal and thermal methods.

Thesis objectives

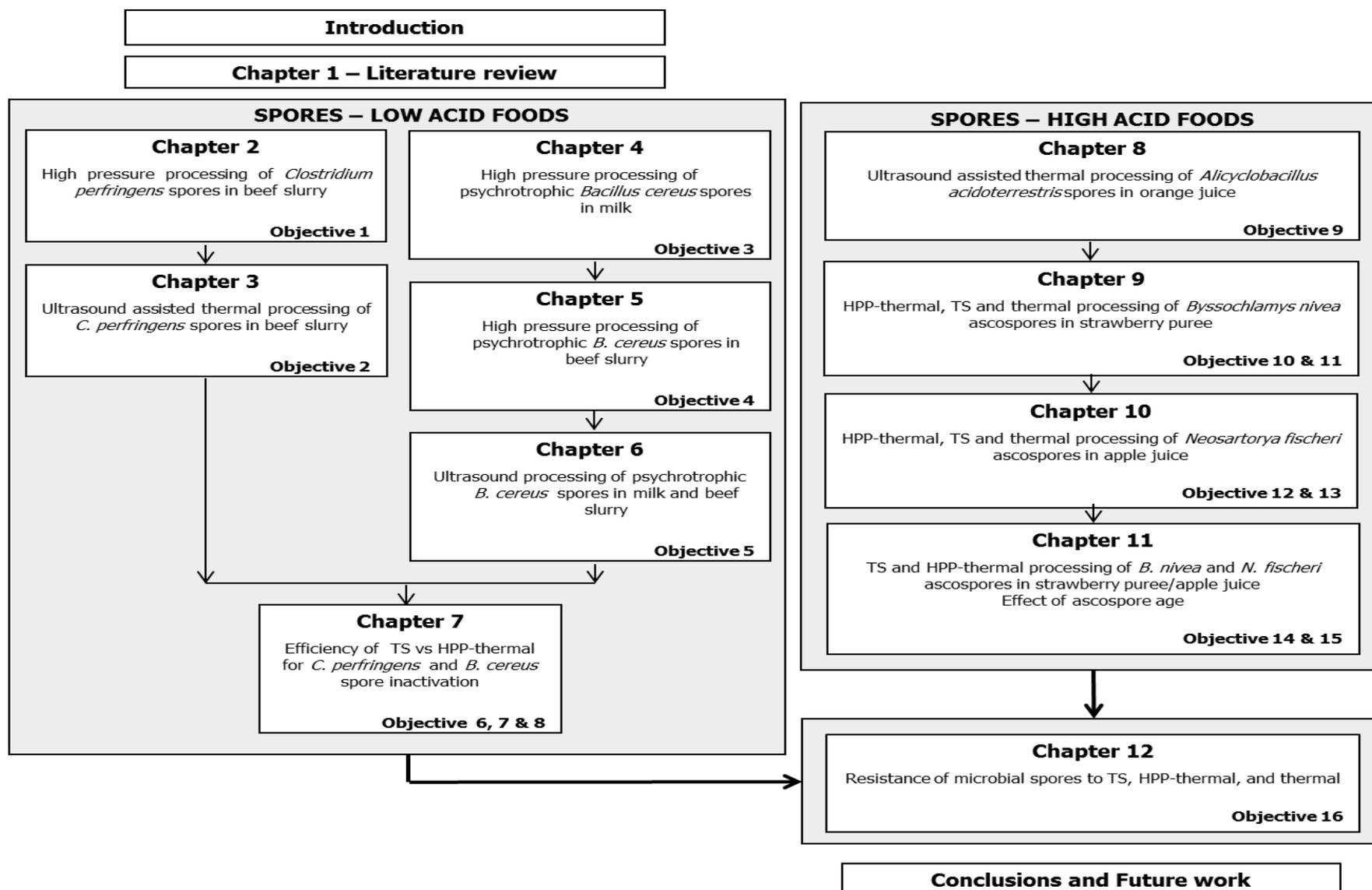
Overall the aim of this research was to assess the inactivation of bacterial spores and moulds ascospores that might be a problem for food industries, by using non-thermal technologies. The effectiveness of HPP and power ultrasound alone and in combination with heat for spore inactivation was investigated and compared with conventional thermal inactivation. The inactivation of *Clostridium perfringens*, psychrotrophic *Bacillus cereus*, *Alicyclobacillus acidoterrestris*, *Neosartorya fischeri*, and *Byssochlamys nivea* spores in several foods were modeled. The specific objectives of this research were:

- 1) To model the inactivation of *C. perfringens* spores in beef slurry by HPP combined with thermal processing;
- 2) To model the *C. perfringens* spores inactivation in beef slurry by thermal processing assisted with ultrasound;
- 3) To model the inactivation of psychrotrophic *Bacillus cereus* spores in skim milk by HPP combined with thermal processing and thermal processing alone;
- 4) To model the inactivation of psychrotrophic *B. cereus* spores in beef slurry by HPP combined with thermal processing and thermal processing alone;
- 5) To model the thermosonication (TS) inactivation of *B. cereus* spores in skim milk and beef slurry;
- 6) To compare HPP-thermal, TS and thermal inactivation of *C. perfringens* spores in beef slurry;
- 7) To compare HPP-thermal, TS and thermal inactivation of psychrotrophic *B. cereus* spores in milk;

- 8) To compare HPP-thermal, TS and thermal inactivation of psychrotrophic *B. cereus* spores in beef slurry;
- 9) To model the TS inactivation of *A. acidoterrestris* spores in orange juice;
- 10) To model the inactivation of *Byssochlamys nivea* ascospores in strawberry puree by HPP-thermal, TS and thermal processing;
- 11) To compare the HPP-thermal, TS and thermal processing to inactivate *B. nivea* ascospores in strawberry puree;
- 12) To model the inactivation of *Neosartorya fischeri* ascospores in apple juice by HPP-thermal, TS and thermal processing;
- 13) To compare the HPP-thermal, TS and thermal processing to inactivate *N. fischeri* ascospores in apple juice;
- 14) To study the effect of ascospore age on the TS and HPP-thermal inactivation of *B. nivea* ascospores in strawberry puree.
- 15) To study the effect of ascospore age on the TS and HPP-thermal inactivation of *N. fischeri* ascospores in apple juice;
- 16) To compare the resistance of different microbial spores species after TS, HPP-thermal, and thermal processing.

Thesis organization

The results obtained in this research are presented in twelve chapters to fit the objectives. The **first chapter** is a literature review focused on HPP and ultrasound technologies fundamentals, and a review on inactivation modeling of bacterial spores and mould ascospores in various food media. **Chapters 2-3** cover the modeling of HPP-thermal and thermal (carried out with ultrasound pretreated spores) inactivation of *Clostridium perfringens* spores in beef slurry. **Chapters 4-6** investigate the modeling of the inactivation of psychrotrophic *Bacillus cereus* spores in skim milk and beef slurry processed by TS, HPP-thermal, and thermal processing. In **Chapter 7**, the efficiency of TS vs HPP-thermal for *C. perfringens* and *B. cereus* spore inactivation were compared. The effect of TS and thermal assisted ultrasound on the inactivation of *Alicyclobacillus acidoterrestris* spores in orange juice is presented in **Chapter 8**. **Chapters 9 and 10** study the effects of TS, HPP-thermal, and thermal processing on the inactivation *B. nivea* ascospores in strawberry puree and *N. fischeri* spores in apple juice, respectively. In **Chapter 11**, the effect of mould spore age on TS and HPP-thermal inactivation of *N. fischeri* spores in apple juice and *B. nivea* ascospores in strawberry puree were investigated and compared. The comparison of microbial spore species resistance to TS, HPP-thermal, and thermal processes was presented in **Chapter 12**. Finally, **the last section** of this thesis is a general conclusion and suggestions for further work. The following scheme demonstrates the relationship of all the chapters in this thesis.



Chapter 1 Literature review

1.1. Microbial spores and their lifecycle

Microbial spores are dormant life forms produced by stressed cells. When there are favorable conditions (e.g. water, nutrients, and germinants), spores may break their dormancy through germination and outgrowth, and cause contaminations, food spoilage, diseases, etc. Bacteria, fungi, and green plants are microbes that are able to produce spores. Among them, bacterial spores and mould ascospores are well known for their resistance against various agents and stresses such as radiation, high temperature, freezing, pressure, desiccation, extreme pH and attack by a wide variety of toxic chemicals (Black et al., 2007a; Reineke et al., 2013; Setlow, 2006; Tournas, 1994), thus are frequently used as targets in pasteurization processes. The resistance of spores is considered to be due to substantial structural specialization developed within a mother cell (Moir & Smith, 1990).

1.1.1 Bacterial spores

Bacterial spores were initially observed and described by Perty (1852), followed by Ferdinand J. Cohn in 1872 who coined the *Bacillus* name for the first time. Bacterial spores (endospores) are highly durable and can germinate even after a million year period of dormancy (Cano & Borucki, 1995; Vreeland et al., 2000). Bacterial endospores comprise of resistant coats made up by several layers in which the core is mainly surrounded by the inner spore membrane, the cortex, the outer membrane and then the spore coat (**Figure 1-1**).

Exosporium, the outermost layer of the spore structure, may be present in certain spore formers and mainly consists of proteins, lipids and carbohydrates (Leggett et al., 2012). The hydrophobicity and pathogenicity of spores are determined by their compositions (Leggett et al., 2012). The inside of the exosporium (if present) is the spore coat containing multiple impermeable protein layers that consist of the basement, the outer and inner coat, and the crust (Setlow, 2012). In the most common studied *Bacillus subtilis* spores, the coat can

1. Literature review

contain ≥ 70 different types of proteins (Setlow, 2012), and many of them are cross-linked via disulfide bonds that contribute to its resistance (Leggett et al., 2012).

The next structure of bacterial endospores is the outer and inner membrane. The outer membrane is considered important for spore formation but the function is not yet clear (Popham, 2002). The cortex resides between the outer and inner membranes containing the spores' thick peptidoglycan layer which has low levels of cross linking (Popham, 2002). The cortex presumably maintains heat resistance and dormancy (Ellar, 1978). The germ cell wall (also a peptidoglycan) is located within the cortex and functions as the cell wall of the bacterium following spore germination (Pedraza-Reyes et al., 2012). The spore's inner membrane is the location for different types of nutrient/germinant receptors, thus serving as a barrier against several chemicals (Setlow, 2003). The cortex together with the spore's inner membrane seems to play a significant role in maintaining the low water content of the spore's core (Popham, 2002).

The spore's core consists of chromosomal DNA embedded in small acid-soluble spore proteins (SASPs), ribosomes, and essential enzymes needed once returned to the vegetative state (Leggett et al., 2012). The unique properties of the spore's core that have long been known to contribute to the high resistance level in bacterial endospores include saturation of the DNA with SASPs, low water content, high levels of minerals and dipicolinic acid/DPA, and decreased core permeability (Pedraza-Reyes et al., 2012).

The simplified life cycle of *Bacillus subtilis* endospore-forming bacterium, the model bacteria used to study endospore formation is outlined in **Figure 1-2**. It mainly consists of vegetative growth, sporulation and germination. Vegetative growth is characterized by symmetric division of a parent cell into two morphologically and genetically identical daughter cells. The sporulation process begins when nutrients become limited to the bacteria and ends when

1. Literature review

the spore dehydrates its cytoplasm and is released from the cell to produce resting endospores. Sporulation involves an asymmetric cell division, engulfment of the smaller cell (pre/forespore) within the mother cell, cortex and proteinaceous coat synthesis, and formation of mature spores (**Figure 1-2**) (Errington, 2003). The endospores can convert back to a vegetative cell through several well-known steps: activation (a treatment that makes spores ready for germination, for example sub-lethal heat, chemical, radiation, high pressure, extreme pH and sonication) (Ray & Bhunia, 2003); germination (a change from a dormant state into a metabolically active state, indicated by release of DPA, rehydration of the spore with water, and hydrolysis of cortex); and finally outgrowth (cell division).

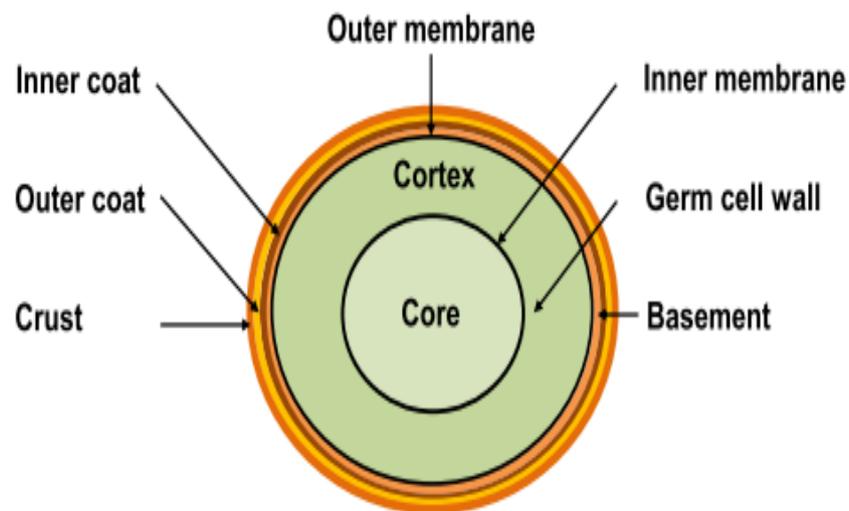


Figure 1-1 Endospore structure of *Bacillus* (Reprinted from Setlow, 2003, and Reineke, 2013, with permission from Elsevier)

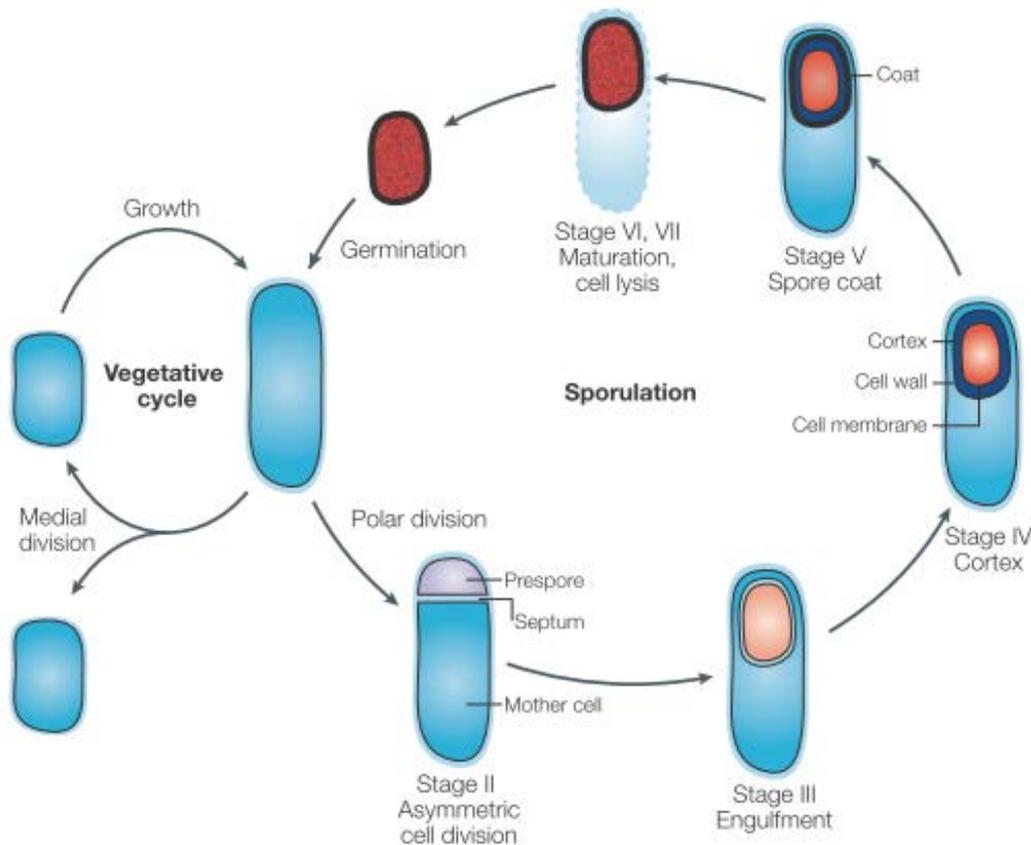


Figure 1-2 Life cycle of *Bacillus subtilis* endospore forming bacterium (Errington, 2003, with permission from Nature Publishing Group).

1.1.2 Fungal spores

Most fungi produce spores as part of their life cycle, which are not only used for survival under unfavorable conditions but also used for reproduction. The taxonomy of fungi is based on the size, shape, color and genesis of their spores. Fungal spores can be formed by either asexual or sexual processes. Asexual spores (genetically similar to their parent) include conidia, chlamydospores, sporangiospores, and zoospores, whereas sexual spores (a result of genetic recombination) include ascospores, basidiospores, oospores, and zygospores.

Zygomycetes, ascomycetes and deuteromycetes are three sub-kingdoms of fungi of significant importance in food spoilage (Pitt & Hocking, 1997). Fungal ascospores, which are one of the interests of this study, belong to the ascomycetes sub-kingdom. Ascospores are

1. Literature review

produced within a sac called ascus and generally mature after incubation for 10 days or more at 25°C (Pitt & Hocking, 1997). Ascospores are often resistant to heat and chemicals, and have a higher survival capability than conidia and the most resistant yeast ascospores during pasteurizing processes given to acidic foods (< pH 4.6) (Dijksterhuis, 2007; Pitt & Hocking, 1997). *Byssochlamys*, *Neosartorya* and *Talaromyces* are examples of the most heat resistant fungal ascospores known.

Ascomycetes are pleomorphic fungi, exhibiting both sexual (teleomorphic) and asexual (anamorphic) reproduction in their life cycle. **Figure 1-3** depicts the life cycle of *Talaromyces* (and thus the life cycle of ascomycetes), which is the best studied fungal ascomycetes. During its sexual reproduction, the core of most ascomycetes exists in haploid state (although sometimes it can be diploid nuclei produced by nuclear fusion). This nucleus undergoes cell division through meiosis and then mitosis to produce typically eight haploid ascospores in the ascus in response to unfavorable conditions such as nutrient depletion. When ascospores mature, the asci breaks and free ascospores are released. The ascospores have a thick wall, highly refractile and are usually decorated (Pitt and Hocking, 1997). Similar to bacterial endospores, a resting ascospore can be returned to a vegetative cell through activation and germination. Heat or chemical, or both in combination can activate fungal spores to germinate under favorable conditions (Sussman, 1976).

Asexual reproduction in ascomycetes is through production of conidia type spores in long chains on conidiophores (**Figure 1-3**). These conidial spores are easily detached from their places and float in the air like dust (Gumerato, 1995). Under certain conditions, conidia may also germinate to produce hyphae and then mycelium.

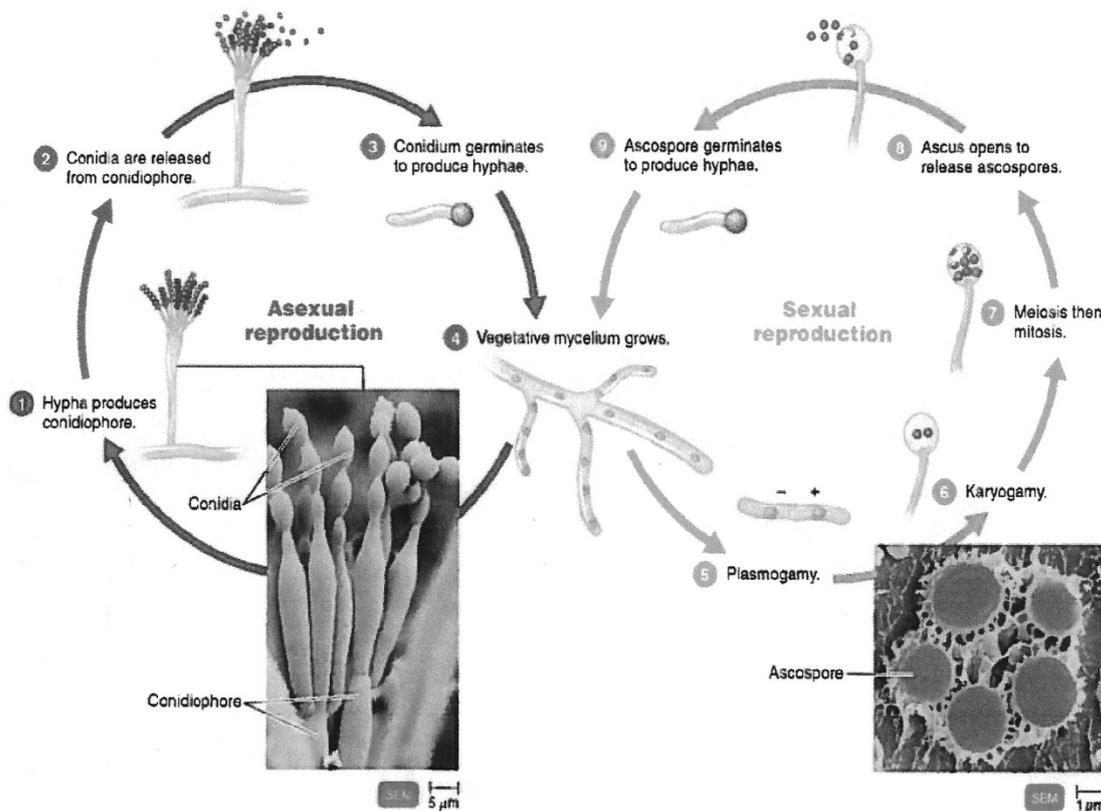


Figure 1-3 The lifecycle of ascomycetes *Talaromyces* (Tortora et al., 2013, with permission from Pearson and Taylor & Francis Group)

1.2 Modeling of microbial inactivation kinetics

Kinetic parameters and models of microbial inactivation are important tools to analyse the effectiveness of different preservation technologies and improve food safety and quality. These are based on the reduction in the number of microbial populations in response to a lethal effect applied. Linear or non-linear (shoulders and tails) models can be used to determine the kinetic parameters of the log survival curves of pathogens and spoilage bacteria in various foods, and these are discussed in the following section.

1.2.1 First order kinetics

Predictive microbiology began when Bigelow (1921), Bigelow and Esty (1920), and Esty and Meyer (1922) proposed the use of first order kinetics to model microbial inactivation by thermal. The model describes a linear decrease in logarithmic cell populations with time as a (constant intensity of) heat is applied. It assumes that different individuals in a population have a general similarity of resistance (often called the mechanistic approach) (Lee & Gilbert, 1918). In this model, decimal reduction time (D_T -values, being the time required at a certain temperature to reduce a microbial population by 90%) was calculated from the reciprocal of the slope as follows (Equation 1-1):

$$\log \frac{N}{N_0} = -\frac{t}{D_T} \quad (1-1)$$

where $\log N/N_0$ is the logarithmic microbial survival ratio, N is the number of survivors (cfu/mL or cfu/g) in the food/medium after being exposed to a lethal treatment for a specific time t (min), and N_0 is untreated/the initial microorganism concentration of raw food/medium. The temperature coefficient, z_T -value ($^{\circ}\text{C}$) (the temperature increase that results in a 10-fold decrease in the D_T -value) was estimated from the negative reciprocal of the slope (Equation 1-2):

$$\log \left(\frac{D}{D_{T_{ref}}} \right) = \frac{T_{ref} - T}{z_T} \quad (1-2)$$

where $D_{T_{ref}}$ is D -value at the reference temperature T_{ref} (can be any reference temperature, $^{\circ}\text{C}$), and T is the temperature of the isothermal treatment. From this equation, $\log [N/N_0]$ versus time $[t]$ is a straight line with $1/D$ as the slope. Solving for D , for $t = D$ and $N = N_0/10$ offers a new correlation between D and k or death rate constant ($D = \ln 10/k = 2.303/k$), thus the equation becomes as follows (Equation 1-3):

$$\log \frac{N}{N_0} = -\frac{k t}{2.303} \quad (1-3)$$

This equation with a slope k has been used as the scientific basis for evaluating and designing thermal processes i.e. canning. The Arrhenius (1889) equation is a useful tool to correlate the corresponding rate constant (k) to the temperature (Equation 1-4) and the Eyring equation (Eyring, 1935a, 1935b) can be used for the pressure-dependence of k (Equation 1-4 and Equation 1-5):

$$\frac{\partial \ln k}{\partial T} = -\frac{E_a}{RT^2} \quad (1-4)$$

$$\frac{\partial \ln k}{\partial P} = -\frac{\Delta V^\ddagger}{RT} \quad (1-5)$$

where E_a represents the activation energy (kJ mol^{-1}), ΔV is the activation volume of the reaction ($\text{cm}^3 \text{mol}^{-1}$), and R is the molar gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$).

1.2.2 Deviations from linearity

Deviations from the linearity such as shoulders and tails can be observed in the survival curves after non-thermal (such as HPP) or even thermal treatments. In contrast to the mechanistic model, Lee and Gilbert (1918) also develop an assumption to describe the observed non-linearities i.e. individuals in a population which are different (vitalistic concept). Later, several reasons for describing shoulders and tails were reported: the existence of cell clumps (Klijn et al., 2001); multi-target inactivation, the existence of sub-lethal injury, and the heterogeneity of resistance in microbial populations after undergoing four to five cycles (Smelt et al., 2002); the presence of activated dormant spores and sub-population with varying resistance (Teixeira & Rodriguez, 2009). However, the most

compelling source of variability in responses to an inactivation treatment is thought to be heterogeneity of heat (or other factors) resistance within a population (Aguirre et al., 2009; Pin & Baranyi, 2006). In other words, microbial inactivation would not necessarily follow first-order kinetics.

1.2.2.1 Weibull model

The Weibull distribution originated in 1951 (Weibull, 1951), and more recently has been used by various investigators due to its simplicity and great accuracy for describing the non-linear microbial inactivation in various foods (Peleg & Cole, 1998; van Boekel, 2002; van Boekel, 2009). Two mathematical forms of the Weibull model that have been used are shown as follows (Mafart et al., 2002; Peleg & Cole, 1998) :

$$\log \frac{N}{N_0} = -bt^n \quad (1-6)$$

$$\log \frac{N}{N_0} = -\frac{t^p}{\delta} \quad (1-7)$$

where b (the scale factor) is a rate parameter which is related to the velocity of the inactivation of the microorganism and n is the survival curve shape factor. $n < 1$ and $n > 1$ correspond to survival curves with concave-upwards (tailings) and concave-downwards (shoulders), respectively. When $n = 1$, the Weibull model becomes the simple first-order kinetics. Equation 1-7 is another Weibull model proposed by Mafart et al. (2002), in which the equation has parameter p and δ . The parameter p also characterizes the shape of the curve ($p > 1$, convex curves; $p < 1$, concave curves), whereas parameter δ is called the first decimal reduction time in the microbial population.

1.2.2.2 Log-logistic model

Chen and Hoover (2003) modified and renamed four parameter (α , ω , σ , τ) logistic function initially proposed by Cole (1993) into a simpler three parameter (A , σ , τ) log-logistic model, which is shown in the following equation:

$$\log \frac{N}{N_0} = \frac{A}{1+e^{4\sigma(\tau-\log t)/A}} - \frac{A}{1+e^{4\sigma(\tau+6)/A}} \quad (1-8)$$

These authors defined σ as the maximum inactivation rate (\log (cfu/mL or cfu/g)/log min), and τ as the time to the maximum inactivation rate (log min), which commonly decreases as the lethal effect increases. Chen and Hoover (2003) also defined a new parameter A , as $\omega - \alpha =$ lower asymptote – upper asymptote (\log cfu/mL or cfu/g) to reduce the number of parameters. A small value of t ($t \sim 10^{-6}$ min) was also used to approximate $t = 0$ due to a undefined $\log t = 0$.

1.2.2.3 Biphasic-linear model

The microbial inactivation kinetics can also be described by the biphasic-linear model (Cerf, 1977; Panagou et al., 2007; Zimmermann et al., 2013) (Equation 1-9):

$$\log \frac{N}{N_0} = \log [f e^{-k_{max1}t} + (1-f)e^{-k_{max2}t}] \quad (1-9)$$

in which parameters f is the fraction of the treatment-sensitive population, $(1-f)$ is the treatment-resistant population, and k_{max1} and k_{max2} are the inactivation rate constant of treatment sensitive and treatment resistant populations (min^{-1}).

1.2.2.4 Modified Gompertz and Baranyi–Roberts models

Gibson et al. (1988) used the modified Gompertz equation (Equation 1-10) to model microbial growth curves, and later it was used to model inactivation kinetics by Linton et al.

(Linton et al., 1996; 1995; Xiong et al., 1999). In the model, M is the time at which the absolute death rate is maximum, B is the relative death rate at M , and C is the difference in the value of the upper and lower asymptotes. The Baranyi–Roberts equations transformed by Xiong et al. (1999) (Equation 1-11 and Equation 1-12) have also been found in some microbial inactivation kinetics reports (Pérez et al., 2007; Saucedo-Reyes et al., 2009).

$$\log \frac{N}{N_0} = C e^{-e^{BM}} - C e^{-e^{-B(t-M)}} \quad (1-10)$$

$$\log \frac{N}{N_0} = \log(q_B + (1 - q_B)e^{-k_{max}(t-B(t))}) \quad (1-11)$$

$$B(t) = \frac{r}{3} \left(\frac{1}{2} \ln \frac{(r+t)^2}{r^2+rt-t^2} + \sqrt{3} \arctan \frac{2t-r}{r\sqrt{3}} + \sqrt{3} \arctan \frac{1}{\sqrt{3}} \right) \quad (1-12)$$

In this model, k_{max} is the maximum death rate; r is the time required for the relative death rate to reach half of the k_{max} and q_B is the tailing ratio. $q_B = N_{min}/N_0$, where N_{min} is the minimum cell concentration remaining in the tailing phase.

1.2.2.5 n^{th} order kinetic model

This model has been used to model *Alicyclobacillus acidoterrestris* and *Bacillus stearothermophilus* spores after non-thermal treatments such as HPP as the function of temperature and pressure (Ardia, 2004) and is shown as follows (Equation 1-13):

$$\log \frac{N}{N_0} = \log(1 + k \cdot N_0^{n-1} \cdot t \cdot (n - 1))^{-\frac{1}{n-1}} \quad (1-13)$$

where t is time (min), N is the number of survivors at time t (cfu/mL), N_0 is the initial spore count (cfu/mL or cfu/g), k is the rate constant (min^{-1}), and n is the reaction order. The model has also been reported by other investigators (Margosch, 2006; Reineke et al., 2012).

1.2.2.6 Models with activation shoulders

Interestingly, kinetic models for the inactivation of heat treated microbial spores with an activation shoulder have also been reported (Peleg, 2002; Sapru et al., 1992, 1993; Shull al., 1963; Rodriguez et al., 1992; van Boekel, 2009). Among the models, the double Weibullian (Equation 1-14) and Peleg (Equation 1-15) model were frequently reported. In this model, a_1 , b_1 , b_2 , n_1 , and n_2 are adjustable temperature dependent parameters (Corradini et al., 2010):

$$\log \frac{N}{N_0} = b_1 t^{n_1} - b_2 t^{n_2} \quad (1-14)$$

$$\log \frac{N}{N_0} = \frac{a_1 t}{b_1 + t} - b_2 t^{n_2} \quad (1-15)$$

1.2.3 Model performance

Model evaluation and validation is also an important step in the modeling process, in which the goodness of fit of the model to experimental data is assessed so that it can be accepted as a predicted model. Residual plots, mean square error (MSE), coefficient of determination (R^2), and accuracy (A_f) are mostly used to assess the proximity of observations to the fitted model (Ross, 1996). The MSE and A_f are shown in the following equations:

$$MSE = \frac{\sum(\text{predicted} - \text{observed})^2}{n - p} \quad (1-16)$$

$$A_f = 10 \left(\frac{\sum \left| \log \left(\frac{\text{predicted}}{\text{observed}} \right) \right|}{n} \right) \quad (1-17)$$

where n stands for the number of observations and p stands for the number of model parameters used. A relatively small MSE, R^2 and A_f values closer to 1, indicate the adequacy of the model for describing the data (Ross, 1996). A_f is a performance indicator which shows the accuracy of the model and indicates how close the fitted values are, on average, to the

observed values. When A_f is equal to 1, there is a perfect agreement between the fitted and observed values.

Establishing appropriate treatment conditions must be taken into considerations when the description of cell death kinetic models is the goal. For example, the inoculation level is required to be able to extract reliable parameter values of the inactivation curve/level needed (Balasubramaniam et al., 2004). The literature has shown approaches to fit kinetic data, describe process variables and design a pasteurization process for a new product (Legan et al., 2002; Manas & Pagán, 2005; Silva et al., 2014).

1.3 High Pressure Processing (HPP)

1.3.1 HPP principles

HPP relies on the use of high pressure generally between 100-600 MPa to process liquid and solid foods (with or without heat, packaged or otherwise) for periods of up to 20 min. According to Le Chatelier's principle, any processes resulting in a decrease of total volume are encouraged by pressure. On the other hand, any processes involving an increase of total volume are inhibited by pressure (Butz & Tauscher, 1998; Patterson et al., 2006). As a consequence, HPP works only by breaking non-covalent bonds in contrast to thermal treatment, leading to damage of the cell membrane and inactivation of pathogenic bacterial cells. The α -helical and β -pleated sheets stabilized by hydrogen bonds are unaffected (Heremans, 2003). The alterations to secondary and tertiary structures of macromolecules which are maintained by electrostatic and hydrophobic interactions depend on the pressure applied (Marcos et al., 2005). Pressure is also transmitted uniformly and rapidly throughout food products regardless of shape, size and geometry of food according to the isostatic principle. During pressurization, the food is uniformly compressed in all directions, which

will cause no/less damage to the product which then will return to its original shape when the pressure has been released (decompressing). These considerations make high pressure processing an attractive food preservation method over thermal processing.

1.3.2 History

HPP treated foods were only seen on the market from the early 1990s (Lau & Turek, 2003; Ludikhuyze et al., 2001; van Loey et al., 2003) although the extension of shelf life of raw milk after exposure to 600 Mpa HPP for 60 min at room temperature was demonstrated by Hite in 1899. Japan was the first country to introduce HPP pasteurized food products, with fruit jams and sauces as product examples. This was then followed by HPP pasteurized guacamole in the United States, fruit juice in France, Mexico, and the UK, and a delicatessen style ham in Spain (Patterson et al., 2006). Since then, HPP has been extended to preserve fruits and vegetables (32%), meat products (27%), seafood (16%), juices and beverages (11%), and other products (14%) (Buckow & Bull, 2012).

Data in the end of 2014 showed that approximately 265 industrial-scale HPP machines have been produced and installed in the worldwide food processing facilities since 2000s (**Figure 1-4**), and is forecasted to exceed 350 in 2015, suggesting an intensified use of this technology (Hiperbaric.com, 2015; Visiongain, 2015). This is mainly because of a greater consumer demand for HPP treated foods and a decline in capital and maintenance costs. The major countries involved in the commercialization of HPP technology are the USA, Mexico, Canada, France, Italy, the UK, Portugal, Spain, the Netherlands, Greece, Germany, Australia New Zealand, Japan, South Korea, and China (Balasubramaniam et al., 2008).

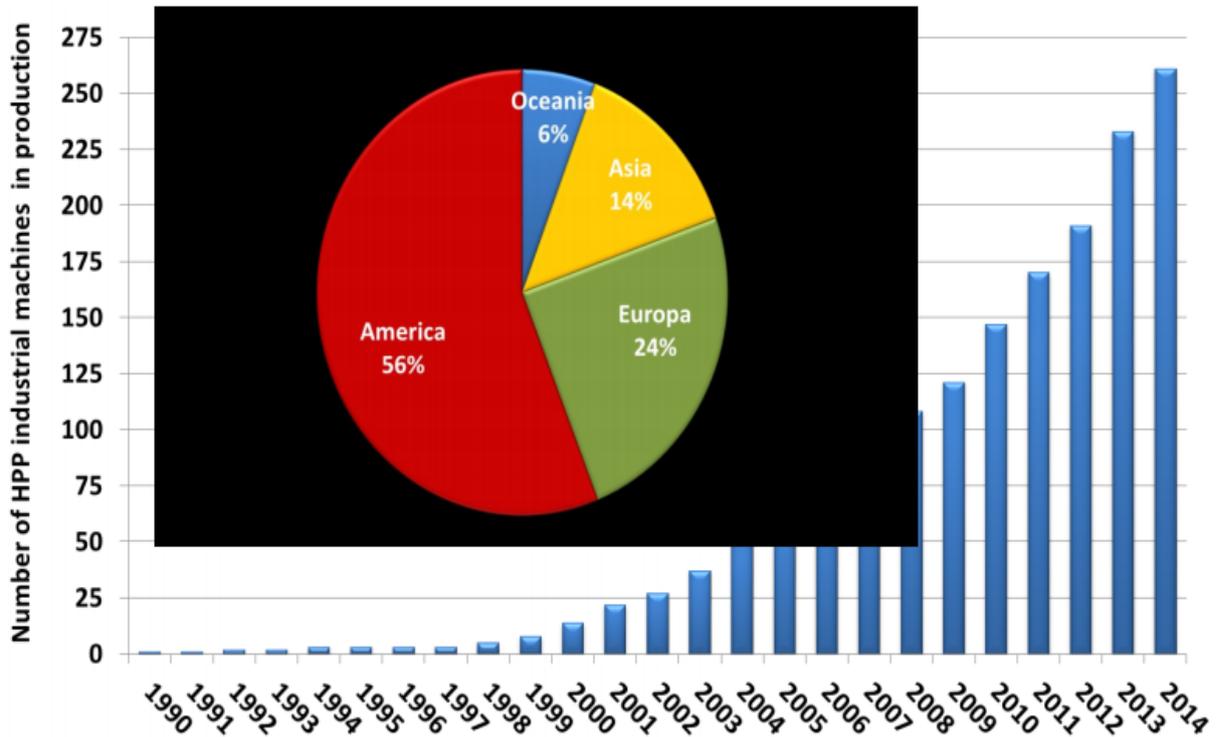


Figure 1-4 Development of number of commercial HPP machines around the world (Hiperbaric, 2015).

The total global production of HPP food has increased constantly over time. The amount of production worldwide have been estimated as reaching 200,000 metric tons/year in 2008 (Samson, 2008), 250,000 metric tons/year in 2009 (Jung et al., 2011) and more than 500,000 metric tons/year in 2014 (Samson, 2014). More than 200 different HPP products are traded by 60 different companies around the world.

1.3.3 Equipment and cost

HPP technology is still considered more expensive than conventional technologies, typically around 0.1-0.2 euros per litre, while thermal treatment may only cost 0.02-0.04 euros per litre (Patterson, 2005). This technology has also been considered more expensive than other non-thermal technologies such as membrane filtration and UV light (Rodriguez-Gonzalez et al., 2015). However, decreases in the processing costs due to the growth in demand for HPP by almost four times from 1996 to 2008 were reported (Hewson, 2008).

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The main components of a HPP system are a pressure vessel, a closure for sealing the vessel, a device to keep the closures in place under pressurization (e.g. yoke), a high-pressure intensifier pump/piston, a controlling and monitoring panel, and a product-handling system (e.g. perforated baskets) for transferring the product to and from the pressure vessel (Balasubramaniam et al., 2008). Usually, the system also incorporates a site for filtering and re-utilizing the pressure compression fluid (water or a food-grade solution). Water is normally used as it is compatible with food materials (Earnshaw, 1996). Commercial-scale HPP vessels are mostly cylindrical and are constructed of low-alloy steel of high tensile strength. The vessels are available in vertical and horizontal configurations, with internal volumes ranging from 55 to more than 500 litres (Hiperbaric, 2015). Depending on the capacity and automation of these systems, the cost of purchase in 2008 varied from 0.5 to 2 million euros (Balasubramaniam et al., 2008). The most well-known manufacturers of commercial-scale HPP equipment around the world are Avure (USA), Hyperbaric (Spain), Multivac (Germany), Kobelco (Japan), and Chic (China). **Figure 1-5** shows one of the industrial HPP machines by Avure.

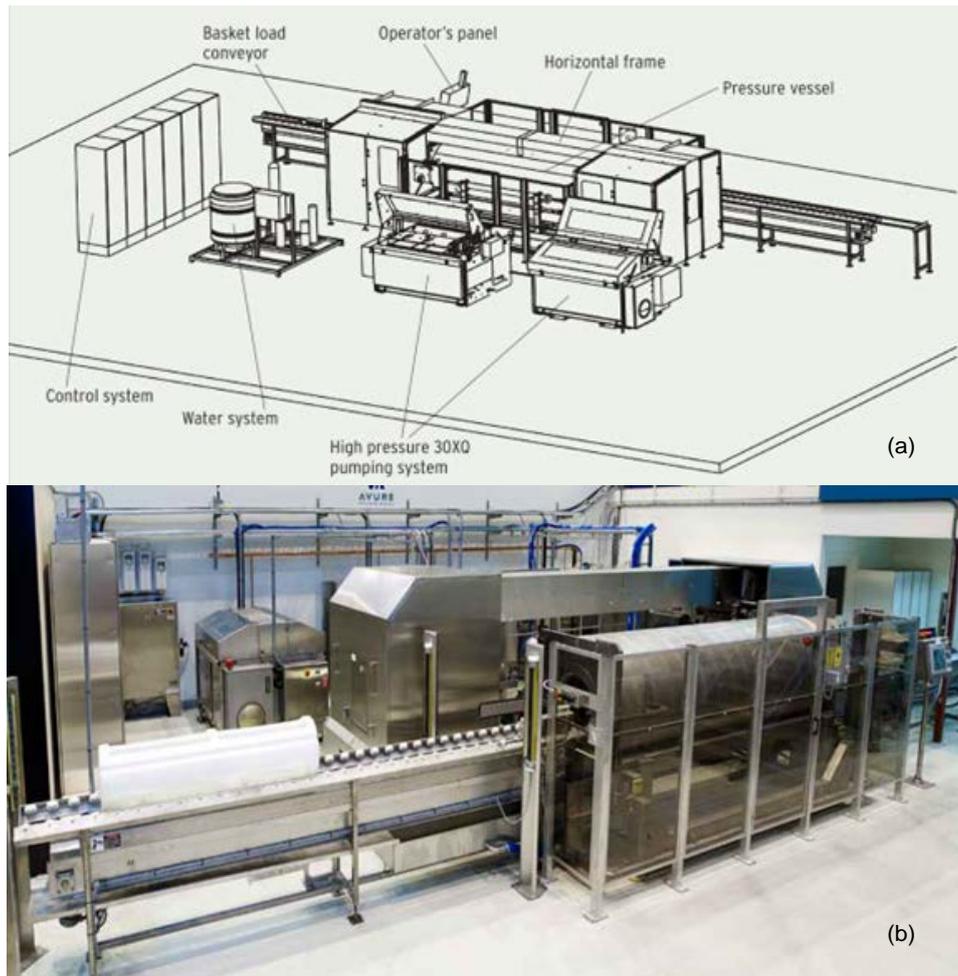


Figure 1-5 Avure HPP QFP 350-600 L: schematic diagram (a), machine (b).

1.3.4 HPP operation

HPP systems can be operated in batch (for packaged liquid and solid foods), semi-continuous or continuous (for unpackaged liquid foods) (Hogan et al., 2005). There are four stages in a batch HPP process: product loading, vessel pre-filling, pressurization, and product unloading. Prior to loading the packaged food products into the high pressure vessel, they are placed in a flexible container (pouches or plastic containers). After loading, the vessel is sealed and filled with a pressure compression fluid. Pressure is applied at its operating pressure by compressing the water surrounding the packaged foods, and the pressure is maintained

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without any further energy input. The pressure is released after an appropriate process holding time, and the processed products are unloaded from the vessel. At this stage, the food products can be stored for distribution. The pressure is generated either by direct or indirect compression. In direct compression, a piston is used to pressurize the fluid, while a high-pressure intensifier pump is used for indirect compression.

The main operation in semi-continuous processing is basically similar to the batch system, but only applies to fluid products such as juices, and requires two or more (parallel) pressure vessels. There is a free-floating piston in each vessel thus splitting the vessel into two compartments. The first compartment is filled with liquid food and the second compartment is filled with the pressure compression fluid. When the fluid in the second compartment is pressurized, the liquid food in the first compartment is compressed. When the desired holding time is reached, the products are discharged from the vessel to a sterile holding tank by compressing the piston. Finally, the liquid food products can be transferred into their sterile containers by an aseptic operation.

Figure 1-6 presents a pressure-time-temperature profile during a HPP batch treatment. The come-up time phase (T_1 - T_2) presents the time required to increase pressure from atmospheric pressure (P_1) to the intended process pressure (P_2). Normally, there is a decrease in the volume of food products as a function of pressure, e.g. pure water undergoes around 15% reduction when subjected to a 600 MPa at ambient temperature. The increase in pressure also results in an increase of food temperature (t_1 - t_2). The time mainly depends on the power of the high pressure pump, the compression rate, and the target process pressure. Most equipment needs one to three minutes to complete this action. The holding time phase (T_2 - T_3) is the time required for holding the products under a constant target pressure (P_2 - P_3). In this phase, the temperature of the products (T_2 - T_3) is independent of the compression rate,

assuming no heat exchange takes place between the product and the surroundings. Commercial-scale operations often take three to ten minutes for the holding time. Reducing the holding time (< 5 min) is considered to increase the economics of commercial HPP operations (Yordanov & Angelova, 2010). The decompression phase (T_3 - T_4) is the time needed to bring the process pressure back down to atmospheric pressure (P_3 - P_4). It usually takes only a few seconds to depressurize. During this phase, products often return to their initial volume (Farkas & Hoover, 2000). As a result of decompression, the temperature (T_3 - T_4) falls to its initial value (in a system with good insulation), or slightly lower than its initial temperature (T_1) due to heat loss to the surroundings (commonly observed).

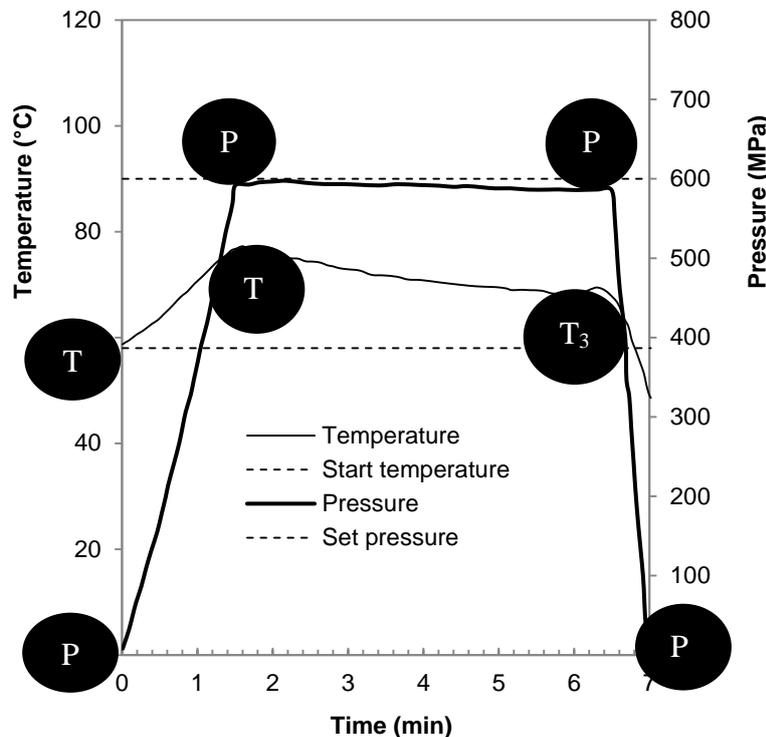


Figure 1-6 A typical pressure-time-temperature profile for a batch HPP treatment

A moderate and uniform temperature increase (i.e. 5-15°C) is typically observed during the pressurization of foods (termed adiabatic heating). Food composition, initial temperature and the final pressure determine the heat of compression of food materials (thus making the difference in heat of compression value of each food material). For instance, the water

temperature increases by 3°C every 100 MPa of compression at 25°C, whereas for fats and oils, it increases about 8-9°C with the same amount of pressure applied (Patazca et al., 2007; Rasanayagam et al., 2006).

1.3.5 Estimation of HPP energy requirements

In HPP, energy is required for preheating the pressurizing fluid (sensible heat) and compression work during pressurization (adiabatic heat). The heat energy required (Q) for preheating can be calculated as follows:

$$Q = mC_p\Delta T \quad (1-16)$$

where m is the mass of the food sample (kg); c_p is the food heat capacity (J/(kg°C)), and ΔT (°C) is difference between temperature of food in the HPP chamber before the pressurization phase and initial food's temperature. Regarding adiabatic heating, the variation of liquid volume in response to pressure changes can be determined by knowing the properties of foods and the fluid compressibility (β) factor (compressibility of pure water is 4.6×10^{-10} m²/N at 25°C):

$$\beta = -\frac{1}{V} \left(\frac{\partial V}{\partial P} \right)_T \quad (1-17)$$

Then, the work (energy) for compression can be estimated by introducing ∂V in Equation 1-17 to Equation 1-18 as follows (Rodriguez-Gonzalez et al., 2015):

$$W = -\int P dV \quad (1-18)$$

where V is the volume (m³) and P is the pressure (Pa). Finally, the specific energy (J/kg or J/L) for HPP process can be obtained by dividing Q and W by the mass (kg or L) of processed food.

1.4 Microbial spore inactivation by HPP

1.4.1 Mechanism of spore inactivation

Microbial spores are distinguishable from vegetative cells in the mechanism of inactivation by high pressure. According to Ananta et al. (2001), Raso and Barbosa-Cánovas (2003), germination is likely to occur initially at 100-300 MPa, followed by inactivation of the germinated forms at pressure of over 1000 MPa and elevated heat. To date, more research has been carried out to explain the mechanism and two-step processes have been widely accepted: the release of DPA during germination causing a loss of heat resistance and the subsequent inactivation by pressure and heat (Black et al., 2005; Margosch et al., 2004; Mathys et al., 2007; Mathys et al., 2009; Reineke, 2012; Reineke et al., 2013).

Reineke (2013) proposed three different pressure temperature combinations to explain the germination and inactivation mechanism of *Bacillus subtilis* spores in ACES-buffer solution (**Figure 1-7**), and this was also further explained by Georget et al. (2015). First region is a pressure of 0.1-600 MPa at 30-50°C for physiological pressure induced germination (dominates between 100-200 MPa), leading to a maximal spore inactivation of only 4 log after >1 h pressure treatment. The germination at this ambient temperature (~ 37°C) occurs by triggering germinant receptors (nGRs), followed by Ca-DPA release and cortex-lytic enzymes (CLEs) activation degrading the cortex and SASP and finally spore inactivation for long pressure dwell times.

The second region is 0.1-600 MPa at $T < 60^\circ\text{C}$, and third region is $P \geq 600$ MPa at $T > 60^\circ\text{C}$, both for non physiological pressure induced germination (dominates between 400-600 MPa). Germination in these regions occurs without the nGRs and bypasses individual germination steps, and no degradation of SASP is observed. The combination of pressure-heat directly affects the inner spore membrane, leading to spore core hydration and

subsequent inactivation (>7 log inactivation), which is fastest in the third region. Nonetheless, the mechanism of the spore inactivation was carried out in buffer, thus more research is needed to elucidate the process in food products.

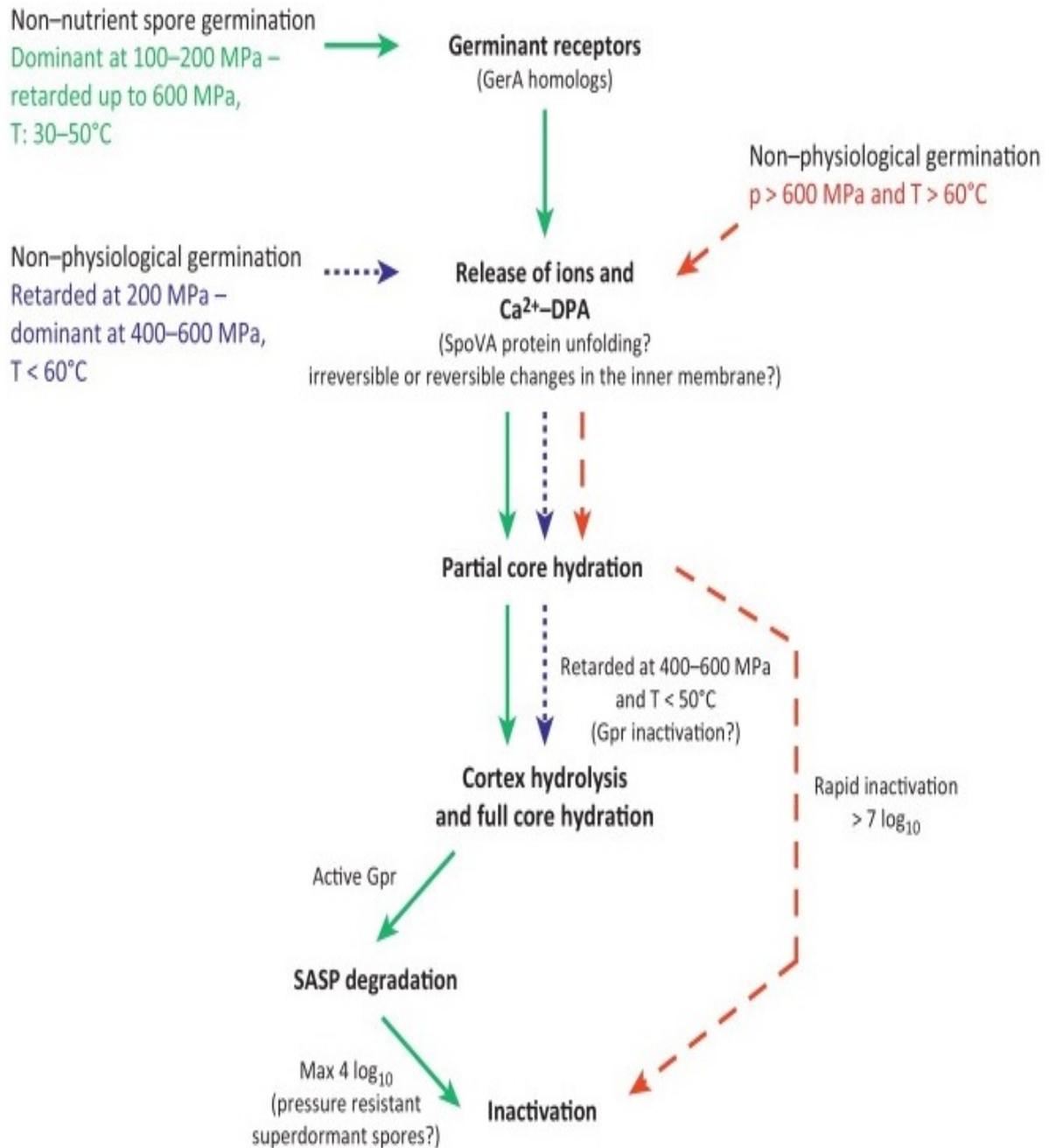


Figure 1-7 Pressure-heat dependence germination and inactivation pathways of *Bacillus subtilis*. Reprinted from Reineke et al. (2013), with permission from Elsevier.

1.4.2 Research areas for spore inactivation

Studies of bacterial spore inactivation by HPP were initially carried out by Hite in 1899 Chlopin with *B. subtilis* spores. This was then followed by an investigation of *Bacillus anthracis* spores by Chlopin and Tammann at the beginning of the 20th century (1903). Their investigations concluded that bacterial spores were resistant to hydrostatic pressure at room temperature. The results were confirmed by Larson et al. (1918) using a broader range of pressure application (up to 1200 MPa). Other than the reason spores may survive, the use of high pressure (>1000 MPa) at ambient temperature has not been recommended because industrial HPP units may face difficulties reaching the high intensity of pressure (Barbosa-Cánovas & Juliano, 2008). Basset and Macheboeuf (1932) pioneered the comparison of the inactivation of vegetative and sporulated cells. During the period 1969-1970, a phenomena of spore germination at <200 MPa, and inactivation of *Clostridium spp.* were reported for the first time (Clouston & Wills, 1969; Gould & Sale, 1970; Sale et al., 1970).

A synergistic effect of high pressure and heat inactivating bacterial spores was reported by Johnson & Zobell in 1948, and continues to be reported until now. Applying pressures above 500 MPa and temperatures above 60°C have been successful to some degree in achieving spore inactivation (Ahn et al., 2007; Daryaei & Balasubramaniam, 2013; Johnson & Balasubramaniam, 2010; Paredes-Sabja et al., 2007; Rajan et al., 2006a, 2006b; Ramaswamy et al., 2010; Reddy et al., 2006; Reineke et al., 2011; Robertson et al., 2008; Silva et al., 2012; van Schepdael et al., 2004; Vercammen et al., 2012; Wang et al., 2009).

Other important points that highlight the research areas on microbial spore inactivation under high pressure are explained as follows. Pressure oscillatory (i.e. reciprocal, cycling or pulse) treatments and the application of a simultaneous or sequential combination of heat and pressure appeared in the period from 1994 to 1998 based on the idea of pressure-induced

germination (Hayakawa et al., 1994a; Hayakawa et al., 1994b; Ludwig et al., 1996; Mills et al., 1998; Sojka & Ludwig, 1994). Combinations with other non-thermal preservation methods such as electroporation, irradiation, ultrasound, food additives or gas (CO₂ and Ar) have also been attempted in order to diagnose a synergy between these methods and allow a wider industrial application of HPP (Crawford et al., 1996; Fujii et al., 2002; Kalchayanand et al., 1994; Roberts & Hoover, 1996; Ross et al., 2003; Shearer et al., 2000; Stewart et al., 2000; Watanabe et al., 2003; Wimalaratne, 2009). Reports also appeared on the inactivation of *Bacillus subtilis* spores when they were exposed to high-pressure treatments at 400 MPa for 30 min after alternating current pretreatment (Shimada, 1992). Although all of the studies mentioned showed there were improvements in the inactivation compared to when the high pressure was applied alone, little effect on further research was seen.

The concept activation of nutrient germinant receptors at low pressure (100-200 MPa), and the release of spores' Ca²⁺-DPA channels for subsequent germination as a result of even higher pressure (500-600 MPa) was introduced by Wuytack et al. (2000) and Paidhungat et al. (2002). Several studies on germination and DPA also appeared in the following years (Black et al., 2007b; Farkas et al., 2003; Subramanian et al., 2006, 2007; Vepachedu et al., 2007).

1.4.3 Kinetics and models for microbial spore inactivation by HPP

Table 1-1 to **Table 1-5** show the inactivation of bacterial and fungal spores in buffer, low acid and high acid foods and their kinetics after HPP-thermal processing, which is the main background of this research. As can be seen from the tables, generally the first order kinetics was the main model used for describing the HPP log survivors. However, non-linear trends could be observed and thus have continued to be reported since 2000.

1.4.3.1 *Clostridium* spores

The kinetic inactivation of *Clostridium* spores by HPP-thermal processes has been shown to be dominated by the first order kinetics (**Table 1-1**), and only one work was found on the non-linear kinetic model which was in combination with a biopreservative (Gao et al., 2011). However, some of the log survivors fitted with the first order model were actually non-linear. For example, Reddy et al.'s *Clostridium botulinum* log survivors showed an upward concavity when the data are plotted. In addition, few HPP-thermal log survivors reported in Margosch et al. (2006), Okazaki et al. (1997), and Ramaswamy et al. (2010) for *Clostridium* spores also exhibited (a slight) deviation to linearity. *Clostridium sporogenes* and *C. botulinum* were the main species reported, thus there is lack information available on the kinetics of other important species.

The evaluation of the efficacy of HPP between 207-1400 MPa in conjunction with heat (35-145°C), and bacteriocin-based biopreservatives (such as pediocin and nisin) on *Clostridium* spores has been attempted. Log reductions of up to 6.3 logs were achieved, although high pressures (>700 MPa) combined with high temperatures (>105°C) or biopreservatives for \leq 14 min were required to obtain close to 6 log or above (Gao et al., 2011; Koutchma et al., 2005; Margosch et al., 2006; Reddy et al., 2013; Sharma et al., 2009). As stated earlier, commercial HPP units typically operate in the pressure range of 100 to 600 MPa and also at temperatures between 5 and 65°C (Balasubramaniam et al., 2008; Bermúdez-Aguirre, & Barbosa-Cánovas, 2011). Thus those more intense processes are still not commercially feasible and more research needs to be conducted.

Table 1-1 First order kinetics for the inactivation of *Clostridium* spores by high pressure processing combined with heat.*

Spores & strain	Medium	First order kinetics parameters							Microbial reduction				Reference	
		pH	P (MPa)	T (°C)	D-value (min)	z-value	P range (MPa)	T range (°C)	P (MPa)	T (°C)	Time (min)	Log reduction		
<i>Clostridium Sporogenes</i> PA 3679	Milk	nr	900	80	13.7	18.9°C*	-	80-100	900	100	5	4.5	Shao et al. (2010)	
				90	7.0									
				100	1.2									
<i>Clostridium Sporogenes</i> ATCC 11437	Milk	nr	900	80	9.1	18.3°C*	700-900	80-100	900	100	2.5	3.5	Ramaswamy et al. (2010)	
				90	3.8									
				100	0.7									
				700	100	1.0	1429 MPa*							
				800	0.8									
	900	0.7												
<i>Clostridium sporogenes</i> ATCC 3679	Meat broth	nr	800	90	5.3	19.3°C*	400-800	90-108	-	-	-	-	Rovere et al. (1999)	
				93	5.3									
				98	2.8									
				108	0.7									
				600	108	1.3	769 MPa*							
				700	0.9									
	800	0.7												
<i>Clostridium sporogenes</i> PA 3679	Ground beef	nr	900	80	7.0	19.1°C*	700-900	80-100	900	100	2	3	Zhu et al. (2008)	
				90	2.3									
				100	0.6									
				700	100	1.5	526 Mpa*							
				800	1.0									
	900	0.6												

Table 1-1 Continued.

<i>Clostridium sporogenes</i> ATCC 7955	Salmon slurry	nr	900	80 90 100 700 800 900	10.9 2.9 0.6 1.3 1.0 0.6	15.8°C 588 MPa	700-900	80-100	900	100	2	2.5	Ramaswamy and Shao (2010)
<i>Clostridium sporogenes</i> PA 3679	Phosphate buffer	7.0	800	91 100 108 600 700 800	4.5 2.3 0.8 1.1 1.0 0.8	23.1°C* 1429 MPa*	600-800	91-108	800	108	3	4.5	Koutchma et al. (2005)
	Egg patties	nr	700	100 105 111 121	1.5 0.9 0.6 0.3	32.4°C*	-	-	700	121	3	6.0	
<i>Clostridium sporogenes</i> PA 3679	ACES buffer	nr	750	105	1.3	-	700		700	105	6	6.3	Reddy et al. (2013)
<i>Clostridium sporogenes</i> PA 3679	Buffer	7.0	0.1	110 200 400	0.7 0.1 0.1	345 MPa*	0.1-400	35-110	400	110	7	<3.0	Okazaki et al. (1996)
<i>Clostridium sporogenes</i> PA 3679	Soymilk	7.0	276	102 121 133 145 207 276	14.4 9.5 4.8 2.9 3.3 2.9	60.6°C* 1250 MPa*	207-276	85-145	276	145	0.4	5.8	Sharma et al. (2009)

Table 1-1 Continued.

<i>Clostridium</i>	Tris-His	5.2	300	120	0.09	400 MPa*	0.1-1400	70-120	800	120	2	5.5	Margosch et al. (2006)**
<i>botulinum</i>	Buffer		600		0.004								
TMW 2.357			900		0.001								
			1200		0.0001								
			1400		0.0000								
<i>Clostridium</i>	ACES buffer	nr	750	105	2.2	-	700		700	105	6	5.4	Reddy et al. (2013)
<i>botulinum</i>													

*The values were calculated based on D-values supplied or interpreted from the graph in the paper; **The log survivors exhibited linearity, although the first order kinetic parameters were reported; nr- not reported.

1.4.3.2 *Bacillus* spores

With respect to *Bacillus spp.* spores, pressures as high as 1400 MPa have also been attempted, and temperatures of up to 130°C between 0.1 and 360 min have been investigated (**Table 1-2** and **Table 1-3**). High variability of the spore inactivation was reported (1.5-8 log reduction), depending on the spore resistance and the conditions applied. Within these research, combinations of pressures >600 MPa and high temperatures (>108°C) for ≥0.1 min (Ardia, 2004; Koutchma et al., 2005; Mathys, 2008; Rajan et al., 2006a) or combination of pressures between >60 MPa and temperatures ≥75°C for long treatment times (>60 min) (Ananta et al., 2001; Clery-Barraud et al., 2004; Furukawa and Hayakawa, 2000) were sometimes needed to obtain ≥6 log of *B. cereus* spores (ACMSF, 1992; CFA, 2006; ECFF, 2006), which also renders them not yet feasible for commercial application. With respect to the kinetics, the log-linear model was still the most frequently reported model for *Bacillus* spores. However, many of the investigators also found that non-linear models (Weibull and log-logistic) were better suited for describing the HPP-thermal log survivor curves (Daryaei et al., 2013; Daryaei and Balasubramaniam, 2013; Mathys, 2008; Rajan et al., 2006b; Wang et al., 2009 Tola and Ramaswamy, 2014), but information on some important species is incomplete.

