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Germination, emergence and resumption of growth of bacterial spores after a heat treatment

Clément Trunet

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Clément Trunet

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de Biodiversité et d'Ecologie Microbienne
LUBEM EA3882

**Germination,
émergence et reprise de
croissance de spores
bactériennes après un
traitement thermique**

Thèse soutenue le 4 juillet 2016

devant le jury composé de :

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*A mon père,
à toute ma famille*

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Avant-propos

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- Le LUBEM qui est spécialisé en microbiologie alimentaire, notamment par l'étude de la biodiversité des flores microbiennes et le développement d'outil de microbiologie prévisionnelle pour assurer la maîtrise de leur comportement ;
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Chapitre 1

Introduction générale

Les bactéries sporulées sont d'une grande importance en écologie microbienne, que ce soit au niveau environnemental, médical ou alimentaire. Elles dominent la flore du sol et peuvent être retrouvées dans l'appareil digestif des mammifères ou des insectes (Postollec *et al.*, 2012). Sous la forme de spores, elles sont capables de résister à de nombreux stress chimiques ou physiques extrêmement intenses. Elles seraient même capables de résister aux températures très élevées qu'elles subiraient lors d'une hypothétique traversée de l'atmosphère (Slobodkin *et al.*, 2015). Elles sont aussi capables de reprendre une croissance après être restées en dormance des millions d'années (Torred *et al.* 2012). Ces capacités de résistance et de persistance en font des contaminants difficiles à maîtriser pour les industriels, d'autant plus que certaines bactéries sporulées ont un caractère pathogène ou sont responsables de l'altération des produits alimentaires. Elle représente donc un risque sanitaire et économique important.

Les bactéries sporulées : contaminants alimentaires

1.1.1. Généralités

Les bactéries sporulées appartiennent à l'ordre des *Bacilliales*, dont sont issus différents genres comme *Bacillus* sp., *Alicyclobacillus* sp., *Geobacillus* sp., *Paenibacillus* sp., ou *Brevibacillus* sp. ; et à la classe des *Clostridia* et *Clostridium* sp., *Moorella* sp. ou *Thermoanaerobacterium* sp. (Bergey's 2013, Fritze, 2004)

Les *Bacilliales* sont ubiquitaires et font notamment partie des colonisateurs naturels de la flore du tractus gastro-intestinal des insectes et de nombreuses espèces d'animaux à sang chaud. Elles présentent une large gamme de caractéristiques phénotypiques et génotypiques et comprennent des souches psychrotolérantes, psychrophiles, mésophiles, thermotolérantes et

thermophiles, avec des capacités à se développer en aérobiose, aéro-anaérobiose, anaérobiose stricte. Les espèces du genre *Bacillus* et apparentées dominent la microflore du sol et sont donc rencontrées au niveau des végétaux, des aliments et des environnements naturels ou créés par l'homme (Guinebretière *et al.*, 2008; Postollec *et al.*, 2012).

Le genre *Bacillus* peut être retrouvé comme contaminant alimentaire à différents stades des procédés de fabrication, depuis les matières premières jusqu'au produit final. Les capacités d'adhésion, de formation de biofilms, et de résistance au stress, par leur forme sporulée, en font un agent important de contamination. Au sein de ce genre, on retrouve *Bacillus cereus*, une cause majeure, avec 309 foyers déclarés en 2013, de Toxi-Infections Alimentaires Collectives (TIAC) en France (Six *et al.*, 2012). Cette espèce est capable d'entraîner deux types d'intoxications alimentaires. Le symptôme dit « émétique » est dû à une toxine, le céréulide, produite dans les aliments et résistante à une large gamme de températures (15-20 minutes à 121°C, voire 90 minutes à 126°C) et de pH (2-11). Le syndrome « diarrhéique » est dû à plusieurs entérotoxines produites pendant la croissance de la bactérie dans l'intestin grêle après ingestion de cellules végétatives ou de spores en quantité suffisante (Carlin and Nguyen-The, 2013). Les niveaux de contaminations des aliments incriminés dans les toxi-infections alimentaires liées à *Bacillus* sont relativement élevés et vont de 10^3 à 10^6 UFC.g⁻¹ (Six *et al.*, 2012). Ainsi et selon la réglementation européenne, le seuil réglementaire est établi à 500 UFC.g⁻¹ pour les aliments déshydratés (Règlement (CE) 2073/2005 et (CE) 1441/2007). D'autres espèces, comme *Bacillus licheniformis*, ont pu être associées à des toxi-infections alimentaires dues à des toxines thermorésistantes (Salkinoja *et al.*, 1999). Les bactéries du genre *Bacillus* sont aussi responsables d'altérations des aliments. Elles entraînent des défauts organoleptiques par la production de gaz, d'acides organiques, d'enzymes ou d'autres composés indésirables (Burgess *et al.*, 2010; Postollec *et al.*, 2012).

On retrouve le genre *Bacillus* dans de nombreux produits comme les aliments prêts à l'emploi, les plats traités thermiquement ou encore les produits cuisinés réfrigérés à durée de vie étendue ou REPFEDs (REfrigerated Processed Food of Extended Durability). Les produits déshydratés tels que le lait en poudre, les soupes déshydratées, les herbes et épices peuvent être contaminés par des spores de *Bacillus* (Hariram and Labbé, 2015; Oomes *et al.*, 2007).

