

***Geobacillus stearothermophilus* spore germination and
inactivation mechanisms during emerging multi hurdle
combinations**

Von der Naturwissenschaftlichen Fakultät der
Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades
Doktorin der Naturwissenschaften (Dr. rer. nat.)

genehmigte Dissertation

von

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2015

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Tag der Promotion: 16.03.2015

This work is dedicated to

My grandfather, Louis André Velay (04/02/1924 -) for his love and care, his support since my infancy, and being a never-ending source of pride and inspiration.

My grandmother, Solange Joséphine Marie Georget (13/10/1909-06/01/2009) for the avant-gardist woman she was throughout her life. She has been a constant reminder that resilience, will and dignity are essential attributes to prevail on challenges.

Acknowledgements

Acknowledgements

I would like to express my gratitude to Professor Ralf Günter Berger for having granted me the opportunity to join the Graduate School of Natural Sciences of the Leibniz Universität Hannover as well as his availability and his support during the preparation of this thesis. To Dr. Volker Heinz, Director of the German Institute of Food Technologies (DIL), many thanks for having welcomed me at DIL, endorsed my research activities for the past three years and for his willingness to review this thesis. My gratitude is also expressed to Professor Thomas Scheper (Institut für Technische Chemie, Leibniz Universität Hannover) and Professor Rudi F. Vogel (Lehrstuhl für Technische Mikrobiologie, Technische Universität München) for having agreed to evaluate this thesis and be part of the evaluation committee. To PD Dr. Ulrich Krings (Institut für Lebensmittelchemie - Leibniz Universität Hannover), many thanks for having accepted to chair the thesis evaluation committee.

The work accomplished within the past three years would not have been possible without the contribution of several colleagues and friends as well as inspiring academic supporters.

I particularly would like to thank Dr. Alexander Mathys, my department head at DIL, for his supervision of the thesis, his trust and the great independence he gave me. He has been both a mentor and a sponsor of my professional development over the past three years.

I gratefully acknowledge Professor Roland Winter from the Technische Universität Dortmund for the access to his lab and many helpful discussions, as well as Professor Cornelia Rauh and Professor Dietrich Knorr from the Technische Universität Berlin for the access to the process lab. I am grateful to Kemal Aganovic, Dr. Kai Reineke, Robert Sevenich and Dr. Shobhna Kapoor for many helpful discussions and their support of my research activities. Many thanks go to my former students Lola, Aselya and Brittany for their help and their hard work.

This research was funded by the Nestlé Research Center, Lausanne, Switzerland, and I thank Dr. Edwin Ananta and Dr. Michael Callanan for their support through the different stages of the project.

The above list is far from complete as I had the opportunity to discuss and collaborate with many interesting and supportive people on multiple occasions. I hereby thank them for having contributed to this work by the inspiration of new ideas or by raising the right questions at the right time.

Acknowledgements

Last but not least, my family and friends are warmly acknowledged on this occasion. While this group is too large to be fully listed and also partially overlaps with the above mentioned, I would like to highlight some persons.

My sanity during the past three years owes a lot to my friends who, from close or far, were there for me. W. Robert, Benno, Flo L. & Flo L., Jens B. (and several unknowing Krav Maga training partners), thank you for your support and friendship throughout this time.

To my family and in particular to my parents, Sylvie and Philippe: Thank you for always having provided for all my needs since the beginning and also during my peregrinations, for having imbued me with your drive, your energy, and the desire to learn. Thank you for your unconditional support, your trust and your understanding during my studies and my doctorate.

To my partner, Tarek: Words do not do justice to your importance in my life and your incredible support throughout the past years. You have been and remain my best friend; you have shared my successes and my fears, dried my tears and laughed with me. Thank you for your love, your support and your patience – *in crime and glory*.

Preface

Preface

This thesis is based on reformatted work which has been published in the following peer reviewed publications:

- Georget, E., Kapoor, S., Winter, R., Reineke, K., Song, Y., Callanan, M., Ananta, E., Heinz, V., Mathys, A. (2014). *In situ* investigation of *Geobacillus stearothermophilus* spore germination and inactivation mechanisms under moderate high pressure. *Food Microbiology*, 41, 8-18.
- Georget, E., Kushman, A., Callanan, M., Ananta, E., Heinz, V., Mathys, A. (2015). *Geobacillus stearothermophilus* ATCC 7953 spore chemical germination mechanisms in model systems. *Food Control*, 50, 141-149.
- Georget, E., Miller, B., Aganovic, K., Callanan, M., Heinz, V., Mathys, A. (2014). Bacterial spore inactivation by ultra-high pressure homogenization. *Innovative Food Science and Emerging Technologies*, 26, 116-123.
- Georget, E., Miller, B., Callanan, M., Heinz, V., Mathys, A. (2014). (Ultra) high pressure homogenization for continuous high pressure sterilization of pumpable foods - a review. *Frontiers in Nutrition*, 1(15), 1-6.

Zusammenfassung

Zusammenfassung

Aufgrund ihrer Resistenz gegenüber herkömmlichen Haltbarmachungsverfahren und dem Potenzial, Rekontaminationen zu verursachen, sind bakterielle Sporen einer der Hauptrisikofaktoren für die Lebensmittelsicherheit und Produktqualität. Innovative Haltbarmachungshürden können eine Keimung von Bakteriensporen auslösen, wodurch deren Widerstand gegen mildere Wärmebehandlungen verringert werden kann. Diese Hürden können dadurch eine direkte Inaktivierung mit verringerter thermischer Belastung ermöglichen. Das Hauptziel der vorliegenden Arbeit war es, innovative technologische Lösungen für die Inaktivierung von Sporen zu entwickeln, welche eine Reduzierung der angewandten thermischen Intensität erlauben. Der Fokus der gewonnenen Erkenntnisse lag darauf, eine schonendere Sterilisation von empfindlichen Produkten zu erreichen, um Sicherheit und Frische für säurearme Lebensmittel bei Raumtemperatur zu gewährleisten. Zum Erfüllen dieser Bedingungen konzentrierten sich die Forschungsaktivitäten auf das Hauptziel des Sterilisationsprozesses – hochresistente bakterielle Sporen. Unter der Voraussetzung, die Inaktivierung von Sporen zu erleichtern, ist diese Arbeit daher an der Schnittstelle zwischen Mikrobiologie und Lebensmittelverfahrenstechnik angesiedelt und zielt auf ein verbessertes Verständnis der Mechanismen während der Anwendung von neuartigen Haltbarmachungshürden ab. Ausgesuchte Hürdenelemente wurden einzeln betrachtet und in Modellpuffersystemen mit physiko-chemischen, mechanischen und thermischen Verfahren kombiniert. Die Arbeit wurde überwiegend unter Verwendung von *Geobacillus stearothermophilus* ATCC 7953 Sporen, des offiziellen Indikators für die Sterilisation durch feuchte Hitze, durchgeführt.

Unter Verwendung neuartiger Untersuchungsmethoden in-situ (FT-IR Spektroskopie in Diamantstempelzellen und Laurdanfluoreszenzspektroskopie) konnten weitere Einblicke in die Mechanismen der Keimung von bakteriellen Sporen gewonnen werden. Im Fokus standen hier die Veränderungen der inneren Sporenmembran. Moderater Hochdruck bei 200 MPa und Kalziumdipicolinsäure führten beide zu einer Keimung von etwa 3 log₁₀ Sporen aufgrund unterschiedlicher Mechanismen. Obwohl signifikant, war dies selbst nicht ausreichend, um kommerzielle Sterilitätsstandards zu erreichen, und die Verfahrensbedingungen waren zum Teil von dem verwendeten Indikatorkeim abhängig. Weitere Untersuchungen auf Basis der Ultraschallhomogenisierung (UHPH) erlaubten die kontinuierliche hochdruckunterstützte thermische Behandlung von pumpfähigen Medien. Der starke Temperaturanstieg an dem Ultraschall-Homogenisierventil (~ 20 ° C / 100 MPa) und die Anwendung von dynamischem Druck über 300 MPa ermöglichten ein Temperaturprofil ähnlich zu jenem, welches durch direkte Dampf-injektion (DSI) erreicht werden kann. Dadurch konnten die verwendeten bakteriellen Sporen

Zusammenfassung

in einem Durchlauf bei Verweilzeiten unter einer Sekunde und Temperaturen über 140 °C inaktiviert werden. Nach Prozessende konnte keine signifikante Keimung detektiert werden.

Die Modellierung der thermischen Abtötungskinetik deutet darauf hin, dass die Inaktivierung vor allem durch thermische Einflüsse verursacht wird.

UHPH kann somit eine indirekte und kontinuierliche Form des druckunterstützten Erhitzens darstellen, welche die Wärmebelastung im Vergleich zu DSI reduzieren und die Verfahrenskosten der Sterilisation senken kann. Dieses Verfahren ermöglicht es auch, die Sterilisation und Homogenisierung von pumpbaren Gütern in einem einzelnen Schritt zu kombinieren, wodurch Verarbeitungszeit, Prozessintensität sowie Ressourcenaufwand weiter verringert werden können.

Schlagwörter: Bakterielle Sporen, Keimung, Inaktivierung

Abstract

Abstract

Bacterial spores are a chief concern for food safety and quality by their resistance to most conventional preservation hurdles and the potential they carry for recontamination. Innovative hurdles can trigger germination of bacterial spores, hence reducing resistance to milder thermal treatments, or enable direct inactivation with a reduced thermal load. The main objective of this work was to develop innovative technological solutions for spore inactivation which could support the reduction of the total thermal load applied to food products. With the knowledge gained, the target is to achieve more gentle sterilization of sensitive products, thus bridging the gap between safety and freshness of ambient stable low acidic food. In order to reach this goal, the research activities focused on the target of sterilization – the bacterial spore. To facilitate the inactivation of the spore, this work is located at the interface between microbiology and food process technologies in order to improve our understanding of the mechanisms at play during the use of novel hurdles.

Several hurdles were considered individually and combined in model buffer systems, including physico-chemical, mechanical and thermal hurdles. The work was mainly focused on *Geobacillus stearothermophilus* ATCC 7953 spores, the official indicator for wet heat sterilization. Using novel *in situ* spore investigation tools (FT-IR spectroscopy in diamond anvil cell and Laurdan fluorescence spectroscopy), new insights in the germination mechanisms of bacterial spores could be gained. The focus was set on the changes occurring in the inner spore membrane. Moderate high pressure of 200 MPa and calcium dipicolinic acid both led to up to about 3 log₁₀ germination by different mechanisms. Though significant, this was not sufficient by itself to achieve commercial sterility and the treatment conditions were partially strain dependent.

Further investigations based on ultra-high pressure homogenization (UHPH), allowed continuous high pressure assisted thermal treatment of pumpable media. The strong temperature increase at the homogenization valve of the UHPH system (~20 °C / 100 MPa) and the use of dynamic pressure above 300 MPa enabled a temperature profile close to what is achieved by direct steam injection (DSI). This could inactivate bacterial spores in a single pass with holding times below one second at temperatures above 140 °C. No significant germination was triggered by this treatment and thermal inactivation kinetics modeling suggested that the inactivation was mainly thermally driven.

UHPH could thus be an indirect and continuous form of pressure assisted heating which could reduce the thermal load by comparison to DSI, along with the operational costs of sterilization. This technology could also offer to combine sterilization and homogenization of pumpable goods, in a single step thereby further reducing processing time, intensity and resource needs.

Keywords: Bacterial spore, germination, inactivation

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List of abbreviations

List of abbreviations

Abbreviation Description

| | |
|-----------|---|
| ACES | N-(2-Acetamido)-2-aminoethanesulfonic acid |
| AFM | Atomic force microscopy |
| AGFK | L-asparagine, D-glucose, D-fructose, potassium ions |
| ATCC | American type culture collection |
| a_w | Water activity |
| <i>B.</i> | <i>Bacillus</i> |
| <i>C.</i> | <i>Clostridium</i> |
| CaDPA | Ca ²⁺ - Pyridine-2,6-dicarboxylic acid complex |
| CFD | Computational fluid dynamics |
| CFU | Colony forming unit |
| CLE | Cortex lytic enzyme |
| CLFE | Cortical fragment lytic enzyme |
| Cr | Spore core |
| Cx | Cortex |
| DAC | Diamond anvil cell |
| DNA | Deoxyribonucleic acid |
| DPA | Pyridine-2,6-dicarboxylic acid |
| DSI | Direct steam injection |
| FDA | Food and drug administration |
| FIB | Focused ion beam |
| FT-IR | Fourier transform infrared |
| <i>G.</i> | <i>Geobacillus</i> |
| GP | Generalized polarization |
| HP | High pressure |
| HPTS | High pressure thermal sterilization |
| Ic | Inner coat |
| IM | Inner membrane |
| IR | Infrared |
| Laurdan | 6-Dodecanoyl-N,N-dimethyl-2-naphthylamine |
| LD50 | Median lethal dose |

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List of abbreviations

| | |
|------|---|
| Oc | Outer coat |
| OD | Optic density |
| OM | Outer membrane |
| PATS | Pressure assisted thermal sterilization |
| PBS | Phosphate buffer system |
| PEF | Pulsed electric fields |
| pH | Decadic logarithm of activity of hydroxonium ions |
| SASP | Small acid-soluble proteins |
| SEM | Scanning electron microscopy |
| TEM | Transmission electron microscopy |
| RNA | Ribonucleic acid |
| Uc | Under coat |
| UHPH | Ultra-high pressure homogenization |
| UHT | Ultra-high temperature |
| UV | Ultra-violet |

| Symbol | Description |
|---------------|---|
| b | Weibull scale parameter |
| D_{θ} | Decimal reduction time at temperature θ |
| E_a | Activation energy |
| k | Rate constant |
| N | Number of microorganisms |
| n | Reaction order |
| n | Weibull shape parameter |
| p | Pressure |
| T / θ | Temperature |
| t | Time |
| z | z-value at a specific temperature for decimal D-value reduction |
| σ | Sigma factor, protein subunit |

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Introduction

1. Introduction

Bacterial endospores are resistant and non-physiologically active forms of vegetative bacteria, the transition to which results from exposure of the spore-forming microorganisms to stress conditions such as nutrient deprivation (Setlow, 2000). Once sporulation is completed, bacterial spores can survive the most extreme conditions or treatments (Horneck, 2006; Moeller et al., 2008; Nicholson et al., 2000). It was further suggested that bacterial spores can survive in this form for millions of years (Vreeland and Rosenzweig, 2000). While spores of most Firmicutes do not lead to disease, some bacilli and clostridia spore formers can induce food spoilage, food-borne diseases and human diseases such as gas gangrene, tetanus, botulism, anthrax, or pseudomembranous colitis (Setlow, 2014). In fact diseases associated to spore-formers are described as early as in ancient literature. One of several examples can be found in the biblical book Exodus 9:1-12. Several biblical scholars believe the fifth plague (death of livestock) to correspond to an outbreak of animal anthrax while the sixth plague is thought to correspond to cutaneous anthrax (Blane, 1890; Hort, 1957).

As far as food is concerned, consumer safety is the prime concern in food industry. In this regard, bacterial spores are a major issue due to their high resistance to preservation hurdles and the potential they carry for recontamination of foodstuffs with resulting spoilage or food-borne diseases. Bacteria such as *Clostridium botulinum*, which produces botulin toxin (LD50 of 1 ng/kg) (Arnon et al., 2001), highlight the necessity of inactivating bacterial spores prior to commercialization of products stored at ambient temperature. For this reason, the food industry has traditionally implemented high thermal loads which insure food safety but also result in organoleptic and nutritional losses (Georget et al., 2013a). Innovative multi-hurdle concepts such as nonthermal technologies, combined with thermal treatments, offer the potential to reach similar results while reducing the thermal loads and thus improving the quality of the finished products based on bacterial spore germination. However, the exact mechanisms behind bacterial spores' germination remain unclear and need to be further investigated.

Theory and literature review background

2. Theory and literature review background

2.1. *Bacillus* and *Geobacillus* endospores

2.1.1. Scientific classification

Perty (1852) was probably the first to report the existence of bacterial spores although he only described them as elliptical, light-refracting bodies in bacteria and did not observe the actual process of sporulation or germination (Morrison and Rettger, 1930). Cohn was the first to characterize the existence of bacterial spores in 1872-1875 (Cohn, 1872, 1875) and to coin the name "*Bacillus subtilis*" with his discovery of the heat resistance of *B. subtilis* endospores (Drews, 2000). Figure 2.1 shows one of the first graphical representations of bacterial spores as represented by Cohn (1875). Cohn observed the light-refracting spore within the cell and monitored the process of germination of spores of *B. subtilis* directly under the microscope (Torred et al., 2012). At the same time, Koch (1876) described the developmental cycle of spore formation of the pathogen *Bacillus anthracis*.

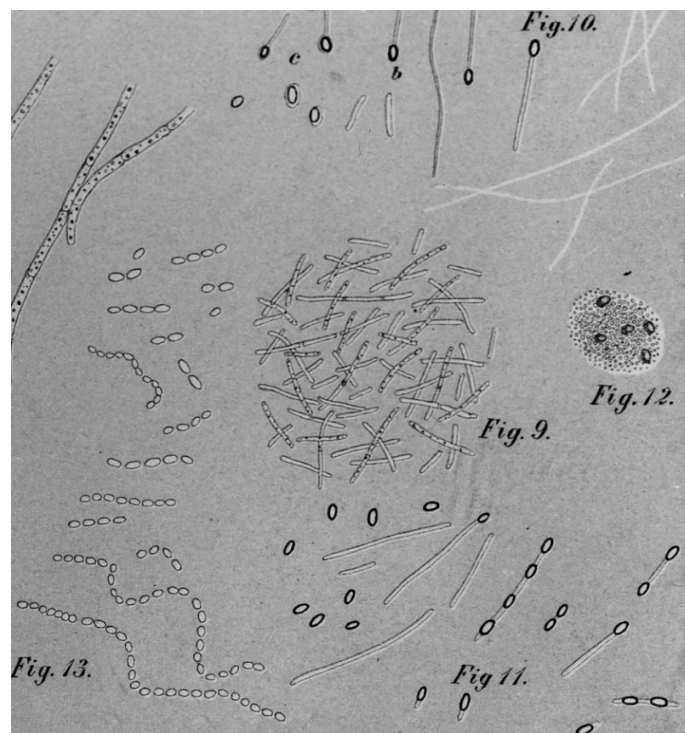


Figure 2.1: Different bacteria, drawn by Cohn. Fig. 9: *Bacillus anthracis*, from blood of a cow which died from anthrax; Fig. 10: mobile bacteria with endospores from rennet; 11: bacilli with endospores from butyric acid fermentation; 12: *Micrococcus* and spores from rennet; 13: *Micrococcus bombycis* from silkworm, sick from flaccid disease. Extract from Cohn (1875).

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Spore-forming bacteria belong mainly to the phylum of Firmicutes. Some bacteria of the phyla Actinobacteria (e.g. genus *Streptomyces*) and Proteobacteria (e.g. genus *Myxococcus*) also form spores, however only the spores of Firmicutes are referred to as endospores (Giglio and Garza, 2012; Swiercz and Elliot, 2012) and are the object of this work. Firmicutes are subdivided in five classes (Parte, 2014) including two main ones: the Clostridia (anaerobic) and the Bacilli (aerobic). Each of these classes is in turn divided in several orders, families, genera, species and strains. The *Bacillus* genus counts 302 accepted species (including subspecies) (Leibniz Institut DSMZ, 2014) and is classified as follow:

Kingdom: Bacteria
Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Family: Bacillaceae
Genus: *Bacillus*

In 2001, a taxonomic study of aerobic thermophilic Bacilli led the species *Bacillus stearothermophilus*, among others, to be transferred to a new genus termed *Geobacillus* (Nazina et al., 2001). A recent study refined this 2001 taxonomy and 18 strains were received as *G. stearothermophilus* (Coorevits et al., 2012). The *Geobacillus* genus counts a total of 23 accepted species (including subspecies) (Leibniz Institut DSMZ, 2014).

In this study, spores of the genus *Bacillus* and *Geobacillus* were investigated, with a focus on spores of the species *G. stearothermophilus*. The strain used in this work was *G. stearothermophilus* ATCC 7953. The reason for the selection of this strain was its use in the industry as validation standard for wet heat sterilization (Albert et al., 1998; European Pharmacopoeia Commission, 1997), making it a potential candidate for the validation of other novel sterilization approaches. Spores of the species *Bacillus subtilis* (PS 832 – courtesy of Prof. Peter Setlow, Connecticut Health Center, USA) were also partially considered as comparison object in order to generalize some of the findings on *Geobacillus stearothermophilus* spores. In fact, *B. subtilis* is to date the best characterized spore former and has been broadly used as paradigm for the investigation of bacterial spore mechanisms. However, the dominant use of *B. subtilis* spores, in particular for germination mechanism investigation, has occasionally raised the question whether all the discoveries in this field could be transferred to other spore species or genera. For instance, *B. subtilis* spores' thermal resistance is significantly inferior to that of *G. stearothermophilus* spores. When targeting food sterilization application, this

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generalization is a necessity in order to prove the decontamination potential of any novel multi-hurdle concept.

2.1.2. Sporulation of spore forming bacteria

Sporulation has been extensively investigated since early on and is a well understood process (Higgins and Dworkin, 2012; Robleto et al., 2012; Schaeffer et al., 1965; Stephenson and Lewis, 2005). Sporulation is a response triggered in spore forming bacteria when facing environmental conditions no longer favorable to growth (nutrient restriction, stress factors such as variation in temperature, pH or salinity). A succession of steps is then initiated which eventually leads to the formation and release of a physiologically inactive, highly resistant, bacterial spore. Sporulation of *B. subtilis* is described by a series of seven stages which are controlled by five sigma factors, leading from the vegetative cell to the release of the mature spore as represented in Figure 2.2 (de Hoon et al., 2010). The sigma factors are proteins required for the initiation of RNA synthesis. They are bacterial transcription initiation factors that enable specific binding of RNA polymerase to gene promoters (Gruber and Gross, 2003).

The initial stage corresponds to the vegetative cell and the occurrence of a non-favorable environment where further growth is not permitted. This leads to stage I and the initiation of the sporulation process which can only be initiated after a round of DNA replication has been completed, so as to ensure that two chromosome copies are available in the pre-divisional cell (Veening et al., 2009). Spo0A, a master transcription factor whose phosphorylation state governs its ability to bind to promoters and thereby regulate gene expression, drives the changes in gene expression which underlie morphological differentiation in both the pre-divisional sporangium and later in the two compartments. Phosphorylation of Spo0A governs the decision to initiate sporulation and is controlled by specific kinases (Stephenson and Lewis, 2005).

Spo0A and the sigma factor σ^H present in the genome of undifferentiated vegetative cells then initiate the formation of an asymmetric cell septum which divides the cell into the forespore and the mother cell (II) (Robleto et al., 2012). The forespore specific σ^F factor controls the expression of 50 genes (Wang et al., 2006) and governs the sequential activation of three other compartment-specific transcription factors. It enables the completion of the translocation of the partially septum trapped genome molecule into the forespore.

Following completion and directed by the σ^F factor, the septum curves and, eventually, the forespore becomes wholly engulfed within the mother cell (III) (Higgins and Dworkin, 2012).

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The next stage involves the synthesis of a thick peptidoglycan layer, the cortex (IV). The gene expression is directed by the remaining σ^E factor from the former mother cell which controls the expression of 262 genes (Eichenberger et al., 2004). During this stage, the forespore loses water and ions such as potassium leading to a stark decrease in volume and a reduction of the pH by about one unit. It further takes up cations (Ca^{2+} , Mg^{2+} or Mn^{2+}) and pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA) synthesized by the mother cell. The parallel accumulation in DPA and dehydration leads to concentrations in DPA above saturation and it is assumed that chelation between calcium and DPA occurs (CaDPA) (Leggett et al., 2012).

σ^G , following activation by σ^E , promotes the maturation of the spore (V) and coordinates the gene expression of more than 90 genes for DNA protection and repair as well as late gene expression in the mother cell via activation of σ^K . During this stage, the spore's resistance properties such as DNA protection by small acid-soluble proteins (SASPs) and germination functions are acquired (Robledo et al., 2012). Finally σ^K activates 75 additional genes (Eichenberger et al., 2004) and promotes the formation of the spore coat, the lysis of the mother cell and the spore release (V-VI-VII) (Robledo et al., 2012). The completion of this cycle takes between 8 and 10 hours for *B. subtilis* (de Hoon et al., 2010). The previous description focused on the sporulation of *B. subtilis*, and it is generally considered that sporulation in other *Bacillus* species follows a similar path. Early work also showed that sporulation of *G. stearothermophilus* can be traced through the same main steps (Walker and Baillie, 1968). However, sporulation in other bacterial classes can substantially differ. *Clostridium difficile* illustrates the existence of these differences and many questions regarding the sporulation of this indicator remain, in particular with regard to the initiation of sporulation and the regulation of the different sigma factors (Paredes-Sabja et al., 2014).

Finally, while the sporulation process takes place naturally, it can also be induced in the laboratory by mean of specific sporulation media. It is important to note that the choice of the sporulation conditions, in particular the temperature and medium, can play a significant role in the resistance and germination behavior of the resulting bacterial spores (Lenz and Vogel, 2014; Melly et al., 2002; Nguyen Thi Minh et al., 2011; Nguyen Thi Minh et al., 2008; Ramirez-Peralta et al., 2012). Sporulation of *G. stearothermophilus* ATCC 7953, the indicator used in this work, had to follow a specific protocol. Obtaining a high concentration of spores for this indicator can prove to be challenging. The large volumes required in this work led to the necessity to establish a reliable sporulation protocol for this strain which allowed for reproducible sporulation rate, resistance and germination behavior (Georget et al., 2014a).

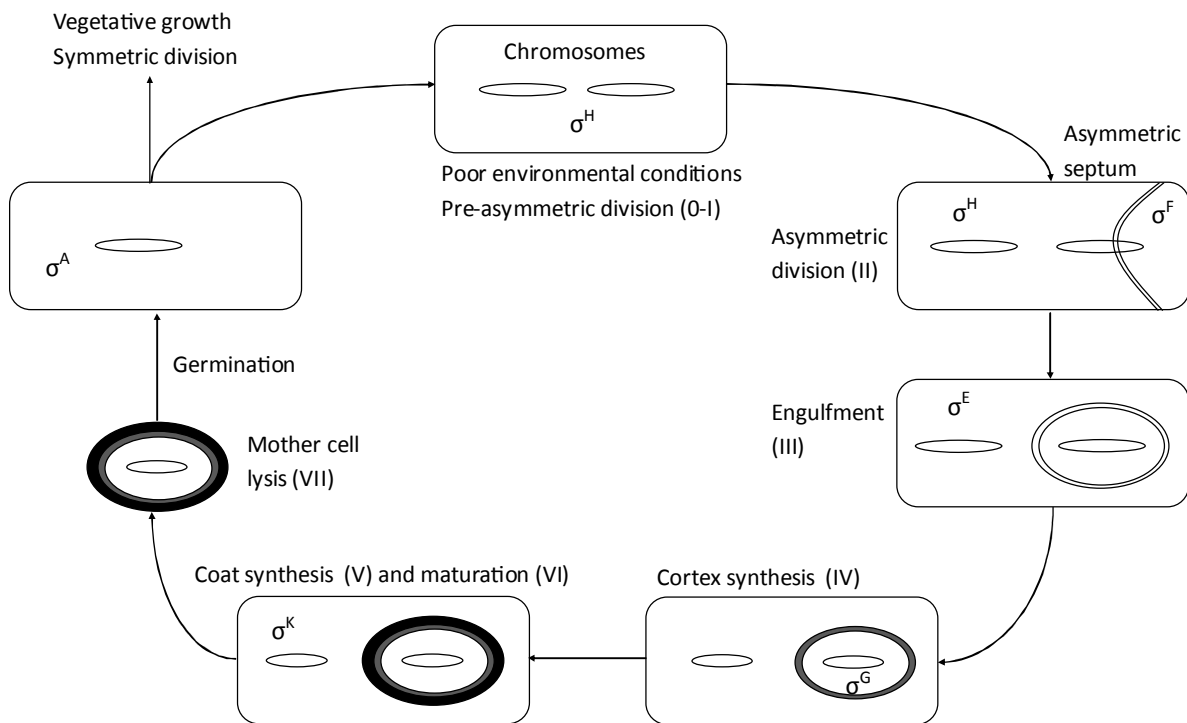


Figure 2.2: Outline of the main steps during sporulation, illustrated with the case of *B. subtilis* (adapted from de Hoon, et al. (2010)).

2.1.3. Structural factors affecting the dormant spore resistance

The final bacterial spore's structure is represented in Figure 2.3. The structure and composition of each layer confer bacterial spores their resistance to a large spectrum of stress factors. Bacterial spores' concentric structure and its role in spore resistance are described hereafter from the outermost layer towards the inside. It is important to mention that bacterial spore detailed structure and sublayers can significantly vary from one species to another and the following description only focuses on the general and common aspects between most bacterial spore species. The exosporium is the outermost layer of some bacterial spores such as spores of *Bacillus cereus* (Aronson, 2012) or *C. difficile* (Paredes-Sabja et al., 2014) but is not a standard feature. It is for instance not present in spores of *B. subtilis* or *G. stearothermophilus* (Figure 2.4) (Aronson, 2012; Blake and Weimer, 1997). The exosporium is a large loose fitting multi-layered shell which surrounds the spore but is not attached to it. It has this far not been associated with any resistance property of spores but was suggested to play a role in the pathogenesis of *C. difficile* (Paredes-Sabja et al., 2014).

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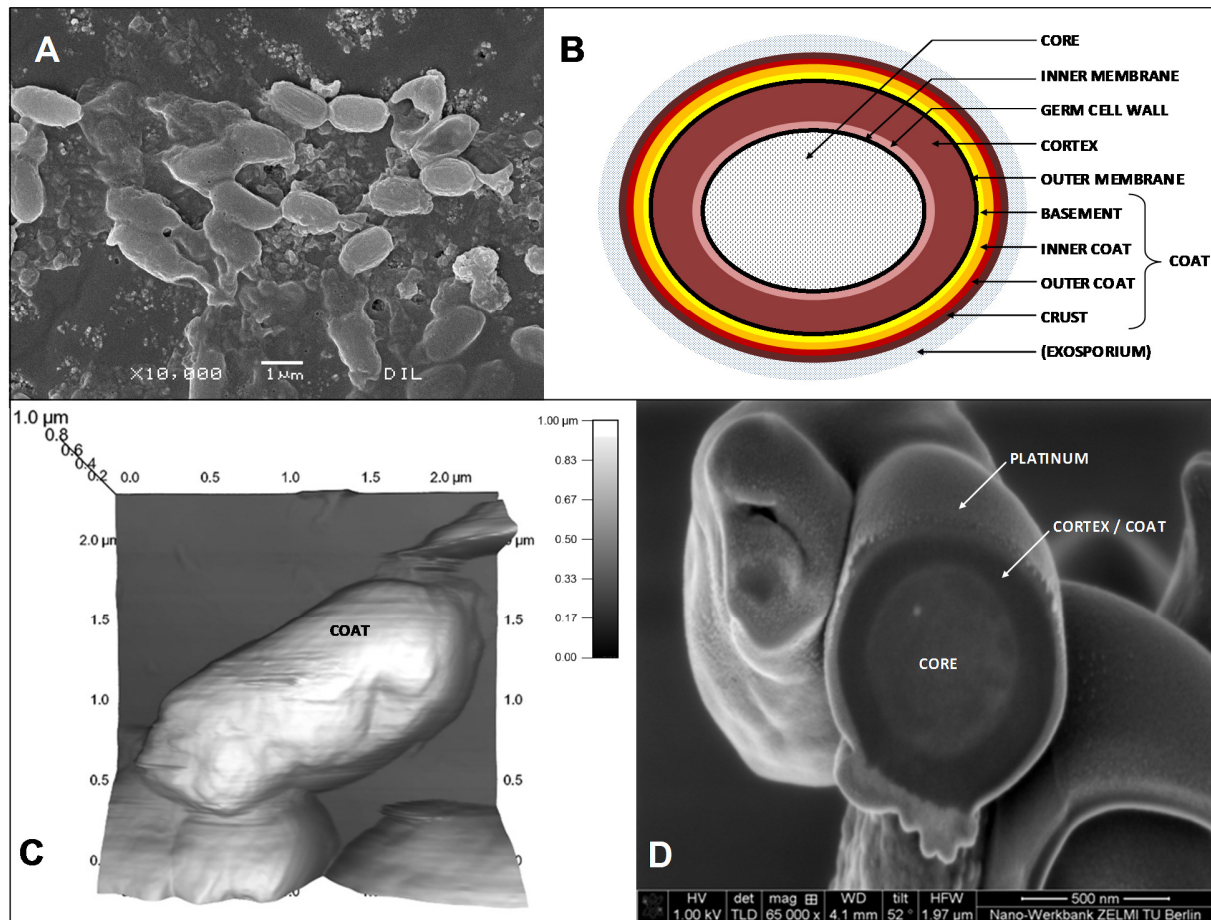


Figure 2.3: Bacterial spore visualization. A) SEM of *G. stearothermophilus* spores (JSM-6460LV SEM with back scattering detector, Jeol LTD, Japan¹). B) Schematic representation of a dormant bacterial spore (adapted from Paredes-Sabja et al. (2011)). The various layers are not represented to scale, and there can be several sublayers in the coat and exosporium. For instance, details of the coat structure corresponding to *B. subtilis* spores (Setlow, 2012). C) *In situ* AFM scanning of the surface of a *G. stearothermophilus* spore fixed on an apolar surface in MilliQ water (MFP-3D SA AFM, Asylum Research, Santa Barbara, California, U.S.A.)². D) SEM of a *G. stearothermophilus* spore sectioned using a focused ion beam – method described in Reineke et al. (2013a).

¹ Spore suspension in distilled water on a carbon conductive tab, air dried and put under vacuum in the chamber. Sample sputtered with a few nm thick layer of gold. Settings: acceleration voltage: 10 KV, working distance: 12 mm, Spot size: 25, Signal: SEI, Vacuum mode: HV.

² Acquisition using standard silicon cantilevers with a nominal spring constant of 2 N/m (OMCL-AC-240TS, Olympus Corporation, Tokyo, Japan). Topography images recorded in intermittent contact mode with resonant frequencies of 20 kHz and a scan speed of 0.4 Hz. Image analysis using IGOR Pro Software (WaveMetrics, Portland, USA).

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When present, it has been shown that the exosporium is separated from the coat by a layer called the interspace, the structure of which seems to be void and whose function is not yet fully clear (Henriques and Moran, 2007; Setlow, 2014).

The spore coat is the next layer inwards. The coat is a multilayered proteinaceous formation whose structure can significantly vary between different species (Aronson and Fitz-James, 1976). For instance, *B. subtilis* spores were established to consist of >70 different proteins organized in a crust an outer and inner coat and a basement (Setlow, 2006, 2012). A recent study suggests an even further refined structure consisting of an outermost amorphous (crust) layer, a rodlet layer, a honeycomb layer, a fibrous layer, a layer of “nanodot” particles, a multilayer assembly, and finally the undercoat/basement layer (Plomp et al., 2014). However, less than 25 % of the spore coat proteins of *B. subtilis* have homologs in *C. difficile* spores, making predictions and knowledge transfer to *Clostridial* spores difficult (Paredes-Sabja et al., 2014).

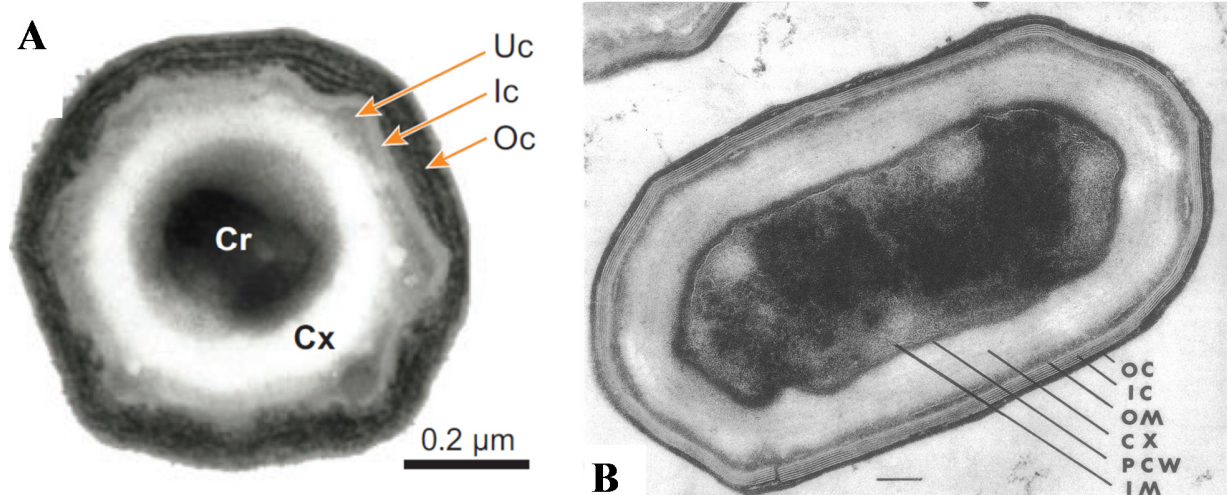


Figure 2.4: A) Thin sectioning electron micrograph of a cross-section of spores of *B. subtilis*. The following spore compartments or structures are : the spore core (Cr), the cortex peptidoglycan layer (Cx), the undercoat region (Uc), the inner (Ic) and the outer (Oc) coat layers (Henriques and Moran, 2007). B) Electron micrograph of a longitudinally medial section of a dormant spore of *G. stearothermophilus* ATCC 7953. The following structures are indicated in representative places: outer coat (OC), inner coat (IC), outer membrane (OM), cortex (Cx), primordial cell wall (PCW = Germ cell wall), and inner membrane (IM). Bar = 100 nm (Beaman et al., 1982).