Table 1-2 First order kinetics for the inactivation of *Bacillus* spores by high pressure processing combined with heat.*

Spores & strain	Medium	First order kinetics parameters							Microbial reduction				Reference	
		pH	P (MPa)	T (°C)	D-value (min)	z-value	P range (MPa)	T range (°C)	P (MPa)	T (°C)	Time (min)	Log Reduction		
<i>Bacillus stearothermophilus</i> ATCC 7953	ACES buffer	6.0	1400	100	46.1	12.1°C*	800-1400	100-120	1400	120	0.1	8.0	Ardia (2004)	
				110	9.2									
		120	1.0											
	Phosphate buffer	6-7	800	90	255.9	37.9°C*	200-1400	90-130	800	130	1	8.0		
				100	135.5									
110				76.8										
			120	46.1										
			130	20.9										
<i>Bacillus stearothermophilus</i>	Broth	nr	800	105	0.3	-	400-800	70-110	-	-	-	-	De Heij et al. (2005)	
<i>Bacillus stearothermophilus</i> ATCC 10149	Water	nr	700	92	0.5	30°C	500-700	92-111	700	111	0.2	1.5	Patazca et al. (2006)	
				105	0.3									
				111	0.1									
				500	0.9	213 MPa								
				600	0.2									
			700	0.1										
<i>Bacillus stearothermophilus</i> ATCC 7953	Egg patties	nr	700	100	0.3	48°C*	-	-	688	108	5	6.0	Koutchma et al. (2005)	
				105	0.3									
				111	0.2									
				121	0.1									
<i>Bacillus stearothermophilus</i> ATCC 7953	Soy milk	6.5	620	70	10.6	41.5°C	550-620	70-90	620	90	7	3.7	Estrada-Giron et al. (2007)	
				80	6.2									
				90	3.5									

Table 1-2 Continued.

			550	90	11.8	143 MPa							
			585		6.5								
			620		3.8								
<i>Bacillus</i>	Mashed broccoli	nr	-	-	-	-	100-600	60-120	600	120	20	6.0	Ananta et al.
<i>stearothermophilus</i>	Cocoa mass	nr	600	70	145.9	18.7°C*	100-600	70-90	600	90	60	5.5	(2001)
ATCC 7953	(70-90%)			80	46.8								
				90	12.4								
			100	90	249.2	400 MPa*							
			200		135.2								
			400		59.6								
			600		12.4								
<i>Bacillus</i>	Distilled water	7.5	-	-	-	34.5°C	500-700	92-110	-	-	-	-	Rodriguez et al.
<i>stearothermophilus</i>						370 MPa							(2004)
ATCC 10149													
<i>Bacillus</i>	Buffer	7.0	200	75	17.0	44.3°C*	30-200	5-100	200	95	6	6.0	Hayakawa et al.
<i>stearothermophilus</i>				85	11.0								(1998)
IFO 12550				95	6.0								
<i>Bacillus</i>	Phosphate	7.0	-	95	-	29.3 MPa	10-60	35-95	60	95	425	≤6.0	Furukawa and
<i>stearothermophilus</i>	buffer		-	95	-	546 MPa	60-100						Hayakawa (2000)
IFO 12550													
<i>Bacillus</i>	Tomato juice	nr	600	75	2.1	33°C*	-	75-105	600	105	0.67	4.0	Daryaei and Bala-
<i>coagulans</i>				85	1.6								subramaniam (2013)
185A				95	0.91								
				100	0.73								
				105	0.19								

Table 1-2 Continued.

<i>Bacillus coagulans</i> ATCC 7050	Buffer	7.0	0.1	110	0.69	434 MPa	0.1-400	50-110	400	110	20	7.0	Okazaki et al. (1996)
				200	0.27								
				400	0.08								
<i>Bacillus cereus</i> ATCC 9818	Cooked rice	6.0	600	60	2.36	71.4°C*	-	60-85	600	85	4	≥7.0	Daryaei et al. (2013)
				70	1.54								
				85	1.04								
<i>Bacillus anthracis</i> RP42	Distilled water	nr	280	75	12	476 MPa*	280-500	20-75	500	75	360	≥8.0	Clery-Barraud et al. (2004)
			400		9								
			500		4								
			500	20	160	34°C*							
				45	19								
				75	4								
<i>Bacillus subtilis</i> 1403	Buffer	7.0	0.1	105	1.13	455 MPa*	0.1-400	25-105	400	105	15	5.0	Okazaki et al. (1996)
				200	0.38								
				400	0.15								
<i>Bacillus subtilis</i> IFO 13722	Water	7.0	100	75	20	-		45-75	100	75	20	5.0	Furukawa et al. (2002)

*The values were calculated based on *D*-values supplied or interpreted from the graph in the paper; nr – not reported.

Table 1-3 Non-linear inactivation of *Bacillus* spores by high pressure processing combined with heat.

Spores & strain	Medium	pH	Kinetic model	Microbial reduction				Reference		
				P range (MPa)	T range (°C)	P (MPa)	T (°C)		Time (min)	Log reduction
<i>Bacillus stearothermophilus</i> ATCC 7953	Phosphate buffer ACES buffer	6 6	Weibull: b = 4.2; n = 0.73 b = 3.7; n = 0.65	500-900	-	900 900	80 80	2.0 2.5	6.0 6.0	Mathys (2008)
<i>Bacillus stearothermophilus</i> ATCC 7953	Cocoa mass (70-90%)	nr	n th order kinetics: c = 1; k = 1.30E-03 s ⁻¹ c = 2.5; k = 3.09E-03 s ⁻¹ c = 1; k = 3.43E-04 s ⁻¹ c = 2.5; k = 6.44E-04 s ⁻¹ c = 1; k = 2.11E-04 s ⁻¹ c = 2.5; k = 2.84E-04 s ⁻¹ c = 1; k = 1.21E-04 s ⁻¹ c = 2.5; k = 1.57E-04 s ⁻¹	100-600	70-90	600 400 200 100	90 90 90 90	60	6.0	Ananta et al. (2001)
<i>Bacillus subtilis</i> ATCC 6633	Mince crabmeat	7.25	Second order polynomial: $\beta_0 = -15.21$; $\beta_1/P = 0.04$ $\beta_4/pH = -1.74$; $\beta_3/time = 1.0$	690-827	60-75	827	75	5	3.5	Balasubramanian and Balasubramanian (2010)
<i>Bacillus amyloliquefaciens</i> Fad 82	Egg patty mince	7.25	Weibull: b = 5.83; n = 0.47	0.1-700	95-121	700	121	1	6.5	Rajan et al. (2006a)
<i>Bacillus coagulans</i> 185A	Tomato juice	4.2	Weibull: b = 1.9; n = 0.68 b = 1.3; n = 0.70 b = 0.9; n = 0.79 Log-logistic: A = -4.0; $\sigma = -4.95$; $\tau = -0.03$ A = -3.8; $\sigma = -4.24$; $\tau = -0.75$	-	95-105	600 600	95 85 75 105 100	0.7 0.7	4.0 4.0	Daryaei and Balasubramanian (2013)

Table 1-3 Continued.

<i>Bacillus coagulans</i> IFFI 10144	Buffer Milk	6.7 nr	Log-logistic: A = 5.9; $\sigma = -1.92$; $\tau = 0.01$ Log-logistic: A = 5.7; $\sigma = -2.86$; $\tau = 0.66$	400-600 500-600	- -	600 600	80 80	30 30	5.0 5.2	Wang et al. (2009)
<i>Bacillus coagulans</i> ATCC 7050	Tomato pulp	4.3	Biphasic linear: f = 1.0; $k_{max_1} = 1.42-1.43$; $k_{max_2} = 0.37-0.45$	300-600	50-60	600	60	15	5.7	Zimmermann et al. (2013)
<i>Bacillus cereus</i> ATCC 9818	Cooked rice	6.0	Log-logistic: A = -3.2; $\sigma = -5.10$; $\tau = -0.08$	-	60-85	600	85	4	≥ 7.0	Daryaei et al. (2013)
<i>Bacillus licheniformis</i>	Carrot juice	4.5 5.5 6.2	Weibull: $\alpha = 0.16$; $\beta = 0.79$ $\alpha = 0.24$; $\beta = 0.67$ $\alpha = 0.66$; $\beta = 0.67$ $\alpha = 0.13$; $\beta = 0.73$ $\alpha = 0.24$; $\beta = 0.63$ $\alpha = 0.56$; $\beta = 0.62$ $\alpha = 0.13$; $\beta = 0.70$ $\alpha = 0.34$; $\beta = 0.64$ $\alpha = 0.74$; $\beta = 0.66$	-	40-60	600 600 600	60 60 60	3 3 3	4.9 4.6 4.1	Tola and Ramaswamy (2014)

nr – not reported.

1.4.3.3 *Alicyclobacillus acidoterrestris* spores

A. acidoterrestris (AAT) spores are problems in high acid food (pH<4.6) industries (Silva & Gibbs, 2001; Silva et al., 2014). Few investigators have started to investigate the kinetics of AAT spore inactivation after HPP (Ardia et al., 2004; Buzrul et al., 2005; Silva et al., 2012; Uchida & Silva, 2015) (**Table 1-4**). A combination of pressures of more than 450 MPa and long treatment times (≥ 25 min) (Buzrul et al., 2005; Uchida & Silva, 2015) or high pressure-high temperature (700 MPa, 95°C) treatment for a short time (1 min) (Ardia et al., 2004) were noted to achieve a minimum of 3 log reduction. Similarly, the processes are still noted to be unfeasible for commercial application. Both linear and non-linear (Weibull and n^{th} order kinetic) models were reported, being the non-linear models more common (Ardia, 2004; Buzrul, 2005; Uchida, 2015).

1. Literature review

Table 1-4 Models for the inactivation of *Alicyclobacillus acidoterrestris* spores by high pressure processing combined with heat.

Strain	Medium	First order kinetics parameters								Microbial reduction				Reference
		pH	°Bx	P (MPa)	T (°C)	D-value (min)	z-value (°C)	P range (MPa)	T range (°C)	P (MPa)	T (°C)	Time (min)	Log reduction	
NZRM 4098	Orange juice	3.8	9.2	600	45	12.9	34.4°C	200-600	45-65	600	65	10	2.0	Silva et al. (2012)
NZRM 4447	Apple juice	3.4	10.6	600	45	8.6	-	-	-	600	45	30	3.5	Uchida (2015)
	Lime juice concentrate	2.5	20.2	600	45	19.9	-	-	-	600	45	45	2.0	
	Blackcurrant concentrate	3.1	30.3	600	45	46.1	-	-	-	600	45	45	1.5	
Non-linear														
NZRM 4447	Malt extract broth	3.8	10	Weibull: b=0.60; n=0.55				600	55-65	600	65	10	2.5	Uchida (2015)
			20	b=1.47; n=0.31						(30°Bx)				
			30	b=0.35; n=0.86										
DMS 2498	Orange juice	3.8	9	n th order kinetics: A ₀ = 44.7 ± 2.1 Ea = -145070 ± 6340 kJkg ⁻¹ A ₁ = -32.3 ± 3.31 kJ kg ⁻¹ MPa ⁻¹ A ₂ = 0.079 ± 0.012 kJkg ⁻¹ MPa ⁻² A ₃ = -7.9*10 ⁻⁵ ± 1.1*10 ⁻⁵ kJkg ⁻¹ MPa ⁻³				100-700	-	700	95	1	5.5	Ardia et al. (2004)
DSM 2492	BAM broth	3.0	nr	Weibull: b=0.059T-1.62; n=0.47-0.73				350-450	35-50	450	50	25	6.0	Buzrul et al. (2005)

nr – not reported.

1.4.3.4 Fungal spores

Similar to AAT, fungal (yeast and mould) spores also cause problem in high acid food products. Milder HPP processes than for bacterial spores (300-600 MPa, 21-60°C, and 0.3-300 min) have been used, and the log reductions obtained were also varied (1-7 log) (**Table 1-5** and **Table 1-6**). Only the first order kinetics were reported although non-linear trends in the HPP-log survivor were often observed (Eicher & Ludwig, 2002; Merkulow et al., 2000; Raso et al., 1998b; Voldřich et al., 2004). Eicher & Ludwig (2002) and Merkulow et al. (2000) also used two-step first order kinetics instead of using a non-linear model (e.g. biphasic linear) to characterize the fungal spore log survivors after HPP-thermal processing. This is an important concern for process design and for food safety and quality. The kinetics of heat resistant fungal spores (*Talaromyces*, *Eurotium* and *Penicillium*) was studied by only a few investigators and very long treatment times (>40 min) were needed (Merkulow et al., 2000; Voldřich et al. 2004). The kinetics of many other important heat resistant spores were not investigated, thus there is still very limited information available.

1. Literature review

Table 1-5 First order kinetic inactivation of mould spores by high pressure processing.*

Spores & strain	Medium		First order kinetics parameters								Microbial reduction				Reference
			P	T	D-value	z-value	P range	T range	P	T	Time	Log			
	pH	°Bx	a _w	(MPa)	(°C)	(min)		(MPa)	(°C)	(MPa)	(°C)	(min)	reduction		
<i>Talaromyces avellaneus</i>	Apple juice	4.5	11	nr	600	17	32	85.5°C*	300-600	17-60	600	60	60	6	Voldřich et al. (2004)
<i>Eurotium repens</i> DSM 62631	NaCl soln.	nr	nr	nr	500	45	7	nr	-	4-45	500	45	50	6	Merkulow et al. (2000)
	Broccoli juice	6.6	2.6	nr	500	45	16	nr	-	4-45	500	45	100	6	
	Apple juice	3.3	12.4	nr	500	45	D ₁ =2 D ₂ =9	nr	-	4-45	500	45	40	6	
<i>Eurotium repens</i> DSM 62631	NaCl soln.	nr	nr	nr	500	25	D ₁ =26 D ₂ =101	nr	-	-	500	25	240	6	Eicher and Ludwig (2002)
<i>Eurotium repens</i> DSM 62631	CsCl soln.	nr	nr	nr	500	25	30	nr	-	-	500	25	300	5	van Almsick et al. (1996)
<i>Penicillium expansum</i> DSM 1994	NaCl Soln.	nr	nr	nr	350	40	<1	nr	-	4-45	350	40	50	7	Merkulow et al. (2000)
	Broccoli juice	6.6			350	40	<1	nr	-	4-45	350	40	125	7	
	Apple juice	3.3			350	40	<1	nr	-	4-45	350	40	125	7	

*The values were calculated based on D-values supplied or interpreted from the graph in the paper; nr – not reported. nr – not reported.

Table 1-6 First order kinetic inactivation of yeast spores by high pressure processing.*

Spores & strain	Medium				First order kinetics parameters						Microbial reduction				Reference
		pH	°Bx	a _w	P	T	D-value	z-value	P range	T range	P	T	Time	Log	
					(MPa)	(°C)	(min)		(MPa)	(°C)	(MPa)	(°C)	(min)	reduction	
<i>Saccharomyces cerevisiae</i>	Orange Juice	3.9	nr	nr	300	25	10.8	117	300-500	-	500	25	1	6	Zook et al. (1999)
					350		2.8	MPa							
					400		1.0								
					450		0.5								
					500		0.2								
	Apple juice	3.8	nr	nr	300	25	10.0	115	300-450	-	500	25	1	6	
					350		2.5	MPa							
					400		0.9								
					450		0.5								
	Model juice Buffer	3.5-	nr	nr	300	25	7.2-10.0	119	300-350	-	500	25	1	6	
		5.0			350		2.2-2.8	MPa							
					400		0.7-1.0								
					450		0.4-0.5								
					500		0.1-0.2								
<i>Saccharomyces cerevisiae</i>	Orange Juice	3.7	11	nr	350	25	1.3	nr	350-500	-	500	25	0.33	4.5	Parish (1998)
						400	Initial T	0.4					Initial T		
						450		0.2							
						500		0.1							
<i>Zacharomyces bailii</i>	Sabouraud dextrose Broth	3.5	nr	0.95	414	21	2.6	222.7	414-517	-	-	-	-	Palou et al. (1997)	
					431		2.1	MPa							
					517		0.9								

Table 1-6 Continued.

				0.98	241	21	13.1	85.8	241-345	-	345	21	2.5	≤3	
					276		4.8	MPa							
					310		2.0								
					345		0.80								
<i>Zacharomyces bailii</i> ATCC 36947	Apple juice	4.1	nr	nr	300	25	15.6	nr	-	-	300	25	25	2.5	Raso et al. (1998b)
	Orange juice	3.9	nr	nr	300	25	10.4	nr	-	-	300	25	25	2.5	
	Pineapple juice	3.4	nr	nr	300	25	37.0	nr	-	-	300	25	25	2.5	
	Cranberry juice	3.5	nr	nr	300	25	14.9	nr	-	-	300	25	25	3	
	Grape juice	3.0	nr	nr	300	25	33.3	nr	-	-	300	25	25	1	

*The values were calculated based on *D*-values supplied or interpreted from the graph in the paper; nr – not reported.

1.5 Power Ultrasound

1.5.1 Ultrasound fundamentals

Ultrasound technology (>20 kHz) utilizes the energy of sound waves that travel through a medium (solid, a liquid, or a gas) at a specific speed or velocity. This technology is known to have a wide variety of application in industrial food processing, including microbial and enzyme inactivation, drying, extraction, crystallization, filtration, freezing, emulsification, degassing, meat tenderization, defoaming, and oxidation processes. Based on the frequency used, ultrasound technology is divided into three categories: power ultrasound (20–100 kHz), high frequency ultrasound (100 kHz–1 MHz), and diagnostic ultrasound (1–500 MHz) (Wu et al., 2013). Power ultrasound with a typical sound intensity between 10 and 1000 W/cm² is a known technique used for disruptive processes, and is the main focus of this research.

Power ultrasound can be used to alter physicochemical properties and improve the quality of foods during processing due to a number of physical, chemical, and biochemical effects arising from acoustic cavitation (Rastogi, 2011). Cavitation refers to the creation, growth, and collapse of microgas bubbles due to regions of pressure change (Chen, 2012; Feng & Yang, 2011b; Piyasena et al., 2004). Stable cavitation develops strong eddies and micro-currents in the surrounding area of the bubbles known as micro-streaming (Bermúdez-Aguirre & Barbosa-Cánovas, 2011). The forces from micro-streaming cause the thinning of the cell membranes due to the separation of the cytoplasm membrane from the cell wall and physical damage (Earnshaw et al., 1995). Transient cavitation generates a strong explosion, releasing a considerable amount of energy and inducing localized extreme conditions, which are considered to make a greater contribution to bacterial cell death (Feng & Yang, 2011b; Gogate, 2011; Wu et al., 2013). The gas in the bubbles is claimed to be able to reach a temperature in excess of 5000 K and pressures of more than 100 MPa at a micro scale

(Suslick & Price, 1999). However, increases in the matrix's overall temperature is considered low ($\sim 5^{\circ}\text{C}$) due to a transient heating and very localized condition (Salazar et al., 2010). The extreme pressure and temperature also leads to sonolysis in water molecules, producing high energy free radicals and H_2O_2 that are known to attack the cell wall, reducing enzyme function (denature protein), thus biological activity (Butz & Tauscher, 2002; Fellows, 2000; Feng et al., 2011a; Jason & Mason, 2008; Piyasena et al., 2004; Riesz & Kondo, 1992). Asymmetrical micro-jets of fluid or bubbles, acceleration, resonance effects and collisions between the microorganism are also other effects known to be produced by cavitation (Loske et al., 1999).

1.5.2 History

Microbial inactivation using power ultrasound was first initiated in the 1920s (Harvey & Loomis, 1929). In the 1960s, research showed that sound waves used in anti-submarine warfare has the potential to kill fish (Earnshaw, 1995). Research then continued to look at the mechanism of ultrasound interaction with microbial cells (Alliger, 1975; Hughes & Nyborg, 1962). It was shown in this period that the separation of the membrane from the cell wall caused a thinning of the cell membrane (Alliger, 1978). During the period 1980-1990s, a combination of power ultrasound with other treatments such as heat (thermosonation/TS), pressure (manosonation/MS), or both (manothermosonation) to inactivate vegetative cells and spores was attempted (Garcia et al., 1989; Pagan et al., 1999; Ordoñez et al., 1987; Raso et al., 1998c; Wrigley & Llorca, 1992). The combined treatments are attractive alternatives over traditional thermal processes due to an increase in anti-microbial potency, and the lower processing temperatures and energy required, thus better food quality (Feng & Yang, 2011b; Mason et al., 1996; Villamiel et al., 1999). The bactericidal effects of ultrasonic treatments depends on the type of the microorganism being treated, the type and volume of foods to be

processed, time of exposure, energy (amplitude) and intensity of the ultrasound, and the sonication conditions (temperature and pressure) (Piyasena et al., 2004; Scherba et al., 1991). Until now however, although ultrasound has shown benefits in inactivating microorganisms, the technology is still in research stages, and reports on inactivation efficiency are limited and give very little information on spores.

1.5.3 Kinetics of microbial spore inactivation by ultrasound

Although the long processing time required for microbial spore inactivation by ultrasound has been mentioned (Ortega-Rivas, 2012), some investigators found that a combination of ultrasound and heat (TS and ultrasound before or after thermal processing) is attractive (Ganesan et al., 2015) due to the possible continuous disruption of spores by the acoustic waves (Belgrader et al., 1999; Chandler et al., 2001). **Table 1-7** summarizes research development on the kinetics of ultrasound assisted inactivation of microbial spores along with their thermal kinetic data. Ultrasound working temperatures between 70 and 112°C, and between 45 and 60°C have been attempted to inactivate bacterial and fungal spores, respectively (Burgos et al., 1972; Garcia et al., 1989; López-Malo et al., 2005; Ordonez and Burgos, 1976). A combination of ultrasound with high temperature (>99°C) (Ordonez and Burgos, 1976) and long processing time (30 min) (Burgos et al., 1972) was required to achieve >5 log inactivation of spores, indicating the process is still not commercially feasible. However, the benefits of this technology were observed by some investigators discussed below.

Regarding thermosonication or TS, Garcia et al. (1989) first reported the lethal effect of TS (20 kHz, 70-111°C, 5 W/mL) in inactivating two strains of *Bacillus subtilis* spores in whole milk and glycerol, and found lower *D*-values than those obtained during thermal processing alone (**Table 1-7**). The authors also found the best inactivation was achieved at around 70°C.

1. Literature review

Similarly, López-Malo et al. (2005) also observed the synergistic effect of ultrasound and heat (20 kHz, 45-60°C) i.e. lower decimal reduction (*D*-) values in the TS on fungal *Aspergillus flavus* and *Penicillium digitatum* conidiospore inactivation.

With respect to the ultrasound assisted (before or after) thermal processing for spore inactivation, heat sensitization of *Bacillus spp.* spores is generally observed after ultrasonic 20 kHz, 0-12°C, 12-15 W/mL treatment (Burgos et al., 1972; Ordonez & Burgos, 1976) (**Table 1-7**). Some other investigators have also found the heat sensitizing effect on *Geobacillus stearothermophilus* spores after ultrasound pretreatment. However, no kinetics were reported (Sanz et al., 1985). In addition, the release of low molecular weight substances from the spore protoplast after the ultrasonic treatments was registered (Palacios et al., 1991).

Although the previous research was shown as promising, further studies were barely seen. All the inactivation kinetic models reported for the bacterial and fungal spore inactivation by ultrasound were from the first order kinetic model. Non-linear inactivation of spores after ultrasound, however, could be observed (Bermúdez-Aguirre, & Corradini, 2012; Condón et al., 2005).

1. Literature review

Table 1-7 First order kinetic inactivation of microbial spores by ultrasound assisted thermal vs. thermal processing alone.*

Spores & strain	Medium	Treatment				First order kinetics parameters			Microbial reduction		Reference
		Frequency (kHz)	Power (W)	Amplitude (μm)	Time (min)	T ($^{\circ}\text{C}$)	D (min)	z ($^{\circ}\text{C}$)	Time (min)	Log reduction	
<i>Bacillus subtilis</i> 189	Ringer soln.	20*	60*	nr	10	112	1.6	5.5	10	5.0	Ordonez and Burgos (1976)
<i>Bacillus cereus</i>	Ringer soln.	-	-	-	-	110	11.5	nr	10	1.0	Burgos et al. (1972)
	Ringer soln.	20*	nr	nr	12	110	1.5	nr	10	5.0	
<i>Bacillus licheniformis</i>	Ringer soln.	-	-	-	-	99	5.5	nr	30	4.0	
	Ringer soln.	20*	nr	nr	12	99	3	nr	30	6.5	
<i>Bacillus subtilis</i> ATCC 6051	Whole Milk	-	-	-	-	100	2.6	9.1	-	nr	Garcia et al. (1989)
	Whole Milk	20	150	nr	nr	100	1.6	9.4	-	nr	
	Whole Milk	20*	150*	nr	nr	100	2.8	9.1	-	nr	
	Glycerol	-	-	-	-	100	40.5	13.4	-	nr	
	Glycerol	20	150	nr	-	100	9.1	14.4	-	nr	
<i>Bacillus subtilis</i> <i>var. niger-40</i>	Whole Milk	-	-	-	-	100	11.4	6.7	-	nr	
	Whole Milk	20	150	nr	nr	100	1.8	6.3	-	nr	
	Whole Milk	20*	150*	nr	nr	100	6.8	6.4	-	nr	
	Glycerol	-	-	-	-	100	37.0	13.5	-	nr	
	Glycerol	20	150	nr	-	100	13.2	13.8	-	nr	
<i>Aspergillus flavus</i>	Broth (pH 3.0, a_w 0.99)	-	-	-	-	60	1.6	5.9	-	nr	López-Malo et al. (2005)
	Broth (pH 3.0, a_w 0.99)	20	nr	120	-	60	0.8	8.1	-	nr	
	Broth (pH 3.0, a_w 0.95)	-	-	-	-	60	2.3	6.3	-	nr	
	Broth (pH 3.0, a_w 0.95)	20	nr	120	-	60	2.2	17.3	10	1.4	
<i>Penicillium digitatum</i>	Broth (pH 3.0, a_w 0.99)	-	-	-	-	53	9.5	10.6	-	nr	
	Broth (pH 3.0, a_w 0.99)	20	nr	120	-	53	3.8	9.5	-	nr	
	Broth (pH 3.0, a_w 0.95)	-	-	-	-	53	14.0	10.5	-	nr	
	Broth (pH 3.0, a_w 0.95)	20	nr	120	-	53	6.3	11.9	10	1.6	

*Ultrasound before or after thermal processing.

1.6 Gaps in the literature

As mentioned previously, microbial spores are a significant problem for human health and the food industry. Mild thermal food pasteurization may not be enough to reduce the number of heat resistant spores and thermal processing at high temperature (generally between 80 and 100°C) often diminishes food constituents. Therefore, there is an interest in studying non-thermal treatments such as High Pressure Processing (HPP) and power ultrasound and in combination with heat.

Table 1-1 to 1-7 showed studies which have been done on the HPP-thermal and ultrasound assisted thermal inactivation of spores of *Clostridium spp.*, *Bacillus spp.*, *A. acidoterrestris*, and fungi, and their kinetics. Although extensive studies have been conducted to achieve the inactivation of microbial spores and to obtain the kinetic parameters, bacterial and fungal spores may vary in their response to a lethal effect such as high pressure due to various factors involved in the inactivation. The previously listed research, however, provides an excellent guide for the selection of the main works of this research as discussed following.

The spores of *C. perfringens*, *A. acidoterrestris*, and psychrotrophic *Bacillus cereus* bacteria, and *Byssochlamys nivea*, and *Neosartorya fischeri* moulds were selected in this study because these spores exhibited high resistance to heat. *C. perfringens* and psychrotrophic *B. cereus* spores are also important pathogens (toxin producers) in low acid foods. More specifically, regarding *C. perfringens*, there was not enough information available about kinetic inactivation by HPP and ultrasound as previously explained. With respect to psychrotrophic *B. cereus*, its ability to grow at low temperature during refrigerated storage is a concern since non-thermal processed foods are generally cold distributed. To the best of the author's knowledge, none has modelled the HPP kinetic inactivation with psychrotrophic *B. cereus* spores.

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The spores of *A. acidoterrestris*, *B. nivea*, and *N. fischeri* are concerns for economic loss in high acid foods due to their ability to grow in high acid products after mild heat treatment, reducing the shelf life and causing food spoilage. More specifically, regarding *A. acidoterrestris*, except with vegetative cells, previous studies have not attempted ultrasound inactivation of the spores. With respect to *B. nivea*, and *N. fischeri* ascospores, because of their heat resistance among other factors, these species are mycotoxin producers. In addition, no research has investigated their kinetics with HPP and ultrasound treatment, and thus this is not yet clear. To conclude, regarding ultrasound, there was scant information on the modeling, whereas for HPP, there was missing information regarding those strains.

Thus the following chapters of the thesis report the investigation of the effect of HPP and ultrasound processes in combination with heat for the inactivation of *C. perfringens*, *A. acidoterrestris*, psychrotrophic *B. cereus*, *N. fischeri* and *B. nivea* spores in several foods and their inactivation kinetic modeling. The investigation on the thermal inactivation of these spores was also carried out to compare the results with non-thermal treatments and to draw better conclusions. The foods chosen (milk, beef slurry, fruit juice/puree) represent low acid and high acid foods and are prone to contamination by these bacterial and fungal spores.

Chapter 2 High pressure thermal processing for the inactivation of *Clostridium perfringens* spores in beef slurry

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2. High pressure processing *C. perfringens* spores in beef slurry

Chapter abstract

The spores of *Clostridium perfringens* can survive and grow in cooked/pasteurized meat, especially during the cooling of large portions. In this study, 600 MPa high pressure thermal processing (HPTP) at 75°C for the inactivation of *C. perfringens* spores was compared with 75°C thermal processing alone. The HPTP enhanced the inactivation of *C. perfringens* spores in beef slurry, resulting in 2.2 log reductions for HPTP vs. no reductions for thermal processing after 20 min. Then, the HPTP resistance of two *C. perfringens* spore strains in beef slurry at 600 MPa was compared and modeled, and the effect of temperature investigated. The NZRM 898 and NZRM 2621 exhibited similar resistance, and Weibull modeled well the log spore survivor curves. The spore inactivation increased when HPTP temperature was raised from 38 to 75°C. The results confirm the advantage of high pressure technology to increase the thermal inactivation of *C. perfringens* spores in beef slurry.

2. High pressure processing *C. perfringens* spores in beef slurry

2.1 Introduction

Clostridium perfringens is an anaerobic, mesophilic, spore-forming bacterium that can grow at temperatures ranging from 15 to 50°C and pH values between 5.0 and 8.0 (McClane, 2007). This bacterium has been identified as one of the major causes of food-related infections arising from low-acid pasteurized foods (Golden et al., 2009; Juneja et al., 1995; Juneja et al., 2006; Juneja & Marmer, 1996; Juneja et al., 2010; Labbé et al., 2014; Scallan et al., 2011; Silva & Gibbs, 2010; Silva et al., 2014). Depending on the isotype, *C. perfringens* strains are classified as A, B, C, D, or E (Uzal & McClane, 2011), with type A food poisoning being the third most common foodborne disease after norovirus and *Salmonella* spp. (CDC, 2011; Havelaar et al., 2012). Illness is caused after ingestion of a large number of cells that subsequently produce toxin, causing symptoms. Around 5% of isolates have been reported to carry *C. perfringens* enterotoxin (cpe) genes, which also cause gastrointestinal infections, antibiotic-associated diarrhea and sporadic diarrhea in humans (Fisher et al., 2005). Foodborne illness typically arises following temperature abuse (either slow or improper cooling) of cooked meat products during which *C. perfringens* spores germinate then grow rapidly due to their short generation times (i.e. 8 min at the optimal growth temperature of 43-45°C) (Labbé, 2000), and has been associated with foods (commonly meat and poultry) prepared in industrial kitchens, e.g. factories, hospitals, school cafeterias, prisons, and nursing homes.

Current strategies to prevent the growth of *C. perfringens* involve rapid cooling of meat and poultry products after cooking/thermal processing down from 55 to 15°C (EFSA, 2005a), however, given cooling practices aren't always followed strictly, elimination of *C. perfringens* spores through processing is an alternative strategy. The thermal inactivation of *C. perfringens* spores in foods has been reported in several works (Byrne et al., 2006; Juneja

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et al., 1995; Juneja et al., 2006; Juneja & Marmer, 1996), and the results showed high resistance to heat: the $D_{100^{\circ}\text{C}}$ -values ranged between 1.9 and 28.1 min, with z -values ranging between 7.2 and 11.4°C depending on the strain and food. $D_{100^{\circ}\text{C}}$ -values of 5.5 and 7.1 min in beef slurry for two strains of *C. perfringens* spores were obtained in our past study (Evelyn & Silva, 2015b), while around 20 min was found in beef gravy (Juneja et al., 2003). The most heat-resistant *C. perfringens* spores were reported to survive for 1 h at 100°C (Labbé et al., 2014). Thus, most thermal inactivation studies were carried out at $\geq 100^{\circ}\text{C}$ (Bradshaw et al., 1977; Orsburn et al., 2008; Sarker et al., 2000). The high temperature heating might cause changes in the meat color and texture, and the formation of undesirable flavours (Jayasena et al., 2013; Kong et al., 2007). These findings prompted research on the *C. perfringens* risk assessment on ready-to-eat and partially cooked meat and poultry products and its spore inactivation in different foods (Akhtar et al., 2009; Crouch et al., 2009; Gao et al., 2011; Golden et al., 2009).

High pressure processing (HPP) is a commercial food preservation technology with less adverse effects on food quality compared with conventional thermal processes alone (Cullen et al., 2012). HPP in the range of 400-600 MPa between 5 and 10 min, with or without additional heat, is typically applied to inactivate spoilage/pathogenic microorganisms and to extend food shelf-life. For most microbial spores and enzymes, inactivation is not achieved by high pressure alone; therefore, the combination of high pressure processing with mild heat, also referred to as high pressure thermal processing (HPP-thermal or HPTP) is required (Evelyn & Silva, 2015c; 2015d; Evelyn et al., 2016; Patterson, 2005; Silva et al., 2012; Sulaiman & Silva, 2013; Sulaiman et al., 2015a). The efficacy of HPP-thermal between 400 and 650 MPa and 55-75°C to inactivate *C. perfringens* spores has been investigated (Gao et al., 2011; Paredes-Sabja et al., 2007). *C. perfringens* spore reductions of up to 6 logs were achieved, depending on the conditions applied and the spore resistance. Among these results,

2. High pressure processing *C. perfringens* spores in beef slurry

Paredes-Sabja et al. (2007) obtained a 6-log cycle reduction of *C. perfringens* F4969 spores in buffer solution (pH 6.5) with processing conditions of 650 MPa and 75°C for a processing time of 15 min. To date, lack information is available on the inactivation kinetics of *C. perfringens* spores after HPP-thermal, which resulted in limited modeling. Therefore, more information on the HPP-thermal inactivation of *C. perfringens* spores with several strains are needed.

The current research investigated HPP-thermal inactivation of *C. perfringens* spores inoculated in beef slurry. The pasteurized beef slurry was chosen for this research since it is similar to beef mince and prone to contamination by *C. perfringens*. The main objectives were: (i) to compare the 600 MPa HPP-thermal with thermal inactivation of *C. perfringens* spores in beef slurry at 75°C; (ii) to compare the resistance of two *C. perfringens* strains to 600 MPa HPP-therma; and (iii) to model the 600 MPa HPP-thermal inactivation kinetics of *C. perfringens* spores in beef slurry.

2.2 Materials and methods

2.2.1 *C. perfringens* microbiology

2.2.1.1 Strains

Two important chromosomal-cpe isolates (NZRM 898 and NZRM 2621) of *C. perfringens* type A strains were sourced freeze-dried from the New Zealand Reference Culture Collection. The NZRM 898 and NZRM 2621 strains were isolated from salted beef and feces, respectively (Stringer, Turnbull, & Gilbert, 1980). The strain NZRM 898 is the same as ATCC 14809 and NCTC 10239, and has been previously referred to as 17450, Bradford

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17450, CECT 563, CN 6953, F 5012, F 5058, Hobbs FH 5012, Hobbs FH 5058, NRRL B-23852 and Smith 17450. The NZRM 2621 is the same as ATCC 12917 and NCTC 8239, and has also been referred to as 3653, 3650, BCRC 17462, CCRC 17462, CCUG 18370, CECT 486, CIP 104880, CN 3417, FH 3653, FH 3650, Hobbs FH 3653, Hobbs FH 3650 and NRRL B-23851.

2.2.1.2 Spore production

The cultures were prepared by suspending the freeze-dried cultures into test tubes containing 10 mL of cooked meat medium (Acumedia, Neogen Europe), and incubated under anaerobic conditions at 37°C for 48 h. Initial growth was obtained by inoculating 0.1 mL of the starter culture into 9.9 mL of thioglycolate broth (Difco, Becton Dickinson, France) and incubated at 37°C overnight. Sporulation was obtained by inoculating 0.2 mL of the overnight culture into 10 mL modified Duncan-strong (DS) sporulation medium (Labbé & Rey, 1979), followed by anaerobic incubation at 37°C for 48 h. The spore formation was monitored and confirmed using a Motic microscope (BA410 Series). The resultant spore suspension was centrifuged at 4°C for 15 min, three times, in a sterile sodium phosphate buffer (0.1 M, pH 7.0) at 4,000g, followed by resuspension in sterile distilled water. The spore suspension was then heated for 20 min at 75°C to remove the remaining vegetative cells, and stored at 2°C until use. A spore concentration of approximately 10⁹ cfu/mL was obtained.

2.2.1.3 Spore enumeration

The *C. perfringens* spore concentration in beef slurry before and after processing was determined by spread plating into brain heart infusion agar (BD Diagnostic Systems, Sparks, MD, USA) and anaerobic incubation at 37°C for 48 h. Beef slurry samples were first homogenized in a stomacher (Masticator stomacher, IUL Instruments, Germany) with an equal volume of 0.1% (w/v) sterile buffered peptone water (BPW) solution as dilution fluid

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for 2 min (this dilution was considered in the calculation of final spore concentration). Samples were serially diluted with BPW and plated twice. Spore suspensions were enumerated using the decimal dilution with BPW. Average results were expressed in cfu per gram (cfu/g) of beef slurry.

2.2.1.4 Beef slurry inoculation

Beef slurries (pH 6.5) were prepared to expedite the HPP and HPP-thermal processes. The major composition of the beef slurry was 76% moisture, 14% protein, 7% fat, 2.6% carbohydrate, and 0.4% ash (determined by an accredited laboratory in New Zealand). A portion (0.1 mL) of spore suspension was inoculated into 2.9 g of beef slurry placed inside food grade sterile pouches (8x8 cm, Cas-Pak, New Zealand) to yield an initial spore concentration of approximately 10^7 cfu/g of beef slurry. The pouches were then vacuum packed, thermosealed (Multivac C200, Germany) and mixed in the stomacher before processing.

2.2.2 High pressure processing of beef slurry

2.2.2.1 High pressure equipment and operation

A high pressure food processing system (QFP 2L-700, Avure Technologies, Columbus, Ohio, USA) was used for pressure and pressure combined heat treatment of *C. perfringens* spores. The maximum pressure and temperature supported by the Avure HPP machine were 600 MPa and 75°C, respectively. For combined pressure–heat treatment applications, the 2 L stainless steel pressure chamber was immersed in a temperature controlled bath, in which propylene glycol acted as the heating medium (heating with propylene glycol was not carried out for the treatment at room temperature). The temperature of the external glycol bath was

2. High pressure processing *C. perfringens* spores in beef slurry

set prior to the combined treatment to achieve the target temperature. Two internal thermocouples were used to monitor the temperature in the distilled water contained in the pressure chamber and another internal thermocouple was used to measure the glycol bath temperature during processing. The come-up time for 600 MPa ranged between 1 and 1.5 min.

2.2.2.2 High pressure and high pressure combined with thermal processing

The pouches containing the 3 g inoculated beef slurry samples were subjected to high pressure at room temperature (HPP) or high pressure combined with moderate temperatures (HPP-thermal) for between 5 and 40 min. The long processing time was chosen in this study due to high resistance of *C. perfringens* spores, so that it can reduce significantly the number of spores. The inactivation kinetics and resistance of the two *C. perfringens* spores in beef slurry at 600 MPa and 38°C [no additional heat], 50°C, 60°C, and 75°C, were investigated and compared. A pressure of 600 MPa was selected not only because this is the maximum working pressure of the Avure HPP unit, but also because previous publications have shown that higher pressures are more effective for the inactivation of *C. perfringens* spores (Akhtar et al., 2009; Gao et al., 2011; Paredes-Sabja et al., 2007). HPP at 600 MPa is also the most frequently pressure used by the food industry. The temperatures selected were lower than those employed in thermal processing ($\geq 100^\circ\text{C}$). Preliminary trials were carried out to determine the initial temperature of the water and beef contained in the HPP chamber. Depending on the processing time, initial temperatures ranging between 30–34°C, 41–46°C, 51–56°C, and 64–69°C were set before starting the high pressure treatments in order to achieve the average working temperatures of 38°C, 50°C, 60°C, and 75°C during the constant pressure phase of the HPP and HPP-thermal cycles, respectively. The maximum temperatures achieved after pressurization were 45–52°C at 38°C, and 81–86°C at 75°C, respectively,

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indicating 3°C increase per 100MPa. Two samples were processed for each processing time and three survival experiments were carried out for each temperature. Pressure and pressure-thermal-treated samples were submerged in an ice water bath prior to spore enumeration. The survivors for *C. perfringens* NZRM 898 under HPP-thermal at 75°C were also compared with previously obtained data on thermal survivors of this strain at 75°C (Evelyn & Silva, 2015b).

2.2.3 Modeling the HPP and HPP-thermal inactivation of *C. perfringens* spore in beef slurry

The HPP and HPP-thermal survival curves (log microbial numbers vs. time) were non linear, and Weibull was an appropriate model (Equation 1-6). TableCurve 2D version 5.01 (SYSTAT Software Inc., USA) was used to fit the Weibull model to the spore survival lines, and estimate the model parameters \pm standard errors (SE). Mean square error (MSE) and coefficient of determination (R^2) were used to compare the performance of different models. R^2 values close to 1 and a relatively small MSE indicated the adequacy of the model to describe the data. For each temperature, three survival experiments were carried out and the model parameters (b , n) were estimated by regression of logarithmic number of survivors ($\log N/N_0$) versus time. N_0 was the spore concentration of the untreated sample (control) and N was the spore concentration in the beef slurry sample at a certain processing time. Then, the parameter mean value \pm standard deviation (SD) were calculated for each HPP temperature. Additionally, the temperature dependence of the estimated parameters was investigated. t -test (Statistica 8.0, Statsoft Inc., USA) was used to compare the log reductions of the two *C. perfringens* strains under the 600 MPa HPTP after a 40 min process.

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2.3 Results and discussion

2.3.1 600 MPa and 75°C vs 75°C thermal inactivation of *C. perfringens* spores in beef slurry

Figure 2-1 shows the log survivors of *C. perfringens* NZRM 898 spores in beef slurry by 600 MPa HPTP and thermal process alone at 75°C. The 75°C HPP process was faster than 75°C thermal inactivation of *C. perfringens* spore in beef slurry. For a 20 min process, 2.2 log reductions in *C. perfringens* spores were obtained with HPP-thermal vs. no reduction with thermal inactivation alone, indicating that pressure might sensitize the spores to subsequent inactivation by heat or pressure treatments. Previous investigators explained that the mechanism of inactivation (loss of resistance) of *C. perfringens* spores under HPP involves calcium 2,6-pyridine dicarboxylic acid or calcium dipicolinic acid (Ca-DPA) release through the GerKA-KC receptor pathway or germination as wild-type spores (Paredes-Sabja et al., 2009a, 2009b). The benefit of HPP-thermal (586 MPa, 73°C, 10 min) vs. thermal (90°C, 20 min) for the inactivation of *C. perfringens* spores has been documented by Akhtar et al. (2009), showing 4-log reductions in spore counts for both processes. However, the processes were carried out with prior spore pretreatment (i.e. addition of 50 mM KCl followed by incubation at 55°C for 15 min). Therefore, our results confirmed that the 600 MPa HPP-thermal of *C. perfringens* spores synergistically enhanced its thermal inactivation. Gao et al. (2011) reported a 2.5-log reduction in *C. perfringens* AS 64701 spores in UHT milk using 600 MPa-65°C HPTP after 12.5 min. Paredes-Sabja et al. (2007) reported a 0.4-log reduction in the same *C. perfringens* spore strain and between 0.1- and 3.1-log reductions for other chromosomal-*cpe* strains using HPP-thermal at 650 MPa and 75°C in citric acid buffer (pH 6.5) after a holding time of 15 min. These results suggest that there is considerable variability between *C. perfringens* spore resistance, and that the extent of *C. perfringens* spore

2. High pressure processing *C. perfringens* spores in beef slurry

inactivation by HPP-thermal is highly dependent on the bacterial strain, the food/medium used, and the HPP-thermal conditions employed during the process.

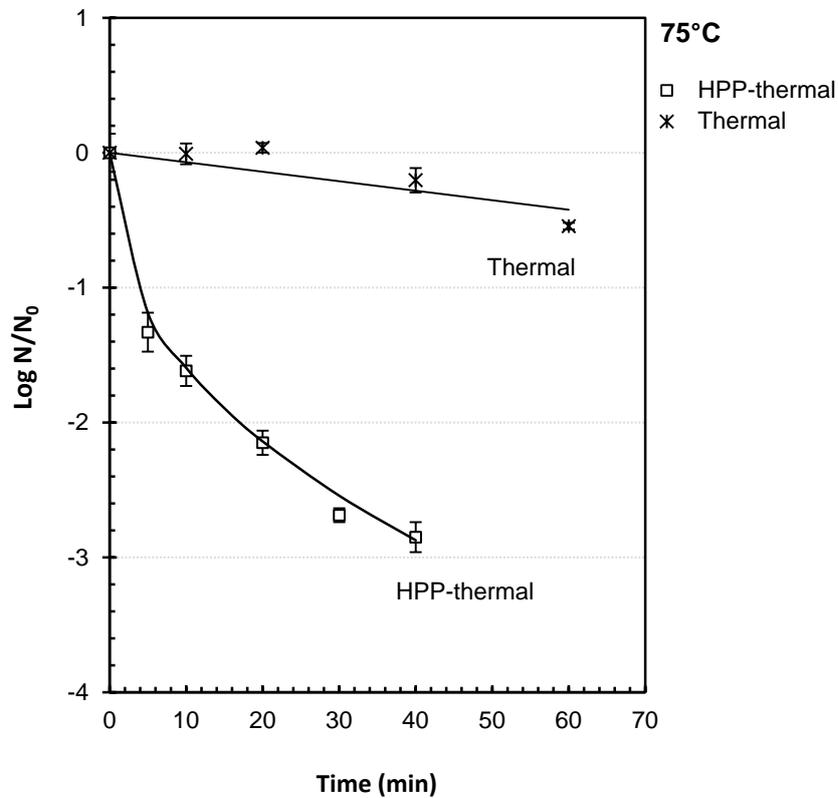


Figure 2-1 Comparison of 600 MPa HPP-thermal and thermal inactivation of *C. perfringens* NZRM 898 (= ATCC 14809, NCTC 10239) spores in beef slurry at 75°C (data points are average \pm standard deviation).

2.3.2 Comparing the spore resistance to HPP-thermal of two strains

Figure 2-2 shows the log survivors of *C. perfringens* NZRM 898 and NZRM 2621 spores in beef slurry by 600 MPa HPP (38°C-no additional heat) and HPP-thermal at three temperatures (50, 60 and 75°C) during up to 40 min of processing. In general, NZRM 898 and NZRM 2621 spores exhibited similar resistance, although the *t*-test confirmed differences at certain processing times for 600 MPa-38°C ($p < 0.05$). Paredes-Sabja et al. (2007) also reported both similarities and difference in resistance among the chromosomal-*cpe* isolates tested (NCTC

2. High pressure processing *C. perfringens* spores in beef slurry

10239, NCTC 8239, FD 1041, E13, SM 101) in citric acid buffer (pH 6.5) under 550 and 650 MPa-75°C HPP-thermal. The inactivation of NZRM 898 spores using HPP at 38°C (**Figure 2-2**) was similar to that achieved using the 75°C thermal process (**Figure 2-1**) after 40 min ($p > 0.05$), indicating that the addition of high pressure technology enables use of a 37°C lower temperature for the same result.

The use of higher HPP-thermal temperatures substantially promoted the inactivation of *C. perfringens* spore (**Figure 2-1** and **Figure 2-2**), indicating an important role of temperature for inactivating these spores. For example, increasing the temperature from 38°C (no heating used) to 75°C at 600 MPa for 40 min increased the *C. perfringens* spore inactivation in beef slurry by approximately 2.5-log for both strains. Similar observations were reported with spores of *C. perfringens* C-cpe (SM 101, FD 1041, E13, NCTC 8239) and P-cpe isolates (F 5603, F 4969, B 40, NB 16) with 650 MPa HPP when the temperature was increased from 55°C to 75°C (Paredes-Sabja et al., 2007). Similarly, Gao et al. (2011) found that the number of *C. perfringens* AS 64701 spores decreased by 3 log when the temperature was increased from 35°C to 95°C.

2. High pressure processing *C. perfringens* spores in beef slurry

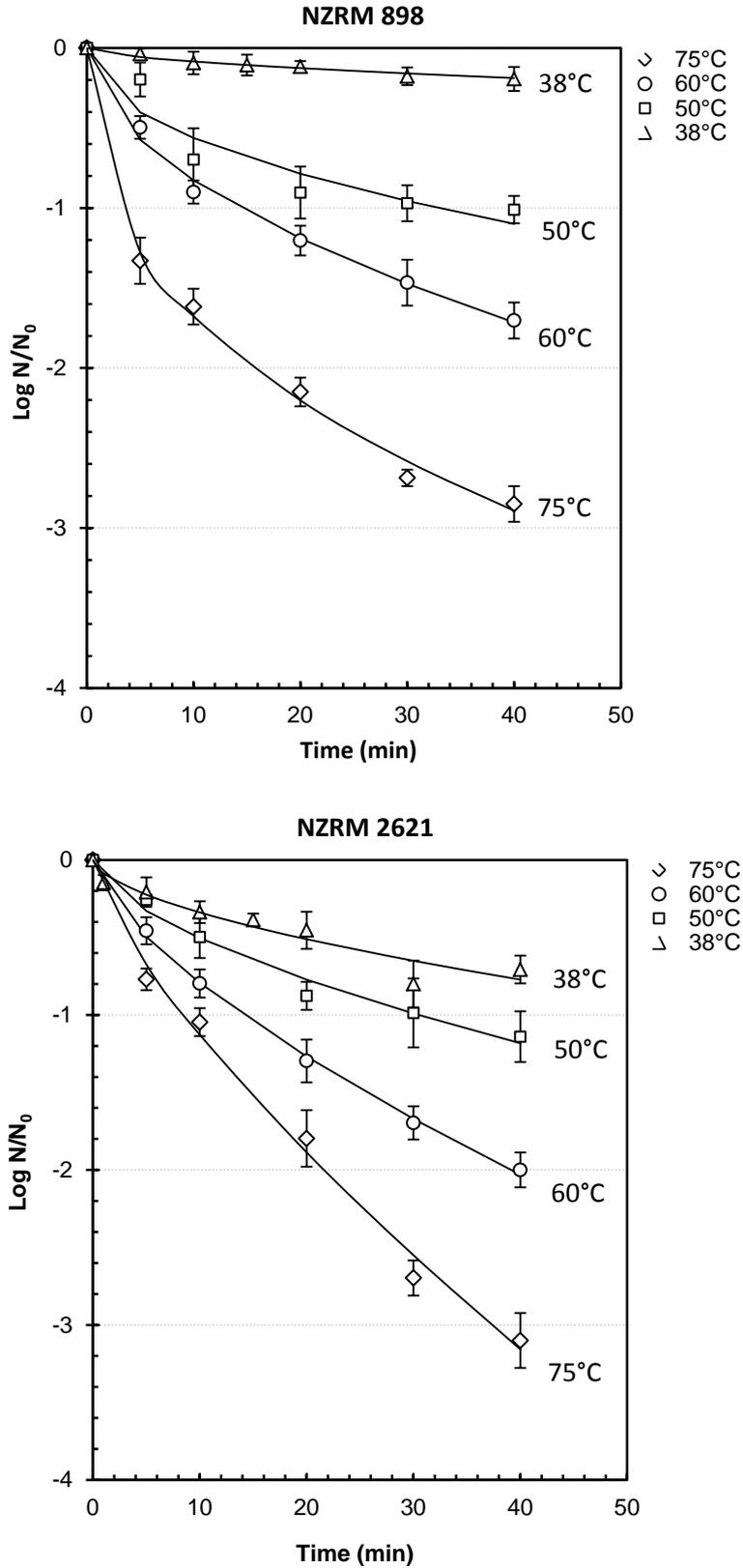


Figure 2-2 The Weibull model fitted to 600 MPa HPP (38°C-no additional heat) and HPP-thermal (50, 60, 75°C) inactivation of *C. perfringens* NZRM 2621 (=ATCC 12917, NCTC 8239, DSM 11874) spores in beef slurry (data points are average \pm standard deviation).

2. High pressure processing *C. perfringens* spores in beef slurry

2.3.3 Modeling the 600 MPa HPP and HPP-thermal inactivation kinetics of *C. perfringens* spores in beef slurry

C. perfringens spore inactivation during HPP, and HPP-thermal showed a non-linear pattern (**Figure 2-1** and **Figure 2-2**), and was therefore not consistent with first-order kinetics. Weibull and log-logistic models were investigated for HPP and HPP-thermal. The log-logistic model presented poor temperature effects on the parameters (not shown), and higher standard deviations for the kinetic parameters estimated at some treatment temperatures. In contrast, the simpler 2-parameter Weibull model showed good performance indices ($0.0002 \leq \text{MSE} \leq 0.031$, $0.890 \leq R^2 \leq 0.999$) (**Table 2-1**), and therefore was selected.

In the Weibull distribution, the b parameter is the scale factor that represents the spore inactivation rate and the n value is the shape factor of the survival curves and the deviation from linearity. As can be seen from the data in **Table 2-1** and **Figure 2-3**, at 600 MPa, the higher the temperature, the higher was the rate of inactivation (b value). The b value increased from 0.02 to 0.68 for NZRM 898 and from 0.09 to 0.20 for NZRM 2621 as the HPP temperature increased from 38°C to 75°C. The dependency of the b -values to temperature was observed, therefore plotting between $\log b$ and temperature was carried out with $0.88 \leq R^2 \leq 0.96$) (**Figure 2-3**). The dependence of inactivation rate (b parameter) on the HPP temperature has also been observed by other investigators for *C. perfringens* (Buzrul, 2015; Buzrul et al., 2005; Daryaei et al., 2013; Juliano et al. 2009; Luu-Thi et al., 2014) and for other spore-formers, such *Bacillus cereus* spores (Evelyn & Silva, 2015c), and *Byssoschlamys nivea* and *Neosartorya fischeri* ascospores (Evelyn & Silva, 2015d; Evelyn et al., 2016).

All the n values were less than 1 (**Table 2-1**), confirming that the spore survivor curves produced by HPP-thermal were concave upward (**Figure 2-2**). This type of concavity

2. High pressure processing *C. perfringens* spores in beef slurry

suggests that there is a mixed resistance of the spore population to the lethal treatment (Peleg & Cole, 1998; van Boekel, 2002), in which the most sensitive spore population is inactivated at a faster rate, followed by the slower and steadier decline of a more resistant population (Tola & Ramaswamy, 2014). The n values for NZRM 898 changed slightly with temperature, varying between 0.39 and 0.73. However, these values for NZRM 2621 showed minimal change (from 0.61 to 0.74). There are inconsistencies in the literature regarding the dependency of n -values to lethal factors (temperature and/or pressure). Cunha et al. (1998) suggested that n should not change with the temperature since it is related to the kinetic pattern, and others reported findings consistent with this, i.e. that Weibull n -values in many instances are independent of temperature and/or pressure (Bermúdez-Aquirre & Corradini, 2012; van Boekel, 2002). In contrast, other authors have reported that Weibull n -parameters are temperature- and/or pressure-dependent (Buzrul, 2015; Chen & Hoover, 2003; Juliano et al., 2009; van Boekel, 2008). Fixing of the n -values for log survivors' fittings was not carried out, so that different HPP-thermal lethal mechanism on each strain can be observed and compared.

2. High pressure processing *C. perfringens* spores in beef slurry

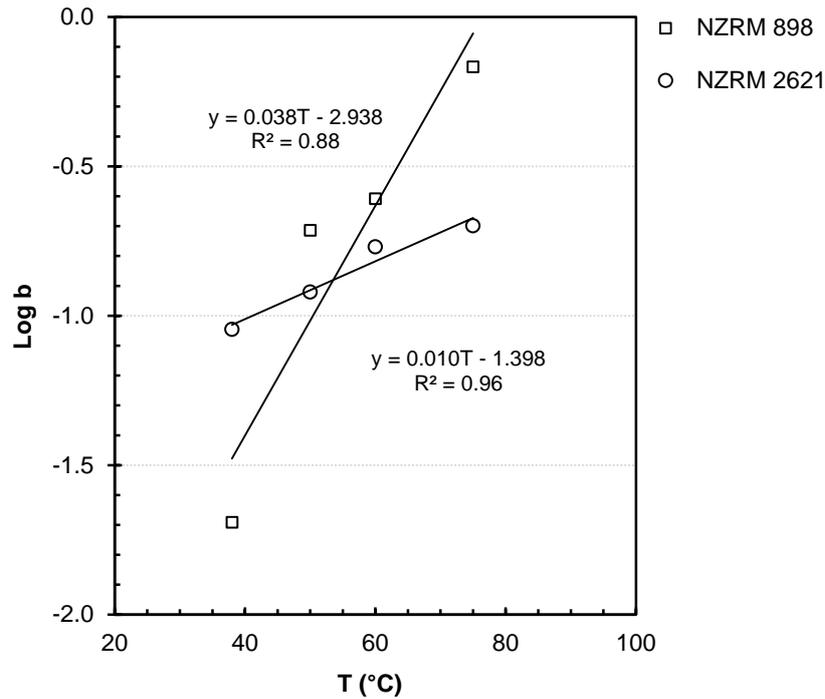


Figure 2-3 HPP combined with thermal processing (HPP-thermal): The effect of temperature on the Weibull b -parameter.

Table 2-1 Weibull model parameters for 600 MPa HPP and HPP-thermal inactivation of *Clostridium perfringens* spores in beef slurry*

T (°C)	NZRM 898		NZRM 2621	
	$b \pm SD$	$n \pm SD$	$b \pm SD$	$n \pm SD$
75	0.68±0.10	0.39±0.03	0.20±0.03	0.74±0.03
60	0.25±0.03	0.53±0.02	0.17±0.04	0.68±0.06
50	0.19±0.10	0.50±0.14	0.12±0.05	0.62±0.10
38	0.02±0.01	0.73±0.28	0.09±0.04	0.61±0.10

**C. perfringens* NZRM 898 and *C. perfringens* NZRM 2621 were used; b and n are the Weibull scale and shape factors, respectively; Parameter values are means±standard deviation (SD) and obtained from three experiments; The Weibull model was supported by good performance indices ($0.0002 \leq MSE \leq 0.031$, $0.890 \leq R^2 \leq 0.999$). Low mean square error (MSE) and R^2 close to 1 are indication of good fit; HPP temperatures of 50, 60, and 75°C were used.

2. High pressure processing *C. perfringens* spores in beef slurry

2.4 Conclusion

The current study demonstrated that the 600 MPa HPP-thermal process was much faster than thermal processing alone to inactivate *C. perfringens* type A spores in beef slurry. Approximately, a 2.2 log reduction in spore count was obtained using 75°C HPP-thermal after 20 min in contrast to no inactivation for 75°C thermal process. However, a long treatment time is still needed to achieve a 6D pasteurization, which is not commercially feasible. Thus HPP-thermal above 75°C is required in addition to rapid cooling of the pasteurized food. The two *C. perfringens* type A strains exhibited similar resistance to 600 MPa HPP-thermal. *C. perfringens* spore inactivation in beef slurry is temperature dependent for 600 MPa HPP-thermal processes, with greater inactivation at higher temperatures. Weibull distribution was a better model than the log-logistic to describe the inactivation of *C. perfringens* spores by the HPP-thermal process. The results confirmed that HPP-thermal was more effective for the inactivation of *C. perfringens* spores in beef slurry compared with thermal processes at the same temperature.

Chapter 3 Use of power ultrasound to enhance the thermal inactivation of *Clostridium perfringens* spores in beef slurry

Evelyn, & Silva, F. V. M. (2015). Use of power ultrasound to enhance the thermal inactivation of *Clostridium perfringens* spores in beef slurry. *International Journal of Food Microbiology*, 206, 17-23.

3. Power ultrasound and thermal processing *C. perfringens* spores in beef slurry

Chapter abstract

Clostridium perfringens is a pathogen of concern in pasteurised foods. The main objective of this study was to use power ultrasound to enhance the thermal inactivation of *C. perfringens* spores in beef slurry. The effect of simultaneous ultrasound and heat (TS, thermosonication) on the spore inactivation in beef slurry was first investigated. At 75°C, a 60 min TS process (24 kHz, 0.33 W/g) resulted in a less than 1.5 log reduction for both *C. perfringens* NZRM 898 and NZRM 2621 spores. Then, the thermal inactivation first order kinetic parameters of *C. perfringens* spores in beef slurry were estimated for the two strains. The $D_{105^{\circ}\text{C}}$ - and z -values were 2.5 min and 10.6°C for NZRM 898 and 1.8 min and 10.9°C for NZRM 2621. After, the effect of a spore heat shock followed by ultrasound on its thermal inactivation in beef slurry was investigated. This heat shock + ultrasound pretreatment was able to double the spore thermal inactivation rate in beef slurry. For example at 95°C D -value of 20.2 min decreased to 9.8 min, demonstrating that spore exposure to heat shock followed by ultrasonication enhanced its thermal inactivation.

3. Power ultrasound and thermal processing *C. perfringens* spores in beef slurry

3.1 Introduction

Clostridium perfringens is an anaerobic mesophilic spore-forming bacterium which grows well at temperatures ranging from 15 to 50°C and pH values between 5.0 and 8.0 (McClane, 2007). This bacterium has been identified as one of the major causes of food outbreaks in low-acid pasteurized foods (Golden et al., 2009; Juneja et al., 2010; Juneja et al., 2006; Juneja & Majka, 1995; Juneja & Marmer, 1996; Labbé et al., 2014; Scallan et al., 2011; Silva & Gibbs, 2010; Silva et al., 2014). *C. perfringens* food poisoning often occurs when large quantities of foods (e.g. meat and poultry) are prepared in industrial kitchens (e.g. factories, hospitals, school cafeterias, prisons, and nursing homes). The slow or improper cooling after cooking the meat products enable the growth of *C. perfringens*, due to their short generation times (i.e. 8 minutes at the optimal growth temperature of 43-45°C) (Labbé, 2000). Depending on the major lethal toxins produced, *C. perfringens* strains are classified as types A, B, C, D, or E (Uzal & McClane, 2011), with type A food poisoning being the second ranked foodborne disease (Scallan et al., 2011). Around 5% of isolates have been reported to carry enterotoxin (cpe) genes which also cause gastrointestinal infections, antibiotic-associated diarrhoea and sporadic diarrhoea in humans (Fisher et al., 2005).

With respect to thermal resistance, the most heat resistant *C. perfringens* spores survived 100°C for 1 h or more (Labbé et al., 2014). This thermal resistance is almost equivalent to the spores of *Clostridium botulinum*, the target microorganism used as reference for commercial sterilization processes ($D_{121.1^{\circ}\text{C}} = 0.2$ min, z -value = 10°C) (Farkas, 2001). $D_{100^{\circ}\text{C}}$ -values around 20 min were determined in beef gravy for *C. perfringens* spores (Juneja et al., 2003), the same D -value of *C. botulinum* spores at 101°C. These findings promoted research on the *C. perfringens* risk assessment on ready-to-eat and partially cooked meat and poultry

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products and its spore inactivation in different foods (Akhtar et al., 2009; Crouch et al., 2009; Gao et al., 2011; Golden et al., 2009; Varga & Szigeti, 2012).