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Spores of *B. cereus* show a thin, cross-hatched appearance in the outer coat covering a thin undercoat layer (Aronson, 2012). On the other hand, spores of *B. subtilis* and *G. stearothermophilus* exhibit a thicker coat (Figure 2.4), the outer layer of which is speculated to be a condensed variation of the exosporium based on the comparison of protein homologs in *B. subtilis* and *B. anthracis* (Pedraza-Reyes et al., 2012). In spite of structural differences, the function of the coat remains seemingly constant. The coat is one of the important structural developments in spores which allow for chemical resistance. The spore coat proteins are thought to react with and detoxify chemical agents and thereby constitute the first protective layer of the spore. However, the coat does not constitute a barrier for small molecules (Setlow, 2006) and is hypothesized to contain multiple GerP proteins facilitating the transfer of nutrients towards the inner membrane (Behravan et al., 2000; Butzin, 2012). The coat is particularly relevant to protect spores against peptidoglycan lytic enzymes which could damage the underlying cortex such as lysozyme. This layer also constitutes a protection against protozoan predation (Klobutcher et al., 2006). Finally, it is thought that the coat might facilitate the permeation of nutrients, necessary for nutrient germination, towards the inner spore membrane (Setlow, 2003). Several enzymes are also present in the coat, in particular, the CwlJ protein which is involved in the cortex degradation as cortex lytic enzyme (CLE) in the early stages of *B. subtilis* germination. While CwlJ was established in *B. subtilis*, the presence of orthologues of the CLE CwlJ was found in several other strains of the *Geobacillus* genus (Paredes-Sabja et al., 2011). This suggests that a (partially) similar coat structure might be present in *G. stearothermophilus* spores.

The outer membrane plays a central role during the sporulation process but may not retain its integrity in mature spores (Popham, 2002). To date no barrier or resistance function has been associated with the outer membrane (Setlow, 2014).

Inwards, the cortex and the germ cell wall are found. Both structures are mainly composed of peptidoglycans. While the structures of the peptidoglycan in the growing cell and in the germ cell wall appear identical, the cortex peptidoglycan exhibits some particularities which are not found in the germ cell wall or the cell wall. In spores of *B. subtilis*, the cortex shows a much lower level of cross-linking between glycan strands and an important number of *N*-acetylmuramic acid residues with a single L-alanine residue attached. It also shows an important percentage of muramic acid residues, present as muramic acid- δ -lactam (M-L) (Warth and Strominger, 1969, 1972) (Figure 2.5). This latter particularity also allows for the specific recognition of the cortex peptidoglycan by the CLEs active during the germination and which, thus, do not attack the germ cell wall which will become the future cell wall (Setlow, 2003).

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Correspondingly, CLEs are found in the cortex of most bacterial spores, regardless of the genus (Popham et al., 2012). For *B. subtilis*, SleB is the corresponding CLE and is additionally found in the inner membrane (Figure 2.6), in association with the YpeB protein needed for the occurrence of SleB in spores (Setlow, 2003).

SleB was also purified in germination exudates from *B. cereus* spores indicating proximity between spore formers of different families. Furthermore, genes encoding for homologues of SleB and YpeB are found almost universally in the genomes of *Bacillus* and *Geobacillus* species, among others (Popham et al., 2012). This suggests that this CLE might also be represented in *G. stearothermophilus* spores. Previous work has shown that the overall composition of the cortex is similar between many spore-forming bacteria, including *B. sphaericus*, *B. cereus*, *B. megaterium*, *G. stearothermophilus* and *C. sporogenes* (Atrih and Foster, 2001).

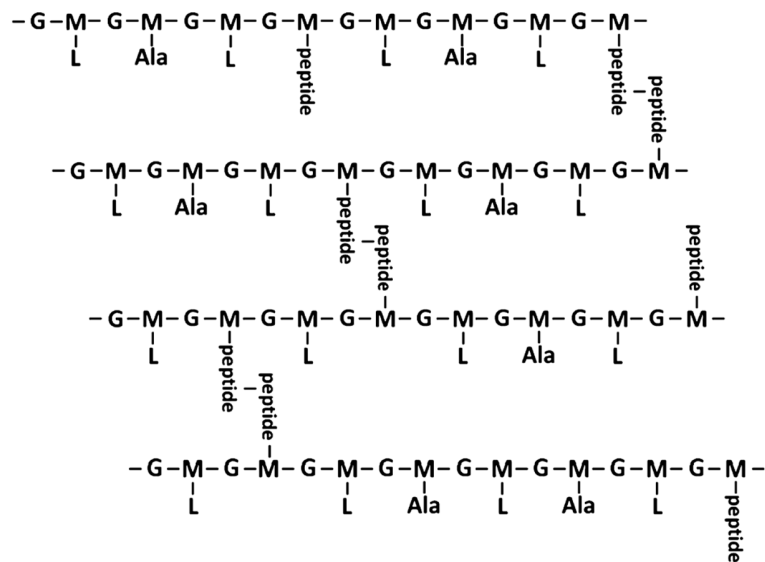


Figure 2.5: Schematic representations of spore peptidoglycan structure. G, N-acetylglucosamine; M, N-acetylmuramic acid; M-L, muramic acid- δ -lactam; Ala, L-alanine; peptide, tri- or tetrapeptide side chains that can form cross-links between glycan strands. With permission from John Wiley and Sons ©Leggett, et al. (2012) Journal of Applied Microbiology © 2012 The Society for Applied Microbiology.

The cortex mucopeptide structure described for *B. subtilis* was also investigated in other spore formers (*B. subtilis*, *B. megaterium*, *B. cereus* and *C. botulinum*) and it was observed that there is a conserved basic structure between peptidoglycan of these species, with the only difference being the level of de-N-acetylation of an amino sugar (Atrih and Foster, 2001). By its structure, the cortex

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acts as a straitjacket around the spore, restricts its expansion and might also be responsible for the very low level of water in the core. Furthermore, it has been suggested that the structure of the cortex plays a significant role in determining the timing and the rate of CaDPA release during *B. subtilis* spore germination (Zhang et al., 2012).

The inner membrane is one of the key structures of bacterial spores and displays several particularities. It contains lipids and proteins, and its lipid composition is similar to the one of a cytoplasmic membrane (Cortezzo and Setlow, 2005). However, the state of compaction of this membrane, foremost maintained by the presence of the surrounding cortex (~2 MPa osmotic potential or more (Bassi et al., 2012)), makes it a strong permeability barrier which thus protects the dormant spore against many DNA-damaging chemicals (Cortezzo and Setlow, 2005). It has been shown that under the cortical pressure in dormant spores, the inner membrane of *B. subtilis*, *B. megaterium* (Cowan et al., 2003; Cowan et al., 2004), *C. beijerinckii* and *C. sporogenes* spores (Hofstetter et al., 2012) is largely immobile. The high viscosity in the inner membrane is probably associated to a lipid gel phase (Loison et al., 2013). This condensed and impermeable membrane (3-4 times less permeable than a prototypical lipid bilayer) most probably helps maintaining the state of dehydration of the core (Cowan et al., 2004; Hamill and Martinac, 2001; Leggett et al., 2012; Sunde et al., 2009). The inner membrane is finally strongly involved in the spore germination by the presence of special proteins which act as nutrient receptors near of or in the inner membrane (Setlow, 2014).

The core is at the heart of the bacterial spore where the DNA, ribosomes, tRNAs and most spore enzymes are localized. It is characterized by a very low water content of 27-55 % of the spore wet mass which is conjectured to be responsible for the absence of physiological activity, in particular of enzymatic activity, and the heat resistance (Leggett et al., 2012). It could also be shown that the water content correlates negatively with the heat resistance. The water content of spores of *G. stearothermophilus* ATCC 7953 was partially measured or calculated for the spore's different compartments and it was suggested that the core of this strain contains no more than 0.21 g H₂O/g dry mass with an equivalent a_w of 0.83 (Algie and Watt, 1984) thereby justifying the extreme thermal resistance of this specific strain. However, it was shown in *B. subtilis* spores that the majority of this small amount of water is freely mobile in the core (Kaieda et al., 2013); but the literature on this aspect is not unanimous and recent work nuanced these results suggesting water in the core is only partially unbound (Friedline et al., 2014). Furthermore, the core of bacterial spores is highly mineralized and contains a large depot (5-15 % of total spore dry mass depending on species) of a spore specific molecule, DPA mainly 1:1 chelated to calcium (CaDPA) (Gerhardt and Marquis, 1989). The DPA content of *G. stearothermophilus* ATCC 7953 spores was estimated at

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83.1·10⁻³ mol/g [dry wt] (Beaman et al., 1982), corresponding to 26.4 % dry mass DPA (Warth et al., 1963). While the concentration of CaDPA per spore can vary between individual spores (e.g. in *B. subtilis* spores from 800 mM – 1 M), it remains in any case well above the solubility limit in water (<100 mM)(Huang et al., 2007). The high concentration of Ca²⁺ and DPA in the core protects the spore against multiple hurdles such as wet heat (Setlow et al., 2006) and damages by UV radiation by promoting an alternative photochemistry of the spore DNA (Douki et al., 2005). Table 2.1 reports the average DPA concentration per spore for *G. stearothermophilus* and *B. subtilis* and it is visible that *G. stearothermophilus* spores contain on average up to nearly five times more DPA in their core, which can account for the increased heat resistance of the ATCC 7953 strain.

The very high concentration, over saturation, of CaDPA in the core, has led to two hypotheses as to the state of the CaDPA in core as well as the mobility of water. The initial suggestion was that the spore core, including water, might be in an amorphous, solid state termed “glassy” state (Ablett et al., 1999; Sapru and Labuza, 1993).

Table 2.1: Average DPA concentration per spore c_s for *G. stearothermophilus* and *B. subtilis*

| Strain | c_s [mol DPA/spore] | Method / Reference |
|--|---|--|
| <i>B. subtilis</i> ATCC 9372 | $0.475 \cdot 10^{-15}$ | Experimental (Ball mill) (Heinz, 1997) |
| <i>G. stearothermophilus</i> ATCC 7953 | $2.192 \cdot 10^{-15}$ | Experimental (Ball mill) (Personal communication, Mathys, 2008). |
| <i>G. stearothermophilus</i> ATCC 7953 | $2.095 \cdot 10^{-15}$ | Experimental (121°/20 min) (Personal communication, Mathys, 2008). |
| <i>G. stearothermophilus</i> ATCC 7953 | $1.810 \cdot 10^{-15} - 1.910 \cdot 10^{-15}$ | Experimental (121 °C/20 min) (Georget, 2012) |

This glassy state could have explained the enzymatic dormancy through the retardation of diffusive molecular motions of the substrates (Pedraza-Reyes et al., 2012). Novel approaches based on spin relaxation rates in H₂O/D₂O exchange in *B. subtilis* spores challenged this theory. It was suggested that water, even bound to proteins hydration layers, has a high mobility throughout the spore, even in the core and that consequently, dormancy might rather result from a dehydration induced conformational change of the enzymes (Sunde et al., 2009). This led to the establishment of the second hypothesis that the core might be in an insoluble heat-stable gel state consisting of a structured macromolecular framework permeated by mobile water. This hypothesis was also supported by recent work (Kaieda et al., 2013). However, unanimity has not yet been reached in the

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scientific community on this point and contradicting evidence remains which suggests that the glassy state might exist in dried spores while the gel state could be present in aqueous suspension (Friedline et al., 2014).

A final specificity of the spore core is the presence of small acid-soluble proteins (SASPs) which bind to and saturate the DNA, thereby modifying its structure and its photochemistry. SASPs are 60-75 amino acids long and are very abundant in spores, making up to 20 % of the core proteins and 3-6 % of the spore proteins in *B. subtilis* spores (Moeller et al., 2009). These SASPs protect the spore DNA against wet heat (Setlow, 2007b), dry heat (Setlow and Setlow, 1995; Setlow, 2006), desiccation (Setlow, 1995), chemical attacks (Melly et al., 2002) and UV-radiation (Setlow, 2006). Mechanisms and structural factors affecting *B. subtilis* spore survival to various physical and chemical treatments have been previously summarized (Reineke, 2012) and are presented in Table A1 in the annexes.

2.1.4. Germination of bacterial spores

Early work looked at the germination of *G. stearothermophilus* spores (Cheung et al., 1998; Cheung et al., 1982; Diep et al., 1972). However, the bulk of the literature focusing on this topic has worked with *Bacillus* or *Clostridium* spores. The germination process has been recently reviewed by several authors (Christie, 2012; Olgún-Araneda et al., 2014; Paredes-Sabja et al., 2011; Setlow, 2013; Setlow, 2014) and is summarized hereafter.

Dormant spores, though metabolically non active keep monitoring their environment for the occurrence of more favorable conditions which would allow resumption of physiological activities. The process which allows spores to return to the initial vegetative state is called germination. Spore germination occurs naturally when bacterial spores detect appropriate nutrients in their environment and is an irreversible process (Yi and Setlow, 2010). Germination is characterized by a succession of steps leading the dormant, highly resistant spore, to a germinated state, much more temperature sensitive, and eventually a vegetative cell. Once germination is initiated, it occurs very fast for most spores and requires no ATP production and no synthesis of macromolecules. During outgrowth RNA and protein synthesis takes place initially supported by the amino acids available post degradation of the SASPs.

Two main pathways of germination can be identified (Setlow, 2014). The “nutrient germination” involves the detection of nutrients by the so-called nutrient receptors as described above. The “non-nutrient germination” refers to germination triggers which can affect the spore independently from the nutrient receptors. These triggers can be of chemical or physical nature.

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2.1.4.1. Nutrient germination

The general mechanism initiating germination appears to be largely conserved between *Bacillus* and *Clostridium* spores. However, multiple different mechanisms relying on GerA, B, C or K-type as well as CspC-type nutrient receptors exist which distinguish germination between *Clostridium* spores from *Bacillus* spores (Olguín-Araneda et al., 2014).

Spores of *Clostridium* and *Bacillus* react to similar germinants. Nutrient germinants which have been identified in the literature are generally single amino acids (L-alanine), sugars (D-glucose) or purine nucleosides (inosine) but also combinations of nutrients such as L-asparagine, D-glucose, D-fructose, potassium ions (AGFK) (Setlow, 2003).

When in presence of the right (mixture of) nutrient germinant(s), spores commit to germinate following a process which is to date not clearly understood and even under altered environment conditions, the germination process will continue. The lag phase before commitment can however significantly vary from spore to spore between minutes and over 24 h. The germination process then occurs in two stages (Figure 2.6):

Stage I:

- 1) Following detection and binding of the germinant(s) to the germinant receptor, the germination signal is hypothesized to be transduced and amplified by the GerD protein. H⁺, monovalent cations and Zn²⁺ are released from the spore core. This leads to an increase of the core pH (~6.5 to 7.7) which will support enzymatic activity once the core has rehydrated (Setlow, 2003).
- 2) The spore core's large depot of DPA is released mostly associated to Ca²⁺ and other divalent cations. *B. subtilis* spore CaDPA release was shown to happen within 0.5-3 min at 37 °C (Zhang et al., 2010). The exact mechanism of this release is not yet fully clear but it was recently shown that the release mechanism might involve, among other SpoVA proteins, the SpoVAC protein in *B. subtilis* spores which exhibits a channel like activity in model systems (Velásquez et al., 2014). SpoVA proteins are encoded in one or more operons in all spore-forming bacilli and clostridia (Paredes-Sabja et al., 2011). This release is accompanied by the release of free amino acids such as arginine and glutamic acid (Setlow et al., 2008). The link between the germinant receptors and these channels remains to date unknown.
- 3) This release of the DPA depot which constitutes ~25 % of the dry mass allows for some water up-take – presumably via the same channel – which in turns lead to a reduction of the spore heat resistance (Cowan et al., 2003). Additionally, it was shown in *B. subtilis* spores

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that the release of CaDPA contributes to the activation of a CLE (CwlJ) which will initiate the degradation of the thick cortex peptidoglycan structure. It is noteworthy that the release of CaDPA and partial water uptake at the end of stage I are not yet sufficient to allow for a resumption of the enzymatic activity in the core.

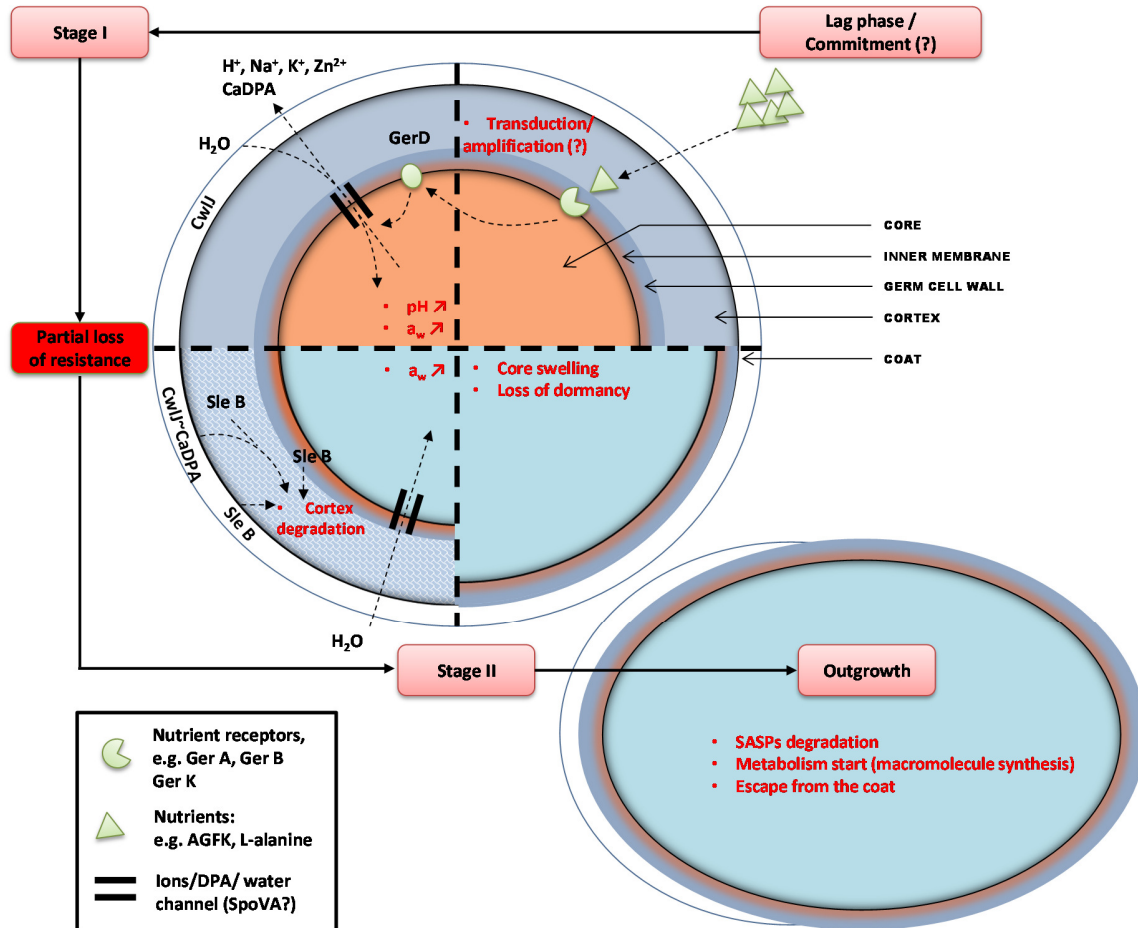


Figure 2.6: Nutrient germination pathway of *Bacillus* spores based on Reineke (2012) and Setlow (2003; 2014).

Stage II:

- 1) The beginning of stage II is marked by the beginning of the spore cortex degradation and further water uptake made possible by the removal of this “straightjacket” from around the core. As mentioned above, the CLEs are specific for cortex peptidoglycan because of their targeted action requiring the muramic acid δ -lactam recognition and cleavage. *Bacillus* spores generally contain two main CLEs, namely CwlJ and SleB, which are likely to be lytic transglycosylases (Setlow, 2013). While it is proven that CaDPA activates CwlJ, it is less clear how SleB is activated and most studies propose that activation by turgor-induced stress on

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the cortex is important for SleB activation (Popham et al., 2012). In addition to the CLEs, cortical fragment lytic enzymes (CFLEs) act on fragments of the spore peptidoglycan. SleL is an example of such CFLEs found both in *B. cereus* and *B. subtilis* spores, which shows no activity on the intact cortex.

- 2) The continued water uptake (up to ~80 % wet mass) leads the spore to become further heat sensitized.
- 3) During germination, the water uptake and core expansion lead to an extension of the inner membrane up to 2-fold without any lipid synthesis, accompanied by an increase in its fluidity (Cowan, et al., 2004).
- 4) The enzymatic activity in the core is resumed which can allow the beginning of the outgrowth.

During the spore outgrowth and thanks to the initiation of the enzymatic activity, the SASPs (~10-20 % of the core proteins) which saturate the DNA can be degraded and hydrolyzed by the germination protein Gpr (Setlow, 2007b).

Interestingly, most of the proteins implicated in spore nutrient germination, including the germinant receptors, GerD, and the SleB CLE, are located in or close to the inner membrane (Korza and Setlow, 2013). This highlights the particular interest of the inner membrane and the need to further expand our understanding of its behavior under various conditions.

2.1.4.2. Non-nutrient germination

Different chemical and physical treatments can lead to germination initiation and are briefly described hereafter.

Lysozyme, exogenous CaDPA, dodecylamine (cationic surfactant) or muropeptide fragments of the cell wall released by growing bacteria can lead to germination following different mechanisms as illustrated in Figure 2.7.

- Lysozyme can only cause germination of decoated spores by degrading the cortex which is sufficient for promoting spore germination by inducing the release of CaDPA and allowing for core hydration.
- Exogenous CaDPA induces germination without interaction with the germinant receptors of spores of the *Bacillus* genus. Recent evidence suggests that a different mechanism might be at stake for *Clostridium* spores involving the receptor GerKC (Olguín-Araneda et al., 2014). It was shown that spores which lack the CwlJ CLE do not germinate with exogenous CaDPA which

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indicates that germination by exogenous CaDPA is mediated by activation of this specific CLE (Paidhungat et al., 2001). To date, all spore formers investigated for this path of germination responded to CaDPA for germination, making of CaDPA one of the most promising substances to trigger a targeted germination aiming at sterilization. The exact mechanism following cortex degradation is however to date unknown. While it is assumed that the degradation of the cortex allows CaDPA release and water uptake, it seems also plausible that the early detection of the cortex degradation by the spore could allow it to release its CaDPA and rehydrate before the cortex degradation is complete. Verifying this hypothesis is one of the objectives presented in section 6.

- Dodecylamine, a surfactant, was identified as triggering spore germination (Setlow et al., 2003) and it was suggested that this mechanism relies on a direct opening of the CaDPA release channels. Spores germinated in such a way did not, however, resume metabolism.
- Peptidoglycan fragments such as muropeptides derived from the breakdown of peptidoglycans from growing cells of related species were able to induce germination of spores of *B. subtilis* and *B. cereus*. An inner membrane associated eukaryotic-like protein kinase (PrkC) recognizes these muropeptides and subsequently phosphorylates serine/threonine residues, kinase activity which is required to trigger germination. The actual mechanism linking the kinase activity to germination is however not known (Shah et al., 2008). A similar pathway and response could be triggered by bryostatins, a modulator of protein kinase C (Wei et al., 2010).

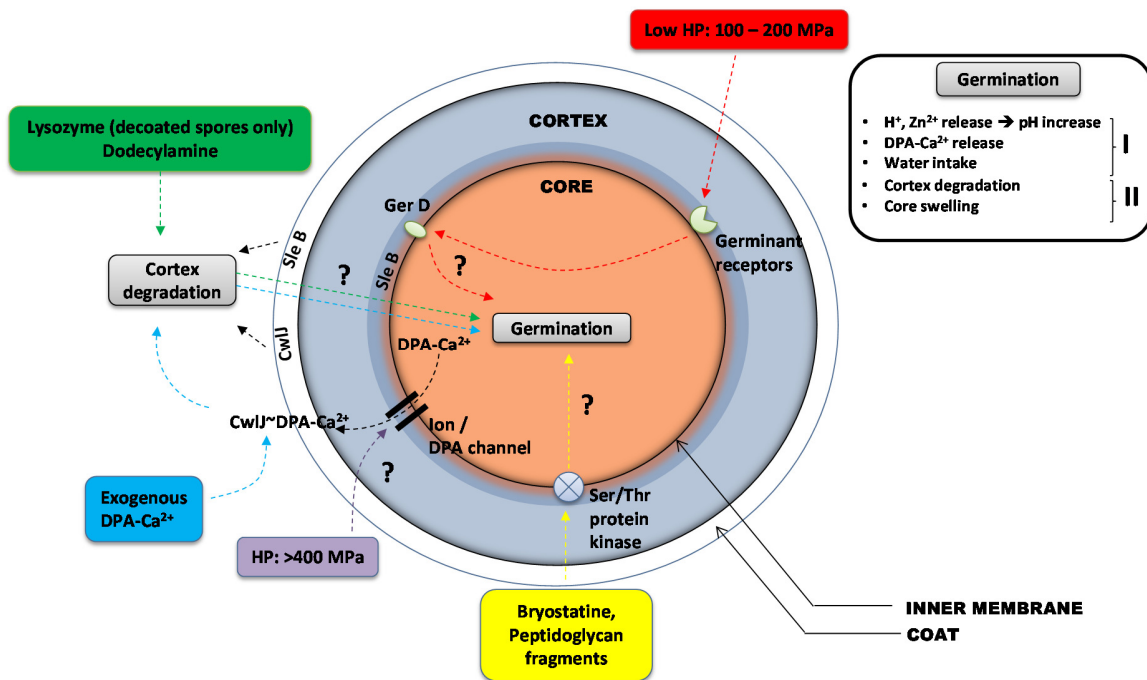


Figure 2.7: Non-nutrient germination pathways of *Bacillus* spores.

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In addition to these non-nutrient chemical means to trigger germination, physical means can also induce germination. Due to its potential application for high pressure thermal sterilization of food, high pressure is the best known physical treatment inducing germination. While the impact of high pressure on bacterial spores is further discussed in section 2.3.2, some basic aspects are presented hereafter. Two different paths of high pressure germination have been suggested.

The first way is germinant receptor dependent and there is a large body of evidences which allows to say that all spore-formers of *Bacillus* genus are triggered to germinate by moderate high pressure of 100-150 MPa (Christie, 2012; Paidhungat et al., 2002; Wuytack et al., 1998).

Within this pressure range, individual germinant receptors show different pressure sensitivity. For instance, GerA in *B. subtilis* spores is more sensitive than GerB, itself more sensitive than GerK, and it was shown that the structure of the receptor is more responsible for this variability in sensitivity than the number of receptors present (Black et al., 2005). Reineke (2012) showed that the high pressure activation of the germinant receptor is dominant at 100-200 MPa. This mechanism, though retarded above this range, is still partially active up to 600 MPa. This path is referred to as non-nutrient germination.

Conversely *B. subtilis* spores which lacked germinant receptors could be germinated with pressure above 500 MPa (Paidhungat et al., 2002). The range >400 MPa was primarily associated with the “unphysiological germination” by Reineke (2012) which does not require the germinant receptors to initiate a rapid release of CaDPA through what is suspected to be an opening of the SpoVA channels. CaDPA release can in turn activate the cortex degradation as in the conventional germination chain reaction. It is worth mentioning that for $p > 600$ MPa and $T > 60$ °C, it was argued that cortex degradation under pressure could be inhibited – possibly by a denaturation of the CLEs (Black et al., 2007b). Reineke et al. (2013c) further showed that in these p,T conditions, the CaDPA release does not seem to allow CwlJ CLE activation as no cortex degradation seems to take place, comforting the previous assumption.

The summary of the different known germination pathways is presented in Table 2.2. Of all means introduced to germinate bacterial spores, nutrients, CaDPA and high pressure appear as interesting approaches to investigate for *G. stearothermophilus* spores and more generally as a way to achieve sterilization by combined germination and pasteurization treatments.

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Table 2.2: Triggers and mechanisms of intact *Bacillus* spore germination. Adapted from Reineke (2012); Setlow (2014)

| Germination trigger | Mechanism activated |
|---|--|
| Nutrients | Germinant receptors |
| CaDPA | CwlJ |
| Dodecylamine | SpoVA channel |
| Peptidoglycan fragments from vegetative cells | Protein kinase |
| HP 100-200 MPa (retarded up to 600 MPa) | Germinant receptors |
| HP > 400 MPa (retarded at 200 MPa) | CaDPA release channel – probably SpoVA |

2.1.4.3. Heterogeneity in spore germination and superdormancy

As mentioned above, spore germination would offer an interesting approach to reduce the resistance of bacterial spores to conventional thermal treatments such as pasteurization. However, this strategy has been largely impaired by the incapacity to fully germinate bacterial spores. This results from the strong heterogeneity between spores which affects among others the size, core water and DPA content of spores significantly (e.g. ± 25 % size variation between individual spores of a same population) (Ghosh and Setlow, 2009; Setlow et al., 2012).

Germination commitment has also shown to be relatively heterogeneous among populations of spores obtained from the same single CFU. The spores which exhibit an extended lag phase before committing to germinate (up to >24 h) have been termed superdormant (Setlow et al., 2012). As introduced above, germination can be achieved by different means and thereby the reasons for superdormancy may be multiple without a single set of characteristics.

Isolation techniques for superdormant spores obtained by different germination paths allowed the recent investigation of such sub-populations.

Superdormancy in nutrient germination has been associated to very low levels of germinant receptors in the inner membrane (Chen et al., 2014; Ghosh et al., 2012; Setlow et al., 2012) and longer commitment and lag times. Yet, it could be shown that when stimulated by different germination triggers (non-nutrient ones or different nutrients) bacterial spores germinated normally (Wei et al., 2010).

Superdormancy to other paths of germination such as CaDPA germination was also investigated and associated to a coat defect and accompanying low levels of the cortex-lytic enzyme CwlJ (Perez-Valdespino et al., 2013).

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Some of the major unanswered questions related to spore germination are being investigated in this work in sections 4 and 6:

- How is the inner membrane impacted by different treatments? This is done with a focus on non-nutrient germination, in particular *in situ* investigation under pressure or during CaDPA germination. Can this improve our understanding of germination and superdormancy phenomena?
- Can spore germination be manipulated efficiently for food preservation applications?

2.2. Impact of bacterial endospore on food safety and resulting issues

Commercial sterility of low-acid food is defined by the Codex Alimentarius Commission (W.H.O./F.A.O.) CAC/RCP 40-1993 as follows:

“Commercial sterility means the absence of microorganisms capable of growing in the food at normal non-refrigerated conditions at which the food is likely to be held during manufacture, distribution and storage.” CAC/RCP 40-1993 (Joint F. A. O. / W. H. O. Codex Alimentarius Commission, 2001).

In this context, bacterial spores constitute a hazard by their strong resistance to chemical and physical hurdles. The presence of spores in food or on processing equipment opens the way to (re)contamination. Following germination in the food matrix, vegetative microorganisms will proliferate again – potentially producing toxins -, jeopardizing consumer safety and product quality (Georget et al., 2013a).

Botulism is a notorious example illustrating the danger of bacterial spores in food. Botulism, an often fatal disease affecting the nervous system, has accompanied mankind since the first attempts at preserving food for keeping at ambient temperature and the first detailed reports of outbreaks are dated from the end of the 18th century (Erbguth, 2004). The link to the botulinum toxin was however only made in 1895 following an important outbreak in Belgium and Emile von Ermengem described it as a heat-labile toxin produced by an anaerobic bacterium initially named *Bacillus botulinus* (later: *Clostridium botulinum*). The toxin itself could only be purified 1928 (Erbguth, 2004). The botulinum toxin comes in different types and is the most acutely lethal toxin known. The exact lethal dose for humans is not known but was estimated for the type A toxin to a

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human median lethal dose (LD50) of 1.3–2.1 ng/kg intravenously or intramuscularly, 10–13 ng/kg when inhaled, and 1 µg/kg orally (Arnon et al., 2001).

From the spoilage perspective, microbial spoilage of canned foods by thermophilic and highly heat-resistant spore-forming bacteria, such as *G. stearothermophilus*, is an additional persistent problem in the food industry. Risks related to *G. stearothermophilus* spores were reported with the example of flat-sour spoilage of canned green beans or asparagus (Lin et al., 1968; Rigaux et al., 2014). Flat-sour spoilage by thermophilic spore formers such as *G. stearothermophilus* can also occur in dairy products. Spore concentrations as high as 10^5 spores.g⁻¹ can occur in milk powder, resulting in it being downgraded to a lower-value product (Seale et al., 2008).

These examples illustrate the imperative for bacterial spores inactivation in packaged food stored at ambient temperature – in particular when considering high water activity and low acidic foods. Conventionally high thermal intensities have been applied to this end and by 1925, outbreaks of Botulism from industrial canned food mostly ceased (Tanner, 1935) thanks to a widespread adoption of standards for minimum heating times and temperature, also known as “botulinum cook” (3 min at 121 °C). This treatment might however not be sufficient to guarantee the inactivation of spores of thermophilic microorganisms. Thus, sterilization processes of food which can be unpackaged (ultra-high temperature (UHT) process) or packaged (autoclave treatment) have been established with a higher thermal intensity. In spite of identical process target, the process conditions of autoclave heating and UHT heating are different (Figure 2.8), accounting for variable heat transfer conditions depending on the food matrix and batch versus continuous processing:

Autoclave sterilization: 109 – 121 °C for 40 - 15 min

UHT heat treatment: 135 – 150 °C for 10 – 1 s

Comparison of the sterilization domain as represented in Figure 2.8 with the lines marking the beginning of “unwanted reactions” such as the change of color or the beginning of nutrients degradation in milk underlines that though safe, sterile products also loose in organoleptic and nutritional properties. However, the lines marking “wanted reactions” such as the destruction of spores also suggest that alternative domains might be possible. This led to the search for alternative processes to control bacterial spores.

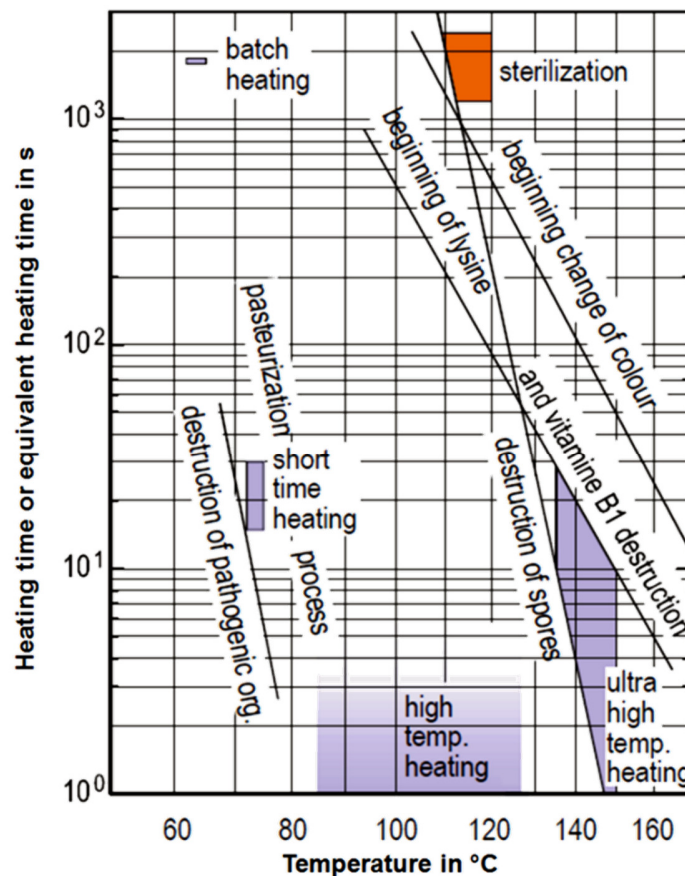


Figure 2.8: Limiting lines for destruction of spores and effects on milk - Source: *Food and Bio Process Engineering: Dairy Technology* (Kessler, 2002) with permission.

2.3. Multi-hurdle concept and novel approaches to food sterilization

2.3.1. The multi-hurdle concept

The multi-hurdle concept is an approach targeting microorganism control or elimination in food by the combination of several “hurdles” (i.e. stress factors affecting the microorganism viability). Each of these hurdles will have to be overcome by the microorganisms to remain viable in the food matrix (Leistner, 2000). The hurdle technology concept was defined by Leistner (1985). The targeted use of the multi hurdle technology has soared in the last 30 years and was driven by an increasing will to improve food freshness and nutritional properties (i.e. reduce the temperature hurdle) without affecting food safety, hence leading to the reinforced use of non-thermal hurdles. This was made possible by the gain in knowledge with regard to the principles of major preservative factors for foods (e.g. temperature, pH, a_w) and their interactions (Leistner, 2000).

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Hurdles applicable to food preservation can be classified in three different sub-categories as represented in Figure 2.9. While biological hurdles such as fermentation have been applied to achieve food preservation as early as the seventh millennium before Christ (e.g. fermented beverage of rice, honey, and fruit from the early Neolithic village of Jiahu in Henan province in China (McGovern et al., 2004)), later approaches valued the use of chemical solutions as well as thermal treatments. The use of solutes such as salt and sugars to reduce the water activity of foods are classical examples. The modern urge to reduce sugar and salt intake originating from always more complexly formulated foods and the disaffection for chemical preservatives by consumers has led the modern food industry to primarily rely on thermal processing to stabilize foods. This is particularly true when considering shelf-stable, low acid foods which offer optimal growth conditions for pathogenic and spoilage microorganisms (Georget et al., 2013a).