The ultrasound technology has a large number of applications in food processing such as defoaming, emulsification, extrusion, extraction, and waste treatment (Feng & Yang, 2011b). Ultrasound assisted inactivation of foodborne pathogens and spoilage enzymes is an alternative to traditional thermal processes due to the lower processing temperatures and energy required, thus better food quality (Feng & Yang, 2011b; Mason et al., 1996; Villamiel et al., 1999). Critical factors determining the efficiency of this technology in terms of microbial inactivation, include the amplitude of the ultrasonic waves, exposure/contact time, the volume and composition of the foods processed, the treatment temperature, and the type of microorganisms (Señorans et al., 2003). To date, few authors have modeled the ultrasound inactivation kinetics of microbial spores in foods. The ultrasonication of spores has shown to induce changes in the *Bacillus* spores such as swelling, surface erosion, and growth stimulation (Burgos et al., 1972). The release of calcium, dipicolinic acid and low molecular weight substances from the bacterial spore protoplast were also reported (Palacios et al., 1991). For simultaneous ultrasound and heat (TS, thermosonication), higher inactivation of *Bacillus subtilis* spores by TS (20 kHz, 70-111°C, 5 W/mL) was obtained than heat alone (Garcia et al., 1989). López Malo et al. (2005) also observed lower decimal reduction (*D*-) values in the TS (20 kHz, 45-60°C) with mould spores. Recently, Evelyn and Silva (2015a) obtained at least 3 fold lower *D*-values with psychrotrophic *Bacillus cereus* spores for TS (24 kHz, 50-70°C, 0.33 W/mL or W/g) in beef slurry and skimmed milk than thermal processing at the same temperature.

With respect to the use of ultrasound in series with thermal processing (before or after) for microbial spore inactivation, Goodenough and Solberg (1972) reported no heat sensitizing

3. Power ultrasound and thermal processing *C. perfringens* spores in beef slurry

effect on *Clostridium perfringens* spores at 90°C after the ultrasonication of spores for 12 min (0°C, 15 W/mL). In 2007, Broda also reported no change on the *Clostridium estertheticum* spore thermal resistance after three 1-min ultrasonic treatments (0°C, 36 W/mL). On the contrary, 100°C thermal resistance of *Bacillus cereus*, *Bacillus licheniformis*, and *Bacillus subtilis* spores decreased markedly after the spore ultrasonication for 1.5 min (20 kHz, 0-12°C, 12-15 W/mL) (Burgos et al., 1972; Ordonez & Burgos, 1976). Sanz et al. (1985) also observed a decrease in the heat resistance of *Geobacillus stearothermophilus* spores after ultrasonic treatment at room temperature. Additionally, *Bacillus coagulans* inactivation by 6 log was observed in skimmed milk after pasteurization (60 min, 30 min) followed by a 10 min ultrasonic pretreatment (20 kHz, 0°C, 250 W/mL) (Khanal et al., 2014). To date, the efficacy of ultrasound assisted thermal inactivation of *Clostridium* spores is under investigation.

The thermal inactivation of *C. perfringens* spores in foods with and without prior heat shock has been reported in several works (Bradshaw et al., 1977; Byrne et al., 2006; Juneja et al., 2006; Juneja & Majka, 1995; Juneja & Marmer, 1996; Juneja et al., 2003; Varga & Szigeti, 2012) and the first order kinetic parameters were reported (**Table 3-1**). The $D_{100^{\circ}\text{C}}$ -values ranged between 1.9 and 21.4 min and z-values ranged between 7.2 and 10.8°C depending on the strain and food.

The search for strategies to enhance the conventional thermal process *C. perfringens* spore inactivation is important due to consumers demand for more food safety and quality. Pasteurized beef slurry was chosen for this research since it is a food prone to contamination by *C. perfringens* and the main objectives were: (i) to study the thermosonication inactivation of *C. perfringens* spores in beef slurry at 75°C; (ii) to estimate the first order spore thermal inactivation parameters in beef slurry, and (iii) to compare the thermal inactivation of

3. Power ultrasound and thermal processing *C. perfringens* spores in beef slurry

untreated vs heat shocked spores in beef slurry (iv) to submit the spores to a heat shock followed by ultrasound, and study the thermal inactivation of pretreated spores in beef slurry.

Table 3-1 First order thermal resistance parameters of *Clostridium perfringens* spores in meat products.

Meat product	Strain*	T (°C)	D-value	D-value	z-value (°C)	T range (°C)	Reference
			(min) No heat shock	(min) Heat shock**			
Beef gravy	NCTC 8798	98.9	31.4	nr	7.2	99-116	Bradshaw et al. (1977)
		104.4	7.2				
		110	0.6				
Beef gravy	NCTC 10240	98.9	27.7		7.4	99-116	
		104.4	7.8				
		110	1.0				
Ground turkey	ATCC 10288, NCTC 8238, NCTC 8239 (NZRM 2621)	99	23.2	nr	nr	nr	Juneja & Marmer (1996)
Beef gravy	NB 16 NCTC 10239 (NZRM 898) B 40 153 FD 1041 C 1841 NCTC 8238 F 4969 222 NCTC 8239 (NZRM 2621)	100	21.4	28.1	nr	nr	Juneja et al. (2003)
		100	20.2	26.4			
		100	19.1	23.2			
		100	18.4	26.0			
		100	18.3	26.0			
		100	18.0	22.0			
		100	16.7	18.4			
		100	16.4	24.0			
		100	15.8	19.7			
Ground beef	ATCC 10288, NCTC 8238, NCTC 8239 (NZRM 2621)	99	19.4	nr	nr	nr	Juneja & Majka (1995)
Pork luncheon roll	DSM 11784, NCTC 10614, NCTC 8237	90	31.0	nr	8.3	90-100	Byrne et al. (2006)
		95	9.7				
		100	1.9				
Marinated chicken breast	ATCC 10288, NCTC 8238, NCTC 8239 (NZRM 2621)	90	14.0	nr	nr	nr	Juneja et al. (2006)
Duck liver	NCTC 1265	80	50.5	nr	10.8	80-95	Varga & Szigeti (2012)
		85	16.7				
		90	6.3				
		95	2.2				

* NCTC 8798 (CCUG 42877, CCUG 42881, CIP 106157); NCTC 10240 (ATCC 14810); NCTC 8238 (ATCC 12916, CECT 7468, KCTC 5101, NCIMB 13079, NRRL B-23850); NCTC 8239 (ATCC 12917, CECT 486, CCUG 18370, CIP 104880, NRRL B-23851, BCRC 17462, CCRC 17462, NZRM 2621); NB 16 (NRRL B-23842); NCTC 10239 (ATCC 14809, CECT 563, NRRL B-23852, NZRM 898); 153 (NRRL B-23844); FD 1041 (NRRL B-23846); C 1841 (NRRL B-23847); F 4969 (NRRL B-23848); 222 (NRRL B-23845); DSM 11784 (8239, C 103); NCTC 8237 (ATCC 13124, ATCC 19408, DSM 756, CECT 376, NCTC 6125, NRRL B-23962).

** Heat shock was 75°C for 20 min.

nr, not reported.

3. Power ultrasound and thermal processing *C. perfringens* spores in beef slurry

3.2 Materials and methods

3.2.1 *C. perfringens* microbiology

C. perfringens type A strains used, and method to produce and enumerate spores were described previously (Chapter 2).

3.2.1.1 Beef slurry inoculation

The major composition of the beef slurry was the same as described in Chapter 2. The spore suspension was inoculated in beef slurry to yield a final concentration of approximately $\sim 10^7$ cfu/g. For TS experiments, a portion (ca. 1 mL) of spore suspension was inoculated in approximately 99 g of beef slurry and mixed with a sterile rod. Regarding thermal experiments, aliquots (0.1 mL) of untreated (no prior treatment) or treated (as described in Section 3.2.3) spores were inoculated into 3 g of beef slurry, samples were vacuum packed and mixed in the stomacher before processing.

3.2.2 Ultrasound equipment

A 200W-UP200S ultrasonic processor by Hielscher (Hielscher-Ultrasonic GmbH, Germany) was used for the induction of ultrasonic waves. The processor generates longitudinal mechanical vibrations through electric excitation with high frequency (24 kHz). Equipment was operated at 100% amplitude and on a continuous cycle mode.

3.2.2.1 Thermosonication inactivation of *C. perfringens* spores in beef slurry

Two strains of *C. perfringens* spores (NZRM 2621 and NZRM 898) were used for the TS experiment to compare their resistance to a new technology. All experiments were carried out

3. Power ultrasound and thermal processing *C. perfringens* spores in beef slurry

using a 3 mm tip-diameter sonotrode of the Hielscher UP200S (460 W/cm², 33 W, 0.33 W/g) (Hielscher, 2007). Prior to the experiment, the probe was disinfected with ethanol and then washed with sterile distilled water, and this procedure was repeated between each run. Initially, a 200 mL round bottom-flask containing 99 g of beef slurry was placed in a water bath and heated by a controlled thermostatic water bath ($\pm 1^\circ\text{C}$) to bring the sample temperature to the processing temperature of 75 °C (maximum temperature supported by the ultrasound probe). The temperature of the beef slurry sample during processing was monitored by inserting a thin temperature probe in the slurry, and the thermostatic water bath was used to keep it at the desired value during the processing. Spores (ca. 1 mL) were inoculated aseptically when the beef slurry reached 75°C and mixed with a sterile rod. The ultrasound probe was then immersed in the centre of the beef sample, more or less 1 cm from the flask bottom and turned on, which further helped in mixing uniformly the spores with the beef slurry and keeping the temperature uniform in the whole sample. Ultrasonication was carried out up to 60 min. For each prespecified time (5, 10, 20, 30, 40, and 60 min), two beef slurry samples were taken and immediately submerged into ice water for enumeration. The experiment was repeated 3 times for each strain. The *C. perfringens* spore average logarithmic reduction ($\log N/N_0$) was calculated for different TS times and plotted.

3.2.3 Pretreatment of *C. perfringens* spores

C. perfringens NZRM 898 spores were selected for the pretreatment study since this strain seems to be more resistant than NZRM 2621. The following spore pretreatments were attempted to improve thermal inactivation: (i) heat shock (HS, 80°C for 10 min) and (ii) HS followed by 1-min ultrasonication. HS can activate the dormant spores of *C. perfringens* and induce the germination of spores (Juneja et al., 2010), leading to a loss of thermostability

3. Power ultrasound and thermal processing *C. perfringens* spores in beef slurry

during the transition to the germinating stage or the first phase of germination (Sojka & Ludwig, 1994), thus could possibly increase sensitivity of the spores to subsequent treatment. The combination of HS and ultrasound for spore pretreatment was carried out since literature results have shown that only ultrasonication prior to the thermal treatments had a negligible effect on the *C. perfringens* spores (Broda, 2007; Goodenough & Solberg, 1972).

For heat shocked spores, test tubes containing 10 mL of spore suspension ($\sim 10^7$ cfu/g) prepared with sterile distilled water were thermally processed at 80°C for 10 min in a water bath. For the ultrasonication pretreatment, a 14 mm tip-diameter sonotrode (105 W/cm², 162 W, 16.2 W/mL) was used (Hielscher, 2007). The probe was disinfected as explained previously, submerged immediately into the 10 mL HS spore suspension contained in a test tube and 1 min ultrasonication was carried out in an ice water bath.

3.2.4 Thermal inactivation of *C. perfringens* spores in beef slurry

Regarding the thermal inactivation studies, first the thermal resistances of two strains (NZRM 898 and NZRM 2621) in beef slurry were determined. In the following experiment, the thermal resistance of NZRM 898 heat shocked spores in beef slurry was estimated. Finally, the NZRM 898 spores were heat shocked and ultrasonicated for 1 min, and its thermal resistance in beef slurry was reassessed.

3.2.4.1 Beef slurry sample preparation

Depending on the experiment, treated or untreated spores were inoculated into the beef slurry and placed inside thin layer (approximately 1–2 mm thick) food grade sterile pouches (8 × 8 cm, Cas-Pak, New Zealand). As a result, the larger area of bags compared to the small mass of food (3 g) allowed a quick heat transfer. The pouches were then vacuum packed,

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thermosealed (Multivac C200, Germany), and placed in a stomacher (Masticator Stomacher, IUL Instruments, Germany) for 2 min to ensure good spore mixing and uniform distribution within the beef slurry.

3.2.4.2 Thermal inactivation experiments

The pouches were submerged in a heated oil bath for various times. The oil bath was maintained at the desired temperatures during the thermal treatment of untreated, heat shocked, and heat shocked followed by 1-min ultrasonicated spores. Preliminary experiments were initially carried out to test the heat penetration into the beef slurry contained in the plastic pouches. Temperatures of 95, 100 and 105°C were used for thermal inactivation of both strains of untreated *C. perfringens* spores (temperatures of 75 and 85°C were not carried out since the other three temperatures showed no difference between the thermal resistance of heat shocked and untreated spores). The inactivation of untreated *C. perfringens* NZRM 2621 spores was carried out to compare the heat resistance of both strains in beef slurry and compare with the literature results. Regarding the thermal inactivation experiments conducted with pretreated spores, temperatures of 75, 85, 95, 100 and 105°C were studied. For each temperature and time, 2 samples were taken for microbial enumeration. After the thermal processing, the pouches were kept in ice water, washed in soap solution, rinsed in sterile distilled water and ethanol. The *D*-value was estimated from the log (N/N_0) vs. time experimental data. Two survival experiments were carried out for each temperature and the average *D*-value \pm SD was calculated.

3. Power ultrasound and thermal processing *C. perfringens* spores in beef slurry

3.2.5 Modeling *C. perfringens* spore inactivation in beef slurry

First order kinetic (Equation 1-1) and Weibull (Equation 1-6) models were fitted to the log spore survival vs. time data, to estimate the TS or thermal resistance parameters of *C. perfringens* NZRM 898 and NZRM 2621 spores in beef slurry.

A one-way analysis of variance (ANOVA) followed by the Tukey's test with a confidence level of 95% ($p < 0.05$) was carried out to compare the log reductions of different strains at the same temperature, and the log reductions of the same strain at different temperatures. The Tukey's test was again used to compare the log reductions of heat shocked spores vs. untreated spores and to compare the log reductions of heat shocked vs. heat shocked + ultrasonicated spores at the same temperature. In addition, Tukey's test was also used to compare the *D*-values at the same temperature for different spore strains or pretreatments.

Statistica 8.0 (Statsoft Inc., USA) was used to fit several models to the spore survival lines, and to perform statistical analysis of the data. Coefficient of determination (R^2) and mean square error (MSE) were used to indicate the performance of the models. The spore resistance parameters were estimated, and temperature dependence of the parameters estimated was checked by plotting logarithmic of the estimated kinetic parameters (*D* for linear and *b* for Weibull model) against the process temperature. For first order, z -value \pm SE was estimated from the reciprocal of the slope of log *D* vs. T line.

3. Power ultrasound and thermal processing *C. perfringens* spores in beef slurry

3.3 Results and discussion

3.3.1 Thermosonication inactivation of *C. perfringens* spores in beef slurry at 75°C

The thermosonication inactivation at 75 °C for two strains *C. perfringens* spores is illustrated in **Figure 3-1**. Following the treatment, the spores reduced steadily with the processing time reaching nearly 1.0 log after 20 min, however it declined more slowly after 20 min. Inactivation results were similar for both strains, only from 40 min a slightly higher resistance of *C. perfringens* NZRM 898 was registered ($p < 0.05$). Although previous investigators (Evelyn & Silva, 2015a; Garcia et al., 1989) concluded that the combination of heat with ultrasound (or thermosonication) was effective for the inactivation of *Bacillus* spores at around 70°C, the process was ineffective for reducing the numbers of the two strains of *C. perfringens* spores, exhibiting 1 to 1.5 log reductions after a 60 min treatment time. These results were found to be in agreement to Ahmed and Russel (1975) that reported the survival of *Clostridium sporogenes* spores in Ringer solution after 30 min thermosonication (20 kHz, 35°C, 500 W). The TS survivor curves for both strains followed Weibull's model, indicated by the low MSE (≤ 0.01) and R^2 (≥ 0.95) values close to 1 (**Table 3-2**). Due to the low inactivation obtained for TS after 60 min at the maximum power and temperature (75°C) supported by the power ultrasound unit, the use of ultrasound as a spore pretreatment to enhance its thermal inactivation in beef slurry was attempted and results are shown in Section 3.3.3.2.

3. Power ultrasound and thermal processing *C. perfringens* spores in beef slurry

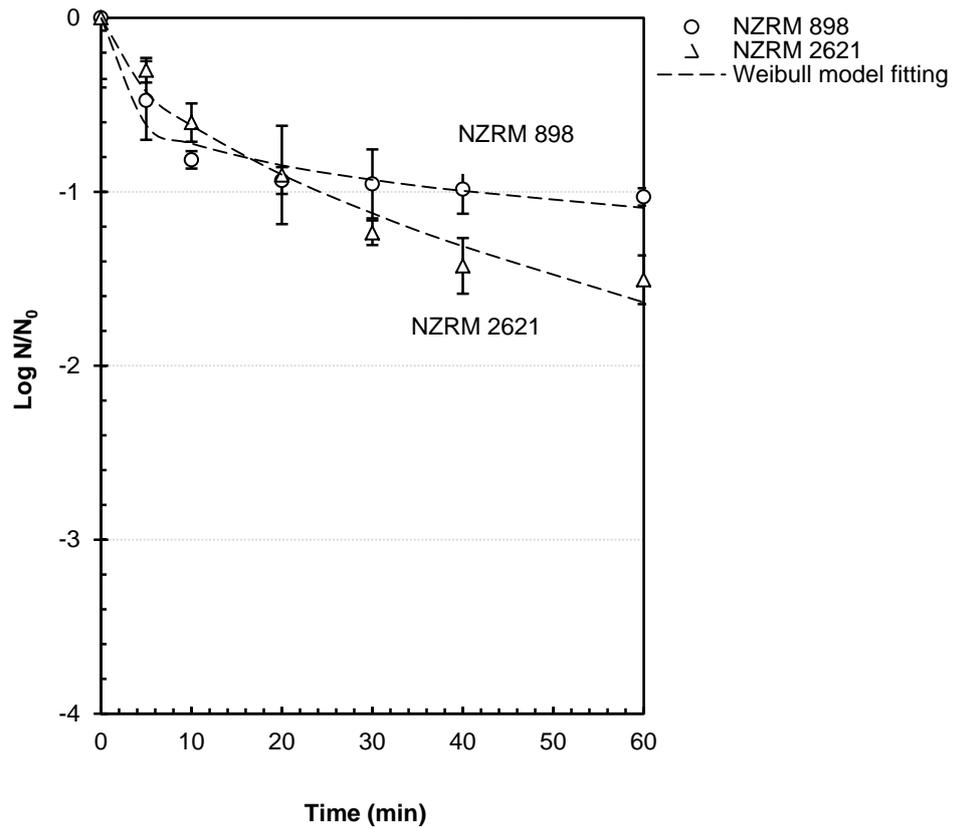


Figure 3-1 *Clostridium perfringens* spores 75°C thermosonication inactivation in beef slurry (24 kHz, 0.33 W/g). Experimental data are average \pm standard deviation (SD) from three survival experiments and dashed line shows the Weibull model fitted to data.

Table 3-2 Thermosonication (24 kHz, 0.33 W/g) inactivation of *Clostridium perfringens* spores in beef slurry: Weibull model performance and parameters estimation.*

Strain	T (°C)	$b \pm SE$	$n \pm SE$	R^2	MSE
NZRM 898	75	0.42 ± 0.01	0.23 ± 0.01	0.950	0.005
NZRM 2621	75	0.18 ± 0.03	0.61 ± 0.05	0.971	0.010

* *C. perfringens* NZRM 898 (=ATCC 14809, NCTC 10239) and NZRM 2621 (=ATCC 12917, NCTC 8239) were used; b and n are the Weibull scale and shape factors, respectively (Equation 1-6); R^2 close to 1 and low mean square errors (MSE) are indication of good fit.

3. Power ultrasound and thermal processing *C. perfringens* spores in beef slurry

3.3.2 Thermal inactivation of untreated *C. perfringens* spores in beef slurry: first order kinetic parameters

Figure 3-2 shows the thermal log survivors of untreated *C. perfringens* NZRM 898 and NZRM 2621 spores in beef slurry for 95, 100 and 105°C. The heat inactivation for the two strains followed first order kinetics as shown by the good performance indices ($0.003 \leq \text{MSE} \leq 0.078$, $0.958 \leq R^2 \leq 0.994$). For NZRM 2621, the *D*-values were 1.8 min at 105°C, 5.5 min at 100°C and 15.0 min at 95°C, whereas values obtained with NZRM 898 were higher: 2.5 min at 105°C, 7.1 min at 100°C and 21.7 min at 95°C (**Table 3-3**). Similar to TS, the strain NZRM 898 seems to be more resistant than NZRM 2621 since the *D*-values were significantly higher for 95 and 105°C ($p < 0.05$). Juneja et al. (2003) also registered a higher $D_{100^\circ\text{C}}$ -value of 20.2 min with NZRM 898 in beef gravy vs. 15.5 min with NZRM 2621. Higher *D*-values in beef gravy than in beef slurry were expected due to the gravy's high fat content. Juneja et al. (2003) estimated $15.5 \text{ min} < D_{100^\circ\text{C}} < 21.4 \text{ min}$ in beef gravy for 10 *C. perfringens* strains and Juneja and Majka (1995) obtained a $D_{99^\circ\text{C}} = 19.4 \text{ min}$ in ground beef using a cocktail of 3 strains. In contrast, lower *D*-values were determined in pork luncheon roll, marinated chicken breast and duck liver with other strains (Byrne et al., 2006; Juneja et al., 2006; Varga & Szigeti, 2012). To conclude, the differences in the *D*-values of *C. perfringens* spores could be attributed to the food composition and/or microbial strain. The *z*-values estimated in our study were 10.6 and 10.9°C (**Table 3-3**), which are within the expected values for bacterial sporeformers.

3. Power ultrasound and thermal processing *C. perfringens* spores in beef slurry

Table 3-3 First order thermal resistance parameters of two strains *Clostridium perfringens* spores in beef slurry*

<i>D</i> -value ± SD (min)				
Temperature (°C)	<i>Untreated spores</i> NZRM 2621	<i>Untreated spores</i> NZRM 898	<i>Heat shocked spores</i> NZRM 898	<i>Heat shock followed by 1-min ultrasonicated spores</i> NZRM 898
105	1.8±0.2	2.5±0.2	2.7±0.7	1.1±0.3
100	5.5±0.8	7.1±1.6	6.2±0.9	3.4±0.4
95	15.0±1.4	21.7±2.8	20.2±1.3	9.8±1.2
85	nd	nd	51.4±2.1	first order not appropriate
75	nd	nd	108.7±4.8	first order not appropriate
<i>z</i> -value±SE (°C)	10.9±0.02	10.6±0.02	11.4±0.02	10.5±0.01
<i>R</i> ²	0.99	0.99	0.99	0.99

**C. perfringens* strains NZRM 898 (=ATCC 14809, NCTC 10239) and NZRM 2621 (=ATCC 12917, NCTC 8239) were used; *D*_T-values were expressed as means±standard deviation (SD) and obtained from two independent experiments; Temperatures between 95 to 105°C were used to estimate the *z*-values; nd – not determined. The first order kinetics was supported by good performance indices ($0.842 \leq R^2 \leq 0.999$, $0.003 \leq \text{MSE} \leq 0.078$). Weibull model described the 75 and 85°C thermal resistance of NZRM 898 spores submitted to prior heat shock followed by 1-min ultrasound ($0.982 \leq R^2 \leq 0.998$, $0.001 \leq \text{MSE} \leq 0.020$).

3. Power ultrasound and thermal processing *C. perfringens* spores in beef slurry

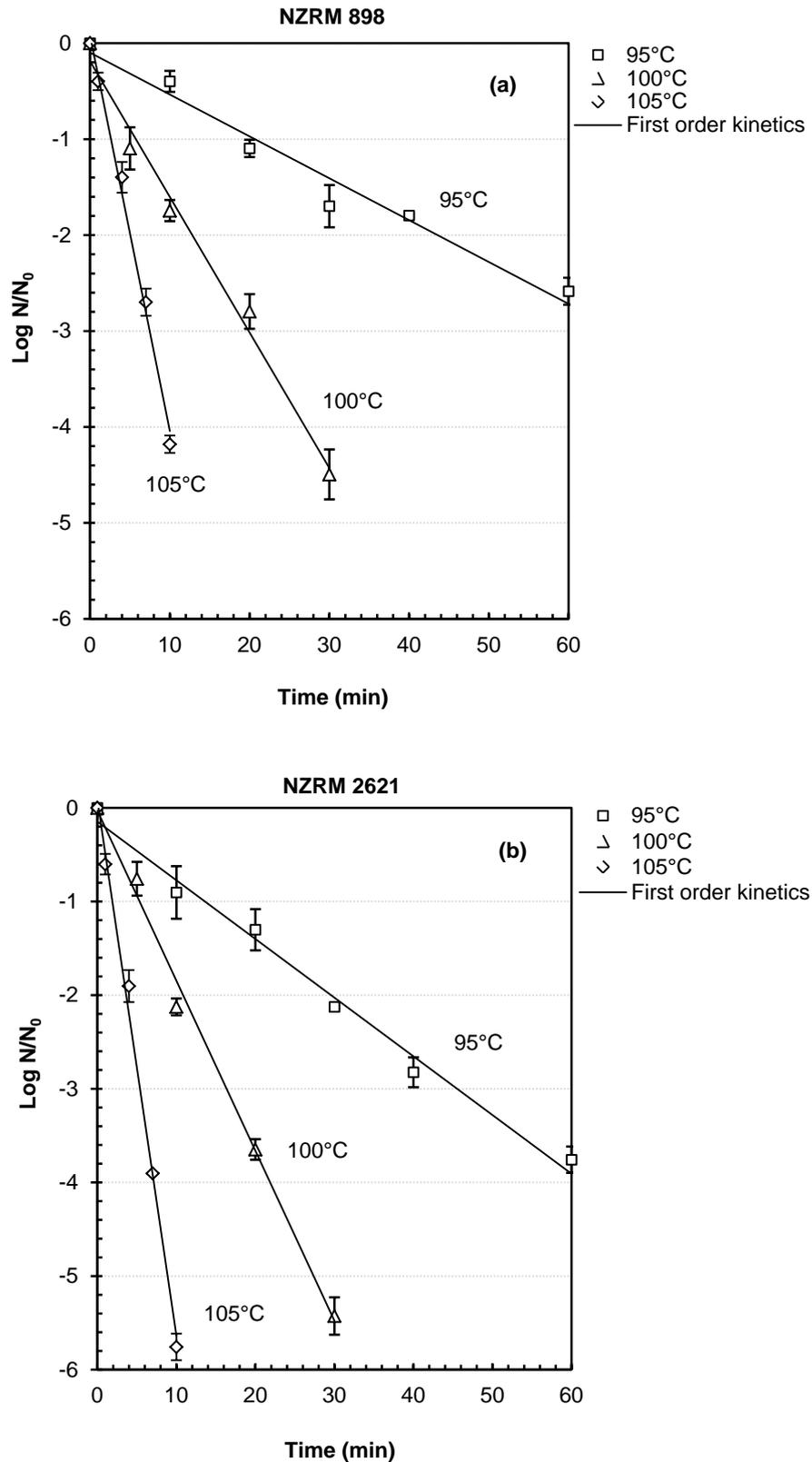


Figure 3-2 Thermal inactivation of untreated *Clostridium perfringens* spores in beef slurry. Values are average \pm standard deviation (SD) from two survival experiments. Solid lines show the first order kinetic model fitted to data

3. Power ultrasound and thermal processing *C. perfringens* spores in beef slurry

3.3.3 Heat shock and ultrasonic pretreatments to enhance the thermal inactivation of *C. perfringens* spores in beef slurry

3.3.3.1 Thermal inactivation of heat shocked vs. untreated spores

The average log reductions of heat shocked (HS — 80°C for 10 min) *C. perfringens* NZRM 898 spores (**Figure 3-3a**) are similar to untreated spores (**Figure 3-2a**). The thermal log survivors of HS spores were linear ($0.009 \leq \text{MSE} \leq 0.033$, $0.842 \leq R^2 \leq 0.994$) thus *D*- and *z*-values were estimated (**Table 3-3**). No significant differences ($p > 0.05$) were registered between the *D_T*-values of HS and untreated NZRM 898 *C. perfringens* spores: 2.5–2.7 min at 105°C, 6.2–7.1 min at 100°C, and 20.2–21.7 min at 95°C. On the contrary, Juneja et al. (2003) observed an increase (up to 1.3 fold) in the *D*_{100°C}-values of 9 out of 10 *C. perfringens* spores. An increase from 20.2 to 26.4 min in the *D*_{100°C} -value was also registered for NZRM 898 strain in beef gravy after HS of 20 min at 75°C (**Table 3-1**). The *z*-value we obtained for HS spores (11.4°C) was similar to the value of untreated spores (10.6°C). The thermal inactivation of HS spores at lower temperatures (75°C, 85°C) was almost none (0.5 log reductions at 75°C and 1.2 log at 85°C after 60 min) (**Figure 3-3a**). However, the study at these low temperatures enabled the comparison with HS + ultrasonicated pretreated spores, as shown in the following section.

3.3.3.2 Thermal inactivation of heat shocked and ultrasonicated spores

Figure 3-3 compares the log survival of spores submitted to heat followed by 1-min ultrasound vs spores submitted to heat shock only for 75, 85, 95, 100 and 105°C. HS followed by 1-min ultrasonication of spores at 95, 100 and 105°C duplicated the spore thermal inactivation, in which the time needed to achieve similar lethality of spores was reduced by half. For example at 95°C, approximately 3 log reduction of *C. perfringens* spores was obtained after 30 min (**Figure 3-3b**) as opposed to 60 min without prior ultrasound

3. Power ultrasound and thermal processing *C. perfringens* spores in beef slurry

(**Figure 3-3a**) ($p < 0.05$). Similarly, at 100°C, close to 15 min were required to achieve 5 log in ultrasonicated spores vs. 30 min for only HS spores. Linear thermal death curves were observed for the log survivors at 95, 100 and 105°C for the HS + ultrasound spores ($0.984 \leq R^2 \leq 0.999$, $0.006 \leq \text{MSE} \leq 0.040$) and also good temperature dependence for the D -values ($R^2 = 0.99$) (**Table 3-3**). A z -value of 10.5°C was similar to the z -values previously estimated for *C. perfringens* in beef slurry. The benefit of ultrasonic pretreatment to enhance the thermal inactivation was confirmed by lower D_T -values for HS + ultrasound pretreated spores: $D_{105^\circ\text{C}}$ -value of 1.1 min vs. 2.7 min, $D_{100^\circ\text{C}}$ -value of 3.4 min vs. 6.2 min, and $D_{95^\circ\text{C}}$ -value of 9.8 min vs. 20.2 min ($p < 0.05$, **Table 3-3**).

With respect to thermal treatments at 75 and 85 °C, higher log reductions were still obtained after 60 min processing when using ultrasound pretreatment ($p < 0.05$) (**Figure 3-3**). However, the non-linearity observed, with tails and log numbers tending to a constant value, confirmed it is more difficult to thermal inactivate the spores at these lower temperatures (**Figure 3-3b**). Note that tails were not observed for the thermal death lines of HS *C. perfringens* NZRM 898 spores at the same temperatures, demonstrating that the non-linearity is most probably caused by distribution of resistance of the population to this treatment. The ultrasound pretreatment may have caused changes in the spore properties which might affect its thermal inactivation in the beef slurry. Non-linear thermal inactivation of *Clostridium* spores at lower temperatures ($\leq 85^\circ\text{C}$) has been observed by Dixit et al. (2005) and Peck et al. (1993). Due to the non-linearity, the Weibull model was found to be more appropriate than the first order kinetic model ($0.982 \leq R^2 \leq 0.998$, $0.001 \leq \text{MSE} \leq 0.020$) with b parameter of 0.51 (at 85°C) and 0.59 (at 75°C), and n parameter of 0.35 (at 85°C) and 0.14 (at 75°C). Interestingly, a similar non-linear trend and spore inactivation of approximately 1 log after 60 min ($p > 0.05$) was obtained by the 75°C thermal process of HS followed by 1-min ultrasound spores (**Figure 3-3b**) and thermosonication process (**Figure 3-1**).

3. Power ultrasound and thermal processing *C. perfringens* spores in beef slurry

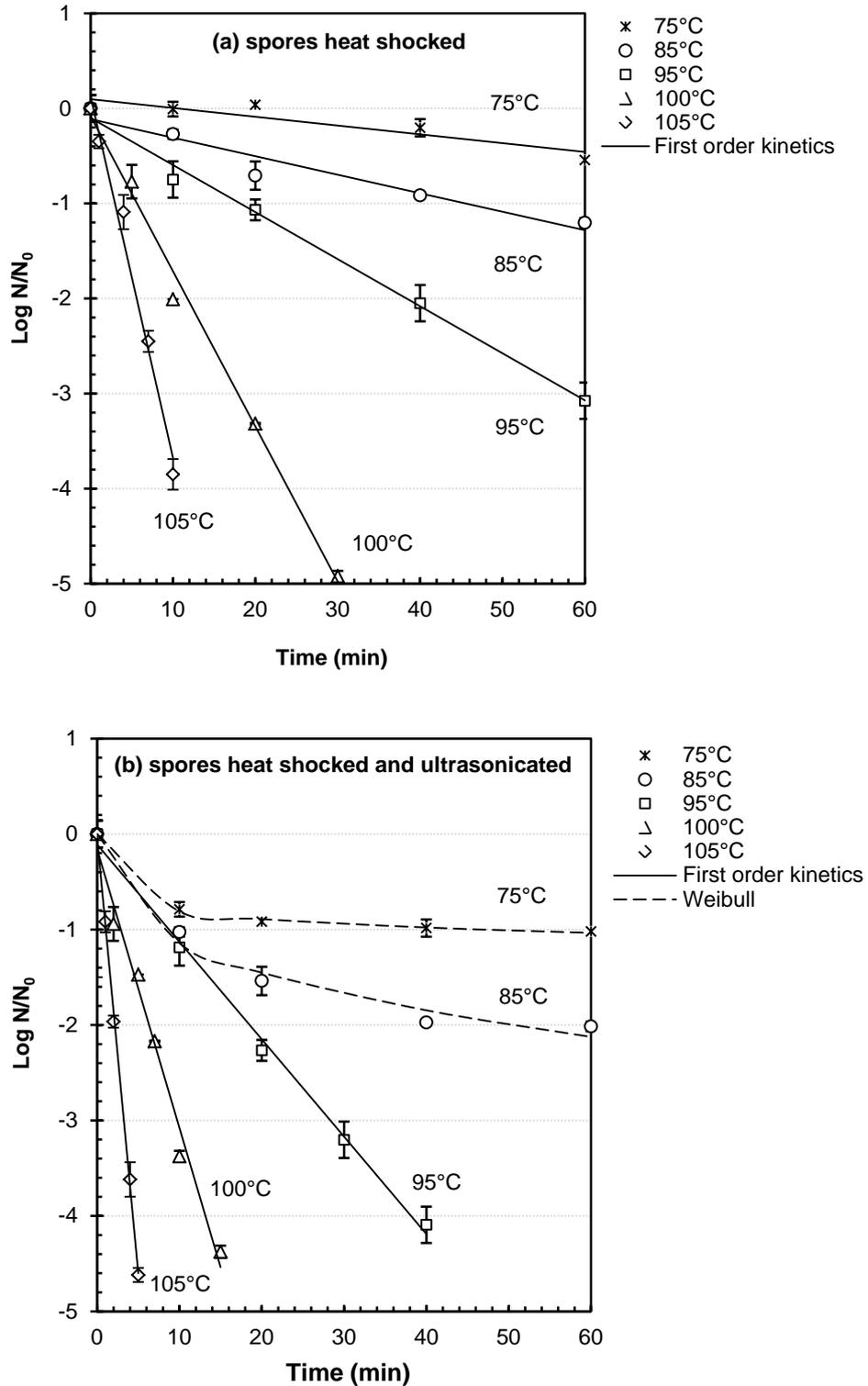


Figure 3-3 Thermal inactivation of *Clostridium perfringens* NZRM 898 spores in beef slurry.

Spores submitted to: heat shock (80°C, 10 min) only (a); and heat shock (80°C, 10 min) followed by 1-min ultrasound (24 kHz, 1.62 W/mL) (b). Values are average \pm standard deviation (SD) from two survival experiments. Solid and dashed lines show the first order kinetics and Weibull model fitted to data, respectively.

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This indicates that both processes had a similar effect on the spore damage, but the mechanisms for the inactivation are still unknown and need to be explored.

The only two studies found with *Clostridium* demonstrated that prior ultrasound processing of *C. perfringens* spores for 12 min at 75 W (Goodenough & Solberg, 1972) and *Clostridium estertheticum* spores for 3 min at 90 W (Broda, 2007) did not decrease the spore thermal resistance. Therefore, the results obtained with *C. perfringens* inactivation in our work show for the first time that the heat shock + ultrasound pretreatment of spores enhanced its thermal inactivation.

3.4 Conclusion

Thermosonication at 75 °C for 60 min was not suitable for *C. perfringens* spore inactivation in beef slurry. The inactivation of spores by TS was not linear and was well described by the Weibull model. The thermal inactivation of *C. perfringens* spores followed the first order kinetics and the *D*- and *z*-values were determined. The NZRM 898 *C. perfringens* spores showed a slightly higher resistance to TS and thermal processes than the NZRM 2621 spores. The application of a 10 min 80°C heat shock did not affect the spore thermal resistance in beef slurry. However, the same heat shock followed by 1 min ultrasound treatment reduced by half the *C. perfringens* spores thermal resistance in beef slurry. The results show a strategy to enhance the thermal inactivation of *C. perfringens* spores, thus allowing lower food processing times and a better food quality.

Chapter 4 High pressure processing of milk: Modeling the inactivation of psychrotrophic *Bacillus cereus* spores at 38–70°C

Evelyn, & Silva, F. V. M. (2015). High pressure processing of milk: Modeling the inactivation of psychrotrophic *Bacillus cereus* spores at 38–70°C. *Journal of Food Engineering*, 165, 141-148.

4. High pressure vs thermal processing psychrotrophic *B. cereus* spores in milk

Chapter abstract

Bacillus cereus is a pathogen that can grow in foods and beverages with low acidity. The main objective of this work was to model the inactivation of psychrotrophic *B. cereus* spores in reconstituted milk treated by high pressure (HPP) combined with a thermal process, and to compare it with thermal inactivation kinetics. First, the effect of HPP pressure (200, 400 and 600 MPa) for up to 40 min at 70°C on *B. cereus* spores was investigated. A pressure increment from 200 to 600 MPa slightly reduced the spore numbers in the reconstituted milk. Then the influence of temperature at 600 MPa on spore inactivation for up to 40 min was studied. Increasing the HPP temperature from 38 to 70°C increased the spore inactivation in milk by 3.5 log. The 600 MPa HPP combined with heat enhanced the spore inactivation in milk, requiring a temperature 20°C lower to achieve the same spore inactivation. However, for a 5 log spore inactivation, the pressure-thermal process required a higher specific energy than the thermal processing. The resistance of two psychrotrophic strains of *B. cereus* spores in milk was also investigated and showed similar results. The Weibull model described spore inactivation by pressure-heat, whereas the first order kinetic model was more appropriate for the thermal inactivation alone. The results of this study confirmed the advantage of HPP technology to further increase the thermal inactivation of *B. cereus* spores in milk.

4. High pressure vs thermal processing psychrotrophic *B. cereus* spores in milk

4.1 Introduction

The growth of psychrotrophic bacteria during cold storage and the distribution of perishable foods is a concern. Low acid foods such as milk are perishable, requiring low temperature to delay spoilage and assure food safety. Among the psychrotrophs, *Bacillus cereus* is a spore-former isolated from low acid, chilled foods (Carlin et al., 2000; Carlin et al., 2000; Dufrenne et al., 1995; Silva & Gibbs, 2010; Silva et al., 2014). The spores of this pathogen can survive the usual pasteurization process and produce enterotoxin (Bennett & Belay, 2001; Granum & Lund, 1997), causing food-borne diseases (e.g. diarrhea and emetic) outbreaks. *B. cereus* was responsible for 5.4 and 32 % of food-borne illnesses in the Netherlands in 2006 and Norway in 2000 respectively (Wijnands, 2008), and 0.7% of the foodborne illnesses in the United States (Scallan et al., 2011).

With respect to thermal resistance, the decimal reduction time (*D*-value) of psychrotrophic *B. cereus* spores can range from 1 to 100 min at 90°C, depending on the strain and heating medium (Dufrenne et al., 1995; Evelyn & Silva, 2015a; Fernández et al., 2001; Wimalaratne, 2009). HPP foods are stored under refrigerated conditions, and since mesophilic strains do not grow below 10°C (Wijnands et al., 2006) it is more appropriate to study the thermal resistance of psychrotrophic strains.

High pressure processing (HPP) is a commercial non-thermal food preservation technology with minimal adverse effects on food quality (Cullen et al., 2012). HPP relies on the use of high pressures (generally 100-600 MPa) to process liquid or solid foods (with or without heat) for times of up to 20 minutes to inactivate spoilage/pathogenic microorganisms and to extend food shelf-life. Inactivation of microbial spores and enzymes by high pressure alone is not feasible. Therefore, a combination of HPP with a mild thermal process is required (Patterson, 2005; Sulaiman & Silva, 2013; Sulaiman et al., 2015a). Depending on the

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resistance of the bacterial spores, high pressure (300-700 MPa) combined with temperature (45-100°C) has been successful in inactivating them (Lado & Yousef, 2002; Ludwig et al., 1996; Luu-Thi et al., 2014; Moerman, 2005; Okazaki et al., 1994; Reineke et al., 2013; Rovere et al., 1998; Sojka & Ludwig, 1997; van Opstal et al., 2004). After processing, HPP foods are commonly stored under refrigerated conditions to prevent spoilage by psychrotrophic bacteria such as some strains of *B. cereus* or by the residual activity of spoilage enzymes. The efficacy of HPP between 100-900 MPa (pulsed or continuous) in conjunction with mild heat, antimicrobial agents (e.g. nisin and sucrose laurate) or an additional control hurdle (e.g. olive powder) to inactivate *B. cereus* spores has been investigated (Aoyama et al., 2005; Arroyo et al., 1997; Daryaei et al., 2013; Fornari et al., 1995; Gola et al., 1996; Ju et al., 2008; Lopez-Pedemonte et al., 2003; Marco et al., 2011; McClements et al., 2001; Meyer, 2000; Raso et al., 1998a; Robertson et al., 2008; Rovere et al., 1998; Scurrah et al., 2006; Shearer et al., 2000; Shigeta et al., 2007; van Opstal et al., 2004). *B. cereus* spore log reductions between 1 and ≥ 7 were achieved, depending on the conditions applied and the spore resistance. McClements et al. (2001), Lopez-Pedemonte et al. (2003), and van Opstal et al. (2004) studied the inactivation of the psychrotrophic strain of *B. cereus* spores in cheese and milk. However, different resistances to pressure-thermal treatment were reported and the kinetics were not modeled. In addition, the market volume of low acid, chill stored foods is increasing by 10% each year. This will increase the risk of psychrotrophic *B. cereus* foodborne infections. Therefore, more information on *B. cereus* spore inactivation and its models with several strains are needed.

In this research, skim milk inoculated with psychrotrophic strains of *B. cereus* spores was processed using HPP combined with heat and thermal processing alone. The effects of temperature and pressure on *B. cereus* inactivation were modeled, and the kinetics of pressure-thermal was compared with thermal alone. The HPP-thermal resistance for two

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psychrotrophic strains was reported for the first time. The objectives were: (i) to investigate the effect of pressure on pressure-thermal assisted spore inactivation; (ii) to compare the 600 MPa pressure-thermal with thermal inactivation of spores; (iii) to compare the resistance of two *B. cereus* strains; and (iv) to model the 600 MPa pressure-thermal and thermal inactivation kinetics of spores.

4.2 Materials and methods

4.2.1 Microbiology

4.2.1.1 Strains

Psychrotrophic *B. cereus* NZRM 984 strain (NCTC 10320, ATCC 11778, DSMZ 345) was obtained from the New Zealand Reference Culture Collection, whereas psychrotrophic *B. cereus* ICMP 12442 strain (ATCC 9139, ATCC 21, BCRC 17036, CECT 5144, LMG 9005, NCCB 48010, NCIMB 11925, VTT E-96727) was obtained from Landcare Research New Zealand. The psychrotrophic behavior (growth at 4°C) of NZRM 984 strain was previously reported (Evelyn & Silva, 2015a; Wimalaratne, 2009), whereas psychrotrophic behavior (growth at 8°C) of ATCC 9139 strain was previously demonstrated by Lopez-Pedemonte et al. (2003). All strains were sourced freeze-dried and revived according to the suppliers' instructions.

4.2.1.2 Sporulation

Sporulation of both *B. cereus* strains was carried out in accordance with the method described by Evelyn and Silva (2015a), except the sporulation of *B. cereus* ICMP 12442 which was

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obtained in 8 days. Both strains were stored in sterile distilled water and maintained at 2°C until use.

4.2.1.3 Skim milk preparation and inoculation

A portion (*ca.* 0.1 mL) of the spore solution was inoculated into 2.9 mL of milk placed inside food grade sterile pouches (8x8 cm, Cas-Pak, New Zealand) to yield an initial food spore concentration of $\sim 10^7$ cfu/mL of milk. Reconstituted skim milk was prepared by diluting New Zealand skim milk powder with 100 mL of sterile distilled water (Evelyn & Silva, 2015a). The milk consisted of (g/100g): moisture (90.5), carbohydrates (4.9) of which 4.7 were sugars, protein (2.7), ash (1.8), and fat (<0.1). The pouches were then vacuum packed and thermosealed (Multivac C200, Germany).

4.2.1.4 Spore enumeration

The *B. cereus* spore concentration in the reconstituted milk before and after pressure-thermal and thermal processing was determined by spread plating onto trypticase soy agar (TSA). 1 mL milk samples were decimal diluted with 9 mL of 0.1% (w/v) sterile buffered peptone water (BPW; Difco, Becton Dickinson, USA). Each tube dilution was mixed repeatedly using a high speed vortex mixer to yield a uniform spore suspension, and plated twice. The TSA plates were then inverted and incubated aerobically at 37°C for 24 to 48 h, until visible colonies were formed. Average colony counts (\pm standard deviation) were calculated and the results were expressed in colony forming units per millilitre (cfu/mL) of milk.

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4.2.2 High pressure processing

4.2.2.1 High pressure equipment and operation

The high pressure food processor and operation previously described for *C. perfringens* spores (Chapter 2) were also used in this study. Previous **Figure 1-6** illustrates an example of the pressure and temperature histories obtained during the HPP process cycle. During the pressurization phase, adiabatic heating occurred. Then during the pressure-holding time phase of the HPP cycle, the chamber temperature dropped steadily towards the initial set temperature. As was explained (Chapter 2), the HPP treatment temperature was approximately the average temperature registered during the constant pressure phase of the HPP cycle. Thus, for each HPP-thermal treatment, preliminary trials were run to select the initial temperature so that the desired average treatment temperature was obtained. Depending on the processing time and temperature, the standard deviations of the mean process temperature were 1.41–2.97°C, 1.97–2.70°C and 0.53–1.29°C, for 200, 400 and 600 MPa, respectively.

4.2.2.2 High pressure and high pressure combined with thermal processing

The pouches containing the 3 mL inoculated milk samples were subjected to high pressure at room temperature or high pressure combined with moderate temperatures for similar times described for *C. perfringens* (5 and 40 min). Initially, the influence of HPP pressure (200, 400 and 600 MPa) at 70°C on the *B. cereus* NZRM 984 spore inactivation in reconstituted milk was studied. Then, the inactivation kinetics of *B. cereus* ICMP 12442 and NZRM 984 spores in reconstituted milk under high pressure at 600 MPa (the highest performance pressure) combined with 38°C and moderate heat (50, 60 or 70°C) was investigated and compared. The temperatures selected were lower than and/or equal to conventional thermal processing temperatures. For this range of pressures and temperatures, initial temperatures

4. High pressure vs thermal processing psychrotrophic *B. cereus* spores in milk

between 30 and 67°C were used. The pressure-thermal resistance of the two psychrotrophic strains were compared. HPP treated samples were submerged in an ice water bath prior to spore enumeration. The logarithmic number of survivors ($\log N/N_0$) versus time was plotted for each survival experiment to estimate the kinetic parameter values and for comparison with the thermal results. For each temperature/pressure, replicates were carried out as described previously (Chapter 2).

4.2.3 Thermal processing

Thermal inactivation experiments with *B. cereus* ICMP 12442 spores in milk were carried out at 70, 80 and 90°C using the method described in Evelyn & Silva (2015a). Two survival experiments were carried out with duplicate samples. The spore logarithmic reduction ($\log N/N_0$) after the thermal treatments was calculated, plotted and compared with HPP-thermal treatments. For each survival experiment, the first order *D*- and *z*-values resistance parameters for the ICMP 12442 strain were estimated.

4.2.4 Modeling the kinetics of *B. cereus* spores inactivation in reconstituted skim milk

A first order kinetics (Equation 1-1) was used to model the thermal inactivation results and did not suit the pressure-thermal log survivors. The Weibull (Equation 1-6) and log-logistic (Equation 1-8) models were used to model the log survivors of pressure and pressure-thermal processing

Table Curve 2D version 5.01 (SYSTAT Software Inc., USA) was used to fit the models to the spore survival lines and estimate the model parameters in order to minimize the errors.

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Mean square error (MSE), coefficient of determination (R^2), standard error (SE) and accuracy factor (A_f) were used to compare the performance of the different models (Evelyn & Silva, 2015a). For each temperature, two or three survival experiments were carried out and the model parameters (*D-value*, b , n , A , τ , σ) were estimated by regression of the logarithmic number of survivors ($\log N/N_0$) versus time. Then the parameters mean value \pm standard deviation (SD) was calculated for each temperature/pressure. Additionally, the temperature and pressure dependence of the estimated parameters was plotted and investigated.

4.3 Results and discussion

4.3.1 Influence of HPP pressure on the inactivation of *B. cereus* spores

The effect of increasing pressure from 200 to 600 MPa in a combined pressure-thermal process at 70°C on the log survivors of *B. cereus* NZRM 984 (= ATCC 11778) spores in reconstituted milk is illustrated in **Figure 4-1**. A higher spore inactivation was obtained with a higher pressure. For example, for a 40 min process, 4.8 log was obtained at 600 MPa compared to 4.2 log at 400 MPa and 3.6 log at 200 MPa. HPP pressures between 100 and 600 MPa and temperatures between 30 and 67°C were reported to germinate *B. cereus* spores, followed by the inactivation of the germinated forms (Sarker et al., 2015; van Opstal et al., 2004). Van Opstal et al. (2004) also observed more spore inactivation of *B. cereus* LMG 6910 (= ATCC 7004) when increasing the HPP pressure from 200 to 600 MPa at 45, 50 and 60°C for a 30 min process. Since the highest spore inactivation was obtained at 600 MPa, the kinetics of *B. cereus* spore inactivation via 600 MPa HPP at different temperatures was modeled in our research and is discussed in the following sections.

4. High pressure vs thermal processing psychrotrophic *B. cereus* spores in milk

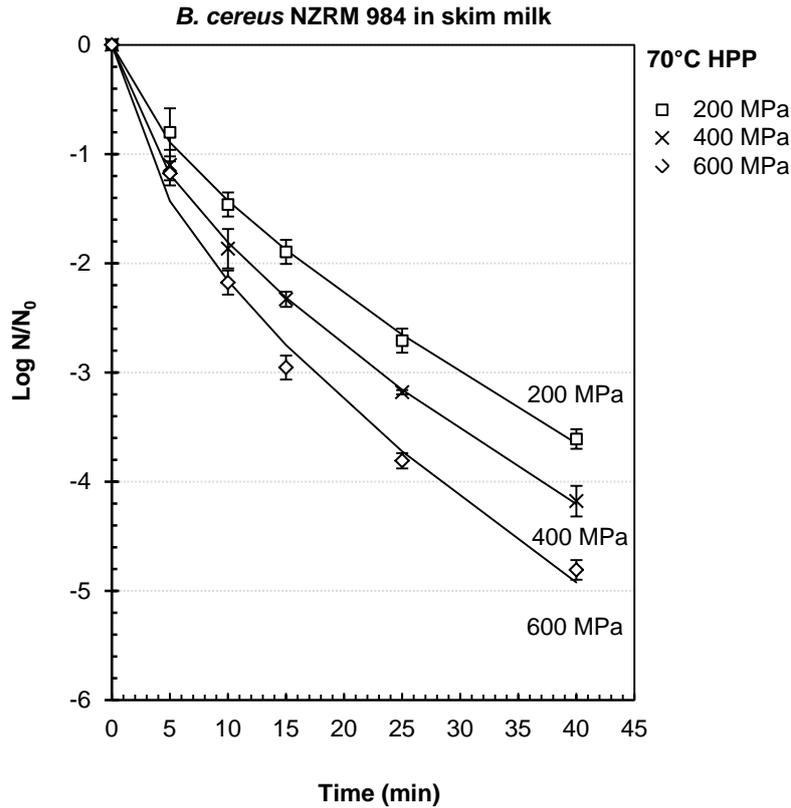


Figure 4-1 Weibull model fitted to the inactivation of psychrotrophic *B. cereus* NZRM 984 (= ATCC 11778) spores in skim milk at high pressure (200, 400, and 600 MPa) combined with 70°C (data points are average \pm standard deviation).

4.3.2 Pressure-thermal vs. thermal inactivation of *B. cereus* spores

Figure 4-2 shows the log survivors of *B. cereus* ICMP 12442 (= ATCC 9139) spores in reconstituted milk with 600 MPa pressure-thermal processing for 50 to 70°C, and thermal processing alone for 70 to 90°C. With respect to the pressure-thermal treatment, the highest spore inactivation of 5.3 log was registered after 40 min at 70°C, while at 38°C (no additional heat), a small effect on the spores was observed (1.8 log reduction) after a 40 min process (**Figure 4-2a**). The higher the HPP process temperature, the higher was the *B. cereus* spore inactivation, indicating the important role of temperature for inactivating the spores. For example, increasing the temperature from 38 to 70°C at 600 MPa for 40 min increased the *B.*

4. High pressure vs thermal processing psychrotrophic *B. cereus* spores in milk

cereus spore inactivation in reconstituted milk by 3.5 log. Similar observations were reported with spores of *B. cereus* LMG 6910 (= ATCC 7004) and ATCC 9818, *Alicyclobacillus acidoterrestris* and *Clostridium botulinum* with 600 MPa HPP in combination with heat (Daryaei et al., 2013; Margosch et al., 2006; Silva et al., 2012; van Opstal et al., 2004; Vercammen et al., 2012).

The pressure-thermal and thermal treatments at 70°C were selected to compare the spore inactivation in reconstituted milk during 600 MPa pressure-thermal treatment and thermal processing alone (**Figure 4-2a** and **Figure 4-2b**). The pressure-thermal process was found to enhance *B. cereus* spore thermal inactivation in reconstituted milk. For a 20 min process at 70°C, 4.0 log reductions were observed for the pressure-thermal process, as opposed to only 0.4 log for thermal, indicating a remarkable advantage of using HPP technology. Similar spore inactivation (≈ 2.5 log) was obtained after 20 min for the 80°C thermal process and pressure-60°C process, thus demonstrating that pressure-thermal methods require a 20°C lower temperature than a thermal process alone to obtain the same level of lethality. The specific energy estimated for a HPP-70°C-40 min process was 439kJ/L and for the equivalent 90°C-10 min thermal process was 348 kJ/L, suggesting a higher energy demand for the pressure-thermal method. Lower temperatures for the pressure-thermal process were also documented by Daryaei et al. (2013) and Luu-Thi et al. (2014) for mesophilic *B. cereus* spores. Daryaei et al. (2013) reported 4.0 log reductions for ATCC 9818 in cooked rice at 600 MPa-85°C-4 min, as opposed to <0.4 log reductions for a thermal process at the same conditions (85°C-4 min).

4. High pressure vs thermal processing psychrotrophic *B. cereus* spores in milk

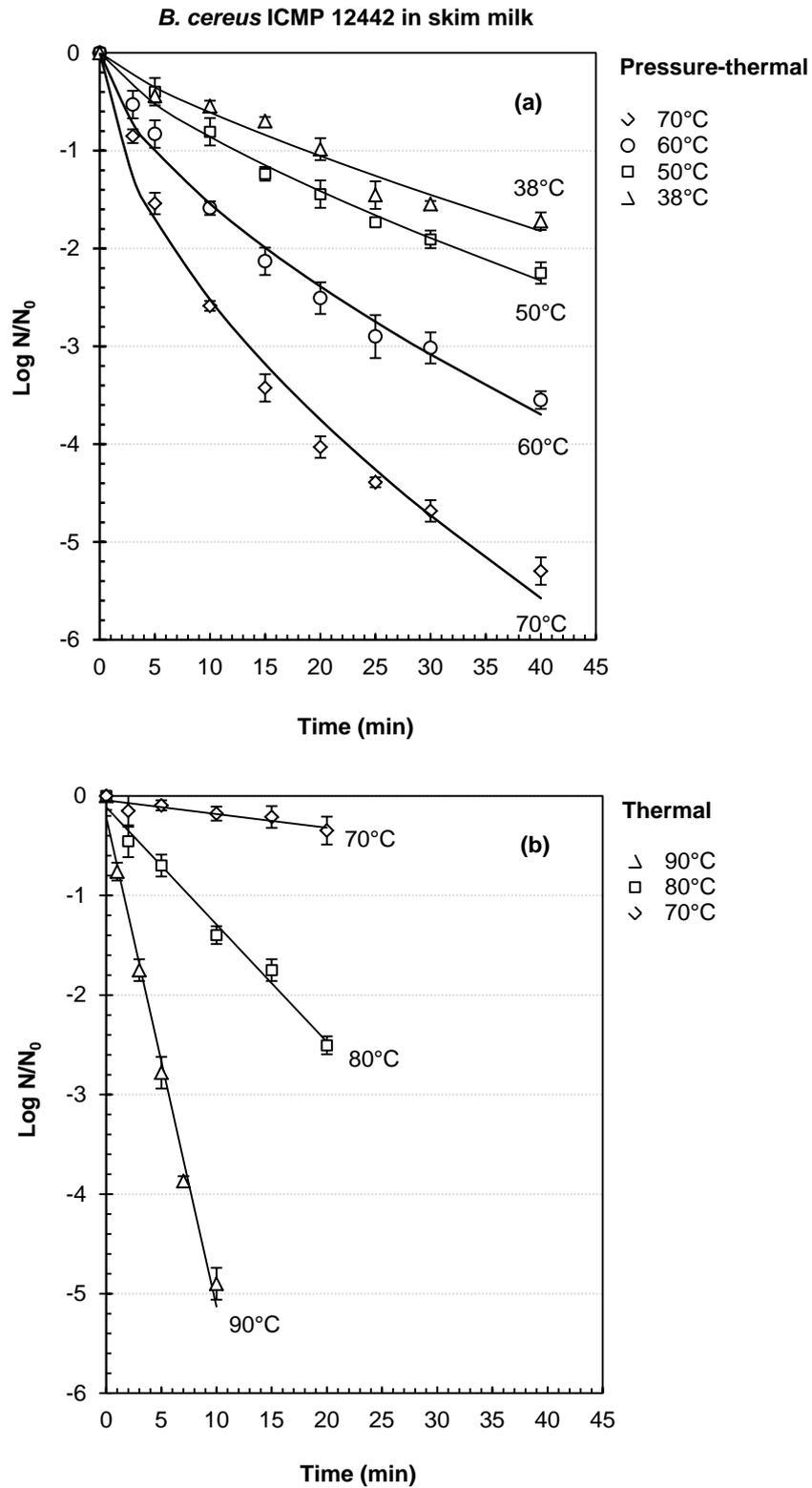


Figure 4-2 Log survivors of psychrotrophic *B. cereus* ICMP 12442 (= ATCC 9139) spores in skim milk: (a) the Weibull model fitted to a 600 MPa pressure-thermal process and (b) the first order model fitted to thermal inactivation (data points are average \pm standard deviation).

4. High pressure vs thermal processing psychrotrophic *B. cereus* spores in milk

4.3.3 600 MPa pressure-thermal resistance of two strains of *B. cereus* spores

Figure 4-3 shows the log survivors of *B. cereus* NZRM 984 spores in reconstituted milk after a 600 MPa HPP process of up to 40 min at four temperatures (38, 50, 60 and 70°C). Those results can be compared with ICMP 12442 (ATCC 9139) in **Figure 4-2a**. NZRM 984 spores seemed to exhibit a slightly lower spore inactivation than ICMP 12442 (0.4–0.5 log lower after 40 min at all temperatures tested). These results suggest that the resistance of the two psychrotrophic strains to pressure-thermal processes is similar. Van Opstal et al. (2004) reported a higher inactivation (7 log) by 600 MPa–60°C–30 min with psychrotrophic *B. cereus* LMG 6910 (= ATCC 7004) spores in reconstituted milk in contrast to 3 log in our study with the strain ICMP 12442 (= ATCC 9139). This work also reported ~6 log reductions with spores of three other *B. cereus* psychrotrophic strains (INRAAV TZ415, INRAAV P21S, INRAAV Z4222) in milk processed at 500 MPa–60°C–30 min. Other investigators processed cheese and milk at 400 MPa–30°C–15 min using the same *B. cereus* strain (ATCC 9139 = ICMP 12442) as our study and registered less than 0.5 log reductions (Lopez-Pedemonte et al., 2003; McClements et al., 2001). Our experiment with the same strain and processing conditions of 600 MPa–38°C–15 min resulted in a 0.7 log reduction. This suggests that HPP-heat resistance is dependent on the *B. cereus* strain.

4. High pressure vs thermal processing psychrotrophic *B. cereus* spores in milk

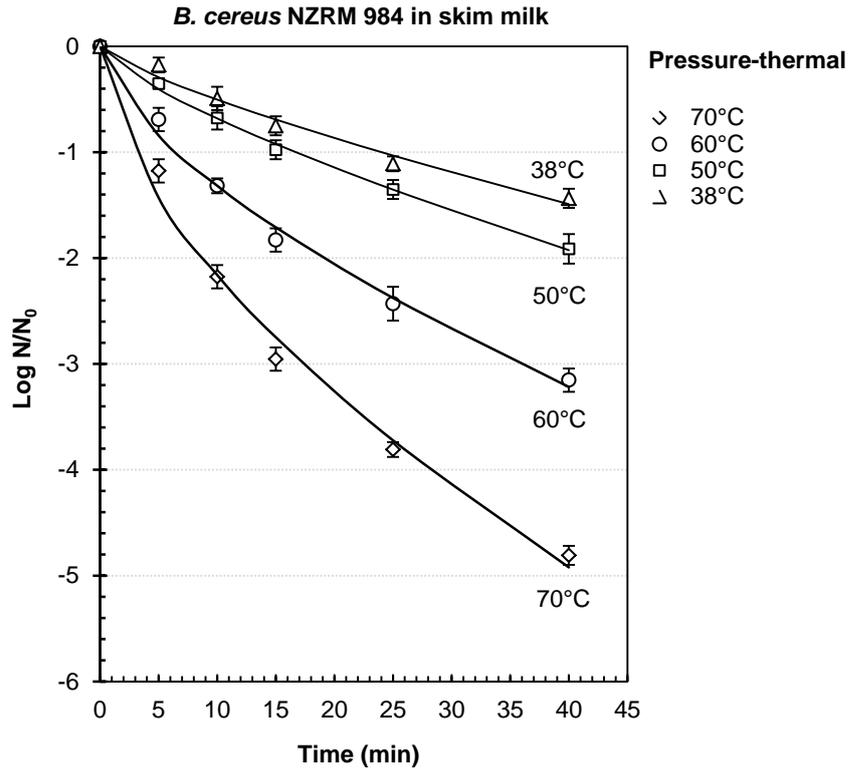


Figure 4-3 Log survivors of psychrotrophic *B. cereus* NZRM 984 (= ATCC 11778) spores in skim milk: the Weibull model fitted to 600 MPa pressure-thermal process (data points are average \pm standard deviation).

4.3.4 Modeling the 600 MPa HPP-thermal inactivation of *B. cereus* spores

The non-linearity observed in the survival curves for both strains (**Figure 4-1**, **Figure 4-2a** and **Figure 4-3**) was confirmed by the high MSE ($0.014 \leq \text{MSE} \leq 0.595$), low R^2 ($0.830 \leq R^2 \leq 0.957$) and high A_f (1.14–1.33) obtained with the first order kinetics. Therefore, the Weibull and Log-logistic models were attempted. Although the log logistic model showed comparable performance indices to the Weibull model (**Table 4-1**), Weibull with two parameters is a simpler model than the three parameter log-logistic model. Additionally, the Log-logistic model presented poor temperature/pressure effects on the parameters (not shown) and higher standard deviations for the parameters estimated at some of the

4. High pressure vs thermal processing psychrotrophic *B. cereus* spores in milk

temperatures (**Table 4-1**). Therefore, the Weibull model resulted in good performance indices ($0.001 \leq \text{MSE} \leq 0.054$, $0.964 \leq R^2 \leq 0.998$, $1.04 \leq A_f \leq 1.13$) and was suggested for describing the data. Weibull has been largely used in the past to describe the non-linear survival curves of spore and vegetative survivors after thermal and non-thermal processes.