The will to reduce thermal processing and reach minimally processed foods with high nutritional value and equivalent level of safety has led to the development of novel – comparatively recent – hurdles to control microorganisms such as high pressure or pulsed electric fields (PEF). The investigation of the impact of these hurdles, alone or combined, on vegetative and sporulated microorganisms has thus been the object of a significant research effort in the recent years (Barbosa-Canovas et al., 2004; Hendrickx and Knorr, 2002). While significant progresses have allowed the transfer of some of these hurdles for pasteurization applications (PEF and high pressure) – the domain of sterilization remains for the time still unreached at the industrial level, both for technological challenges and lack of understanding of the impact of these novel hurdles on bacterial spores. In particular, one remaining scientific and industrial question is: Can spore germination be instrumentalized as an efficient way to achieve food safety? (Setlow, 2014). This approach, originally referred to as tyndallization (Gould, 2006), would rely on the combined use of a spore germination means (leading to thermal sensitization) and a mild heat shock (pasteurization level) to inactivate bacterial spores. One object of this work was to focus on the potential of natural chemical compounds and high pressure hurdles in combination with a reduced thermal load in order to achieve bacterial spore inactivation.

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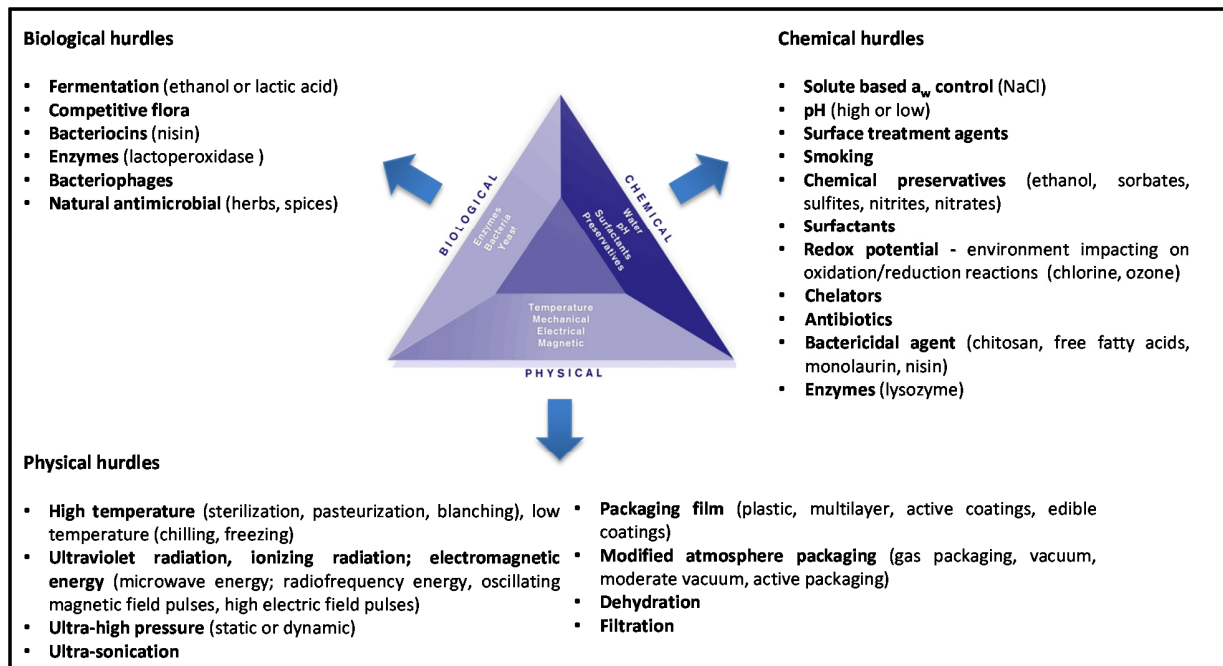


Figure 2.9: Schematic representation of the three sub-categories of hurdles applicable to achieve food preservation and non-exhaustive list of examples (DIL, 2011).

2.3.2. High pressure and its impact on bacterial spores

2.3.2.1. Isostatic high pressure

The first study which inferred the high pressure resistance of bacterial spores is dated from 1914 (Hite et al., 1914) and early studies could show that survivors of *B. subtilis* spores were found after up to 14 h at 1200 MPa (Basset and Macheboeuf, 1932; Larson et al., 1918). Inactivation of bacterial spores by high pressure was achieved when this hurdle was combined with medium or high temperature, following mechanisms which partly differ from the ones found in vegetative microorganisms. The history of high pressure inactivation of bacterial spores was recently extensively reviewed and the impact of pressure on several spore formers such as *B. subtilis*, *B. licheniformis* or *G. stearothermophilus* was assessed (Mathys, 2008; Reineke, 2012). Resistance of bacterial spores to high pressure as such has been associated to different roots, such as, the inactive metabolism, the presence of multiple protective layers, SASPs protecting the DNA and the low water activity in the spore core (see section 2.1.3). While thermal spore inactivation is considered as a single step process, the inactivation of bacterial spores under combined temperature and pressure has been suggested to be a two stage process (Heinz and Knorr, 2002; Margosch et al., 2004; Mathys et al., 2007a; Reineke et al., 2011a; Wuytack et al., 1998).

Theory and literature review background

Three pressure temperature domains were suggested in a recent study on the mechanisms of *B. subtilis* spore germination and inactivation during high pressure processing in ACES buffer (Reineke, 2012) and are represented in Figure 2.10.

1. 0.1-600 MPa and 30-50 °C: This domain can trigger non-nutrient pressure induced spore germination, which subsequently leads to maximum 4 log₁₀ spore inactivation after long pressure dwell times (>1 h). This pathway is hypothesized to be germinant receptor dependent (Reineke et al., 2013b).
2. 0.1-600 MPa and < 60 °C : The combined pressure and temperature affects the inner spore membrane and / or membrane channel proteins in an uncharacterized manner, leading to the spore core hydration and a subsequent inactivation (Reineke et al., 2013b).
3. > 600 MPa and > 60 °C: Rapid inactivation likely due to a loss of barrier properties of the inner spore membrane and CaDPA release. CLEs might also be inhibited thus retarding or inhibiting the cortex degradation (Reineke et al., 2013b; Reineke et al., 2011b).

In path 1) and 2) inactivation post germination could also be achieved by a pasteurization treatment instead of longer holding times at high pressure thereby combining high pressure and mild temperature to achieve sterile conditions. It remains that the extent of germination is, among others, organism dependent (Black et al., 2005). The incapacity to trigger a full spore germination has further hindered the transfer of this mechanistic insight into industrial application for food sterilization (Gould, 2006; Setlow, 2007a).

Multiple questions regarding the high pressure germination of spores remain. Regardless of the pathway (1 or 2) considered, the inner spore membrane constitutes a key player in the mechanism chain and the current gap of knowledge on the impact of high pressure on this structure is, thus, critical. Path 3) highlights the synergy existing between pressure and temperature leading to bacterial spore inactivation at much lower temperatures than temperature alone. The synergy between pressure and temperature for spore inactivation was also previously demonstrated in *G. stearothermophilus* ATCC 7953 (Mathys, 2008) and was confirmed in this work during preliminary investigations (Figure 2.11).

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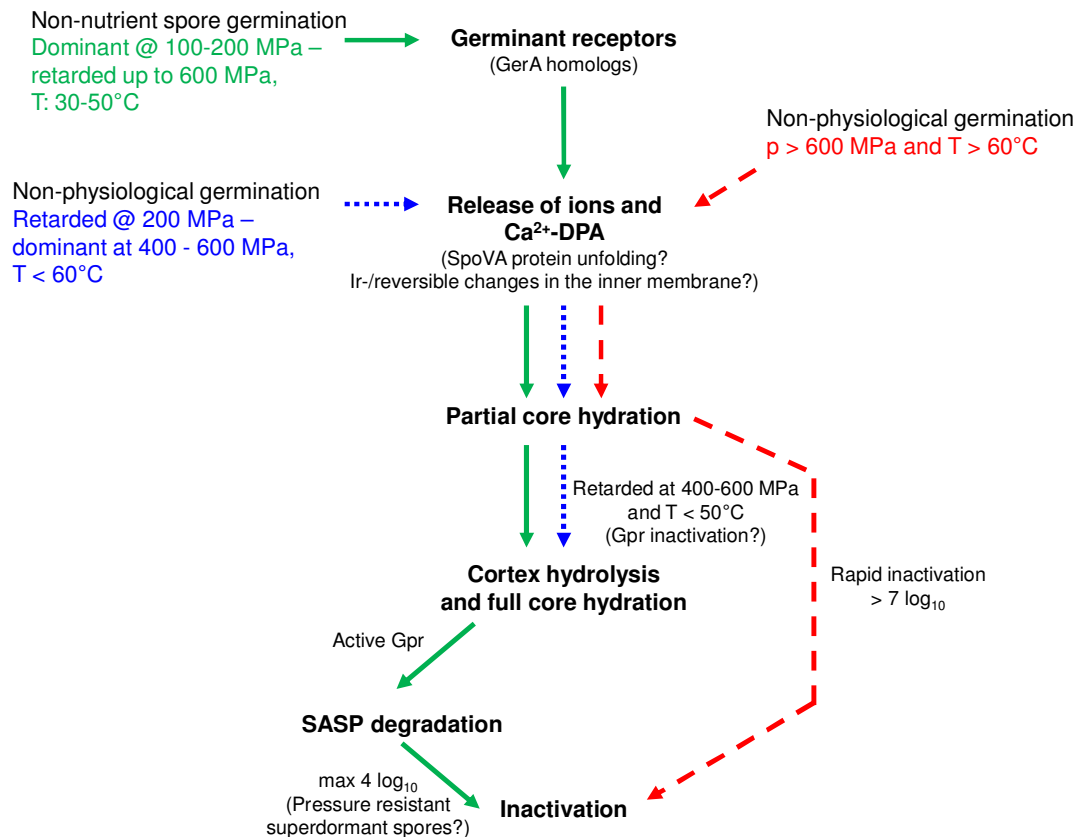


Figure 2.10: Proposed germination and inactivation pathways of *Bacillus subtilis* in dependence of the applied pressure temperature conditions (Reprinted from Reineke, et al., 2013b, with permission from Elsevier).

High pressure can also be used as a mean to reach spore lethal temperatures faster than conventional heating through adiabatic heating. This offers the chance to reduce the thermal load by comparison to conventional processing and thus minimises deleterious changes. This approach led to two sterilization concepts which both rely on adiabatic heating to bring the product to sterilization temperature:

- Pressure Assisted Thermal Sterilization (PATS) (Illinois Institute of Technology, 2009) which only considers the thermal sterilization temperature being reached during pressure dwell time as the lethal factor.
- High Pressure Thermal Sterilization (HPTS) which also takes into account the impact of pressure on spore inactivation (Mathys et al., 2009).

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Even if the process of PATS for mashed potatoes filed at the FDA was accepted in 2009 (Illinois Institute of Technology, 2009), no industrial application of HPTS or PATS exists yet.

One reason for this is the current technological challenge to develop high pressure vessels allowing for a proper thermal control. The theoretical adiabatic heating is derived from the first law of thermodynamics (Bridgman, 1912) and indicates the maximal achievable temperature change. In a uniform material, the temperature rise happens simultaneously in the whole product.

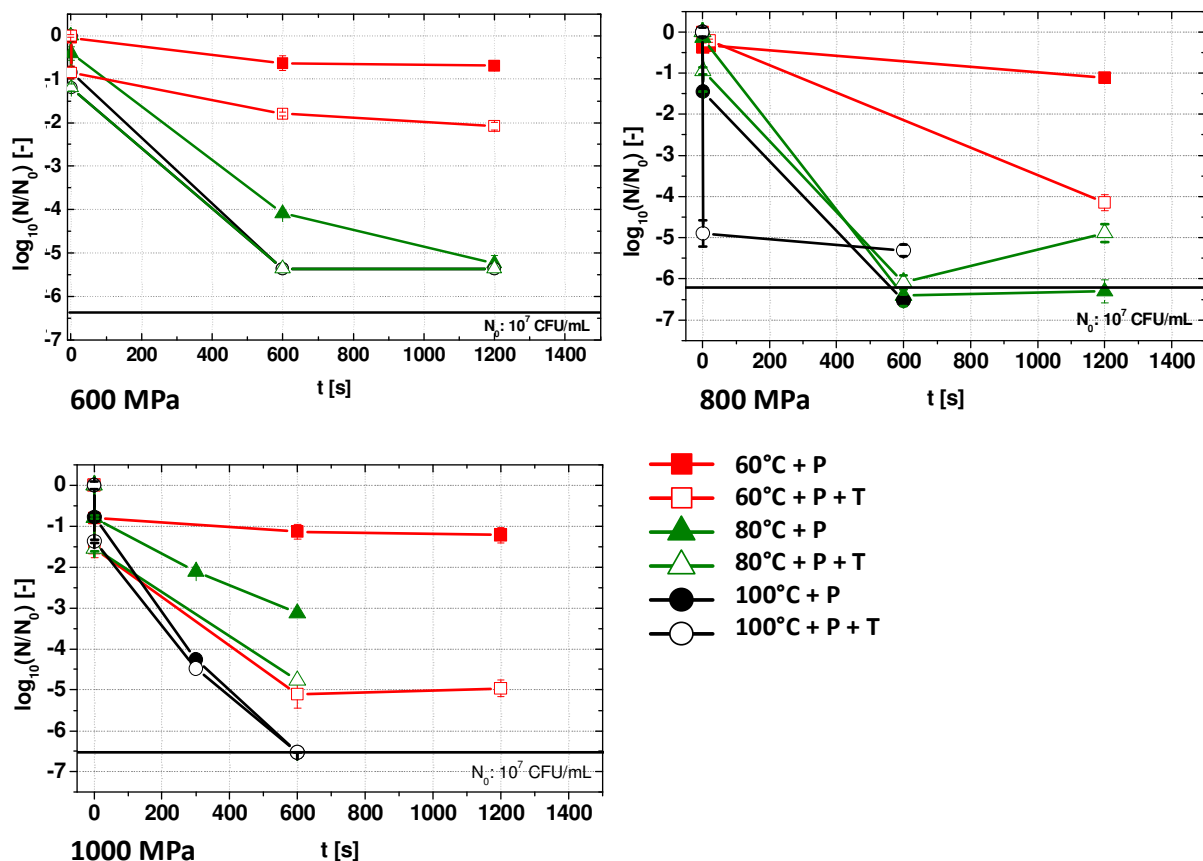


Figure 2.11: Inactivation of spores of *G. stearothermophilus* ATCC 7953 suspended in ACES buffer after given holding times at high pressure. The temperatures indicated correspond to the temperature at final pressure (P). + T signifies the application of a pasteurization step (80 °C, 20 min) post high pressure processing.

However, the extent of the temperature rise depends on the material properties (adiabatic heating depending on the compressibility and the specific heat of the substance) and, most importantly, on the overlapping heat transfer phenomena (non-adiabatic conditions) which lead to non-isothermal conditions. The temperature lack of uniformity during high pressure high temperature processing with industrial vessels was reported by numerous research groups and is explained by different

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adiabatic heating of the product, pressure medium and the heat transfer from the vessel wall (Grauwet et al., 2012; Juliano et al., 2009). This latter aspect is, to date, a key restriction preventing the implementation of high pressure high temperature as means of bacterial spore inactivation for food sterilization.

The question of the microbial indicator for such a process has also divided the scientific community. *Clostridium* spores (*C. botulinum*, *C. sporogenes* and *Clostridium perfringens*), *Bacillus* spores (*Bacillus amyloliquefaciens*) and *G. stearothermophilus* spores are pointed out as very highly pressure and temperature resistant (Ahn et al., 2015; Juliano et al., 2009; Margosch et al., 2006; Rajan et al., 2006). The biggest challenge in selecting a reference spore former is that some of the thermal sterilization reference strains are pressure sensitive. On the other side, some less thermo-resistant microorganisms can become very resistant under high pressure conditions (Margosch et al., 2006; Sevenich et al., 2013).

2.3.2.2. Dynamic high pressure: high pressure homogenization³

Mechanical homogenization was defined as the capability of producing a homogeneous size distribution of particles suspended in a liquid, by forcing the liquid under the effect of high pressure through a disruption valve (Donsì et al., 2009). The first occurrence of homogenization for the stabilization of food and dairy emulsions is dated from 1900 at the Paris World's Fair and was first patented in 1899 by Auguste Gaulin as an invention for "intimately mixing milk" using pressures up to 30 MPa (French Patent no. 295.596) (Gaulin, 1899). Since then conventional homogenization extended the pressure range until 50 MPa. High pressure homogenization (HPH), also known as dynamic high pressure homogenization, has been repeatedly highlighted for its potential for pasteurization of food matrices at reduced thermal loads (Belloch et al., 2012; Diels and Michiels, 2006; Poliseli-Scopel et al., 2013; Popper and Knorr, 1990). HPH enables pressures 10 to 15 times higher than traditional homogenizer and covers the range 100 – 400 MPa within which the upper pressure range from 300 to 400 MPa has been referred to as ultra-high pressure homogenization (UHPH) (Dumay et al., 2013). Here, the range 100-200 MPa is referred to as HPH and the range >200 MPa as UHPH. Inactivation of vegetative microorganisms has been demonstrated (Popper and Knorr, 1990) and is achieved through a combined action of cavitation³, shear stress, turbulence,

³ The following section is taken from: Georget E, Miller B, Callanan M, Heinz V and Mathys A (2014) (Ultra) high pressure homogenization for continuous high pressure sterilization of pumpable foods – a review. *Front Nutr.* 1:15, p1-6. doi: 10.3389/fnut.2014.00015 - © Georget, E., Miller, B., Callanan, M., Heinz, V., Mathys, A., 2014. (Ultra) high pressure homogenization for continuous high pressure sterilization of pumpable foods - a review. *Frontiers in Nutrition* 1, 1-6.

Theory and literature review background

impingement and high pressure leading to disruption of the vegetative microorganism (Kleinig and Middelberg, 1998). It was shown that the efficiency of UHPH for vegetative microorganisms inactivation increased with the pressure level, the number of passes, and the inlet and valve temperatures, but the exact mechanisms and interaction between parameters remain however to date to be elucidated (Dumay et al., 2013). The valve temperature (T_{valve}) showed a variable contribution to inactivation. While for $T_{\text{valve}} < 60$ °C, the mechanism of inactivation suggested a synergetic action of all stress factors, $T_{\text{valve}} > 80$ °C seemed to lead to a temperature driven inactivation (Dumay et al., 2013).

The progression towards UHPH has also opened the door to new sterilization opportunities which might go past the initial theory that it might not be possible to inactivate spores by HPH (Popper and Knorr, 1990). Bacterial spores, whose resistance to lethal treatments intensifies the concern of their threat to food microbial safety (Georget et al., 2013a), and their behavior when processed through UHPH are of prime interest. Comparatively, promising results could be achieved using high isostatic pressure thermal sterilization in model systems or products such as baby food (Mathys, 2008; Sevenich et al., 2014). Synergetic effects of temperature and isostatic pressure were demonstrated enabling a reduction of the total thermal load. However as mentioned above, this process is, to date, not available at industrial scale, and remains a batch process which leads to significantly higher production costs than conventional thermal processes. Moreover, the minimum pressure required to achieve spore inactivation at high temperature is significantly higher than the UHPH pressure range (> 500 MPa) (Mathys, 2008; Sevenich et al., 2014). The existing literature focusing on bacterial spore inactivation by UHPH is more recent and still faces inhomogeneity in conclusions. A recent extensive review of the technological aspects and potential applications of UHPH is available for the reader but only succinctly touched upon the topic of inactivation of bacterial spores by UHPH (Dumay et al., 2013). Hereafter, the existing literature focusing on bacterial spore inactivation by HPH and UHPH in different matrices as well as the main learnings reached in terms of sterilization potential are reviewed. A particular attention is given to the individual process parameters and target organisms in correlation with the achieved inactivation.

Detailed work has been reported on the impact of high pressure on bacterial spores (Mathys, 2008; Reineke, 2012; Reineke et al., 2013b). It could be shown that high pressure (>600 MPa) and temperature (>60 °C) have a synergistic impact on bacterial spore inactivation (Mathys et al., 2009). High pressure germination of bacterial spores was also achieved in the range 150-300 MPa / 30-55 °C (Georget et al., 2014a; Reineke et al., 2011a). Yet, it was shown that very short exposure to high pressure might not be sufficient to trigger germination if immediately followed by atmospheric pressure as was shown for *Bacillus subtilis* spores treated at 150 MPa / 37 °C for a few seconds

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(Kong et al., 2013). Furthermore, several investigations reported with HPH/UHPH and spores failed at showing significant inactivation potential (Diels and Michiels, 2006). However, a more in depth analysis of the work conducted this far on bacterial spore inactivation by HPH/UHPH shows that, often, a suboptimal process window might be responsible for this result.

The first study on the impact of HPH on bacterial spores was reported by Feijoo et al. (1997). While the inlet and outlet temperatures were given, the actual maximal temperature of processing was not stated. A maximum spore reduction of 68 % - that is 0.55 log₁₀ - was reported for 200 MPa and 50 °C inlet temperature and though not successful in full spore inactivation, this work opened a new field of investigation.

Several following investigations within the HPH or UHPH domain also reported failure to strongly inactivate bacterial spores of different genus, species and strain in model systems or food matrices (Bevilacqua et al., 2007; Bevilacqua et al., 2012; Chaves-López et al., 2009; Chen et al., 2013; Pereda et al., 2007; Pinho et al., 2011) (Table 2.3). A common point to all these investigations is that the maximum valve temperature achieved was not stated, and/or relatively low, when one considers the applied inlet temperatures and estimates the valve temperature on the basis of ~20 °C increase per 100 MPa (Popper and Knorr, 1990). While some authors attributed spore resistance to a lower exposure area of proteins in the spore, as well as inner structure cross protection by DPA (Bevilacqua et al., 2007), one might rather suspect that the thermal load necessary to achieve spore inactivation was not achieved and/or that the contribution of the other stress factors was insufficient to trigger sufficient inactivation.

Some authors attempted to combine HPH or UHPH with additional hurdles such as low pH (Bevilacqua et al., 2007; Chaves-López et al., 2009), dimethyl dicarbonate 250 ppm (Chen et al., 2013), or sodium benzoate (Bevilacqua et al., 2012). While Bevilacqua et al. (2012) suggested that an interaction between HPH and sodium benzoate (80 mg/L) could occur in some cases (e.g. apple juice) for a specific strain of *Alicyclobacillus acidoterrestris* at low inoculums, this could not be generalized and all in all, it appears that none of these treatments were sufficient to achieve a cumulative or synergetic response in the spore inactivation by HPH or UHPH.

In the work of Chaves-López et al. (2009), inactivation improved through multiple cycles and three cycles led to 5 log₁₀ reduction of *Bacillus cereus* spores (SV3, SV98, SV50 and SV108) and a corresponding DPA release of up to 52 %. This led the authors to suggest that the ultra-rapid depression during HPH treatments might cause a mechanical disruption of the coat and cortex, allowing DPA to leak out. However, it is reasonable to wonder why, with over 99.99 % of spores inactivated, the DPA release did not reach 100 %. This difference in result suggests that other mechanisms might be at stake, leading to spore incapacity to grow post multiple cycles of HPH.

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Moreover, the mention that during multiple cycles, samples were successively treated without any storage suggests that the new inlet temperature might have been close to the first outlet temperature estimated at 45 °C and likely even higher for the third cycle. No mention is made of the valve temperature and it is hard to estimate what might have been the most drastic conditions applied to the spores. An increasing processing valve temperature could be one possible explanation in the increasing inactivation observed. This hypothesis would also support why later work on thermo-resistant spores of *Geobacillus stearothermophilus* ATCC 7953 did not show any inactivation with up to 16 passes at 300 MPa and a maximum valve temperature of 84 °C (Pinho et al., 2011). The interest of the work done by Pinho et al. (2011) lies in the choice of strains and inoculation level, *G. stearothermophilus* ATCC 7953 and *C. sporogenes* PA 3679 both at 10⁵ spore/mL, the former being the reference strain for wet heat sterilization and thus highly relevant when looking at UHPH sterilization. The study conducted at pressures between 100 and 300 MPa however, concluded on an absence of inactivation of spores of both strains based on an inlet temperature of 45 °C and maximum valve temperature of 84 °C. Furthermore, treatment did not change the D and z-values of *G. stearothermophilus* and *C. sporogenes*, indicating that UHPH treatment at 300 MPa did not sensitize the spores to thermal treatments nor cause germination. These results contradicted the results of Chaves-López et al. (2009) and seem more likely considering the very short exposition time to high pressure (<1 s). The authors did not investigate higher inlet temperatures and therefore did not reach higher temperature at the valve either. The conclusion on the absence of sterilization potential of UHPH might, here again, be linked to treatments done at too low valve temperatures to lead to inactivation.

Yet multiple successful attempts at bacterial spore inactivation by HPH/UHPH were also reported (Table 2.4) (Amador-Espejo et al., 2014a; Amador-Espejo et al., 2014b; Cruz, 2008; Cruz et al., 2007; Ferragut et al., 2011; Polisel-Scopel, 2012; Polisel-Scopel et al., 2012, 2014; Valencia-Flores et al., 2013). Most of this recent work was conducted in animal and vegetal milks and with higher pressures and inlet temperature ranges. Although the reported holding time at high temperatures varies between studies, it could overall be approximated to <1 s. In spite of this very short time, one must consider the corresponding valve temperature (Table 2.4). For all studies where a full and durable inactivation of the native or inoculated spore flora could be achieved, the maximum temperature achieved (directly after the valve) was above 130 °C and the pressure at 300 MPa. These recent studies are to date the best examples that UHPH is a promising technology to achieve commercial sterility of pumpable foods at the condition of using sufficiently high homogenization pressures and high inlet temperature. A patent was published on January 26, 2012, which focuses on the use of UHPH for simultaneous sterilization and homogenization of pumpable foods and using

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the case of soy milk (Guamis et al., 2012). The results in this patent correspond to the ones obtained in the studies introduced here above and confirm the potential of UHPH to inactivate endogenous spore formers. It nonetheless remains that the role of various stress factors during UHPH has not been investigated and the inactivation mechanisms remain to be established. While Valencia-Flores et al. (2013) claims that the thermal treatment in combination with physical forces led to inactivation of endogenous vegetative cells and spores, no formal proof is given. The resulting high valve temperature seems to be a pre-requisite to any successful UHPH inactivation of mesophilic spore strains whose thermal resistance is limited. Using the indicated residence time at high temperature as well as the D and z values of isolated strains within the product, it would have been interesting to estimate how much inactivation could be associated to the thermal load only. Additionally, assessing the full inactivation potential via inoculated samples would also have been interesting and was not conducted in the work of Valencia-Flores et al. (2013). Also little work was conducted with highly thermo-resistant bacterial strains (Pinho et al., 2011). While the non-inoculated samples sterility could be assessed by shelf-life studies, the absence of high thermostable strains suggests a potential hazard in the process validation. Very recent work by Amador-Espejo et al. (2014a) initiated exploration of UHPH thermophilic spore inactivation with inoculation of whole UHT milk and showed that with an inlet temperature of 85 °C, *G. stearothermophilus* spores could be inactivated. However the strain used in this work was not ATCC 7953, the official wet heat sterilization indicator. If UHPH proves to be a thermally driven process, *G. stearothermophilus* ATCC 7953 would be recommended as indicator due its high resistance to wet heat inactivation (Albert et al., 1998).

Table 2.3: Overview of literature on non-successful HPH/UHPH inactivation of bacterial spores (Georget et al., 2014c)

| Equipment | Matrix | Spore strain | Initial count (spore/mL) | Maximal reduction ($\log_{10} (N/N_0)$) | Pressure (MPa) | T _{inlet} (°C) | Max T _{valve} (°C) | Source |
|--------------------|--|--|--------------------------|--|----------------|-------------------------|-----------------------------|-----------------------------|
| Microfluidizer® | Ice cream | <i>B. licheniformis</i> ATCC 14580 | 2.00E+04 | 0.55 | 200 | 50 | ? | (Feijoo et al., 1997) |
| Niro homogenizer | Soavi Double distilled water | <i>B. cereus</i> SV3, SV98, <i>B. subtilis</i> SV50, SV108 | 1.00E+07 1.00E+08 | - <0.5 with single pass - 5 with 3 cycles | 150 | 20 | ? | (Chaves-López et al., 2009) |
| Panda Soavi - Niro | Laboratory medium at pH 4.5 and 3.5 | <i>A. acidoterrestris</i> DSMZ 2498, Γ4 and c8 | 1.00E+05 | 0.67 (140-170 MPa) | 140-170 | ? | ? | (Bevilacqua et al., 2007) |
| Panda Soavi - Niro | Malt Extract broth (pH 4.5) and apple juice (pH 3.7) | <i>A. acidoterrestris</i> DSMZ 2498 and Γ4 | 1.00E+05 | 0.82 ± 0.07 | 140 | ? | ? | (Bevilacqua et al., 2012) |
| SFP FPG 12500 | Broth pH 4 | <i>A. acidoterrestris</i> N-1100, N-1108, N-1096, SAC, OS-CAJ | 1.00E+06 | <0.5 | 100, 200, 300 | ? | ? | (Chen et al., 2013) |
| SFP 7400H:350 FPG | Skim milk | <i>G. stearothermophilus</i> ATCC 7953, <i>C. sporogenes</i> PA 3679 | 1.00E+05 | 0.67 (16 passes - 300 MPa) | 100-300 | 45 | 84 | (Pinho et al., 2011) |
| SFP FPG 11300 | Milk 3.5 % fat | Naturally present spores | 5.00E+01 | 1.1 (200-300 MPa) | 100, 200, 300 | 30, 40 | 103 | (Pereda et al., 2007) |

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Table 2.4: Overview of literature on successful HPH/UHPH inactivation of bacterial spores (Georget et al., 2014c)

| Equipment | Matrix | Spore strain | Initial count (spore/mL) | Maximal reduction (log ₁₀ (N/N ₀)) | Pressure (MPa) | T _{inlet} (°C) | Max T _{valve} (°C) | Source |
|----------------------------------|--------------------------------|--|--|---|----------------|-------------------------|-----------------------------|---------------------------------|
| SFP Benchtop Homogenizer nG12500 | UHT whole milk | <i>B. cereus</i> (CECT 5144), <i>B. licheniformis</i> (DSMZ 13), <i>B. sporothermodurans</i> (DSMZ 10599), <i>B. coagulans</i> (DSMZ 2356), <i>G. stearothermophilus</i> (CECT 47), <i>B. subtilis</i> (CECT 4002) | ~1.00E+06 | >5 (for all strains at 300 MPa/85 °C) | 300 | 55, 65, 75, 85 | 139.0 ± 1.3 | (Amador-Espejo et al., 2014a) |
| SFP 11300 | FPG Soy milk | Naturally present spores | 2.34E+02 | 2.13 (300 MPa) | 200, 300 | 40 | 108 | (Cruz, 2008; Cruz et al., 2007) |
| SFP 11300 | FPG Almond Beverage | Naturally present spores (mesophilic and <i>B. cereus</i>) | 1.00E+04 1.00E+03 (<i>B. cereus</i>) | ND after 30 °C/20 d (300 MPa/65-75 °C) | 200, 300 | 55, 65, 75 | 129.3 ± 12.6 | (Valencia-Flores et al., 2013) |
| SFP 11300 | FPG Almond milk - Soy milk | Naturally present spores | 1.51E+03 (S)- 1.62E+04(A) | ND (200 MPa/75 °C - 300 MPa /65-75 °C) | 200, 300 | 55, 65, 75 | 135.7 ± 1.5 | (Ferragut et al., 2011) |
| SFP 11300 | FPG Almond beverage - Soy milk | <i>Paenibacillus taichungensis</i> , <i>B. cereus</i> , <i>B. subtilis</i> , <i>Lysinibacillus spp.</i> | 1.00E+05 1.00E+06 | ND for all except <i>B. cereus</i> : ~5 | 300 | 55, 65, 75, 85 | 138.0 ± 1.4 | (Poliseli-Scopel, 2012) |
| SFP 11300 | FPG Soy milk | Naturally present spores (mesophilic and <i>B. cereus</i>) | 2.88E+03- 3.55E+03 (<i>B. cereus</i>) | ND after 30 °C/20 d (300 MPa/75 °C) | 200, 300 | 55, 65, 75 | 135.7 ± 1.5 | (Poliseli-Scopel et al., 2012) |
| SFP 11300 | FPG Milk 3.5 % fat | Naturally present spores | 1.00E+01 | ND after 30 °C/15 d and 45 °C/ 7d (300 MPa/75-85 °C) | 200, 300 | 55, 65, 75, 85 | 139.0 ± 2.7 | (Amador-Espejo et al., 2014b) |
| SFP 11300 | FPG Soy milk | Naturally present spores (mesophilic and <i>B. cereus</i>) | 1.51E+02 1.95E+02 (<i>B. cereus</i>) | ND after 30 °C/20 d and 55 °C/ 10 d | 300 | 80 | 144 | (Poliseli-Scopel et al., 2014) |

ND: not detected

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2.4. Modeling of germination and inactivation kinetics

Kinetic modeling is frequently used to describe the inactivation of vegetative microorganisms and bacterial spores as well as intermediate events in spore inactivation such as germination (Georget et al., 2015b). The interest in modeling arises from the need to reliably and precisely quantify surviving populations but also to a certain extent, to predict these in order to optimize processing parameters and guarantee consumers' safety. Several modeling approaches have been proposed and mostly belong to two classes: mechanistic and vitalistic models (Lee and Gilbert, 1918; Watson, 1908).

Mechanistic modeling

The mechanistic conception is based on the hypothesis that the microorganism's inactivation is similar to a chemical reaction and time dependent (Lee and Gilbert, 1918). This led to the most commonly employed expression of the microbial inactivation following a first order inactivation kinetic derived from the integration of (1). In this case, the number of surviving microorganisms is logarithmically plotted versus time and the exponential inactivation curve becomes a linear function with the rate constant k as slope (2).

$$\frac{dN}{dt} = -k \cdot N \quad (1)$$

$$\ln\left(\frac{N}{N_0}\right) = -k \cdot t \text{ or } \log_{10}\left(\frac{N}{N_0}\right) = \frac{-k \cdot t}{2.303} = \frac{-t}{D_\theta} \quad (2)$$

The second form of (1) is often selected in relation to the expression of the decimal reduction time (D-value) which corresponds to the time required at a given temperature θ to achieve a reduction of the microorganism concentration by 1/10 of the initial value.

While (1) proved to be useful to model inactivation of microorganisms, recent work also showed that it could conveniently be used to model intermediate stages of the high pressure high temperature inactivation process of bacterial spores involving germination (Mathys et al., 2007a; Reineke et al., 2011a).

Subsequently, a linear Arrhenius-type equation (3) has been commonly used to assess the temperature dependency of the inactivation rate constant k .

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$$\ln k(T) = -\frac{E_a}{R \cdot T} + \ln k_0 \quad (3)$$

This model is the basis of thermal processing evaluation (e.g. canning) for treatments with variable temperatures and led to the 12 D-concept, which is still commonly used in the industry (Kessler, 2002).

However, inactivation kinetics often do not strictly follow a first order inactivation kinetic (Cheftel, 1995; Heinz and Knorr, 1996; Heinz and Knorr, 1998; Metrick et al., 1989). An initial lag phase (“shoulder”) and/or a leveling off for extended treatment times (“tailing”) are common deviations to the log linear microbial inactivation. An n^{th} -order model can be used instead of a first order model, which allows for tailing behaviors modeling but not shoulders (van Boekel, 2010). This was shown practically for high pressure high temperature spore inactivation for instance by Reineke (2012). In addition to this, the n parameter has no mechanistic background (van Boekel, 2010).

Vitalistic modeling

Vitalistic models rest on the theory that all individual microorganisms are different in their response to stresses such as temperature and pressure (Lee and Gilbert, 1918). It further stipulates that the survival curve represents a distribution of lethal events in time. Vitalistic models account well both for shoulder and tailing behaviors and multiple variants are found in the literature (Peleg and Cole, 1998; van Boekel, 2010). An example of vitalistic modeling is the Weibull-like model which was derived from reliability engineering. Weibull-like models allow for a great fitting of microbial germination (Collado et al., 2006; Paredes-Sabja and Torres, 2010) and inactivation curves (Ahn et al., 2015). A commonly used form of Weibull model for inactivation modeling is presented in (4).

$$\log_{10} S(t) = -b \cdot t^n \quad (4)$$

where $S(t)$ represents $N(t)/N_0$, b is the scale parameter, n is the shape parameter.

The shape parameter characterizes the progression of the inactivation curve while giving indications about the inactivation behavior of the microorganisms. For $n < 1$, the inactivation curve flattens in time (tailing) which implies that the remaining microorganisms which are still alive have an increased resistance by comparison to those which have been inactivated. For $n = 1$, the first order kinetic is found assuming an equal resistance in time of all microorganisms. For $n > 1$, the

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inactivation rate increases with time and is associated with decreasing resistance of the remaining microorganisms (shouldering). This simple approach allows describing shouldering or tailing behaviors in inactivation. An adaptation of this model, the double Weibullian model (5), has been suggested to model non-log linear inactivation of vegetative and sporulated microorganisms including both a tailing and shouldering behavior.

$$\log_{10} S(t) = b_1 \cdot t^{n_1} - b_2 \cdot t^{n_2} \quad (5)$$

This model was assessed by Reineke et al. (2011a) to model isorate lines for the high pressure inactivation of *B. subtilis* spores. Although each kinetic showed good individual fit, no systematic pressure and temperature dependence of the model parameters could be found and the inactivation pressure temperature isorate lines could not be obtained. This limited the application of these models for predictive applications. Instead, Reineke et al. (2011a) proposed that a multiresponse kinetic modeling, based on simple differential equations linking the different states of the bacterial spores (dormant, germinated, and inactivated), could offer an alternative, mechanistic based, modeling of germination and inactivation under high pressure high temperature. Alternatively, Heinz and Knorr (1996) showed that a combination of the Weibull distribution and a mechanistic model (1st order kinetic) in a two-step-model which considered distributed resistance mechanisms, could be applied to model the high pressure inactivation of cells of *B. subtilis*. In this case, regressively derived parameters showed a log-linear behavior as function of pressure, which supported the capacity of this model to represent vegetative microorganism's high pressure inactivation.