In Weibull distribution, the b parameter is the scale factor that is related to the spore inactivation rate. As can be seen from **Table 4-1**, **Figure 4-2a** and **Figure 4-3**, the higher the temperature or pressure, the higher the b value. For the two strains modeled, the b value increased from 0.08–0.10 to 0.55–0.67 as the temperature increased from 38°C to 70°C. Daryaei et al. (2013) also obtained an increase of b from 0.81 to 1.66 as the 600 MPa HPP temperature was increased from 60 to 85°C. The $\log b$ was found to be linearly dependent on the pressure-thermal process temperature ($0.97 \leq R^2 \leq 0.99$) and pressure ($R^2 = 0.95$) (**Figure 4-4**).

The n value in the Weibull distribution presents the shape factor of the survival curves and the deviation from linearity. All the n values were less than 1 (**Table 4-1**), indicating that all the spore survivor curves for pressure-thermal processes were concave-upward (**Figure 4-1**, **Figure 4-2a**, and **Figure 4-3**). This type of concavity suggests that there is a mixed resistance of the spore population to the lethal treatment (Peleg & Cole, 1998; van Boekel, 2002), in which the most sensitive spore population is inactivated at a faster rate, followed by the slower and steady decline of a more resistant population (Tola & Ramaswamy, 2014). The n values decreased and became farther than 1 with increasing HPP temperature and pressure (**Table 4-1**), indicating a more pronounced deviation from linearity. This was shown by a dramatic initial reduction at higher temperatures, followed by tailing as time increased, leaving a small resistant population. From **Table 4-1**, the n values, which ranged from 0.57–0.59 to 0.78–0.79 in reconstituted milk, also seem to show temperature dependence for

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both strains with $0.95 \leq R^2 \leq 0.99$ (**Figure 4-5a**). Similarly, the n was also a linear function of the HPP pressure (**Figure 4-5b**) with $R^2 = 0.91$ for NZRM 984 strain. The dependence of b and n on the HPP temperature and pressure was also reported by Juliano et al. (2009) and Buzrul (2015).

4.3.5 Modeling thermal inactivation of *B. cereus* spores

Thermal survival of psychrotrophic *B. cereus* ICMP 12442 spores in reconstituted skim milk was linear and well modeled by the simple first order kinetic model (**Figure 4-2b**) (0.001– 0.054 MSE, 0.973–0.985 R^2 , and 1.09–1.15 A_f). Therefore, the kinetic parameters were estimated (**Table 4-2**). The D -values obtained for ICMP 12442 spores were 2.0 min at 90°C, 8.5 min at 80°C and 78.5 min at 70°C, and a z -value of 12.5°C. The z -value obtained in our work with ICMP 12442 spores is in the range of Johnson et al. (1982) obtained with strain F4165/75 in buffer and Montville et al. (2005) with ATCC 7004, ATCC 4342, ATCC 9818 in milk and orange juice. A wide range of *B. cereus* spore heat resistance was reported in the literature, highly dependent on the strain, type of medium/food and sporulation conditions (Evelyn & Silva, 2015a; Mazas et al., 1995; Montville et al., 2005). The high variability of *B. cereus* spores' thermal resistance is a food safety concern, due to existence of survivors after thermal pasteurization (Silva & Gibbs, 2010). Therefore, an appropriate safety margin for *B. cereus* spore elimination in foods should be established when designing thermal processing conditions. For example, the extremely high $D_{70^\circ\text{C}}$ -value obtained (78.5 min) in our experiment with ICMP12442, demonstrates that common milk HTST pasteurization at 72°C-15 s is not enough for the pasteurization of milk contaminated with this strain. A temperature of 110°C for 15 s would be a better option, since it delivers a 5 log reduction in psychrotrophic *B. cereus* spores in milk.

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Table 4-1 Weibull and log-logistic model parameters for pressure-thermal inactivation of psychrotrophic *Bacillus cereus* spores in skim milk.*

P (MPa)	T (°C)	Weibull				Log-logistic					
		ICMP 12442		NZRM 984		ICMP 12442			NZRM 984		
		<i>b</i> ± SD	<i>n</i> ± SD	<i>b</i> ± SD	<i>n</i> ± SD	<i>A</i> ± SD	τ ± SD	σ ± SD	<i>A</i> ± SD	τ ± SD	σ ± SD
600	70	0.67±0.10	0.57±0.05	0.55±0.08	0.59±0.04	-7.12±0.33	1.21±0.04	-4.67±0.09	-7.24±0.74	1.34±0.09	-4.55±0.15
	60	0.36±0.05	0.63±0.04	0.30±0.04	0.65±0.04	-5.27±0.43	1.33±0.07	-3.39±0.11	-5.19±0.62	1.40±0.11	-3.25±0.15
	50	0.16±0.03	0.72±0.05	0.12±0.02	0.75±0.03	-3.63±0.40	1.43±0.08	-2.56±0.11	-5.58±3.38	1.90±1.32	-3.07±0.95
	38	0.10±0.03	0.79±0.10	0.08±0.02	0.78±0.08	-5.31±9.11	1.88±1.31	-3.11±3.91	-1.90±0.09	1.30±0.03	-1.73±0.04
400	70	nd	nd	0.46±0.02	0.61±0.02	nd	nd	nd	-11.27±2.86	1.86±0.21	-5.82±1.20
200	70	nd	nd	0.30±0.02	0.68±0.02	nd	nd	nd	-9.88±2.03	1.87±0.17	-5.05±0.72

**B. cereus* ICMP 12442 (=ATCC 9139, ATCC 21, BCRC 17036, CECT 5144, LMG 9005, NCCB 48010, NCIMB 11925, VTT E-96727), and *B. cereus* NZRM 984 (=NCTC 10320, ATCC 11778, DSMZ 345) were used; nd – not determined; *b* and *n* are the Weibull scale and shape factors (Equation 1-6), respectively. The Weibull model exhibited low MSE values (0.001– 0.054), high R^2 (0.964–0.998), and A_f close to 1.00 (1.04–1.13); *A*, τ and σ are the log-logistic model parameters (Equation 1-8). Log-logistic model was also suitable with comparable performance indices (MSE: 0.001– 0.033; R^2 0.965–0.999; A_f 1.03–1.13); The parameters' values were expressed as means±standard deviation (SD) and obtained from three experiments.

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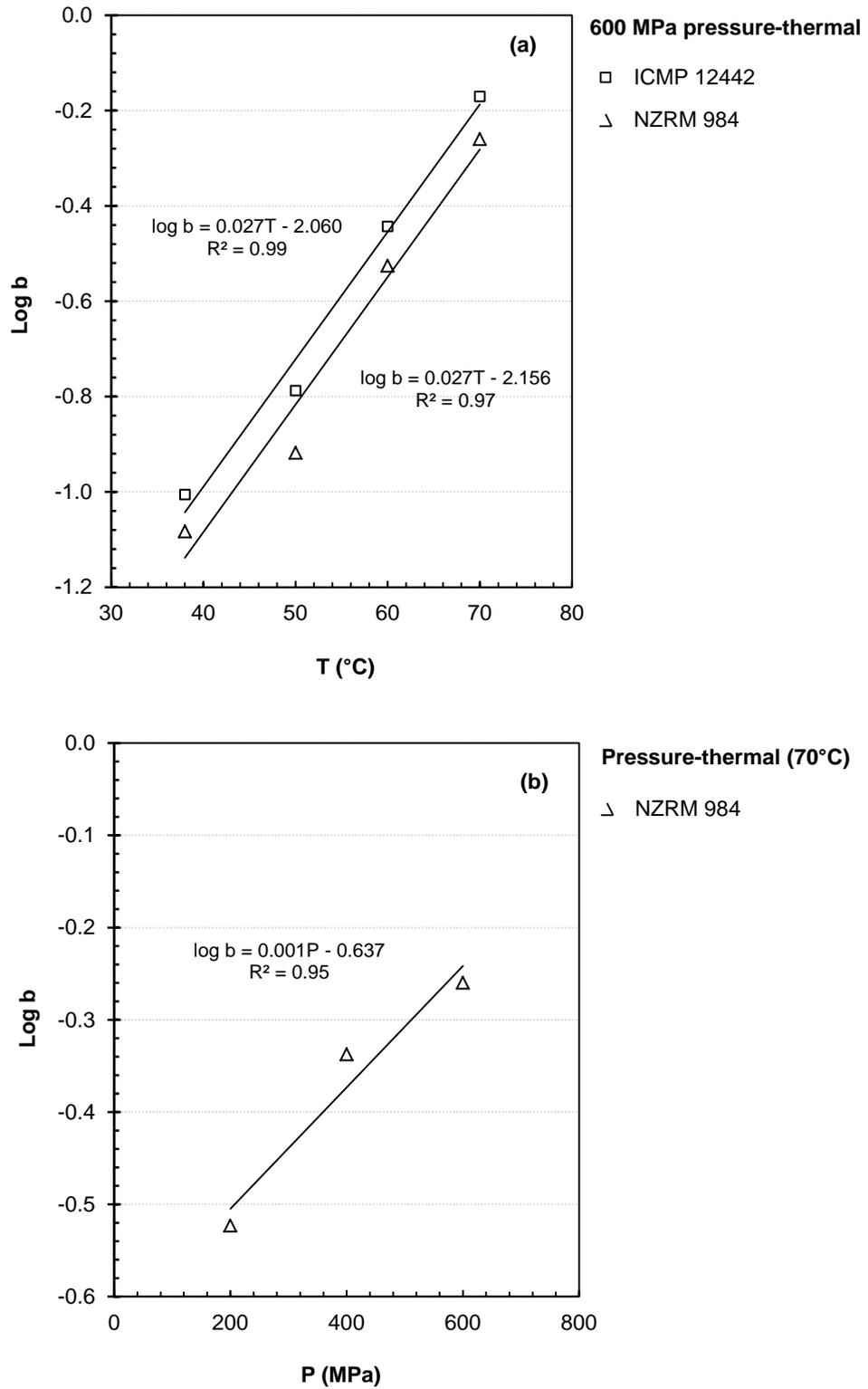


Figure 4-4 High pressure combined with thermal processing: The effect of temperature (a) and pressure (b) on the Weibull *b* parameter.

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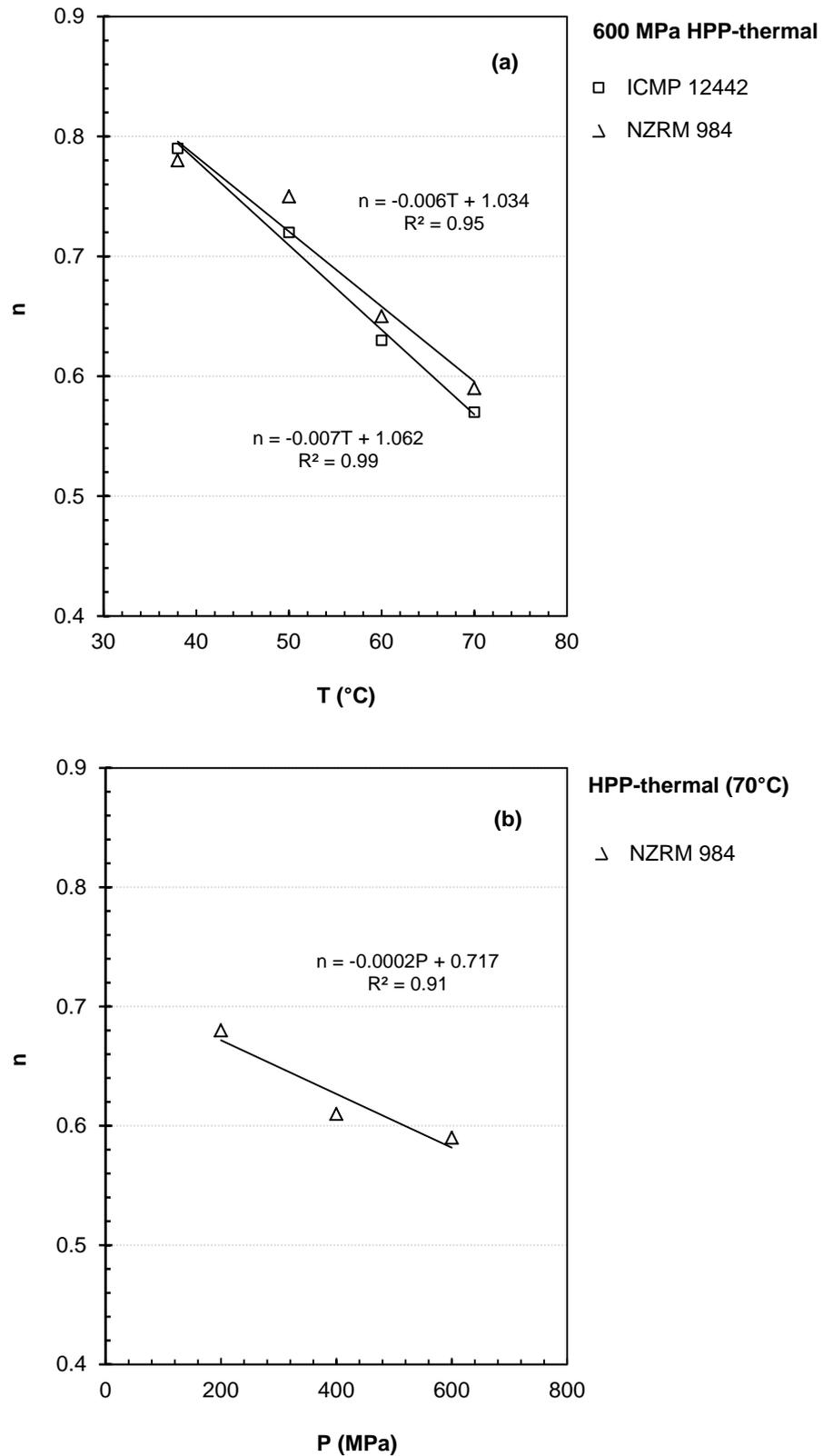


Figure 4-5 HPP combined with thermal processing: The effect of temperature (a) and pressure (b) on the Weibull n parameter.

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Weibull also gave good fit for thermal inactivation of the *B. cereus* spore and the b and n parameters were also estimated (Table 4-2) to compare with the pressure-thermal Weibull parameters (Table 4-1). The b for a 90°C thermal process (0.7) is similar to the b obtained with a 600 MPa-70°C process. This result also confirms the need of lower temperatures in the case of a HPP pressure-thermal process. The Weibull model has also been used to describe the thermal log survivors of two psychrotrophic spore strains of *Bacillus cereus* (Fernandez et al., 1999).

Table 4-2 First order and Weibull model parameters for thermal inactivation of psychrotrophic *Bacillus cereus* ICMP 12442 spores in skim milk.*

T (°C)	First order		Weibull	
	D_T -value \pm SD (min)	z -value \pm SE (°C)	$b \pm$ SD	$n \pm$ SD
90	2.0 \pm 0.20	12.5 \pm 0.20 $R^2 = 0.98$	0.74 \pm 0.05	0.83 \pm 0.04
80	8.5 \pm 0.04		0.19 \pm 0.04	0.85 \pm 0.08
70	78.5 \pm 0.30		0.02 \pm 0.01	0.98 \pm 0.17

**B. cereus* ICMP 12442 is the same as ATCC 9139, ATCC 21, BCRC 17036, CECT 5144, LMG 9005, NCCB 48010, NCIMB 11925, VTT E-96727; D_T is the decimal reduction time at certain temperature and z -value is the temperature required that results in a 10-fold decrease in the D_T -value (Equation 1-1 and Equation 1-2). D_T -values are means \pm standard deviation (SD) and obtained from two experiments; b and n are the Weibull scale and shape factors, respectively (Equation 1-6); Both models worked well presenting low MSE values (0.001–0.054), high R^2 (0.973–0.998), and A_f (1.02–1.15) close to 1.

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4.4 Conclusion

This study has shown that psychrotrophic *B. cereus* spore inactivation in reconstituted skim milk is pressure dependent for a pressure-thermal process at 70°C, being higher at higher pressure. The spore inactivation also increased as the temperature increased from 38 to 70°C at 600 MPa. The 600 MPa pressure-thermal process enhanced *B. cereus* spore thermal inactivation allowing less 20°C lower treatment temperature for the same spore inactivation. The two psychrotrophic spores exhibited more or less the same resistance under a HPP pressure-thermal process. The Weibull distribution was a better mathematical model than the Log-logistic model to describe the inactivation of psychrotrophic *B. cereus* spores via a pressure-thermal process, whereas the thermal inactivation followed the first order kinetics. The results demonstrated the benefit of the HPP technology and allowed a better understanding of the kinetics of pressure-induced inactivation of psychrotrophic *B. cereus* spores in milk.

Chapter 5 High pressure processing combined with 38-70°C to inactivate psychrotrophic *Bacillus cereus* spores in beef slurry: Modeling the HPP and thermal inactivation

Content submitted for publication

5. High pressure processing psychrotrophic *B. cereus* spores in beef slurry

Chapter abstract

The growth of psychrotolerant *Bacillus cereus* in pre-prepared cooked chilled foods and refrigerated processed foods of extended durability (REPFED) is a concern. High pressure processing (HPP) is an established food processing technology that retains the flavor and nutrients in the processed food. In this study, the efficacy of 600 MPa HPP in combination with 70°C for the inactivation of *B. cereus* ICMP 12442 spores in beef slurry was investigated and compared with 70°C thermal processing alone. The HPP-70°C process enhanced the *B. cereus* spore thermal inactivation in beef slurry, resulting in 4.9 log reductions after 20 min vs. 0.5 log for thermal processing. Then, the effect of temperature at 600 MPa on the spore inactivation up to 40 min was studied, and the log survivors vs. time were modeled. Increasing the HPP temperature from 38 to 70°C, increased the spore inactivation in beef slurry up to 3 log. Weibull model described the spore inactivation by HPP-thermal at 38, 50, 60 and 70°C. Lastly, the thermal inactivation of the spores was investigated and compared with the HPP-thermal results. The thermal inactivation of spores in beef slurry required 30°C higher temperatures to achieve the same spore inactivation. The first order kinetic model was more appropriate for the thermal inactivation of spores ($D_{90^\circ\text{C}}$ -value = 0.98 min, z -value = 11.9°C). The results of this study confirm the advantage of HPP technology for the inactivation of *B. cereus* spores in beef slurry.

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5.1 Introduction

Increasing consumer demand for pre-prepared cooked chilled foods (including refrigerated processed foods of extended durability or REPFEDs) has led tremendous research in the area of food safety microbiology to reduce the risk associated with this type of convenience foods (Afchain et al., 2008; Carlin et al., 2000; Carlin et al., 2000; Choma et al., 2000; Daelman et al., 2013; Evelyn & Silva, 2015a; Guinebretiere, 2003; Lopez-Pedemonte et al., 2003; Malakar et al., 2004; Membré et al., 2006; van Opstal et al., 2004). Spore forming and psychrotolerant bacteria has been linked to the safety and stability of these foods because of its ability to survive the normal heat treatment (pasteurization and/or cooking) and grow at low temperature during the chilled storage (Membré et al., 2006; Silva & Gibbs, 2010; Silva et al., 2014). As a result, the foods are spoiled and reduced in their shelf life, and there is a risk of foodborne diseases caused by the outgrowth of pathogenic spore formers such as psychrotolerant *Bacillus cereus* and non-proteolytic strains of *Clostridium botulinum* (Silva & Gibbs, 2010).

B. cereus is a Gram-positive, rod-shaped, spore-forming facultative anaerobic bacterium which is able to grow over a wide range of temperatures (4–55 °C), pH (4.9–9.3), and water activities values (0.92–1.0) (EFSA, 2005b). The psychrotrophic strains of *B. cereus* are able to regenerate to large numbers at refrigerated temperatures (Choma et al., 2000; Christiansson et al., 1989; Valero et al., 2007), produce toxins in foods (Samapundo et al., 2011), and cause diarrhea or emesis food poisoning and fatal meningitis (Dierick et al., 2005; Evreux et al., 2007; Luby et al., 1993; Schoeni, 2005; Slaten et al., 1992). Prevalence (16.8% population per package) and concentrations at the time of consumption (2.5% of the packages contaminated with >6.7 log cfu/g) of psychrotolerant *B. cereus* group II in REPFED foods indicate this group as a high potential food risk (Hendrickx, 2011). The following

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contaminated foods have been reported in outbreaks of *B. cereus* around the world: meat (Luby et al., 1993; Slaten et al., 1992), raw and pasteurized milk (Ahmed, 1983; Røssland et al., 2005), starchy foods (for instance rice, potato, pasta) and cheese products (USFDA, 2012a), vegetable puree as well as other chilled-foods containing vegetables (Carlin et al., 2000; Carlin et al., 2000; Jenson et al., 2003), and cake and other desserts (Ghelardi et al., 2002; Granum & Lund, 1997). With respect to thermal resistance, the decimal reduction time (*D*-value) of psychrotrophic *B. cereus* spores can range from 0.22 to 3.1 min at 100°C, depending on the strain and heating medium (Evelyn & Silva, 2015a; 2015c; Fernández et al., 2001; Wimalaratne, 2009).

High pressure processing (HPP) is a commercially non-thermal food preservation technology with less adverse effects on food quality (Cullen et al., 2012). HPP relies on the use of high pressures (generally 100-600 MPa) to process liquid and solid foods (with or without heat) for times up to 20 min, to inactivate spoilage/pathogenic microorganisms and to extend food shelf-life. Inactivation of microbial spores and enzymes by high pressure alone is not feasible. Therefore, combination of HPP with a mild heat is required (Evelyn & Silva, 2015c, 2015d, 2016b; Evelyn et al., 2016; Patterson, 2005; Silva et al., 2012; Sulaiman & Silva, 2013; Sulaiman et al., 2015a). With respect to *B. cereus* spores, the efficacy of HPP between 100-900 MPa (pulsed or continuous) in conjunction with mild heat, antimicrobial agents (e.g. nisin and sucrose laurate), or an additional control hurdle (e.g. olive powder) to inactivate *B. cereus* spores has been investigated (Aoyama et al., 2005; Arroyo et al., 1997; Daryaei et al., 2013; Evelyn & Silva, 2015c; Fornari et al., 1995; Gola et al., 1996; Ju et al., 2008; Lopez-Pedemonte et al., 2003; Luu-Thi et al., 2014; Marco et al., 2011; McClements et al., 2001; Meyer, 2000; Raso et al., 1998a; Robertson et al., 2008; Rovere et al., 1998; Scurrah et al., 2006; Shearer et al., 2000; Shigeta et al., 2007; van Opstal et al., 2004). *B. cereus* spore log reductions between 1 and ≥ 7 were achieved, depending on the conditions applied and the

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spore resistance. Among these results, Daryaei et al. (2013) and Luu-Thi et al. (2014) modeled the HPP inactivation kinetics with mesophilic *B. cereus* spores. With respect to psychrotrophic *B. cereus* spores, McClements et al. (2001), Lopez-Pedemonte et al. (2003), and van Opstal et al., (2004) studied its inactivation in milk and cheese, however the kinetics were not modeled. We have previously modeled the inactivation of psychrotolerant *B. cereus* spores in milk by HPP-thermal and found that Weibull distribution was a better model to describe the log survivors. Due to ability of this bacteria to grow in minced beef stored under refrigeration, and high variability in the spore resistance, HPP-thermal inactivation of psychrotrophic *B. cereus* spores in beef slurry was investigated. The objectives were as follows: (i) to compare the 600 MPa HPP-thermal with thermal inactivation of psychrotrophic *B. cereus* spores in beef slurry at 70°C; (ii) to model the 600 MPa HPP-thermal inactivation of psychrotrophic *B. cereus* spores in beef slurry; and (iii) to model the thermal inactivation of psychrotrophic *B. cereus* spores in beef slurry.

5.2 Materials and methods

5.2.1 Microbiology

5.2.1.1 Strain

Psychrotrophic *B. cereus* ICMP 12442 described previously in Chapter 4 (Evelyn & Silva, 2015c) was also used in this study.

5.2.1.2 Sporulation and spore enumeration

The same sporulation and enumeration procedure for this strain was described previously (Evelyn & Silva, 2015a, 2015c). The *B. cereus* spore concentration in beef slurry before and

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after HPP-thermal and thermal processing was determined by spread plating onto trypticase soy agar (TSA) and aerobic incubation at 37°C for 48 h. Beef slurry samples were first homogenized in a stomacher (Masticator stomacher, IUL Instruments, Germany) with equal volume of 0.1% (w/v) sterile buffered peptone water as dilution fluid for 2 min (this dilution was considered in the calculation of final spore concentration). Samples were further decimal diluted ten times in 0.1% (w/v) sterile buffered peptone water, mixed repeatedly with a vortex mixer, and plated twice. Average results were expressed in cfu per gram (cfu/g) of beef slurry. The spores were stored in sterile distilled water and maintained at 2°C until use.

5.2.1.3 Beef slurry preparation and inoculation

Pasteurised beef was also chosen in this study since it is similar to beef mince and prone to contamination by *B. cereus* (Thippareddi et al., 2009). The beef preparation and composition were described in Evelyn and Silva (2015a) and previous chapter (Chapter 2). For HPP-thermal and thermal experiments, a portion (*ca.* 0.1 mL) of spore suspension was inoculated into 3 g of beef slurry placed inside 8×8 cm food grade sterile pouches (Cas-Pak, New Zealand) to yield a final concentration of approximately $\sim 10^7$ cfu/g.

5.2.2 High pressure processing of beef slurry

The high pressure food processing equipment, operation, and procedure used for pressure and pressure-thermal treatment of *B. cereus* spores in beef slurry followed the method previously described for *B. cereus* spores in skim milk (Chapter 4). Pressure of 600 MPa was selected since it is the maximum pressure supported by the Avure HPP machine, and it showed the best performance for the same *B. cereus* spore strain inactivation in milk (Evelyn & Silva, 2015c, Chapter 4).

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5.2.3 Thermal processing of beef slurry

The same procedure and temperatures (70, 80 and 90°C) described for milk was used (Chapter 4) for the thermal inactivation experiments with *B. cereus* ICMP 12442 spores in this study. Two survival experiments were carried out with duplicate samples.

5.2.4 Modeling the kinetics of psychrotrophic *B. cereus* spore inactivation in beef slurry

Based on the aspect of the survival curves (log microbial numbers vs. time), first order kinetics (Equation 1-1) was fitted to the thermal inactivation data, whereas Weibull (Equation 1-6) and log-logistic (Equation 1-8) mathematical models were fitted to the HPP-thermal log survivors of psychrotrophic *B. cereus* spores in beef slurry.

TableCurve 2D version 5.01 (SYSTAT Software Inc., USA) was used to fit the models to the spore survival lines and estimate the model parameters. Mean square error (MSE), coefficient of determination (R^2), and accuracy factor (A_f) were used to compare the performance of different models. Additionally, temperature dependence of the parameters estimated was checked. For each temperature two or three survival experiments were carried out and the model parameters (*D-value*, b , n , A , τ , σ) were estimated by regression of logarithmic number of survivors ($\log N/N_0$) versus time. Then, the parameters' mean \pm standard deviation (SD) were calculated for each temperature. Additionally, the parameter's temperature dependence was investigated.

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5.3 Results and discussion

5.3.1 Comparing 600 MPa HPP-70°C vs 70°C thermal inactivation of psychrotrophic *B. cereus* spores in beef slurry

Figure 5-1 shows the log survivors of *B. cereus* ICMP 12442 (= ATCC 9139) spores in beef slurry by 600 MPa HPP-70°C and 70°C thermal process. The HPP-thermal inactivation was much faster than thermal inactivation alone. For a 20 min process at 70°C, 4.9 log reduction for HPP-thermal vs. 0.5 log reduction for thermal alone were obtained, indicating a remarkable advantage when using HPP technology. The benefit of HPP-thermal vs. thermal was also documented by Daryaei et al. (2013) and Luu-Thi et al. (2014) for mesophilic *B. cereus* spores, and in our previous results carried out in milk (Evelyn & Silva, 2015c). Daryaei et al. (2013) reported similar log reductions (~3.5 log) for 600 MPa HPP in cooked rice with *B. cereus* ATCC 9818 at 75°C HPP temperature and 4 min holding time. Luu-Thi et al. (2014) observed less than 1.5 log for *B. cereus* F4430/73 in ethanesulfonic acid (MES) buffer at the same process conditions. These results indicate that strain and spore suspending medium play an important role for the inactivation of *B. cereus* spores by HPP-thermal. The 4 log reductions previously obtained in milk (Evelyn and Silva, 2015c) was lower than 4.9 log obtained with beef slurry using the same *B. cereus* strain, after 20 min process at 70°C. This could be due to the baroprotective effect of milk components such as sucrose on the spore inactivation by HPP (Gervilla et al., 2000; Patterson et al., 1995; Simpson & Gilmour, 1997; Styles et al., 1991).

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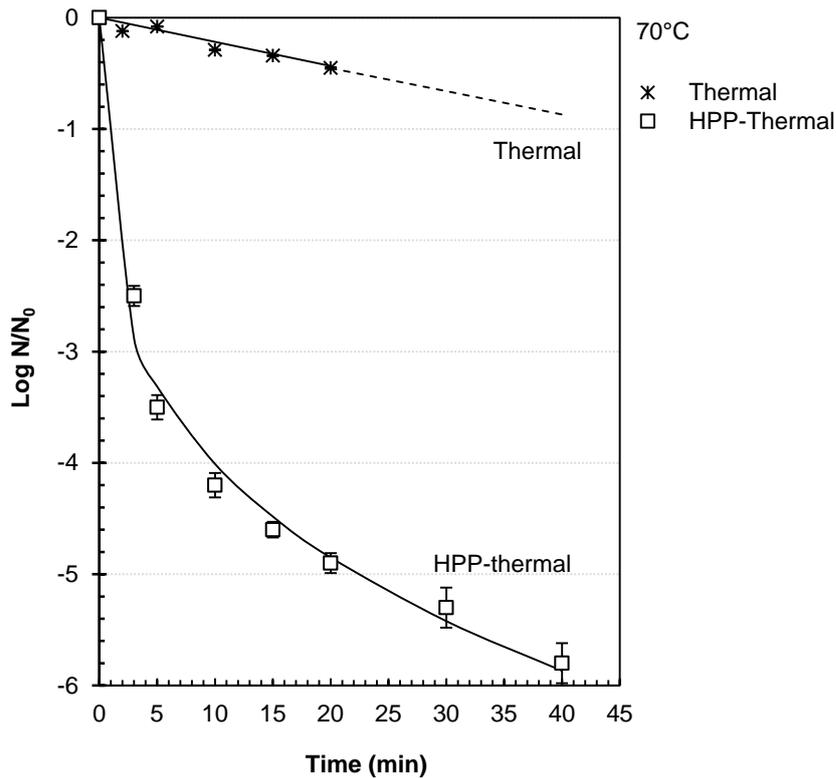


Figure 5-1 Comparison of 600 MPa HPP-thermal and thermal inactivation of psychrotrophic *B. cereus* ICMP 12442 spores in beef slurry at 70°C (data points are average \pm standard deviation). Dashed line represents predicted thermal log survivors up to 40 min at 70°C.

5.3.2 Modeling the 600 MPa HPP-thermal inactivation kinetics of psychrotrophic *B. cereus* spores in beef slurry

The log survivors of *B. cereus* ICMP 12442 spores in beef slurry by 600 MPa HPP and 600 MPa HPP-thermal for 50 to 70°C are illustrated in **Figure 5-2**. The higher the HPP process temperature, the higher was the *B. cereus* spore inactivation, confirming the significant effect of HPP temperature for inactivating the spores. For example, increasing the temperature from 38°C (no heating used) to 70°C at 600 MPa for 40 min increased the *B. cereus* spore inactivation in beef slurry by 3 log. Our previous results in milk showed 3.5 log reductions for this strain (Evelyn & Silva, 2015c). An increase in the log reductions with temperature was also reported with spores of psychrotrophic *B. cereus* LMG 6910 (= ATCC 7004),

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mesophilic *B. cereus* ATCC 9818 and F4430/73, *Alicyclobacillus acidoterrestris*, and *Clostridium botulinum* using 600 MPa HPP-thermal (Daryaei & Balasubramaniam, 2013; Luu-Thi et al., 2014; Margosch et al., 2006; Silva et al., 2012; van Opstal et al., 2004; Vercammen et al., 2012) and mould spores (Evelyn & Silva, 2015d, 2015d; Evelyn et al., 2016).

The non-linearity (fast inactivation followed by a slower inactivation) observed in the *B. cereus* spore survival curves (**Figure 5-2**) was confirmed by the low R^2 (≤ 0.760) obtained for first order kinetics thus not being appropriate to describe the spore survivor experimental data. Then, Weibull (2 parameters) and log logistic (3 parameters) models were attempted. Although the log logistic model exhibited better performance indices than the Weibull model (Table 5-1), the log logistic model showed poor fit for temperature effect on the estimated parameters and is a more complex model. Therefore, the Weibull model was still selected for the fitting ($0.020 \leq \text{MSE} \leq 0.036$, $0.983 \leq R^2 \leq 0.990$, $1.04 \leq A_f \leq 1.09$) (Table 5-1).

In Weibull distribution, the b parameter is the scale factor that relates with the spore inactivation rate. As can be seen from the **Table 5-1**, at 600 MPa, the higher the temperature, the higher was the value of b . The b increased from 0.56 to 2.13 as the temperature increased from 38 to 70°C. Similarly, our past results with same strain in milk showed the increase of b from 0.10 to 0.67 under the same conditions (Evelyn & Silva, 2015c). Our results in beef slurry also show similarities with the results obtained by Daryaei et al. (2013), in which the b in cooked rice increased from 0.81 to 1.66 as the 600 MPa HPP temperature was increased from 60 to 85°C. **Figure 5-3** shows a plot of $\log b$ as a function of the HPP temperature, which increased linearly with temperature ($R^2 = 0.97$). Similar results were obtained previously in milk (Evelyn & Silva, 2015c).

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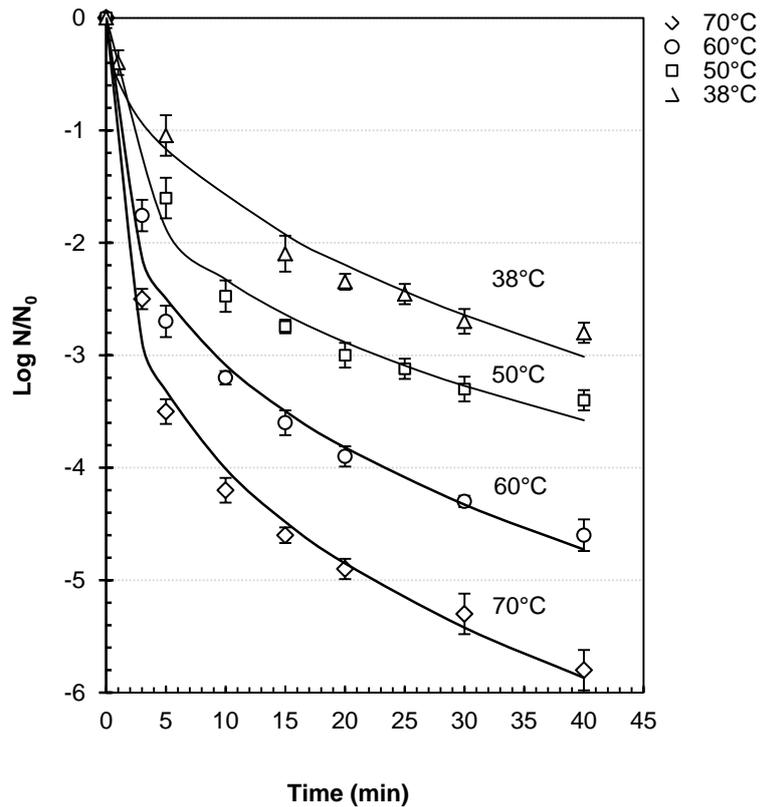


Figure 5-2 Weibull model fitted to psychrotrophic *B. cereus* ICMP 12442 (=ATCC 9139, ATCC 21) log survivors after 600 MPa HPP alone (38°C) and HPP-thermal processing (50, 60, 70°C) (data points are average \pm standard deviation).

The n parameter in the Weibull distribution presents the shape factor of the survival curves and the deviation from linearity. All the n values were less than 1 (**Table 5-1**), indicating that the spore survivor curves by HPP-thermal process were concave upward (**Figure 5-2**). This type of concavity suggests that there is a mixed resistance of the spore population to the lethal treatment (Peleg & Cole, 1998; van Boekel, 2002), in which the most sensitive spore population is inactivated at a faster rate, followed by the slower and steady decline of a more resistant population (Tola & Ramaswamy, 2014). Although at 38°C n was higher, the n did not change within 50 to 70°C HPP temperature (**Table 5-1**). Cunha et al. (1998) has mentioned that n should not change with temperature as it is related with the kinetic order.

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Table 5-1 Weibull and log-logistic model parameters for 600 MPa HPP-thermal inactivation of psychrotrophic *Bacillus cereus* ICMP 12442 spores in beef slurry.*

T (°C)	Weibull		Log-logistic		
	<i>b</i> ± SD	<i>n</i> ± SD	<i>A</i> ± SD	τ ± SD	σ ± SD
70	2.13±0.15	0.28±0.02	-7.28±1.09	0.82±0.20	-2.93±0.28
60	1.52±0.13	0.30±0.03	-5.75±0.85	0.86±0.18	-2.58±0.25
50	1.14±0.14	0.31±0.04	-3.73±0.13	0.79±0.03	-2.63±0.18
38	0.56±0.09	0.46±0.05	-3.81±0.39	1.10±0.11	-2.06±0.10

**B. cereus* ICMP 12442 is the same as ATCC 9139, ATCC 21, BCRC 17036, CECT 5144, LMG 9005, NCCB 48010, NCIMB 11925, VTT E-96727; *b* and *n* are the Weibull scale and shape factors, respectively (Equation 1-6); . The Weibull model exhibited low MSE values (0.020– 0.036), high R^2 (0.983–0.990), and A_f close to 1.00 (1.04–1.09); *A*, τ and σ are the Log-logistic model parameters (Equation 1-8). Log-logistic model was also suitable with good performance indices (MSE: 0.002– 0.016; R^2 0.994–0.996; A_f 1.01–1.05); The parameters' values were expressed as means±standard deviation (SD) and obtained from three experiments.

5.3.3 Modeling the thermal inactivation kinetics of psychrotrophic *B. cereus* spores in beef slurry

The thermal log survivors of the psychrotrophic *B. cereus* ICMP 12442 spores in beef slurry are presented in **Figure 5-4**. Similar spore inactivation (≈ 3.0 log) was obtained after 20 min for the 80°C thermal process (**Figure 5-4**) and 50°C HPP process (**Figure 5-1**), thus demonstrating that HPP-thermal required a 30°C lower temperature than a thermal process alone to obtain the same level of lethality. The HPP-70°C process specific energy required was 421 kJ/kg, while the equivalent 90°C thermal process required was 352 kJ/kg, suggesting a slightly higher energy demand for the equivalent pressure-thermal process.

Thermal survival lines of *B. cereus* ICMP 12442 spores in beef slurry were linear (**Figure 5-4**), which are supported by good performance indices (MSE \leq 0.002, $R^2\geq$ 0.998, A_f between 1.01 and 1.03) and temperature dependence of the *D*-values ($R^2=0.998$) (**Table 5-2**). The *D*-values obtained for ICMP 12442 spores were 1.0 min at 90°C, 6.9 min at 80°C and 46.0

5. High pressure processing psychrotrophic *B. cereus* spores in beef slurry

min at 70°C. Our previous results with this strain showed a slightly higher thermal resistance in milk with $D_{90^{\circ}\text{C}} = 2.0$ min, $D_{80^{\circ}\text{C}} = 8.5$ min and $D_{70^{\circ}\text{C}} = 78.5$ min (Evelyn & Silva, 2015c), probably also due to protective effect of milk components mentioned previously. Byrne et al. (2006) determined $D_{90^{\circ}\text{C}}$ -value of 10 min with *B. cereus* spores cocktail in pork roll as opposed to 1 min in beef slurry obtained in our study. Our previous studies with NZRM 984 spores in beef slurry (Evelyn & Silva, 2015a) demonstrated a similar $D_{90^{\circ}\text{C}}$ -value (1 min). A wide range of *B. cereus* spore heat resistance was reported in the literature, highly dependent on the strain, type of medium/food and sporulation conditions (Mazas et al., 1995; Montville et al., 2005). The z -value obtained in our work (11.9°C) with ICMP 12442 spores was in the range of *B. cereus* z -values obtained with strain ATCC 9818, F4165/75, CRA 1787 and F4165/75 in nutrient broth or distilled water modified and buffer (Ababouch & Busta, 1987; Casadei et al., 2001; Johnson et al., 1982), and in foods such as milk and orange juice with *B. cereus* ATCC 7004, ATCC 4342, ATCC 9818 spores (Montville et al., 2005).

Table 5-2 First order kinetic parameters for thermal inactivation of psychrotrophic *Bacillus cereus* ICMP 12442 spores in beef slurry.*

T (°C)	Thermal				
	D_T -value \pm SD (min)	z -value \pm SE (°C)	MSE	R^2	A_f
90	0.98 \pm 0.02	11.9 \pm 0.01	0.002	0.998	1.03
80	6.93 \pm 0.06	$R^2 = 0.98$	0.0001	0.999	1.01
70	46.03 \pm 0.80		0.0001	0.999	1.01

**B. cereus* ICMP 12442 is the same as ATCC 9139, ATCC 21, BCRC 17036, CECT 5144, LMG 9005, NCCB 48010, NCIMB 11925, VTT E-96727; D_T - and z -values are the first order kinetic parameters; D_T -values are means \pm standard deviation (SD) and obtained from two experiments; Low mean square error (MSE), R^2 and A_f close to 1 are indication of good fit.

5. High pressure processing psychrotrophic *B. cereus* spores in beef slurry

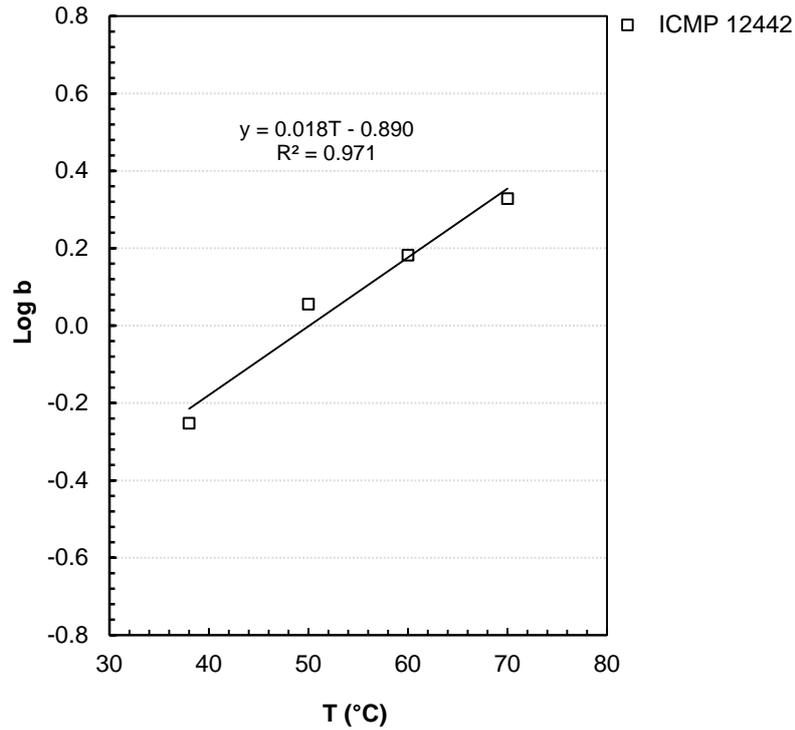


Figure 5-3 HPP combined with thermal processing for the inactivation of *B. cereus* ICMP 12442 spores: The effect of temperature on the Weibull b parameter.

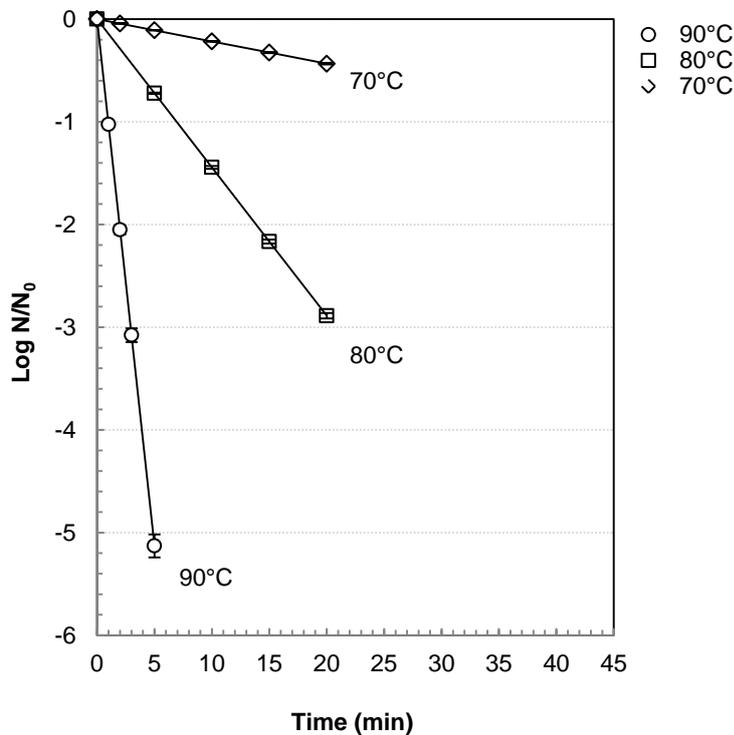


Figure 5-4 First order kinetics fitted to psychrotrophic *B. cereus* ICMP 12442 (=ATCC 9139, ATCC 21) log survivors after thermal processing (70, 80, 90°C) (data points are average \pm standard deviation).

5. High pressure processing psychrotrophic *B. cereus* spores in beef slurry

5.4 Conclusion

Current study demonstrated that psychrotrophic *B. cereus* spore inactivation in beef slurry is temperature dependent for 600 MPa HPP-thermal processes, being higher for higher temperature. The 600 MPa HPP-thermal process enhanced *B. cereus* spore thermal inactivation and reduced the treatment temperature to less 30°C for the same spore inactivation. Weibull distribution was the best mathematical model to describe the inactivation of psychrotrophic *B. cereus* spores in beef slurry by HPP-thermal process, whereas the thermal inactivation alone followed the conventional first order kinetic model. The results demonstrated the benefit of the HPP technology and allowed a better understanding of the kinetics of pressure-thermal induced inactivation of psychrotrophic *B. cereus* spores in beef slurry.

Chapter 6 Thermosonication versus thermal processing of skim milk and beef slurry: Modeling the inactivation kinetics of psychrotrophic *Bacillus cereus* spores

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6. Thermosonication vs thermal processing psychrotrophic *B. cereus* spores

Chapter abstract

Non-thermal processed foods are generally cold stored and distributed. The use of ultrasound for food preservation has attracted the interest of many research groups. In the current study, the thermosonication (TS, simultaneous ultrasound and thermal process) inactivation of psychrotrophic *Bacillus cereus* spores was investigated (24 kHz, 210 μm , 0.33 W/mL or W/g). First, the effectiveness of a 1.5 min TS process at 70 °C in skim milk, beef slurry, cheese slurry, and rice porridge was investigated. The TS was more effective than sole thermal treatment in reducing *B. cereus* spores in rice porridge, beef slurry and cheese slurry by 7, 6, and 4 fold, respectively. Then, the first-order *D*- and *z*-values for TS and thermal processing in skim milk and beef slurry, and the best model to fit TS inactivation of *B. cereus* spores in beef slurry were determined. The $D_{70^{\circ}\text{C}}$ -values in skim milk were 2.9 min for TS and 8.6 min for the thermal treatment. And in beef slurry, values of 0.4 min for TS and 2.3 min for thermal were estimated. It was found that the log-logistic model better described the TS spore inactivation in beef slurry. The ultrasound technology required 20–30°C lower temperatures for the same spore inactivation, which resulted in better food quality and energy saving gains.

6. Thermosonication vs thermal processing psychrotrophic *B. cereus* spores

6.1 Introduction

Psychrotrophic microorganisms can grow at temperatures of 7°C or less, regardless of the optimum growth temperature (Collins, 1981). Psychrotrophic bacteria are of great concern in pasteurized, low-acid, cold distributed foods (pH>4.6, e.g. milk and dairy products) and also chilled prepared foods such as sous vide and cook-chill foods (Carlin et al., 2000; Carlin et al., 2000; Silva & Gibbs, 2010; Silva et al., 2014). The population of bacteria can increase when foods are prepared under poor hygienic conditions and during distribution (Barbano et al., 2006). Spore-forming psychrotrophic pathogens such as non-proteolytic *Clostridium botulinum* and certain strains of *Bacillus cereus* have been linked to the microbiological safety of this type of foods because they can survive pasteurization, grow under refrigerated conditions and cause food poisoning (Silva & Gibbs, 2010). Yet control of psychrotolerant *B. cereus* spores in this class of food products is one of the most important concerns (Choma et al., 2000; Dierick et al., 2005; Evreux et al., 2007; Ghelardi et al., 2002; Luby et al., 1993; Silva & Gibbs, 2010; Slaten et al., 1992).

B. cereus is a Gram-positive, rod-shaped, spore-forming facultative anaerobic bacterium which is able to grow over a wide range of temperatures (4–55°C), pH (4.9–9.3), and water activities values (0.92–1.0) (EFSA, 2005b). This organism is able to regenerate to large numbers at refrigerated temperatures (Choma et al., 2000; Christiansson et al., 1989; Valero et al., 2007), and produce toxins in foods (Samapundo et al., 2011). When the level exceeds 10⁵ cfu/g, food intoxication by *B. cereus* can cause diarrhea or emesis depending on the type of toxin produced (Dierick et al., 2005; Luby et al., 1993; Schoeni, 2005; Slaten et al., 1992). Fatal meningitis has also been reported (Evreux et al., 2007). Contamination is difficult to detect since the organoleptic properties of the foods do not change (Christiansson et al., 1989). In addition, mild, short duration and self-limiting symptoms, and infrequent routine

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laboratory analysis, result in a low number of reported incidents (FSANZ, 2013). Nevertheless, the following contaminated foods have been reported in outbreaks of *B. cereus* around the world: starchy foods (for instance rice, potato, pasta) and cheese products (USFDA, 2012a), raw and pasteurized milk (Ahmed, 1983; Rovere et al., 1999), meat (Luby et al., 1993; Slaten et al., 1992), vegetable puree as well as other chilled-foods containing vegetables (Carlin et al., 2000; Carlin et al., 2000; Jenson et al., 2003) and cake and other desserts (Ghelardi et al., 2002; Granum & Lund, 1997). The decimal reduction times of psychrotrophic strains of *B. cereus* spores at 90°C ($D_{90^{\circ}\text{C}}$ -value) ranged from 2.2 to 9.2 min in buffer (Dufrenne et al., 1995) and from 4.4 to 6.6 min in reconstituted skim milk (Shehata & Collins, 1973), while others have reported values of 4 and 10 min in water and pork roll, respectively (Byrne et al., 2006; Fernández et al., 2001). According to Silva and Gibbs (2010), psychrotrophic *B. cereus* appears to have a higher heat resistance than psychrotrophic non-proteolytic *C. botulinum* strains, exhibiting D -values in the magnitude of seconds at $>95^{\circ}\text{C}$ for *B. cereus* and at lower temperatures (85°C to $>95^{\circ}\text{C}$) for *C. botulinum*.

The use of non-thermal technologies has emerged as an alternative to minimize changes of the food sensory properties induced by heating. The inactivation of bacteria using ultrasound was first initiated in the 1920s (Harvey & Loomis, 1929). This technology relies on the application of pressure waves (frequency ranging from 20 to 100 kHz) to the food/beverage, causing microbial cell death. This phenomenon, called cavitation (Chen, 2012; Feng et al., 2009; Feng & Yang, 2011b; Piyasena et al., 2004), creates microgas bubbles due to regions of pressure change. Some authors may also use the term cavitation to describe the bubble growth and subsequent collapse with considerable energy release, inducing localized extreme conditions, which leads to bacterial cell death (Feng & Yang, 2011b; Gogate, 2011; Wu et al.). Microbial killing involves the thinning of the cell membranes, localized heating, and the production of free radicals (Butz & Tauscher, 2002; Fellows, 2000; Piyasena et al., 2004),

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which induces adverse chemical changes in the DNA or protein denaturation (Riesz & Kondo, 1992).

The effect of ultrasound alone has been considered ineffective for the inactivation of bacterial spores (Butz & Tauscher, 2002). Hence, the combination with other treatments such as temperature, pressure, or both heat and pressure to increase the lethal effect has been investigated. The combination of ultrasound with chemicals and ionizing radiation has also been studied. Spore inactivation was significantly enhanced and the treatment times were also significantly reduced compared to ultrasound, chemical or radiation alone (Ahmed & Russell, 1975; Sagong et al., 2012; Sierra & Boucher, 1971). Regarding ultrasound assisted (before or after) thermal processing for spore inactivation, heat sensitization of *B. cereus*, *Bacillus licheniformis*, *Bacillus subtilis* and *Geobacillus stearothermophilus* spores is generally observed after ultrasonic treatments (Burgos et al., 1972; Ordonez & Burgos, 1976; Sanz et al., 1985). In addition, the release of low molecular weight substances from the spore protoplast after ultrasonication was registered (Palacios et al., 1991).

With respect to the simultaneous use of ultrasound and heat, often referred to as thermosonication (TS), a process of 20 kHz, 5 W/mL and 70°C was much more effective for the spore inactivation of two strains of *B. subtilis* in milk than thermal processing alone (Garcia et al., 1989). López-Malo et al. (2001) and López-Malo et al. (2005) also observed the synergistic effect of ultrasound and heat on fungal spore inactivation which resulted in lower TS decimal (*D*-) reduction values (20 kHz, 40–60°C) than the corresponding thermal *D*-values. To date, there have been only two publications on TS microbial spore inactivation, and there is almost no documentation regarding the kinetic modeling of bacterial spore inactivation by TS. Due to the importance of psychrotrophic *B. cereus* spores and the lack of reports on TS inactivation modeling, as well as the benefits over thermal inactivation alone,

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in this research, *B. cereus* spore inactivation by TS in skim milk, beef slurry, cheese slurry, and rice porridge was investigated as follows: (i) to study the effectiveness of 1.5 min of thermosonication (TS) vs. thermal processing at 70°C to reduce the spores in different foods; (ii) to model the thermosonication and thermal inactivation kinetics in skim milk and beef slurry; (iii) to compare the first-order kinetic parameters (*D*- and *z*-values) for TS and thermal inactivation of spores in skim milk and beef slurry.

6.2 Materials and methods

6.2.1 Food sample preparation

Skim milk, beef, cheese, and rice were chosen for this research since they are prone to contamination by *B. cereus* (USFDA, 2012b), and the major composition was determined by an accredited laboratory in New Zealand (**Table 6-1**). All foods under study were commercial products to avoid variability of food composition between each treatment. The foods were also prepared in the same way for all the treatments.

6.2.1.1 Skim milk

Reconstituted skim milk was prepared by diluting New Zealand skim milk powder with 100 mL of sterile distilled water (SDW).

6.2.1.2 Beef slurry

Sirloin beef mince was stored overnight at 4°C before use. After autoclaving, the beef was mixed with SDW in a sterile laboratory scale blender (100 mL of SDW was added to every 100 g of minced meat).

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6.2.1.3 Cheese slurry

Cheese slurries were prepared by mixing and blending 100 g of New Zealand grated cheddar cheese with 100 mL SDW.

6.2.1.4 Rice porridge

Raw jasmine rice was cooked with water (ratio of 1:7) to make porridge. The cooked rice porridge was immediately blended.

Table 6-1 The composition and pH of foods processed in this study.*

	Composition (g/100 g)			
	Skim milk	Beef slurry	Cheese slurry	Rice porridge
Moisture	90.5	76.0	68.2	75.9
Fat	<0.1	7.0	16.1	<0.1
Protein	2.7	14.0	10.6	1.9
Carbohydrate	4.9	2.6	3.3	22.1
Sugars	4.7	0.3	0.2	<0.1
Ash	1.8	0.4	1.8	0.1
pH	6.5	6.5	5.8	6.7

*The analyses were carried out by an accredited laboratory and the average values are presented.

6.2.2 *B. cereus* microbiology

6.2.2.1 Strain

B. cereus strain NZRM 984 previously described in Chapter 4 was selected for this study since preliminary tests with NZRM 984 and other psychrotrophic ATCC 9139 demonstrated

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higher heat resistance for NZRM 984. The psychrotrophic behavior (growth at 4°C) of NZRM 984 was previously demonstrated (Wimalaratne, 2009). The original freeze-dried culture was initially suspended into test tubes containing 5 mL of Brain–Heart Infusion (BHI) broth (Oxoid, Hampshire, UK) for 20 min. The culture was then inoculated into a larger volume (50 mL) of BHI broth and grown overnight at 37 °C with continuous shaking at 200 rpm in an orbital shaker.

6.2.2.2 Sporulation

The cells from the overnight culture in BHI were used as a starting culture for sporulation. Aliquots of 0.1 mL were spread plated onto tryptic soy agar (TSA; Difco, Becton Dickinson, USA) supplemented with 0.05 g/L $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ to induce the sporulation. The plates were incubated aerobically for 14 days at 37°C for sporulation. The level of sporulation was monitored using a phase contrast microscope (Motic Microscope BA410 Series, Canada) by initially smearing, fixing and drying the bacteria on a glass slide. Then this slide was stained with 5% w/v malachite green solution and counterstained with 5% w/v safranin solution. Once sporulation level had reached at least 90%, the spores were collected by flooding the surface of the culture plates with 1–2 mL sterile SDW and by scraping it with a sterile bent glass rod. Spore pellets were obtained by centrifuging and washing three times with sterile phosphate buffer (pH 7.2) at 4000 g for 15 min and 4°C (Centrifuge Sigma 4K15, UK). The spores were resuspended in SDW, subjected to 60°C for 10 min to kill any remaining vegetative cells, and stored at 4°C until use. Although heat shock may germinate *B. cereus* spores (Collado et al., 2006), microscopic observation revealed no spore germination, which was confirmed by the thermal resistance values obtained in this study. Byrne et al. (2006) registered a huge difference in *B. cereus* vegetative cell and spore thermal resistance: while

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50°C for vegetative cells gave a *D*-value of ~33 min, 85°C was required to obtain the same *D*-value with the spore for the microorganism.

6.2.2.3 Food inoculation

For TS experiments, a portion (ca. 1 mL) of the spore solution was inoculated in approximately 99 mL of milk or 99 g of food slurries/porridge to yield an initial food spore concentration of $\sim 10^7$ cfu/mL of milk or cfu/g of slurry or porridge. Regarding thermal experiments, aliquots (0.5 mL) of the spore suspension were inoculated into 3 mL milk or 3 g of slurry/porridge to obtain approximately the same initial concentration of spores.

6.2.2.4 Spore enumeration

The *B. cereus* spore concentration in foods before and after processing was determined by spread plating into nutrient agar (NA) supplemented with 0.1% soluble starch. Milk samples (1 mL) were decimal diluted with 9 mL of 0.1% (w/v) sterile buffered peptone water (BPW; Difco, Becton Dickinson, USA). Each tube dilution was mixed repeatedly using a high speed vortex mixer to yield a uniform spore suspension, and plated twice. The NA plates were then inverted and incubated at 37°C for 24–48 h until visible colonies were formed. Plates with 10 to 100 colonies were used for enumeration, and average colony counts (\pm standard deviation) were calculated.

With respect to beef and cheese slurries, and rice porridge, samples were placed in 110 × 230 mm sterilized stomacher bags (Interscience, France) and decimal diluted ten times with 0.1% (w/v) BPW (USFDA, 2012b). These dilutions were considered in the calculation of the final spore concentration. Samples were then homogenized in the stomacher (Masticator Stomacher, IUL Instruments, Germany) for 2 min prior to plating onto NA plates. Spore

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concentration was expressed in colony forming units per milliliter or per gram (cfu/mL or cfu/g) of food sample.

6.2.3 Experimental design and data analysis

In the first experiment, the effectiveness of *B. cereus* spore reduction under TS vs. thermal processing at 70°C for 1.5 min was investigated in skim milk, beef slurry, cheese slurry, and rice porridge as this is the maximum temperature that can be used with the ultrasound probe. It has also been successfully employed for the inactivation of *B. subtilis* spores in whole milk and glycerol (Garcia et al., 1989). The short processing time of 1.5 min was selected to be close to potential commercial reality. Each treatment was carried out twice. The *B. cereus* spore logarithmic reduction ($\log N_0/N$) after thermosonic and thermal treatments was calculated and plotted for each food. Significant differences in the microbial log reductions among the eight treatments (4 foods thermosonication, 4 foods thermal) were investigated by performing a one-way analysis of variance (ANOVA) followed by Tukey's test, with a confidence level of 95% ($p < 0.05$) (Statistica 8, Statsoft Inc., USA).

Results from the first experiment showed a very different inactivation between high solid content foods (beef and cheese slurries and rice porridge) and milk, especially for TS. Therefore, beef slurry was selected to represent the solid foods in the second set of experiments, which involved several *B. cereus* survival experiments in beef slurry and skim milk for TS and thermal inactivation kinetic modeling. *B. cereus* spore inactivation by TS was determined at three temperatures (50, 60, and 70°C), since ~70°C was the maximum temperature supported by the ultrasound probe. Regarding thermal inactivation experiments, 70, 80, 90, and 100°C were selected based on preliminary experiments with this strain. Sole thermal inactivation of spores in skim milk and beef slurry was determined at four

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temperatures and compared with thermosonication. The logarithmic number of survivors ($\log N/N_0$) versus time was plotted for each survival experiment to estimate the first-order kinetic D_T -value. Two samples were processed for each time and two survival experiments were carried out for each temperature. Then, the average D_T -value \pm standard deviation (SD) was calculated for each temperature. The kinetic parameters of non-linear models were estimated for each temperature by fitting the models to all data points at that temperature (Statistica 8, Statsoft Inc., USA).

6.2.4 Thermosonication inactivation of *B. cereus* spores

6.2.4.1 Ultrasonic processor

Unless stated, equipment used for the induction of ultrasonic waves in this study was the same as described above (Chapter 3). All the inactivation experiments were done using a standard sonotrode of a 3 mm tip-diameter coupled to a Hielscher UP200S unit at 100% amplitude (210 μm , 460 W/cm^2 , 0.33 W/mL or W/g) and operating in pulse mode (acoustic power discharged 0.5 s, no power discharged 0.5 s). Therefore the processing time is actually double the real treatment time measured when the ultrasound power is being delivered to the sample. The sonotrode coupled to the ultrasonic processor via the horn amplified the vibrations and transferred them to the medium to be sonicated. As mentioned, the maximum temperature supported by the sonotrode was $\sim 70^\circ\text{C}$.

6.2.4.2 Thermosonication experiments

In general, the procedure was the same to the previously described in Chapter 3. Briefly for each temperature, a round bottom-flask containing the food sample (100 mL or 100 g) was placed in a thermostatic water bath to bring the sample temperature to the designated

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temperature. Inoculation of spores was carried out aseptically when the sample reached the designated temperature, by adding a small volume inoculum (ca. 1% v/v or v/m) as described in Section 6.2.2.3. The inoculated food was initially mixed with a sterile rod, and then the ultrasound was turned on, which further helped to uniformly mix the spores with the food (Hielscher-Ultrasonic, 2014). Ultrasonic treatments were carried out for up to 20 min, depending on the thermosonication temperature. Food samples were removed from the water bath at pre-specified intervals, and spore survivor counts were immediately performed.

6.2.5 Thermal inactivation of *B. cereus* spores

The inoculated food samples were vacuum packed using food grade sterile pouches (8 × 8 cm, Cas-Pak, New Zealand), which were then thermosealed (Multivac C200, Germany). The pouches were placed in the stomacher (Masticator Stomacher, IUL Instruments, Germany) for 2 min to ensure good spore mixing with the food and uniform distribution. The pouches were then compressed into a very thin layer (approximately 1–2 mm thick). Thus the large surface area of the bags (8×8 cm) compared to the small volume of the food (3 g slurry/porridge or 3 mL of milk) allowed a very quick heat transfer. For the first experiment comparing thermosonication with thermal at 70°C in the four foods, the packed samples were submerged in a 70°C water bath for 1.5 min.

Regarding the thermal inactivation experiment for the kinetic modeling, the packed food samples were processed for up to 20 min (depending on the treatment temperature) in a thermostatic water bath with the temperature set to the processing temperature (70, 80, 90 and 100°C). Treated samples were taken out at different time intervals and kept in an ice water bath until microbial enumeration.

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6.2.6 Modeling the kinetics of *B. cereus* spores inactivation in skim milk and beef slurry

Based on the aspect of the survival curves (log microbial numbers vs. time), first-order kinetic (Equation 1-1) and log-logistic (Equation 1-8) equations were fit to the spore survival data. Then, the kinetic parameters were estimated. Statistica 8.0 software (Statsoft Inc., USA) was used to fit several models to the spore survival lines, and to perform statistical analysis of data. Residual plots, mean square error (MSE), coefficient of determination (R^2), accuracy factor (A_f), and the temperature dependence of the parameters estimated were used to compare the performance of the above models (Chapter 1).