In this work, the vitalistic modeling approach was also considered but rejected on the basis of the evaluation below. In Section 8, Figure 8.2 represents the thermal inactivation kinetics of bacterial spores of *B. subtilis* and *G. stearothermophilus* which were used as basis to assess the thermal contribution to inactivation by ultra-high pressure homogenization (Georget et al., 2014b). There, it can be seen that the thermal inactivation kinetics partially display shouldering or tailing behaviors. In this regard, (4) could have been a more precise alternative to the first order inactivation kinetic used. (4) was fitted to the experimental data and $R^2 > 0.99$ were obtained for all kinetics. The set of n and b parameters obtained for both strains at different temperatures are represented in Figure 2.12 in dependence of temperature. Based on graphs A and B, it is visible that no obvious correlation between n, b and the temperature could be found common to both strains. Thereby, the need for extrapolation to higher temperatures could not be fulfilled by the Weibull-like model in this work.

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It results that although less accurate in terms of fitting, the mechanistic approach was more suitable for the assessment of the thermal inactivation of the indicators considered. Regarding the modeling of the germination mechanisms of bacterial spores in Section 6, a multiresponse kinetic [(7)-(9)] modeling derived from (6) was deemed the most flexible tool and also allowed to account for the link between different subpopulations which occur in a multiple stage germination pathway.

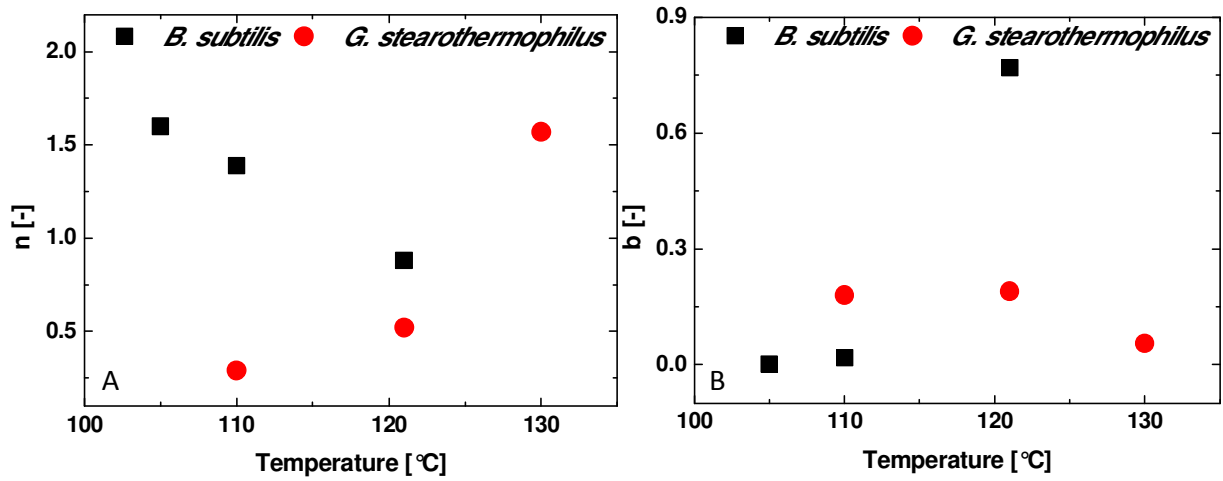
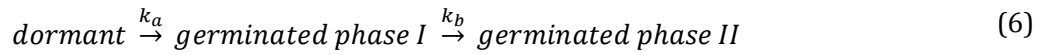


Figure 2.12: Temperature dependence of the shape parameter n (A) and the scale parameter b (B) (based on raw data from Georget et al. (2014b)).



$$\frac{d[\text{dormant}]}{dt} = -k_a[\text{dormant}] \quad (7)$$

$$\frac{d[\text{germinated I}]}{dt} = k_a[\text{dormant}] - k_b[\text{germinated I}] \quad (8)$$

$$\frac{d[\text{germinated II}]}{dt} = k_b[\text{germinated I}] \quad (9)$$

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2.5. Objectives of the work

The main objective of this work was to develop innovative technological solutions for spore inactivation which could support the reduction of the total thermal load applied to food products. With the knowledge gained, the target is to achieve more gentle sterilization of sensitive products. In order to reach this goal, the research activities focused on the target of sterilization – the bacterial spore. To facilitate the inactivation of the spore, this work had to be at the interface between microbiology and food process technologies in order to improve our understanding of spore behavior under novel hurdles.

The initial focus of the process toolbox was set on mechanical and thermal treatments (e.g. high pressure germination/pasteurization, high pressure thermal sterilization, ultra-high temperatures between 150-170 °C with ms holding time). Additionally, means of chemical germination were also investigated for potential combination with pasteurization treatments and inactivation of germinated spores. In order to allow for in depth parallel investigation of the spore physiology and corresponding toolbox development, the processing window was centered on pressure, chemical and thermal treatments.

As mentioned, an important target was, not only to germinate or directly inactivate spores, but also to understand how the various treatments applied impacted on the bacterial spores. This target was an essential aspect of this work in order to allow the establishment of more global solutions which might be effective on bacterial spores of different genera. In order to achieve this, novel tools of investigation also had to be developed and implemented for the indicator of this work *G. stearothermophilus* ATCC 7953 in order to monitor germination and inactivation.

Preface

3. Preface to the publication “*In situ* investigation of *Geobacillus stearothermophilus* spore germination and inactivation mechanisms under moderate high pressure”

The following work follows a long existing line of research focusing on bacterial spore germination and inactivation under high pressure (Heinz, 1997; Mathys, 2008; Reineke, 2012).

This cumulative work has significantly advanced the understanding of the spore physiology post high pressure processing and in particular the pressure dependence of different germination/inactivation pathways post high pressure.

While the high pressure high temperature domain ($P > 600$ MPa, $T > 60$ °C) showed very high potential for sterilization with strong spore inactivation, the ideal of achieving germination at pressures below 600 MPa (economical and industrial feasibilities) and at low temperatures (< 60 °C) is still limited in its extent by a significant superdormant population. The existence of these spores which cannot be germinated by high pressure underlined the need for further investigation of this physiological like path of germination.

One of the focuses of this work was to shift the investigation of spore germination from the *ex situ* to the *in situ* domain. In fact, although the events occurring after decompression have been very well investigated, the events occurring during the high pressure treatment itself have been seldom investigated, partially due to a lack of adequate tools to allow for *in situ* investigations.

The objective of the work below was to develop and adapt the necessary toolbox to *G. stearothermophilus* ATCC 7953 spores in order to achieve *in situ* investigation of this strain under moderate high pressure and temperatures leading to germination.

***In situ* investigation of *Geobacillus stearothermophilus* spore germination and inactivation mechanisms under moderate high pressure**

4. *In situ* investigation of *Geobacillus stearothermophilus* spore germination and inactivation mechanisms under moderate high pressure

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Food Microbiology, 41, p. 8-18, 2014 – with permission from Elsevier

Abstract

Bacterial spores are a major concern for food safety due to their high resistance to conventional preservation hurdles. Innovative hurdles can trigger bacterial spore germination or inactivate them. In this work, *Geobacillus stearothermophilus* spore high pressure (HP) germination and inactivation mechanisms were investigated by *in situ* infrared spectroscopy (FT-IR) and fluorometry. *G. stearothermophilus* spores' inner membrane (IM) was stained with Laurdan fluorescent dye. Time-dependent FT-IR and fluorescence spectra were recorded *in situ* under pressure at different temperatures. The Laurdan spectrum is affected by the lipid packing and level of hydration, and provided information on the IM state through the Laurdan generalized polarization. Changes in the $-\text{CH}_2$ and $-\text{CH}_3$ asymmetric stretching bands, characteristic of lipids, and in the amide I' band region, characteristic of proteins' secondary structure elements, enabled evaluation of the impact of HP on endospores lipid and protein structures. These studies were complemented by *ex situ* analyses (plate counts and microscopy). The methods applied showed high potential to identify germination mechanisms, particularly associated to the IM. Germination up to 3 log₁₀ was achieved at 200 MPa and 55 °C. A molecular-level understanding of these mechanisms is important for the development and validation of multi-hurdle approaches to achieve commercial sterility.

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4.1 Introduction

Bacterial endospores constitute a risk for food processors due to their strong resistance to both chemical and physical hurdles (Esty and Meyer, 1922; Heinz and Knorr, 1996; Sale et al., 1970; Setlow, 2000, 2006). Presence of bacterial spores in a food matrix or on processing equipment may lead to food (re)contamination following germination, and jeopardize consumer safety as well as product quality. Exemplarily, bacteria such as *Clostridium botulinum*, which produces botulin toxin (LD50 of 1 ng/kg), highlight the necessity of inactivating bacterial spores prior to commercialization (Black et al., 2007a; Esty and Meyer, 1922).

To counter this problem, inactivation strategies both for food and equipment surfaces have been developed based on thermal or a combination of thermal and chemical treatments (Georget et al., 2013a). Though effective, these strategies often rely on incomplete knowledge of the mechanisms at stake leading to over processing and/or low product quality.

Alternative, innovative hurdles have been suggested to achieve food safety while improving product quality (Koutchma et al., 2005). Notably, high pressure represents a promising alternative for pasteurization (Palou et al., 1999). It has been implemented for several years now in different sectors of the food industry (Mújica-Paz et al., 2011). However, it was highlighted from early on that high pressure alone is insufficient to achieve bacterial spore inactivation, a prerequisite to sterilization and ambient temperature storage (Basset and Macheboeuf, 1932; Hite et al., 1914; Larson et al., 1918). The potential of high pressure as a sterilizing technology was highlighted only later on through its capacity to trigger bacterial spore germination (Gould and Sale, 1970; Heinz, 1997; Heinz and Knorr, 1998; Reineke, 2012) or inactivation when combined with temperature (Ananta et al., 2001; Heinz and Knorr, 1998; Knoerzer et al., 2007; Mathys, 2008; Mathys et al., 2009). The inability to stimulate full germination and equipment limitations for high pressure thermal sterilization are still limiting the implementation of HP technology for food sterilization. In particular, the incomplete knowledge of the pathways at stake during HP germination, a key step in reducing spore thermal resistance, has been pointed out (Abel-Santos, 2012) and hinders the validation of this technology for the food industry (Hendrickx and Knorr, 2002; Mathys, 2008; Reineke, 2012).

To date, most studies conducted to improve the understanding of germination mechanisms could only be conducted via *ex situ* analyses and little is known about the transformations occurring during the high pressure process itself. Additionally, most of the knowledge gathered in the literature focuses on germination of *Bacillus subtilis* (Black et al., 2005; Heinz, 1997; Reineke, 2012;

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Setlow, 2003, 2007a; Vepachedu et al., 2007; Wuytack, 1999), which is one of the best known strains, but not necessarily the most relevant for the food industry.

Reviews of the current knowledge on bacterial spore germination have strongly emphasized the key role played by the inner membrane in the regulation of the associated mechanisms even though many unknowns remain, in particular regarding the first phase of the germination (Paidhungat and Setlow, 2002; Setlow, 2003; Stewart et al., 1981; Yi et al., 2011). Considering the importance of the inner membrane in germination, the current lack of knowledge about this structure is a limitation.

The inner membrane of bacterial spores was reported to have a composition very close to the cytoplasmic membranes of growing cells (Cortezzo and Setlow, 2005). One of the key structural models established so far is the Fluid Mosaic Model which represents the biological membrane essentially as a two-dimensional fluid with embedded proteins (Singer and Nicolson, 1972). The proportion of proteins in such membranes is between 20 % and 35 % (Stevens and Arkin, 2000) and these proteins are localized in a lipid environment. Besides its unique properties, the inner membrane has a set of unique proteins, including the nutrient receptors and proteins, such as those encoded by the *spoVA* operon, possibly involved in movement of core molecules across the inner membrane. As these proteins function while being localized in the inner membrane, they are likely to be largely immobile in this environment (Setlow, 2008b). Membrane lipids and proteins may influence each other directly as a result of their biochemical nature and in response to environmental changes (Winter and Jeworrek, 2009). This scenario is particularly relevant for bacterial spores' inner membrane, where the presence of proteins plays a key role in the germination process under pressure (Setlow, 2007a). It has been suggested that the highly and unusual compact and impermeable state of this membrane impacts on the proteins' mobility as well as functionality (Cowan et al., 2003; Cowan et al., 2004). However, *in situ* pressure studies are still very scarce. The investigation of the pressure effects on Na⁺,K⁺-ATPase reconstituted into phospholipid bilayers showed that HP above 220 MPa irreversibly changed the protein conformation, probably because of the dissociation and partial unfolding of the subunits (Powalska et al., 2007), highlighting the need for further studies *in situ* of the bacterial spores' inner membrane under pressure.

In this work, we investigated the germination mechanisms of *Geobacillus stearothermophilus* spores, an industrial thermal sterilization indicator, by means of high pressure (200 MPa) and temperature up to 55 °C, using a combination of *in* and *ex situ* analyses to improve the understanding of the phenomenon at stake under high pressure conditions. A series of methods was developed or

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adapted to this bacterium to achieve the *in situ* analysis under pressure of the inner membrane, a key player in germination pathways, as well as of the protein secondary structures.

4.2 Material and methods

4.2.1 Bacterial strain

The strain used in this work was *G. stearothermophilus* ATCC 7953 obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, and stored at -80 °C at the German Institute of Food Technologies (DIL).

G. stearothermophilus spores were obtained following successful development of a sporulation method for this indicator enabling >95 % spores, as described hereafter.

4.2.2 Sporulation method

By modifying a method described elsewhere for *Bacillus subtilis* (Nicholson and Setlow, 1990; Paidhungat and Setlow, 2000) and adapting it to *G. stearothermophilus*, a high sporulation ratio could be achieved. Repeatable sporulation was achieved in less than a week. A single colony of *G. stearothermophilus* grown on nutrient agar over night was used to inoculate 10 mL of TSB medium (Tryptic Soy Broth Fluka T8907-500G from Sigma-Aldrich Company, Germany - 30 g/L in distilled water). The TSB cultures were incubated at 55 °C and 250 rpm in a shaking incubator for a minimum of 5 h. After 5 h, the OD₆₀₀ was checked every 30 min until the required density was reached (1.6-1.8 / control with cell density meter Ultrospec 10 from Amersham Biosciences GmbH, Germany). A 200 µL aliquot of the culture was then spread onto Difco agar plates (for 1 L distilled water: pepton 5 g, meat extract 3 g, Agar 20 g, KCl 1 g, MgSO₄·7 H₂O 0.12 g, 1 M Ca(NO₃)₂ 1 mL*, 0.01 M MnCl₂ 1 mL*, 1 mM FeSO₄ 1 mL*⁴). The plates were sealed in plastic bags and incubated at 55 °C for a minimum of 3 days. Sporulation was monitored via phase brightness with a transmitted-light microscope daily. When more than 95 % sporulation was observed, plates were removed from the bags and left at room temperature for drying. The plates were controlled under phase contrast microscope each day until remaining vegetative cells were dried out; following which the spores were collected using 4 °C distilled water and cleaned by repeated centrifugation at 4800 g, 4 °C for 30 min until the supernatant was clean. Following sporulation, spores were stored at -80 °C until further use.

⁴ Components marked with an * were sterile filtered and added post autoclaving.

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4.2.3 Laurdan staining of the inner membrane

Based on the method previously developed (Hofstetter et al., 2012; Hofstetter, 2012) detailing the use of the fluorescent probe Laurdan to label and measure inner membrane fluidity of endospores of *Clostridium* spp., fluorescent staining of *G. stearothermophilus* spore inner membrane was conducted with the sporulation method described above. Laurdan was selected as fluorescent dye because of its non-cytotoxicity (Owen et al., 2012), enabling further evaluation of the physiological state of spores by plate count, for instance.

Endospores containing Laurdan were generated by plating 400 μ L of saturated Laurdan (6-Dodecanoyl-N,N-dimethyl-2-naphthylamine, purchased from Sigma-Aldrich, Germany) suspended in 100 % ethanol onto Difco agar plates, allowing the ethanol to evaporate in the absence of light. 200 μ L of a fully-grown culture of *G. stearothermophilus* (OD_{600} between 1.6 and 1.8) were plated on Laurdan-containing Difco agar plate and the remaining of the sporulation protocol was conducted as described above.

A single batch of stained spores of *G. stearothermophilus* was used for this study. Using a Thoma chamber, the spore percentage was estimated to be 94.1 % \pm 1.5 % (average of 4 different counts and corresponding standard deviation).

4.2.3.1 Generalized Polarization (GP)

Lipid packing/order can be quantified by fluorospectroscopy of an environment-sensitive fluorophore such as Laurdan (Molina-Höppner et al., 2004; Sezgin et al., 2012). The fluorescent naphthalene moiety of the Laurdan molecule possesses an electrical dipole moment, which increases upon excitation, and may cause reorientation of the surrounding solvent dipoles. Solvent reorientation results in a decrease of the excited state energy of the probe, which is reflected in a red shift of the probe's emission spectrum (Sánchez et al., 2007). Consequently, Laurdan emission spectra exhibit a red shift in the liquid-crystalline fluid-like phase of lipid membranes compared with the ordered gel phase which is characterized by a dehydration of the lipid's upper chain region due to dense lipid packing. For Laurdan, these two extreme conditions of lipid bilayers have been studied in detail and the wavelength maxima for the two extremes were found to be at 440 nm for ordered gel phases and at 490 nm for disordered liquid-crystalline phases (Harris et al., 2002; Owen et al., 2012; Parasassi et al., 1991).

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Since wavelength displacements may be subtle and hard to characterize only by peak wavelengths, the so-called General Polarization, *GP*, presents a more precise parameter to quantify the polarity change (i.e., shift of the emission maximum). The concept of *GP* was introduced in detail elsewhere (Parasassi et al., 1990). Using the intensity data collected at different excitation and emission wavelengths, the *GP* is defined by Equation (10) (Parasassi et al., 1991) and was used to characterize the lipid bilayer state in the bacterial spore membrane during the different treatments.

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \quad (10)$$

Here, I_{440} and I_{490} correspond to the fluorescence emission intensities measured at 440 nm and 490 nm, respectively, following excitation at 360 nm. The *GP* values are limited between +1 and -1 but practically, values range from -0.3 to 0.6 (Hofstetter, 2012; Parasassi et al., 1998).

In this study, a FP-8500 Fluorescence Spectrometer (Jasco, Germany) and K2 multifrequency phase and modulation fluorometer (ISS Inc., Champaign, IL USA) were used. Excitation light (at 360 nm) was obtained with a Xe lamp source and emission spectra collected from 400 to 550 nm. For the monitoring of the *GP* under pressure, Laurdan-stained spores were suspended in ACES buffer (pH 7.4, 0.05M) to a final OD₆₀₀ of 0.5.

4.2.4 Fourier Transform Infrared (FT-IR) spectroscopy

The temperature- and pressure-dependent FT-IR spectra were recorded with Nicolet 5700 and Magna IR 550 spectrometers (Thermo Fisher Scientific Inc., USA) equipped with liquid nitrogen cooled MCT (HgCdTe) detectors, respectively (Czeslik et al., 1998; Herberhold et al., 2004; Herberhold and Winter, 2002; Nicolini et al., 2006; Squires et al., 2002). For the pressure-dependent measurements, the infrared light was focused by a spectral bench onto the pin hole of a diamond anvil cell (DAC) (with type IIa diamonds from Diamond Optics, Tucson, USA) and powdered barium sulfate as a calibrant was placed in the hole of the steel gasket of the DAC. Changes in pressure were quantified by the shift of the band at 983 cm⁻¹ assigned to a barium sulfate stretching vibration (Table 4.1). The freeze-dried spore suspension was resuspended in heavy water (D₂O) to achieve a high spore concentration of approx. 10⁹ CFU/mL. 10 μL of the sample was spread on the DAC and dried with a stream of nitrogen gas, following resuspension in the same volume of D₂O before the DAC was sealed. Each spectrum was obtained by co-adding 256 scans at a spectral resolution of 2 cm⁻¹ and was apodized with a Happ-Genzel function, and

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corresponding processing was performed using GRAMS software (Thermo Fisher Scientific Inc., USA). After background subtraction, the spectra were baseline corrected and normalized by setting the area between 1700 and 1600 cm^{-1} to 1 to allow for a quantitative analysis of the time evolution of protein secondary structural changes. The changes in the $-\text{CH}_2$ and $-\text{CH}_3$ asymmetric stretching bands characteristic of lipids were also monitored following base lining and normalization.

4.2.4.1 FT-IR spectra analysis

For the analysis of the proteins' secondary structural changes, second derivative and Fourier self-deconvolution (FSD) were applied to the normalized spectra to identify the components of the amide-I' band region. These peaks were then fitted to the normalized raw spectra using a Levenberg-Marquardt curve fitting routine with bands of Voigt line shape. All spectra were fitted with a similar set of peaks and parameters.

Table 4.1: Assignment of bands in the FT-IR spectra

| Wave number (cm^{-1}) | Functional group assignment | Reference |
|----------------------------------|---|------------------------|
| ~1700-1600 | Amide I' band of proteins | |
| ~1620/1883 | Intermolecular β -pleated sheets | (Byler and Susi, 1986; |
| ~1627-1637 | Intramolecular β -pleated sheets | Naumann et al., 1991) |
| ~1640-1645 | Random coils/unordered structures | |
| ~1650-1655 | α -helices | |
| ~1670-75 | Turns and loops | |
| ~3100-2800 | Lipid domain | |
| ~2960 | $-\text{CH}_3$ asymmetric stretching in acyl chains | |
| ~ 2925-2916 | $-\text{CH}_2$ asymmetric stretching in acyl chains | |
| ~2873 | $-\text{CH}_3$ symmetric stretching in acyl chains | |
| ~ 2855-2849 | $-\text{CH}_2$ symmetric stretching in acyl chains | |
| ~983 | Barium sulfate stretching vibration (pressure calibration in DAC) | |

The area under each peak represented the fraction of the respective component (assuming similar transition dipole moments for the different conformers) and was finally used to determine the percentages of the secondary structure components. To analyze the membranous structures, the position and shape of $-\text{CH}_2$ and $-\text{CH}_3$ stretching vibrations were taken into account (Table 4.1).

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4.2.5 Germination induction via high pressure

A high pressure treatment of 200 MPa was applied using respectively a diamond anvil cell (DAC) (as described above for the FT-IR measurement) or a ISS High Pressure Cell System (ISS Inc., USA) connected to a K2 multifrequency phase and modulation fluorometer (ISS Inc., USA) for the Generalized Polarization (*GP*) analysis during compression (see section 4.2.3.1). For plate count estimation of germination, a HP unit (0101-7000-S, Sitec Sieber Engineering AG, Switzerland), with a maximum vessel volume of 100 mL and a compression rate of 4 MPa s⁻¹ was used for pressure treatment of the plastic tubes (1.6 mL), with water as pressure-transmitting media. Treatments were conducted at 30 °C and 55 °C. An example of a *T, p* profile as measured in the geometrical center of the sample tube during HP processing, can be found in Figure 4.1.

Following the HP trials, plate counts were performed before and after a thermal treatment at 80 °C during 20 min to inactivate the potentially sensitized spores and establish the extent of the germination. Samples were plated in triplicate on nutrient agar and incubated at 55 °C for 48 h.

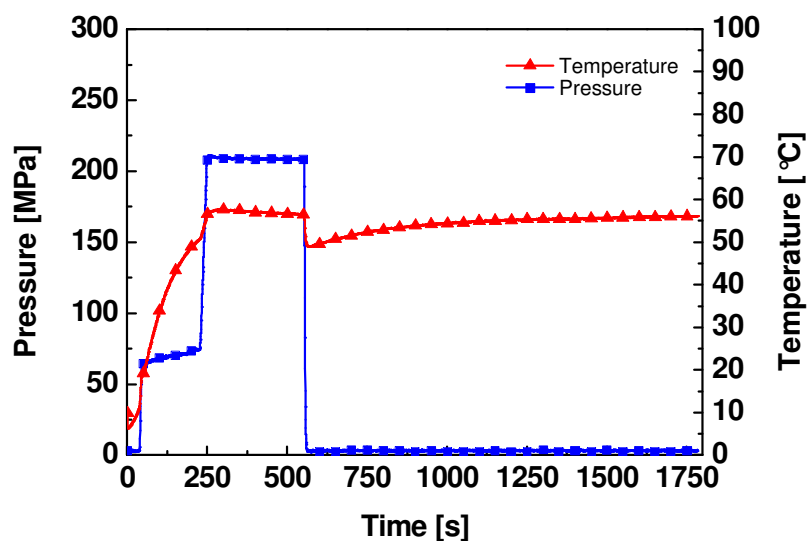


Figure 4.1: Pressure temperature profile in the Sitec High Pressure Processing unit, example for 200 MPa at 55 °C for 5 min.

These counts were compared to calcium dipicolinate DPA-Ca²⁺ release as measured by HPLC using a method developed elsewhere (Heinz, 1997). Additionally, phase contrast microscopic analyses were performed. For all pressure treatments except in the DAC, the spores were suspended in pressure-stable N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer (pH 7.4, 0.05 M). For the

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fluorescence trial, the spore sample was suspended with an optical density (OD) set at 0.5 and filled in quartz vials, sealed and placed in the pre-tempered pressure vessel. The HP treatments were applied for 1 s (shoot), 5 min, 20 min and 40 min dwell time before decompression and were done at least in duplicate. Following decompression, the samples were kept at experimental temperature for 20 min prior to plate count. The *in situ* data acquisition was conducted before, during and after treatment up to 20 min to evaluate changes occurring during and after compression.

4.2.6 Scanning Electron Microscopy (SEM) imaging combined with Focused Ion Beam (FIB) sectioning

The FIB–SEM method followed the method developed for *B. subtilis* spores by Reineke et al. The spore samples were critical point dried to avoid creating structural damage to the spores. For focused ion beam (FIB) sectioning and scanning electron microscopic (SEM) imaging, a Helios NanoLab 600 dual-beam microscope (FEI, Hillsboro, USA) was used at the department of electron microscopy at the Technische Universität Berlin, Germany (ZELMI). Prior to sectioning, samples were covered by a 50 nm thick platinum (Pt) layer as described elsewhere (Reineke et al., 2013). For the SEM of FIB sectioned spores between 10 and 40 spores were analyzed from each sample.

4.3 Results and discussion

4.3.1 Laurdan staining of *G. stearothermophilus* spore inner membrane

The first part of this work dealt with assessing the inner membrane state in the non-treated spores of *G. stearothermophilus*, based on the successful application of the Laurdan staining method. Staining of *G. stearothermophilus* spores using Laurdan was successfully achieved (Figure 4.2) following the procedure previously used for Clostridial spores (Hofstetter, 2012). To confirm the location of this stain in the inner membrane, decoating trials were conducted using an adaptation of a method described elsewhere (Fitz-James, 1971) with all incubations conducted at 55 °C. Decoating has been previously used in the literature to remove the outer layers of the spores including the outer membrane which would also be expected to contain Laurdan. The decoating success was controlled by measuring the inactivation of decoated spores by a lysozyme treatment at 55 °C as described elsewhere (Klobutcher et al., 2006). A complete inactivation of decoated spores compared to non-decoated spores was observed (results not shown).

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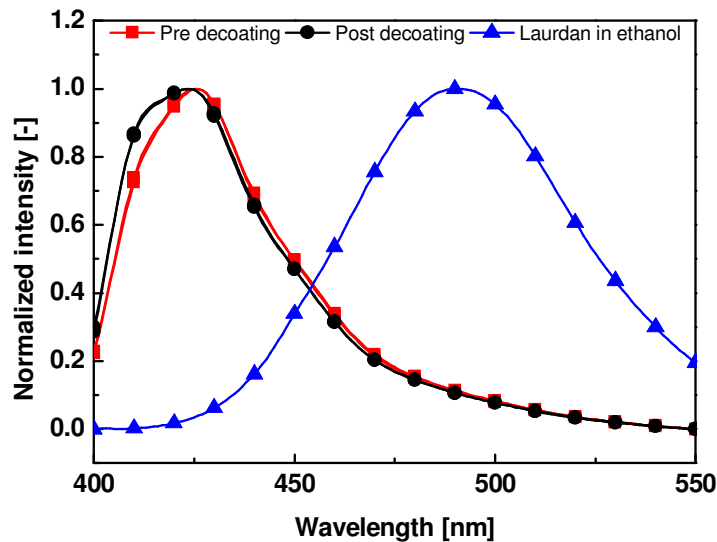


Figure 4.2: Fluorescent signal intensity in *G. stearothermophilus* spores pre- and post-decoating (measurements in duplicate – two curves of the same color overlapping). OD adjusted to 0.5 prior to the measurement. Non stained spores showed no fluorescence signal while Laurdan spectra diluted 1:1000 after saturation in 100 % ethanol showed a maximum of emission at 490 nm as expected for Laurdan in the free solution state.

Using equation (10), the Laurdan *GP* value calculated before and after decoating amounted to 0.68 and 0.68 respectively (average of two repetitions). While *GP* in lipid liquid-like phases ranges from -0.3 to +0.3, *GP* in gel lipid phases generally ranges from 0.5 to 0.6 (Parasassi et al., 1998). Values of 0.77 and 0.73 have been reported for *C. beijerinckii* and *C. sporogenes* spores (Hofstetter et al., 2012), indicating that the range obtained in this study, though higher than for the conventional gel phases, is consistent with the results obtained on spores of other species. It can additionally be mentioned that the outer spore membrane structure was reported to be the one of a vegetative cell membrane (liquid crystalline). If the contribution to the *GP* value was significant, the emission profile of dormant spores should be stronger at 490 nm which was not the case. The *GP* values obtained therefore emphasize the high degree of order and compressed state of the lipids in the inner spore membrane, also highlighted in other studies (Cowan et al., 2004; Sunde et al., 2009). The results of this section confirm that the *GP* values obtained are the outcome of the Laurdan emission of the inner membrane and are not resulting of the emission of the outer spore membrane.

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4.3.2 Germination of *G. stearothermophilus* spores under low high pressure

4.3.2.1 Quantification by plate counts and microscopic observation

Stained *G. stearothermophilus* spores were suspended in ACES buffer (0.05 M, pH 7.4) to an optical density of 0.5 for all GP measurements. Plate count results can be found in Figure 4.3 and notably over 2 log₁₀ could be heat sensitized after 5 min at 200 MPa / 55 °C. Similarly, 200 MPa for 40 min at 55 °C led to an inactivation of 3 log₁₀ following the subsequent inactivation of the heat sensitized population of *G. stearothermophilus* spores. Treatments at 30 °C led to a more moderate heat sensitization/inactivation of spores. These results were complemented by phase contrast microscopy, showing spores having lost their phase brightness associated to DPA-Ca²⁺ release (Setlow, 2003) and confirmed a HP triggered germination of spores. Additionally, DPA-Ca²⁺ release was quantified in HP treated spores by HPLC and was consistent with the germination results indicated by plate counts. Pressure treatment at 200 MPa, 55 °C led to a rapid full release of DPA-Ca²⁺ while the spores treated at 30 °C displayed only partial release of the total content matching partial germination.

The key role played by temperature is in favor of a physiological-like germination at 200 MPa in accordance with results obtained for *Bacillus subtilis* (Reineke, 2012; Setlow, 2008b). One of the main conclusions of Reineke's work was that physiological-like germination of *B. subtilis* is dominant in the range 100-200 MPa for temperatures between 30 and 50 °C. The results of this study nuance this result with regard to the temperature dependency of germination. For *G. stearothermophilus* spores, a minimum temperature requirement of 55 °C, which may be related to the strain's optimal growth temperature, seemed necessary to achieve germination at 200 MPa. The higher inactivation after 40 min than after 5 min suggested a significant impact to the physiological fitness of a part of the germinated population associated with prolonged exposure to pressure. The results were also in agreement with the findings obtained for *B. subtilis* (Reineke, 2012), where count reduction of spores was reported after 60 min under pressure of 150 MPa.

It was also noted that the extent of the germination achieved between the 5 min and 40 min treatments was not very different, suggesting that the commitment and germination trigger of the bacterial spores occurred rapidly under pressure, likely through activation of the nutrient receptors as suggested in previous work (Black et al., 2005; Wuytack et al., 2000).

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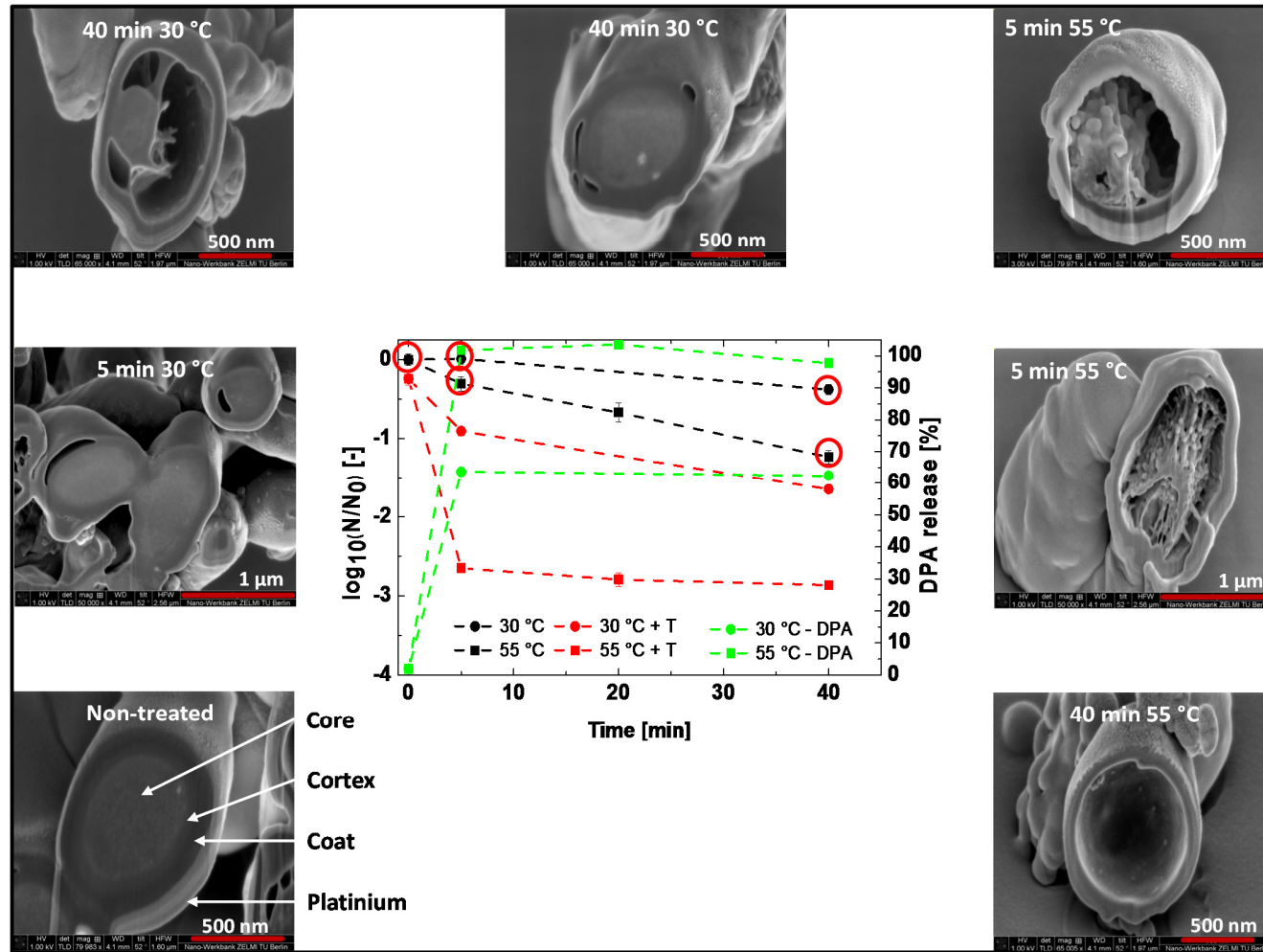


Figure 4.3: Plate counts of samples treated with 200 MPa, corresponding DPA-Ca²⁺ release and representative FIB/SEM pictures of spores snap frozen (-196 °C) directly after decompression. All treatments were performed at 200 MPa and 30 °C or 55 °C. The bars correspond to standard deviations obtained from plate counts in triplicates. + T indicates a subsequent thermal inactivation step (80 °C, 20 min) killing heat sensitized spores. Decompression after 5, 20, and 40 min respectively. N₀: 2.3x10⁸ CFU/mL.

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4.3.2.2 SEM-FIB observation of HP treated spores

To track the impact of different HP treatment duration and temperature particularly on DPA-Ca²⁺ release and cortex degradation without the influence of the post decompression incubation time, SEM-FIB was performed on snap frozen samples following decompression and showed various structures Figure 4.3. Non treated spores showed a full core and well developed cortex structure around it. Based on picture analysis, spores with different core state could be counted (full, partially empty, empty). Spores treated at 30 °C showed a maximum of 59 and 69 % of empty or partially empty core (corresponding to DPA-Ca²⁺ release) and partially degraded cortex after 5 and 40 min, respectively. Spores treated at 55 °C showed the highest percentage of empty or partially empty core with 73 and 91 % of the spores observed after 5 and 40 min, respectively. Additionally, the spores treated for 5 min at 55 °C showed a particular core structure suggesting initiation of fusion of the core and transition of the DPA-Ca²⁺ core to a liquid state. This might correspond to one of the first steps of DPA-Ca²⁺ release and water uptake.

4.3.2.3 Fluorescence measurements in and ex situ under pressure and at different temperatures

To evaluate the impact of the various thermal treatments applied in this study and the capacity of Laurdan *GP* to detect changes in *G. stearothermophilus* spores' inner membrane structure, *ex situ* measurements were initially conducted following different thermal treatments of 55 °C for 120 min (HP treatment at highest temperature), at 80 °C for 20 min (used for heat sensitized spores inactivation) and an autoclave treatment (121 °C, 20 min – full inactivation reference). Laurdan *GPs* are shown in Figure 4.4 in correlation to the corresponding log₁₀ reduction.