6.3 Results and discussion

6.3.1 Thermosonication versus thermal processing to inactivate *B. cereus* spores in different foods

The effect of thermosonication (TS, 24 kHz, 210 μm , 0.33 W/mL or W/g) and thermal treatments of skim milk and slurries/porridge at 70°C for 1.5 min on *B. cereus* spores in different foods is illustrated in **Figure 6-1**. TS was better than thermal processing (1.5 min) in the inactivation of *B. cereus* spores in beef slurry (4.2 vs. 0.7 log reductions), rice porridge (4.1 vs 0.6 log reductions), and cheese slurry (3.2 vs 0.8 log reductions) ($p < 0.05$). The hydrophobic character of *Bacillus spp.* spore surfaces (Peng et al., 2001) could potentially attract the cavitation bubbles generated by the ultrasonic waves (Gao et al., 2014), and enhance their damage by heat and shear stress. Regarding skim milk, less than 0.5 log reductions were observed for both TS and thermal treatments. A longer TS time was needed to achieve higher log reductions (see **Figure 6-2a**, e.g. 10 min — 3.5 log). Based on the

6. Thermosonication vs thermal processing psychrotrophic *B. cereus* spores

determined food composition, the higher sugar content of the milk (4.7%, lactose), compared with the other foods tested ($\leq 0.3\%$), might have caused such a difference in the log reduction result (Table 6.1). The sonoprotective effect of milk lactose on the spore reductions had been previously observed by Gera and Doores (2011). In addition, skim milk presents a lower solid content (or high moisture content) than the slurries (Table 6-1). Past studies have shown an increased effect of the ultrasonic waves for higher total solid concentration (Sala et al., 1995; Santamaria & Castellani, 1952). Lower reductions in the cheese slurry than those in beef slurry and rice porridge could be due to the protective effect of fat in this media (Senhaji & Loncin, 1977).

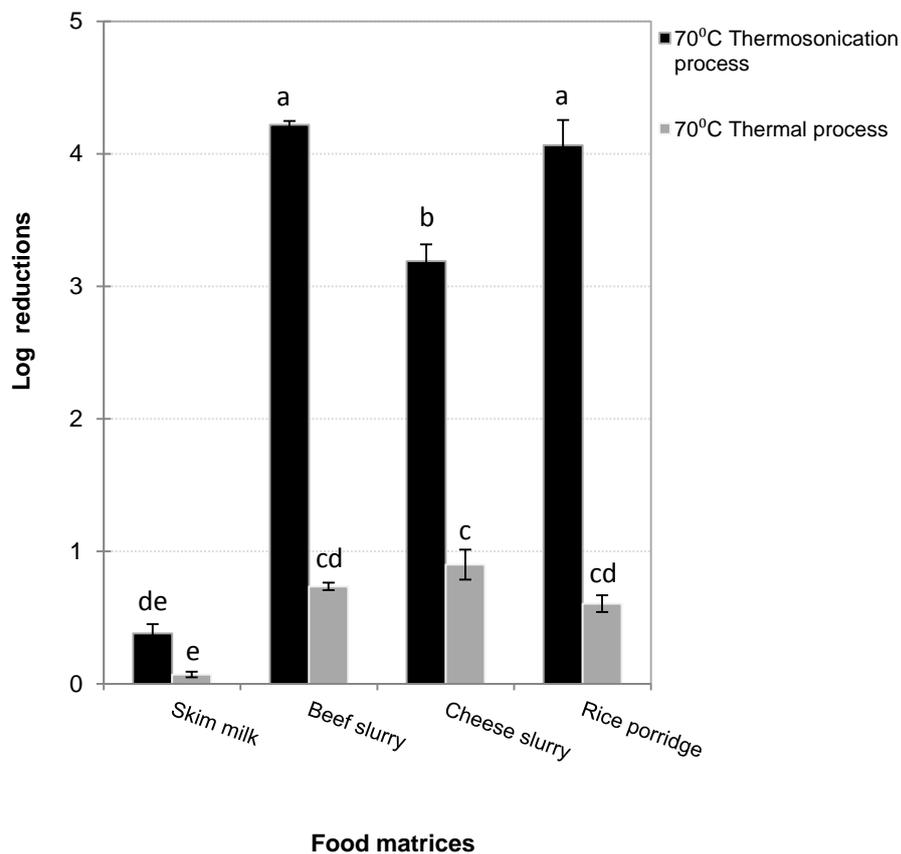


Figure 6-1 Psychrotrophic *B. cereus* spore log reductions in different foods after thermosonication (24 kHz, 210 μm , 0.33 W/mL or W/g) and thermal processing, both at 70°C and for 1.5 min (error bars are standard deviation; treatments/foods with different letters are significantly different).

6. Thermosonication vs thermal processing psychrotrophic *B. cereus* spores

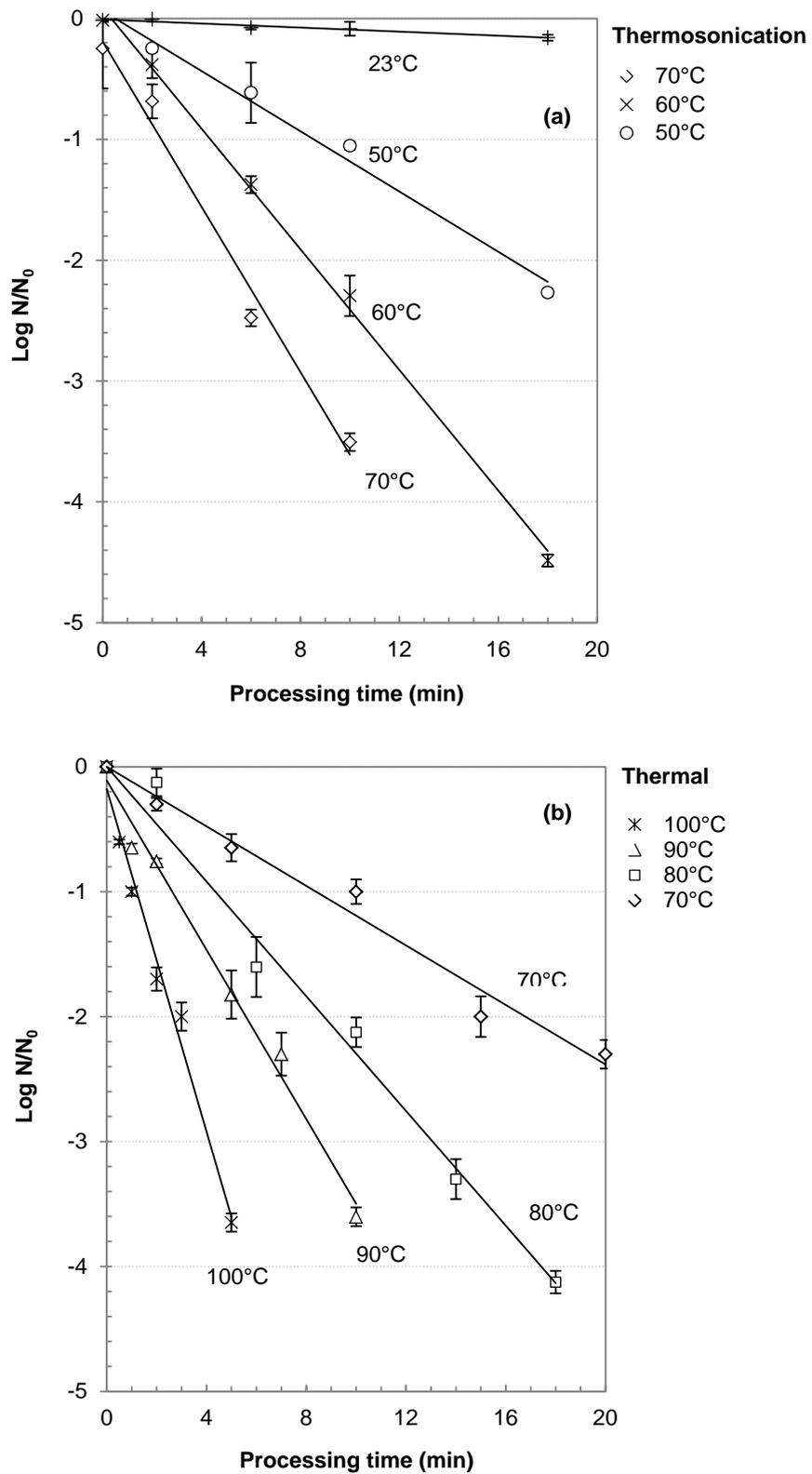


Figure 6-2 Log survivors of psychrotrophic *B. cereus* spores in skim milk: the first order model fitted to direct contact thermosonication (TS, 24 kHz, 210 μ m, 0.33 W/g, pulse mode) (a) and thermal inactivation (b) (data points are average \pm standard deviation).

6. Thermosonication vs thermal processing psychrotrophic *B. cereus* spores

6.3.2 Modeling the thermosonication and thermal inactivation kinetics in skim milk and beef slurry

The log survivors of *B. cereus* spores under TS and thermal processing in skim milk and beef slurry are shown in **Figure 6-2** and **6-3**. As expected, the higher the temperature, the higher the spore inactivation rate.

6.3.2.1 Model performance

Initially, the first-order was attempted to model the spore survivors in beef and milk after the thermosonication and thermal processes as shown in **Figure 6-2** and **6-3**. The performance of the adjustments is also presented in **Table 6-2**. The linearity observed in the TS and thermal plots for milk and thermal inactivation in beef slurry was confirmed by the random residuals, low MSE value (0.01–0.09), high R^2 (0.980–0.998), A_f close to 1 (1.07–1.30), and also good fit for the effect temperature on D -values. The non-linear models were inappropriate for milk (TS and thermal) and beef thermal inactivation and presented higher A_f values and poor fit for the effect of temperature on the parameter estimated (results not shown). Thus, the first-order kinetic parameters were estimated for both skim milk and beef slurry, and discussed in the following section. Linearity was also observed for different *B. cereus* spore thermal inactivation by Luu-Thi et al. (2014).

6. Thermosonication vs thermal processing psychrotrophic *B. cereus* spores

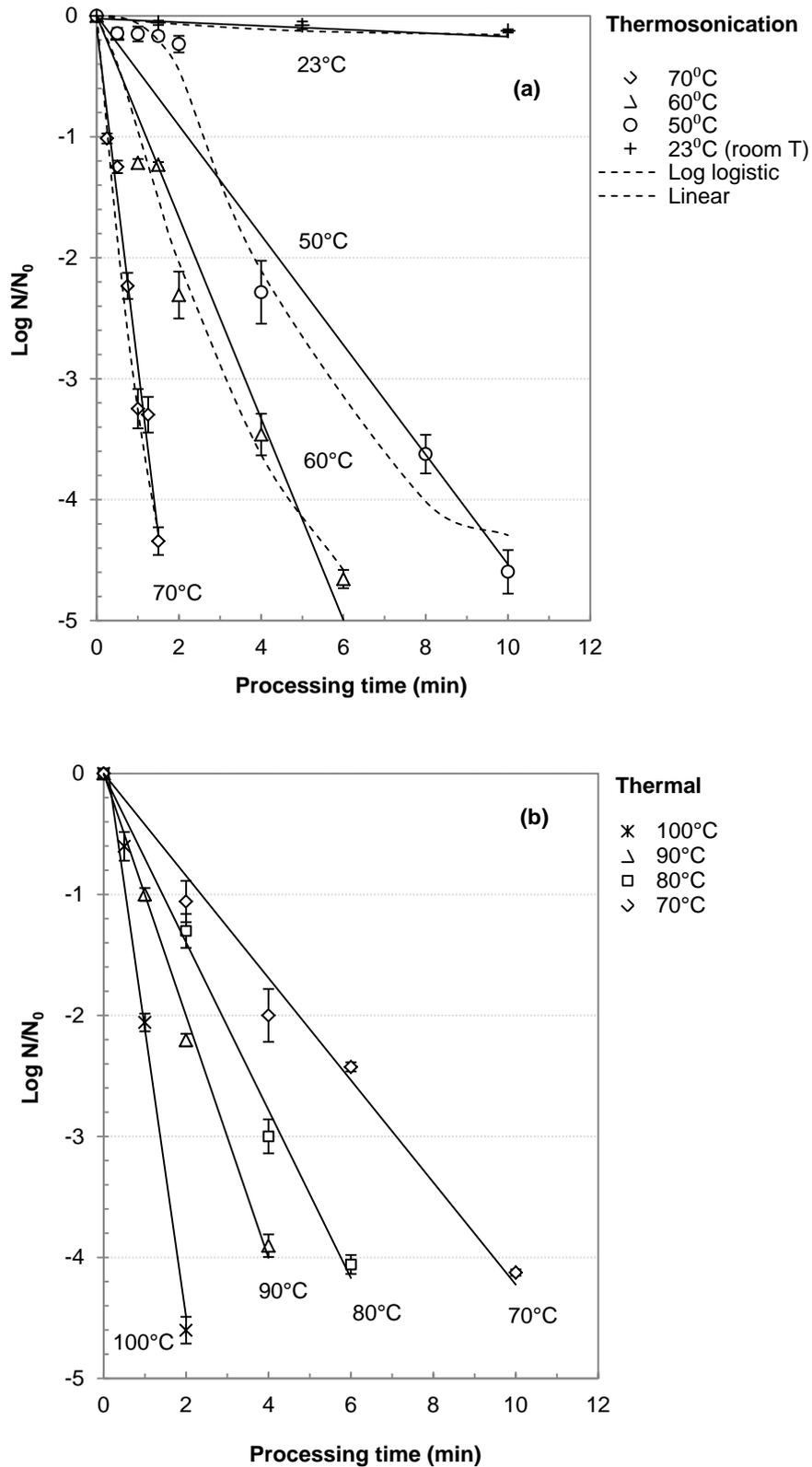


Figure 6-3 Log survivors of psychrotrophic *B. cereus* spores in beef slurry: the first order and log-logistic models fitted to the direct contact thermosonication inactivation (TS, 24 kHz, 210 μm , 0.33 W/g, pulse mode) (a), and the first order model fitted to the spore thermal inactivation (data are average \pm standard deviation).

6. Thermosonication vs thermal processing psychrotrophic *B. cereus* spores

Table 6-2 The performance of the first-order and non-linear models for the survival of *Bacillus cereus* spores after thermosonication (24 kHz, 210 μ m, 0.33 W/mL or W/g, pulse mode) and thermal processing in skim milk and beef slurry.*

Process	Food and model	MSE	R^2	A_f	Residual plot	Remarks
Thermal	<i>Skim milk:</i>					
	First-order	0.02–0.03	0.980–0.994	1.18–1.25	Random	High R^2 , low MSE and A_f Good fit for temperature effect on D - values
TS	First-order	0.01–0.03	0.980–0.996	1.07–1.15	Random	High R^2 , low MSE and A_f Good fit for temperature effect on D - values
Thermal	<i>Beef slurry:</i>					
	First-order	0.03–0.09	0.983–0.998	1.16–1.30	Random	High R^2 , low MSE and A_f Good fit for temperature effect on D - values
TS	First-order	0.06–0.11	0.960–0.980	1.12–1.74	Random	Poor fit, higher MSE and A_f , lower R^2
	Weibull	0.02–0.13	0.977–0.998	1.04–1.59	Random	Higher MSE and A_f
	Gompertz	0.02–0.13	0.977–0.998	1.05–1.52	Random	Higher MSE and A_f
	Log-logistic	0.02–0.07	0.990–0.998	1.04–1.17	Random	Higher R^2 , lower MSE and A_f Good fit for temperature effect on τ - values

*Low mean square error (MSE), R^2 and A_f close to 1, and random residuals are an indication of good fit. For TS, temperatures of 23, 50, 60, and 70 °C were used and thermal inactivation was carried out at 70, 80, 90 and 100 °C.

With respect to TS in beef slurry, shoulders and tails were observed in the survival curves of the spores, especially at 50°C (**Figure 6-3a**). Interaction between ultrasound, microstructure, and physical properties of beef slurry at this lower temperature, might be attributed to the more pronounced non-linearity observed. Although the first-order model seems to work for 70°C (the higher temperature tested) and 23°C (the lower temperature tested), overall it

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showed poorer fit with lower R^2 (0.96–0.98) and higher A_f (1.12–1.74). Therefore, the Weibull, modified Gompertz, and log-logistic non-linear models were attempted. Log-logistic showed the best fitting (**Figure 6-3a, Table 6-2**) with $MSE \leq 0.07$, $R^2 \geq 0.99$ and A_f ranging between 1.04 and 1.17. The parameter τ estimated for each temperature, also showed a good temperature dependence and results are presented in Section 6.3.2.3. Survival curves displaying shoulders may relate to a combination of activation and inactivation in the beginning of the treatment which become more negligible as the temperature increases (Russell, 1971). These behaviors often occur with vegetative cells but not with spores. For example, Joyce et al. (2003) observed non-linearity with vegetative cells of *B. subtilis* species that were treated by ultrasound. According to Feng and Yang (2011b), a gradual change in the properties of medium during sonication may also cause non-linearity of the survival plots. It was concluded that the kinetic model of *B. cereus* spore inactivation by thermosonication could depend not only on the food product, but also on the treatment temperature (**Figure 6-2 and Figure 6-3**) because in beef the shoulders and tails were only observed at 50 and 60°C. This confirms Daryaei et al. (2013) and Feng and Yang's (2011b) findings.

6.3.2.2 *First-order kinetic parameters (D- and z-values) for thermosonication vs. sole thermal treatments in skim milk and beef slurry*

The estimated first-order kinetic parameters for TS and sole thermal treatment in skim milk and beef slurry are shown in **Table 6-3**. The TS and thermal D -values for milk were ≥ 3 fold higher than the values obtained in beef at the same temperature. Higher D -values for the spores in milk than those in the beef slurry could be due to the higher concentration of sugar in milk (Silva et al., 1999; Stumbo, 1965). As was mentioned, simultaneous ultrasonic and thermal effects were higher in the high solid content foods such as beef slurry (24% w/w)

6. Thermosonication vs thermal processing psychrotrophic *B. cereus* spores

compared with milk (9% w/w) (**Table 6-1**). Ultrasound propagation speed (=velocity) in solid medium is higher than that in liquid medium (Awad et al., 2012) due to reduced compressibility in the solid medium. A combination of longitudinal wave (particle motion is parallel to the motion of wave) and shear waves (particle motion is perpendicular to the motion of wave) can occur in the solid medium (Ortega-Rivas, 2012). This could result in more shear stress which induces a greater sensitivity to the heat action compared to only the longitudinal wave that travels through the liquid medium.

Table 6-3 D_T - and z -values for *Bacillus cereus* spores in skim milk and beef slurry for thermosonication (24 kHz, 210 μ m, 0.33 W/mL or W/g, pulse mode) and sole thermal processing.*

T (°C)	Skim milk		Beef slurry	
	D_T -value \pm SD (min)		D_T -value \pm SD (min)	
	Thermosonication	Thermal	Thermosonication	Thermal
100	nd	1.45 \pm 0.04	nd	0.42 \pm 0.04
90	nd	3.15 \pm 0.35	nd	1.03 \pm 0.10
80	nd	4.28 \pm 0.02	nd	1.39 \pm 0.03
70	2.93 \pm 0.03	8.64 \pm 0.30	0.36 \pm 0.001	2.32 \pm 0.02
60	4.00 \pm 0.04	nd	1.27 \pm 0.02	nd
50	8.05 \pm 0.11	nd	2.09 \pm 0.10	nd
23 (Room T)	119.3 \pm 4.38	nd	140.9 \pm 3.25	nd
z -value \pm SE (°C)	45.7 \pm 0.07 $R^2=0.96$	40.7 \pm 0.06 $R^2=0.98$	25.8 \pm 0.14 $R^2=0.94$	42.4 \pm 0.08 $R^2=0.96$

**B. cereus* strain NZRM 984 (=NCTC 10320, ATCC 11778, DSM 345); nd-not determined; D -values are means \pm standard deviation from 2 survival experiments (Equation 1-1); for ultrasound processing, room temperature was not used to estimate the z -value (Equation 1-2).

6. Thermosonication vs thermal processing psychrotrophic *B. cereus* spores

In order to compare TS with thermal results, 70°C was used, the only temperature tested for both TS and thermal. The $D_{70^{\circ}\text{C}}$ -values for skim milk were 2.93 min for TS and 8.64 min for thermal. Similar D -values (≈ 3 min) were obtained for the 90°C thermal process and 70°C TS process, indicating the benefit of ultrasound. Likewise the thermal 80°C D -value (4.28 min) was similar to the TS D -value at 60°C (4.00 min), thus demonstrating that ultrasound required 20°C less temperature to obtain the same rates of inactivation of an exclusively thermal process (**Table 6-3**). For the spore inactivation in beef slurry (**Figure 6-3a** and **6-3b**), the $D_{70^{\circ}\text{C}}$ -value for TS (0.36 min) was more than 6 fold lower than thermal (2.32 min) (**Table 6-3**), indicating a remarkable advantage of using the ultrasound technology. In fact, similar D -values were obtained for TS at temperatures of 30°C lower than those employed for the simple thermal process. For example, the $D_{70^{\circ}\text{C}}$ -value for TS was 0.36 min compared to the $D_{100^{\circ}\text{C}}$ -value for the thermal of 0.42 min. Similarly, the TS value at 60°C (1.27 min) was close to the thermal value at 90°C (1.03 min). The lower processing temperature required for TS represents huge energy and food quality gains. Garcia et al. (1989) worked with temperatures between 70 and 95°C and also reported lower D_{T} -values for TS compared to thermal processing for *B. subtilis* spore inactivation in whole milk. Advantages of thermosonication (lower process temperatures and processing times to achieve the same lethality values, with less impact in the food quality) compared to thermal processes for food preservation have also been reported by Mason et al. (1996) and Villamiel et al. (1999).

The *B. cereus* spore thermal D -values obtained ($D_{90^{\circ}\text{C}}$ -value = 3.15 min and $D_{100^{\circ}\text{C}}$ -value = 1.45 min in skim milk) were similar to Bassen et al. (1989) in custard mostly containing skim milk ($D_{90^{\circ}\text{C}}$ -value = 3.6 min), Lekogo et al. (2010) in broth ($D_{100^{\circ}\text{C}}$ -value = 1.7 min) and González et al. (1999) in buffer ($D_{100^{\circ}\text{C}}$ -value-value = 2 min). Shehata and Collins (1973) and Desai and Varadaraj (2010) reported a $D_{90^{\circ}\text{C}}$ -value of 5.8 min and 6.2 to 12.5 min in skim

6. Thermosonication vs thermal processing psychrotrophic *B. cereus* spores

milk, respectively. Byrne et al. (2006) determined a $D_{90^{\circ}\text{C}}$ -value of 10 min in pork roll as opposed to 1 min in beef slurry obtained in our study.

With respect to z -values, 45.7°C and 40.7°C for TS and thermal processing of milk were obtained, respectively. Regarding inactivation in beef slurry, z -values of 25.8°C for TS and 42.4°C for thermal inactivation were estimated (**Table 6-3**). The z -values obtained in this study were very high, indicating low microbial susceptibility to temperature change. Other authors have also reported high z -values (ranging between 20.4 and 36.5°C) for thermal inactivation of spores of other strains of psychrotrophic *B. cereus* (ATCC 9818, CFR 1521, CFR 1532, CFR 1534) in distilled water, brain–heart infusion broth, and skim milk (Desai & Varadaraj, 2010; Novak et al., 2005). Thermal z -values ranging from 22.3 to 29.2°C have been reported with the same *B. cereus* strain in pumpkin soup (Wimalaratne, 2009). Garcia et al. (1989) also obtained similar z -values in the *B. subtilis* spore TS and thermal destruction in milk. Once again the reason for the relatively lower and better z -value obtained in our work beef TS could be the higher solid content interference with the ultrasound waves. To conclude, changes in the properties of suspending medium during the heating and acoustic cavitation process may be attributed to the cavitation intensity produced (Mason et al., 1996), and hence the z -values obtained.

6.3.2.3 Log-logistic kinetic parameter estimation for thermosonication inactivation in beef slurry

The estimated log-logistic parameters for TS inactivation in beef slurry are shown in **Table 6-4** and the model fitting is shown in **Figure 6-3a**. The τ -values (the log time to achieve the maximum inactivation rate) decreased from 1.45 for room temperature ultrasound to 0.23 for TS at 70°C . This result is consistent with Log-logistic models used in other studies, in which

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the lethal effect of temperature reduced the τ -values (Chen & Hoover, 2003; Daryaei & Balasubramaniam, 2013). Additionally, there was no substantial effect of temperature on σ -values (Chen & Hoover, 2003). There has been little to no research carried out on kinetic modeling of bacterial spores by thermosonication and therefore it was difficult to find other results to compare. However, the Log-logistic model proved to be very useful in fitting survival curves of *Bacillus* spores with upward or downward concavities for many non-thermal technologies such as pulsed electric field and high pressure processing (Daryaei & Balasubramaniam, 2013; Pina-Pérez et al., 2009).

Table 6-4 The log-logistic (Equation 6-3) parameters estimation of *Bacillus cereus* spore inactivation by thermosonication (24 kHz, 210 μm , 0.33 W/g, pulse mode) and room temperature ultrasound in beef slurry.

Temperature (°C)	Log-logistic		
	<i>A</i>	σ	<i>T</i>
70	-9.14±1.12	-5.28±0.27	0.23±0.03
60	-7.03±1.91	-5.60±0.59	0.58±0.19
50	-4.64±0.37	-7.88±1.13	0.63±0.04
23	-0.35±0.61	-0.11±0.11	1.45±2.31

6. Thermosonication vs thermal processing psychrotrophic *B. cereus* spores

6.4 Conclusion

The current study demonstrated that thermosonication (simultaneous ultrasound and thermal processing, TS) inactivated psychrotrophic *B. cereus* spores in skim milk and beef slurry, and was much more effective at reducing the numbers of spores than thermal processing alone, at the same temperature. For a short process of 1.5 min at 70°C, TS could effectively inactivate the psychrotrophic *B. cereus* spores in the beef slurry to achieve a 4.2 log reduction. However, more than 10 min were needed to obtain the same result in skim milk. With the exception of beef slurry TS at 50 and 60°C, the first-order kinetic model described well the TS and thermal inactivation kinetics in skim milk and beef slurry. This technology might provide a technological alternative to the traditional thermal pasteurization/sterilization of liquid foods, in order to obtain higher quality food due to the mild heating process conditions employed. Ultrasound also enables savings on the process energy required, especially if operated in pulse mode as occurred in this research.

Chapter 7 Efficacy of thermal, thermosonication and HPP-thermal for *Clostridium perfringens* and psychrotrophic *Bacillus cereus* spores inactivation

7. Efficiency of TS vs HPP-thermal for *C. perfringens* and *B. cereus* spore inactivation

7.1 Introduction

In Chapters 2 and 3, high pressure combined with thermal (HPP-thermal), thermosonication (TS) and thermal processing were used to inactivate *Clostridium perfringens* spores in beef slurry. In Chapter 4, 5 and 6, same technologies were used to inactivate psychrotrophic *Bacillus cereus* spores in reconstituted milk and beef slurry. In the current chapter, a comparison of the efficacy of the three technologies in terms of *C. perfringens* and *B. cereus* spores inactivation was carried out.

7.2 *Clostridium perfringens* spore inactivation

Figure 7-1 show replots of the effects of 600 MPa HPP-thermal, TS, and thermal processing at 75°C on *C. perfringens* NZRM 898 spores for up to 60 min. The 600 MPa HPP-thermal was the best technique for the spore inactivation, since the spores reduced much faster with the processing time, allowing 2.2 log after 20 min. On the contrary, a slower inactivation rate of *C. perfringens* spores was observed for the 75°C TS, only reaching nearly 1 log after the same processing time. Regarding the thermal alone, there was almost negligible effect on spores with approximately 0.5 log reductions after 60 min process. With respect to *C. perfringens* NZRM 2621 strain, the 600 MPa HPP-thermal was also better than TS for spore inactivation (the thermal inactivation of spores was not carried out) (**Figure 7-2**). For a 20 min process, the spores decreased almost linearly with the HPP processing time allowing 1.8 log reductions. TS inactivation was non-linear with only 0.9 log reductions after 20 min and 1 log reductions after 60 min, being much less effective than HPP-thermal at the same temperature.

7. Efficiency of TS vs HPP-thermal for *C. perfringens* and *B. cereus* spore inactivation

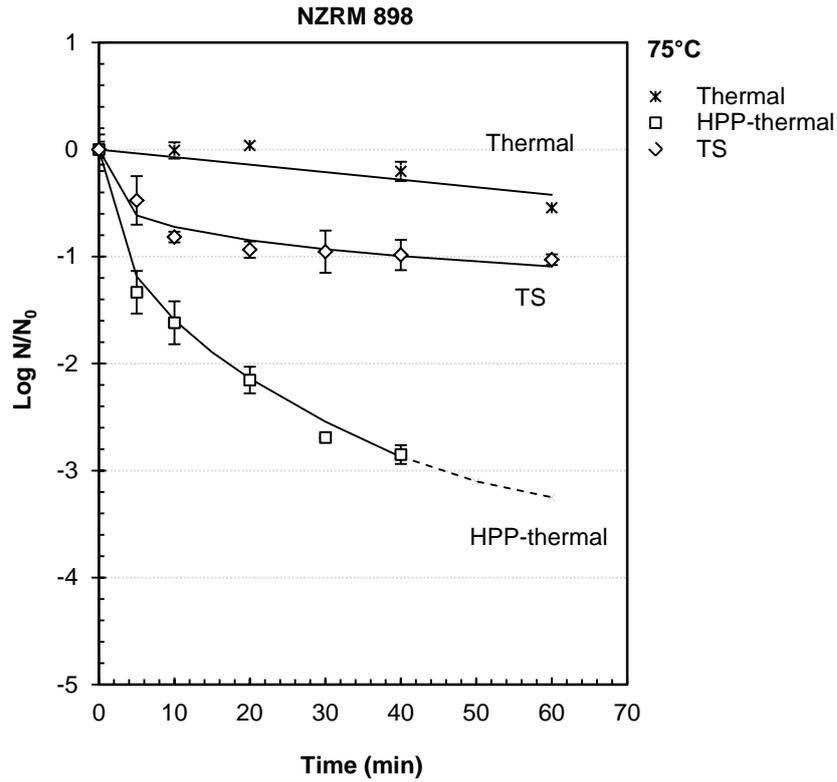


Figure 7-1 600 MPa HPP-thermal, thermosonication (24 kHz, 0.33 W/ml), and thermal inactivation of *Clostridium perfringens* NZRM 898 spores in beef slurry at 75°C.

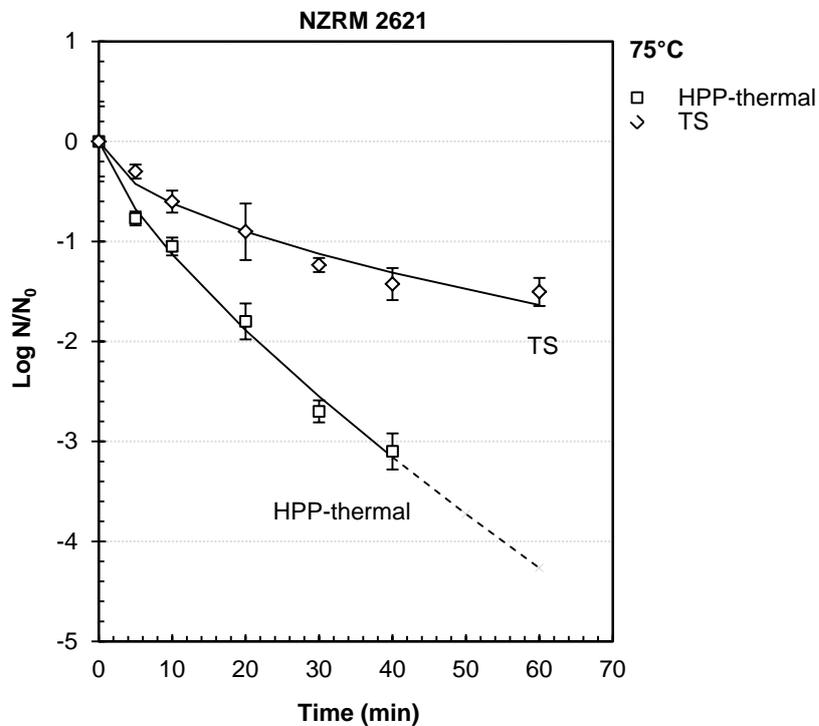


Figure 7-2 600 MPa HPP-thermal and thermosonication (24 kHz, 0.33 W/ml) inactivation of *Clostridium perfringens* NZRM 2621 spores in beef slurry at 75°C.

7. Efficiency of TS vs HPP-thermal for *C. perfringens* and *B. cereus* spore inactivation

Overall, for a 20 min process at 75°C, an average reduction of 2 log in the *C. perfringens* spores was obtained for HPP-thermal vs. 1 log reductions for TS, and no inactivation for thermal. The higher spore inactivation by HPP-thermal could be due to spore germination triggered by the pressure followed by subsequent death of germinated spores which became more susceptible to the pressure-heat treatment).

7.3 *Bacillus cereus* spore inactivation

Figure 7-3 to **7-6** present replots the effects of 600 MPa HPP-thermal, TS, and thermal processing at 70°C on two strains of psychrotrophic *B. cereus* spores. The results demonstrated that the combination of high pressure or ultrasound with heat spores was more efficient than thermal processing alone for inactivating *B.cereus* spores. However, among the two non-thermal methods, results from **Figure 7-3** suggested TS was a better method than HPP-thermal, which was in contrast to the previous results obtained for *C. perfringens* spores. For NZRM 984 spores in milk, the only strain submitted to the three technologies, the sensitivity of *B. cereus* spores to TS can be seen from the lowest $D_{70^{\circ}\text{C}}$ -value obtained (2.93 min) translated in the highest spore reduction after 15 min (>5 log) (**Figure 7-3**). On the contrary, HPP-thermal and thermal showed ~5 log reduction only after 40 min processing. The D -value of 8.64 min was obtained for the 70°C thermal. The better spore inactivation by TS vs. thermal was also shown by results in beef slurry with NZRM 984 strain (**Figure 7-5**), in which the $D_{70^{\circ}\text{C}}$ -value of 0.36 min and >5 log after 2 min were obtained for TS and D -value of 2.32 min and only 1 log after 2 min for thermal. Much faster spore inactivation was obtained under 600 MPa HPP-70°C than 70°C thermal in milk and beef slurry with ICMP 12442 spores (**Figure 7-4** and **Figure 7-6**). For example after 10 min, the 600 MPa HPP-

7. Efficiency of TS vs HPP-thermal for *C. perfringens* and *B. cereus* spore inactivation

70°C produced an average 3.7 log reduction in the psychrotrophic spores compared to only 0.2 log for the thermal.

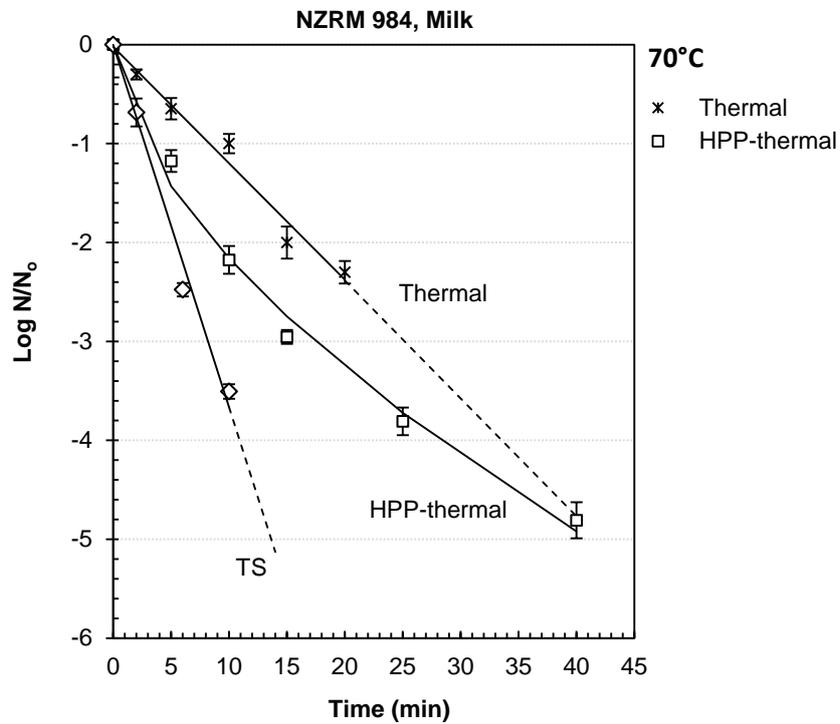


Figure 7-3 Thermosonication (24 kHz, 0.33 W/ml), 600 MPa HPP-thermal, and thermal inactivation of psychrotrophic *Bacillus cereus* NZRM 984 spores in milk at 70°C.

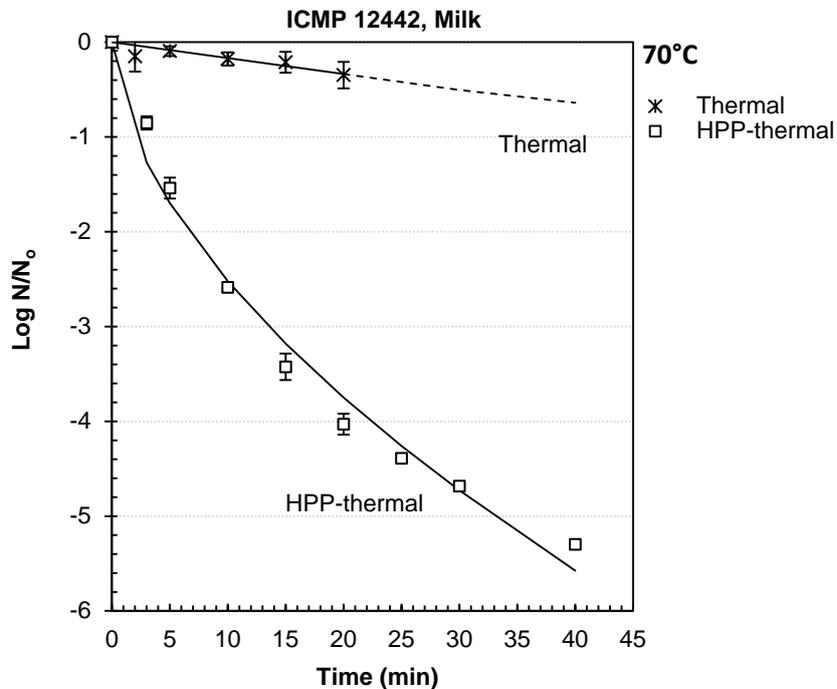


Figure 7-4 600 MPa HPP-thermal and thermal and inactivation of psychrotrophic *Bacillus cereus* ICMP 12442 spores in milk at 70°C.

7. Efficiency of TS vs HPP-thermal for *C. perfringens* and *B. cereus* spore inactivation

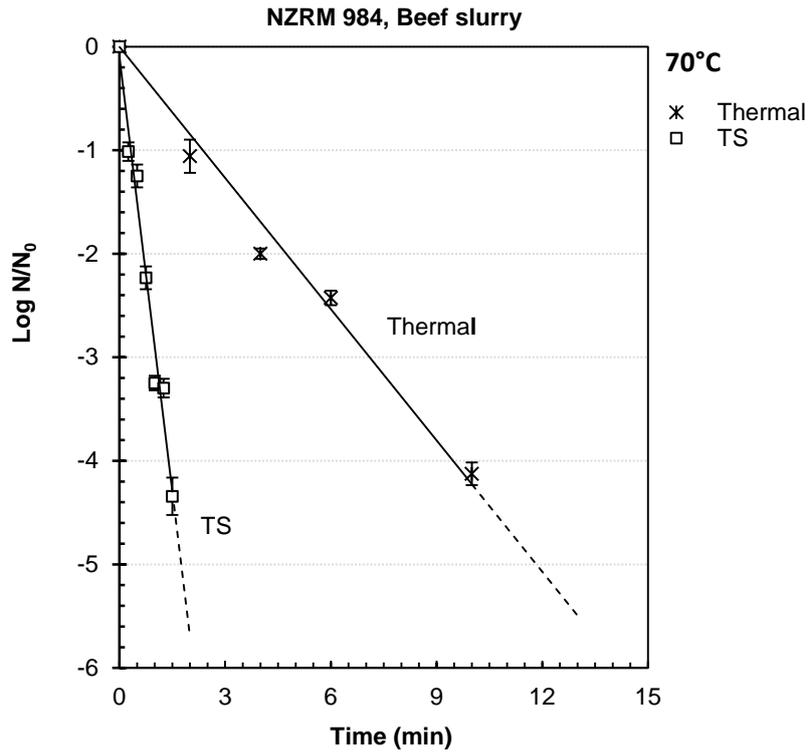


Figure 7-5 Thermosonication (24 kHz, 0.33 W/ml) and thermal inactivation of psychrotrophic *Bacillus cereus* NZRM 984 spores in beef slurry at 70°C.

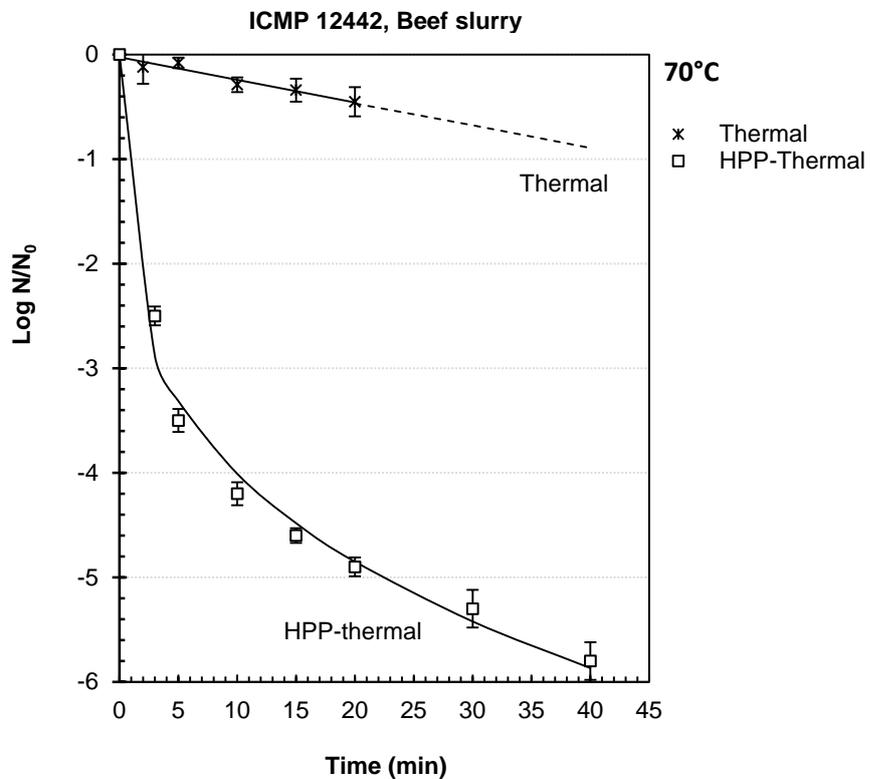


Figure 7-6 600 MPa HPP-thermal and thermal inactivation of psychrotrophic *Bacillus cereus* NZRM ICMP 12442 spores in beef slurry at 70°C.

Chapter 8 High pressure processing pretreatment enhanced the thermosonication inactivation of *Alicyclobacillus acidoterrestris* spores in orange juice

Evelyn, & Silva, F. V. M. (2016). High pressure processing pretreatment enhanced the thermosonication inactivation of *Alicyclobacillus acidoterrestris* spores in orange juice. *Food Control*, 62, 365-372.

8. Thermosonication vs thermal processing *Alicyclobacillus acidoterrestris* spores

Chapter abstract

The spoilage of high acid fruit juices and nectars by *Alicyclobacillus acidoterrestris* is a major concern to juice manufacturers around the world since it is difficult to detect. In this study, thermosonication (ultrasound and heat, TS) and thermal inactivation of *A. acidoterrestris* spores in pretreated orange juice were carried out and resistance parameters were estimated. First, the effect of TS acoustic energy density (AED, 0.3-20.2 W/mL) on the inactivation at 75°C was investigated. Then, the influence of TS temperature (70-78°C) on the spore inactivation (AED, 0.3-20.2 W/mL) was studied. Next, we explored the effect of high pressure processing (HPP) pretreatment of juice on the 20.2 W/mL TS inactivation at the best temperature (78°C). Lastly, the thermal inactivation of spores in juice heat shocked + 1 min sonicated vs. untreated juice was also investigated.

Results of TS showed higher spore inactivation for higher AED ($D_{75^{\circ}\text{C}}$ -value of 49 min for 20.2 W/mL vs. 217 min for 0.33 W/mL). Lower D -values were obtained at higher temperatures ($D_{78^{\circ}\text{C}}$ -value of 28 min vs. $D_{70^{\circ}\text{C}}$ -value of 139 min at 20.2 W/mL). The TS $D_{78^{\circ}\text{C}}$ -value (at 20.2 W/mL) decreased further from 28 min to 14 min when the orange juice was previously submitted to 600 MPa for 15 min. Thermal treatment alone at 78°C resulted in almost no spore inactivation, whereas the heat shock + ultrasound pretreatment of juice enhanced the thermal inactivation of spores ($D_{85^{\circ}\text{C}}$ -value decreased from 69 to 29 min). To conclude, HPP-assisted TS provided the best method for spore inactivation, indicating the benefit of high pressure and power ultrasound technology in addition to heat. TS required at least 8°C lower temperatures than thermal treatments to achieve the same spore inactivation, which could enhance juice quality and energy savings.

8. Thermosonication vs thermal processing *Alicyclobacillus acidoterrestris* spores

8.1 Introduction

Alicyclobacillus acidoterrestris (AAT) is an aerobic, rod-shaped, gram-positive, endospore-forming bacterium which is able to grow at a pH range of 2.0–7.0 and a temperature range of 25–60°C. Optimal growth occurs at a pH of around 4.0–4.5, and a temperature around 40–45°C (Bevilacqua et al., 2008a). The spores of AAT survive the thermal pasteurization (generally between 80 and 100°C) employed by the fruit beverage industry, and exhibit very high heat resistance compared with major spoilage microbes of high-acid shelf-stable foods ($1.0 \text{ min} < D_{95^\circ\text{C}} < 5.3 \text{ min}$ and $6.0 \text{ min} < D_{90^\circ\text{C}} < 23.0 \text{ min}$) (Silva & Gibbs, 2001; Silva et al., 2014). AAT spore germination and growth up to a level of 10^5 – 10^6 cfu/mL can occur after pasteurization (cycle of up to 5 days) in high-acid fruit juices when the storage and distribution temperatures are around 40°C (Splittstoesser et al., 1994). Product spoilage is difficult to detect visually since AAT does not produce gas during growth. However, juice/beverage spoilage is evident by the off flavour, caused by guaiacol and other halophenols (ppb) (Gocmen et al., 2005). Therefore, AAT was suggested as reference microorganism for pasteurization processes in high-acid fruit products (Silva & Gibbs, 2001, 2004).

Large-scale AAT spore germination and spoilage was first reported in 1982 in aseptically packaged apple juice (Cerny et al., 1984). Since then, other incidents have been reported in USA, Europe and Japan (Jensen, 2000) and in different types of fruit products such as lemonade carbonated fruit juice drinks, shelf-stable ice tea containing berry juice, fruit pulps, and canned diced tomatoes (Duong & Jensen, 2000; Pettipher & Osmundson, 2000; Walls & Chuyate, 1998). Today, food and beverage spoilage by AAT spores has become an industrial issue.

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The effectiveness of heat alone or combined with antimicrobials for inactivating AAT has been investigated: nisin was added to fruit juices (Komitopoulou et al., 1999), chlorine dioxide was added to the surface of apples (Lee et al., 2004), grape polyphenols were added to grape juice (Oita & Kohyama, 2002), enterocin AS-48 was added to fruit juices (Grande et al., 2005), ascorbic acid was added to apple juice (Bahçeci & Acar, 2007), and eugenol and cinnamaldehyde were added to acidified malt extract broth (Bevilacqua et al., 2008b).

A number of different non-thermal technologies and their combination with heat have also been investigated for microbial spore inactivation in juices, fruit products and other foods (Evelyn et al., 2016). These include high hydrostatic pressure combined with heat or HPP-thermal (Evelyn & Silva, 2015d; Lee et al., 2002; Shearer et al., 2000; Silva et al., 2012; Sokołowska et al., 2012), high pressure carbon dioxide (Bae et al., 2009; Casas et al., 2012), and radiation (Nakauma et al., 2004). Power ultrasound is another non-thermal method that has been studied for microbial spore (Evelyn & Silva, 2015a, 2015b) and enzyme inactivation (Sulaiman et al., 2015). Ultrasonic waves at sufficient intensity can cause microbial cell death by a phenomenon called cavitation (Chen, 2012). The microgas bubbles are formed during the rarefaction cycle of the acoustic wave within a liquid, collapse violently during the compression cycle (Leong et al., 2011), and create micro-mechanical shocks leading to disruption of cellular components and hence cell lysis (Guerrero et al., 2001). Lower decimal reduction values (*D*-values) of bacterial and mould spores were registered after simultaneous use of ultrasound and heat (thermosonication [TS]) and ultrasound-assisted (before or after) thermal processing (Burgos et al., 1972; Evelyn & Silva, 2015a, 2015d; Evelyn et al., 2016; Garcia et al., 1989; López-Malo et al., 2005; Ordonez & Burgos, 1976).

To date, limited information is available on the inactivation of AAT by power ultrasound, especially on spores (Ferrario et al., 2015; Wang et al., 2010; Yuan et al., 2009). Therefore, in

8. Thermosonication vs thermal processing *Alicyclobacillus acidoterrestris* spores

this research, orange juice inoculated with AAT spores was processed by TS. The effects of varying energy density, temperature, and juice pretreatments were investigated, and the spore first-order TS resistance parameters (*D*- and *z*-values) were determined and compared with thermal inactivation processes. The specific objectives were: (i) to determine the best acoustic energy density (AED) for TS inactivation at 75°C; (ii) to determine the effect of TS temperature on the *D*-values, (iii) to study the effect of high pressure pretreatment on the TS spore inactivation and compared with thermal inactivation alone; (iv) to compare the thermal resistance of spores in orange juice pretreated with ultrasound vs. no pretreatment; and (v) to recommend optimal TS conditions for the pasteurization of orange juice.

8.2 Materials and methods

8.2.1 *A. acidoterrestris* microbiology

8.2.1.1 Strain

Alicyclobacillus acidoterrestris type strain NZRM 4447 (same as ATCC 49025 and NCIMB 13137) was obtained from the New Zealand Reference Culture Collection. This strain was isolated from apple juice concentrate. It was precultured on potato dextrose agar (PDA, Difco North Ryde, Australia) adjusted to a pH 4.0 with 10% w/v (0.1 g/mL) tartaric acid. The PDA plates were incubated at 45°C for 3 days and used as source of inoculum for sporulation.

8.2.1.2 Sporulation

The sporulation procedure described by Silva et al. (2012) was used. Briefly, the fresh cells from the initial culture were inoculated on PDA (pH 5.6) and incubated for 21 days at 45°C to obtain spores. The spores were confirmed by phase contrast microscopy (Motic

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microscope BA410 Series, Canada). Then, the spores were harvested by flooding the plates with 1–2 mL of sterile distilled water and dislodging the spores from the agar surface with a sterile bent glass rod. After harvesting, the spores were washed three times by centrifugation with sterile distilled water (Centrifuge Sigma 4K15, UK) at 4,000g and 4°C for 10 min, resuspended in 50 mL sterile phosphate buffer (pH 7.2), and stored at 2°C until use.

8.2.1.3 Orange juice inoculation

The orange juice used in this study (pH 3.8, 9.5±0.1°Brix) was purchased from a local supermarket and used as the treatment medium for the AAT spore inactivation. The juice contained added pulp, flavour, food acid (citric acid), and preservatives (potassium sorbate). For 0.33 W/mL TS experiments, a small portion (ca. 1–2 mL) of spore solution was inoculated into 99 mL of orange juice, whereas for thermal and other TS experiments, 1 mL of the spore solution was inoculated into 49 mL of orange juice. A final spore concentration of approximately 10⁶ or 10⁷ cfu/mL was obtained in orange juice.

8.2.1.4 Spore enumeration

The *A. acidoterrestris* spore concentration in the juice before and after processing was determined by spread plating into acidified (pH 4) PDA plates. The spore concentration before processing was determined after a heat shock treatment (80°C, 10 min) of 5 mL juice in a thermostatic water bath to eliminate any vegetative cells. Orange juice samples were decimal diluted ten times with 0.1% (w/v) sterile buffered peptone water (Difco, Becton Dickinson, USA). Each tube dilution was mixed repeatedly using a high-speed vortex mixer to yield a uniform spore suspension, and plated twice. The PDA plates were then inverted inside a sealed plastic bag, to avoid drying of the medium and keep the moisture away from the agar surface, and incubated at 45°C for 3–5 days. Plates showing 30 to 300 colonies were

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used for enumeration, and spore concentration was expressed in colony forming units per milliliter (cfu/mL) of juice sample after calculations for corresponding dilution.

8.2.2 Experimental design and data analysis

8.2.2.1 Experimental design

The first experiment examined the effect of TS acoustic energy density (AED) at 75°C on AAT spore inactivation in orange juice. The AED of 0.33, 4.10 and 20.20 W/mL were used and evaluated, with 20.20 W/mL being the maximum energy of the equipment for the tip and juice volume used in the TS process. Because AED 20.20 W/mL was also the best performing AED, the following TS experiments were carried out at 20.20 W/mL. In the next experiment, TS inactivation of AAT spores was carried out at three temperatures (70, 75, and 78°C) for up to 60 min; 78°C is the maximum temperature recommended for this ultrasound unit by the manufacturer. Thirdly, we investigated the effect of TS on AAT spore inactivation with and without juice HPP pretreatments, and compared with TS and thermal inactivation alone at 78°C, the best temperature. Two 15 min HPP pretreatments at 200 and 600 MP were attempted in order to improve the TS inactivation of *A. acidoterrestris* spores.

Lastly, thermal inactivation of AAT spores in orange juice was carried out at three temperatures (78, 85, and 95°C) for orange juice pretreated with a heat shock followed by 1-min ultrasound (16.20 W/mL, see Section 2.5.1) and untreated orange juice. The results were also compared with the TS results.

8.2.2.2 Data analysis

TableCurve 2D version 5.01 (SYSTAT Software Inc., USA) was used to fit the 1st order kinetics to the linear spore survival lines, and estimate the kinetic parameters (D_T - and z -

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values). D_T -value is the time required at a certain temperature to reduce the microbial population by 90%, whereas z_T -value ($^{\circ}\text{C}$) is the temperature change that results in a 10-fold change in the D_T -value. Mean square error (MSE) and coefficient of determination (R^2) were used to evaluate the goodness of the fit. For each temperature/method at least two survival experiments were carried out and the D -values were estimated by linear regression of logarithmic number of survivors ($\log N/N_0$) versus time. Then, the average D_T -value \pm standard deviation (SD) was calculated for each temperature/treatment. A t -test with significance assigned at $p < 0.05$ was used to compare any two D -values or any two log reductions for different processing conditions/methods (Statistica 8, Statsoft Inc., USA).

8.2.3 Power ultrasound unit

An UP200S ultrasonic processor by Hielscher (Hielscher-Ultrasonic GmbH, Germany) was used for the induction of ultrasonic waves. The processor has a high frequency (24 kHz) and was operated at 100% amplitude and continuous energy supply. Sonotrodes with a tip-diameter of 3 mm (460 W/cm^2 , 33 W) and 14 mm (105 W/cm^2 , 162 W) were used to generate different AEDs (Hielscher, 2007). The sonotrode coupled to the ultrasonic processor via the horn, amplified the vibrations and transferred them to the orange juice to be sonicated. The maximum temperature supported by the sonotrode was 78°C .

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*8.2.4 Thermosonication inactivation of *A. acidoterrestris* spores in orange juice*

8.2.4.1 HPP pretreatment of orange juice

A high-pressure food processing system (QFP 2L-700, Avure Technologies, Columbus, Ohio, USA) was used for pressure treatment of AAT spores. The maximum pressure handled by the distilled water (the working fluid) was 690 MPa. Pressure come-up times were ≤ 1.5 min and depressurization took less than 30 s. Plastic pouches (16 × 16 cm, Cas-Pak, New Zealand) containing the inoculated orange juice (50 mL) were vacuum packed and thermosealed (Multivac C200, Germany). Then, the juices were submitted to 200 or 600 MPa HPP for 15 min at a temperature below 39°C. The treated juices were subsequently submitted to thermosonication as explained in the following section.

8.2.4.2 Thermosonication of orange juice

Three different AED levels were used: 0.33, 4.10 and 20.20 W/mL. For 0.33 W/mL, the TS experiment was carried out using the procedure described in Evelyn and Silva (2015a) with the 3 mm tip-diameter sonotrode. The ultrasonic treatments were carried out inside the biosafety cabinet (ESCOAC2-E/S, Singapore) to prevent contamination of the sample. Briefly, 100 mL of orange juice was added to a round-bottomed flask which was placed inside a thermostatic water bath for better control of the temperature during the process. After preliminary trials to obtain the working temperature for the TS treatment, the juice was preheated to an initial temperature. Next, aseptic inoculation was carried out by adding 1–2 mL of inoculum and ultrasound was switched on for the sonication treatment. Treatment times included the time to reach 75°C (≤ 2 min of sonication). This temperature was maintained constant ($\pm 1^\circ\text{C}$) throughout the experiments. Juice samples (0.5 mL) were taken

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from the flask at specific processing times after inoculation and cooled in an ice water bath for spore survivor counts.

With respect to 4.10 and 20.20 W/mL TS, the Hielscher's stainless steel D14K temperature-controlled flow vessel was filled with 8 mL orange juice containing the spores. A probe with a 3 mm diameter tip was used to obtain 4.10 W/mL, whereas a 14 mm diameter probe was used to generate the AED of 20.20 W/mL. The vessel was also initially preheated up to an initial temperature (also determined from preliminary trials), filled with unprocessed juice, and then the ultrasonication treatment time count started. The times to reach the desired temperatures were quick (≤ 1 min), and these were included in the TS treatment times. The juice was maintained at the desired temperature ($\pm 0.5^\circ\text{C}$), which was recorded by a thermocouple (connected to a computer) at the vessel outlet. This temperature was kept constant by continuously running cooling water from the tap through the jacket of the vessel. The juice was treated for specific processing times, cooled in an ice water bath and spore survivor counts were immediately performed. The procedure was repeated for other specific processing times.

8.2.5 Thermal inactivation of *A. acidoterrestris* spores in orange juice

8.2.5.1 Pretreatment of orange juice

Heat shock (HS, 80°C for 10 min) followed by 1-min ultrasonication of juice containing the spores was attempted to improve thermal inactivation, since past results showed HS + ultrasonication of *Clostridium perfringens* spores enhanced its thermal inactivation (Evelyn & Silva, 2015b). Briefly, for the heat shock, a test tube containing 10 mL of juice with the *A. acidoterrestris* spores ($\sim 10^7$ cfu/mL) was thermally processed at 80°C for 10 min in a water

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bath and then placed in an ice water bath and submitted to 1-min ultrasound by submerging the 14 mm tip ultrasound sonotrode in the juice. The resulting AED was 16.20 W/mL.

8.2.5.2 Thermal processing of orange juice

Thermal processing of orange juice was carried out for treated (HS followed by 1-min ultrasound) and untreated orange juice. Transparent food grade sterile pouches (8 × 8 cm, Cas-Pak, New Zealand) were filled with 3 mL of treated or untreated juices, and thermally processed at 78, 85, and 95°C for various times in a water bath. The thermally treated samples were taken out from the water bath at different time intervals and kept in an ice water bath until microbial enumeration.

8.3 Results and discussion

8.3.1 Effect of TS acoustic energy density on *A. acidoterrestris* spore inactivation in orange juice at 75°C

The effect of TS acoustic energy densities (0.33, 4.10 and 20.20 W/mL) on the AAT spore reduction in orange juice at 75°C for 60 min is illustrated in **Figure 8-1**. The first order kinetics of the TS inactivation was supported by the low MSE value (0.0001–0.004) and high R^2 (0.850–0.997). Our results showed higher spore inactivation for higher AED (D -values decreased with the increase in AED): a $D_{75^\circ\text{C}}$ -value of 49 min for 20.20 W/mL compared to 97 min for 4.10 W/mL and 217 min for 0.33 W/mL. The z -value for the effect of AED on D -values was 36 W/mL ($R^2=0.85$). López-Malo et al. (2005) also found lower D -values for mould spores at the highest amplitude of 120 μm at 20 kHz: $D_{60^\circ\text{C}}$ -value of 0.8 min for *Aspergillus flavus* and $D_{52.5^\circ\text{C}}$ -value of 3.8 min for *Penicillium digitatum* in

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Sabouraud broth. Previous investigators have also observed that greater log reductions in AAT vegetative cells occur during TS (23-25 kHz) when the energy was increased from 200 to 600 W at 25-50°C (Wang et al., 2010; Yuan et al., 2009). Linear, biphasic linear and Weibull models have been used to predict vegetative AAT cells (DSM 3922 and DSM 14558) in apple juice (Wang et al., 2010; Yuan et al., 2009).

Except for the aforementioned data on vegetative cells, there have been no reports on TS inactivation of AAT spores or other bacterial spores for different AED. Since the highest spore inactivation was obtained with 20.20 W/mL, the TS inactivation of AAT at 20.20 W/mL at different temperatures was further investigated and results are discussed in the following section.

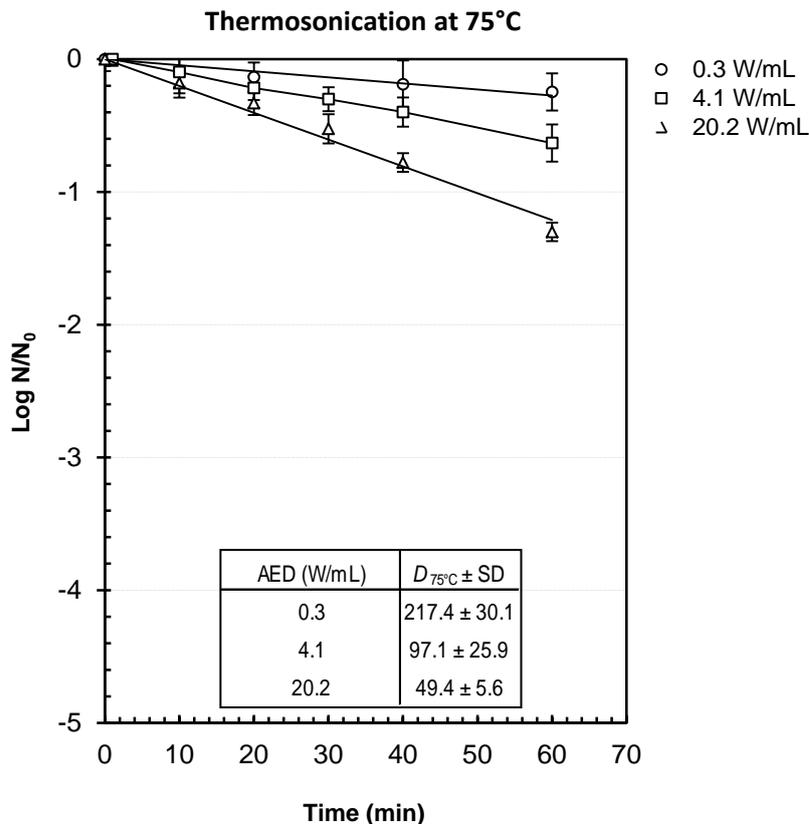


Figure 8-1 Effect of acoustic energy density (AED) on the thermosonication inactivation of *A. acidoterrestris* spores in orange juice at 75°C.

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8.3.2 Effect of temperature on the TS inactivation of *A. acidoterrestris* spores in orange juice

The log survivors of AAT spores under 20.20 W/mL TS processing in orange juice at three temperatures (70, 75 and 78°C) are shown in **Figure 8-2**. As expected, TS temperature plays a significant role in spore inactivation, with greater spore inactivation at higher temperatures. The 20.20 W/mL TS *D*-values obtained were 28 min at 78°C, 49 min at 75°C and 139 min at 70°C, with *z*-value of 11.5°C (**Table 8-1**). The first-order kinetic model showed again good performance indices (0.0001–0.013 MSE, 0.959–0.998 R^2), and also good fit for the effect of temperature on *D*-values ($R^2=0.99$), confirming the linearity of the TS death curves. The higher spore inactivation at the higher temperature for the survival curves are in agreement with our past results with *Bacillus cereus* spores in skim milk (Evelyn & Silva, 2015a) and the result of Garcia et al. (1989) with *Bacillus subtilis* spores in whole milk and glycerol.