From these results, it was confirmed that heating of the spores over long periods of time at 55 °C and 80 °C did not impact significantly the state of the inner membrane. Conventional sterilization (121 °C, 20 min) led to a full inactivation of *G. stearothermophilus* spores as expected, accompanied by a reduction of the Laurdan *GP* to 0.45, indicating changes in the structure of the inner membrane, likely disruption of the inner membrane and/or denaturation of the proteins within the inner membrane.

The measurement of the Laurdan *GP* values was also conducted *in situ* during the HP treatments at 30 and 55 °C (Figure 4.5). Steady *GP* values of 0.68 ± 0.01 at 55 °C and 0.66 ± 0.01 at 30 °C in ACES buffer were determined at atmospheric pressure. Treatment at 200 MPa led to a slight *GP* decrease

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to 0.63 ± 0.00 and 0.61 ± 0.01 , respectively, while decompression brought the *GP* value back to its initial value. Compression up to 40 min did not lead to different observations with a steady *GP* under HP reversibly returning to the initial higher values following decompression. The *GP* value post decompression also showed no significant changes up to 20 min.

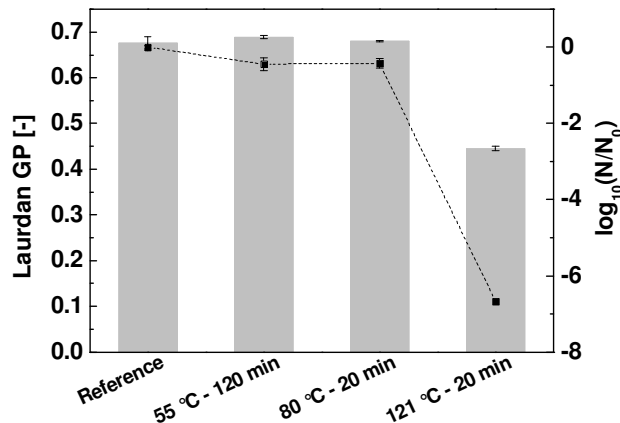


Figure 4.4: Impact of various thermal treatments on *G. stearothermophilus* spore inner membrane state (Laurdan *GP*, bars) and correspondence to inactivation (\log_{10} reduction, squares). Standard deviations were obtained from 3 Laurdan *GP* measurements. For *GP*: OD adjusted to 0.5, excitation 360 nm, samples were cooled down to room temperature prior to *GP* acquisition. Spore count, reference population N_0 : 7.5×10^8 CFU/mL. Detection limit: $-7.2 \log_{10}$.

In both cases, the changes observed under pressure were fully reversible. The reduction of the Laurdan *GP* corresponds to a slight red shift which is usually associated to an increase of the fluidity. However, this change being quasi instantaneous under pressure, reversible and very small, it could not be attributed to a state change of the inner membrane (i.e., fluidization) upon germination since this phenomenon takes place only during the phase II of germination – a few minutes after the germination trigger. The high values of Laurdan *GP* measured under pressure indicate the retention of the ordered gel-like phase status in agreement with results obtained for Clostridial spores elsewhere (Hofstetter et al., 2013), where it was noticed that the transition to the fluid state of lipid membranes observed under high temperature (90 °C) seemed to be counteracted by pressure of 200 MPa. It remains unclear, however, what might have led to the slight reversible Laurdan *GP* reduction under compression and to which extent this might be associated with the trigger of the germination. It might be due to minor changes in the membrane's lateral organization, only, leading to a slightly different packing of the lipid membrane upon increasing the pressure to 200 MPa. The

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appearance of different high-pressure gel phases has in fact been reported for model biomembrane systems (Winter, 2001; Winter and Czeslik, 2000). The addition of proteins in these model membranes further modifies the phases co-existence and phase transition regions, and it was concluded that not only the lipid bilayer structure and T , p -dependent phase behavior depends on the protein, but also the protein's conformation (and thus function) can be considerably influenced by the lipid environment (Zein and Winter, 2000). Typically, no pressure-induced unfolding of polypeptides was observed up to 1000 MPa in Zein and Winter's work. However, for large integral and peripheral proteins, pressure-induced changes in the membrane physical state may lead to a weakening of protein-lipid interactions and to protein dissociation (Winter and Jeworrek, 2009) and thus, altered functionality.

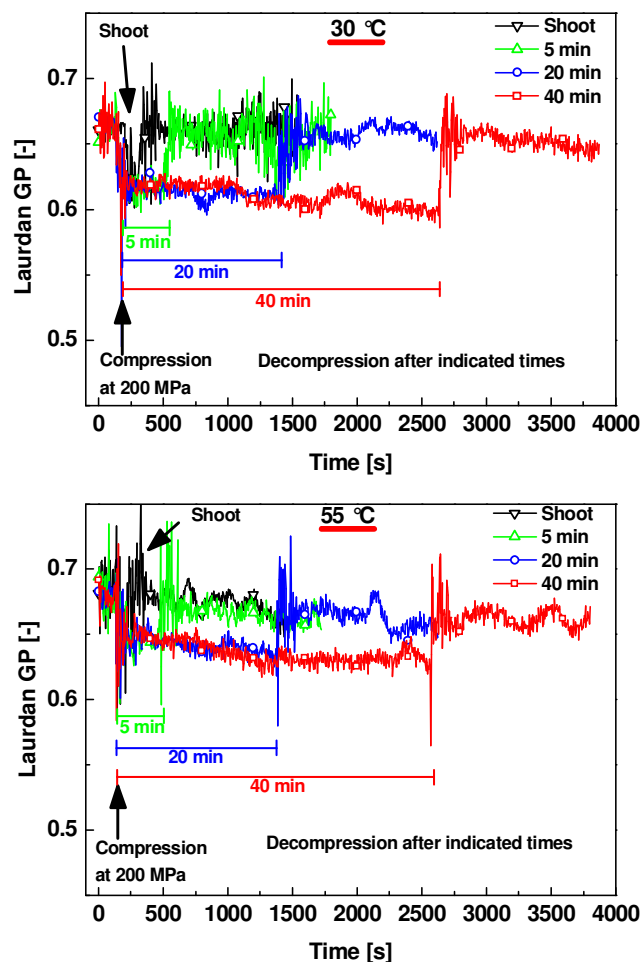


Figure 4.5: GP index of *G. stearothermophilus* spores stained with Laurdan and compressed at 200 MPa for 1 s (shoot), 5, 20 and 40 min at 30 °C (above) and 55 °C (below), respectively.

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Furthermore, it can be hypothesized that the high pressure of 200 MPa applied on the inner membrane of the bacterial spores induced a reversible minor modification of the lipid phase and possibly of protein conformations in and at the periphery of the inner membrane. Such a reversible conformational change could be associated with an activation of the germinant receptors under low high pressure (Figure 4.6). Germination under pressure by activation of the nutrient receptors localized in the inner membrane has been shown before but the nature of the changes occurring could not be determined so far, notably due to the difficulty to isolate nutrient receptors at sufficient concentration for *ex situ* investigations. The results in Figure 4.5 would hint towards a reversible conformational change in the (proteins of the) inner membrane leading to germination under pressure.

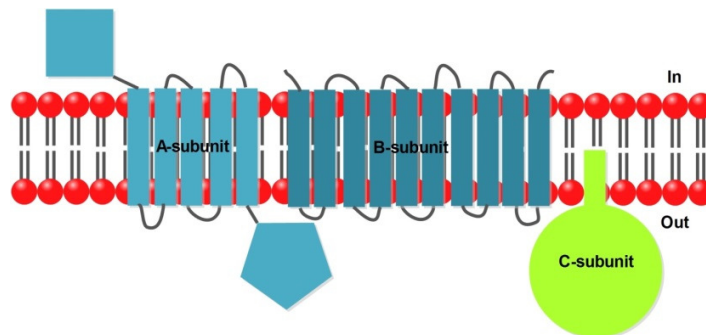


Figure 4.6: Schematic representation of the three proteins that constitute a typical GerA-type receptor in the inner membrane (Adapted from Christie (2012)). The presence of several domains at the periphery of the membrane might expose them to a reversible conformation modification under HP up to 200 MPa.

The absence of changes in Laurdan *GP* following decompression indicates that the impact of the HP treatment, though leading to minimal inactivation, is different from autoclave inactivation since it could be shown that autoclaved spores exhibit a much lower Laurdan *GP* (Figure 4.4). This observation and the results in Figure 4.3 support the hypothesis of a two-step mechanism consisting of germination followed by inactivation, most likely due to an impact on the physiological fitness of spores. The same absence of changes in Laurdan *GP* following decompression also raised the question of whether the phase II of germination had been completed as the cortex degradation is a necessary step to the inner membrane extension which takes place only during the phase II (Paidhungat and Setlow, 2002). Completion of germination would lead to an increase in the inner

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membrane fluidity as well as a surface extension up to 2 fold (Cowan et al., 2004), and an increase in the inner membrane permeability which could be expected to impact the Laurdan *GP* values.

In the work of Hofstetter et al., *in situ GP* measurements of Clostridial endospores heated to 90 °C at 200 MPa highlighted an antagonistic effect of pressure on the fluidization of membranes and a high degree of order was maintained (Hofstetter et al., 2013). No information was given on the corresponding inactivation / germination impact on the spore populations investigated in this pressure range. One can nonetheless suspect that 90 °C was too high to trigger significant germination.

4.3.2.4 FT-IR measurements in situ under pressure and/or at 55 °C

Both the lipid and protein responses under pressure and following decompression were monitored *in situ* in *G. stearothermophilus* spores using FT-IR spectroscopy. This allowed complementary analyses to the ones made by fluorescence spectroscopy. Notably, analysis of the amide I' band enabled *in situ* tracking of the changes of the spore proteins' secondary structures. Since *G. stearothermophilus* spores showed the highest germination under pressure at 55 °C, this temperature was chosen for the FT-IR spectroscopic investigation. FT-IR spectra were first measured at 55 °C in D₂O under atmospheric pressure. A very good signal was obtained (Figure 7) for the asymmetric and symmetric -CH₃ and -CH₂ lipid stretching vibrations (Kapoor et al., 2011). From Figure 4.7, it can be seen that no significant changes took place with respect to the -CH₂ and -CH₃ asymmetric stretching bands up to 40 min (signal at 2930 and 2960 cm⁻¹ respectively), suggesting an absence of a temperature effect alone on the lipid structures over prolonged times. Similarly, the amide I' band (1700-1600 cm⁻¹) was monitored at 200 MPa, 55 °C for 40 min dwell time (Figure 4.8). Again, no significant changes with time at this temperature could be noticed.

Comparatively, in Figure 4.9, spectra under pressure for 40 min dwell time showed reversible modifications in the -CH₃ and -CH₂ asymmetric stretching bands and small irreversible changes in the amide I' band. In Figure 4.9, a broadening and a slight reversible shift towards higher wavenumbers of the asymmetric and symmetric -CH₃ and -CH₂ bands is visible.

The shift of the lipid stretching vibrational bands under pressure towards higher wavenumbers was expected due to the pressure-induced ordering and denser packing of lipid molecules within the membrane, leading to stronger inter-CH repulsion between the neighboring -CH₂/-CH₃ groups in lipid chains (Kapoor et al., 2011). However, the broadening of the same bands under pressure indicates co-existence of various conformations, implying co-existence of different pressure-induced

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phases in the membrane of the bacterial spores. This innocuous suggestion would be in agreement with the *GP* values and also suggests the plausible induction of different gel-phases in the membrane (Figure 4.9). The reversible nature of the changes observed also matched the ones observed via the Laurdan *GP* method. Interestingly, in both cases, the release of pressure did not trigger any further changes than a return to initial conditions. No additional modification of the membrane structure could be monitored, also confirming the Laurdan *GP* results, while plate counts highlighted a significant germination/inactivation under this pressure/temperature/time condition.

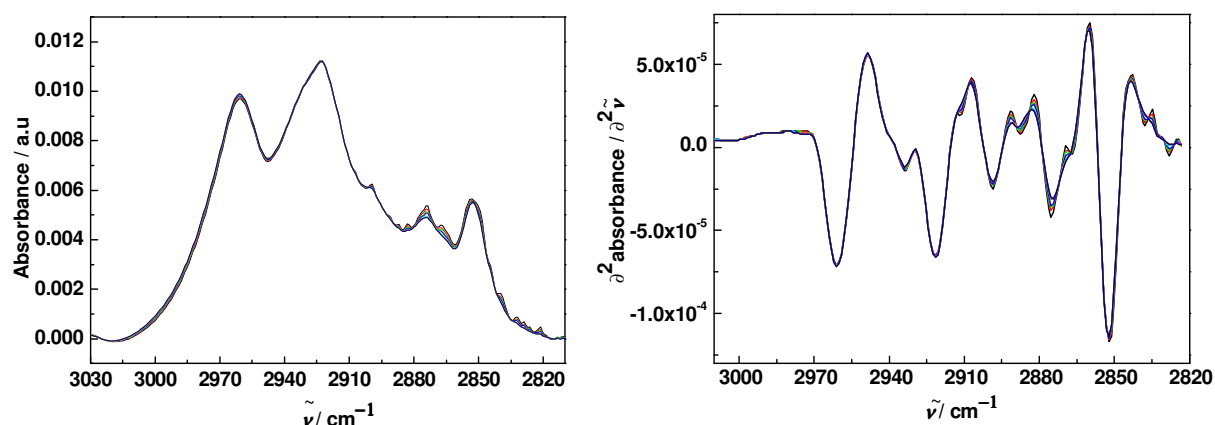


Figure 4.7: Asymmetric and symmetric -CH₃ and -CH₂ stretching bands of *G. stearothermophilus* spores suspended in D₂O at 55 °C up to 40 min at atmospheric pressure, one spectra every 5 min. Spectra baselined and normalized (left) – Second derivative (right).

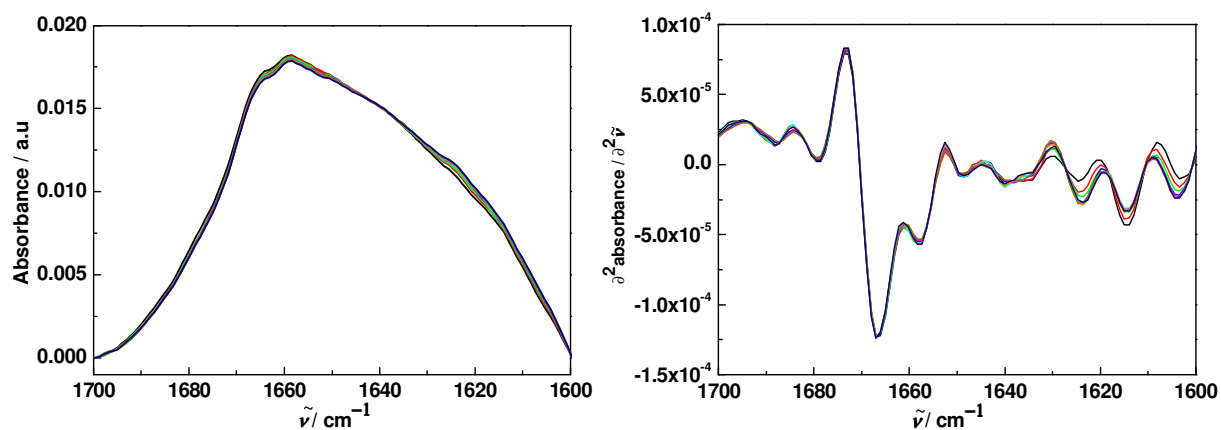


Figure 4.8: Amide I' IR bands of *G. stearothermophilus* spores at 55 °C suspended in D₂O up to 40 min at atmospheric pressure, one spectrum every 5 min. Spectra baselined and normalized (left) – Second derivative (right).

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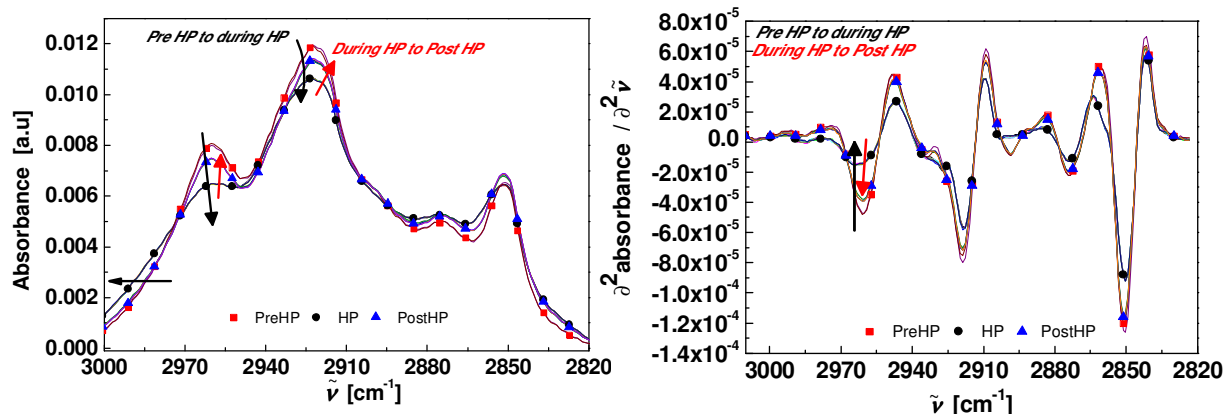


Figure 4.9: Asymmetric and symmetric $-\text{CH}_2$ and $-\text{CH}_3$ stretching bands of *G. stearothermophilus* spores before, during and following compression at 200 MPa for 40 min at 55 °C. Spectra baselined and normalized (left) – Second derivative (right).

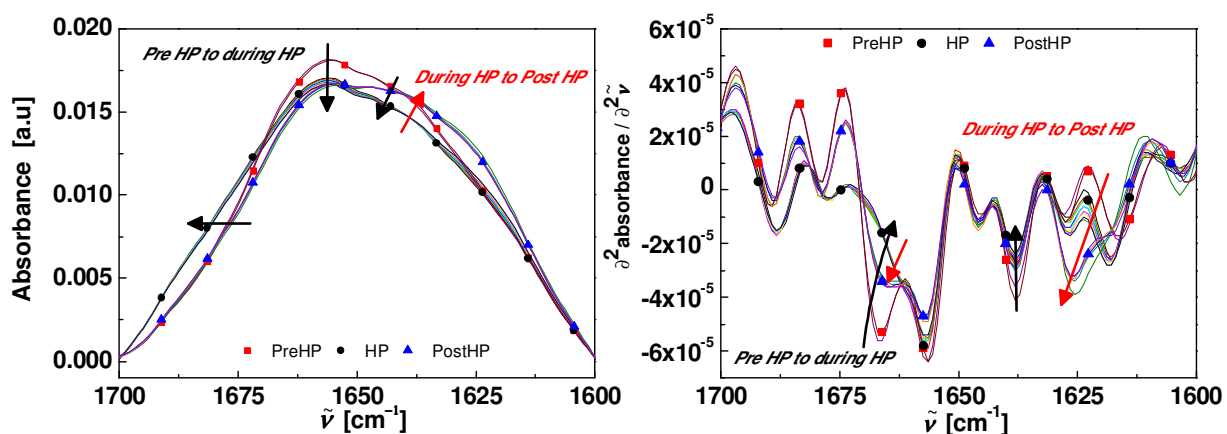


Figure 4.10: Amide I' IR band of *G. stearothermophilus* spores before, during and following compression at 200 MPa for 40 min at 55 °C. Spectra baselined and normalized (left) – Second derivative (right).

The amide I' band region at ambient pressure and 55 °C displayed predominant amounts of ordered secondary structural elements such as helices (appearing at $\sim 1655 \pm 5 \text{ cm}^{-1}$) and intramolecular β -sheets (at about $1635 \pm 5 \text{ cm}^{-1}$). Upon instantaneous application of hydrostatic pressure (200 MPa), some reduction of helical and intramolecular β -sheet structures took place, depicted by a reduction in the amide I' subband intensities in those wavenumber regions. This was coupled with a concomitant increase in the intensity at low wavenumbers ($\sim 1620 \text{ cm}^{-1}$) with time, suggesting

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formation of solvent exposed structural motifs, especially (possibly aggregated) intermolecular beta sheets (aggregated structures are generally prone to be induced at high temperatures), along with an increase in turns and loops (at $\sim 1650\text{-}1670\text{ cm}^{-1}$). All in all, Figure 4.10 indicates that the interplay of temperature and pressure induced an increased solvation of secondary structural motifs, especially of beta sheets. The absence of a massive broadening of the amide I' band region implied a high thermal stability of the spore proteins, at least up to 55 °C. However, some aggregation seemed still to take place, the aggregation being largely suppressed by high pressure, however. Upon decompression, the hydration of the secondary structural motifs was reduced again, resulting in a marked increase of less solvated intermolecular beta sheet structures (at $\sim 1626\text{ cm}^{-1}$, $+81.5\% \pm 4.2\%$). The level of intramolecular beta sheets (at $1635 \pm 5\text{ cm}^{-1}$) increased after decompression ($+8.9\% \pm 1.8\%$), but remained still less compared to the pre-HP conditions ($-5.5\% \pm 1.8\%$). Overall, following decompression at 55 °C, random structures ($1645 \pm 4\text{ cm}^{-1}$) appeared to have slightly increased ($+12.7\% \pm 2.6\%$), pressure-induced solvent exposed structures converted to less solvated beta sheets and aggregated structures (1620 cm^{-1}), and the helical as well as turn and loop structures decreased ($-23.3\% \pm 4.0\%$). This resulted in a band shape that was broader at the beta sheet/random coil regions and narrower at the turn and loop regions. This trend is more clearly seen by following the changes in the second derivative spectra (right side) during the compression and decompression cycles. Overall, it can nonetheless be concluded that the pressure and temperature induced changes affecting the proteins' secondary structures appeared to have been minor, suggesting that the majority of the spore's proteins were quite stable with respect to the treatment applied.

For all trials conducted under pressure, it was observed that no drastic changes in the FT-IR or Laurdan *GP* signal occurred during the different treatment phases (i.e., during compression and decompression, respectively). The changes monitored upon compression or decompression were likely due to minor structural rearrangements enabling an overall lower free energy state and satisfying Le Chatelier's principle, i.e. end up in an overall smaller volume of the system at high pressure conditions. Notably, although significant germination could be triggered, no drastic structural changes (i.e., changes in the packing and hydration of the inner membrane, degradation of proteins) during phase II of germination was detected by the methods implemented. Remarkably, the changes observed were, in the case of lipids, fully reversible regardless of the duration of the compression or temperature of the treatment.

To date, only a few studies have conducted FT-IR investigations of bacterial spores and none were found by the authors on *in situ* monitoring of HP triggered germination. Two studies reported the

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use of FT-IR spectroscopy to investigate pressure assisted thermal inactivation (PATS) of spores (Subramanian et al., 2006; Subramanian et al., 2007). Significant changes in secondary structure of proteins were observed which were associated with denaturation and aggregation of protein and correlated with bacterial spore inactivation under PATS (121 °C at 700 MPa). Interestingly, the authors also found that most structural changes taking place during the treatment applied happened initially (before the set temperature and final pressure were attained), which is consistent with the observations made in this work. Additionally, the different and minor structural changes observed suggest that inactivation results more likely from a different mechanism than what was observed during PATS, and would support the hypothesis of a physiological-like germination followed by a loss of physiological fitness.

Investigations of chemical germination of *B. subtilis* by L-alanine using FT-IR spectroscopy (Cheung et al., 1999) can also be compared to our results. The authors reported a degradation of spore proteins during germination beginning immediately following the initiation of germination, and approximately 26 % of the spore protein was hydrolyzed in the first 15 min in wild-type spores. Their results match other authors' observations that about 15-20 % of the total protein in spores is degraded in the first 20 min of nutrient germination (Setlow, 1983). In contrast, the changes associated with HP-induced germination reported here are of different nature. Partial protein disordering and aggregation was observed, only. Changes in lipid packing upon compression and decompression or by temperature changes were fully reversible in the temperature and pressure range covered.

4.4 Conclusions

In this work, germination and inactivation of *G. stearothermophilus* spores under pressure was investigated at 200 MPa and various temperatures, using *ex situ* and *in situ* analytics. Partial inactivation (up to 1 log₁₀) could be achieved during the treatment itself, but comparison with other studies revealed that the structural changes are different to those observed during PATS (Subramanian et al., 2007). In this study, inactivation most likely resulted from a combination of germination and loss of physiological fitness taking place during prolonged pressure exposure or the pasteurization step post HP. Monitoring of the inner membrane's lipid state through Laurdan *GP* as well as secondary protein structural changes recorded by FT-IR spectroscopy suggest reversible or minor changes affecting the overall spore structure under low high pressure conditions (up to 200 MPa), including the induction of a different ordered gel-like lipid phase of the inner membrane.

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Additionally, a key impact of temperature could be identified: a temperature of 55 °C appeared to increase significantly germination under pressure. These results strongly support the hypothesis of a pressure-induced physiological-like germination through reversible structural changes occurring in the inner spore membrane. The pressure-induced formation of a gel phase with different packing properties and lateral organization could contribute to the activation of germinant receptors which are known to consist of several subunits. The pressure range covered is consistent with the pressure range where reversible changes in conformational properties of membrane proteins have in fact been observed in reconstituted membrane systems (Winter and Jeworrek, 2009).

The reason why no further structural changes in lipids and proteins were monitored post decompression is still open for investigation and could be linked to either incompleteness of the phase II of germination which includes expansion of the inner membrane and degradation of the small acid-soluble proteins.

5. Preface to the publication “*Geobacillus stearothermophilus* ATCC 7953 spore chemical germination mechanisms in model systems”

Following the investigation of spore germination in the moderate high pressure domain, it was concluded that pressure alone, when used in the physiological like domain of spore germination, could not allow full germination. The *in situ* investigation showed changes occurring in the inner lipid membrane which could in all likelihood affect the conformation of the nutrient receptors present in this membrane. The absence of complete germination could then be associated with either a randomly occurring absence of proper reconfiguration of these receptors, thus failing at triggering germination or an absence (or reduced number) of a particular (set of) pressure activated receptor(s).

However, if combined to an additional hurdle such as a chemical germinant, which could further germinate spores which were not germinated by high pressure, the fraction of germinated spores might become sufficiently large to allow for potential food application.

Chemical germination of spores has been significantly investigated in the literature as described in the following section. However, little data is available on the indicator used in this work. Furthermore, most studies focused on achieving the first log of germination which only covers 90 % of the spore population and is thus of little interest for food applications.

In the following work, a large screening of substances which have been reported previously as nutrient germinant or chemical germinant has been conducted on *G. stearothermophilus* ATCC 7953 spores. The mechanisms of germination were further investigated *in situ* for the most successful candidate, and using the toolbox developed in the previous section.

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6. *Geobacillus stearothermophilus* ATCC 7953 spore chemical germination mechanisms in model systems

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Food Control, 50, p.141-149, 2015 – with permission from Elsevier

Abstract

Bacterial endospores through their strong resistance to both chemical and physical hurdles constitute a risk for food industry. Inactivation strategies are based on thermal and/or chemical treatments but rely on incomplete knowledge of the mechanisms of inactivation. Alternative strategies were suggested to achieve food safety while improving product quality. One of them relies on the successive germination and inactivation by pasteurization of bacterial spores. However, to date, a gap of knowledge on bacterial spore germination remains and hinders such an application for food sterilization. *Geobacillus stearothermophilus* ATCC 7953 spore germination mechanisms were investigated by *in situ* fluorometry combined with plate counts. *G. stearothermophilus* spores' inner membrane was stained with Laurdan fluorescent dye. While nutrient pathways showed no strong germination with the combinations tested, successful germination up to 3 log₁₀ could be achieved using 60 mmol.l⁻¹ calcium-dipicolinic acid (CaDPA) at 55 °C for 2 h. A model for the CaDPA germination mechanism in two phases could be derived which suggested a potential key role of cortex fragments in the germination path, before completion of the cortex degradation. Additionally, it was confirmed that the germination potential of CaDPA, which does not rely on nutrient receptors, is a widespread germination trigger across spore formers. Understanding germination mechanisms and the limitations of different germination paths is important for the development of multi-hurdle approaches to achieve commercial sterility with reduced thermal load.

6.1 Introduction

Many members of the gram positive bacterial orders are able to survive starvation by forming dormant, resistant spores. Bacterial endospores through their strong resistance to both chemical and physical hurdles constitute a risk for the food industry (Esty and Meyer, 1922; Heinz and Knorr, 1996; Sale et al., 1970; Setlow, 2000, 2006). Inactivation strategies for food and equipment surfaces have been based on thermal and chemical treatments but rely mostly on incomplete knowledge of the mechanisms at stake, leading to over processing and/or low product quality (Georget et al., 2013a). Alternative hurdles have been suggested to achieve food safety while improving product quality, which do not solely depend on very strong thermal treatments (Knorr and Heinz, 2001). One of them is based on germination and successive inactivation by pasteurization of bacterial spores (Gould, 2006). Even being dormant, these spores constantly monitor their environment and under conditions advantageous for growth, in particular the presence of nutrients, they can germinate, outgrow and ultimately become growing vegetative cells (Setlow, 2003). Germination is an early and important step in this process and is of high interest since it weakens the bacterial spore to pasteurization treatments through core partial rehydration. Unfortunately, the inability to stimulate full germination of spores is still limiting the implementation of this approach for food sterilization. In particular, the incomplete knowledge of the pathways at stake during germination has been mentioned (Abel-Santos, 2012) and hinders the validation of this approach for the food industry (Mathys, 2008; Reineke, 2012).

Nutrient germinants of low molecular weight include amino acids, purine derivatives, and sugars. These nutrient germinants usually trigger the germination of bacterial spores in the environment through what is speculated to be stereospecific binding to nutrient receptors localized on the inner spore membrane (Paidhungat and Setlow, 2000; Paredes-Sabja et al., 2011). Most commonly, L-Alanine in combination with *Bacillus subtilis* spores has been extensively investigated (Stewart et al., 1981). There is some evidence that specific spore coat proteins make it easy for such exogenous germinants to pass through the outer layers of *Bacillus* spores, but how this is accomplished is not known (Behravan et al., 2000; Carr et al., 2010). This germination path is mostly referred to as nutrient or physiological germination. To date, the best established fact is that the initial interaction of the germinant with the spore forms the trigger reaction and irreversibly commits the spore to undergo the complex series of germination events (Setlow, 2003; Stewart et al., 1981). Moreover, sub lethal heat treatments also were suggested to enable what was described as “heat activation” which increases the rate and extent of nutrient germination (Curran and Evans, 1945; Finley and

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Fields, 1962; Stewart et al., 1981). While activation is seen as a reversible process (Keynan et al., 1964), the mechanism of spore activation is still not fully understood (Aiba and Toda, 1966; Paidhungat and Setlow, 2002). Most clearly, the inner spore membrane appears as one of the key spore structures where the current knowledge gap is critical. The inner membrane of bacterial spores was reported to have a composition very close to the cytoplasmic membranes of growing cells (Cortezzo and Setlow, 2005) where the proportion of proteins is expected to be between 20 % and 35 % by comparison to the Fluid Mosaic Model (Singer and Nicolson, 1972; Stevens and Arkin, 2000). These proteins are localized in a lipid environment and the inner membrane carries a set of unique proteins, including the nutrient receptors and proteins, such as those encoded by the *spoVA* operon, possibly involved in movement of core molecules across the inner membrane. As these proteins function while being localized in the inner membrane, they are expected to be largely immobile in this environment (Setlow, 2008b). It was indeed suggested that the unusual highly compact and impermeable state of this membrane impacts on the proteins' mobility as well as functionality (Cowan et al., 2003; Cowan et al., 2004). However, *in situ* studies are still very scarce. Non-nutrient germination pathways also exist which do not require nutrient receptors. The best characterized is exogenous dipicolinic acid and its 1:1 calcium chelate (CaDPA), a substance naturally present in the bacterial spore core and responsible for the low water content of the spore core as well as the resulting wet heat resistance (Paidhungat and Setlow, 2000). It is believed that CaDPA triggers spore germination by a process that does not require nutrient receptors but instead activates germination in *B. subtilis* spores by activation of the cortex lytic enzyme CwlJ which initiate cortex degradation (Cheung et al., 1982; Ghosh and Setlow, 2010). Cortex hydrolysis has been suggested to lead to a reduction of the pressure on the inner-membrane and spore core, leading to the release of ions, CaDPA and full core hydration (Setlow, 2003). However, this hypothesis could to date not be verified. Moreover, later work showed that vegetative cell wall peptidoglycan fragments at concentration inferior to 1 pg.mL⁻¹ could also trigger spore germination by binding to an inner membrane-bound protein kinase (Shah et al., 2008; Wei et al., 2010). This led to wonder whether the initiation of cortex degradation might trigger germination through activation by the resulting peptidoglycan fragments, before mechanical rehydration occurs due to full cortex degradation (Setlow, 2008a).

A literature overview has finally shown that in recent times, little attention has been given to the investigation of *G. stearothermophilus* spore germination mechanisms (Campbell and Williams, 1953; Cheung et al., 1982; O'Brien and Campbell, 1957). Most of the studies on going in this field have focused on different strains and thus, the current state of the art in terms of mechanistic

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studies of spore germination was related to spores of *Bacillus subtilis*, *Bacillus cereus*, *Bacillus megaterium* or spores the *Clostridium* genus (Nerandzic and Donskey, 2013; Paredes-Sabja et al., 2011; Pelczar and Setlow, 2008; Ramirez and Abel-Santos, 2010). Some of these studies in particular suggested the use of combined germination and decontamination either through antimicrobials or pasteurization as a mean of sterilization (Nerandzic and Donskey, 2013; Worthington, 2011). In order to validate such approaches, a sufficiently universal substance needs to be identified in order to eradicate a large spectrum of spores of different strains and families.

With regard to the above introduced facts, further investigations of the mechanisms of spore germination are needed to understand and control this process. Applications of this knowledge could be to heat-sensitize spores in food matrices or, conversely, to prevent germination to take place, targeting the control of spores rather than inactivation. In this work, chemical germination of *Geobacillus stearothermophilus* ATCC 7953 spores, an industrial thermal sterilization indicator (European Pharmacopoeia Commission, 1997), was investigated at 55 °C (optimal growth temperature of the indicator) by nutrient and non-nutrient chemicals. A series of *in* and *ex situ* analyses previously used for this bacterium (Georget et al., 2014a) was applied to achieve the *in situ* analysis of the inner membrane, a key player in germination pathways, during germination.

6.2 Material and methods

6.2.1 Bacterial strain

The strain used in this work was *G. stearothermophilus* ATCC 7953 obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), and stored at -80 °C at the German Institute of Food Technologies (DIL, Quakenbrueck, Germany).

6.2.2 Sporulation method

Sporulation of *G. stearothermophilus* was achieved in less than a week following the method described elsewhere (Georget et al., 2014a). A single colony of *G. stearothermophilus* grown on nutrient agar over night was used to inoculate 10 mL of TSB medium (Tryptic Soy Broth Fluka T8907-500G from Sigma-Aldrich Company, Germany - 30 g.L⁻¹ in distilled water). The TSB cultures were incubated at 55 °C and 250 rpm in a shaking incubator for a minimum of 5 h. After 5 h, the OD₆₀₀ was checked every 30 min until the required density was reached (1.6-1.8 / control with cell density meter Ultraspec 10 from Amersham Biosciences GmbH, Germany). A 200 µL aliquot of the culture was then spread onto Difco sporulation plates (Schaeffer et al., 1965). The plates were

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sealed in plastic bags and incubated at 55 °C for a minimum of three days. Sporulation was monitored by controlling the phase brightness with a transmitted-light microscope daily. When more than 95 % sporulation was observed by Thoma chamber, plates were removed from the bags and left at room temperature for drying. The plates were monitored under phase contrast microscope each day until remaining vegetative cells were dried out; following which the spores were collected using 4 °C distilled water and cleaned by repeated centrifugation at 4800 g, 4 °C for 30 min until the supernatant was clean. Following cleaning, the spores were suspended in distilled water and kept at -20 °C until further use within a time window of six months.

6.2.3 Laurdan staining

Lipid packing/order can be quantified by fluorometry of an environment-sensitive fluorophore such as 6-Dodecanoyl-N,N-dimethyl-2-naphthylamine (Laurdan) (Molina-Höppner et al., 2004; Sezgin et al., 2012). The fluorescent naphthalene moiety of the Laurdan molecule possesses an electrical dipole moment, which increases upon excitation, and may cause reorientation of the surrounding solvent dipoles. Solvent reorientation results in a decrease of the excited state energy of the probe, which is reflected in a red shift of the probe's emission spectrum (Sánchez et al., 2007).

Based on an adaptation of the method developed previously (Hofstetter et al., 2012; Hofstetter, 2012), Laurdan fluorescent staining of *G. stearothermophilus* spores' inner membrane was achieved with the sporulation method described above and as explained elsewhere (Georget et al., 2014a). Laurdan (Sigma Aldrich, Hamburg, Germany) was selected as fluorescent dye because of its non-cytotoxicity (Owen et al., 2012), enabling further evaluation of the physiological state of spores by plate count, for instance.

A single batch of stained spores of *G. stearothermophilus* was used for this study. Using a Thoma chamber, the spore percentage was estimated to be 94.1 % \pm 1.5 % (average of four counts and corresponding standard deviation). Additionally, and to establish a reference in terms of membrane fluidity, vegetative cells of *G. stearothermophilus* were stained with Laurdan adapting a method described elsewhere (Molina-Höppner et al., 2004). Overnight cell culture in TSB broth were centrifuged at 2700 g for 5 min and suspended in sterile 0.9 % saline twice. Laurdan suspended in ethanol was added to the cell suspension to a final concentration of 40 μ M. The staining was conducted for 30 min at 55 °C in the dark following which cells were washed twice by centrifuging at 2700 g for 5 min and finally re-suspended in sterile saline 0.9 %.

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6.2.4 Chemical germination

6.2.4.1 Nutrient germination

Previous genomic analyses underlined the presence of homologs of the *B. subtilis* germinant receptors genes as well as *gerD* and *spoVAB, C, D* genes in other strains of the *Geobacillus* genus, thus suggesting potential for germination of *G. stearothermophilus* spores with L-Alanine or AGFK (Paredes-Sabja et al., 2011; Pelczar and Setlow, 2008).

Therefore, the potential of individual amino acids to trigger *G. stearothermophilus* spore germination was assessed. 16 L-amino acids (L-Alanine, L-Aspartic acid, obtained from Fluka, Sigma Aldrich, Hamburg, Germany and L-Leucine, L-Arginine, L-Lysine, Glycine, L-Histidine, L-Methionine, L-Proline, L-Threonine, L-Isoleucine, L-Tryptophan, L-Tyrosine, L-Glutamine, L-Valine, L-Phenylalanine, obtained from Carl Roth, Karlsruhe, Germany) were diluted individually in Phosphate buffer (PBS), pH 7 at a concentration of 50 mmol.l⁻¹; inosine at a concentration of 5 mmol.l⁻¹, and a sample containing PBS buffer only was used as a reference. These high concentrations were selected to avoid the absence of germination due to a lack of germinant in the medium. An incubator (Eppendorf Thermomixer comfort, Eppendorf AG, Hamburg, Germany) was used for the heat activation of the spores, the germination, and subsequent inactivation at 80 °C. A suspension of spores was distributed between two falcon tubes, one was placed on ice, and the second one exposed to the heat activation at 80 °C during 30 min. After heat activation, the sample was placed on ice 15 min for cooling and enumerated by plate count. A maximum reduction of 0.5 log₁₀ by comparison to the initial count was detected which was within the precision of the method. Heat activated and non-heat activated spore suspensions were distributed between the Eppendorf tubes, centrifuged at 4800 g and 4 °C for 5 min and re-suspended each with the respective nutrient solutions to be tested. All samples were germinated for 2 h at 55 °C. During germination, the Eppendorf tubes were shaken to prevent sedimentation. After germination, the CFU.mL⁻¹ for each sample was determined by plate count before and after heat inactivation at 80 °C for 20 min to quantify the germinated population.

Furthermore, additional experiments were conducted with combined L-Alanine and inosine, previously underlined as co-germinant (Dodatko et al., 2009; Luu et al., 2011; Pinzón-Arango et al., 2010), as well as with AGFK which consisted of 100 mmol.l⁻¹ L-Asparagine, 10 mmol.l⁻¹ D-glucose-monohydrate, 10 mmol.l⁻¹ D-fructose and 50 mmol.l⁻¹ KCL (AGFK) in PBS buffer, pH 7 (Cabrera-Martinez et al., 2003; Paredes-Sabja et al., 2008; Pelczar et al., 2007; Ramirez and Abel-Santos, 2010; Wuytack et al., 2000).

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For L-Alanine and inosine, different heat activation temperatures and incubation durations were tested (80 or 100 °C) taking over early work on *G. stearothermophilus* spores (Finley and Fields, 1962; Foerster, 1983) and incubation up to 24 h. In all cases, germination was assessed by the plate count method with or without heat inactivation post germination and by the measure of the optic density (OD) during the germination. For OD controls, the trials were conducted in reagent tubes with the spore suspension set at OD ~1 in the preheated nutrient solutions. All samples were germinated during 2 h at 55 °C and the OD of samples was measured at 600 nm along time (Ultrspec 10 Cell Density Meter, classic, GE Healthcare, Germany).

6.2.4.2 Non nutrient germination by dipicolinic acid and its chelates

CaDPA, Na₂DPA and pure DPA were considered as potential germinant for *G. stearothermophilus* spores following suggestions of previous studies (Fields and Frank, 1969; Perez-Valdespino et al., 2013).

DPA (Sigma-Aldrich, Hamburg, Germany) and chloride salts (CaCl₂, NaCl Carl Roth, Karlsruhe, Germany) were diluted with Tris buffer at a concentration of 60 mmol.l⁻¹ and adjusted to the pH level 8.0 following the optimized conditions described elsewhere (Ghosh and Setlow, 2010; Gould, 1971; Magge et al., 2008). The potential effect of heat activation was evaluated for each condition by treating in parallel non-heat activated spores and spore suspensions that had been heated at 80 °C for 30 min and cooled down on ice. Heat activated and non-heat activated spore suspension were centrifuged at 4800 g, 4 °C for 5 min and re-suspend with the 60 mmol.l⁻¹ DPA chelate solutions tested. All samples were germinated during 2 h at 55 °C and were plated before and after heat inactivation of germinated spores at 80 °C for 20 min.

The CaDPA germination kinetic was determined through heat inactivation after different incubation times. In order to determine the kinetic of phase I germination by CaDPA as suggested in the section 6.3 (CaDPA release and partial core hydration leading to heat sensitization resulting from activation by cortex fragments), a small sample (80 µL) was taken out of the spores' suspension at given time intervals and subjected to rapid inactivation at 80 °C during 20 minutes in preheated tubes and then cooled and kept in ice, with subsequent plate count. Additionally, the spores used for this work were Laurdan stained and monitoring the generalized polarization (*GP*) signal enabled to follow the second suggested phase of CaDPA germination (inner membrane extension upon completed cortex degradation). A sample was extracted from the experimental spore suspension at given time intervals for spectra acquisition.

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All graphical representations in this work were plotted with OriginPro (Version 8.0724, B724; OriginLab Corporation, Northampton, MA, USA). The bars in the figures represent the standard deviations associated to triplicates.

6.3 Calculation

6.3.1 Generalized Polarization

As introduced in the section 6.2.3, Laurdan emission spectra exhibits a red shift in the liquid-crystalline fluid-like phase of lipid membranes compared with the ordered gel phase which is characterized by a dehydration of the lipid's upper chain region, due to dense lipid packing. For Laurdan, these two extreme conditions of lipid bilayers have been studied in detail and the wavelength maxima for the two extremes were found to be at 440 nm for ordered gel phases and at 490 nm for disordered liquid-crystalline phases (Harris et al., 2002; Owen et al., 2012; Parasassi et al., 1991). Since wavelength displacements may be challenging to characterize only by peak wavelengths, the *GP* presents a more precise parameter to quantify the polarity change (i.e., shift of the emission maximum). The detailed concept of *GP* was introduced elsewhere (Parasassi et al., 1990) and the reader is referred to this work for additional details. *GP* is defined by (11) (Parasassi et al., 1991) and was used to characterize the lipid bilayer state in the bacterial spore membrane during the different treatments.

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \quad (11)$$

I_{440} and I_{490} correspond to the fluorescence emission intensities measured at 440 nm and 490 nm, respectively, following excitation at 360 nm. The *GP* values are limited between +1 and -1 but practically, values range from -0.3 to 0.6 (Hofstetter, 2012; Parasassi et al., 1998).

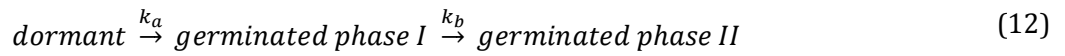
A FP-8500 Fluorescence Spectrometer (Jasco, Germany) was used in this study. Excitation light (at 360 nm) was obtained with a Xe lamp source and emission spectra collected from 400 to 550 nm. Laurdan-stained spores were suspended to a final OD_{600} of 0.5.

6.3.2 Modeling approach for CaDPA germination

A two phase kinetic model was tested to represent the CaDPA isothermal germination kinetics of *G. stearothermophilus* spores. The aim was to assess whether a full cortex degradation (and corresponding core and inner membrane extension) was occurring before or after the release of CaDPA and partial core hydration. In this model, the first step (termed phase I) corresponds to the

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release of CaDPA from the core and partial core hydration as could be assessed by inactivation at 80 °C 20 min post germination and plate counts. The second step (termed phase II) accounts for the full cortex degradation, resulting in a core swelling and inner membrane extension upon further hydration, as could be monitored through Laurdan *GP* kinetics. Although these two stages are by now accepted for nutrient induced germination, CaDPA germination (a non-nutrient triggered germination) might follow a different mechanism which has to date not been fully elucidated (Bassi et al., 2012). It was previously suggested that the cortex degradation might be an initiating stage leading to rehydration (Cowan et al., 2003) and the reverse hypothesis is being considered in this work. The objective was to derive and compare the different kinetic rates for these two phases and test whether the heat sensitization of spores occurs before or after the complete cortex degradation. Based on the *in* and *ex situ* data collected, a multi-response kinetic model was tested to derive a global model for CaDPA germination. The two step model in (12) was suggested, based on a mechanistic model for chemical reactions, and solved with a non-linear multi-response regression. The set of the differential equations used was (13), (14) and (15), with k_a and k_b as rate constants.



$$\frac{d[dormant]}{dt} = -k_a[dormant] \quad (13)$$

$$\frac{d[germinated\ I]}{dt} = k_a[dormant] - k_b[germinated\ I] \quad (14)$$

$$\frac{d[germinated\ II]}{dt} = k_b[germinated\ I] \quad (15)$$

The set of differential equations (13)–(15) was solved with the differential equation solver Berkeley Madonna (Version 8.0.1, R.I. Mackey & G.F. Oster, University of California at Berkeley, CA, USA) and the rate constants optimized based on the experimental data obtained from plate counts and Laurdan *GP*. The modeled equations and source data were plotted with OriginPro (Version 8.0724, B724; OriginLab Corporation, Northampton, MA, USA). The final form of the integrated differential set of equations can be seen in (16)–(18).

$$[dormant] = [dormant]_0 \cdot e^{-k_a t} \quad (16)$$

$$[germinated\ I] = \frac{k_a}{k_b - k_a} \cdot [dormant]_0 \cdot (e^{-k_a t} - e^{-k_b t}) \quad (17)$$

$$[germinated II] = [dormant]_0 \cdot \left[1 - \frac{k_b \cdot e^{-k_a t} - k_a \cdot e^{-k_b t}}{k_b - k_a} \right] \quad (18)$$

6.4 Results

6.4.1 Laurdan staining of *G. stearothermophilus* spores' inner membrane and impact on the heat resistance

Laurdan staining allowed for the assessment of the inner membrane state in the non-treated spores of *G. stearothermophilus*. It was achieved as described in previous work (Georget et al., 2014a). In this work, it was compared to the one of the vegetative cells of the same strain (Figure 6.1).

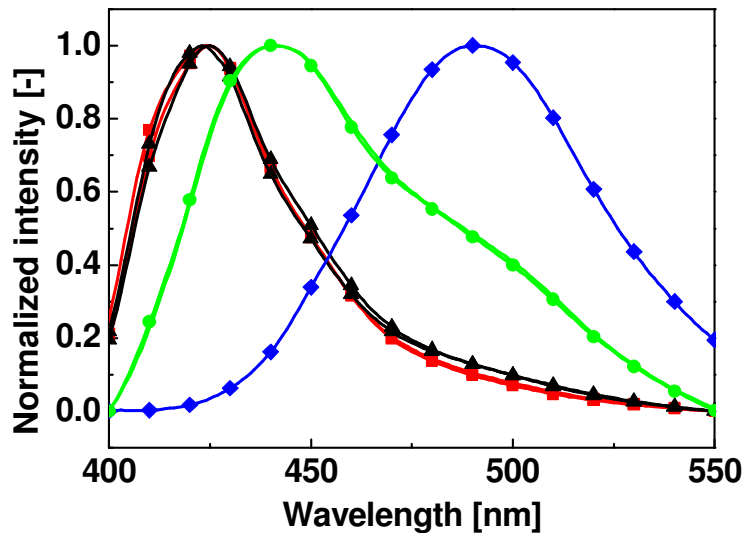


Figure 6.1: Fluorescent signal intensity in *G. stearothermophilus* spores pre- (■) and post-decoating (▲) (measurements in duplicate – two curves of the same color overlapping $GP = 0.68$). The OD was adjusted to 0.5 prior to the measurement. Laurdan spectra diluted 1:1000 after saturation in 100 % ethanol showed a maximum of emission at 490 nm as for Laurdan in a polar environment (◆) (Data from Georget, et al., 2014). Data compared to vegetative cells signal (●) corresponding to Laurdan in a lipid liquid crystalline membrane with emissions both at 440 and 490 nm – $GP = 0.31$.

To confirm the location of the stain in the inner membrane, decoating trials were conducted using an adaptation of a method described elsewhere (Fitz-James, 1971) with all incubations conducted at 55 °C. Decoating removes the outer layers of the spores including the outer membrane which would also be expected to contain Laurdan. To measure the efficiency of the decoating, the inactivation of decoated spores by a lysozyme treatment at 55 °C was assessed as described elsewhere (Klobutcher

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et al., 2006) and a complete inactivation of decoated spores compared to non-decoated spores was achieved. Finally, the spectra obtained for vegetative cells of *G. stearothermophilus* are also shown and give a benchmark for the liquid crystalline membrane of this organism (Figure 6.1).

Using equation (11), the Laurdan *GP* value calculated before and after decoating amounted to 0.68 and 0.68 respectively (average of two repetitions). *GP* of vegetative cells of *G. stearothermophilus* amounted to 0.31 which corresponds to *GP* expected in lipid liquid crystalline-like phases (ranges from -0.3 to +0.3). A comparison of the decimal reduction time (*D*) and *z* values of stained and non-stained spores of *G. stearothermophilus* at sterilization temperature was conducted using the capillary method as described elsewhere (Mathys, 2008). A linear Arrhenius-type equation was used to fit the experimental rate constants *k*(*T*) obtained at 110, 121 and 130 °C in equation (19) and derive the energy of activation *E_a*.

$$\ln k(T) = -\frac{E_a}{R \cdot T} + \ln k_0 \quad (19)$$

The aim was to ensure that the staining protocol applied did not compromise the spore characteristics, notably its inner membrane properties, which could have resulted in biased further results. The results presented in Table 6.1 show no major difference in the *D*, *z*-value and *E_a* between stained and non-stained spores and if any would only suggest a slight increased resistance of stained spores at higher temperature.

Table 6.1: Comparison of *D* and *z*-value of stained and non-stained spores of *G. stearothermophilus* at sterilization temperatures 121 and 130 °C (standard deviation under brackets)

| | T (°C) | 121 | 130 |
|--|-------------|--------------|------------|
| <i>D</i> (s) | Non stained | 89.7 (10.1) | 5.1 (1.5) |
| | Stained | 128.7 (33.4) | 10.5 (0.3) |
| <i>E_a</i> (kJ.mol ⁻¹) | Non stained | 386.4 (16.9) | |
| | Stained | 405.7 (40.6) | |
| <i>z</i> -value (°C) | Non stained | 7.6 (0.4) | |
| | Stained | 8.4 (1.4) | |

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6.4.2 Germination of *G. stearothermophilus* spores by single or combined nutrients

A broad range of individual L-amino acids and one purine base were tested for their potential to trigger nutrient germination of *G. stearothermophilus* spores. However, regardless of the use of heat activation or not, no individual amino acid or purine base tested had a significant advantage over the reference treatment (PBS buffer alone) (Figure A1 appendix). In the best case, between 0.5 and 1 log₁₀ germination could be achieved. Although significant from a microbiological perspective, these results remained largely insufficient for any food application and failed at underlining an individual component which could induce a full nutrient germination when included in a food matrix for instance. Combined L-Alanine and inosine at different heats of activation (80 °C or 100 °C) also showed no benefits in terms of germination and increase in the overall incubation duration up to 24 h also did not show any increased germination. The OD decrease was not different in the PBS reference and in the samples incubated with L-Alanine (with and without inosine) reached a maximum of approximately 25 % OD reduction which matches the <0.2 log₁₀ germination established by plate counts. Finally, germination with the AGFK mixture over 2 h led to a germination of <0.2 log₁₀ which was once more not sufficient for the potential application considered (data not shown).

6.4.3 Germination of *G. stearothermophilus* spores with DPA chelates

DPA chelates (Ca²⁺ and Na⁺) and pure DPA (60 mmol.l⁻¹) were prepared in Tris buffer pH 8.0, as described previously (Ghosh and Setlow, 2009; Paidhungat and Setlow, 2000). Figure 6.2 shows the results of germination with DPA and DPA chelates during 2 h. Germination with CaDPA and successive thermal inactivation gave the best results and reached about 3 log₁₀ spore reduction following inactivation of the heat sensitized population. The difference between heat activated and non-heat activated spores was not significant. CaDPA having shown the most complete germination, it was further investigated for the determination of germination kinetics as described in section 6.3. Figure 6.3 shows the normalized fluorescent signal intensity as function of the wavelength in nanometers. The highest intensity laid in the boundaries of 420 and 440 nm in agreement with previous data (Figure 6.1). Along with germination, the relative intensity at 490 nm increased in time and for easier data analysis, these results are presented in terms of Laurdan *GP* vs. time of incubation in Figure 6.4. Figure 6.4 presents the log₁₀-reduction corresponding to germinated population in time as well as the Laurdan *GP* value in time of the spore batch being germinated. In model lipid membranes, *GP* values, which are equal or higher than 0.5, correspond to a gel phase of

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the lipids in the inner membrane. Values which are below 0.3 imply a liquid crystalline phase (Sánchez et al., 2007). These values were used as benchmark to assess the changes observed in this work.

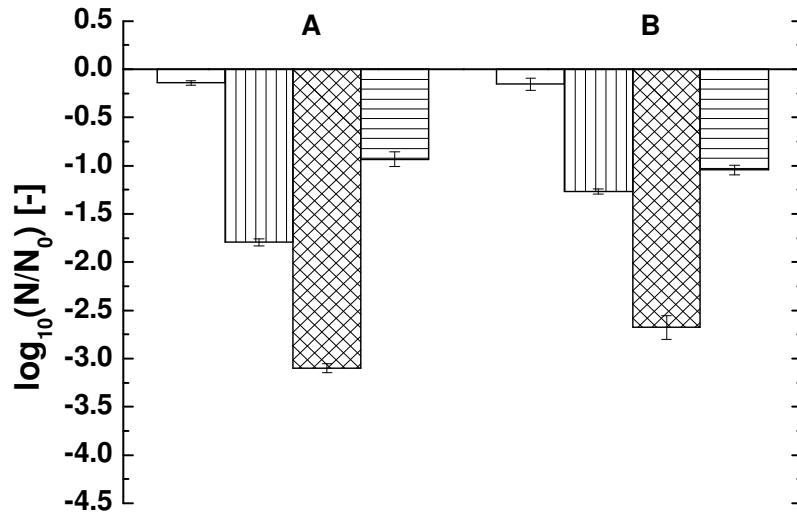


Figure 6.2: Germination with 60 mmol.l⁻¹ CaDPA [hatched], Na₂DPA [vertical lines], DPA [horizontal lines] and Tris reference trial [white] during 2 hours at 55 °C (initial concentration of spores: 10⁷ CFU.mL⁻¹) and heat inactivation step at 80 °C for 20 min. The logarithmic reduction shows the germinated heat sensitized spores. A: non-heat activated spores, B: heat activated spores at 80 °C for 30 min.

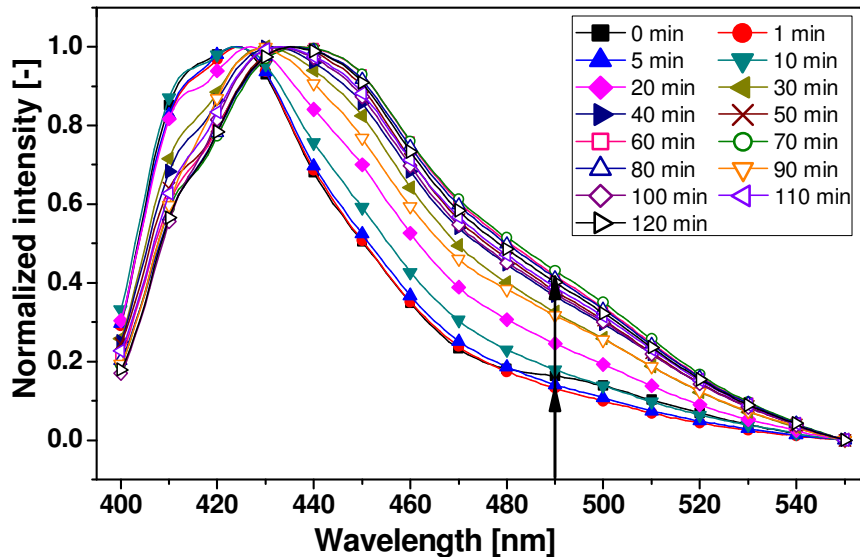


Figure 6.3: Normalized fluorescent signal intensity in *G. stearothermophilus* spores germinated with 60 mmol.l⁻¹ CaDPA. OD was adjusted to 0.5 at the beginning of the measurement. Black arrows mark the relative increase at 490 nm in time.

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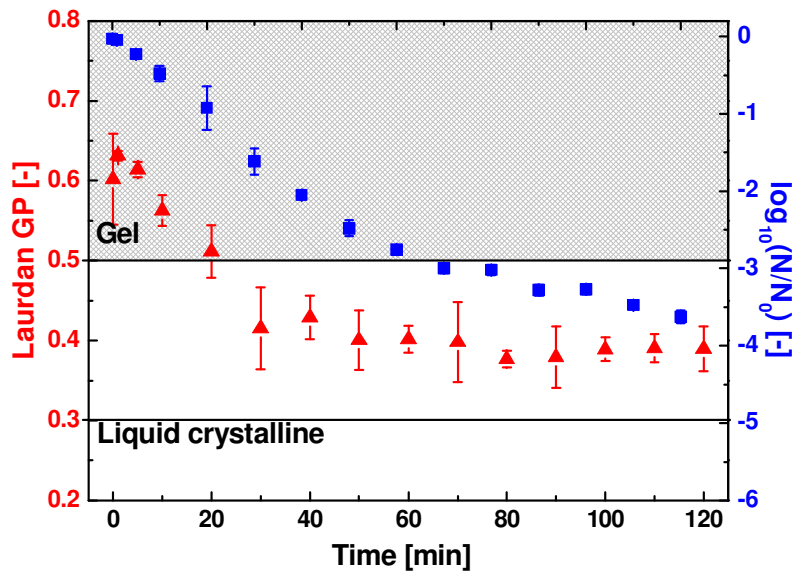


Figure 6.4: Germination and inactivation of heat sensitized spores by pasteurization step 80 °C 20 min after incubation in 60 mmol.l⁻¹ CaDPA (initial concentration of spores 10⁸ CFU.mL⁻¹) (right axis ■). Laurdan *GP* vs. duration of incubation – sample set at OD 0.5 (left axis ▲).

As previously described, dormant spores are characterized by the presence of a gel phase of the phospholipids, while the lipid structure of the vegetative cells represents the liquid crystalline phase (Figure 6.1) (Cowan et al., 2004; Georget et al., 2014a). After 20 min already about 90 % of the spores have become heat sensitive ($\sim 1 \log_{10}$ reduction), and a part of this population had also fully degraded its cortex (decrease of the *GP* value parallel to the log reduction). The *GP*, which is an average of the signal emitted by all spores, starts decreasing from $\sim 5-8$ min onwards after the beginning of germination and it can be inferred that this is due to a fraction of spores whose relative emission has shifted towards 490 nm, due to completed cortex degradation and resulting inner membrane extension. The reason why the absolute *GP* value is still in the gel phase domain is because only a limited fraction of the spores have shifted to germination phase II while the others have not yet. These results suggest a strong heterogeneity of the spore population in germination commitment within the first 20 min of germination. After 20 min already about 90 % of the spores have completed the first phase of germination ($\sim 1 \log_{10}$ reduction), and it is noticeable that a part of this population has also begun to transit to phase II of the germination (decrease of the *GP* value parallel to the log reduction). After 30 min no further shift in Laurdan *GP* is measured and it can be concluded that >90 % of spores have also completed the full cortex degradation and full core hydration/inner membrane extension. This occurs about 10 min after reaching the first log of heat

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sensitized spores, and suggests that the hydration of the core takes place before the full cortex degradation.

In order to model the kinetics of the suggested germination phase I and II and based on the information above, the data had to be put on a comparative scale and were represented as percentages. Typically, the log reduction could be expressed in percent of remaining spores after germination and inactivation of heat sensitized spores, which corresponds to the dormant population. The Laurdan *GP* values were normalized and expressed as percentages of the highest *GP* value vs. time. This percentage was associated with the fraction of spores that were still dormant and/or spores that had not yet fully degraded their cortex and reflected their proportion in the sample at *t*. The complementary population of spores emitting a lower *GP* – i.e. the spores in phase II of germination could thus be deduced. Finally, using the percentage of dormant spores (plate count) and the percentage of dormant spores and spores in phase I (Laurdan *GP*), the population in phase I could be estimated. The two-step kinetic model, based on differential chemical equations and presented in section 6.3, was used to derive a global model from the data generated. This model, used in Figure 6.5, fits the experimental data extracted from the Laurdan *GP* values and plate counts with the optimized constants $k_a = 0.11 \text{ min}^{-1}$ and $k_b = 0.07 \text{ min}^{-1}$.

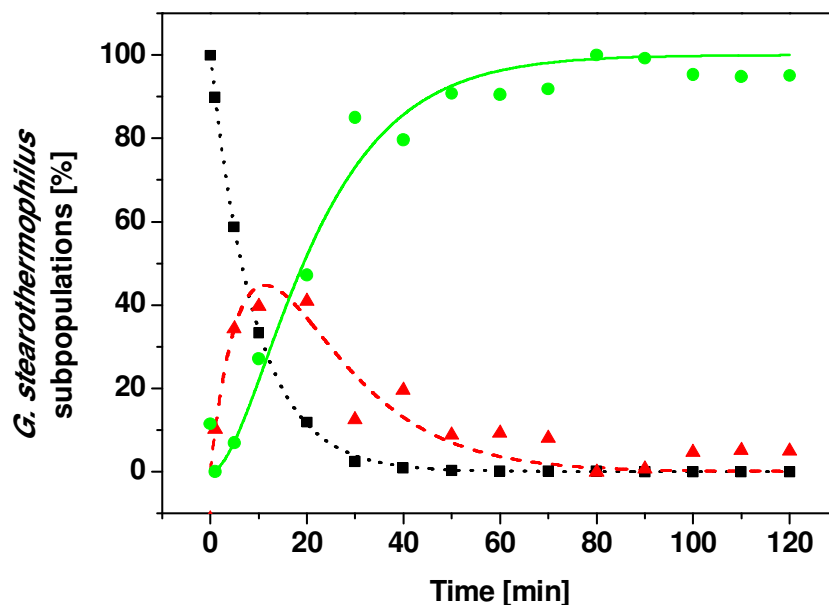


Figure 6.5: Kinetic modeling of germination phase I (- - -) and II (—) of *G. stearothermophilus* dormant spores (····) germinated with 60 mmol.l^{-1} CaDPA. Experimental data points – Dormant spores: ■, Germinated I: ▲, Germinated II: ●.

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6.5 Discussion

6.5.1 Laurdan staining of *G. stearothermophilus* spores' inner membrane

GP in gel lipid phases generally ranges from 0.5 to 0.6 (Parasassi et al., 1998). Values of 0.77 and 0.73 were reported for *C. beijerinckii* and *C. sporogenes* spores (Hofstetter et al., 2012). This indicates that the range obtained in this study, though higher than for conventional gel phases, is in agreement with the results obtained on spores of other genera. These *GP* values emphasize the high degree of order and compressed state of the lipids in the inner spore membrane pointed out in other studies (Cowan et al., 2004; Sunde et al., 2009).

The *D*, *z*-value and *E_a* results were comparable between stained and non-stained spores (Table 6.1), and if any, would suggest only a minor increased resistance of stained bacterial spores over non-stained ones at high temperatures. This could however also be due to a batch to batch variability. *D* and *z*-value determination for the same strain of *G. stearothermophilus* was done in another study using certified NAMSAs (NAMSAs, Northwood, Ohio, USA) and showed $D_{121} = 126$ s and $D_{130} = 12.4$ s, *z*-value= 7.6 °C and $E_a = 389.5$ kJ.mol⁻¹ (Mathys, 2008) which confirmed that the range found above both for stained and non-stained spores is in agreement with the expected thermal resistance of these spores and suggest that the presence of Laurdan in the inner membrane is not affecting its properties, in particular its impermeability and capacity to retain the CaDPA core dehydrated.

6.5.2 Germination of *G. stearothermophilus* spores by individual and combined nutrients

None of the approaches tested to trigger nutrient germination of *G. stearothermophilus* spores were successful to induce a strong germination response, which could be used in the perspective of a decontamination treatment. Commonly investigated germinants in previous studies, for either clostridial spores or spores of the *Bacillus* genus, proved to lead to limited germination of spores of *G. stearothermophilus* within the combination tested and by comparison to PBS alone. Individual amino acids, combinations of inosine and L-Alanine, as well as complex mixes (AGFK) applied at the optimal growth temperature of the indicator (55 °C) for up to 24 h failed at showing germination >1 log₁₀. This confirmed the results obtained in a recent study on single *G. stearothermophilus* NGB101 spores using L-Valine and AGFK, in spite of different heat activation strategies (Zhou et al., 2013). Zhou et al. (2013) suggested that the absence of strong nutrient germination might be linked the incubation temperature being too low. However, this last suggestion would be in opposition with what is known to be the optimal growth requirement of the strain used in this work.

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The absence of strong nutrient germination response might result from the absence or only partial presence of corresponding nutrient receptors on *G. stearothermophilus* inner membrane which could recognize the nutrients used, or a high fraction of superdormant to this germination path. Alternatively, specific co-germinants or nutrient combinations, unidentified to date, might be needed to lead to significant germination of this strain.

One might furthermore add that many studies looking at nutrient germination of *Bacillus* spores reported below 2 log₁₀ germination (Dodatko et al., 2009; Ghosh and Setlow, 2010) and then logically opted for a tracking of germination by percentage rather than log₁₀ reduction post inactivation of the heat sensitized fraction. This approach was not retained for this study as the authors aimed at identifying a substance capable of triggering a large (or full) germination of spores with the objective of sterilization. Additionally, different strategies of heat activation were tested as suggested in previous work (Finley and Fields, 1962), but none seemed to effectively impact on the overall germination of the bacterial spores of *G. stearothermophilus*. The variety of reports of heat activation (or respectively triggered superdormancy) is highlighting the need for more research on this side as the mechanisms seem to be still poorly understood.

Overall, it can be argued that the variety of responses to nutrient germination (and likely of nutrient receptors) in the different spore formers as well as the limited germination achieved is a restriction to any decontamination strategy based on nutrient germination and pasteurization or antimicrobials in opposition to what was previously suggested (Nerandzic and Donskey, 2013; Worthington, 2011). The likelihood of finding a common nutrient (combination) to all strains, leading to strong germination (> 1 log₁₀) is small and thus would always leave a risk for non-complete sterilization.

For future work in the field of nutrient germination, case to case studies on food matrices could be helpful when considering decontamination by germination in food matrices. One might indeed expect that the spore formers naturally present in a given food system also react to nutrients present in this matrix. Thus a pre-incubation between 30 and 50 °C might help germinating spores present and reduce their number eventually by pasteurization. One would however not be looking at a sterilization strategy anymore but rather a shelf life extension approach and the current data available on nutrient germination of spores generally showed quite large superdormant sub-populations.

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6.5.3 Germination of *G. stearothermophilus* spores with DPA chelates

It was expected that CaDPA might lead to a more complete spore germination prior to a comparatively mild decontamination regimen (Perez-Valdespino et al., 2013). However, to date only few studies investigated the impact of DPA chelates on *G. stearothermophilus* spore germination (Fields and Frank, 1969; Zhou et al., 2013) and it was of interest to find out whether this germination pathway is commonly spread among different strains and families of spore formers. Based on the results of this work, it seems reasonable to suppose that for this germination pathway, heat activation does not play any role, in agreement previous work for other spore formers (Ghosh and Setlow, 2010; Keynan and Halvorson, 1962; Paidhungat et al., 2001; Setlow, 2003).

Simultaneous measurement of the \log_{10} -reduction post heat inactivation of the germinated spores and the *GP* value in time enabled the observation of the two stage germination kinetic suggested in this work (Figure 6.4). The first stage was conditioned by the excretion of potassium and hydrogen ions, the excretion of CaDPA, partial water uptake and the loss of heat resistance. The second stage was characterized by the full cortex hydrolysis, core water uptake and expansion, restoration of inner membrane lipid mobility and restoration of core protein mobility (Setlow, 2003) as traced by variations in *GP*.

A first observation based on the Laurdan *GP* shift and absolute value as described in section 6.3, suggested that within one spore batch, the germination commitment varied strongly. While some spores had already proceeded towards phase II (cortex degradation), a large subpopulation had not yet gone through phase I. However, once initiated, the succession between phase I and II occurred rapidly confirming at the scale of the population the results obtained on single spores by Zhou et al. (2013).

It appears that the phase preceding the start of germination phase II (cortex completed degradation / inner membrane extension) is a very short one. Indeed, k_a is nearly twice the value of k_b , confirming that the cortex full hydrolysis occurs very rapidly after the initiation of germination (Dowd et al., 2008) but is still preceded by another short step leading to the heat sensitization of the spores, likely partial hydration. How this hydration is triggered can only be hypothesized at this stage of the work but might be the result of a receptor activation on the inner membrane, sensing cortex fragments as was suggested before (Setlow, 2008a). An alternative hypothesis would be that upon CaDPA activation of a cortex lytic enzyme and corresponding initiation of cortex degradation, the pressure on the inner-membrane and spore core is locally reduced and leads to the release of ions and CaDPA by stimulating mechano-sensitive proteins in the inner membrane. This second

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hypothesis however raises number of questions, and in particular the need for a preferential degradation of the cortex from the inside towards the outside to allow for an early activation of these mechano-sensitive proteins permitting the release of the core CaDPA depot shortly before the completion of the cortex degradation.

On the other hand, the activation of germination by peptidoglycan fragments was already shown using fragments released by vegetative cells and it was shown that this is an extremely sensitive path, with picograms per milliliter being sufficient to activate it (Shah et al., 2008). The germination potential of spore cortex fragments was however not assessed to date. Dowd et al. (2008) characterized the muropeptides released during germination and cortex degradation but did not attempt germination using these extract. They found a total of 38 muropeptides in *Bacillus anthracis* of which most were already present in the dormant spores. However, some novel muropeptides associated with germination-specific cortex hydrolysis were found. It seems thus plausible that upon initiation of the cortex degradation following CaDPA activation of a cortex lytic enzyme, the spore inner membrane could rapidly detect the presence of the novel peptidoglycan fragments in its vicinity and initiates the first steps towards germination before completion of the cortex degradation.

Additionally, individual spores of *B. cereus* and *B. subtilis* were investigated (Kong et al., 2011; Kong et al., 2010). The kinetics of germination with CaDPA were observed by uptake of the nucleic acid dye SYTO 16. The dormant spore core could not be penetrated by SYTO 16. However, when spores were completely germinated, SYTO 16 could cross the spores' inner membrane, bind nucleic acids, and exhibit strong green fluorescence. It was shown that during CaDPA germination of *B. subtilis* there was a 4-7 min delay after time of CaDPA release (first stage) before fluorescence of dye began to increase (second stage) which would match the findings of this work on *G. stearothermophilus* spores.

This would also be in agreement with the results obtained in another work looking at CaDPA germination of single *G. stearothermophilus* spores. There, it was shown via differential interference contrast microscopy that germination of individual spores followed a two stage mechanism, namely CaDPA release followed by full cortex degradation (Zhou et al., 2013). The results presented in this study allow extending this mechanistic hypothesis at the level of an entire spore population.

6.6 Conclusions

A non-nutrient path of germination, through DPA chelates proved to be successful, in particular with CaDPA as was observed for *G. stearothermophilus* spores and for spore formers of other families (Paidhungat et al., 2001; Setlow, 2003). Up to 3 log₁₀ of spores could be germinated and subsequently inactivated. This work underlined two main points:

- Spores of *G. stearothermophilus* could be germinated by CaDPA, which confirmed that the CaDPA activation mechanism is broadly spread across different species and families, possibly as was suggested, through a cortex lytic enzyme (possibly CwlJ) which would be CaDPA activated. This is in agreement with the presence of orthologues of CLE CwlJ of *B. subtilis* in several other strains of the *Geobacillus* genus (Paredes-Sabja et al., 2011). Either the mechanism or the enzyme would thus seem to be widely spread among spore formers of different species.

- Within one spore batch, the germination kinetics between phase I and II varied. While some spores had already proceeded towards phase II (cortex degradation), a large subpopulation had not yet gone through phase I. However, once initiated, the succession between phase I and II occurred rapidly.

As far as food sterilization goes, it is finally relevant to consider that CaDPA is not a GRAS ingredient, it is therefore, to date, not usable in food systems and toxicological validation studies would be needed prior to use in food. Future work would also need to investigate whether individual, purified cortex peptidoglycan fragments could trigger strong germination and thus replace CaDPA as germination trigger. Also, the questions around the superdormancy remain. While 3 log₁₀ could be germinated and inactivated, a remaining population was not affected by this non-nutrient germinant, perhaps due to an absence of necessary cortex lytic enzyme as was suggested by Perez-Valdespino et al. (2013). Therefore, this non-nutrient germination might benefit from a combination with an additional hurdle leading to nutrient like germination, for instance moderate high pressure germination (Reineke et al., 2013b).

7. Preface to the publication “Bacterial spore inactivation by ultra-high pressure homogenization”

Based on the two previous investigations of the spore germination mechanisms, it was found that most pathways were highly physiology dependent and could not easily be used to generate a sterilization process which could be used in a generic manner.