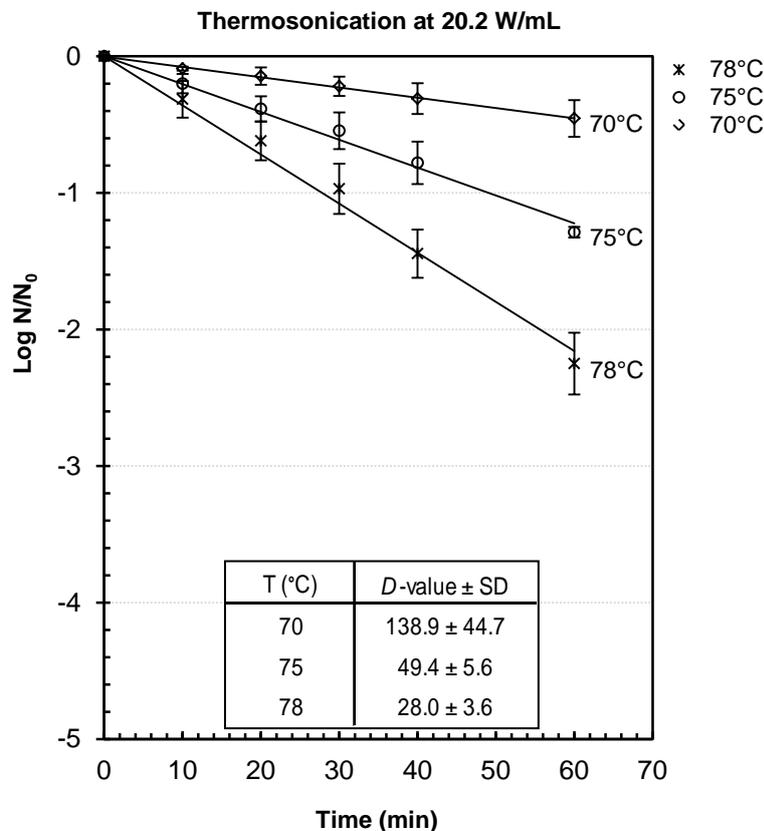


Figure 8-2 Effect of temperature on the thermosonication (20.2 W/mL) inactivation of *A. acidoterrestris* spores in orange juice.

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Table 8-1 D_T - and z -values for thermosonication (20.2 W/mL) and thermal inactivation of *Alicyclobacillus acidoterrestris* spores in orange juice*

Mean D_T -value ± SD (min) at:	No pretreatment of juice		Heat shock (80°C, 10 min) followed by 1-min ultrasound pretreatment of juice
	Thermosonication	Thermal	Thermal
95°C	nd	1.5 ± 0.2	0.8 ± 0.1
85°C	nd	69.4 ± 2.3	29.3 ± 1.2
78°C	28.0 ± 3.6	175.4 ± 11.2	103.1 ± 8.1
75°C	49.4 ± 5.6	nd	nd
70°C	138.9 ± 44.7	nd	nd
z -value ± SE (°C)	11.5 ± 0.3 $R^2 = 0.99$	8.0 ± 0.3 $R^2 = 0.95$	7.9 ± 0.3 $R^2 = 0.97$

**A. acidoterrestris* strain NZRM 4447 (ATCC 49025, NCIMB 13137) was used; D -values are means±standard deviation and obtained from two survival experiments; The first order kinetic parameters for both processes showed good performance indices (0.0001–0.120 MSE and 0.910–0.998 R^2); nd = not determined; SD = standard deviation; SE = standard error.

8.3.3 Effect of high pressure pretreatment on *A. acidoterrestris* spore inactivation at 78°C

Figure 8-3 shows the log survivors of AAT spores at 78°C up to 60 min with HPP juice and its comparison with thermosonication (untreated) and thermal alone. For untreated juice at 78°C (the maximum temperature supported by the ultrasound probe), AAT log reductions by TS (2.3 log) was far higher than after the thermal process (0.3 log) of 60 min ($p < 0.05$). The $D_{78^\circ\text{C}}$ -values were 14–28 min for TS and 175 min for thermal processing, indicating the remarkable advantage of ultrasound technology. The higher spore inactivation by TS than by thermal processing at the same temperature is in agreement with previous experiments carried out with other spore species (Evelyn & Silva, 2015a; Garcia et al., 1989; López-Malo et al., 2005; Wordon et al., 2012).

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Both HPP pretreatments enhanced the AAT spore inactivation in orange juice by TS. HPP at 600 MPa showed a better performance (4.4 log after 60 min, $D_{78^{\circ}\text{C}}\text{-value} = 14$ min) than 200 MPa HPP (2.7 log after 60 min, $D_{78^{\circ}\text{C}}\text{-value} = 23$ min) ($p < 0.05$), followed by TS without pretreatment, which produced inactivation that was similar to using 200 MPa pretreatment (2.3 log after 60 min, $D_{78^{\circ}\text{C}}\text{-value} = 28$ min). It is known that pressures between 100 and 800 MPa can initiate the germination of spores (Gould & Sale, 1970; Paidhungat & Setlow, 2002; Wuytack et al., 1998), thus making the spores more susceptible to subsequent inactivation treatments (Black et al., 2007; Sarker et al., 2015). However, long treatment times (>10 min) were still needed to significantly reduce the number of AAT spores in juice.

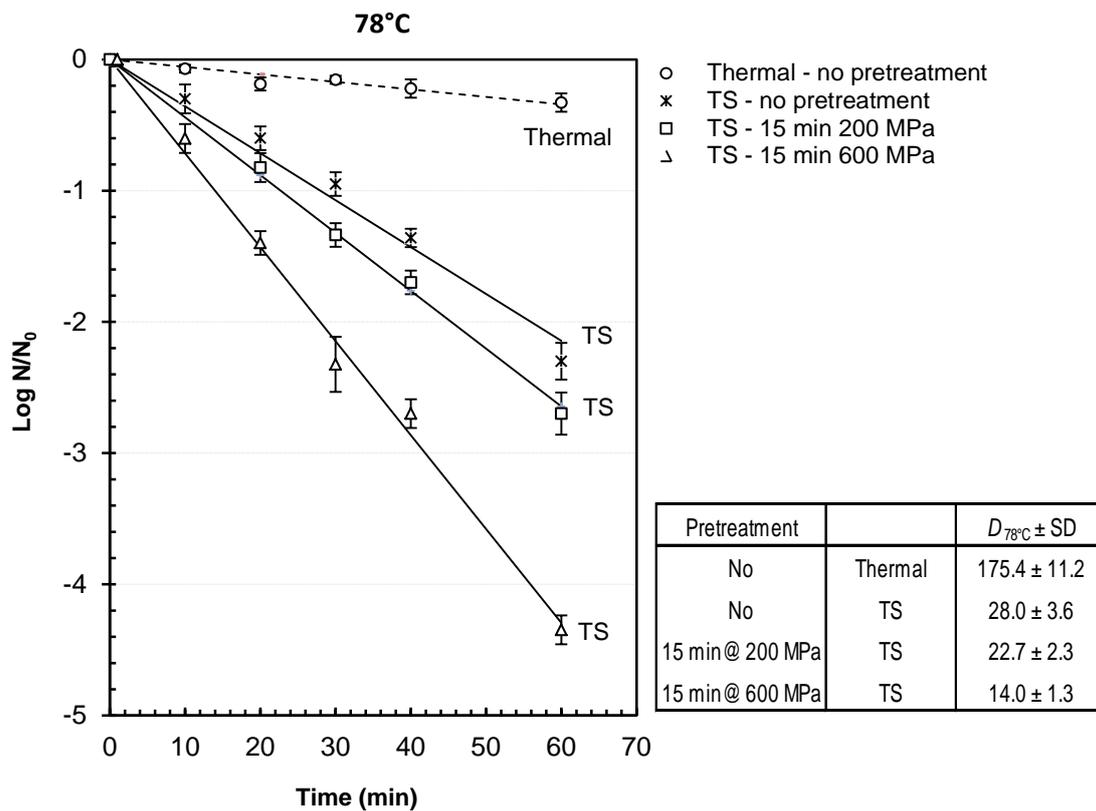


Figure 8-3 Effect of high pressure processing pretreatments on the thermosonication (20.2 W/mL, 78°C) inactivation of *A. acidoterrestris* spores in orange juice.

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8.3.4 Thermal inactivation of *A. acidoterrestris* spores in orange juice pretreated with ultrasound vs. no pretreatment

Figure 8-4 compares the log AAT survivors for juice submitted to HS (80°C, 10 min) followed by 1-min ultrasound (16.2 W/mL) vs untreated juice at temperatures of 78, 85, and 95°C. HS followed by 1-min ultrasonication of AAT spores in the juice decreased ($p < 0.05$) the thermal resistance of AAT spores approximately by half at 85 and 78°C. For example, the $D_{85^\circ\text{C}}$ -value was reduced from 69.4 to 29.3 min, and the $D_{78^\circ\text{C}}$ -value decreased from 175.4 to 103.1 min (**Table 8-1**), confirming the benefit of HS + ultrasound to enhance the thermal inactivation. At 95°C the difference was less marked. Damage of AAT spores might occur after HS + ultrasound thus further sensitizing the spores to subsequent heat treatment. We have previously shown that HS + 1 min ultrasound pretreatment before thermal processing doubles the rate of *C. perfringens* spore inactivation in beef slurry (Evelyn & Silva, 2015b) vs untreated spores: $D_{105^\circ\text{C}}$ -value of 1.1 min vs. 2.5 min, $D_{100^\circ\text{C}}$ -value of 3.4 min vs. 7.1 min, and $D_{95^\circ\text{C}}$ -value of 9.8 min vs. 21.7 min. The mechanisms for the inactivation are still unknown and need to be explored. Thermal inactivation experiments of juices pretreated with 1-min ultrasound but not heat shocked were also carried out. However, a negligible effect was observed on the spore thermal susceptibility, with $D_{95^\circ\text{C}}$ -value = 1.3 min similar to 1.5 min of untreated spores, and z -value of 8.0°C similar to 7.8°C obtained with untreated spores. Previous investigators found no change on the thermal resistance of *Clostridium* spp. spores after exposure to 15–36 W/mL ultrasound treatments alone (Broda, 2007; Evelyn & Silva, 2015b; Goodenough & Solberg, 1972). However, other investigators have reported a decrease in the spore thermal resistance of several species of *Bacillus* after the 12–15 W/mL ultrasound treatments (Burgos et al., 1972; Ordonez & Burgos, 1976), indicating that different species respond differently to the inactivation process.

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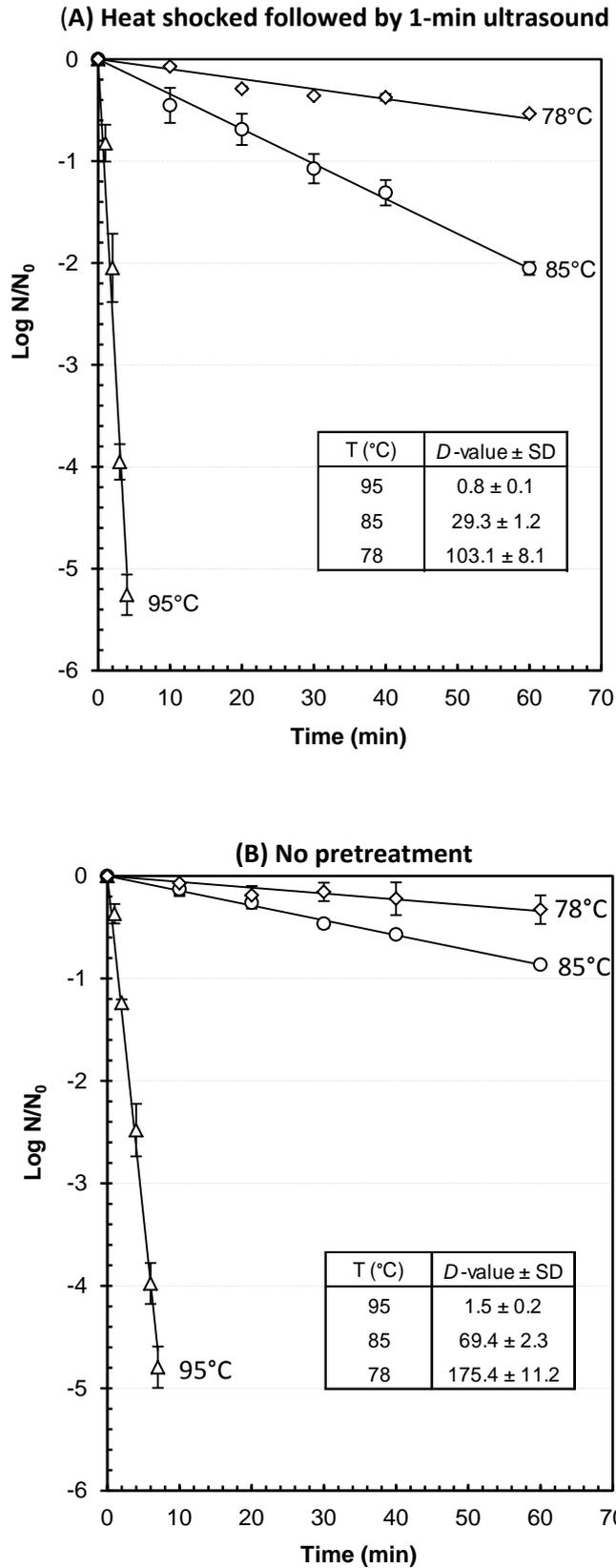


Figure 8-4 Thermal inactivation of *A. acidoterrestris* spores in orange juice (A - heat shock followed by 1-min ultrasound pretreatment of juice; B - no pretreatment of juice).

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The thermal log survivors for pretreated and untreated juice were also linear with 0.0004–0.120 MSE, 0.910–0.996 R^2 , and the D -values temperature dependence ($R^2 \geq 0.95$) (**Figure 8-4, Table 8-1**). The $D_{85^\circ\text{C}}$ -value of 69 min for untreated orange juice was similar to the value obtained (66 min) by Silva et al. (1999) with the same strain in orange juice. $D_{85^\circ\text{C}}$ -values between 50 and 94.5 min were determined with spores from AAT strains 46, 70, 145 and DSM 2498 in orange juice (Eiroa et al., 1999). The $D_{95^\circ\text{C}}$ -value of 1.5 min was also in the range of previous reported values (1.0-5.3 min) (Baumgart et al., 1997; Eiroa et al., 1999; Komitopoulou et al., 1999; Splittstoesser et al., 1994; Walls, 1997). The estimated z -values of 7.9–8.0°C were comparable to the values found (7.7–7.8°C) by other investigators (Pontius et al., 1998; Silva et al., 1999; Splittstoesser et al., 1994).

8.3.5 TS vs thermal inactivation of *A. acidoterrestris* spores in orange juice

The first-order kinetic parameters for TS and thermal treatment in orange juice are shown in **Table 8-1**. TS was a better process than thermal processing for AAT spore inactivation, producing a 6-fold reduction in the D -value at the same temperature: $D_{78^\circ\text{C}}$ -value of 28 min for TS vs 175 min for thermal ($p < 0.05$). Similar D -values were obtained for TS at 8°C lower temperatures than for thermal processes ($p > 0.05$). For example, the TS $D_{70^\circ\text{C}}$ -value was not significantly different from thermal $D_{78^\circ\text{C}}$ -value. Past reports have shown the benefit of ultrasound technology on thermal spore and vegetative cell inactivation (lower temperatures and processing times to achieve the same lethality values), suggesting less negative impact of heat in the food quality (Evelyn & Silva 2015a, 2015d; Garcia et al., 1989; López-Malo et al., 2005; Wordon et al., 2012).

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The lower thermal z -value (8.0°C) than TS z -value (11.5°C) obtained for AAT spores in this study (**Table 8-1**) means the spores are more susceptible to temperature changes in thermal processes than TS processes. The result is in agreement to our previous result using *B. cereus* spores in milk (Evelyn & Silva, 2015a). Garcia et al. (1989) also reported an increase in the TS z -values (9.1 to 9.4°C in milk, 13.4 to 14.4°C in glycerol), which is thought to be related to the changes in the properties of medium used during heating and ultrasound processes (Evelyn & Silva, 2015a; López-Malo et al., 2005; Mason et al., 1996).

8.4 Conclusion

TS of orange juice pretreated with 600 MPa HPP for 15 min was the best technique to inactivate *A. acidoterrestris* spores, allowing a 3 log reduction after 42 min. TS AED and temperature are important determinants of AAT spore inactivation, with greater inactivation occurring at higher AED/temperature. The pretreatment of juice with a heat shock (80°C, 10 min) followed by ultrasound, duplicated the spore thermal inactivation. However, overall TS was 6-fold more effective than thermal treatments in reducing AAT spores in orange juice and required an 8°C lower temperature to obtain the same inactivation rates as the conventional thermal process. The thermosonication and thermal spore inactivation followed first order kinetics. The results demonstrated the advantage of high pressure-assisted thermosonication for the inactivation of *A. acidoterrestris* spores in orange juice. However, further studies are needed to investigate the impact of the long processing time on the juice sensory/quality attributes and to design ultrasound probes that can withstand higher temperatures, thus allowing lower processing times for the same spore inactivation.

Chapter 9 Inactivation of *Byssochlamys nivea* ascospores in strawberry puree by high pressure, power ultrasound and thermal processing

Evelyn, & Silva, F. V. M. (2015). Inactivation of *Byssochlamys nivea* ascospores in strawberry puree by high pressure, power ultrasound and thermal processing. *International Journal of Food Microbiology*, 214, 129-136.

Chapter abstract

Byssochlamys nivea is a mould that can spoil processes fruit products and produce mycotoxins. In this work, high pressure processing (HPP, 600 MPa) and power ultrasound (24 kHz, 0.33 W/mL) in combination with 75°C for the inactivation of four week old *B. nivea* ascospores in strawberry puree for up to 30 min was investigated and compared with 75°C thermal processing alone. TS and thermal processing can activate the mould spores, HPP-75°C resulted in 2.0 log reductions after a 20 min process. For a 10 min process, HPP-75°C was better than 85°C alone in reducing *B. nivea* spores (1.4 vs 0.2 log reduction), demonstrating that a lower temperature in combination with HPP is more effective for spore inactivation than heat alone at higher temperature. The ascospore inactivation by HPP-thermal, TS and thermal processing was studied at different temperatures and modeled. Faster inactivation was achieved at higher temperatures for all the technologies tested, indicating the significant role of temperature in spore inactivation, alone or combined with other physical processes. The Weibull model described the spore inactivation by 600 MPa HPP-thermal (38, 50, 60, 75°C) and thermal (85, 90°C) processing, whereas the Lorentzian model was more appropriate for TS treatment (65, 70, 75°C). The models obtained provide a useful tool to design and predict pasteurization processes targeting *B. nivea* ascospores.

9. Power ultrasound, high pressure and thermal processing *Byssochlamys nivea* spores

9.1 Introduction

Byssochlamys species are abundant in soil and recognized as important spoilage moulds in fruit and fruit products (Beuchat, 1998; Pitt & Hocking, 1997; Silva et al., 2014). *Byssochlamys* can produce 8 spores, called ascospores, inside an ascus. The ascospores of *Byssochlamys fulva*, *Byssochlamys nivea* and *Byssochlamys spectabilis* are very heat resistant, and may require an inactivation temperature above 90°C (Silva & Gibbs, 2004; Silva & Gibbs, 2009; Silva et al., 2014; Tournas, 1994). Bayne and Michener (1979) reported that seven out of 25 strains of *Byssochlamys* were able to survive heating at 90°C for 25 min or longer, when initial numbers were frequently near 10^6 /ml. The ascospores also show great resistance to acids and chemicals such as chlorine and alcohol (Tournas, 1994). A combination of heat with non-thermal processes for spore inactivation and food pasteurization can better retain the food's sensory properties and quality (Alexandre et al., 2011; Krebbers et al., 2003; Vervoort et al., 2012).

B. nivea (anamorph *Paecilomyces niveus*) is able to grow at temperatures between 11 and 43°C (the optimal temperature is around 30°C), water activity between 0.892 and 0.992 (Panagou et al., 2010), over a wide range of pH (3–8) (Pitt & Hocking, 1997), and under reduced oxygen conditions inside food packs and in carbonated beverages (Taniwaki et al., 2009). Month old of *B. nivea* ascospores survived in thermally treated pineapple nectar at 103°C for 7 min (Ferreira et al., 2011). The most resistant strain of month old ascospores of *B. nivea* (strain 162) also survived 90°C for 20 min in tomato juice (Kotzekidou, 1997). Temperature time combinations of 87.5°C-10 min and 90°C-2 min did not kill *B. nivea* in processed canned fruits (Luthi & Hochstrasser, 1952; Put & Kruiswijk, 1964). Other investigators also reported the isolation of *B. nivea* from pasteurized fruit concentrates ((Ferreira et al., 2011; Kotzekidou, 1997; Palou et al., 1998; Pitt & Hocking, 1997;

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Zimmermann et al., 2011)). Milk and milk products (Engel & Teuber, 1991), cucumber brine (Yates & Ferguson, 1963), cream cheese (Pitt & Hocking, 1997) and palm wine (Eziashi et al., 2010) have also been contaminated by this species. In addition to the high thermal resistance, *B. nivea* is also a concern to human and animal health since it can produce the mycotoxins patulin (Roland & Beuchat, 1984; Sant'Ana et al., 2010b; Taniwaki et al., 2009), byssochlamic acid (Escoula, 1974), and byssotoxin A (Beuchat & Rice, 1979). These toxins may act upon the central neural system causing sustained tremors and convulsions (Tournas, 1994).

Preservation of foods by non-thermal technologies such as high pressure processing (HPP) and power ultrasound are attractive alternatives because they have little or minimal effects on the nutrients and taste of food (Cullen et al., 2012; Farkas & Hoover, 2000). HPP technology, generally with pressures between 400 and 600 MPa at ambient or chilled temperatures, and processing times under 10 min, have been used commercially for the preservation of acidic fruit juices and beverages (Cheftel, 1995). In general bacterial spores, mould ascospores and enzymes in foods are difficult to inactivate by HPP alone (Evelyn & Silva, 2015c; Larson et al., 1918; Patterson, 2005; Sulaiman & Silva, 2013; Sulaiman et al., 2015a; Timson & Short, 1965). However, most of the heat resistant spores, including pathogenic species, do not germinate and grow in the acidic environment ($\text{pH} < 4.6$) of the fruit juices (Silva & Gibbs, 2004). The combination of HPP with a mild heat treatment (HPP-thermal) is usually required to inactivate microbial spores (Evelyn & Silva, 2015c; Sarker et al., 2015; Wilson et al., 2008). Unlike bacterial spores, only a few reports about the inactivation of ascospores of *B. nivea* mould by HPP-thermal are available in the literature and none modeled the kinetics. High pressures of up to 900 MPa (oscillatory or continuous) in conjunction with temperatures of 20–90°C have been used to inactivate *B. nivea* ascospores in buffer, juice or concentrate, and the degree of inactivation was dependent on the strain, ascospore age, and °Brix or water

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activity of the suspending medium (Butz et al., 1996; Chapman et al., 2007; Ferreira et al., 2009; Maggi et al., 1994; Palou et al., 1998). HPP prior to or followed by thermal treatment has also been attempted, with prior thermal treatment being less effective than thermal treatment after the HPP processes (Butz et al., 1996; Maggi et al., 1994).

With respect to power ultrasound, cavitation (20–100 kHz) combined with heating causes bacterial spore (Evelyn & Silva, 2015a; Feng & Yang, 2011b) and enzyme inactivation (Sulaiman et al., 2015b). Similar to HPP technology, power ultrasound alone is also ineffective for inactivation of these spores (Butz & Tauscher, 2002; Evelyn & Silva, 2015a). Thus a combination with thermal treatment, often referred to as thermosonication (TS), is needed. The inactivation of the conidia of *Penicillium digitatum* and *Aspergillus flavus* by TS (20 kHz, 40-60°C) has been reported (Coronel et al., 2011; Jimenez-Munguia et al., 2001; López-Malo et al., 2005), being higher when using heat. The TS inactivation and kinetic modeling of *B. nivea* ascospores have not been reported.

In this research, the HPP-thermal, TS and thermal methods of inactivation of *B. nivea* ascospores in strawberry puree were compared for the first time and the inactivation was modeled. The main objectives were: (i) to compare the HPP-thermal, TS and thermal methods of inactivation of ascospores at 75°C for up to 20 min processing; (ii) to study the 600 MPa HPP-thermal inactivation of ascospores at different temperatures; (iii) to study the TS inactivation of ascospores at different temperatures; and (iv) to study the thermal inactivation of ascospores at different temperatures.

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9.2 Materials and methods

9.2.1 Microbiology

9.2.1.1 Mould

B. nivea JCM 12806 (= CBS 696.95) was obtained from the Japan Collection of Microorganisms. This strain was isolated from pasteurized strawberry in the Netherlands.

9.2.1.2 Ascospore production

Ascospores of *B. nivea* were obtained after a growth period of four weeks at 30°C on potato dextrose agar (PDA pH 5.6-Difco, North Ryde, Australia). The spores were collected by flooding the surface of the culture plates with 5 mL sterile distilled water (SDW) and gently rubbing biomass from the agar surface with a sterile bent glass rod. The spore suspension was subsequently filtered through layers of gauze to remove any remaining hyphal fragments. Spore pellets were obtained after centrifugation in SDW at 4,000×g for 15 min at 4°C and the procedure was repeated three times. The final spore suspension was then stored at 2°C in SDW containing glass beads until use. Microscopic observation revealed that a mixture of free ascospores and asci were present. The survivor experiments were carried out without any attempt to free ascospores from their asci. Under natural conditions, the ascospores in fruits would be expected to be as a mixture of asci and free ascospores so carrying out the inactivation experiments in this way, should reflect the reality of fruit juice processing.

9.2.1.3 Strawberry puree inoculation and packaging

Strawberries from Phil Greig Strawberry Gardens New Zealand were pureed (pH 3.4, 8.1±0.1°Brix) in a sterile laboratory scale blender and used as the medium to suspend and process the *B. nivea* ascospores. For HPP-thermal and thermal experiments, aliquots (ca. 0.1

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mL) of *B. nivea* spore solution were inoculated into 3.4 mL of strawberry puree to yield an initial spore concentration of $\approx 10^5$ – 10^6 cfu/mL of puree. The inoculated puree was packed into 8×8 cm food grade retort pouches (Cas-Pak, New Zealand) composed of polyester coated with silicon oxide and laminated to nylon and cast polypropylene (PET-SIOX(12)//ON(15)//RCPP(70)). The pouches can withstand temperatures of up to 130°C, so were suitable for thermal processing and high pressure applications. Regarding TS experiments, the *B. nivea* spore solution was inoculated aseptically by adding a small volume of inoculum to the strawberry puree contained in a 200 ml round-bottom flask (3 mL of spore solution into 97 mL of strawberry puree) before the pretreatment and TS treatment. The initial spore concentration was approximately 10^5 – 10^6 cfu/mL of puree.

9.2.1.4 Spore enumeration

The mould ascospore concentration in strawberry puree before and after processing (thermal, HPP and TS) was determined by spread plating onto PDA. A heat shock (75°C, 5 min) of raw unprocessed strawberry was required to obtain the initial count (N_0) in the untreated strawberry for HPP and thermal processes (Katan, 1985; Splittstoesser et al., 1993). This procedure allows the growth of colonies and ascospore enumeration in the untreated raw puree (N_0). With respect to TS, N_0 was determined in already thermally pretreated strawberry (see Section 10.2.2.3), so there was no need for additional heat shock for enumeration in this case. Prior to plating, the whole content of strawberry contained in the pouches (3.5 mL, HPP and thermal treatments) and in the flask (100 mL, ultrasound processing) was placed in 110×230 mm sterilized stomacher bags (Interscience, France) and diluted 2-fold with 0.1% (w/v) buffered peptone water. Samples from HPP and thermal treatments were then homogenized in a stomacher bag for 1 min, whereas larger volume samples from ultrasound processing were manually mixed. Homogenized half diluted strawberry samples (1 mL)

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were then decimal diluted using 9 mL of 0.1% (w/v) sterile buffered peptone water (Difco, Becton Dickinson, USA). Each tube dilution was mixed repeatedly using a high speed vortex mixer to yield a uniform spore suspension, and 0.1 mL from each tube dilution was plated on two PDA plates. The plates were then incubated at 30°C for 3 to 5 days until visible colonies were formed. Plates with 20 to 100 colonies were used for enumeration. Ascospore concentration was expressed in cfu per milliliter (cfu/mL) of strawberry puree.

9.2.2 Processing

A heat pretreatment of 80°C for 15 min was applied to the inoculated strawberry puree before the TS survival experiments, since preliminary experiments revealed that this procedure allowed a reduction of 15 min in the TS treatment time for the same spore inactivation in strawberry puree. Butz et al. (1996) have demonstrated no effect of prior heat treatment (80°C, 30 min) on the 700 MPa-HPP inactivation of *B. nivea* spores. Thus, the inoculated puree was not heat pretreated before HPP-thermal and exclusively thermal experiments.

9.2.2.1 Experimental design

In the first experiment, the *B. nivea* ascospore inactivation in strawberry puree by 600 MPa HPP-thermal, TS and sole thermal processing at 75°C for treatment times of up to 30 min was investigated. At least two independent survival experiments were carried out for each technology and duplicate samples were processed for each treatment time. Tukey's test was used to compare the log ascospore numbers ($\log N/N_0$) by different methods at the same processing time to check if different methods (TS, HPP-thermal and thermal) resulted in significantly different survivors (Statistica 8, Statsoft Inc., USA). N was the microbial count after processing.

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In the other survival experiments, HPP-thermal, TS and thermal treatments of strawberry puree were carried out at different temperatures, as described in the following sections. The logarithmic number of survivors ($\log N/N_0$) versus time was plotted for each temperature to model and estimate the inactivation parameters. Two samples were processed for each time and three survival experiments were carried out for each treatment temperature.

9.2.2.2 HPP-thermal processing

The same procedure of HPP-thermal described previously for *N. fischeri* ascospores were followed (Chapter 8). The 600 MPa HPP was selected for the inactivation experiments because literature shows that high pressure ≥ 600 MPa was more effective for the inactivation of heat resistant ascospores (Butz et al., 1996; Chapman et al., 2007; Palou et al., 1998), and 600 MPa was the maximum working pressure of the Avure HPP unit. Briefly, HPP at 600 MPa combined with 50, 60 and 75°C was used with processing times of up to 40 min. 600 MPa high pressure treatments without heating (38°C) was also carried out. The HPP treated samples were submerged into an ice water bath for subsequent survivor enumeration.

9.2.2.3 Thermosonication

Thermosonication inactivation experiment was also carried out using the method described previously (Chapter 8), except, the flask containing the inoculated puree was thermally processed at 80°C for 15 min in a water bath inside a biosafety cabinet prior to TS experiments (class II, type A2, AC2 – 4E1, Esco Micro Pte. Ltd., Singapore). Ultrasonic treatments were carried out for up to 60 min depending on the TS treatment temperatures. For each pre-specified processing time, the whole sample of strawberry puree was taken from the water bath, cooled in ice water, and survivors were enumerated.

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9.2.2.4 Thermal processing

The experiments for determining the thermal resistance of *B. nivea* ascospores were carried out using the same method and temperatures (75, 85 and 90°C) described previously for *N. fischeri* spores (see Section 9.2.2.4).

9.2.3 Modeling the *B. nivea* ascospore inactivation in strawberry puree

A non-linear Weibull model (Equation 1-6) was used to describe *B. nivea* ascospore inactivation by HPP-thermal and thermal processes, while the Lorentzian model from TableCurve 2D (version 5.01, SYSTAT Software Inc., Chicago, USA) (Equation 9-1) was used for the TS (Hubbert, 1956; Lorentz, 1875; Peng & Lu, 2006; Systat., 2002):

$$\log \frac{N}{N_0} = a + 4b + \frac{e[-(\frac{t-c}{d})]}{[1+e[-(\frac{t-c}{d})]]^2} \quad (9-1)$$

where a is the baseline of the distribution (that corresponds to the residual-survival-potentially observed); b is the rate of microbial inactivation; c is the the time (t) value at which the inactivation starts; d is the width of the distribution. The logarithmic microbial survival ratio was evaluated with $\log N/N_0$ and expressed as mean \pm standard deviations (SD). N was the ascospore concentration (cfu/mL) in the juice after processing for a specific time t (min), and N_0 was the initial spore concentration of raw juice (HPP, thermal) and preheated juice (before TS). The models were compared with random residual, MSE, R^2 values, and the temperature dependence of the estimated parameters.

9.3 Results and discussion

9.3.1 Activation shoulders and increase in spore numbers

Figure 9-1 to **9-4** show the effects of different food preservation technologies on the log number of *B. nivea* ascospores. With fungal spores, the increase in their numbers is a mechanism caused by the application of heat, a chemical or another factor under certain conditions, which causes the breaking of the spore dormancy for germination, leading to an increase in the viable counts by several logs (Dijksterhuis, 2007; Sussman, 1976; Tournas, 1994).

Activation shoulders were observed during thermosonication treatments (**Figure 9-1** and **Figure 9-3**). The shoulders of 1 log after 5 min TS at 75°C and 3 log after 15 min TS at 65°C demonstrated that the mould spores are more sensitive to the ultrasound plus heat than heat alone. Sonication is recognized as a tool to release the fungal spores from the asci and to produce suspensions of free ascospores (Amaeze, 2012; Beuchat, 1986; Michener & King, 1974). The combination of ultrasound and heat (TS) can further facilitate the increase in the free spore numbers. With respect to thermal processing, a steady increase of *B. nivea* ascospores was registered with processing time at 75°C, reaching 1.2 log after 30 min (**Figure 9-1**). On the contrary, only inactivation was observed at 85 and 90°C thermal processing for up to 60 min. Ferreira et al. (2011) reported a 0.5 log increase of *B. nivea* ascospores in fruit nectars after treatment at 85°C for 10 min. Increases in ascospore numbers have also been observed with other thermally processed heat resistant fungal ascospores such as *Neosartorya* and *Talaromyces spp.* (70–85°C, 7–30 min) (Beuchat, 1986; Dijksterhuis and Teunissen, 2004; Katan, 1985), and thermally processed bacterial spores such as *Bacillus stearothermophilus* (105–120°C, 5–30 min) (Corradini et al., 2010).

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No increase in ascospores was observed after non-thermal 600 MPa HPP at 38°C and 600 MPa HPP processes at temperatures ranging between 50 and 75°C (**Figure 9-2**). This observation is in agreement with previous work with *B. nivea* ascospores employing 600 MPa HPP combined with a 40–70°C temperature (Butz et al., 1996; Ferreira et al., 2009). Other authors reported that 600–900 MPa without heat ($\leq 21^\circ\text{C}$) for ≤ 25 min treatment times increased ascospore numbers up to 2.4 log in four week old *B. nivea*, *Neosartorya*, and *Talaromyces macrosporus* ascospores (Chapman et al., 2007; Hocking et al., 2004; Maggi et al., 1994; Palou et al., 1998), which was attributed to the release of spores from the ascus.

9.3.2 HPP-thermal, TS and thermal inactivation of *B. nivea* ascospores in strawberry puree at 75°C

The effects of a 600 MPa high pressure process combined with thermal (HPP-thermal), thermosonication (TS), and thermal processing at 75°C on *B. nivea* ascospores for up to 30 min are illustrated in **Figure 9-1**. For a 75°C and 10 min process, 1.4 log reductions in *B. nivea* ascospores was obtained for HPP-thermal vs. no reductions for TS and thermal ($p < 0.05$). As mentioned in the previous section the spore numbers increased with TS, reaching a maximum (+1.0 log) at 5 min ($p < 0.05$), which was followed by a steady linear inactivation. For thermal processing, an increase in spore numbers was also observed, with a 0.5 log spore increase after 20 min processing and continued to increase to 1.2 log after 30 min processing ($p < 0.05$). As opposed to TS and thermal treatments, the HPP-thermal process reduced the spores steadily, reaching nearly 2.0 log reduction after 15 min and 3.4 log at 40 min. It is known from the literature that HPP can activate and germinate the dormant spores, rendering them more susceptible to inactivation treatments. For example, short time treatments at pressures between 400 and 800 MPa induced *T. macrosporus* mould spore activation and

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quicker germination (transformation from a dormant state with low metabolic activity to one of high activity in which spores lose much of their extreme resistance), followed by inactivation of the germinated form by pressure or mild heat (Black et al., 2007a; Dijksterhuis & Teunissen, 2004). The results obtained in our research showed that long treatment times (>40 min) are needed to achieve 5 log reduction recommended by the US Food, Drug, and Administration (USFDA, 2001) for fruit juice pasteurization with a 600 MPa HPP-75°C process, which is not feasible for commercial application. This process could be sufficient for lower initial loads of microbial contamination in the strawberry puree. Better results could be achieved at higher HPP temperatures, but currently commercial HPP machines cannot reach very high temperatures.

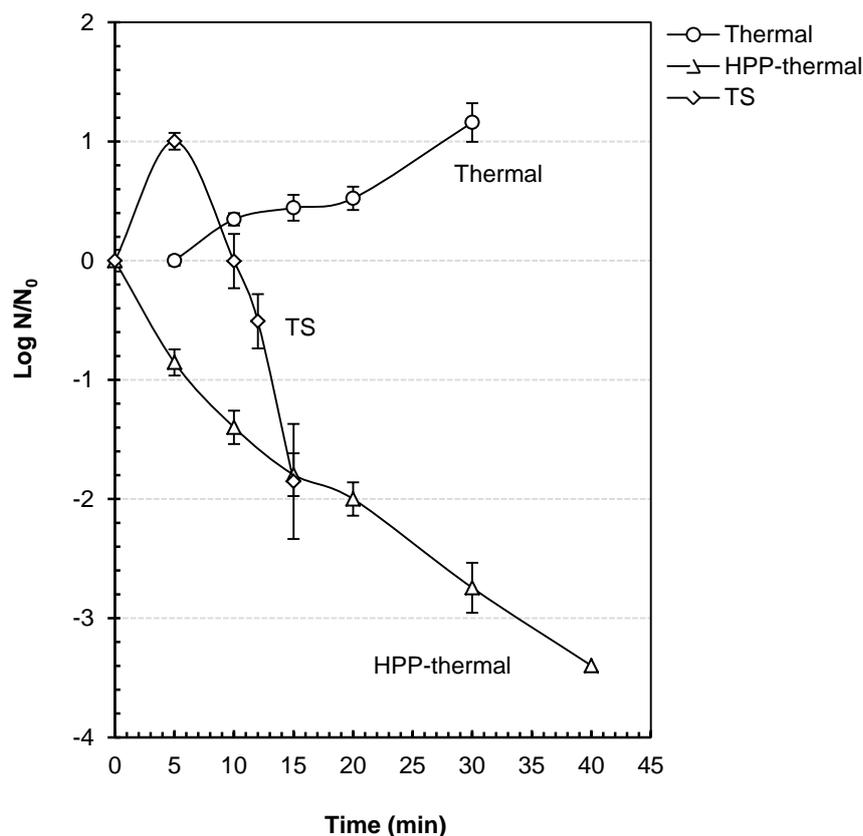


Figure 9-1 Thermal, 600 MPa HPP-thermal and thermosonication (24 kHz, 0.33 W/ml) inactivation of four week old *Byssochlamys nivea* ascospores in strawberry puree at 75°C.

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With respect to TS at 75°C, the ascospore activation shoulder registered during the first 10 min prior to inactivation, resulting in long processing time for the TS treatment. This makes TS not yet applicable for commercial applications, which require shorter times for better industrial productivity. However, ≥ 15 min TS process showed comparable or higher inactivation results than the HPP-thermal (1.8 log, 15 min). The results indicate higher susceptibility of the spores after the TS treatment, demonstrated by a higher inactivation rate with a sharp decrease in TS log survivors after the maximum activation shoulder peak was reached. The spore inactivation by 75°C TS as opposed to spore viable count increase by 75°C thermal treatment alone could be explained by the cell membrane damage caused by the cavitation bubbles generated by the ultrasonic waves which was enhanced by the heat, therefore resulting in spore destruction (Earnshaw, 1998; Evelyn & Silva, 2015a; Garcia et al., 1989; Nayak, 2014). López-Malo et al. (2005) also found the benefit of TS such as lower *D*-values for *A. flavus* and *P. digitatum* conidia inactivation in Saboraud broth between 45 and 52.5°C. Higher spore inactivation by TS vs. thermal was also found at around 70°C by Garcia et al. (1989) with *Bacillus subtilis* spores in milk and Evelyn and Silva (2015a) with psychrotrophic *Bacillus cereus* spores in skim milk and beef slurry. The benefit of ultrasound pretreatment to enhance the thermal inactivation of heat resistant bacterial spores such as *Clostridium perfringens* has also been reported (Evelyn & Silva, 2015b).

9.3.3 Modeling the 600 MPa HPP-thermal, thermosonication and thermal inactivation kinetics of *B. nivea* spores in strawberry puree

9.3.3.1 Modeling the 600 MPa HPP-thermal inactivation

The log survivors of *B. nivea* ascospores after 600 MPa HPP-thermal processing for up to 40 min are illustrated in **Figure 9-2**. The number of *B. nivea* spores reduced with processing

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time for all the temperatures (38, 50, 60, 75°C), with the fastest reduction occurring at 75°C. For example, after 10 min, reductions of 1.4 log, 0.8 log, 0.7 log, and 0.5 log at 75, 60, 50 and 38°C respectively were achieved. These results demonstrate the significant effect of high pressure temperature on the *B. nivea* ascospore inactivation. At 40 min the effect of temperature is more evident: while 0.7 log was registered at 38°C, a value of 3.4 log was observed for HPP at 75°C. These results confirm the benefit of the 600 MPa HPP-thermal method as a successful approach aiming at the inactivation of the heat resistant mould *B. nivea* ascospores in strawberry puree. HPP combined with $\geq 75^\circ\text{C}$ heat for a short processing time will be needed to achieve 5-log inactivation, suggesting a better quality of strawberry puree than exclusively thermally treated puree.

Based on the non-linearity observed in the log survivors, Weibull (Equation 9-1) and three parameter log logistic (Chen & Hoover, 2003) were initially attempted to model the log spore survival data. The log logistic model showed large standard errors in the estimated parameters at some temperatures (results not shown), thus the Weibull model was selected and the model performance and parameters estimated (b and n) are presented in **Table 9-1**. The Weibull model showed an $\text{MSE} \leq 0.013$ and $0.940 \leq R^2 \leq 0.997$. The Weibull b values (scale factors) increased from 0.15 at 38°C to 0.29 at 75°C, demonstrating that this parameter is temperature dependent ($R^2 = 0.90$). The Weibull n values (shape factors) were between 0.46 and 0.66 (≤ 1), indicating an upward concavity. These results are in agreement with past reports with other microorganisms, also obtaining non-linear inactivation and showing the Weibull model capable of predicting the inactivation results (Evelyn & Silva, 2015; Serment-Moreno et al., 2014; van Boekel, 2009; Wang et al., 2009). The inactivation of *Talaromyces avellaneus* mould ascospores by 500-600 MPa HPP at 17-60°C, also seemed to follow a non-linear pattern. However, fitting was only carried out with the conventional first order kinetics (Voldřich et al., 2004).

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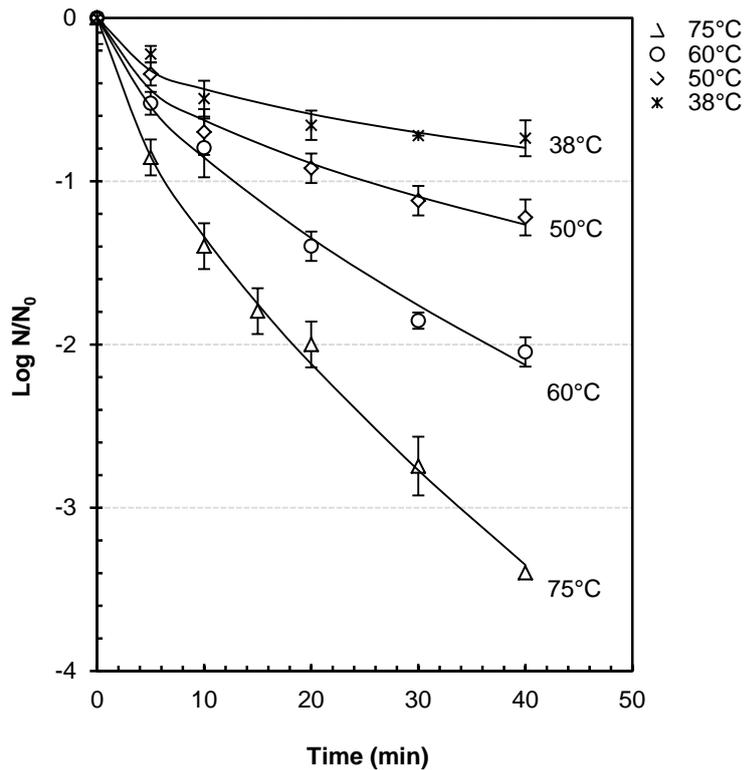


Figure 9-2 Weibull curve fitting for 600 MPa HPP-thermal inactivation of four week old *Byssochlamys nivea* ascospores in strawberry puree.

Table 9-1 Weibull model parameters estimation for the survival of four week old *Byssochlamys nivea* ascospores in strawberry puree after 600 MPa HPP-thermal processing.*

Temperature (°C)	<i>b</i>	<i>n</i>	<i>R</i> ²	MSE
75	0.29±0.02	0.66± 0.03	0.997	0.006
60	0.19±0.03	0.65±0.05	0.993	0.005
50	0.16±0.01	0.57±0.01	0.959	0.013
38	0.15±0.05	0.46±0.11	0.940	0.007

**b* and *n* are the Weibull scale and shape factors, respectively; low mean square errors (MSE) and *R*² close to 1 are indication of good fit; all the temperatures tested showing random residuals.

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9.3.3.2 Modeling the thermosonication inactivation

The log survivors of thermal pretreated (80°C, 15 min) strawberry puree by TS are shown in **Figure 9-3**. TS at 75°C, the maximum temperature supported by the ultrasound equipment, was the best. Short treatments did not inactivate the spores, since activation shoulders were observed for all TS temperatures tested (75, 70, and 65°C) with a maximum of 5 min at 75°C, 10 min at 70°C and 15 min at 65°C. The peaks in the log counts were followed by approximately linear spore inactivation. A higher spore increase for longer periods was obtained when lowering the TS temperature. Overall, while 15 min at 75°C achieved ≈ 2 log reductions, 35 min at 70°C and > 60 min at 65°C were required to obtain the same spore inactivation.

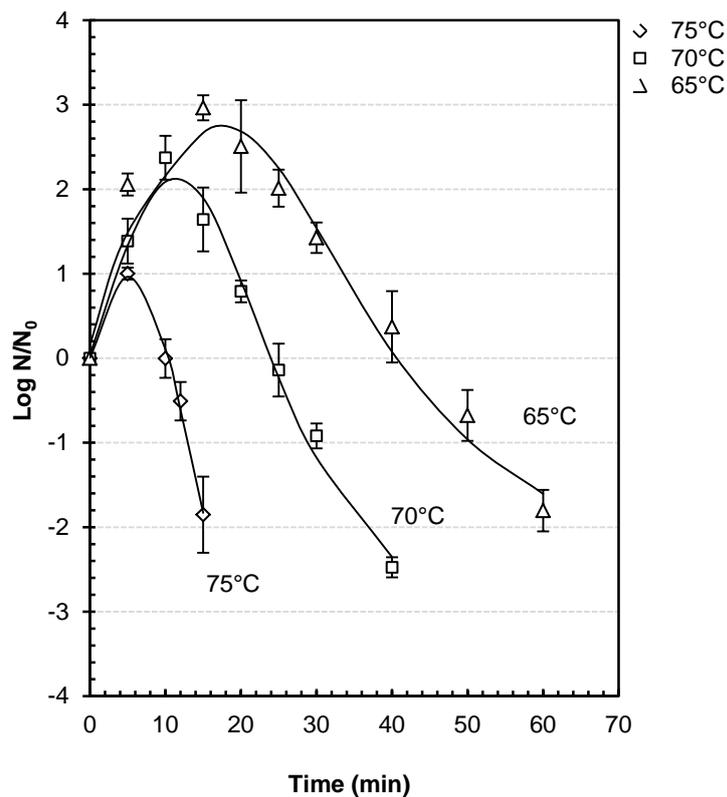


Figure 9-3 Lorentzian curve fitting for thermosonication (24 kHz, 0.33 W/ml) inactivation of four week old *Byssochlamys nivea* ascospores in strawberry puree submitted to a heat shock pretreatment.

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Due to the activation shoulders observed in all the TS spore survival curves, the first order kinetics was not appropriate and modeling was a challenge. Initially, four non-linear models (double Weibullian, Peleg, logistic and Lorentzian) were attempted. However, the double Weibullian and Peleg models (Equation 1-14 and Equation 1-15) suggested by Corradini et al. (2010) for heat activated *Bacillus* spores were inappropriate, presenting high standard errors for the estimated parameters (results not shown). On the contrary, the four parameter logistic (TableCurve 2D, version 5.01, SYSTAT Software Inc., Chicago, USA) and Lorentzian (Equation 9-2) models worked well. The Lorentzian distribution was a better model (0.025–0.248 MSE and 0.940–0.994 R^2), although the performance slightly decreased at 65°C (Table 9-2). The Lorentzian b parameters increased from 5.7 to 10.1 as the temperature was increased from 65 to 75°C, whereas the Lorentzian a , c , and d parameters decreased with the TS temperature, exhibiting temperature dependence ($R^2 \geq 0.81$). There has been little to no research carried out on the kinetic modeling of heat resistant mould ascospores such as *Byssochlamys spp.* by TS. López-Malo et al. (2005) and Coronel et al. (2011) attempted TS without prior thermal pretreatment and obtained first order kinetics for *P. digitatum* and *A. flavus* conidia spore inactivation as opposed to the non-linear Lorentzian model used in our study (Figure 9-3). This confirms that conidia spores are less resistant than sexually produced ascospores (Pitt & Hocking, 1997).

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Table 9-2 Lorentzian model parameters estimation for the survival of four week old *Byssochlamys nivea* ascospores in strawberry puree after thermosonication (24 kHz, 0.33 W/ml).*

Temperature (°C)	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>R</i> ²	MSE
75	-9.1± 6.1	10.1± 6.0	5.1± 0.3	15.9± 6.5	0.994	0.025
70	-3.9± 0.7	6.1± 0.6	11.6± 0.6	16.7± 2.5	0.983	0.072
65	-2.7± 1.2	5.7± 1.1	17.7± 1.5	24.0± 6.5	0.940	0.248

*The spores were submitted to heat shock pretreatment (80°C, 15 min).

a, *b*, *c* and *d* are the Lorentzian temperature dependent parameters (Equation 9-2): *a* is the baseline of the distribution; *b* is the rate of microbial inactivation; *c* is the the time (*t*) value at which the inactivation starts; *d* is the width of the distribution. Low mean square errors (MSE) and *R*² close to 1 are indication of good fit; all the temperatures tested showed random residuals.

9.3.3.3 Modeling the thermal inactivation

The log survivors of *B. nivea* ascospores after thermal processing at 85 and 90°C were plotted in **Figure 9-4**. The 90°C thermal process was successful in inactivating ≈ 5 log of *B. nivea* ascospores in strawberry puree after 8 min. This makes the commercial pasteurization conditions suggested for fruit juice preservation such as 85°C for 20 min inadequate for pasteurization processes aimed at *B. nivea* mould ascospores. Thus *B. nivea* requires 90-95°C for fruit pasteurization, which negatively affects the fruit product sensory quality (Silva et al., 2000) and may result in the loss of raw fruit nutrients such as antioxidants.

Since thermal survival curve at 85°C departed from linearity, only the first-order kinetic parameter at 90°C (i.e. 8 min) was able to be determined and this was compared with literature results. Aragão (1989) reported higher *D*_{90°C}-values (6.4 min) for *B. nivea* ascospores (isolated from strawberry pulp) in 15°Brix strawberry pulp as opposed to the 8°Brix puree used in our study. Kotzekidou (1997) found higher *D*_{90°C}-values (3.5 min) with

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B. nivea spore strains 102 and 162 in 16°Brix tomato juice. Engel and Teuber (1991) found much lower $D_{90^{\circ}\text{C}}$ -values (0.05–0.07 min) for *B. nivea* spores 6607 and 6611 in cream. These results indicate the influence of the strain and the food on the thermal resistance of *B. nivea* ascospores.

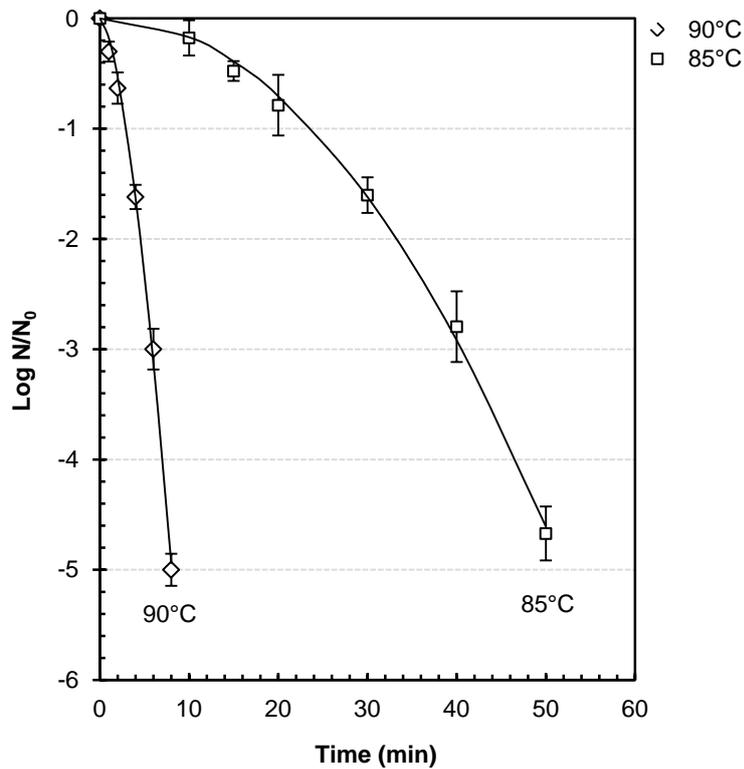


Figure 9-4 Thermal inactivation kinetics and Weibull curve fitting of four week old *Byssochlamys nivea* ascospores in strawberry puree.

The non-linear Weibull model was a better choice for modeling for both temperatures (85 and 90°C), thus the Weibull parameters were estimated (**Table 9-3**). The Weibull n values (shape factor) were more than 1, indicating downward concavity. Weibull distribution function was also reported by Sant'Ana et al. (2010a) to model *B. fulva* ascospore inactivation in clarified apple juice by thermal process.

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Table 9-3 Weibull model parameters estimation for the survival of four week old *Byssochlamys nivea* ascospores in strawberry puree after thermal processing.*

Temperature (°C)	<i>b</i>	<i>n</i>	<i>R</i> ²	MSE
90	$1.8 \times 10^{-1} \pm 3 \times 10^{-2}$	1.6 ± 0.01	0.998	0.010
85	$2 \times 10^{-3} \pm 4 \times 10^{-4}$	2.1 ± 0.07	0.998	0.007

* *b* and *n* are the Weibull scale and shape factors, respectively; low mean square errors (MSE) and *R*² close to 1 are indication of good fit; all the temperatures tested showed random residuals.

9.4 Conclusion

600 MPa HPP-75°C for a short time period (≤ 10 min) was a better method than 75°C thermosonication (TS) and 75°C thermal for the inactivation of *B. nivea* ascospores in strawberry puree, confirming the benefit of HPP technology. A reduction of approximately 1.4 log was obtained for HPP-75°C after 10 min in contrast to no inactivation for 75°C TS and 75°C thermal processes. A longer treatment time is still needed to achieve a 5 log inactivation at 600 MPa HPP-75°C processing conditions, thus a temperature of $\geq 75^\circ\text{C}$ should be used. However, the HPP-thermal process could be sufficient for typical loads (below 10^5 cfu/mL) of ascospore contamination in strawberry puree. The TS process might be applicable at longer treatment times (≥ 15 min) and higher temperatures ($>75^\circ\text{C}$). However, further research must be conducted to reduce the activation shoulders and design an ultrasound probe that can withstand higher temperatures. With respect to exclusively thermal processes, temperatures $\geq 90^\circ\text{C}$ are still required to achieve the efficient inactivation of spores. The Weibull model described the inactivation of *B. nivea* ascospores by the 600 MPa HPP-thermal and thermal processes, while Lorentzian model was more suitable for the TS. The results from this study show that the 600 MPa HPP-thermal process might be a better option for the preservation of fruit products prone to *B. nivea* ascospore contamination.

Chapter 10 Modeling the inactivation of *Neosartorya fischeri* ascospores in apple juice by high pressure, power ultrasound and thermal processing

Evelyn, Kim, H. J., & Silva, F. V. M. (2016). Modeling the inactivation of *Neosartorya fischeri* ascospores in apple juice by high pressure, power ultrasound and thermal processing. *Food Control*, 59, 530-537.

10. Power ultrasound, high pressure and thermal processing *Neosartorya fischeri* spores

Chapter abstract

Neosartorya fischeri is a mould that spoils acid foods and can produce mycotoxins. In this work, the efficacy of high pressure processing (HPP, 600 MPa) and power ultrasound (24 kHz, 0.33 W/mL) in combination with 75°C for the inactivation of four week old *N. fischeri* ascospores in apple juice was investigated and compared with 75°C thermal processing alone. The HPP-75°C process was the most effective technique for inactivating *N. fischeri* spores, resulting in 3.3 log reductions after 10 min vs. no inactivation for thermosonication (TS) and thermal processing. Unexpectedly, activation shoulders were observed during the TS process. Then, the effect of different temperatures on the ascospore inactivation in apple juice by HPP-thermal, TS and thermal processing was investigated, and the log survivors vs. time were modeled. Faster inactivation was achieved at higher temperatures for all the technologies tested, indicating the significant role of temperature for the spore inactivation, alone or combined with other processes. The Weibull model described the spore inactivation better by 600 MPa HPP-thermal (50, 60, 75°C) and thermal (85, 90°C), whereas Lorentzian was more appropriate for the TS treatment (65, 70, 75°C). In conclusion, HPP is the best food preservation technology due to higher spore inactivation in apple juice at the same temperature.

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10.1 Introduction

Extremely heat resistant ascospores from moulds *Byssochlamys*, *Neosartorya*, and *Talaromyces* have been found (Hocking & Pitt, 1984; Silva & Gibbs, 2004; Silva & Gibbs, 2009; Silva et al., 2014). These are often associated with the spoilage of pasteurized fruit products such as juices, purees, jellies, jams, and canned fruits (Beuchat, 1998; Pitt & Hocking, 1997; Silva et al., 2014). *Neosartorya fischeri* (anamorph *Aspergillus fischerianus*) is also a public health concern because of its capacity to produce mycotoxins terrein, fumitremorgins A and B, and verruculogen (Frisvad & Samson, 1991; Misawa et al., 1962; Nielsen et al., 1989; Tournas, 1994). This species is widely distributed in soil (Pitt & Hocking, 1997) and was first isolated from canned strawberries in 1963 (Kavanagh et al., 1963). *N. fischeri* can grow at temperatures between 10 and 52°C (the optimal temperature is around 26–45°C), in oxygen levels as low as 0.1 % at 25°C (Nielsen et al., 1989), and a broad range of pH (3 to 8) as most fungi (Pitt & Hocking, 1997). The extremely heat resistant ascospores formed by the teleomorphs or sexual reproductive stage survive 85°C for 10 min (Houbraken et al., 2012) and drought (< 0.5% relative humidity) (Wyatt, 2014). Pitt and Hocking (1997) reported that the degree of heat resistance of ascospores of *N. fischeri* is comparable with that of many bacterial spores, and is higher than that of *Byssochlamys fulva* ascospores, the most heat resistant mould ascospores known. The heat resistance of *N. fischeri* also increased with the ascospores age (Slongo et al., 2009), with 25 day old ascospores exhibiting changes in their ultrastructure and chemical composition when compared with 11 day old ascospores (Conner et al., 1987). Based on the ascospore ornamentation, three varieties of *N. fischeri* (*var. fischeri*, *var. glabra*, and *var. spinosa*) have been identified (Samson et al., 1990).

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Temperatures between 85 and 95°C are commonly used to prolong the shelf life of fruit juices (Sant'Ana et al., 2010a). However, it has been recognized that the thermal process may activate the dormant ascospores of moulds which subsequently cause deterioration, hence resulting in economic loss (Katan, 1985; Slongo & Aragão, 2006; Splittstoesser et al., 1993). Increasing the intensity (temperature or processing time) of the heat treatment is not desirable, due to quality reasons and consumer demands for 'fresh-like' fruits. Food preservation by non-thermal methods such as high pressure processing (HPP) and power ultrasound in combination with mild heat have been investigated due to reduced treatment temperatures and processing times (Evelyn & Silva, 2015a, 2015b, 2015c). HPP is an established commercial food processing technology and can be combined with temperature for the inactivation of resistant microbial spores (Sarker et al., 2015; Wilson et al., 2008) and enzymes (Sulaiman et al., 2015a). With respect to the heat resistant mould ascospores such as *Byssochlamys fulva*, *Byssochlamys nivea*, *N. fischeri*, *Neosartorya spinosa*, *Talaromyces avellaneus*, *Talaromyces macrosporus*, the efficacy of 600-900 MPa of HPP pressure (cycle, oscillatory or continuous) in conjunction with heat (25–90°C) using 3 to 15 week old spores was up to 5.7 log reductions (Butz et al., 1996; Chapman et al., 2007; Ferreira et al., 2009; Hocking et al., 2004; Maggi et al., 1994; Palou et al., 1998; Reyns et al., 2003; Voldřich et al., 2004). Among the research studies, only Voldřich et al. (2004) modeled the inactivation kinetics for *Talaromyces* spores, reporting a first order kinetics and a decrease in the decimal reduction time (*D-value*) at 600 MPa as the temperature increased from 17 to 60°C.

Power ultrasound (frequency ranging from 20 to 100 kHz) is a promising non-thermal technology for food preservation. This technology relies on the application of pressure waves called cavitation to the food/beverage, causing microbial cell death (Feng & Yang, 2011b; Piyasena et al., 2004). Power ultrasound has been combined with mild heat (thermosonication, TS) to inactivate bacterial and fungal vegetative cells and spores, and a

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synergistic effect was observed (Earnshaw et al., 1995; Evelyn & Silva, 2015a; Garcia et al., 1989; López-Malo et al., 2005; Ordoñez et al., 1987; Zenker et al., 2003). With respect to mould ascospores, Jimenez-Munguia et al. (2001) reported that the inactivation of *Penicillium digitatum* and *Aspergillus flavus* ascospores by TS (20 kHz, 40-45°C) in sabouraud broth increased with the treatment time and amplitude. The addition of boiling chips and air bubbles to the broth medium reduced the *D*-values. López-Malo et al. (2005) found lower *D*-values for TS (20 kHz, 40-60°C) inactivation of *P. digitatum* and *A. flavus* ascospores in sabouraud broth compared to the thermal treatment alone. The authors also concluded an increase in the ultrasound amplitude and decrease in pH resulted in lower *D*-values. Coronel et al. (2011) proposed the Weibull model for the inactivation of *A. flavus* ascospores in broth by TS combined with vanillin. No studies have been carried out on the TS inactivation and kinetic modeling of heat resistant mould ascospores relevant to the fruit industry, such as *N. fischeri*. In particular, no work using fruit products has been reported, being broth inoculated with microorganisms the medium processed.

Due to the importance of *N. fischeri* spores in high acid fruit products, more research is needed to provide models for the HPP-thermal and TS inactivation and design appropriate processes. Therefore, in this research the inactivation of *N. fischeri* ascospores in apple juice by HPP-thermal and TS processes were carried out, and the main objectives were as follows: (i) to compare the HPP-thermal, TS and thermal inactivation of ascospores at 75°C; (ii) to model the 600 MPa HPP-thermal inactivation of ascospores; (ii) to model the TS inactivation of ascospores; and (iv) to model the thermal inactivation of ascospores.

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10.2 Materials and methods

10.2.1 Microbiology

10.2.1.1 Mould

N. var fischeri JCM 1740 was obtained from the Japan Collection of Microorganism (= ATCC 1020, DSM 3700, CBS 101.12, IAM 13864). This strain was isolated from canned apples in the USA.

10.2.1.2 Ascospore production

Ascospores of *N. fischeri* were obtained after growth for four weeks at 30°C on malt extract agar (MEA, pH 5.5-Difco, North Ryde, Australia). The spores were collected by flooding the surface of the culture plates with 5 mL sterile distilled water (SDW), and gently rubbing the agar surface with a sterile bent glass rod. The spore suspension was subsequently filtered through layers of gauze to remove any remaining hyphal fragments. Spore pellets were obtained after centrifugation in sterile SDW at 4,000×g, 15 min, 4°C and the procedure was repeated three times. The final spore suspension (containing asci and ascospores) was then stored at 2°C in SDW containing glass beads until use.