While moderate high pressure allows for germination up to about 3 logs, this result is depending on the use of a physiologically compatible temperature, hence potentially reducing the opportunities to stimulate other spore formers which might germinate preferentially at different temperatures. The nutrient germinants tested were unsuccessful to largely trigger germination in this strain and are receptor dependent which thus would always leave a risk to find another pathogenic/spoilage strain which does not respond to this path. Instead, combinations with a chemical germinant such as CaDPA would possibly offer a means to extend this result since the CaDPA path is non-receptor dependent. However CaDPA, though promising, would still require validation as GRAS in order to serve as ingredient in a food preparation.

In this third chapter, we looked at a non-germination dependent alternative which could lead to successful spore inactivation by combining dynamic high pressure and high temperatures in a successive manner. The idea in this third section was to preferentially focus on a hurdle combination which could enable spore inactivation without relying on a spore weakening by a previous germination step while still allowing for a reduction of the overall thermal intensity.

The technology considered hereafter is ultra-high pressure homogenization, allowing for continuous high pressure processing up to 350 MPa. In the following work, the high pressure hurdle is combined with shear and high valve temperatures. This approach, if successful, could become an interesting alternative to high pressure thermal sterilization relying on commercially existing equipment.

In addition, we aimed to test whether this treatment could lead to spore germination post UHPH, especially in non-lethal regimens. This is an important factor to consider as it could, as shown in section 4, allow for a rapid weakening of bacterial spores and thus open new opportunities for continuous sterilization.

Bacterial spore inactivation by ultra-high pressure homogenization

8. Bacterial spore inactivation by ultra-high pressure homogenization

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Innovative Food Science and Emerging Technologies, 2014, 26, 116-123– with permission of Elsevier

Abstract

A new generation of high pressure homogenizers reaching up to 400 MPa offers opportunities for spore inactivation and high pressure sterilization of foods. A Stansted Fluid Power ultra-high pressure homogenization (UHPH) unit was tested to inactivate bacterial spores in a model buffer system. *Bacillus subtilis* PS 832 and *Geobacillus stearothermophilus* ATCC 7953 spores' thermal resistances were assessed (D , z -value and E_a). The pressure and valve temperature were monitored during UHPH. Residence times under pressure and high temperature were estimated and enabled comparison with thermal inactivation, indicating the estimated thermal contribution to inactivation. Spore germination was also assessed but no germination was observed. Up to five \log_{10} *B. subtilis* and two \log_{10} *G. stearothermophilus* spores were inactivated by the harshest treatments (>300 MPa $-T_{\text{valve}} >145$ °C for ~ 0.24 s). The inactivation profiles were similar to the predicted thermal inactivation suggesting that the valve temperature might be a dominant parameter leading to bacterial spore inactivation.

Industrial relevance

This work showed the UHPH potential for spore inactivation and the need of highly thermoresistant surrogates for process validation, for instance, *G. stearothermophilus* ATCC7953 spores. Both these findings are highly relevant for industrial application as, to date, no surrogate for this process was suggested while the first patents for the technology transfer to industry are being issued.

Bacterial spore inactivation by ultra-high pressure homogenization

8.1 Introduction

Mechanical homogenization has been defined as the ability to generate a distribution of particles of homogeneous size, in a liquid, by forcing the liquid under high pressure through a disruption valve (Donsì et al., 2009). High pressure homogenization (HPH), also known as dynamic high pressure homogenization, utilizes pressures 10 to 15 times higher than those of conventional homogenizers. It covers the full range of 100 to 400 MPa and the range 300-400 MPa is generally referred to as ultra-high pressure homogenization (UHPH). HPH has confirmed its potential for low temperature pasteurization of food matrices (Diels and Michiels, 2006; Dumay et al., 2013; Popper and Knorr, 1990) and the disruption of vegetative microorganisms was suggested to result from a combination of temperature, cavitation, shear, turbulence, impingement and high pressure (Dumay et al., 2013; Kleinig and Middelberg, 1998). However, previous work showed that bacterial spores resist treatments at low homogenization pressure and/or low valve temperature (Bevilacqua et al., 2007; Feijoo et al., 1997), thus limiting applications to the domain of pasteurization.

UHPH extension of the homogenization pressure range offers a new area to investigate bacterial spore inactivation and to attempt to achieve commercial sterility through a single, continuous step combining dynamic high pressure and other stress factors, such as temperature, shear and cavitation. Bacterial spores are resistant to most conventional treatments and are the main reason for the use of high thermal intensity treatments to sterilize food (Georget et al., 2013a). Detailed investigations were conducted on the impact of high hydrostatic pressure on bacterial spores and it was demonstrated that pressure and temperature have a synergetic impact on bacterial spore inactivation (Heinz, 1997; Mathys, 2008; Reineke, 2012; Reineke et al., 2013b). Fewer investigations have looked at the use of UHPH for spore inactivation partially due to the previous unavailability of the equipment. Also, some of the studies looking at spore inactivation by UHPH focused on the control of the homogenization pressure but did not consider the temperature at the valve, thus making it hard to assess the causes for the absence of inactivation (Chen et al., 2013). Existing studies showing successful inactivation of bacterial spores through UHPH were reviewed (Georget et al., 2014c), and it was concluded that the best inactivation could be achieved by combining high homogenization pressures and high inlet and, consequently, valve temperatures (Amador-Espejo et al., 2014b; Cruz, 2008; Cruz et al., 2007; Polisel-Scopel, 2012; Valencia-Flores et al., 2013). In most of the studies inactivation of bacterial spores was hypothesized to be the result of a synergetic effect of pressure, shear, cavitation, temperature and turbulence but this hypothesis was not demonstrated. Although a direct association could be made with the processing temperature, no studies attempted to quantify the expected thermal inactivation through the decimal reduction time

Bacterial spore inactivation by ultra-high pressure homogenization

D_0 , z value and energy of activation E_a of the strains combined with the residence time. This approach could provide a rapid estimation of the effective contribution of temperature to spore inactivation by UHPH. The absence of a clear understanding of the factors leading to bacterial spore inactivation has also prevented the scientific community from reaching an agreement on the most relevant surrogate to use for future work and process parameters validation. Most studies in food matrices focused on *Bacillus cereus* spores or endogenous mesophilic spore strains and claimed sterility based on the full inactivation of these strains. This was without clear establishment of the contribution of different process parameters which is necessary to select the appropriate surrogate, and without high level of inoculation necessary to prove the robustness and reliability of the process (Amador-Espejo et al., 2014b; Polisele-Scopel et al., 2012; Valencia-Flores et al., 2013). A study conducted with *Geobacillus stearothermophilus* spores, a particularly thermostable strain, showed that high pressure of 300 MPa and 84 °C at the UHPH valve were not sufficient to inactivate this strain, but unfortunately did not assess higher valve temperatures (Pinho et al., 2011). Recently published work initiated exploration of UHPH thermophilic spore inactivation with inoculation of whole UHT milk and showed that with an inlet temperature of 85 °C, *G. stearothermophilus* (CECT 47) spores could be inactivated (Amador-Espejo et al., 2014a). Further studies with this indicator and higher inlet and valve temperatures could be useful to validate the UHPH sterilization over a broader range of resistant spore formers and improve our understanding of the role of different process parameters.

In this work, the authors investigated the inactivation of spores of two bacterial strains, *Bacillus subtilis* PS832 and *G. stearothermophilus* ATCC 7953. Both strains were inoculated at high concentration in a model buffer system and UHPH was applied with different combinations of homogenization pressure ($350 \text{ MPa} \geq P \geq 200 \text{ MPa}$) and inlet temperature in order to assess the role of both parameters in the inactivation. In particular, the thermal inactivation kinetics of both strains were also assessed in order to model the impact of temperature during UHPH spore inactivation. For clarity, the pressure range investigated in this work is referred throughout the manuscript to UHPH.

8.2 Material and methods

8.2.1 Bacterial strains and sporulation

The strains used in this work were *G. stearothermophilus* ATCC 7953 obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures, and *B. subtilis* PS832, courtesy of Professor Peter Setlow, University of Connecticut Health Center, USA.

Bacterial spore inactivation by ultra-high pressure homogenization

G. stearothermophilus spores were obtained following a sporulation method enabling >95 % spores, as established in previous work (Georget et al., 2014a). Sporulation was induced at 55 °C on solid Difco medium agar plates without antibiotics. *B. subtilis* spores were obtained using a method described elsewhere (Nicholson and Setlow, 1990; Reineke et al., 2011b). Sporulation was induced at 37 °C on solid 2xSG medium agar plates without antibiotics. In both cases, the spore suspensions were cleaned by repeated centrifugation until the supernatant was clear (minimum 3-fold at 4800 g), washed with cold distilled water (4 °C), and were treated twice with sonication for 1 min (35 kHz - 160 W_{eff}) (Bandelin Sonorex RK 510H, Berlin, Germany). The cleaned spore suspensions contained >95 % phase bright spores without agglomerates as was verified by phase contrast microscopy and were stored in the dark at 4 °C until use.

8.2.2 UHPH unit and treatment conditions

For the high pressure homogenization treatment, a Stansted UHPH unit, model FPG11300 (Stansted Fluid Power Ltd, Harlow, UK) was used as depicted in Figure 8.1 (numbers under brackets [1], [1'] and [2]-[6] in the text hereafter refer to Figure 8.1). This unit consisted of two pistons generating the homogenization pressure – the first homogenization valve [3] – a cooling step [4] – the second homogenization valve [5] and the product outlet for sampling [6]. The Stansted first valve [3] design was such that the fluid streamed axially under high pressure along the mobile part of the valve (valve spindle) and then flowed at high velocity through the radial narrow gap formed between the valve seat and the spindle, before leaving the valve seat at the low pressure maintained by the second homogenization valve (~10 MPa). Bacterial spores were re-suspended in PBS buffer 0.01 M (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄) at pH 7.0, at initial concentrations of 10⁶ CFU/mL for *B. subtilis* spores and 10⁴ CFU/mL for *G. stearothermophilus* spores [1]. PBS buffer was selected for its high stability at the high temperatures which were targeted in this experimental work and avoided potential pKa variation at high temperatures as was reported in previous work (Reineke et al., 2011c).

The parameters controlled were the inlet temperature and the operating pressure before the first valve. The target inlet temperatures were 37 and 80 °C for *B. subtilis* spores and 55 and 80 °C for *G. stearothermophilus* spores. The low temperature corresponds to the optimal growth temperature of the respective indicators and was selected to assess a potential germination under pressure during the high pressure stage of the process. For 37 and 55 °C, the inlet temperature was directly adjusted by means of a water thermostat and plate heat exchanger (DIL e.V., Quakenbrueck, Germany) connected ahead of the UHPH unit [2]. For the trials at high inlet temperature, the spore suspension

Bacterial spore inactivation by ultra-high pressure homogenization

was first preheated by recirculation through a separate plate heat exchanger (DIL e.V., Quakenbrueck, Germany) and then processed through the heat exchanger and UHPH set-up as described above, allowing for an inlet temperature of ~ 80 °C [1']. The cooler consisted of a tubular heat exchanger localized at the outlet of the first valve and connected to a cooling unit using glycol water at -10 °C as the cooling agent [4]. The cooling step allowed for a rapid cooling after the first valve, bringing the process medium from the valve temperature down to temperatures below 50 °C within < 1 s.

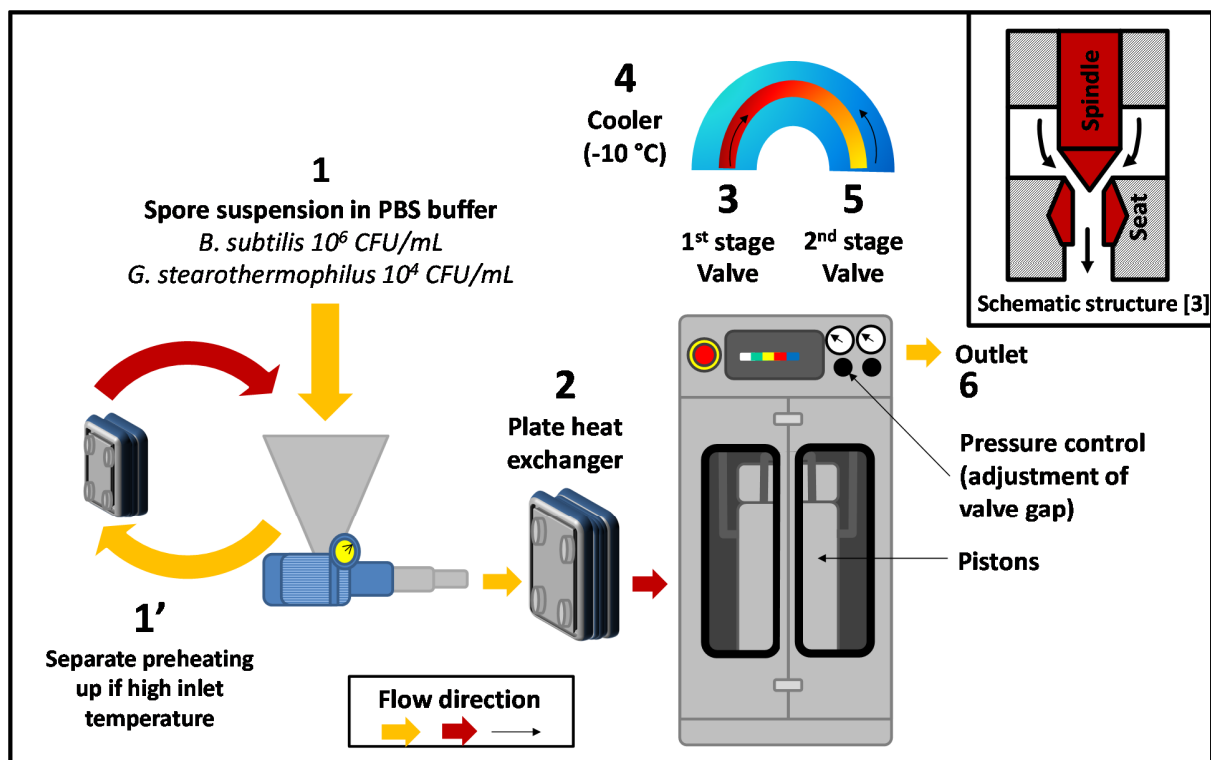


Figure 8.1: Schematic representation of the experimental setup - Stansted UHPH unit, model FPG11300 (Stansted Fluid Power Ltd, Harlow, UK).

For each temperature, three homogenization pressures were tested: 200, 300, and 350 MPa. The second valve was present on the UHPH unit in order to maintain a minimal pressure (~ 10 MPa) after the decompression at the first valve, thus minimizing the formation of steam at the first valve and cavitation phenomena. The valve temperature was recorded during the trial. This parameter directly resulted from the homogenization pressure and the inlet temperature selected and enabled comparison to thermal inactivation kinetics (via D_0 , z value and E_a see section 8.3).

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Each trial was done at least in triplicate using a single batch for both indicators and the bars in the graphs correspond to the standard deviation. The same spore batches were used for the determination of the thermal inactivation kinetics as described hereafter.

8.2.3 Microbiological analyses

Following the UHPH trials, plate counts were performed before and after a thermal treatment at 80 °C during 20 min to inactivate the potentially heat-sensitized spores and establish the extent of germination. Samples were plated at least in triplicate on nutrient agar and incubated at 37 °C for 72 h (*B. subtilis* spores) or 55 °C for 48 h (*G. stearothermophilus* spores).

All graphical representations in this work were obtained with OriginPro (Version 8.0724, B724; OriginLab Corporation, Northampton, MA, USA).

8.3 Calculation

The destruction of microorganisms is usually well described as a first order reaction, although the mechanism may be more complex, resulting in non-log-linear curves (Kessler, 2002; Maroulis and Saravacos, 2003). In a first order reaction, the integration of the change in microorganism concentration, at a given temperature, can be expressed as shown in (20) where N_0 is the initial spore population, N_t is the surviving spore population at time t , and k , the velocity constant which can be obtained by linear regression when plotting the logarithmic reduction as a function of time. D_θ (21) is the time necessary at a specific temperature to reduce the number of microorganism to a tenth of the original value and is temperature (θ) dependent (Kessler, 2002; Maroulis and Saravacos, 2003). The effect of the absolute temperature on the inactivation velocity constant k , and thus on the D_θ , is given by the Arrhenius equation (22) where E_a is the energy of activation in J/mol, R is the universal gas constant = 8.314 J/mol.K, k_0 is the velocity constant in s^{-1} for $1/T=0$ and T is the temperature in Kelvin. The Arrhenius diagram allows for the determination of the energy of activation E_a (independent of temperature) and can be used to calculate the z -value which expresses the increase in temperature necessary to obtain the same inactivation effect in one tenth of the time (23).

Bacterial spore inactivation by ultra-high pressure homogenization

$$\log\left(\frac{N_t}{N_0}\right) = -t \cdot \frac{k}{2.303} \quad (20)$$

$$D_\theta = \frac{2.303}{k} \quad (21)$$

$$\ln\left(\frac{k}{k_0}\right) = -\frac{E_a}{R} \cdot \frac{1}{T} \quad (22)$$

$$z = \frac{2.303 \cdot R \cdot T^* \cdot T}{E_a} \quad (23)$$

Thin wall glass capillaries, enabling quasi isothermal conditions, were used as means to determine D_θ , the z-value and E_a of *B. subtilis* and *G. stearothermophilus* spores. The method was adapted for bacterial spore thermal inactivation kinetics determination and is reported elsewhere (Mathys et al., 2007b). Thin wall glass capillaries with an inner diameter of 1.0 mm, an outer diameter of 1.3 mm and a length of 300 mm were used (Kleinfeld Labortechnik GmbH, Gehrden, Germany). The capillaries were filled with a spore suspension volume of 100 μ L. The capillaries were heated in a thermostat (Haake DC50, Karlsruhe, Germany) with silicon oil SIL 180 (Thermo Fisher Scientific, Karlsruhe, Germany) as heating medium. After the defined heating time, the samples were rapidly cooled in an ice bath.

B. subtilis spores D_θ , z-value and E_a were established at 105, 110 and 121 $^\circ$ C and *G. stearothermophilus* spores at 110, 121 and 130 $^\circ$ C. Each D_θ , z-value and E_a were determined in triplicates.

The strong thermo-sensitivity of the spores used in this work at higher temperatures ($T > 121$ $^\circ$ C or $T > 130$ $^\circ$ C respectively), made the experimental determination of the D_θ at higher temperatures impossible as the very short holding times (< 1 s) required would not allow to reach the final target temperature within the glass capillary (Mathys et al., 2007b). In order to estimate heat inactivation at high temperatures, D_θ at temperatures achieved during UHPH processing were extrapolated based on the linear regressions of experimental $\ln(k)$ versus the inverse of the temperature and the energy of activation E_a ((21) and (22)).

8.4 Results and discussions

8.4.1 Characterization of the thermal inactivation kinetics

The thermal inactivation kinetics of both strains showed linear behavior as a function of the holding time at the treatment temperature (Figure 8.2). All inactivation kinetics could be well represented by a first order inactivation kinetic and allowed for the determination of the D_θ at $\theta = 105, 110$ and 120 $^\circ$ C for *B. subtilis* spores and at $\theta = 110, 121$ and 130 $^\circ$ C for *G. stearothermophilus* spores (Figure

Bacterial spore inactivation by ultra-high pressure homogenization

8.2). Bacterial spores D_{θ} , z-values can slightly vary from batch to batch. These parameters were determined for the batch used in this work in order to characterize its thermal resistance. The D_{θ} , z-values and E_a can be found in Table 8.1 and were in agreement with the values found in previous studies (Mathys, 2008).

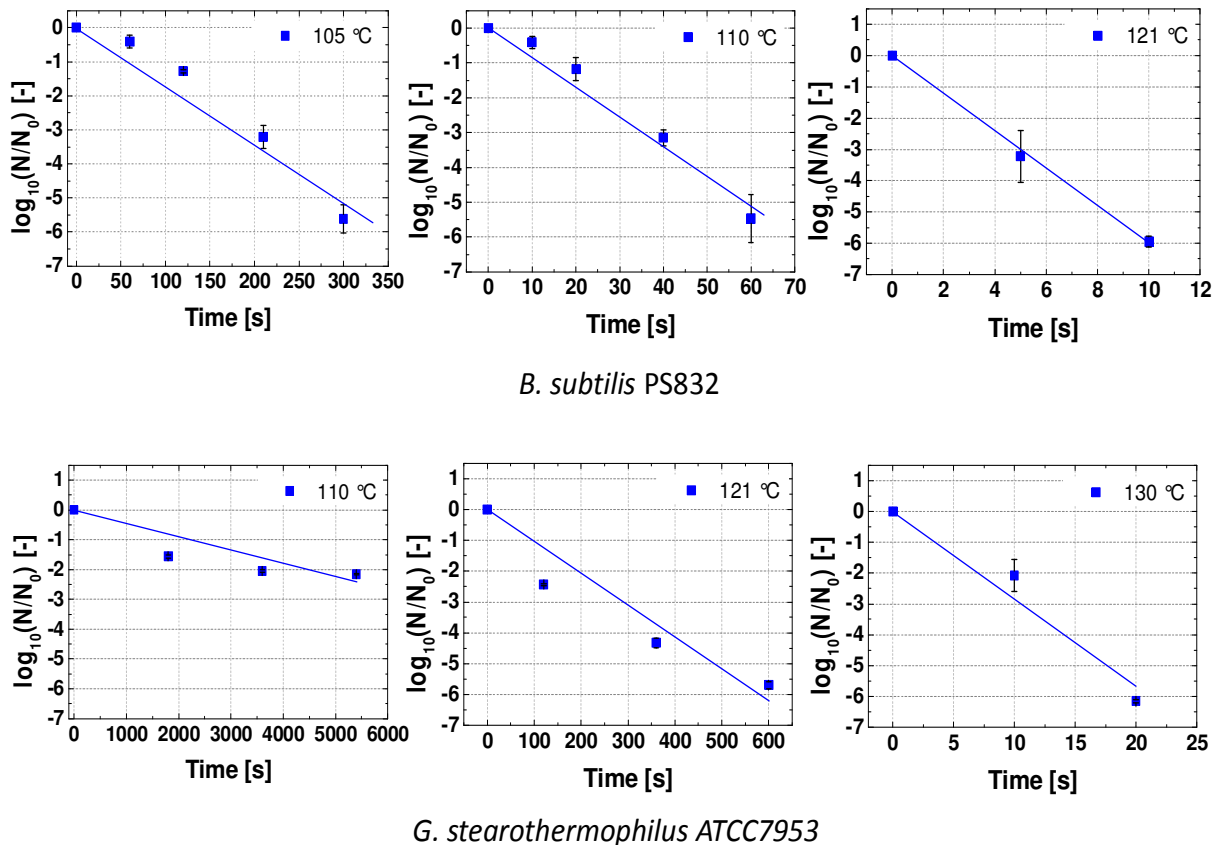


Figure 8.2: Inactivation kinetics at $\theta = 105, 110$ and 120 °C for *B. subtilis* spores and at $\theta = 110, 121$ and 130 °C for *G. stearothermophilus* spores.

Based on the experimental thermal inactivation kinetics, the logarithm of the velocity constant of *B. subtilis* PS832 and *G. stearothermophilus* ATCC 7953 as a function of the inverse of the temperature could be plotted, and showed a linear behavior (Figure 8.3). The linear regression and corresponding $\ln(k_0)$ as well as the E_a were used to extrapolate the D_{θ} at higher temperatures reached during UHPH processing using equation (2) and (3). Although extrapolation includes a risk of deviation from the real behavior, it is important to mention that there is, to date, no experimental means to obtain D_{θ} at temperatures where the decimal reduction value is below 2 s because the glass capillaries, though extremely thin, need 2 seconds before reaching isothermal conditions (Mathys, 2008).

Bacterial spore inactivation by ultra-high pressure homogenization

Table 8.1: D_θ , z-value and E_a of *B. subtilis* and *G. stearothermophilus* spores in PBS buffer as determined by the glass capillary method (Mathys, 2008) – Standard deviation derived from triplicate determinations

| <i>B subtilis</i> PS 832 | | |
|--|----------------|--------------------|
| θ (°C) | D_θ (s) | Standard deviation |
| 105 | 60.2 | 5.2 |
| 110 | 11.9 | 1.5 |
| 121 | 1.7 | 0.1 |
| z-value (°C) | 10.6 | 0.3 |
| E_a (kJ/mol) | 269.8 | 6.6 |
| <i>G. stearothermophilus</i> ATCC 7953 | | |
| θ (°C) | D_θ (s) | Standard deviation |
| 110 | 2080.5 | 22.5 |
| 121 | 89.7 | 10.1 |
| 130 | 3.5 | 0.1 |
| z-value (°C) | 7.6 | 0.3 |
| E_a (kJ/mol) | 407.9 | 2.3 |

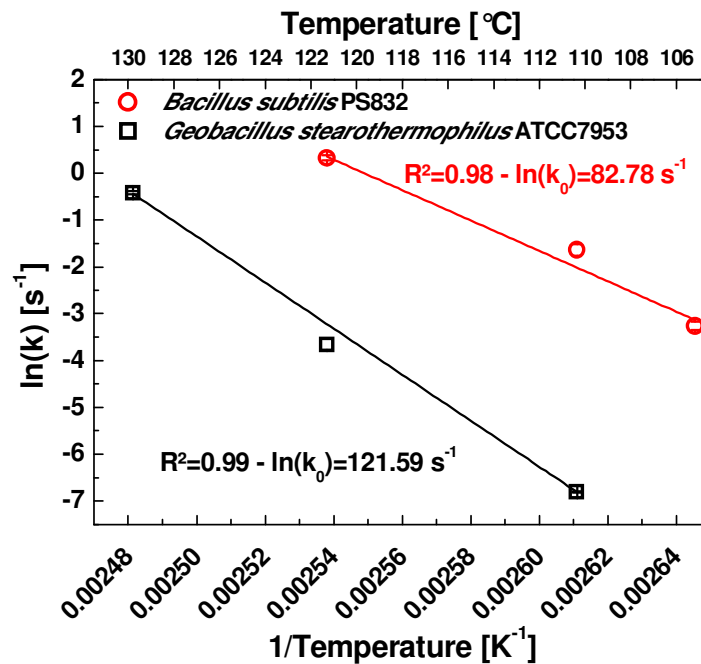


Figure 8.3: Expression of $\ln(k)$ of *B. subtilis* PS832 and *G. stearothermophilus* ATCC7953 as function of the inverse of the temperature. $R^2 > 0.98$ and > 0.99 were calculated by linear regression for both strains respectively.

Bacterial spore inactivation by ultra-high pressure homogenization

8.4.2 UHPH inactivation versus pressure and valve temperature

UHPH was applied at different homogenization pressure and inlet temperatures. In an attempt to characterize the role of these individual factors in the inactivation of spores by UHPH, the logarithmic reductions obtained post treatment were plotted versus the homogenization pressure (Figure 8.4 and Figure 8.5) and the valve temperature (highest temperature reached during UHPH) (Figure 8.6 and Figure 8.7).

The results in

Figure 8.4 and Figure 8.5 showed no clear correlation between homogenizing pressure and inactivation of either strain. However, it appeared that a minimal threshold pressure had to be achieved (in this set of experiments, 300 MPa), in order to enable inactivation. At 300 and 350 MPa, the experimental points showing no inactivation corresponded to low inlet temperatures (~ 37 or ~ 55 °C for *B. subtilis* and *G. stearothermophilus* spores respectively) while the ones showing inactivation corresponded to the high inlet temperature (~ 80 °C).

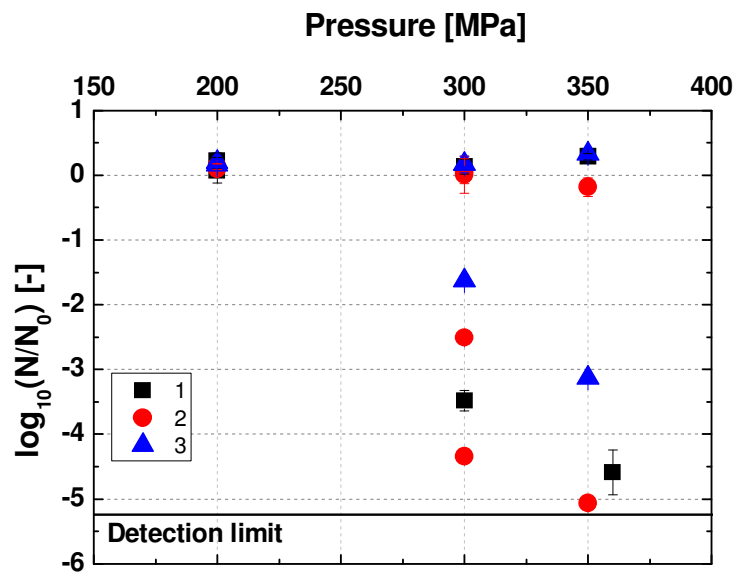


Figure 8.4: *B. subtilis* spore inactivation as function of the homogenization pressure. 1, 2 and 3 correspond to three independent repetitions of the trial with the same initial batch of spores. The valve temperature for each of these data points is not considered here – all log₁₀ reductions were represented exclusively as function of the pressure – independently from the valve temperature achieved.

Bacterial spore inactivation by ultra-high pressure homogenization

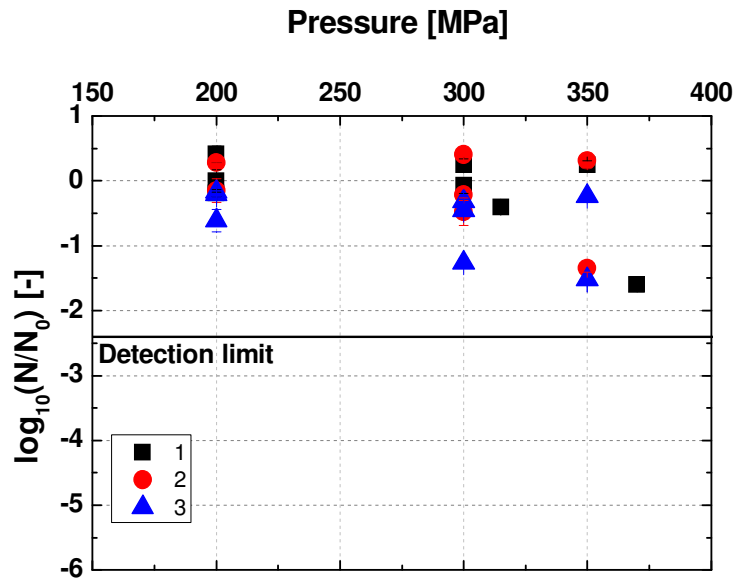


Figure 8.5: *G. stearotherophilus* spore inactivation as function of the homogenization pressure. 1, 2 and 3 correspond to three independent repetitions of the trial with the same initial batch of spores. The valve temperature for each of these data points is not considered here – all \log_{10} reductions were represented exclusively as function of the pressure – independently from the valve temperature achieved.

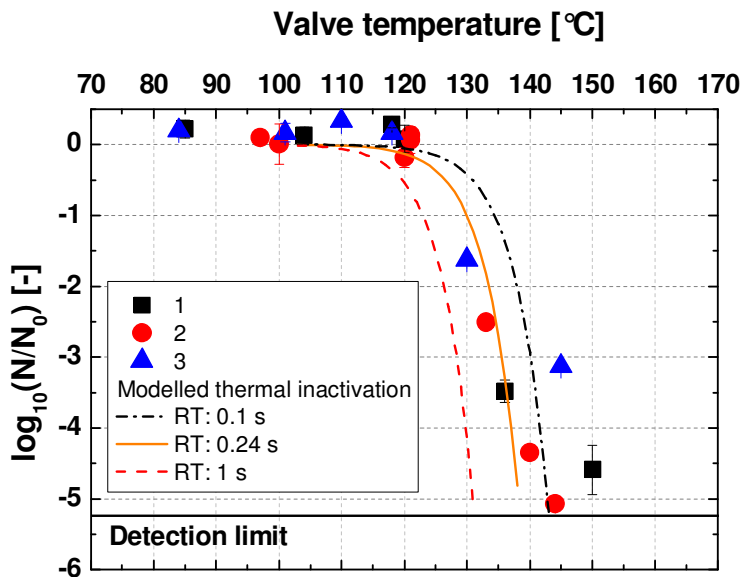


Figure 8.6: *B. subtilis* spore inactivation as function of the valve temperature. The results correspond to three independent repetitions of the trial with the same initial batch of spores. 1, 2 and 3 correspond to three independent repetitions of the trial with the same initial batch of spores. Data represented independently from the homogenization pressure.

Bacterial spore inactivation by ultra-high pressure homogenization

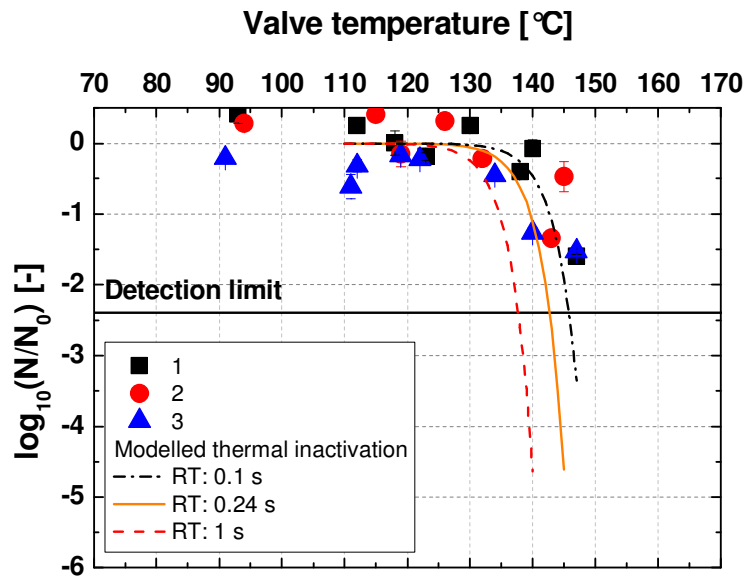


Figure 8.7: *G. stearothermophilus* spore inactivation as function of the valve temperature. The results correspond to three independent repetitions of the trial with the same initial batch of spores. 1, 2 and 3 correspond to three independent repetitions of the trial with the same initial batch of spores. Data represented independently from the homogenization pressure.

A higher inlet temperature and a higher homogenization pressure will lead to higher valve temperatures. Previous work has established that when using a Stansted Fluid Power UHPH valve design (needle valve), an increase of 22 °C / 100 MPa of homogenization pressure could be achieved at the valve (Floury et al., 2004). The results in Figure 8.6 and Figure 8.7 show the logarithmic reduction as a function of the valve temperature and underline a correlation between valve temperature and inactivation for both strains.

The residence time post valve at high temperature and before cooling was estimated to be 0.24 s based on the measured flow rate and path through the equipment. Based on this estimated residence time at high temperature, the temperature at the valve, and the experimental (up to 121 or 130 °C depending on the strain) as well as extrapolated D_0 at higher temperatures, thermal inactivation profiles matching the temperature conditions in the UHPH could be established. These modeled thermal inactivation profiles matched the UHPH inactivation profiles obtained for both *B. subtilis* and *G. stearothermophilus* spores in a reproducible manner. Two other holding times were also considered (0.1 and 1 s) to assess how closely the inactivation was related to this holding time at the valve temperature. Interestingly, it appeared that some of the data points obtained were closer to the expected inactivation at 0.1 s holding time or even below rather than the estimated

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0.24 s. This suggests the possible existence of flow defects (e.g. short cut) post valve which could significantly alter the estimated residence time and which would need to be modeled in future work. Based on these observations, it can be argued that the temperature at the valve, in spite of very short holding times, appears to be a main contributor to spore inactivation by UHPH. This hypothesis is also reinforced by the comparatively higher inactivation achieved for *B. subtilis* spores at lower valve temperatures than for *G. stearothermophilus* spores – a recognized indicator for wet heat sterilization, due its resistance to thermal inactivation (Albert et al. 1998). The results presented in this work would thus contrast with the hypothesis of recent work which suggested that spore inactivation must be the result of combined high temperature and other physical forces at the valve (Valencia-Flores et al., 2013).

Computational fluid dynamics (CFD) modeling of a similar valve design in another Stansted UHPH unit was performed (Floury et al., 2004) and indicated absence of impingement at the exit of the valve due to a very quick decrease in the fluid velocity after leaving the valve gap. Their modeling also highlighted the existence of two strong zones of recirculation post valve which could contrast with short cuts in the central part of the flow. This could explain the observation made in this work that some experimental inactivation data points at very high valve temperatures were closer to the expected thermal inactivation alone and a residence time of 0.1 s or below, instead of the estimated 0.24 s.

8.4.3 UHPH triggered germination

Previous work has suggested possible HPH induced spore germination at 150 MPa (Chaves-López et al., 2009) whereas other studies reached different conclusion (Pinho et al., 2011), and no clear consensus has been reached thus far.

In order to assess whether processing by UHPH could induce germination of the strains used in this work, the trials at low inlet temperature were done as close as possible to the optimal germination and growth temperature for *B. subtilis* and *G. stearothermophilus* spores. Plate counts, post UHPH, were conducted with or without a pasteurization step (80 °C 20 min). Germinated spores would be characterized by increased heat sensitivity, resulting from the release of the calcium-dipicolinic depot present in the spore core and water uptake, making them sensitive to this thermal treatment while dormant spores would not be inactivated. The results of this comparison are presented in Figure 8.8 (*B. subtilis* spores) and Figure 8.9 (*G. stearothermophilus* spores) and suggest that, in both cases, no significant germination was induced by the UHPH treatment. The inlet temperature and different pressure levels did not alter this result.