10.2.1.3 Apple juice inoculation and preparation

Apple juice (pH 3.7, 10.6 ± 0.1°Brix) was obtained from a local supermarket and used as the treatment medium to suspend *N. fischeri* ascospores. For HPP-thermal and thermal experiments, aliquots (ca. 0.5 mL) of *N. fischeri* spore solution were inoculated into 3.0 mL of apple juice to yield an initial juice spore concentration of $\approx 10^5$ – 10^6 cfu/mL of juice. The inoculated juice was packed in 8x8 cm food grade retort pouches (Cas-Pak, New Zealand) composed of polyester coated with silicon oxide, and laminated to nylon and cast

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polypropylene (PET-SIOX(12)//ON(15)//RCPP(70)). The pouches can withstand temperatures of up to 130°C which are suitable for thermal processing and high pressure applications. Regarding the TS experiments, *N. fischeri* spore solution was inoculated aseptically by adding a small volume of inoculum to the apple juice contained in a round-bottom flask (5 mL of spore solution into 95 mL of apple juice) before the TS thermal pretreatment. The initial spore concentration after the thermal pretreatment and before TS was $\approx 10^5$ – 10^6 cfu/mL of juice.

10.2.1.4 Spore enumeration

The mould ascospore concentration in apple juice before and after processing (thermal, HPP and TS) was determined by spread plating onto MEA. A heat shock (75°C, 5 min) of raw unprocessed apple juice was required to obtain the initial ascospore count (1.3×10^7 cfu/mL) in the untreated juice for HPP and thermal processes (Katan, 1985; Splittstoesser et al., 1993). N_0 for TS process was thermally pretreated apple juice at 80°C for 30 min. Prior to plating, spore samples were decimal diluted using 9 mL 0.1% (w/v) sterile buffered peptone water (BPW; Difco, Becton Dickinson, USA). Each tube dilution was mixed repeatedly using a high speed vortex mixer to yield a uniform spore suspension, and plated twice. The plates were then incubated at 30°C for 3 to 5 days until visible colonies were formed. Plates with 20 to 100 colonies were used for enumeration. Ascospore concentration was expressed in cfu per milliliter (cfu/mL) of juice sample.

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10.2.2 Processing

10.2.2.1 Experimental design

In the first experiment, the effectiveness of mould ascospores inactivation in apple juice at 75°C by 600 MPa HPP-thermal and TS vs. sole thermal processing for treatment times up to 40 min were compared. The long processing time was chosen for HPP-thermal and TS due to heat resistance of the spores, so that the processes were able to reduce significantly the number of spores. At least two independent experiments were carried out for each HPP-thermal, thermal or TS condition, and duplicate samples were processed for each treatment time. A *t*-test was used to compare the ascospore numbers ($\log N/N_0$) by different methods at the same processing time and to check if the processing time for each processing method (TS, HPP-thermal and thermal) resulted in significantly different survivors (Statistica 8, Statsoft Inc., USA).

In the other experiments, HPP-thermal, TS and thermal treatments of apple juice were carried out at different temperatures and treatment times, as described in the following sections. The logarithmic number of survivors ($\log N/N_0$) versus time was plotted for each survival experiment to model and to estimate the kinetic parameters. Two samples were processed for each time and three survival experiments were carried out for each treatment temperature. Detailed procedures for apple juice HPP-thermal, TS and thermal treatments are explained as follows.

10.2.2.2 High pressure combined thermal (HPP-thermal) processing

A QFP 2L-700 high pressure food processing system from Avure Technologies-USA distilled water as the working fluid was also used for the HPP-thermal treatments (Chapter 2, 4, 5, and 7). High pressure at 600 MPa combined with temperatures of 50, 60 and 75°C were

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used with processing times up to 40 min. 600 MPa high pressure treatment at room temperature (38°C) was also carried out. No prior heat treatment of the inoculated apple juice was carried out before the HPP experiments, since 500-600 MPa pressures have been recognized as the most effective method for activation of *T. macrosporus* ascospores (Dijksterhuis & Teunissen, 2004; Reyns et al., 2003). Only the constant pressure phase was accounted for HPP processing time. The inoculated apple juice contained in the plastic pouches was submitted to the different high pressure processing conditions. The HPP treated samples were submerged into an ice water bath prior to spore enumeration.

10.2.2.3 Thermosonication

Ultrasonic processor described previously in Chapter 3 and 6 with a sonotrode tip of 3 mm was used for all the thermosonication (TS) experiments (210 μm , 460 W/cm^2 , 0.33 W/mL apple juice). Prior to the TS experiments, the round bottom-flask containing 100 mL of apple juice inoculated with the mould was thermally processed at 80°C for 30 min in a water bath, inside a laminar flow hood, to avoid aerial contamination. A 200 mL round bottom-flask with a narrow neck was used for TS to minimize the water evaporation from the juice sample. This heat shock process can break the dormant states of mould spores and increase the number of spores able to germinate (Sussman, 1976), leading to a loss of stability during the transition to the germinating stage (Eicher & Ludwig, 2002). This could possibly increase sensitivity of the moulds to the TS treatments. Preliminary experiments revealed that this procedure allowed a reduction of 15 min in the TS treatment time for the same spore inactivation in apple juice. Then, the TS treatments were carried out in the thermostatic water bath inside the laminar flow hood. The temperature of the juice sample during processing was monitored and the thermostatic water bath was used to keep it at the desired value during the process. At each temperature (65, 70, and 75°C), the flask containing the pre-heated apple juice sample

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was placed in the water bath and the pre-sterilized sonotrode was readily submerged in the juice more or less 1 cm from the flask bottom. Ultrasonic treatments were carried out for up to 70 min depending on the TS treatment temperatures. Juice samples (0.5 mL) were taken from the flask at pre-specified intervals, cooled in an ice water bath for subsequent spore survivor counts, which were immediately carried out.

10.2.2.4 Thermal processing

Thermal resistance of *N. fischeri* ascospores was carried out using the same thermal method described above (Evelyn & Silva, 2015a, Chapter 6), except at temperatures of 75, 85 and 90°C. Initially, the thermostatic water bath was heated until the treatment temperature was reached. The inoculated apple juice samples contained in the plastic pouches were then submerged into the preheated thermostatic water bath, and heated for various times. Treated samples were taken out at different time intervals and kept in an ice water bath until microbial enumeration.

10.2.3 Modeling the *N. fischeri* ascospore inactivation in apple juice

First order kinetics (Equation 1-1) was used to model the thermal inactivation results in order to compare with literature results, whereas Weibull equation was attempted to model the log survivors by HPP and thermal (Equation 1-6). The three parameters of the log-logistic (A, σ, τ) model are shown in Equation 1-8, and also used to compare the Weibull model for modeling the HPP inactivation results. The four parameter Lorentzian distribution (Equation 9-1) and logistic (Equation 10-1) curve peak functions (TableCurve 2D, version 5.01, SYSTAT Software Inc., Chicago, USA) were also attempted for describing log survivors of the mould ascospores to compare double Weibullian and Peleg models used (Equation 1-14

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and Equation 1-15, respectively). MSE, R^2 , A_f , and the temperature dependence of the parameters estimated described previously in Chapter 1 were used to evaluate the performance of those models.

$$\log \frac{N}{N_0} = a + \frac{b}{1 + \left(\frac{t-c}{d}\right)^2} \quad (10-1)$$

where the parameters a , b , c , and d of the logistic function was the same with previously described for the Lorentzian equation (Chapter 9).

10.3 Results and discussion

10.3.1 Ascospore activation and activation shoulders

Figure 10-1 to 10-4 show the effects of different food preservation technologies on the log number of *N. fischeri* ascospores. With respect to thermal processing, a slight activation (increase in the number of spores with the processing rather than reduction) in the *N. fischeri* ascospores was registered at 75°C (0.6 log-15 min, **Figure 10-1**), but not at 85 and 90°C. With fungal spores, activation is a mechanism caused by the application of heat, a chemical or other factor under certain conditions, which causes breaking of the spore dormancy for germination, leading to an increase in the viable counts by several logs (Dijksterhuis, 2007; Sussman, 1976; Tournas, 1994). Beuchat (1986) also observed *N. fischeri* FRR 1833, FRR 2334, and FRR 110483 ascospore activation up to 4.5 logs in buffer by 75 and 80°C thermal processes during the first 15 to 45 min. Other investigators reported lower spore activation (0.25–0.8 log) between 10 and 50 min with *N. fischeri* (E7, C3, and isolated from spoiled papaya fruit) spores in buffer at the same temperatures which is similar to our results (Amaeze & Ugwuanyi, 2011; Rajashekhara et al., 1996). Slongo and Aragão (2006) found

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that temperature of 85°C between 10 to 20 min was required for maximum activation of *N. fischeri* ascospores in pineapple and papaya nectar, however the level of activated spores was not mentioned. Activation of spores has also been reported with thermal processed (105–120°C, 5–30 min) bacterial spores such as *Bacillus stearothermophilus* spores (Corradini et al., 2010; Finley & Fields, 1962).

Regarding thermosonication treatments, we have registered activation shoulders of at least +2.4 log after TS (65, 70 and 75°C) during the first 10 to 30 min of processing (**Figure 10-1** and **Figure 10-3**), demonstrating the mould spores are more sensitive to the ultrasound + heat than heat alone. Sonication is recognized as a tool to help separate fungal spore clusters and to produce suspensions of free ascospores (Amaeze, 2012; Beuchat, 1986; Michener & King, 1974). This can also lead to higher activation and viable counts in the TS compared to thermal alone. In sum, ultrasound played a significant role in the spore activation.

No activation was observed in the 600 MPa HPP-thermal survival lines (**Figure 10-1** and **Figure 10-2**). This observation is in agreement with previous inactivation works with *B. nivea* ascospores employing sustained pressure treatment of 600 MPa HPP and 40–70°C thermal (Butz et al., 1996; Ferreira et al., 2009) although almost no spore reduction observed at 40°C in Ferreira et al. (2009). Some authors reported that the combination of 600–900 MPa with temperatures $\leq 21^\circ\text{C}$ for treatment time ≤ 25 min activated up to 2.4 log of not only four weeks but also older spores of *Byssochlamys spp.*, *Neosartorya spp.*, and *T. macrosporus* (Chapman et al., 2007; Hocking et al., 2004; Maggi et al., 1994; Palou et al., 1998), which was attributed to the spores release from the ascus. However, this behavior was not observed in our experiments, probably due to the combination of HPP with heat ($\geq 21^\circ\text{C}$).

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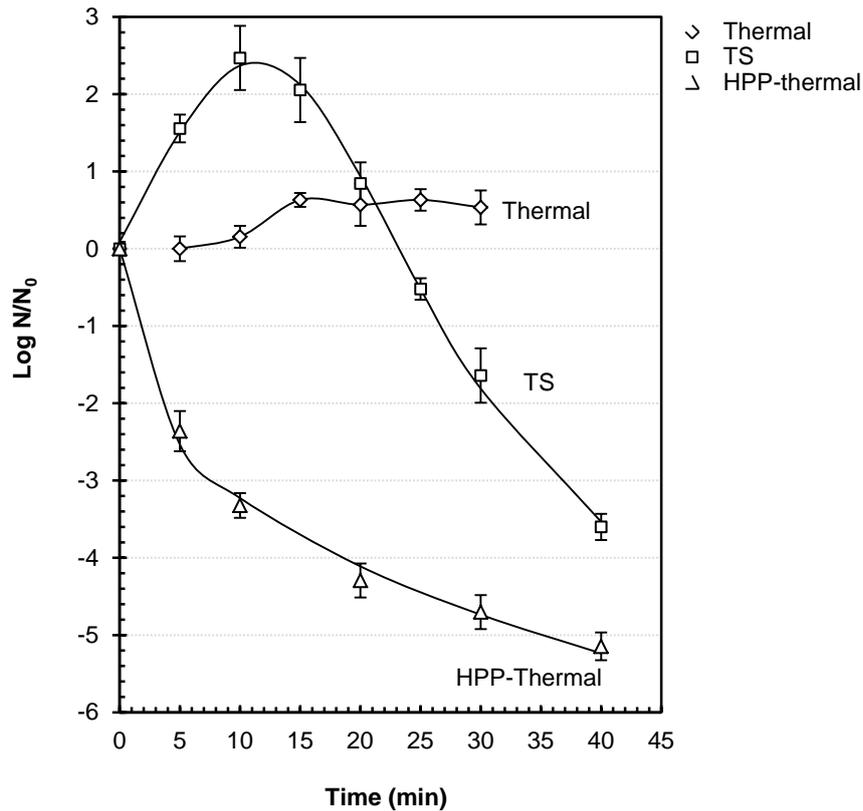


Figure 10-1 Thermal, 600 MPa HPP-thermal and thermosonication (24 kHz, 0.33 W/ml) inactivation of four week old *Neosartorya fischeri* ascospores in apple juice at 75°C.

10.3.2 HPP-thermal, TS and thermal inactivation of *N. fischeri* ascospores in apple juice at 75°C

The effects of 600 MPa high pressure combined with thermal (HPP-thermal), thermosonication (TS) with prior thermal treatment, and thermal processing at 75°C on *N. fischeri* ascospores for up to 40 min are illustrated in **Figure 10-1**. The HPP-thermal was the best method for the ascospore inactivation, since the spores reduced steadily with the processing time reaching nearly 4.3 log after 20 min ($p < 0.05$), and declining more slowly after 20 min. Regarding the 75°C TS, the spore numbers increased (activation), reaching a maximum (+2.4 log, $p < 0.05$) at 10 min, which was followed by a steady linear inactivation. For thermal, a significant spore activation was observed after 15 min ($p < 0.05$), and then

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remained constant until 30 min. Overall, for a 30 min process at 75°C, 4.7 log reductions in *N. fischeri* spores for HPP-thermal vs. 1.6 log reductions for TS vs. 0.5 log increase for thermal were obtained ($p < 0.05$). It has been known that short time treatments at very high pressures (400–800 MPa) can induce maximal spore activation and quicker spore germination, followed by inactivation by mild heat or pressure (Black et al., 2007a; Dijksterhuis & Teunissen, 2004). This could be the cause of the quicker inactivation by HPP-thermal treatment than TS or thermal processing. However, for 600 MPa-75°C long treatment times (>40 min) are still needed to achieve 5 log inactivation, as recommended by US Food Drug and Administration (USFDA, 2001) for fruit juice pasteurization.

With respect to TS at 75°C, the spore activation shoulder registered during the first 10 min, makes the TS not feasible for a commercial application, which requires shorter times for better industrial productivity. However, ≥ 25 min TS process showed higher inactivation results (0.5 log) than thermal (no inactivation). The results indicate higher susceptibility of the spores after the TS activation. Higher spore inactivation by 75°C TS as opposed to activation by 75°C thermal alone could be explained by the cell membrane damage caused by the cavitation bubbles generated by the ultrasonic waves, which was enhanced by the heat, therefore resulting in spore killing (Evelyn & Silva, 2015a; Garcia et al., 1989; Nayak, 2014). López-Malo et al. (2005) also observed the effectiveness of TS with *A. flavus* ascospores in sabouraud broth. Higher spore inactivation by TS vs. thermal was also found at around 70°C by Garcia et al. (1989) with *Bacillus subtilis* spores in milk and Evelyn and Silva (2015) with psychrotrophic *Bacillus cereus* spores in skim milk and beef slurry. The benefit of ultrasound pretreatment to enhance the thermal inactivation of heat resistant bacterial spores such as *Clostridium perfringens* has also been reported (Evelyn & Silva, 2015b; Feng & Yang, 2011b). Although overall the 75°C was the best temperature for the TS inactivation, long

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spore activation (around 10 min) makes the TS process not feasible for a commercial application, as opposed to HPP technology.

10.3.3 Modeling the 600 MPa HPP-thermal inactivation of *N. fischeri* ascospores in apple juice

The log survivors of *N. fischeri* ascospores by 600 MPa HPP-thermal for up to 40 min is illustrated in **Figure 10-2**. The number of *N. fischeri* spores reduced with the processing time for each treatment temperature (38, 50, 60, 75°C), with the fastest reduction occurring at 75°C. For example, 3.3 log after 10 min at 75°C compared to 1.0–1.6 log at 38–60°C. These results demonstrate the significant effect of high pressure combined with thermal on the *N. fischeri* ascospores. As the processing temperature increased from 38 to 75°C, the spore inactivation after 40 min also increased from 1.4 log to 5.2 log, also indicating the significant role of temperature on the spore inactivation. These results confirm the benefit of the 600 MPa HPP-thermal as a successful approach for a commercial application aiming the inactivation of the heat resistant mould *N. fischeri* ascospores in apple juice.

Based on the non-linearity observed in the survival lines, Weibull (Equation 1-6) and three parameter log logistic (Equation 1-8) were attempted to model the spore survival data. Both worked well, the Weibull model performance and the parameters estimated (b and n) are presented in **Table 10-1**. The Weibull model showed $MSE \leq 0.021$, $R^2 \geq 0.982$, and A_f values of 1.01-1.07. The Weibull b values (scale factors) increased from 0.40 at 50°C to 1.44 at 75°C, demonstrating this parameter is temperature dependent within this range of temperatures ($R^2 = 0.92$). The Weibull n values (shape factors) were between 0.20 and 0.48 (≤ 1), indicating an upward concavity. These results are in agreement with past reports with

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other microorganisms. A weak temperature dependence of n values was observed, which is in agreement with Bermúdez-Aquirre and Corradini (2012), and van Boekel (2002). When using HPP technology, non-linear inactivation of spores is observed and Weibull can often predict the inactivation results well (Buzrul et al., 2005; Serment-Moreno et al., 2014; van Boekel, 2009; Wang et al., 2009). The inactivation of *T. avellaneus* mould ascospores by 500-600 MPa HPP at 17-60°C, the only kinetic modeling reported with the mould ascospores, also seemed to follow a non-linear pattern. However, fitting was only carried out with the conventional first order kinetics (Voldřich et al., 2004).

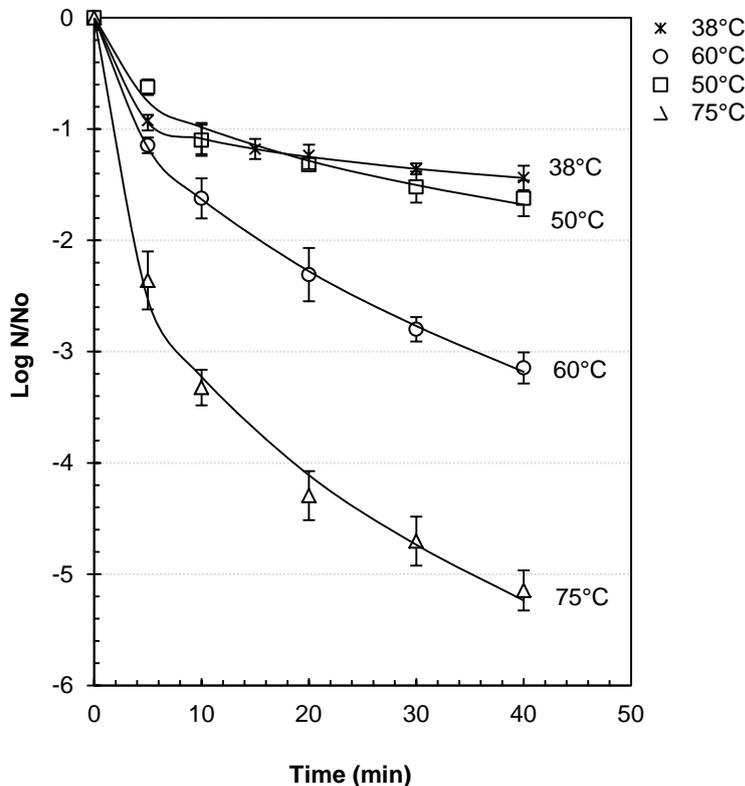


Figure 10-2 Weibull curve fitting for 600 MPa HPP-thermal inactivation of four week old *Neosartorya fischeri* ascospores in apple juice.

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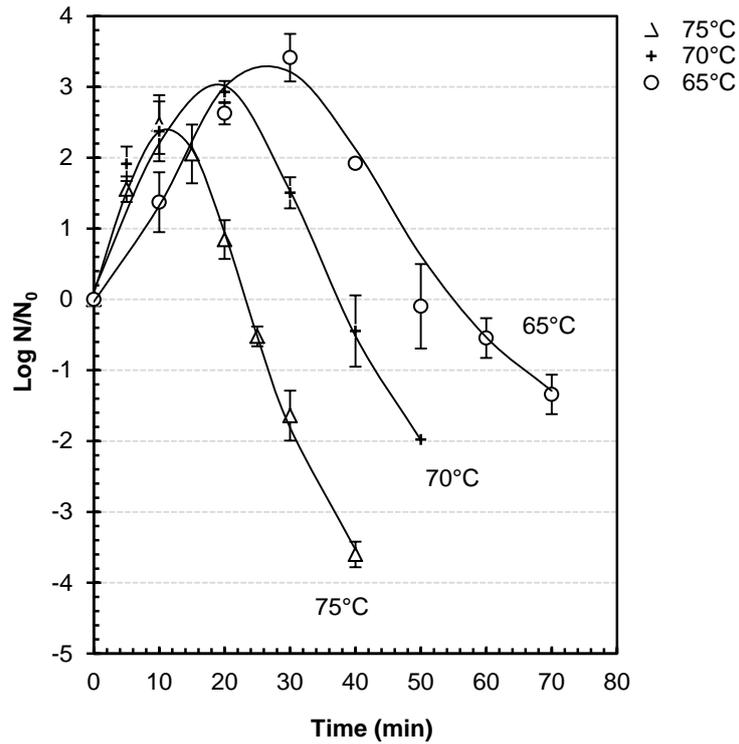


Figure 10-3 Lorentzian curve fitting for thermosonication (24 kHz, 0.33 W/ml) inactivation of four week old *Neosartorya fischeri* ascospores in apple juice.

Table 10-1 Weibull model parameters estimation for the survival of four week old *Neosartorya fischeri* ascospores in apple juice after 600 MPa HPP-thermal processing.*

Temperature (°C)	<i>b</i>	<i>n</i>	<i>R</i> ²	MSE	<i>A_f</i>
75	1.44±0.12	0.35±0.03	0.996	0.021	1.03
60	0.54±0.02	0.48±0.01	0.999	0.001	1.01
50	0.40±0.07	0.39±0.05	0.982	0.009	1.07
38	0.67±0.02	0.20±0.01	0.999	0.0003	1.01

**b* and *n* are the Weibull scale and shape factors, respectively (Equation 1-6); low mean square errors (MSE), and *R*² and *A_f* close to 1 are indication of good fit, with all the temperatures tested showed random residuals.

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10.3.4 Modeling the TS inactivation of *N. fischeri* ascospores in apple juice

The log survivors of thermal pretreated (80°C, 30 min) apple juice containing the *N. fischeri* ascospores by TS are shown in **Figure 10-3**. As mentioned, the maximum temperature supported by the ultrasound equipment (TS at 75°C), was the best temperature. Activation shoulders were observed for all TS temperatures tested (75, 70, and 65°C) with a maximum at 10 min for 75°C, 20 min for 70°C and 30 min for 65°C, followed by approximately linear spore inactivation until 40 min. Longer and higher spore activation were obtained as the TS temperature was reduced from 75 to 65°C. The better spore reduction by 75°C TS compared to 65°C TS and 70°C TS shows the role of temperature in this process. Overall, while 30 min at 75°C achieved ≈ 2 log reductions, 50 min at 70°C and > 80 min at 65°C were required to obtain the same spore inactivation.

Due to the activation shoulders as well as tails observed in all the TS spore survival curves, the first order kinetics was not appropriate. Initially, four non-linear models (double Weibullian, Peleg, log-logistic and Lorentzian) were attempted to describe the ascospore inactivation in apple juice. However, the double Weibullian and Peleg's models (Equation 1-14 and Equation 1-15) suggested by Corradini et al. (2010) for heat activated *Bacillus* spores were inappropriate presenting high A_f and standard errors for the estimated parameters (results not shown). On the contrary, logistic (four parameters, Equation 9-1) and Lorentzian (Equation 9-2) worked well. The Lorentzian distribution was a better model, presenting 0.02–0.09 MSE, 0.971–0.997 R^2 , and 1.04–1.26 A_f (**Table 10-2**). The Lorentzian b parameters increased from 6.3 to 8.5 as the temperature was increased from 65 to 75°C, whereas the Lorentzian a , c , and d parameters decreased with the processing temperatures, exhibiting temperature dependence ($R^2 \geq 0.90$). There has been little to no research carried out on kinetic modeling of heat resistant mould ascospores such as *Neosartorya spp.* by TS,

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therefore there are no other results to compare. López-Malo et al. (2005) and Coronel et al. (2011) attempted TS without prior thermal pretreatment and demonstrated the first order kinetics for *P. digitatum* and *A. flavus* ascospore inactivation as opposed to the non-linear Lorentzian model used in our study as a result of the activation shoulders and tails registered in the *N. fischeri* spore survival curves (**Figure 10-3**). This suggests those mould spores are less resistant and dormant than the *N. fischeri* spores.

Table 10-2 Lorentzian model parameters estimation for the survival of four week old *Neosartorya fischeri* ascospores in apple juice after thermosonication (24 kHz, 0.33 W/ml).*

Temperature (°C)	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>R</i> ²	MSE	<i>A_f</i>
75	-6.1± 0.40	8.5± 0.37	11.5± 0.22	18.6±1.06	0.997	0.018	1.04
70	-4.9± 0.80	7.9± 0.75	18.3±0.45	23.9±2.70	0.997	0.029	1.05
65	-3.1± 0.63	6.3± 0.57	27.6±0.67	26.4±3.38	0.971	0.091	1.26

*The spores were submitted to heat shock pretreatment (80°C, 15 min).

a, *b*, *c* and *d* are the Lorentzian temperature dependent parameters (Equation 9-2); *a* is the baseline of the distribution (that corresponds to the residual-survival-potentially observed); *b* is the rate of microbial inactivation; *c* is the the time (t) value at which the inactivation starts; *d* is the width of the distribution.; low mean square errors (MSE), and *R*² and *A_f* close to 1 are indication of good fit; all the temperatures tested showed random residuals.

10.3.5 Modeling the thermal inactivation of *N. fischeri* ascospores in apple juice

The log survivors of *N. fischeri* ascospores after thermal processing at 85 and 90°C were plotted in **Figure 10-4**. The 90°C thermal process was able to inactivate 4.8 log of *N. fischeri* ascospores in apple juice after 20 min. This result indicates the commercial pasteurization conditions of 77 to 88°C for 25 to 30 s, suggested for apple juice preservation (Moyer & Aitken, 1980), are far from the minimum pasteurization required for pasteurizing apple juice prone to contamination by *N. fischeri*. Thus, this mould requires 90°C and ≥20 min or

10. Power ultrasound, high pressure and thermal processing *Neosartorya fischeri* spores

equivalent for apple juice pasteurization. This process negatively affects the fruit product sensory quality and may result in the loss of raw fruit nutrients such as antioxidants (Silva et al., 2000).

The non-linear Weibull model was a good model to describe the survivors ($0.998\text{--}0.999 R^2$, $0.001\text{--}0.005$ MSE, $1.05\text{--}1.36 A_f$), and the parameters were estimated (**Table 10-3**). The Weibull n values (shape factor) were more than 1, indicating downward concavity. The Weibull model has been used by Sant'Ana et al. (2010a) for the thermal inactivation of *Byssochlamys fulva* ascospores in apple juice. The majority of previous thermal studies found non-linearities for *N. fischeri* log survivors (Gumerato, 1995; Salomão et al., 2007; Slongo & Aragão, 2006; Tournas & Traxler, 1994), but used first order or linearized first order model proposed by Alderton & Snell (1979) to estimate the kinetic parameters. Although the error is higher and fitting not so good ($0.924 R^2$, 0.365 MSE, $1.27 A_f$), the first order $D_{90^\circ\text{C}}$ of 4.9 min was also determined to be able to compare with past results. This value was lower than the values (between 9.9 and 23.4 min) reported earlier for *N. fischeri* ascospores in tomato and pineapple juice (Amaeze, 2012; Kotzekidou, 1997), confirming the influence of the suspending medium and the strain on the heat resistance of *N. fischeri* ascospores.

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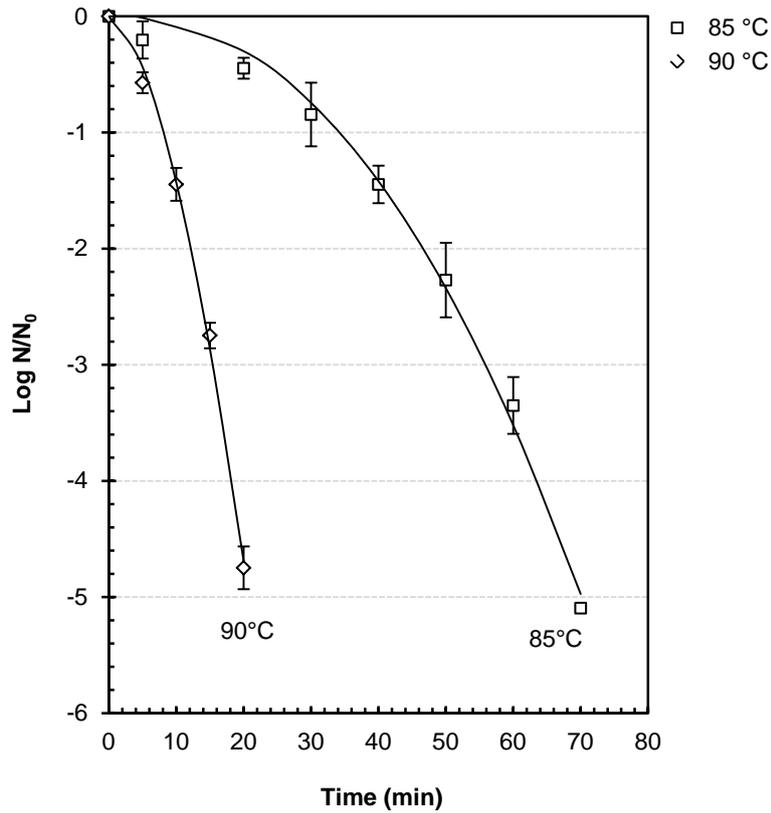


Figure 10-4 Thermal inactivation kinetics and Weibull curve fitting of four week old *Neosartorya fischeri* ascospores in apple juice.

Table 10-3 Weibull model parameters estimation for the survival of four week old *Neosartorya fischeri* ascospores in apple juice after thermal processing.*

Temperature (°C)	<i>b</i>	<i>n</i>	<i>R</i> ²	MSE	<i>A_f</i>
90	$3 \times 10^{-2} \pm 7 \times 10^{-3}$	1.71 ± 0.09	0.997	0.012	1.07
85	$4 \times 10^{-4} \pm 2 \times 10^{-4}$	2.24 ± 0.12	0.995	0.019	1.52

* *b* and *n* are the Weibull scale and shape factors, respectively; low mean square errors (MSE), and *R*² and *A_f* close to 1 are indication of good fit.

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10.4 Conclusion

600 MPa HPP-75°C was a better method than 75°C thermosonication (TS) and sole 75°C thermal for the inactivation *N. fischeri* ascospores in apple juice, confirming the benefit of HPP technology. Approximately 3.3 log reduction was obtained for HPP-75°C after 10 min in contrast to no inactivation for 75°C TS and 75°C thermal processes. Long treatment time is still needed to achieve a 5 log inactivation under the 600 MPa HPP-75°C. Thus, future studies should focus on the optimization of processing parameters to inactivate the *N. fischeri* spores or combination with other method (hurdles) such as addition of preservatives to ensure the juice stability. A TS process at 75°C for 10 min induced *N. fischeri* activation followed by approximately 2 log reduction per 10 min processing time. The TS process might be applicable at higher temperatures. However, further research must be undertaken to design an ultrasound probe that can withstand higher temperatures. With respect to the exclusively thermal process, temperatures $\geq 90^\circ\text{C}$ are still required to achieve the inactivation of spores. Weibull modeled the inactivation of *N. fischeri* ascospore by the 600 MPa HPP-thermal processes, while Lorentzian was more suitable for the TS modeling. The results from this study show that 600 MPa HPP-thermal is a better option for the preservation of fruit juices prone to *N. fischeri* ascospores contamination.

Chapter 11 Resistance of *Byssochlamys nivea* and *Neosartorya fischeri* spores to high pressure thermal processing and thermosonication: effect of spore age

Content submitted to publication

11. TS and HPP-thermal processing of *N. fischeri* and *B. nivea* spores: Effect spore age

Chapter abstract

Byssochlamys nivea and *Neosartorya fischeri* are heat-resistant moulds that spoil acid foods and can produce mycotoxins. This study investigated the efficacy of 600 MPa high pressure processing (HPP) and thermosonication (TS, 24 kHz, 0.33 W/mL) at 75°C for the inactivation of 4-12 week old ascospores of *B. nivea* in strawberry puree and *N. fischeri* in apple juice. The HPP-thermal process was more effective than TS for spore inactivation. The 12-week *B. nivea* and *N. fischeri* spores had similar resistance to HPP. TS caused activation shoulders, which were more pronounced for *N. fischeri*. Spore resistance increased with increasing age for both species, with differences in the extent of inactivation of up to 2 log between spore age groups. The kinetics of mould ascospore inactivation by HPP-thermal and TS were well described by Weibull and Lorentzian models, respectively. The Weibull's scale factor b decreased with spore age for *N. fischeri*, and the Lorentzian parameter b also decreased with spore age for both moulds, indicating more resistance. The results demonstrated that mould spore age is a serious issue for fruit product manufacturers, since spores contaminating fruits can be very old, exhibiting higher spore resistance.

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11.1 Introduction

Byssochlamys nivea (anamorph *Paecilomyces niveus*) and *Neosartorya fischeri* (anamorph *Aspergillus fischerianus*) moulds produce extremely heat-resistant spores inside an ascus, (ascospores). The moulds are able to grow over a wide range temperatures (11 to 43°C for *B. nivea*; and 10 to 52°C for *N. fischeri*), pH (3-8) (Nielsen et al., 1989; Panagou et al., 2010; Pitt & Hocking, 1997), and water activities (0.89 to 0.99, *B. nivea*; 0.90 to 0.99, *N. fischeri*). They can also grow in reduced oxygen conditions inside fruit packs and carbonated beverages, causing spoilage (Taniwaki et al., 2009). The ascospores are often associated with the spoilage of pasteurized fruit products such as juices, purees, jellies, jams, and canned fruits (Beuchat, 1998; Pitt & Hocking, 1997; Silva & Gibss 2004, 2009; Silva et al., 2014). The thermal decimal reduction times at 90°C ($D_{90^{\circ}\text{C}}$ -value) ranged from 1.5 to 23.4 min for *N. fischeri* in fruit juices (Amaeze, 2012; Evelyn et al., 2016; Salomão et al., 2007) and from 1.8 up to 6.3 min for *B. nivea* in strawberry pulp (Aragão, 1989; Engel & Teuber, 1991; Evelyn & Silva, 2015d). *B. nivea* and *N. fischeri* are also a concern for human and animal health, because of their capacity to produce mycotoxins: terrein, fumitremorgins A and B, and verruculogen in *N. fischeri*; patulin, byssochlamic acid, and, byssotoxin A in *B. nivea* (Frisvad & Samson, 1991; Misawa et al., 1962; Nielsen et al., 1989; Tournas, 1994).

Spore age relates to the period of a spore's dormancy in which the hypometabolic state and respiratory rate of the microorganism is very slow (Goddard, 1935; Sussman et al., 1966). The spore ultra or nanostructure (cell, tissue, or organ) and chemical composition can be affected by its age (Conner et al., 1987). The age of an ascospore can affect its thermal and pressure resistance, being generally higher for older spores (Beuchat, 1988; Chapman et al., 2007; Delgado et al., 2012; Dijksterhuis and Teunissen, 2004; Slongo et al., 2006, 2009). For example, Slongo et al. (2009) found a 13% increase in the thermal inactivation D -values of *N.*

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fischeri in papaya juice in 3-month-old spores relative to 1-month-old spores. Chapman et al. (2007) observed ≤ 2.5 log reduction in the number of 5-week-old spores of *Byssoschlamys fulva*, *B. nivea*, *N. fischeri*, and *Neosartorya spinosa* in citrate buffer (pH 4 and 6) after treatment with 600 MPa for 10 min, whereas only a 1 log or less reduction was obtained with the same treatment of 9- to 15-week-old spores. Previous studies have also attempted to relate the ascospore age with growth parameters (such as maximum specific growth rate, μ_{\max} and maximum diameter of the colony) of *B. nivea* and *N. fischeri* in pineapple juice (Zimmermann et al., 2011a, 2011b), although there does not appear to be a significant relationship between ascospore age and these parameters.

Thermal processing is a common method of pasteurization used in industrial food production, but recent studies have investigated supplementing this process with high pressure processing (HPP) and power ultrasound to reduce treatment temperatures and/or processing times (Evelyn & Silva, 2015a, 2015b, 2015c, 2015d, 2015e, 2016a, 2016b; Evelyn et al., 2016a). This in turn will increase the process productivity and product quality. Usually, the combination of HPP with heat (HPP-thermal or HPTP-high pressure thermal processing) is needed to ensure inactivation of resistant microbial spores (Evelyn & Silva, 2015c, 2015d, 2016b; Evelyn et al., 2016; Sarker et al., 2015; Silva et al., 2012; Wilson et al., 2008) and enzymes (Sulaiman & Silva, 2013; Sulaiman et al., 2015a). HPP-thermal of up to 900 MPa (oscillatory or continuous) between 20 and 90°C have been used to inactivate *B. nivea* and *N. fischeri* ascospores (Butz et al., 1996; Chapman et al., 2007; Dijksterhuis & Teunissen, 2004; Evelyn & Silva, 2015d; Evelyn et al., 2016; Ferreira et al., 2009; Hocking et al., 2004; Maggi et al., 1994; Palou et al., 1998; Reyns et al., 2003; Voldřich et al., 2004). According to past results, ≥ 600 MPa pressures combined with $\geq 50^\circ\text{C}$ temperatures are required to inactivate heat resistant ascospores of *B. nivea* and *N. fischeri* (Butz et al., 1996; Evelyn & Silva, 2015d; Evelyn et al., 2016; Ferreira et al., 2009; Maggi et al., 1994). A Weibull model has

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been successfully described the HPP-thermal inactivation of *B. nivea* and *N. fischeri* spores (Evelyn & Silva, 2015a; Evelyn et al., 2016). Chapman et al. (2007) and Dijksterhuis and Teunissen (2004) studied the effect of spore age on the spore inactivation of *Byssoschlamys fulva*, *B. nivea*, *N. fischeri*, *Neosartorya spinosa*, and *Talaromyces macrosporus* at 200-1000 MPa and room temperature ($\sim 20^{\circ}\text{C}$), and found that spore inactivation was dependent on the microbial species and spore age. However, no researchers have modelled the effect of spore age on inactivation kinetics.

Power ultrasound (frequency ranging from 20 to 100 kHz) relies on the application of pressure which generates cavitation to the food/beverage, causing microbial cell death (Feng & Yang, 2011b; Piyasena et al., 2004). Power ultrasound has been combined with mild heat (thermosonication, TS) to inactivate bacterial and fungal vegetative cells and spores, and a synergistic effect was observed (Earnshaw et al., 1995; Evelyn & Silva, 2015a, 2015e, 2016a; Garcia et al., 1989; López-Malo et al., 2005; Ordoñez et al., 1987; Zenker et al., 2003). It is also very effective for polyphenoloxidase enzyme inactivation in fruits (Sulaiman et al., 2015b). With respect to mould spores, our recent results showed that TS at temperatures $\geq 75^{\circ}\text{C}$ could be used to inactivate *B. nivea* and *N. fischeri* ascospores (Evelyn & Silva, 2015d; Evelyn et al., 2016). For TS at 75°C , approximately 2 log reductions were obtained for *B. nivea* after 15 min, and after 30 min for *N. fischeri*, and a Lorentzian model successfully described the effect of TS on spores. TS has also been shown to be more effective than thermal processing alone for the inactivation of conidiospores. Jimenez-Munguia et al. (2001) and López-Malo et al. (2005) reported that the decimal (*D*-) reduction values for *Penicillium digitatum* and *Aspergillus flavus* conidiospore inactivation were 50-85% lower with TS (20 kHz, 120 μm , 40–60 $^{\circ}\text{C}$) than the corresponding thermal *D*-values.

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With the exception of our recent results with *B. nivea* and *N. fischeri*, no study on TS inactivation has been carried out with these moulds. Furthermore, the effect of ascospore age on the TS and HPP-thermal inactivation pattern of mould ascospores has not yet been studied. Therefore, due to the concern of increasing mould spore resistance with their age, the spores were processed by 600 MPa HPP and TS at 75°C, and the main objectives were: (i) to compare HPP-thermal and TS inactivation of 12-week mould spores; (ii) to compare the resistance of 12-week *B. nivea* and *N. fischeri* spores; (iii) to study the effect of mould spore age on the HPP-thermal and TS inactivation; and (iv) to model the HPP-thermal and TS inactivation of *B. nivea* and *N. fischeri* ascospores of different ages.

11.2 Materials and methods

11.2.1 Microbiology

11.2.1.1 Moulds

N. fischeri var fischeri JCM 1740 and *B. nivea* JCM 12806 previously described (Chapter 9 and 10) were also used in this study.

11.2.1.2 Ascospore production, enumeration and inoculation

The procedures described in previous chapters for ascospore production, inoculation and enumeration (Chapter 9 and 10) were used. The final spore suspension after harvesting was stored at 2°C in SDW containing glass beads until use to avoid dense clusters of spores.

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11.2.2 HPP-thermal and thermosonication

Unless stated, HPP-thermal and thermosonication inactivation experiments used the method described previously for *B. nivea* (Chapter 9) and *N. fischeri* (Chapter 10), except the experiment was carried out only at 75°C using 4, 8, 10, and 12 week spores. Temperature of 75°C was chosen since this is the maximum temperature supported by ultrasound probe, and this temperature was the best performed temperature in the past experiments (Evelyn & Silva, 2015d; Evelyn et al., 2016). The HPP-75°C was carried out for up to 40 min, whereas the ultrasonic treatments were carried out for up to 70 min depending on spore age used. Two TS and HPP survival experiments were carried out for each spore age with duplicate samples processed for each time.

11.2.3 Modeling the mould ascospore inactivation in apple juice

TableCurve 2D (version 5.01, SYSTAT Software Inc., Chicago, USA) was used to fit a Weibull model (Equation 1-6) to HPP-thermal and a Lorentzian model (Equation 9-1) to TS spore survival lines, and to estimate the model parameters for *B. nivea* and *N. fischeri* spores in puree/juice. The models had proven to work well in previous spore inactivation research with these moulds (Evelyn & Silva, 2015d; Evelyn et al., 2016). The model parameters were described previously (Chapter 9 and 10).

For each spore age, two survival experiments were performed for HPP-thermal and TS treatments. For each survival experiment, duplicate samples were processed for each time. Then, the $\log N/N_0$ were expressed as mean \pm standard deviation (SD), which includes the result of four different processed samples and the average data were plotted in a chart. A *t*-test (Statistica 8.0, Statsoft Inc., USA) was used to compare the log reductions of the two

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mould spores at the same/different spore age, after a specific processing time. Then, for each spore age the kinetic parameters \pm standard errors (SE) were estimated by fitting the models to the average of four samples at each processing time. Mean square error (MSE) and coefficient of determination (R^2) were used to compare the goodness of fit of the models. A relatively small MSE and R^2 values close to 1 indicate the adequacy of the model to describe the survival data. Additionally, the spore age dependence of the parameters estimated was checked.

11.3 Results and discussion

11.3.1 Comparing the 12-week spore resistance to HPP-thermal and TS processing

Figure 11-1 shows the inactivation of 12-week-old ascospores of *B. nivea* and *N. fischeri* to 600 MPa HPP-thermal and TS processing at 75°C. The HPP-thermal process was more effective than TS for spore inactivation at the same temperature (75°C) due to the activation shoulders observed with TS. For example, there was no inactivation of *B. nivea* and a more than 3-log activation shoulder for *N. fischeri* after 20 min of TS processes, whereas the same duration of HPP-thermal processing resulted in a reduction in ascospore number of approximately 2 log for both moulds ($p < 0.05$). The combination of HPP with heat ($\geq 50^\circ\text{C}$) could readily inactivate the mould ascospores, in agreement with past works (Butz et al. 1996; Evelyn & Silva, 2015d; Evelyn et al., 2016; Ferreira et al., 2009).

Figure 11-1 also allows the comparison of the 2 moulds' resistance. The HPP-thermal inactivation of both moulds' spores was similar until 30 min, whereas after 30 min, a significant difference ($p < 0.05$) in the spore resistance was observed (2.5 log for *B. nivea* vs. 2.9 log for *N. fischeri* after 40 min). Chapman et al. (2007) observed a one log reduction for

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11- and 13-week-old spores of *N. fischeri* FRR 3494 in phosphate buffer (pH 4-6) by HPP at 20°C for 10 min, which is similar to the results in this study (1.5 log) with a much higher temperature (75°C). The TS resulted in activation shoulders and tailings for both moulds with a more pronounced activation shoulder for *N. fischeri*, indicating higher resistance of this mould. For example, after 20 min, there was a 3.5-log spore increase for *N. fischeri* vs. 0.2-log reduction for *B. nivea*. The above results suggest that the response to TS and HPP-thermal was dependent on the microbial species, as demonstrated previously by Chapman et al. (2007).

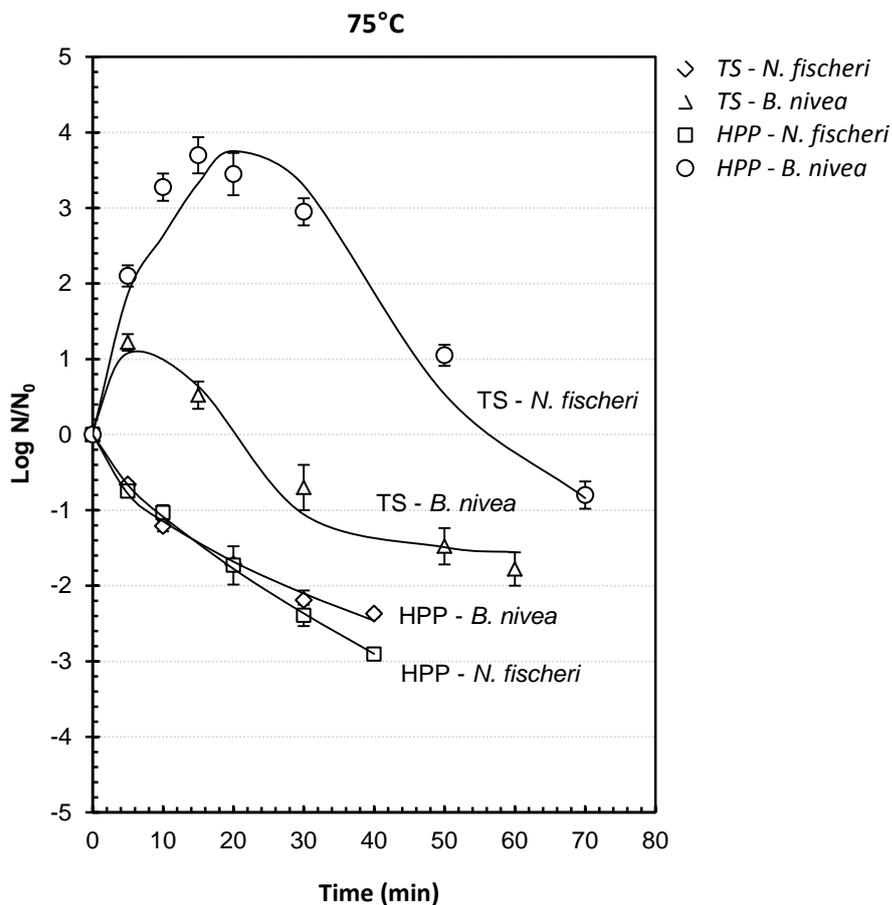


Figure 11-1 Inactivation of 12 week spores of *Byssoschlamys nivea* and *Neosartorya fischeri* by HPP-thermal (600 MPa-75°C) and TS (0.33 W/mL at 75°C).

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11.3.2 Effect of mould spore age on HPP-thermal inactivation at 75°C and modeling

Figure 11-2 shows the effect of mould spore age from 4 to 12 weeks on the 600 MPa HPP-75°C inactivation of *B. nivea* and *N. fischeri* ascospores. In general, spore numbers decreased by around 2 log for *B. nivea* and by 2 to 4 log for *N. fischeri* after 20 min, indicating a more accentuated effect of spore age for *N. fischeri*. The older the spores, the lower the inactivation. Additionally, tails were more pronounced and appeared at higher survival ratios for older spores, implying higher microbial counts and higher risks. It is thought that changes in the spore ultra- or nanostructure through formation of multilayers in the ascospore's wall contribute to the higher resistance for older ascospores (Chapman et al., 2007; Dijksterhuis & Teunissen, 2004; Nguyen, 2012).

Although no effect of *B. nivea* spore age was perceived in the first 20 min of process, increasing the treatment time for up to 40 min increased the extent of spore inactivation from 2.8 to 3.4 log (4-week-old spores) (**Figure 11-2a**). Ferreira et al. (2009) worked with a strawberry-isolated *B. nivea* strain, in which 1.5- to 2-log reductions were obtained with 4-week-old spores suspended in pineapple juice and nectar at HPP temperature of 70°C after 15 min, which is similar to our results. Nonetheless, there were no significant differences in the log reductions of 8-, 10- and 12-week-old spores after 40 min ($p>0.05$). This might be attributed to the effect of spore age on their physiological condition. Chapman et al. (2007) observed a 4-log reduction in the number of 3-week-old *B. nivea* FRR 3798 spores in phosphate buffer (pH 4-6) processed at 600 MPa and 20°C for 10 min, whereas the same process resulted in a 1- to 1.5-log increase in 5- to 9-week-old spores, and a 0.5- to 1-log increase for 11- to 15-week-old spores.

Regarding *N. fischeri*, for the same HPP-thermal processing time 12-week-old spores were more resistant to inactivation than 4-week-old spores, resulting in a reduction in spore

11. TS and HPP-thermal processing of *N. fischeri* and *B. nivea* spores: Effect spore age

number that was 2-log lower for the older than the younger spores (**Figure 11-2b**). However, similar to *B. nivea* results, the reduction in 10- and 12-week-old spores presented no difference ($p>0.05$). Chapman et al. (2007) reported that the inactivation of 5- to 7-week-old *N. fischeri* FRR 3494 spores in phosphate buffer (pH 4-6) using 600 MPa HPP-20°C for 10 min was approximately 1 log more than inactivation of 9- to 15-week old spores.

11.3.2.1 Modeling the spore inactivation by HPP-thermal

The past results have shown that the Weibull model had a great accuracy for describing log survivors of *B. nivea* and *N. fischeri* spores after HPP-thermal processes (Evelyn & Silva, 2015a; Evelyn et al., 2016), therefore Weibull was also used in this study. The Weibull model showed good performance indices for both moulds aged between 4 and 12 weeks (0.006–0.011 MSE and 0.992-0.997 R^2 for *B. nivea*; 0.003–0.061 MSE and 0.980–0.998 R^2 for *N. fischeri*) (**Table 11-1**). The Weibull b values decreased linearly with the spore age for *N. fischeri* ($R^2=0.90$), but the effect of spore age on b parameters was less evident for *B. nivea*, with little change across the spore age range (**Table 11-1**).

The estimated Weibull n parameters were less than 1, indicating that all the spore survivor curves for HPP-thermal processes were concave-upward (**Figure 11-2**), independent of spore age. Concave-upward curves with tailing suggests that there is a mixed resistance of the spore population to the lethal treatment (Peleg & Cole, 1998), in which the most sensitive spore population is inactivated at a faster rate, followed by the slower and steady decline of a more resistant population (Tola & Ramaswamy, 2014). For *N. fischeri*, the shape factor n increased with spore age (0.35 for 4 week to 0.71 for 12 week, **Table 11-1**). However, the n parameter for *B. nivea* showed minimal change (from 0.66 for 4 week to 0.55 for 12 week) (**Table 11-1**). These inconsistencies on the dependency or independency of n from external

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factors have been reported previously (Bermúdez-Aquirre & Corradini, 2012; Buzrul, 2015; Chen & Hoover, 2003; Cunha et al., 1998).

Table 11-1 Weibull model parameters for the survival of *Byssochlamys nivea* and *Neosartorya fischeri* ascospores of different age after 600 MPa HPP-75°C processing* .

Mould	Spore age (week)	Weibull			
		$b \pm SE$	$n \pm SE$	R^2	MSE
<i>B. nivea</i>	4	0.29±0.02	0.66± 0.03	0.997	0.006
	8	0.42±0.05	0.49±0.04	0.993	0.008
	10	0.27±0.04	0.68±0.04	0.995	0.011
	12	0.32±0.05	0.55±0.05	0.992	0.009
<i>N. fischeri</i>	4	1.44±0.12	0.35±0.03	0.996	0.021
	8	0.44±0.11	0.61±0.08	0.980	0.061
	10	0.27±0.04	0.68±0.04	0.995	0.011
	12	0.21±0.02	0.71±0.03	0.998	0.003

* *B. nivea* JCM 12806 (= CBS 696.95) and *N. fischeri* var *fischeri* JCM 1740 (= ATCC 1020) were used; b and n are the Weibull scale and shape factors, respectively; b and n -values \pm standard errors (SE) were obtained from 2 survival experiments (with 2 replicates for each time) for each spore age; R^2 close to 1 and low mean square errors (MSE) are indication of good fit.

11. TS and HPP-thermal processing of *N. fischeri* and *B. nivea* spores: Effect spore age

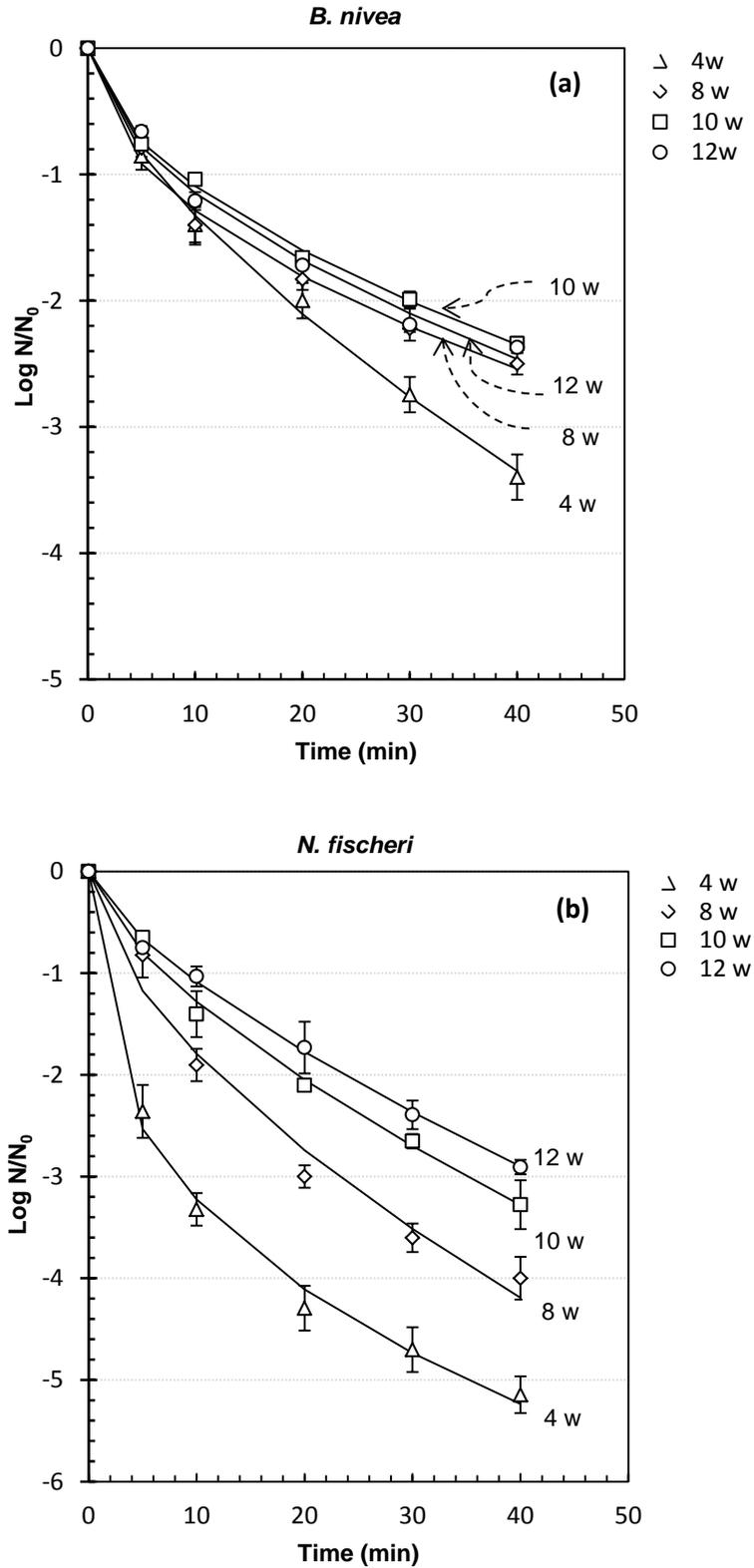


Figure 11-2 Effect of spore age on the 600 MPa HPP-75°C inactivation of *Byssoschlamys nivea* ascospores in strawberry puree (a); and *Neosartorya fischeri* ascospores in apple juice (b): fitting with Weibull model.

11. TS and HPP-thermal processing of *N. fischeri* and *B. nivea* spores: Effect spore age

11.3.3 Effect of mould spore age on TS inactivation at 75°C and modeling

Activation shoulders were observed followed by inactivation in all spores tested (4–12 weeks) (**Figure 11-3**), which is in agreement with our past results with 4-week-old spores at different temperatures (Evelyn & Silva, 2015a; Evelyn et al., 2016). Similar to the observations with HPP-thermal, spore age also affected resistance to TS, being higher for older spores. *B. nivea* exhibited a tail for 12-week-old spores after 30 min, with a much smaller rate in the spore inactivation. Additionally, lower activation shoulder times were observed for younger spores. The same was found by Slongo et al. (2006) with 85°C thermal treatments of *N. fischeri* in pineapple and papaya nectars. Overall, for *B. nivea* spores, 13 min of TS treatment was required for 1 log reduction of 4-week-old spores, whereas 20, 25, and 29 min were needed for the same spore inactivation with 8-, 10-, and 12-week-old spores, respectively (**Figure 11-3a**). Even longer processing times were required for *N. fischeri* spores: 26 min for a 1-log reduction in 4-week-old spores, and 38, 61, and 74 min to obtain the same spore inactivation for 8-, 10-, and 12-week-old spores, respectively (**Figure 11-3b**). For *B. nivea*, spore inactivation was 2 log after 15 min for 4-week-old spores, 3 log after 30 min for 8-week-old spores, 2.5 log after 40 min for 10-week-old spores, 0.8 log after 60 min for 12-week-old spores, respectively, whereas for *N. fischeri* we obtained the maximum inactivation of 3.5 log after 40 min (4-week-old spores), 2.4 log after 50 min (8-week-old spores), 1.6 log after 70 min (10-week-old spores), and 0.8 log after 70 min (12-week-old spores).

11.3.3.1 Modeling the spore inactivation by TS

The Lorentzian equation was used to describe the TS survival curves (Evelyn and Silva, 2015a; Evelyn et al., 2016). The Lorentzian model resulted in good performance indices for

11. TS and HPP-thermal processing of *N. fischeri* and *B. nivea* spores: Effect spore age

B. nivea (0.025–0.135 MSE and 0.965–0.994 R^2) and *N. fischeri* (0.018–0.607 MSE and 0.880–0.997 R^2) (Table 11-2). The Lorentzian b parameters decreased from 10.1 to 3.1 for *B. nivea*, and from 8.5 to 5.9 for *N. fischeri* as the spore age was increased from 4 to 12 weeks, indicating less overall microbial inactivation for older spores. The c and d values did not exhibit any pattern. There has been no research carried out on the effect of mould ascospore age on the thermosonication kinetics, thus no other results to compare.

Table 11-2 Lorentzian model parameters for the survival of *Byssoschlamys nivea* and *Neosartorya fischeri* ascospores of different age after 75°C-thermosonication (0.33 W/ml)*.

Mould	Spore age (week)	Lorentzian					
		$a \pm SE$	$b \pm SE$	$c \pm SE$	$d \pm SE$	R^2	MSE
<i>B. nivea</i>	4	-9.1± 6.1	10.1±6.0	5.1± 0.3	15.9±6.1	0.994	0.025
	8	-5.3±1.6	6.5±1.5	7.2±1.0	17.2±5.2	0.977	0.135
	10	-3.3±0.7	4.6±0.7	8.9±1.3	16.0±4.2	0.977	0.125
	12	-1.7±0.3	3.1±0.5	8.8±1.2	10.7±4.0	0.965	0.120
<i>N. fischeri</i>	4	-6.1±0.4	8.5±0.4	11.5±0.2	18.6±1.7	0.997	0.018
	8	-5.0±1.1	7.8±1.0	15.2±0.7	23.5±5.3	0.979	0.114
	10	-3.3±1.7	6.9±1.7	22.2±2.2	27.7±9.2	0.934	0.385
	12	-2.1±1.9	5.9±1.7	22.4±2.9	24.8±11.2	0.880	0.607

*The spores were submitted to heat shock pretreatment (80°C, 15 min).

a , b , c and d are the Lorentzian spore age dependent parameters: a is the baseline of the distribution (that corresponds to the residual-survival-potentially observed); b is the rate of microbial inactivation; c is the the time (t) value at which the inactivation starts; d is the width of the distribution.; the parameters \pm standard errors (SE) were obtained from from 2 survival experiments (with 2 replicates for eah time) for each spore age; R^2 close to 1 and low mean square errors (MSE) are indication of good fit.

11. TS and HPP-thermal processing of *N. fischeri* and *B. nivea* spores: Effect spore age

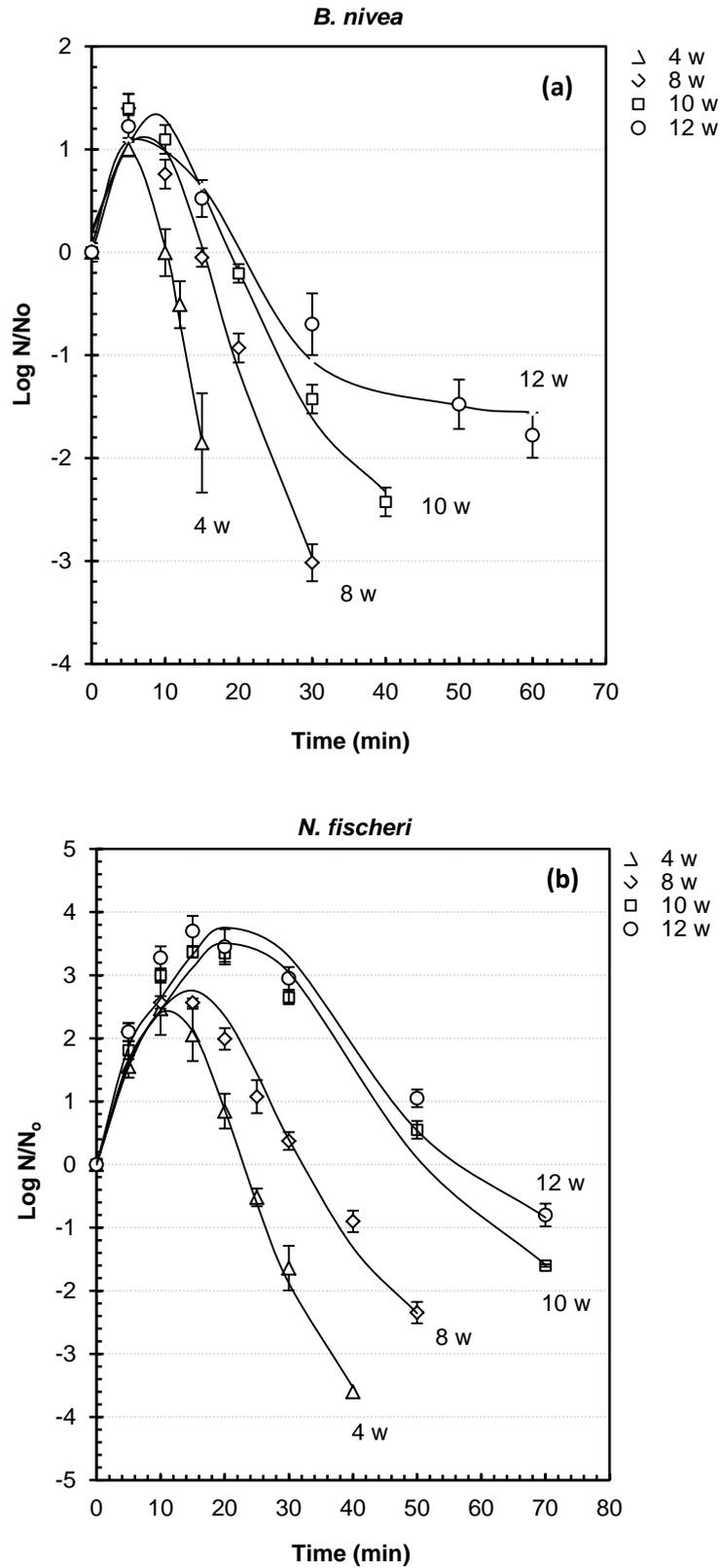


Figure 11-3 Effect of spore age on the 75°C-thermosonication (0.33 W/ml) inactivation of *Byssoschlamys nivea* ascospores in strawberry puree (a); and *Neosartorya fischeri* ascospores in apple juice (b): fitting with Lorentzian model.

11. TS and HPP-thermal processing of *N. fischeri* and *B. nivea* spores: Effect spore age

11.4 Conclusion

The current study showed that HPP-thermal and TS resistance of mould ascospores was species dependent. For example, *N. fischeri* was more resistant than *B. nivea* to TS processes. The HPP-thermal was more effective than TS, since TS exhibited activation shoulders. Lower inactivation was obtained with older spores. For *B. nivea*, a reduction of 2.7 log for 4 week spores vs. 2 log for 12 week spores after 30 min was obtained with HPP-thermal, whereas a 2-log difference in the inactivation of 8- and 12-week-old *N. fischeri* spores was noted. The Weibull model described well the HPP-thermal inactivation of 4-12 week old *B. nivea* and *N. fischeri* spores, in which a more pronounced effect of spore age (*b* scale factor) was observed for *N. fischeri*. The Lorentzian model described TS inactivation with more tailings for older spores. These results allow a better understanding of the influence of mould ascospore age and species (*B. nivea* and *N. fischeri*) on the HPP and TS inactivation in fruit puree/juice.

**Chapter 12 Microbial spores resistance to HPP-thermal, TS,
and thermal treatments**

12. Resistance of microbial spores to TS, HPP-thermal, and thermal

12.1 Introduction

This current chapter aims to compare the resistance of all the microbial spores used in previous studies (*Clostridium perfringens*, psychrotrophic *Bacillus cereus*, *Alicyclobacillus acidoterrestris*, 4 week old *Neosartorya fischeri* and *Byssochlamys nivea*) to HPP-thermal, TS and thermal processing. *B. cereus* and *C. perfringens* are pathogens that can spoil low acid foods such as milk and meat products, whereas *A. acidoterrestris* and moulds are able to grow and spoil high acid foods for example fruit juices.

12.2 Strains

The spores of *A. acidoterrestris* NZRM 4447 in orange juice, *C. perfringens* NZRM 898 in beef slurry, *B. cereus* NZRM 984 in milk and beef slurry, and *B. cereus* ICMP 12442 in beef slurry, *N. fischeri* JCM 1740 in apple juice, and *Byssochlamys nivea* JCM 12806 in strawberry puree were used.

12.3 Spore thermal resistance

The thermal inactivation of selected microbial spores is illustrated in **Figure 12-1**. Regarding *B. cereus*, the log survivors ICMP 12442 strain in beef slurry was presented since it used the same food as *C. perfringens*. Increase in the spore log numbers was observed for both mould spores tested during 30 min processing time, thus making them as the most thermal resistant spores in. It is possible for the mould spore numbers to decrease with a prolonged thermal processing time as reported by Ferreira et al. (2011) after ten minutes processing. With respect

12. Resistance of microbial spores to TS, HPP-thermal, and thermal

to bacterial spores, *A. acidoterrestris* and *C. perfringens* showed higher thermal resistance (75-78°C) than psychrotrophic *B. cereus* at 70°C.

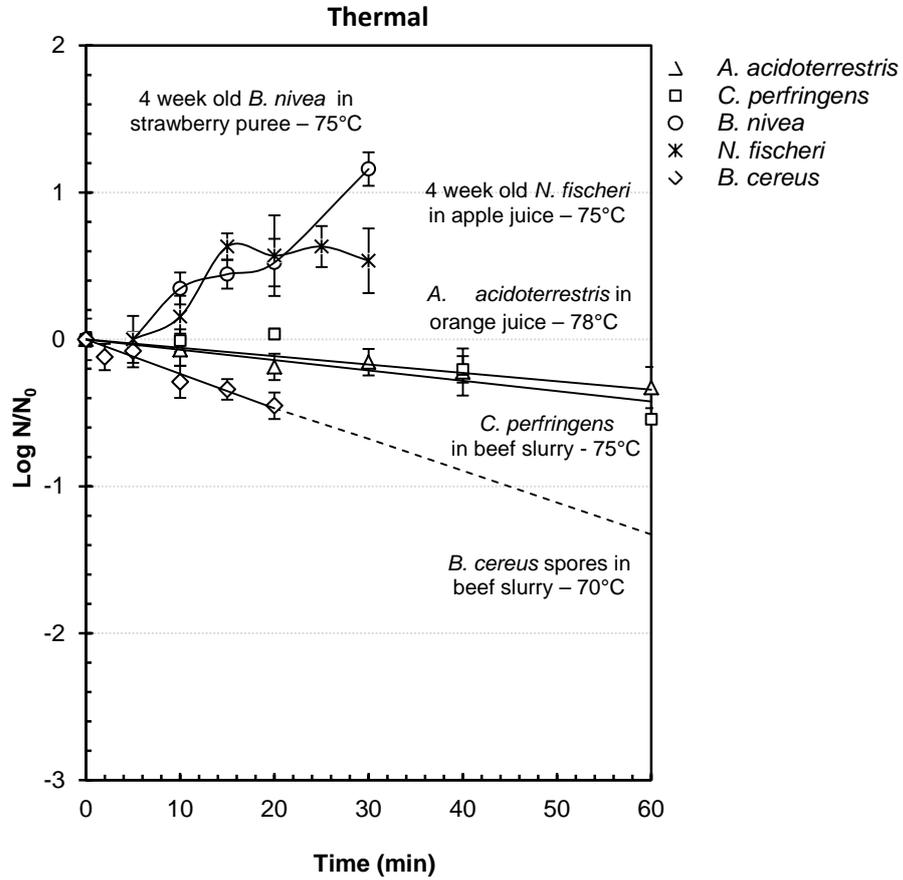


Figure 12-1 Thermal spore resistance of *Byssosclamyces nivea* and *Neosartorya fischeri* moulds, *Alicyclobacillus acidoterrestris*, *Clostridium perfringens*, and psychrotrophic *Bacillus cereus* bacteria.

12. Resistance of microbial spores to TS, HPP-thermal, and thermal

12.4 Spore resistance to HPP-thermal processing

Figure 12-2 presents replots of the 600 MPa HPP-thermal inactivation of microbial spores from selected strain and age used in this work for ease of comparison. *C. perfringens* NZRM 898 in beef slurry was presented since the spores exhibited a slightly higher HPP-thermal resistance than NZRM 2621 strain (Chapter 2). *B. cereus* ICMP 12442 was used since it was also suspended in the same type of food (beef slurry) as *C. perfringens*, and demonstrated a similar HPP-thermal resistance to NZRM 984 (Chapter 4), the other strain used. Regarding *A. acidoterrestris*, the HPP-thermal study have not been carried out, so it is not presented in Figure 12-2.

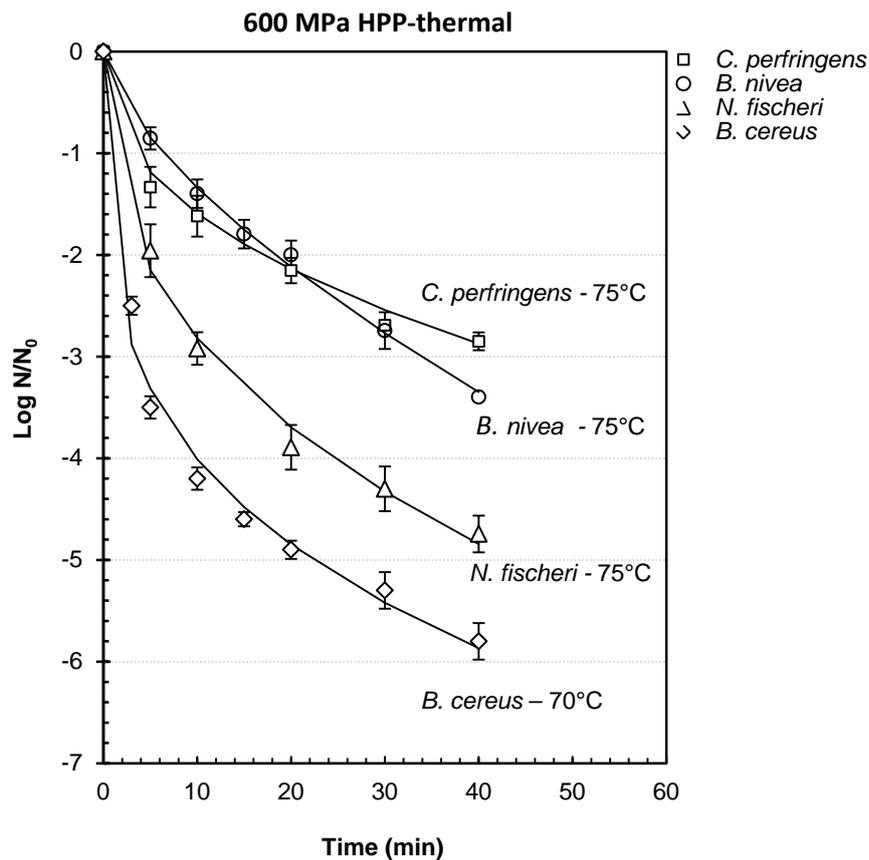


Figure 12-2 600 MPa HPP-thermal spore resistance of *Clostridium perfringens* and psychrotrophic *Bacillus cereus* bacteria, and *Byssoschlamys nivea* and *Neosartorya fischeri* moulds.

12. Resistance of microbial spores to TS, HPP-thermal, and thermal

The figure shows that the spores of *C. perfringens* had the highest resistance to the 600 MPa HPP-75°C (2.9 log after 40 min). The four week old *B. nivea* ascospores appeared exhibiting a similar resistance to *C. perfringens* spores for up to 30 min (2.7 log) at similar temperature, but then showed a lower resistance after 40 min treatment (3.4 log). The next resistant spores to HPP-thermal were 4 week old spores of *N. fischeri* at 75°C with 4.7 log after 40 min, followed by psychrotrophic *B. cereus* spores at 70°C (5.8 log after 40 min).

12.5 Spore resistance to thermosonication

In **Figure 12-3**, TS (0.33 W/mL) resistance of *A. acidoterrestris* NZRM 4447 spores in orange juice, *C. perfringens* NZRM 898 spores in beef slurry, *N. fischeri* JCM 1740 ascospores in apple juice, *B. nivea* JCM 12806 spores in strawberry puree, and psychrotrophic *Bacillus cereus* NZRM 984 spores in milk were compared. *C. perfringens* NZRM 898 strain in beef slurry was presented because of the same reason explained above, and *B. cereus* NZRM 984 spores in milk were selected the spores demonstrated the highest TS resistance in this medium. The chart suggests that AAT spores were mostly insensitive to the TS treatment, with almost no spore reduction after 60 min (≤ 0.3 log). This was followed by *C. perfringens* (1 log, 60 min), then a month old *N. fischeri* and *B. nivea* with prior activation shoulder (5 log after 60 min for *N. fischeri*, 5 log after 25 min for *B. nivea*). Psychrotrophic *B. cereus* showed the least resistance microorganisms to TS (5 log after 2 min at 70°C).

12. Resistance of microbial spores to TS, HPP-thermal, and thermal

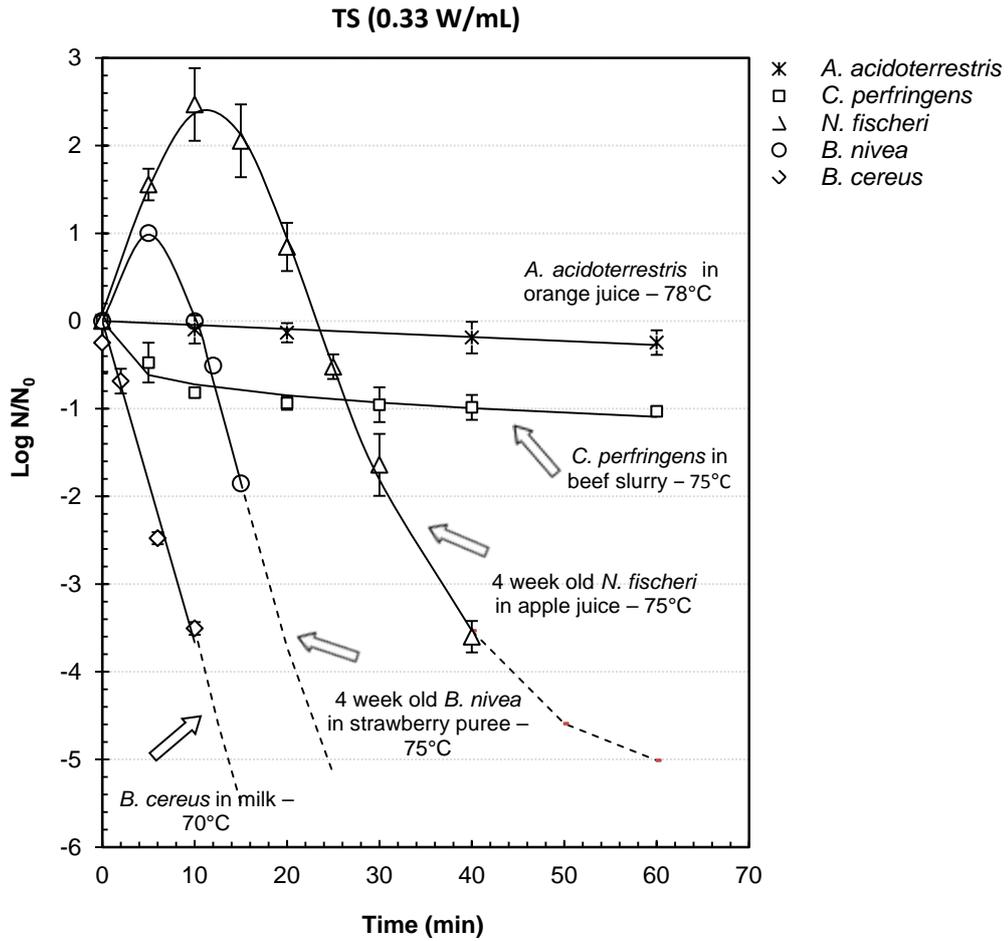


Figure 12-3 Thermosonication (0.33 W/mL or W/g) spore resistance of *Byssochlamys nivea* and *Neosartorya fischeri* moulds, *Alicyclobacillus acidoterrestris*, *Clostridium perfringens*, and psychrotrophic *Bacillus cereus* bacteria.

12. Resistance of microbial spores to TS, HPP-thermal, and thermal

12.6 Effect of food matrix on spore resistance

The effect of suspending media/foods on the thermosonication and HPP-thermal resistance of psychrotrophic *B. cereus* spores were shown in **Figure 12-4** to **Figure 12-6**. A 40 min HPP-thermal process resulted in the same spore inactivation in milk and beef slurry (5.8 log) (**Figure 12-4**). Similarly, a 20 min thermal process caused the same spore inactivation in both foods (4.3 log). On the contrary, a 2 min TS process caused 0.7 log in milk vs. close to 6 log in beef slurry (**Figure 12-5**), which might be attributed to microstructure or composition of the foods used. This demonstrated that suspending medium has a much higher effect on TS inactivation compared with thermal and HPP-thermal inactivation.

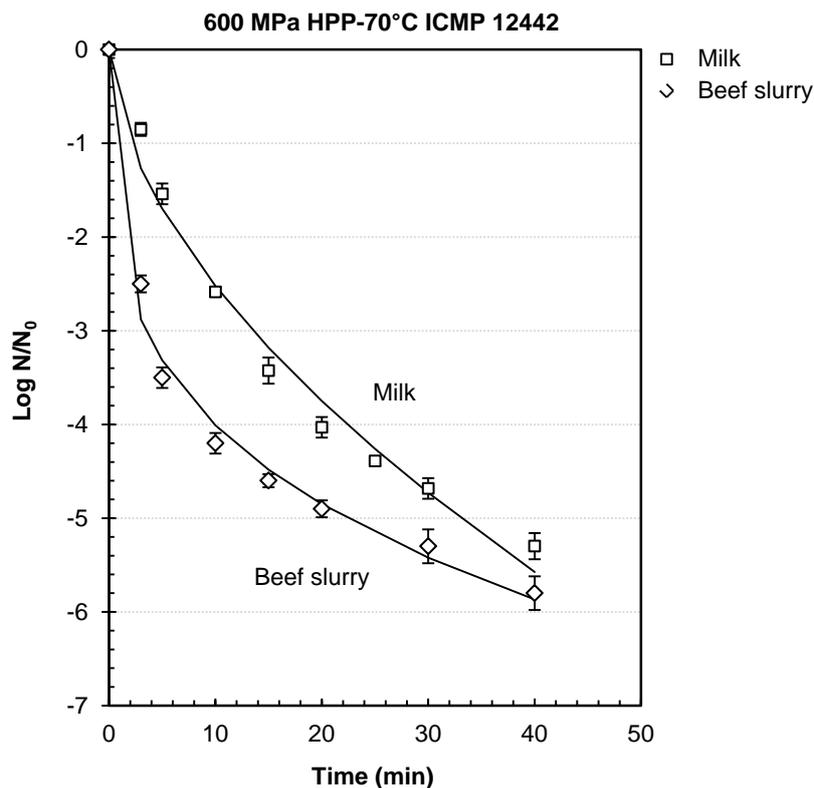


Figure 12-4 Effect of suspending medium (food) on the 600 MPa HPP-70°C inactivation of psychrotrophic *Bacillus cereus* ICMP 12442 spores.

12. Resistance of microbial spores to TS, HPP-thermal, and thermal

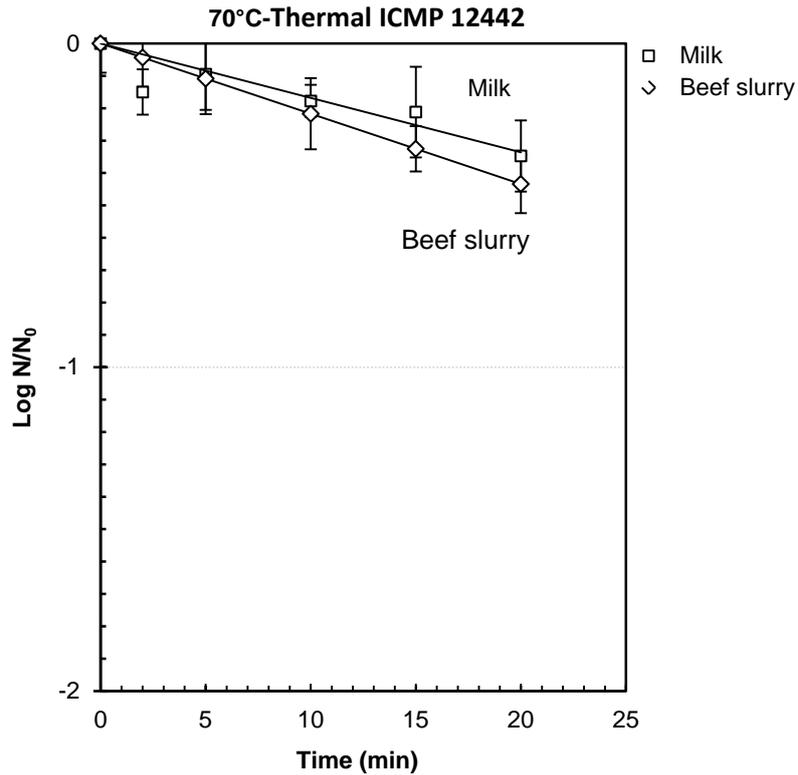


Figure 12-5 Effect of suspending medium (food) on the thermal inactivation of psychrotrophic *Bacillus cereus* ICMP 12442 spores

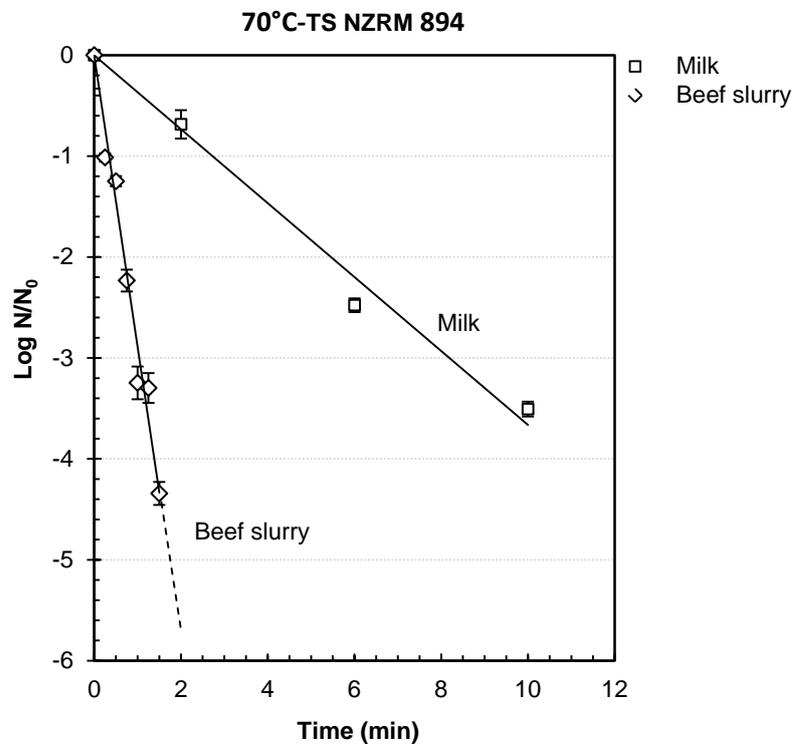


Figure 12-6 Effect of suspending medium (food) on the thermosonication (24 kHz, 0.33 W/ml) inactivation of psychrotrophic *Bacillus cereus* NZRM 894 spores.

12. Resistance of microbial spores to TS, HPP-thermal, and thermal

12.7 Comparison of spore resistance to different technologies

A general conclusion can be derived from **Figure 12-1** to **12-6** after the treatments at around 75°C. The figures suggest that psychrotrophic *B. cereus* spores were the least resistant to all technologies/processes, being readily inactivated by TS, followed by HPP-thermal. The thermal processing alone was not enough for *B. cereus* inactivation. The spores of four week old moulds (*B. nivea* and *N. fischeri*) were more resistant to thermal process than TS and HPP-thermal processes. While for thermal the moulds were more resistant than *A. acidoterrestris* and *C. perfringens* during 30 min processing, *A. acidoterrestris* and *C. perfringens* were more difficult to inactivate by TS and *C. perfringens* was more difficult to inactivate by HPP-thermal. *A. acidoterrestris* spores exhibited a similar resistance to the two technologies tested (thermal and TS), and showed the most resistant spores to TS processing. With respect to *C. perfringens*, *N. fischeri* and *B. nivea* spores, the 600 MPa HPP-thermal showed the best method for the inactivation of these microbial spores. Food has a substantial effect on the spore inactivation by TS but not on thermal and HPP-thermal.

12.8 Recommendations of TS, HPP-thermal and thermal pasteurization conditions

Although there are limitations regarding the maximum temperature allowed by the ultrasound and HPP units (in which temperature above 78°C for ultrasound and above 75°C for HPP can not be applied), attempts were made to estimate the efficiency of the TS and/or HPP-thermal processes to inactivate *B. cereus* spores in milk, *C. perfringens* spores in beef slurry and *A. acidoterrestris* spores in orange juice. Thus, the treatment conditions (temperature-time) resulting in specific log reduction were estimated and discussed in the following sections.

12. Resistance of microbial spores to TS, HPP-thermal, and thermal

However, the recommended treatments should be used with precaution, it being needed experimental validation of the model predictions.

12.8.1 Minimum pasteurization conditions for milk and beef slurry

The F_0 -value of 1.3 min for non-proteolytic *C. botulinum* to deliver 6 decimal reductions (6D) at 121°C is acceptable in refrigerated foods (ACMSF, 1992; CFA, 2006; ECFF, 2006). Thus, it was used as a reference for the psychrotrophic *B. cereus* spores. Due to the best spore inactivation for *B. cereus* spores was obtained with thermosonication, and based on the log survivors of psychrotrophic *B. cereus* NZRM 984 spores predictions in milk (the highest resistance) from previous results (Chapter 6), therefore a TS of at least 97°C for 1.3 min is recommended to pasteurize low acid foods contaminated by 6 log of the psychrotrophic *B. cereus* spores.

The typical level of *C. perfringens* contamination of meat and poultry is around 10^6 – 10^7 cfu/g (EFSA, 2005a), thus a pasteurization value of at least 6D in low-acid chilled foods has been suggested (Betts & Gaze, 1992; Silva & Gibbs, 2010; Silva et al., 2014). The US Food and Drug Administration cooking recommendations suggest pasteurization of 6.5-7.0D (Juneja & Thippareddy, 2004; USDA-FSIS, 1999). Therefore, based on our results with *C. perfringens* NZRM 898 spores (the most resistant strain in this study) (Chapter 2), high pressure processing using 600 MPa and a temperature of 82°C for 1.3 min could be an option to deliver at least 6D reduction of *C. perfringens* spores in beef slurry. Thus, HPTP could be used as an approach to achieve the recommended 6D reduction of *C. perfringens* spores in meat and poultry to below 15°C (USDA-FSIS, 1999).

12. Resistance of microbial spores to TS, HPP-thermal, and thermal

12.8.2 Minimum pasteurization conditions for juices/purees

The typical level of microbial contamination of fruit juice is around 10^2 – 10^3 cfu/mL (Ho et al., 2010). Therefore, and to ensure total microbial inactivation, a 5 or 6D pasteurization is often recommended for fruit juices (Gaze & Betts, 1992; Sant'Ana et al., 2014; USFDA, 1998). However, given the extreme thermal resistance of AAT spores compared with other fruit spoilage organisms, Silva & Gibbs (2001; 2004) recommended a minimum pasteurization of 2 to 3D. High temperature short time (HTST) pasteurization at 90–95°C for 15–30 s are normally used to pasteurize orange juice (Braddock, 1999). These thermal pasteurization conditions are not sufficient to inactivate AAT spores ($D_{95^\circ\text{C}} = 1.5$ min), since only ≥ 4.5 min at 95°C will deliver 3D (3 log reductions in AAT spores). With respect to TS, 78°C was the maximum temperature allowed by the ultrasound unit. However, the D_T -value of the orange juice TS pasteurization can be estimated for higher temperatures based on the TS z -value = 11.5°C (Chapter 8): 6.89 min at 85°C, 2.53 min at 90°C, 0.93 min at 95°C, 0.34 min at 100°C and 0.13 min at 104°C. Consequently, a TS of at least 2.8 min at 95°C or 23 s at 105°C are recommended for orange juice pasteurization by TS (20.2 W/mL), which will ensure the minimum 3 log reduction on the *A. acidoterrestris* spores in orange juice.

General conclusions and future work recommendations

Conclusions

Microbial spores are known for their extreme resistance to various chemical and physical methods. Non-thermal technologies have been applied in the processing of food and beverages due to improved retention of sensory and nutritional attributes. It is well known that combination of high pressure and heat (HPP-thermal) could germinate and inactivate microbial spores. However, information on microbial spore inactivation and the kinetic modeling of key microbes relevant for food safety and stability is lacking. A combination of power ultrasound with heat (thermosonication, TS) for inactivating microbial spores has also been suggested and attempted in this research. However, there was almost no documentation on microbial inactivation and modeling. Therefore, this study was carried out to investigate the inactivation of *Clostridium perfringens*, *Alicyclobacillus acidoterrestris*, psychrotrophic *Bacillus cereus*, *Neosartorya fischeri*, and *Byssoschlamys nivea* spores in foods by using TS and HPP-thermal processes and to model the kinetics. Additionally, a comparison with thermal processing alone was performed.

The results of this study demonstrated that TS and HPP-thermal treatments are better food preservation techniques than thermal processing alone, presenting higher spore inactivation in foods at the same temperature (i.e. 70, 75, or 78°C). Temperature plays a significant role in spore inactivation by TS and HPP-thermal processes, which increased with temperature.

With respect to *C. perfringens* spores, the 600 MPa HPP-thermal was a better technique than TS and thermal processing for their inactivation in beef slurry. For 20 min at 75°C, an average reduction of 2 log in the *C. perfringens* spores was obtained for HPP-thermal vs. 1 log reduction for TS, and no inactivation for thermal techniques.

Conclusions and recommendations for future work

The *B. cereus* spores suspended in milk and beef slurry were better inactivated by TS than HPP-thermal and exclusively thermal treatments at 70°C. For the spores in milk, 5 log reductions were obtained by 70°C TS after 15 min, which could only be achieved after 40 min with HPP-thermal and thermal processes. Regarding the spores suspended in beef slurry, the difference was more accentuated. For example, a 2 min 70°C TS process reduced >5 log *B. cereus* spores compared to only 1 log for the thermal.

Thermosonication of HPP (200 MPa, 600 MPa) pretreated *A. acidoterrestris* spores in orange juice for 15 min at 78°C was a better technique than exclusively TS or thermal processing, allowing a 4.4 log reduction after 60 min. Acoustic power density (AED) plays an important role in the inactivation of *A. acidoterrestris* spores, being higher with a higher AED.

A heat shock pretreatment followed by 1-min ultrasonication of *C. perfringens* and *A. acidoterrestris* spores was able to enhance their thermal inactivation. For *C. perfringens* at 95°C, the *D*-value of 20.2 min decreased to 9.8 min. For *A. acidoterrestris* thermal inactivation at 85°C, the *D*-value of 69 min decreased to 29 min.

The 600 MPa HPP-75°C thermal was also the best method to inactivate *N. fischeri* spores in apple juice (3.3 log after 10 min) and *B. nivea* spores in strawberry puree (1.4 log after 10 min), whereas TS and thermal processing increase the *N. fischeri* and *B. nivea* spore numbers. However, the spore inactivation was registered after a long processing time.

For the same processing temperature of 75°C, a high variability of microbial spore resistance to thermal, TS, and HPP-thermal processes was observed, depending on the species. The spores of *C. perfringens* in beef slurry and *B. nivea* in strawberry puree were the most resistant to HPP-thermal treatment, followed by *N. fischeri* ascospores in apple juice, and lastly the psychrotrophic *B. cereus* spores in beef slurry. TS was very effective on *B. cereus*

Conclusions and recommendations for future work

spores in beef slurry, exhibiting >4 log in less than 2 min processing. TS was not appropriate for the mould spores due to the activation shoulders registered (+1 to +2.5 log increase). *A. acidoterrestris* spores in orange juice exhibited almost no change with TS, and *C. perfringens* only went down by 1 log after 60 min TS. Thermal processing alone at 75-78°C was not effective for spore destruction. Overall, the spores of psychrotrophic *B. cereus* showed the lowest resistance to all technologies tested, and the 4 week old mould ascospores were more resistant than the psychrotrophic *B. cereus* spores. The 600 MPa HPP-75°C and 75°C TS resistance of mould ascospores were species and spore age dependent, being higher for older spores.

The high pressure inactivation for all microbial spores in the foods was non-linear and described by the Weibull model. TS inactivation of psychrotrophic *B. cereus* spores in skim milk and *A. acidoterrestris* spores in orange juice followed the simple first order kinetics, whereas the log logistic and Weibull models described the TS inactivation of psychrotrophic *B. cereus* and *C. perfringens* spores in beef slurry, respectively. The Lorentzian distribution was a better model for describing the log survivors of *N. fischeri* and *B. nivea* ascospores by TS due to the activation shoulders observed.

To conclude, HPP-thermal treatment may be a suitable option for preservation of food products contaminated by *C. perfringens*, *N. fischeri*, and *B. nivea* spores, requiring higher pressures and/or temperatures. Although *B. cereus* spores were readily inactivated by TS, further research should be carried out on this technology as discussed below.

Recommendations for future work

More research on non-thermal food processing and searches for strategies to enhance conventional thermal process are needed due to consumers' demand for foods with higher safety and quality. Therefore, further understanding and more data are required on the inactivation of the key sporeformers important for specific foods.

The inactivation of psychrotrophic *B. cereus* spores by TS, and heat shock followed ultrasonication of spores/food enhancing *C. perfringens* and *A. acidoterrestris* spore thermal inactivation were shown promising and have the potential to be used in the food industry. However, the mechanisms of inactivation with these technologies are not yet described and need to be elucidated. Hence more fundamental research on the physiological phenomena involved before and after processing could provide further understanding of spore inactivation. Complementary data to explain the observed phenomena such as microscopic observations and proteomic data, could be developed in future research. Bacterial spore inactivation by TS in beef slurry was non-linear, exhibiting tailing. The mould spore exhibited activation shoulders after TS, thus a greater understanding this phenomena would help food processors to develop processes for various food products. The TS spore survivors seem to be different depending on the foods and species/strain employed. Investigation of other foods and strains would further supplement the available inactivation kinetic data. More research in engineering/equipment design is also required to increase spore inactivation rates, since the temperature that can be used for the probe is around 70°C.

The results of this study have shown that a significant microbial spore inactivation is only possible at high pressure (600 MPa) and moderate temperatures. However, long treatment times (>20 min) are still needed to obtain the ≥ 5 log reductions recommended for commercial pasteurization. Thus, more studies need to be carried out to optimize the processing

Conclusions and recommendations for future work

parameters such as by increasing the HPP temperature to achieve the desired inactivation (the use of high pressure-high temperature). However, quality of and capital investment on HPP and HPP maintenance costs are challenges. Other possibilities for future work on HPP-thermal processes may include an alternative strategy through combination with germinants and antimicrobial compounds as an approach to increasing the lethality to these spores.

Modeling the spore inactivation in foods provides the basis for process design. Appropriate sampling for microbiological analysis should be done. For example, the use of a single sample from each food could generate more sources of variability in the results. Further validation studies with new data to test the accuracy of the model are recommended: to re-evaluate the goodness of fit; to decide on re-identifying the proposed model structure/parameters for better accuracy; and to consider the necessity of generating additional data. Future work should also consider the estimation of model parameters under dynamic processing conditions due to inhomogeneous temperature distribution (eg. compression heating for HPP).

Other factor to consider for future works includes the quality of foods after processing. Food quality is another area that needs to be explored, especially after ultrasound processing such as cavitation may cause changes in the food properties during processing. Further research is also needed to investigate microbial growth potential after processing and during storage.

Publications and presentations based on thesis work

Publications and presentations based on thesis work

Refereed articles

Evelyn, & Silva, F. V. M. (2016). High pressure thermal processing for the inactivation of *Clostridium perfringens* spores in beef slurry. *Innovative Food Science & Emerging Technologies*, 33, 26-31.

Evelyn, & Silva, F. V. M. (2016). High pressure processing pretreatment enhanced the thermosonication inactivation of *Alicyclobacillus acidoterrestris* spores in orange juice. *Food Control*, 62, 365–372.

Evelyn, Kim, H. J., & Silva, F. V. M. (2016). Modeling the inactivation of *Neosartorya fischeri* ascospores in apple juice by high pressure, power ultrasound and thermal processing. *Food Control*, 59, 530–537.

Evelyn, & Silva, F. V. M. (2015). Inactivation of *Byssochlamys nivea* ascospores in strawberry puree by high pressure, power ultrasound and thermal processing. *International Journal of Food Microbiology*, 214, 129-136.

Evelyn, & Silva, F. V. M. (2015). High pressure processing of milk: Modeling the inactivation of *Bacillus cereus* spores at 38–70°C. *Journal of Food Engineering*, 165, 141-148.

Evelyn, & Silva, F. V. M. (2015). Use of power ultrasound to enhance the thermal inactivation of *Clostridium perfringens* spores in beef slurry. *International Journal of Food Microbiology*, 206, 17-23.

Evelyn, & Silva, F. V. M. (2015). Thermosonication versus thermal processing of skim milk and beef slurry: Modeling the inactivation kinetics of psychrotrophic *Bacillus cereus* spores. *Food Research International*, 67, 67-74.

Submitted articles

Evelyn, & Silva, F. V. M. (2015). High pressure processing combined with 38-70°C to inactivate psychrotrophic *Bacillus cereus* spores in beef slurry: Modeling the HPP and thermal inactivation. Submitted to *Food and Bioproduct Processing*.

Evelyn, & Silva, F. V. M. (2015). Resistance of *Byssoschlamys nivea* and *Neosartorya fischeri* spores to high pressure thermal processing and thermosonication on the high pressure-thermal and thermosonication inactivation: effect of spore age. Submitted to *International Journal of Food Microbiology*.

Conference presentations

Evelyn, & Silva, F. V. M. (2015). Power ultrasound, high pressure and thermal processing for the inactivation of microbial spores. In *ICEF12 (International Congress of Food and Engineering)*, 14–18 June, Quebec City, Canada.

Scientific meetings

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Appendices

These appendices contain examples of the observed data and modeling results presented in Chapter 2 (Appendix A), 5 (Appendix B), and 8 (Appendix C).

APPENDIX A

This appendix summarizes an example of the observed data and Weibull modeling results after HPP-THERMAL processing. Results for two strains *C. perfringens* spores at 75, 60, 50, and 38°C in Chapter 2 (**Figure 2-2**, **Figure 2-3**, and **Table 2-1**) are presented below. The MSE values were calculated using Equation 1-16.

In order to plot the HPP-thermal lines of *C. perfringens* spores, results ($\log N/N_0$) from three replicates were averaged \pm SD (each replicate was the mean value of two processed samples). For example for HPP-75°C *C. perfringens* NZRM 898 spores and 40 min in **Figure 2-2**, average of -2.85 $((-2.73-2.87-2.95)/3)$ and SD of 0.11 were used.

The Weibull parameter (b, n) values were obtained for each replicate and an average value \pm SD was presented. For example for HPP-75°C NZRM 898, the Weibull b -parameters for Replicate 1, 2, and 3 were 0.57, 0.71, and 0.76, respectively. The mean value from these replicates was 0.68 ± 0.10 (**Table 2-1**).

The MSE and R^2 values were also obtained for each replicate and the values were in the range listed in the footnote of **Table 2-1**. For example for HPP-75°C NZRM 898, the MSE values obtained ranged from 0.005 to 0.008, and R^2 values obtained ranged from 0.994 to 0.997. These values were in the range of the values presented in **Table 2-1** ($0.0002 \leq \text{MSE} \leq 0.031$, $0.890 \leq R^2 \leq 0.999$).

Time (min)	HPP-75°C - NZRM 898					
	N (cfu/g)					
	Rep. 1A	Rep. 1B	Rep. 2A	Rep. 2B	Rep. 3A	Rep. 3B
0	6.45E+07	6.45E+07	6.45E+07	6.45E+07	6.30E+07	6.30E+07
5	5.12E+06	3.51E+06	3.23E+06	2.24E+06	2.40E+06	2.09E+06
10	2.29E+06	1.85E+06	1.44E+06	1.38E+06	1.29E+06	1.23E+06
20	5.88E+05	5.36E+05	5.24E+05	3.94E+05	3.63E+05	3.63E+05
30	1.62E+05	1.41E+05	1.23E+05	1.35E+05	1.17E+05	1.17E+05
40	1.38E+05	1.05E+05	9.11E+04	8.20E+04	7.57E+04	6.60E+04
Time (min)	Rep. 1 (Mean)		Rep. 2 (Mean)		Rep. 3 (Mean)	
0	6.45E+07		6.45E+07		6.30E+07	
5	4.32E+06		2.74E+06		2.24E+06	
10	2.07E+06		1.41E+06		1.26E+06	
20	5.62E+05		4.59E+05		3.63E+05	
30	1.52E+05		1.29E+05		1.17E+05	
40	1.21E+05		8.66E+04		7.09E+04	
Time (min)	Log N/N ₀		Log N/N ₀		Log N/N ₀	
0	0.00		0.00		0.00	
5	-1.17		-1.37		-1.45	
10	-1.49		-1.66		-1.70	
20	-2.06		-2.15		-2.24	
30	-2.63		-2.70		-2.73	
40	-2.73		-2.87		-2.95	
	<i>b</i>	<i>b</i> ± SD	<i>n</i>	<i>n</i> ± SD	MSE	R ²
Rep. 1	0.57	0.68 ± 0.10	0.43	0.39 ± 0.03	0.008	0.994
Rep. 2	0.71		0.38		0.006	0.996
Rep. 3	0.76		0.37		0.005	0.997

Time (min)	HPP-60°C - NZRM 898						
	N (cfu/g)						
	Rep. 1A	Rep. 1B	Rep. 2A	Rep. 2B	Rep. 3A	Rep. 3B	
0	3.70E+07	3.70E+07	3.70E+07	3.70E+07	2.44E+07	2.44E+07	
5	1.37E+07	1.44E+07	1.12E+07	9.11E+06	7.72E+06	7.34E+06	
10	5.73E+06	5.47E+06	4.45E+06	4.45E+06	2.80E+06	2.55E+06	
20	2.94E+06	2.44E+06	2.56E+06	2.56E+06	1.22E+06	1.17E+06	
30	1.77E+06	1.47E+06	1.62E+06	1.28E+06	6.13E+05	5.34E+05	
40	1.17E+06	7.98E+05	7.21E+05	6.58E+05	3.87E+05	3.87E+05	
Time (min)	Rep. 1 (Mean)		Rep. 2 (Mean)		Rep. 3 (Mean)		
0	3.70E+07		3.70E+07		2.44E+07		
5	1.41E+07		1.01E+07		7.53E+06		
10	5.60E+06		4.45E+06		2.68E+06		
20	2.69E+06		2.56E+06		1.20E+06		
30	1.62E+06		1.45E+06		5.73E+05		
40	9.84E+05		6.90E+05		3.87E+05		
Time (min)	Log N/N ₀		Log N/N ₀		Log N/N ₀		
0	0.00		0.00		0.00		
5	-0.42		-0.56		-0.51		
10	-0.82		-0.92		-0.96		
20	-1.14		-1.16		-1.31		
30	-1.36		-1.41		-1.63		
40	-1.58		-1.73		-1.80		
Rep.	<i>b</i>	<i>b</i> ± SD	<i>n</i>	<i>n</i> ± SD	MSE	R ²	
	Rep. 1	0.21	0.25 ± 0.03	0.55	0.53 ± 0.02	0.004	0.991
	Rep. 2	0.26		0.50		0.031	0.993
	Rep. 3	0.26		0.53		0.005	0.991

Time (min)	HPP-50°C - NZRM 898						
	N (cfu/g)						
	Rep. 1A	Rep. 1B	Rep. 2A	Rep. 2B	Rep. 3A	Rep. 3B	
0	2.20E+07	2.20E+07	2.20E+07	2.20E+07	2.20E+07	2.20E+07	
5	1.75E+07	1.75E+07	1.75E+07	1.16E+07	1.13E+07	1.02E+07	
10	7.63E+06	6.20E+06	6.06E+06	2.82E+06	3.41E+06	2.31E+06	
20	3.82E+06	3.82E+06	2.97E+06	2.97E+06	1.45E+06	2.23E+06	
30	2.71E+06	2.83E+06	2.71E+06	2.71E+06	2.10E+06	1.39E+06	
40	2.47E+06	1.96E+06	2.83E+06	2.31E+06	1.75E+06	1.75E+06	
Time (min)	Rep. 1 (Mean)		Rep. 2 (Mean)		Rep. 3 (Mean)		
0	2.20E+07		2.20E+07		2.20E+07		
5	1.75E+07		1.45E+07		1.07E+07		
10	6.91E+06		4.44E+06		2.86E+06		
20	3.82E+06		2.97E+06		1.84E+06		
30	2.77E+06		2.71E+06		1.74E+06		
40	2.21E+06		2.57E+06		1.75E+06		
Time (min)	Log N/N ₀		Log N/N ₀		Log N/N ₀		
0	0.00		0.00		0.00		
5	-0.10		-0.18		-0.31		
10	-0.50		-0.70		-0.89		
20	-0.76		-0.87		-1.08		
30	-0.90		-0.91		-1.10		
40	-1.00		-0.93		-1.10		
Rep.	<i>b</i>	<i>b</i> ± SD	<i>n</i>	<i>n</i> ± SD	MSE	R ²	
	Rep. 1	0.09	0.19 ± 0.10	0.67	0.50 ± 0.14	0.011	0.947
	Rep. 2	0.19		0.45		0.024	0.916
	Rep. 3	0.30		0.38		0.031	0.900

Time (min)	HPP-38°C - NZRM 898						
	N (cfu/g)						
	Rep. 1A	Rep. 1B	Rep. 2A	Rep. 2B	Rep. 3A	Rep. 3B	
0	2.80E+07	2.80E+07	2.80E+07	2.80E+07	2.80E+07	2.80E+07	
5	2.80E+07	2.80E+07	2.80E+07	2.33E+07	2.44E+07	2.28E+07	
10	2.67E+07	2.67E+07	2.33E+07	2.12E+07	2.03E+07	1.85E+07	
15	2.44E+07	2.67E+07	2.38E+07	1.97E+07	2.08E+07	1.71E+07	
20	2.33E+07	2.33E+07	2.28E+07	1.94E+07	2.03E+07	1.94E+07	
30	2.03E+07	2.22E+07	1.98E+07	1.72E+07	1.77E+07	1.54E+07	
40	2.12E+07	2.12E+07	1.94E+07	1.69E+07	1.69E+07	1.34E+07	
Time (min)	Rep. 1 (Mean)		Rep. 2 (Mean)		Rep. 3 (Mean)		
0	2.80E+07		2.80E+07		2.80E+07		
5	2.80E+07		2.56E+07		2.36E+07		
10	2.67E+07		2.23E+07		1.94E+07		
15	2.56E+07		2.18E+07		1.89E+07		
20	2.33E+07		2.11E+07		1.98E+07		
30	2.13E+07		1.85E+07		1.65E+07		
40	2.12E+07		1.81E+07		1.51E+07		
Time (min)	Log N/N ₀		Log N/N ₀		Log N/N ₀		
0	0.00		0.00		0.00		
5	0.00		-0.04		-0.07		
10	-0.02		-0.10		-0.16		
15	-0.04		-0.11		-0.17		
20	-0.08		-0.12		-0.15		
30	-0.12		-0.18		-0.23		
40	-0.12		-0.19		-0.27		
Rep.	<i>b</i>	<i>b</i> ± SD	<i>n</i>	<i>n</i> ± SD	MSE	R ²	
	Rep. 1	0.003	0.02 ± 0.01	1.07	0.73 ± 0.28	0.0003	0.923
	Rep. 2	0.02		0.61		0.0002	0.971
	Rep. 3	0.04		0.53		0.0005	0.946

Time (min)	HPP-75°C - NZRM 2621						
	N (cfu/g)						
	Rep. 1A	Rep. 1B	Rep. 2A	Rep. 2B	Rep. 3A	Rep. 3B	
0	2.60E+07	2.60E+07	2.60E+07	2.60E+07	2.60E+07	2.60E+07	
5	5.31E+06	5.11E+06	4.73E+06	4.12E+06	3.85E+06	3.67E+06	
10	2.60E+06	2.16E+06	2.85E+06	2.85E+06	1.97E+06	1.80E+06	
20	6.24E+05	5.46E+05	3.94E+05	5.59E+05	2.92E+05	2.32E+05	
30	7.00E+04	7.00E+04	5.19E+04	4.52E+04	4.73E+04	3.76E+04	
40	2.79E+04	1.60E+04	3.43E+04	2.60E+04	1.68E+04	9.59E+03	
Time (min)	Rep. 1 (Mean)		Rep. 2 (Mean)		Rep. 3 (Mean)		
0	2.60E+07		2.60E+07		2.60E+07		
5	5.21E+06		4.42E+06		3.76E+06		
10	2.38E+06		2.85E+06		1.89E+06		
20	5.85E+05		4.76E+05		2.62E+05		
30	7.00E+04		4.85E+04		4.24E+04		
40	2.19E+04		3.01E+04		1.32E+04		
Time (min)	Log N/N ₀		Log N/N ₀		Log N/N ₀		
0	0.00		0.00		0.00		
5	-0.70		-0.77		-0.84		
10	-1.04		-0.96		-1.14		
20	-1.65		-1.74		-2.00		
30	-2.57		-2.73		-2.79		
40	-3.07		-2.94		-3.29		
Rep.	<i>b</i>	<i>b</i> ± SD	<i>n</i>	<i>n</i> ± SD	MSE	R ²	
	Rep. 1	0.17	0.20 ± 0.03	0.78	0.74 ± 0.03	0.010	0.994
	Rep. 2	0.20		0.74		0.030	0.982
	Rep. 3	0.24		0.71		0.006	0.997

Time (min)	HPP-60°C - NZRM 2621						
	N (cfu/g)						
	Rep. 1A	Rep. 1B	Rep. 2A	Rep. 2B	Rep. 3A	Rep. 3B	
0	2.64E+07	2.64E+07	2.64E+07	2.64E+07	2.64E+07	2.64E+07	
5	1.26E+07	1.02E+07	9.37E+06	8.25E+06	8.16E+06	7.44E+06	
10	4.92E+06	4.92E+06	5.03E+06	4.18E+06	3.32E+06	3.32E+06	
20	2.10E+06	1.38E+06	1.39E+06	1.48E+06	1.00E+06	8.74E+05	
30	7.27E+05	6.63E+05	5.15E+05	4.93E+05	5.03E+05	3.64E+05	
40	3.17E+05	2.89E+05	3.32E+05	2.89E+05	2.20E+05	1.74E+05	
Time (min)	Rep. 1 (Mean)		Rep. 2 (Mean)		Rep. 3 (Mean)		
0	2.64E+07		2.64E+07		2.64E+07		
5	1.14E+07		8.81E+06		7.80E+06		
10	4.92E+06		4.61E+06		3.32E+06		
20	1.74E+06		1.44E+06		9.39E+05		
30	6.95E+05		5.04E+05		4.34E+05		
40	3.03E+05		3.11E+05		1.97E+05		
Time (min)	Log N/N ₀		Log N/N ₀		Log N/N ₀		
0	0.00		0.00		0.00		
5	-0.36		-0.48		-0.53		
10	-0.73		-0.76		-0.90		
20	-1.18		-1.26		-1.45		
30	-1.58		-1.72		-1.78		
40	-1.94		-1.93		-2.13		
Rep.	<i>b</i>	<i>b</i> ± SD	<i>n</i>	<i>n</i> ± SD	MSE	R ²	
	Rep. 1	0.13	0.17 ± 0.04	0.74	0.68 ± 0.06	0.001	0.999
	Rep. 2	0.21		0.63		0.002	0.998
	Rep. 3	0.17		0.67		0.003	0.996

Time (min)	HPP-50°C - NZRM 2621						
	N (cfu/g)						
	Rep. 1A	Rep. 1B	Rep. 2A	Rep. 2B	Rep. 3A	Rep. 3B	
0	2.75E+07	2.75E+07	2.75E+07	2.75E+07	2.64E+07	2.64E+07	
5	1.55E+07	1.46E+07	1.58E+07	1.50E+07	1.45E+07	1.45E+07	
10	1.38E+07	1.09E+07	7.93E+06	8.70E+06	7.61E+06	5.17E+06	
20	4.56E+06	4.36E+06	3.09E+06	2.81E+06	3.82E+06	3.32E+06	
30	5.49E+06	4.79E+06	1.99E+06	2.14E+06	2.83E+06	1.27E+06	
40	2.18E+06	2.18E+06	1.05E+06	1.60E+06	2.70E+06	2.58E+06	
Time (min)	Rep. 1 (Mean)		Rep. 2 (Mean)		Rep. 3 (Mean)		
0	2.75E+07		2.75E+07		2.64E+07		
5	1.50E+07		1.54E+07		1.45E+07		
10	1.24E+07		8.31E+06		6.39E+06		
20	4.46E+06		2.95E+06		3.57E+06		
30	5.14E+06		2.06E+06		2.05E+06		
40	2.18E+06		1.32E+06		2.64E+06		
Time (min)	Log N/N ₀		Log N/N ₀		Log N/N ₀		
0	0.00		0.00		0.00		
5	-0.26		-0.25		-0.26		
10	-0.35		-0.52		-0.62		
20	-0.79		-0.97		-0.87		
30	-0.73		-1.12		-1.11		
40	-1.10		-1.32		-1.00		
Rep.	<i>b</i>	<i>b</i> ± SD	<i>n</i>	<i>n</i> ± SD	MSE	R ²	
	Rep. 1	0.08	0.12 ± 0.05	0.69	0.62 ± 0.10	0.011	0.949
	Rep. 2	0.11		0.68		0.006	0.983
	Rep. 3	0.18		0.50		0.015	0.936

Time (min)	HPP-38°C - NZRM 2621					
	N (cfu/g)					
	Rep. 1A	Rep. 1B	Rep. 2A	Rep. 2B	Rep. 3A	Rep. 3B
0	2.64E+07	2.64E+07	2.64E+07	2.64E+07	2.64E+07	2.64E+07
1	2.20E+07	2.10E+07	1.67E+07	1.83E+07	1.83E+07	1.78E+07
5	2.10E+07	2.00E+07	1.52E+07	1.74E+07	1.15E+07	1.52E+07
10	1.45E+07	1.42E+07	1.15E+07	1.32E+07	1.03E+07	1.05E+07
15	1.23E+07	1.13E+07	1.03E+07	1.18E+07	9.81E+06	9.81E+06
20	1.18E+07	1.08E+07	1.05E+07	1.08E+07	8.16E+06	5.78E+06
30	7.44E+06	4.92E+06	4.48E+06	3.64E+06	3.17E+06	2.89E+06
40	6.33E+06	5.52E+06	5.91E+06	5.78E+06	4.38E+06	3.82E+06
Time (min)	Rep. 1 (Mean)		Rep. 2 (Mean)		Rep. 3 (Mean)	
0	2.64E+07		2.64E+07		2.64E+07	
1	2.15E+07		1.75E+07		1.81E+07	
5	2.05E+07		1.63E+07		1.34E+07	
10	1.43E+07		1.24E+07		1.04E+07	
15	1.18E+07		1.10E+07		9.81E+06	
20	1.13E+07		1.06E+07		6.97E+06	
30	6.18E+06		4.06E+06		3.03E+06	
40	5.92E+06		5.84E+06		4.10E+06	
Time (min)	Log N/N ₀		Log N/N ₀		Log N/N ₀	
0	0.00		0.00		0.00	
1	-0.09		-0.18		-0.16	
5	-0.11		-0.21		-0.30	
10	-0.26		-0.33		-0.40	
15	-0.35		-0.38		-0.43	
20	-0.37		-0.39		-0.58	
30	-0.63		-0.81		-0.94	
40	-0.65		-0.65		-0.81	
Rep. 1 Rep. 2 Rep. 3	<i>b</i>	<i>b</i> ± SD	<i>n</i>	<i>n</i> ± SD	MSE	R ²
	0.05	0.09 ± 0.04	0.72	0.61 ± 0.10	0.002	0.966
	0.09		0.56		0.011	0.890
	0.12		0.54		0.008	0.928

APPENDIX B

This appendix summarizes an example of the observed data and first order modeling results after THERMAL processing. Results for *B. cereus* ICMP 12442 spores at 90, 80, and 70°C in Chapter 5 (**Figure 5-4** and **Table 5-2**) are presented below. The A_f values were calculated using Equation 1-17.

In order to plot the thermal lines of *B. cereus* spores, results ($\log N/N_0$) from two replicates were averaged \pm SD (each replicate was the mean value of two processed samples). For example for 90°C-thermal *B. cereus* ICMP 12442 spores and 5 min in **Figure 5-4**, average of -5.13 $((-5.21-5.05)/2)$ and SD of 0.11 were used.

The first order kinetic parameter (D -) values were obtained for each replicate and an average value \pm SD was presented. For example for 90°C-thermal ICMP 12442, the D -values for Replicate 1, and 2 were 0.99, and 0.96, respectively. The mean value from these replicates was 0.98 ± 0.02 (**Table 5-2**).

The MSE, R^2 , and A_f values were obtained from the averaged $\log N/N_0$ values and were presented for each temperature (**Table 5-2**). For example for 90°C-thermal ICMP 12442, the MSE, R^2 , and A_f values were 0.002, 0.998, and 1.03, respectively (**Table 5-2**).

Time (min)	Thermal at 90°C - ICMP 12442				
	N (cfu/mL)				
	Rep. 1A	Rep. 1B	Rep. 2A	Rep. 2B	
0	1.80E+07	1.80E+07	1.80E+07	1.80E+07	
1	1.72E+06	1.55E+06	1.76E+06	1.76E+06	
2	1.80E+05	1.64E+05	1.53E+05	1.46E+05	
3	1.50E+04	1.19E+04	1.68E+04	1.68E+04	
5	1.27E+02	9.67E+01	1.80E+02	1.39E+02	
Time (min)	Rep. 1 (Mean)		Rep. 2 (Mean)		
0	1.80E+07		1.80E+07		
1	1.64E+06		1.76E+06		
2	1.72E+05		1.50E+05		
3	1.34E+04		1.68E+04		
5	1.12E+02		1.60E+02		
Time (min)	Log N/N ₀		Log N/N ₀		
0	0.00		0.00		
1	-1.04		-1.01		
2	-2.02		-2.08		
3	-3.13		-3.03		
5	-5.21		-5.05		
Rep. 1 Rep. 2	<i>D</i>	<i>D</i> ± <i>SD</i>	MSE	R ²	<i>A_f</i>
	0.99 0.96	0.98 ± 0.02	0.002	0.998	1.03

Time (min)	Thermal at 80°C - ICMP 12442				
	N (cfu/mL)				
	Rep. 1A	Rep. 1B	Rep. 2A	Rep. 2B	
0	1.80E+07	1.80E+07	1.80E+07	1.80E+07	
5	3.38E+06	3.38E+06	3.35E+06	3.55E+06	
10	6.54E+05	6.90E+05	6.34E+05	6.54E+05	
15	1.16E+05	1.22E+05	1.27E+05	1.27E+05	
20	2.16E+04	2.28E+04	2.37E+04	2.48E+04	
Time (min)	Rep. 1 (Mean)		Rep. 2 (Mean)		
0	1.80E+07		1.80E+07		
5	3.38E+06		3.45E+06		
10	6.72E+05		6.44E+05		
15	1.19E+05		1.27E+05		
20	2.22E+04		2.43E+04		
Time (min)	Log N/N ₀		Log N/N ₀		
0	0.00		0.00		
5	-0.73		-0.72		
10	-1.43		-1.45		
15	-2.18		-2.15		
20	-2.91		-2.87		
Rep. 1	D	D ± SD	MSE	R ²	A _f
	6.88	6.93 ± 0.06	0.0001	0.999	1.01
Rep. 2	6.97				

Time (min)	Thermal at 70°C - ICMP 12442				
	N (cfu/mL)				
	Rep. 1A	Rep. 1B	Rep. 2A	Rep. 2B	
0	3.20E+07	3.20E+07	3.20E+07	3.20E+07	
2	2.89E+07	2.89E+07	2.90E+07	2.90E+07	
5	2.48E+07	2.48E+07	2.50E+07	2.50E+07	
10	1.93E+07	1.93E+07	1.97E+07	1.88E+07	
15	1.50E+07	1.46E+07	1.53E+07	1.53E+07	
20	1.22E+07	1.07E+07	1.16E+07	1.19E+07	
Time (min)	Rep. 1 (Mean)		Rep. 2 (Mean)		
0	3.20E+07		3.20E+07		
2	2.89E+07		2.90E+07		
5	2.48E+07		2.50E+07		
10	1.93E+07		1.93E+07		
15	1.48E+07		1.53E+07		
20	1.14E+07		1.18E+07		
Time (min)	Log N/N ₀		Log N/N ₀		
0	0.00		0.00		
2	-0.04		-0.04		
5	-0.11		-0.11		
10	-0.22		-0.22		
15	-0.33		-0.32		
20	-0.45		-0.43		
Rep. 1	D	D ± SD	MSE	R ²	A _f
	45.50	46.03 ± 0.80	0.0001	0.999	1.01
Rep. 2	46.60				

APPENDIX C

This appendix summarizes an example of the observed data and first order modeling results after THERMOSONICATION. Results for *A. acidoterrestris* NZRM 4447 spores at 78, 75, and 70°C in Chapter 8 (**Figure 8-3** and **Table 8-1**) are presented below.

In order to plot the thermosonication lines of *A. acidoterrestris* spores, results ($\log N/N_0$) from two replicates were averaged \pm SD (each replicate was the mean value of two processed samples). For example for 78°C-TS *A. acidoterrestris* spores and 60 min in **Figure 8-3**, average of -2.25 ((-2.41-2.09)/) and SD of 0.23 were used.

The first order kinetic parameter (*D*-) values were obtained for each replicate and an average value \pm SD was presented. For example for 78°C-TS *A. acidoterrestris*, the *D*-values for Replicate 1, and 2 were 25.50 and 30.56, respectively. The mean value from these replicates was 28.0 ± 3.60 (**Table 8-1**).

The MSE and R^2 values were also obtained for each replicate and the values were in the range listed in the footnote of **Table 8-1**. For example for 78°C-TS *A. acidoterrestris*, the MSE values obtained ranged from 0.003 to 0.013, and R^2 values obtained ranged from 0.978 to 0.996. These values were in the range of the values in **Table 8-1** (0.0001–0.120 MSE and 0.981–0.998 R^2).

Time (min)	Thermosonication at 78°C - NZRM 4447			
	N (cfu/mL)			
	Rep. 1A	Rep. 1B	Rep. 2A	Rep. 2B
0	3.50E+07	3.50E+07	2.40E+07	2.40E+07
10	1.63E+07	1.12E+07	1.59E+07	1.32E+07
20	7.82E+06	5.62E+06	8.71E+06	5.93E+06
30	3.66E+06	1.89E+06	3.80E+06	3.16E+06
40	1.26E+06	6.08E+05	1.02E+06	1.26E+06
60	1.78E+05	9.26E+04	2.51E+05	1.35E+05
Time (min)	Rep. 1 (Mean)		Rep. 2 (Mean)	
0	3.50E+07		2.40E+07	
10	1.38E+07		1.46E+07	
20	6.72E+06		7.32E+06	
30	2.78E+06		3.48E+06	
40	9.34E+05		1.14E+06	
60	1.35E+05		1.93E+05	
Time (min)	Log N/N ₀		Log N/N ₀	
0	0.00		0.00	
10	-0.41		-0.22	
20	-0.72		-0.52	
30	-1.10		-0.84	
40	-1.57		-1.32	
60	-2.41		-2.09	
Rep. 1	D	D ± SD	MSE	R ²
	25.50	28.0 ± 3.60	0.003	0.996
Rep. 2	30.56		0.013	0.978

Thermosonication at 75°C - NZRM 4447				
Time (min)	N (cfu/mL)			
	Rep. 1A	Rep. 1B	Rep. 2A	Rep. 2B
0	2.82E+07	2.82E+07	2.82E+07	2.82E+07
10	1.59E+07	1.59E+07	2.19E+07	1.82E+07
20	1.05E+07	9.56E+06	1.51E+07	1.20E+07
30	5.89E+06	7.08E+06	1.20E+07	8.15E+06
40	3.16E+06	4.17E+06	7.59E+06	4.59E+06
60	1.45E+06	1.26E+06	1.66E+06	1.45E+06
Time (min)	Rep. 1 (Mean)		Rep. 2 (Mean)	
0	2.82E+07		2.82E+07	
10	1.59E+07		2.00E+07	
20	1.00E+07		1.36E+07	
30	6.49E+06		1.01E+07	
40	3.67E+06		6.09E+06	
60	1.35E+06		1.55E+06	
Time (min)	Log N/N ₀		Log N/N ₀	
0	0.00		0.00	
10	-0.25		-0.15	
20	-0.45		-0.32	
30	-0.64		-0.45	
40	-0.89		-0.67	
60	-1.32		-1.26	
Rep. 1	D	D ± SD	MSE	R ²
	45.45	49.4 ± 5.55	0.0003	0.998
Rep. 2	53.31		0.008	0.959

Thermosonication at 70°C - NZRM 4447				
Time (min)	N (cfu/mL)			
	Rep. 1A	Rep. 1B	Rep. 2A	Rep. 2B
0	2.82E+07	2.82E+07	2.82E+07	2.82E+07
10	2.29E+07	2.19E+07	2.35E+07	2.35E+07
20	1.91E+07	1.74E+07	2.29E+07	2.14E+07
30	1.66E+07	1.38E+07	1.82E+07	2.00E+07
40	1.32E+07	1.00E+07	1.70E+07	1.59E+07
60	8.32E+06	7.47E+06	1.51E+07	9.75E+06
Time (min)	Rep. 1 (Mean)		Rep. 2 (Mean)	
0	2.82E+07		2.82E+07	
10	2.24E+07		2.35E+07	
20	1.82E+07		2.22E+07	
30	1.52E+07		1.91E+07	
40	1.16E+07		1.64E+07	
60	7.89E+06		1.24E+07	
Time (min)	Log N/N ₀		Log N/N ₀	
0	0.00		0.00	
10	-0.10		-0.08	
20	-0.19		-0.10	
30	-0.27		-0.17	
40	-0.39		-0.23	
60	-0.55		-0.36	
Rep. 1	D	D ± SD	MSE	R ²
	107.32	138.9 ± 44.70	0.0001	0.997
Rep. 2	170.54		0.0002	0.989

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