Bacterial spore inactivation by ultra-high pressure homogenization

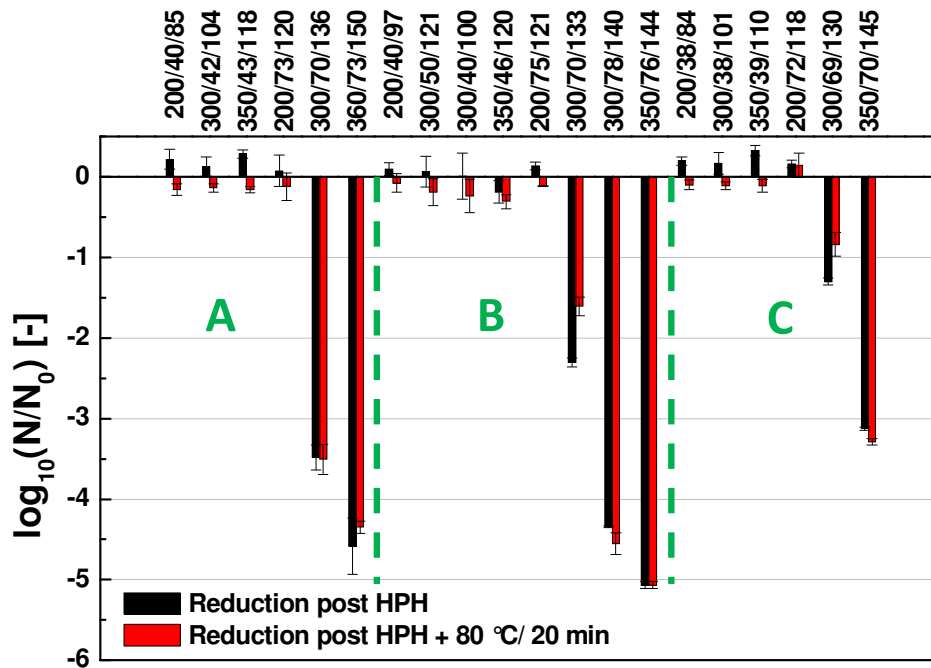


Figure 8.8: *B. subtilis* spore inactivation post UHPH and post UHPH + 80 °C 20 min. The x axis represents the treatment conditions and can be read as Pressure [MPa] / T_{inlet}/T_{valve} [°C]. A, B and C correspond to three independent repetitions of the trial with the same initial batch of spores.

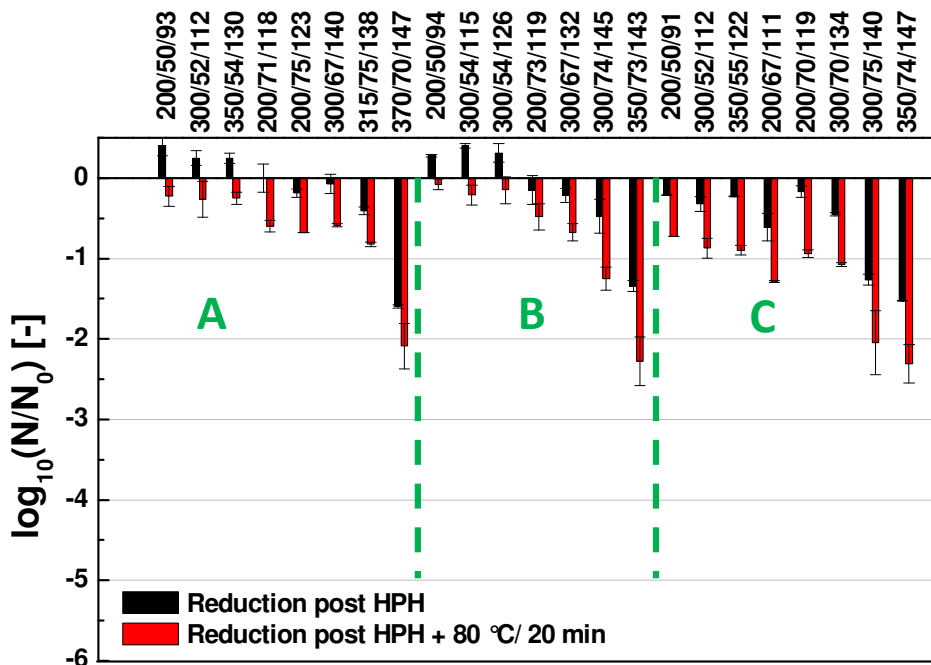


Figure 8.9: *G. stearotherophilus* spore inactivation post UHPH and post UHPH + 80 °C 20 min. The x axis represents the treatment conditions and can be read as Pressure [MPa] / T_{inlet}/T_{valve} [°C]. A, B, and C correspond to three independent repetitions of the trial with the same initial batch of spores.

Bacterial spore inactivation by ultra-high pressure homogenization

In previous work, hydrostatic pressure of 200 to 600 MPa has shown to induce bacterial spore germination by a mechanism designated as “physiological like” germination, referring to the high pressure activation of the nutrient receptors of the bacterial spores, localized on the inner membrane (Georget et al., 2014a; Heinz, 1997; Reineke et al., 2013b). This pressure range is achieved by UHPH, with the difference that UHPH corresponds to the application of dynamic pressure. One might nonetheless wonder what could prevent the triggering of the germination of bacterial spores in this context. A first hypothesis would be the very short residence time under high pressure in this work, estimated at ~ 0.7 s. Previous *in situ* investigation of the impact of high pressure on bacterial spores of *G. stearothermophilus* showed that high pressure was inducing a phase transition in the inner spore membrane which was monitored only for treatments with a minimum of 5 min holding time under pressure. The results of this study also showed that a pressure pulse which could be compared to the pressure holding time under UHPH was not sufficient to allow for this phase transition to be observed. This transition also corresponded to a significant germination of spores after 5 min at 200 MPa of up to three \log_{10} . It was thus suggested that pressure might play a key role in shifting the nutrient receptors’ proteins conformation towards an activated state in a direct manner, as well as indirectly by shifting the lipid structure of the inner membrane under pressure (Georget et al., 2014a). These results agreed with recent work where it was shown that short exposure to high pressure (few seconds) might not be sufficient to trigger germination if immediately followed by atmospheric pressure (Kong et al., 2013). The most likely explanation would be that the necessary time to allow for a thermodynamic reconfiguration at the new equilibrium and lowest energy state leading to the “activation” of the nutrient receptor is not reached by short treatment times.

8.5 Conclusions

The results presented in this work offer a new perspective on the potential of UHPH as a sterilization process. While numerous studies have focused on reducing the processing temperature and achieved only limited inactivation of *Bacillus cereus* spores or other mesophilic spore strains, this work focuses on the ability of UHPH to reach extremely high temperatures quasi instantaneously and with a very short holding time, where spore inactivation can be achieved. It also suggests that most of the inactivation reported in previous work and obtained at lower temperatures might result from the choice to focus on the endogenous flora (no inoculation – low

Bacterial spore inactivation by ultra-high pressure homogenization

counts) and/or thermo-sensitive strains (Poliseli-Scopel, 2012; Poliseli-Scopel et al., 2012; Valencia-Flores et al., 2013).

This study suggests that future work on validation of UHPH as a sterilization process should use *G. stearothermophilus* ATCC 7953 spores as surrogate because of this strains status as reference for wet heat sterilization (European Pharmacopoeia Commission, 1997). The scope and outcome of this work is particularly relevant for today's implementation of UHPH as described in recently filed patents for UHPH sterilization (Guamis et al., 2012). In addition, the relevant authorities and the scientific community will need to reach an agreement on the validation protocol for this sterilization process, in particular with regard to (a) potential surrogate(s). There again, this work brings evidence based suggestions for the selection of such a surrogate.

In order to exclude contributions of other stress factors to the UHPH inactivation of bacterial spores, future work would also need to establish a CFD modeling of the valve and post valve sections of the UHPH unit in order to assess in particular the exact residence time distribution, as was done previously for other valve designs (Floury et al., 2004). This modeling could be supported by an experimental determination of the residence time distribution in this area of the system similar to previous work done in micro-scale systems (Georget et al., 2013b).

Finally, all the trials in this work were performed using a pilot scale equipment and scalability of the UHPH equipment to industrial level needs to be considered along with the valve designs used which can significantly impact the UHPH performance on spore inactivation. GEA Niro Soavi seems, to date, to be close to reach this objective with the UHP4000 prototype equipped with an aseptic design which allows for, SIP and CIP cleanability on both the process side and on the aseptic containment system for sterile product processes. This system operates from 100 up to 500 L/h (Gandini and Grandi, 2007; Grasselli et al., 2007). Homogenizers performing at 310 MPa up to 1500 L/h are commercialized by BEE international (BEE international, 2014), while an equipment with aseptic filling applying 350 MPa and 1000 L/h is advertised by the spin-off Ypsicon, founded in 2013 (Ypsicon, 2014). Nevertheless, no example of the use of these two pieces of equipment for commercial products by industry was found to date by the authors.

The advantages of UHPH versus standard UHT in terms of product quality (organoleptic and nutritional properties) and energy requirements (economic assessment) will also need to be assessed based on a larger range of food matrices and in conditions allowing for commercial sterility. In this context some studies already focused on the quality parameters of vegetal milks after UHPH and showed a benefit of UHPH at 300 MPa on the color and colloidal stability of soy milks by comparison to UHT (Cruz et al., 2007; Poliseli-Scopel et al., 2012, 2014). It was also

Bacterial spore inactivation by ultra-high pressure homogenization

established that lower furan concentrations were achieved by UHPH at 300 MPa than by UHT processing (Poliseli-Scopel, 2012).

Based on the validation of benefits for product quality by homogenization and achievement of commercial sterility, one could hope to fuse two energy consuming steps (sterilization and homogenization) as a single unit operation, thus reducing the overall processing complexity and costs.

Conclusion and outlook

9. Conclusion and outlook

The main objective of this work was to develop innovative technological solutions for spore inactivation which could support the reduction of the total thermal load applied to food products. In order to achieve this goal, two strategies were considered and are synthesized in Figure 9.1 and Figure 9.2. The first one consisted in weakening the spore by a germination treatment and followed by a mild pasteurization step. The second one focused on attaining a lethal regimen for dormant spores while still allowing for thermal load reduction.

The physiological, non-nutrient induced high isostatic pressure germination (200 MPa), though interesting by the moderate target pressure, showed a strong dependency on temperature. This was in agreement with the physiological path associated with this germination trigger. While this is a clear constraint on the generalization of this approach for sterilization applications, it allowed us to show that moderate high pressure germination is dependent on both physiological and physical aspects. Regardless of the temperature applied, the impact on the lipid inner membrane was the same from a structural perspective. The reconfiguration of the germinant receptors should then happen regardless of the temperature applied. However, it can be suspected that the cascade of reactions following - in particular enzymatic reactions - is inhibited by non-adequate temperature (in the case of this work, out of the optimal growth temperature domain of the strain). If used, this approach might lead to very slow germination of strains which are out of their optimal growth conditions and should thus be complemented by another approach.

The use of nutrient germinants would be attractive but proved to be equally limited by strain specificities. Therefore, a non-nutrient chemical germinant resulted as the best alternative with the use of CaDPA. CaDPA is a good candidate as universal chemical germinant and the improved understanding of the associated germination mechanisms could allow for the establishment of an alternative chemical (possibly a specific peptidoglycan combination) which could be validated as GRAS.

In the outlook phase of this work, the combination of high pressure germination followed by CaDPA germination was attempted (Annexes: Figure A2). While one might expect that CaDPA could easily germinate spores which were superdormant to moderate high pressure germination, these first results showed no improvement in the total germination achieved and a plateau at approximately 3 log₁₀. This observation could suggest that the succession of both treatments might lead to some form of induced superdormancy of the remaining population or that superdormant spores to both treatments share common attributes and somehow overlap.

Conclusion and outlook

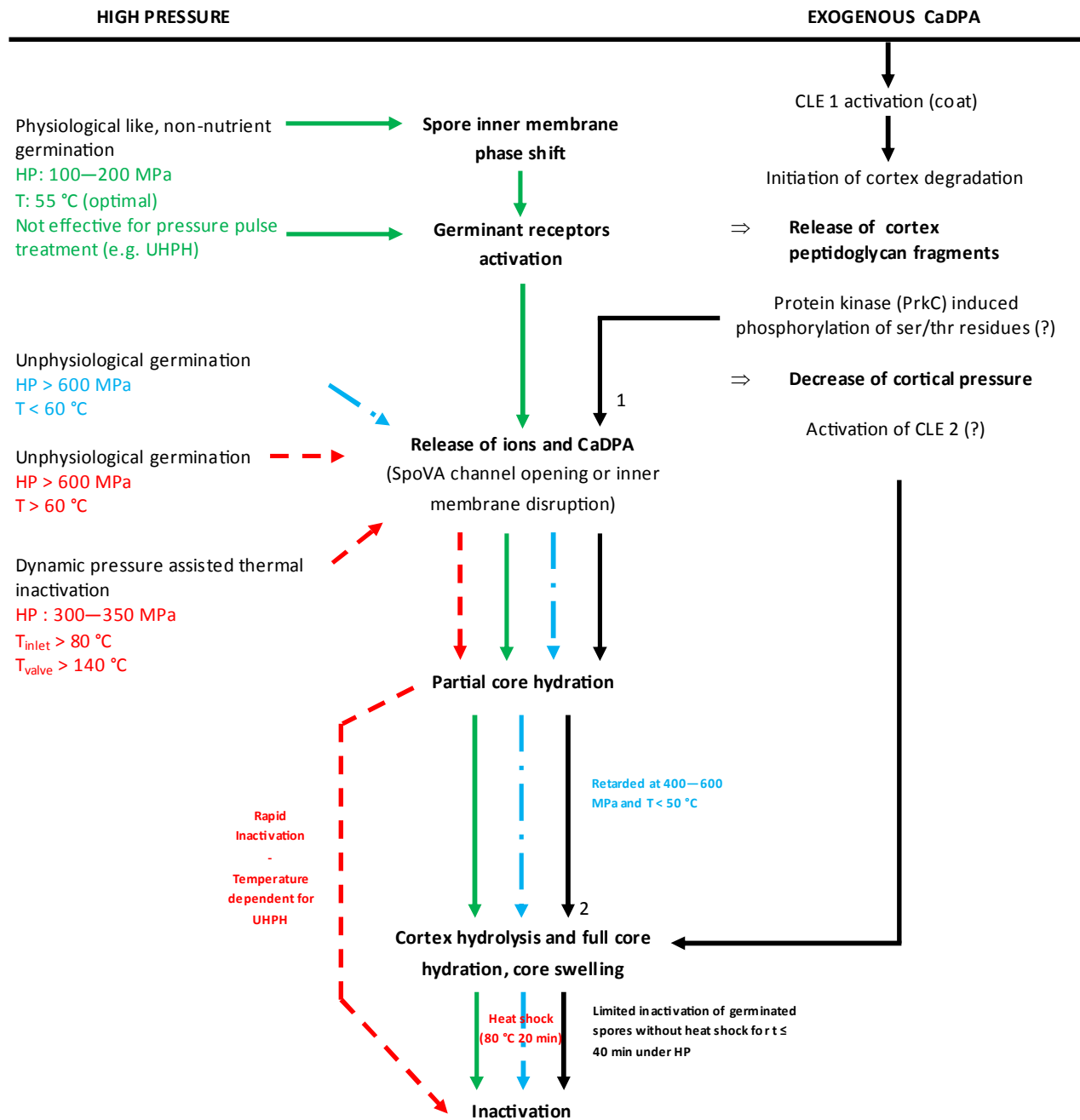


Figure 9.1: Proposed germination and inactivation pathways of *G. stearothermophilus* spores with pressure, thermal and/or chemical hurdles. ---> Direct inactivation path; → Physiological germination dependent inactivation path; → Non-nutrient, chemical germination dependent inactivation path; ----> Non-nutrient, physical germination dependent inactivation path. Based on Mathys (2008), Reineke (2012), Georget et al. (2014a), (2015a), (2014b).

Conclusion and outlook

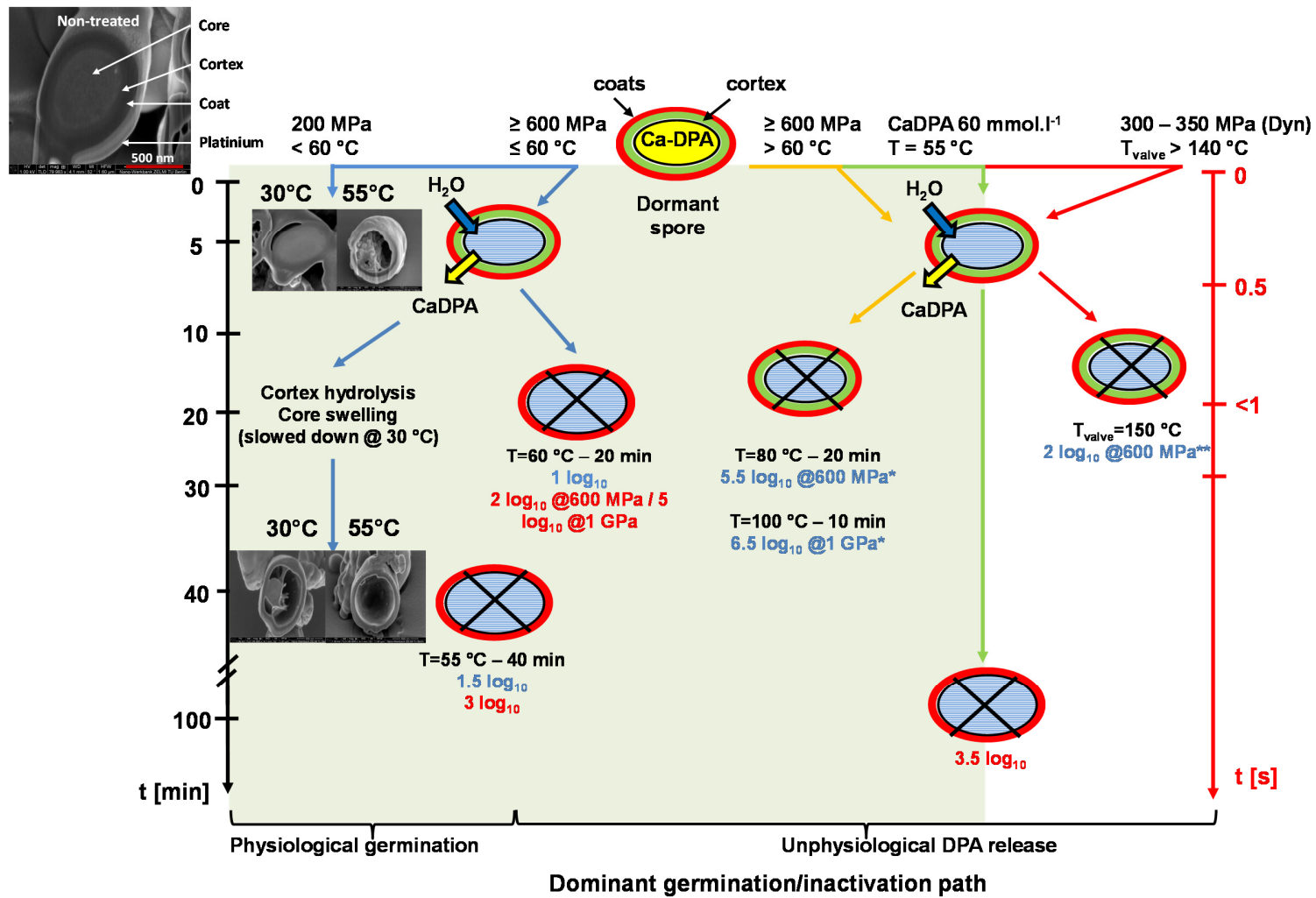


Figure 9.2: Extent of the *G. stearothermophilus* ATCC 7953 spore inactivation achieved depending on the processing conditions and duration.

*, **: no additional inactivation was achieved with heat shock. X: inactivated spores. Maximal inactivation: log₁₀: without heat shock, log₁₀: with heat shock.

Conclusion and outlook

However, this aspect will need to be the object of future in depth investigations. Additional outlook experiments also showed the possibility to germinate *G. stearothermophilus* spores with 600 MPa and 40 °C up to 3 log₁₀. However, this pressure range did not allow for extensive *in situ* investigations and remains to be researched for this indicator. Furthermore, significant research effort will be needed in understanding the impact of sporulation conditions on the germination behavior of *G. stearothermophilus* spores and how can this be tailored to produce the best indicators for germination studies.

The alternative approach was to target hurdle combinations which could induce a lethal regimen for dormant spores. In this work, dynamic pressure and temperature were combined in a successive manner through UHPH. The use of high inlet temperatures which are matching, for instance, the ones used as inlet temperature for steam injection, allowed reaching quasi instantly and for very short holding times, valve temperatures at which bacterial spores could be easily inactivated. The results in this work suggested that this process induces spore inactivation mainly through thermal inactivation and would thus make of *G. stearothermophilus* ATCC 7953 an adequate surrogate for future process validation protocols. Selecting an adequate process indicator is essential to the development of this technology and has not yet been agreed upon. However, future validations will need to establish the exact residence time distribution profile at the valve and temperature distribution to exclude the contribution of other parameters such as shear or impingement. In the light of the results gathered in this work, the best inactivation could be achieved by combining the highest pressures (> 300 MPa) and high inlet (> 80 °C) and valve temperatures (> 140 °C) for short holding times (inferior to one second). This makes of UHPH a technology competitive with direct steam injection in terms of time/temperature profiles but without adjunction of water to the product. UHPH would remain an indirect form of heating which could thus reduce operational costs. This technology could also offer to combine sterilization and homogenization in a single step thereby further reducing the overall processing time, intensity and costs. This approach is however limited to pumpable, particle free matrices.

Based on this work, the need for future work can be summarized as follows:

- Establish whether CaDPA could be GRAS or find a reaction intermediate of the germination pathway which could endorse this function on an equally universal level. If such an intermediate could be validated, attempt combination with UHPH in order to increase the inactivation level.

Conclusion and outlook

- Focus on high isostatic pressure germination *in situ* investigations in the range of 600 MPa. Recently published work managed to show the mechano-sensitive channel function of SpoVAC proteins (supposedly involved in CaDPA release) in vesicle systems. If such vesicle could be used in a DAC at pressures associated to non-physiological germination, one could validate *in situ* the impact of high pressure of the denaturation, and possibly opening of these channels. This pressure range, though higher, could, thus, enable unphysiological germination and therefore open up opportunities for reduction of spore populations in non pumpable foods which could not be treated with UHPH.
- Spore inactivation by UHPH will need to be tested in real food systems and at inlet temperature above 80 °C to ensure the safety of this process and validate the process window suggested in this work. Most of the inactivation mechanisms presented in this work were derived in simple aqueous systems such as buffer solutions, and can be affected by food constituents. The food system itself could have a protective effect on the spores because certain ingredients, such as, fats, sugars, salts, can interact with bacterial spores in a protective way as was already shown for high pressure high temperature inactivation of spores (Georget et al., 2015b).
- Equipment development: all UHPH trials were conducted using a pilot scale equipment and scalability of the UHPH equipment to the industrial level is not yet given. While some companies / start-ups are on this path (GEA Niro Soavi UHP4000 prototype 100 up to 500 L/h, BEE international homogenizers 310 MPa up to 1500 L/h, Ypsicon 350 MPa and 1000 L/h), to date, no example of the use of these pieces of equipment for commercial products by the industry was found. In particular, the optimal design, the stability and the life span of the UHPH valves will need to be proved for the extreme conditions required for spore inactivation.

Annexes

Annexes

Table A1: Mechanisms of endospore survival against physical and chemical treatments. From Reineke (2012) with permission.

| Sporicidal treatment | Defense mechanism / factor influencing the resistance | Reference |
|----------------------------------|---|---|
| Wet heat | <ul style="list-style-type: none"> • Sporulation temperature • Core's level of DPA and Ca²⁺ • α- and β- type SASP • Low water content in the core | (Raso et al., 1995; Melly et al., 2002) (Setlow et al., 2006) (Setlow, 2007) (Gerhardt and Marquis, 1989) |
| Dry heat | <ul style="list-style-type: none"> • DNA protection by α- and β- type SASP • DNA repair enzymes ExoA and Nfo (active during germination) | (del Huesca Espitia et al., 2002) (Setlow, 2006) (Salas-Pacheco et al., 2005) (Barraza-Salas et al., 2010) |
| Desiccation | <ul style="list-style-type: none"> • DNA protection by α- and β- type SASP | (Setlow, 1995) |
| Chemical resistance | | |
| Sodium chloride/ Hypochloride | <ul style="list-style-type: none"> • Spore coat – coat proteins react and detoxify | (Setlow et al., 2006) |
| Hydrogen peroxide | <ul style="list-style-type: none"> • DNA protection by α- and β- type SASP | (Melly et al., 2002) |
| Ionizing radiation | <ul style="list-style-type: none"> • DNA repair enzymes ExoA and Nfo (active during germination) • Decreased level of core water • Sulphur-rich spore coat proteins and DPA • Increased levels of Mn²⁺ and other divalent cations | (Salas-Pacheco et al., 2005) (Barraza-Salas et al., 2010) (Nicholson et al., 2000) (Nicastro et al., 2002) (Granger et al., 2011) |
| UV - radiation | <ul style="list-style-type: none"> • UV-Photochemistry of DPA - DNA – formation of “spore photoproduct” • Error free repair “spore photoproducts” • DNA protection by α- and β- type SASP • DNA repair enzymes ExoA (active during germination) • Specific DNA repair system for “spore photoproduct” | (Nicholson et al., 2000) (Douki et al., 2005a) (Douki et al., 2005b) (Setlow et al., 2006) (Nicholson et al., 2000) (Nicholson et al., 2000) |
| High pressure | <ul style="list-style-type: none"> • Sporulation temperature • Demineralization of the core • Ability to retain DPA | (Raso et al., 1998b) (Igura et al., 2003) (Margosch et al., 2004) (Reineke et al., 2012c) |

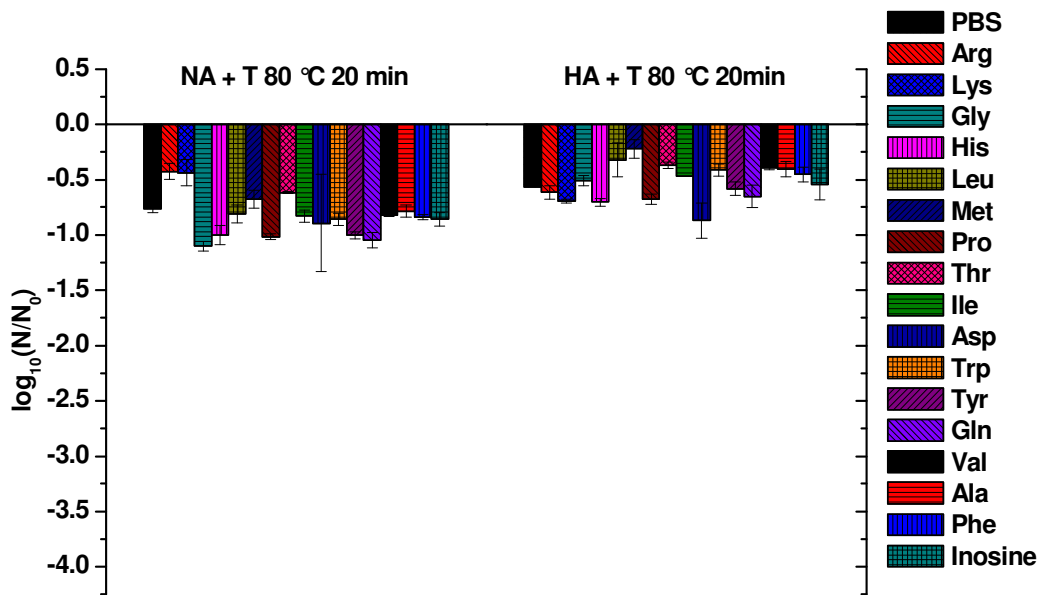


Figure A1: Germination of *G. stearothermophilus* spores with different amino acids (L-amino acids, 50 mmol.l⁻¹ in PBS buffer, pH 7) and inosine (5 mmol.l⁻¹ in PBS buffer, pH 7) (initial concentration of spores: 10⁷ CFU.mL⁻¹). T 80 °C 20 min indicates the thermal treatment applied post incubation in germinants to assess the germinated population. NA: non-heat activated – HA: heat activated 80 °C for 30 min (Georget et al. (2015a) Food Control, 50, p.141-149 – with permission from Elsevier).

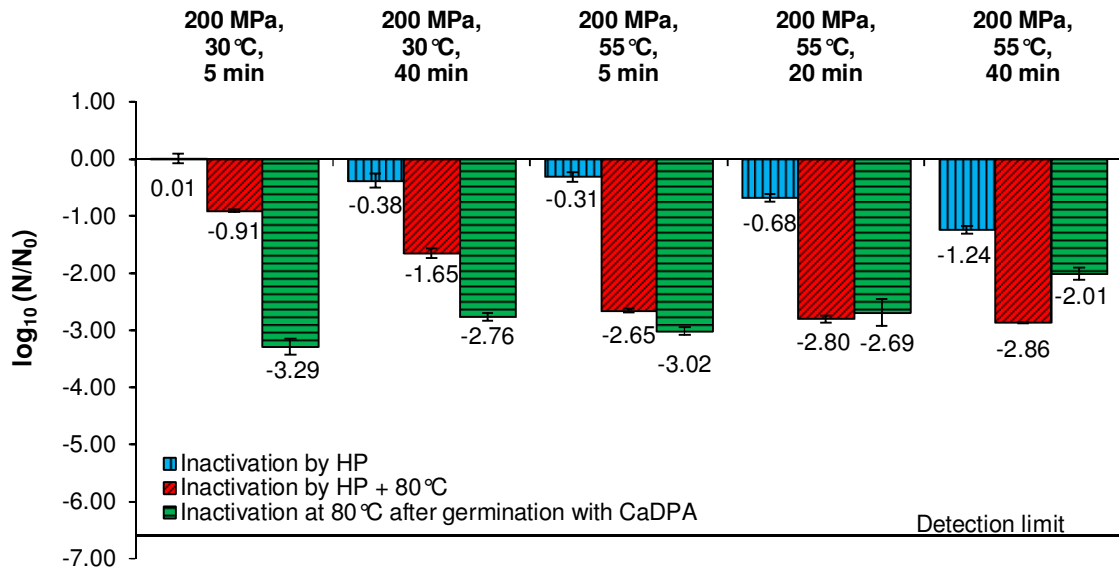


Figure A2: Germination and inactivation of *G. stearothermophilus* spores by HP at 200 MPa, before and after heat shock at 80°C 20 min. CaDPA corresponds to a subsequent germination of the HP treated and heat shocked spores with 60 mM CaDPA and inactivation with temperature at 80 °C 20 min (initial concentration of spores $\approx 10^8$ CFU/ml).

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Curriculum vitae

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LIST OF PUBLICATIONS

Peer reviewed publications

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2. Georget E., Kushman A., Callanan M., Ananta E., Heinz V., Mathys A. (2015) *Geobacillus stearothermophilus* ATCC 7953 spores chemical germination mechanisms in model systems. *Food Control*, 50, 141-149.
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5. Georget E., Kapoor S., Winter R., Reineke K., Song Y., Callanan M., Ananta E., Heinz V., Mathys A. (2014) *In situ* investigation of *Geobacillus stearothermophilus* spores germination and inactivation mechanisms under moderate high pressure. *Food Microbiology*, 41, 8-18
6. Georget E., Sauvageat J.L., Burbidge A., Mathys A. (2013) Residence time distributions in a modular micro reaction system. *Journal of Food Engineering*. 116(4), 910-919.

Chapters in edited books

7. Bolumar T., Georget E., Mathys A. High pressure processing. In: Electron beam pasteurization and complementary food processing technologies. Eds: Pillai S. D. and Shayanfar S. Publisher: Woodhead Publishing Series in Food Science, Technology and Nutrition No. 271. ISBN 1 78242 100 9 – November 2014.
8. Georget E., Reineke K., Heinz V., Knorr D., Ananta E., Mathys A. Spore inactivation mechanisms during industrial food and equipment sterilization. In: *Encyclopedia of Biotechnology in Agriculture and Food*, 2013. Eds: Heldman D.R., Hoover D.G., Wheeler M.B. Publisher: Taylor and Francis: New York, 1-6.

Other publications

9. Georget E., Heinz V., Mathys A. Spore inactivation by novel multi-hurdle approaches: new hopes for the sterilization of sensitive products. *eFOOD-Lab International magazine*, 01/2015, 18-21.
 10. Mathys A., Georget E., Heinz V. Schonende Sterilisation für hochqualitative Nahrungsmittel. *Rundschau für Fleischhygiene und Lebensmittelüberwachung RFL* 65, 12/2013, 450-452.
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PRESENTATIONS

Invited oral presentations

1. Georget E., Miller B., Callanan M., Mathys A., Heinz V. Germination and inactivation mechanisms of *Geobacillus stearothermophilus* spores by emerging multi hurdle combinations. 28th EFFoST Conference, Uppsala, Sweden.
2. Georget E. (2014). Ultra-high pressure homogenization and its potential for sterilization of pumpable foods. IFT Annual Meeting 2014, New Orleans, LA, USA.
3. Georget E. (2014). Inactivation of *Geobacillus stearothermophilus* spores through combined hurdles: from the mechanistic study to application. IAFP's 10th European Symposium on Food Safety, Budapest, Hungary.
4. Mathys A., Georget E., Waldeck P., Petrusan J., Heinz V. (2013). Microalgae as alternative protein source in a sustainable bioeconomy. 19th International Scientific Conference "Capabilities of vegetable proteins", Potsdam, Germany.
5. Töpfl S. and Georget E. (2012). Minimal Processing of Heat Sensitive Liquids by Pulsed Electric Fields and Antimicrobial Ingredients. IFT Annual Meeting 2012, Las Vegas, NV, USA.

Oral presentations

6. Georget E. (2014). *Geobacillus stearothermophilus* spore germination and inactivation mechanisms during innovative multi hurdle combinations for food sterilization. 8th European Workshop on Food Engineering and Technology, Quakenbrück, Germany.
7. Georget E. (2013). Breaking the wall of food safety. 2013 Falling Walls Lab, Berlin, Germany.
8. Georget E., Kushman A., Heinz V., Callanan M., Ananta E., Mathys A. (2013). *Geobacillus stearothermophilus* spore inactivation mechanisms as key for new multi hurdle preservation combinations. 2013 EFFoST Annual Meeting: Bio-based Technologies in the Context of European Food Innovation Systems, Bologna, Italy.
9. Mathys A., Georget E., Waldec P., Petrusan J., Heinz V. (2013). Sustainable protein supply by algae biorefinery concepts. 2013 EFFoST Annual Meeting: Bio-based Technologies in the Context of European Food Innovation Systems, Bologna, Italy.
10. Georget E., Kushman A., Heinz V., Callanan M., Ananta E., Winter R., Mathys A. (2013). *Geobacillus stearothermophilus* germination and inactivation pathways investigated under combined hurdles. iFOOD2013 conference, Hanover, Germany.
11. Georget E., Kapoor S., Winter R., Ananta E., Heinz V., Mathys A. (2012). High pressure germination mechanisms of *Geobacillus stearothermophilus* spores investigated by *in* and *ex situ* techniques. 7th International Conference of High Pressure Bioscience and Biotechnology, Otsu, Japan.
12. Georget E., Kapoor S., Lampe D., Mathys A., Ananta E., Reineke K., Töpfl S., Bindrich U., Winter R., Heinz V. (2012). *In situ* investigation of *Geobacillus stearothermophilus* spores germination and inactivation under high pressure. 50th EHPRG Meeting, Thessaloniki, Greece.
13. Mathys A., Georget E., Sauvageat J. L., Wermeille A., Ulrich S. (2011). Micro Process Engineering in Food and Biotechnology. Oral presentation at iFOOD2011 conference, DIL Quakenbrueck, Germany.

Curriculum vitae

Poster presentations

14. Georget E., Song Y., Kapoor S., Ananta E., Heinz V., Winter R., Mathys A. (2013). High Pressure Germination Mechanisms of *Geobacillus stearothermophilus* Spores Investigated by in situ FTIR and Fluorometry. Poster presentation at IFT Annual Meeting 2013, Chicago, IL, USA.
15. Vorwerk D., Georget E., Figura L., Heinz V., Mathys A. (2013). Potential for micro processing equipment in production of water-in-oil emulsions. Poster presentation at the iFOOD2013 conference, Hanover, Germany.
16. Bourdin G., Georget E., Mathys A. (2012). Micro process engineering for thermal preservation. Poster presentation at the 6th Central European Congress on Food, Novi Sad, Serbia.
17. Aganovic K., Georget E., Ananta E., Heinz V., Mathys A. (2012). Impact of isostatic and dynamic pressure on *Geobacillus stearothermophilus* bacterial spores germination and inactivation. Poster presentation at the 7th International Conference of High Pressure Bioscience and Biotechnology, Otsu, Japan.
18. Georget E., Sauvageat J. L., Burbidge A., Mathys A. (2012). Impact of Residence Time Distributions in a Modular Micro Reaction System. Poster presentation at IFT Annual Meeting 2012, Las Vegas, NV, USA.

HONORS AND AWARDS

- 2014 Winner of the IFT Nonthermal Processing Division Graduate Paper competition at the IFT Annual Meeting in New Orleans, USA.
- 2014 Nonthermal Processing Division's Outstanding Volunteer Award 2013-14.
- 2014 Winner of the IAFP European Symposium Student Travel Scholarship Award at the European Symposium 2014 in Budapest, Hungary.
- 2014 2nd Place of the Julius-Maggi Research Competition at the 8th European Workshop on Food Engineering and Technology, Quakenbrueck, Germany
- 2013 Winner of the GNT award for young scientists at the EFFoST 2013 Annual Meeting, Bologna, Italy.
- 2013 A.T. Kearney Scholarship for the Falling Walls Conference 2013, Germany.
- 2013 2nd Place of the Institute of Food Technologists IFT13 Nonthermal Processing Division Graduate Paper competition at the IFT Annual Meeting 2013, Chicago, USA.
- 2012 3rd Place of the "Charles R. Stumbo Student Paper Competition" of the Institute For Thermal Processing Specialists at the Annual Meeting 2012, San Antonio, USA.