Différentes espèces de *Bacillus* peuvent être retrouvées dans ces aliments. Or ces différentes espèces ont des comportements différents vis-à-vis des conditions environnementales et notamment de la température de conservation. Au sein du groupe *Bacillus cereus sensu lato*, on retrouve sept groupes phylogénétiques qui peuvent être discriminés par leur gamme de température de croissance (Tableau 1). Le groupe *Bacillus cereus sensu lato* rassemble des espèces pathogènes (*B. anthracis*, *B. cereus sensu stricto*) mais aussi des espèces utilisées en industrie comme *B. thuringiensis* en tant que biopesticide au sein de six groupes. *Bacillus weihenstephanensis* appartient au groupe VI, qui comprend des espèces psychrotolérantes. Ces espèces sont donc mésophiles, avec une température optimale de croissance entre 20 et 40°C mais sont capables de croître à des températures basses (jusqu'à 5°C). On retrouve dans ce groupe *Bacillus weihenstephanensis*.

Un second groupe d'intérêt majeur chez le genre *Bacillus* est le groupe *Bacillus subtilis*, ou complexe *Bacillus subtilis*. Ce groupe contient 13 espèces et on y retrouve *Bacillus licheniformis* qui est thermotolérante et peut être responsable d'altération sur les produits laitiers. Elles peuvent être utilisées en tant que probiotiques en alimentation animale, comme *Bacillus subtilis*, ou pour leur capacité à produire des métabolites d'intérêts, comme *Bacillus licheniformis* (Rooney *et al.*, 2009; Zohra *et al.*, 2015).

Les groupes *Bacillus cereus sensu lato* et *Bacillus subtilis* ont reçu une attention particulière au vu de leur grand potentiel d'application. De plus, leur potentiel de contamination est très élevé puisque ces bactéries sont capables de résister aux traitements thermiques puis de germer et reprendre une croissance dans une large gamme de température.

Tableau 1 : Gamme de croissance et thermorésistance des différents groupes phylogénétiques du groupe *Bacillus cereus sensu lato* (d'après (Afchain *et al.*, 2008; Guinebretière *et al.*, 2008; Luu-Thi *et al.*, 2014).

Groupe phylogénétique	Température minimale de croissance	Température maximale de croissance	Valeur moyenne de thermo résistance à 90°C (minutes) ¹	Valeur moyenne de thermo sensibilité (°C) ¹
VII	20	50	90.3	5.7
III	15	45	39	8.4
IV	10	45	25.7	10.5
I	10	43	N/A	N/A
V	8	40	20.5	11.0
II	7	40	30.5	10.2
VI*	5	37	1.7	12.0

* groupe comprenant *Bacillus weihenstephanensis*

¹La thermo résistance, notée D , correspond à la durée nécessaire pour réduire la population bactérienne initiale d'un facteur 10.

²La thermo sensibilité, notée z_T , est l'élévation de température de traitement thermique nécessaire pour réduire la thermo résistance d'un facteur 10.

Comment ces bactéries peuvent-elles être retrouvées dans des produits ayant subis des traitements thermiques ? Les cellules sporulées peuvent être retrouvées dans les sols (Postollec *et al.*, 2012), Carlin, 2011). Les matières premières (légumes, céréales, lait...) peuvent alors être contaminées par des spores, il est alors difficile de connaître leur origine ou précisément les conditions de formation des spores (Hariram and Labbé, 2015; Iurlina *et al.*, 2006; Reyes *et al.*, 2007). Certains procédés industriels comportent des temps de stockage entre deux étapes permettant le développement des bactéries sporulées et la formation de nouvelles spores. Dans ce cas, les caractéristiques de l'environnement de sporulation en termes de température, de pH, d' a_w et même de substrats peuvent être plus précisément connues. Ces éléments peuvent être très importants car ils vont conditionner les caractéristiques des spores. Il est envisageable

de déduire les caractéristiques des spores formées, comme leur thermorésistance à partir de ces conditions, notamment par l'utilisation d'outils de microbiologie prévisionnelle (Baril *et al.*, 2012).

Les traitements thermiques que subissent les produits au cours du procédé de fabrication vont inactiver toutes les cellules végétatives (quelques minutes à 80°C suffisent (Irudayaraj, 2001)). Ici, le traitement thermique peut être un traitement d'inactivation, comme une stérilisation ou une pasteurisation, ou bien une cuisson. Ces traitements peuvent être de 15-30 minutes à 60-65°C pour une pasteurisation basse à un traitement équivalent à plusieurs minutes à 121.1°C pour les produits en conserves ou les produits UHT (Ultra Haute Température) (Bazinet and Castaigne, 2011). Les différentes espèces retrouvées au sein des bactéries sporulées sont plus ou moins résistantes aux traitements thermiques. Par exemple, un traitement de 4 à 5 minutes à 121°C est nécessaire pour réduire la population de 90 % de *Geobacillus stearothermophilus* contre seulement 0,05 minutes à la même température pour *Bacillus subtilis* (Condon *et al.*, 1992). Suite à de tels traitements, une partie des spores peuvent aussi être inactivée mais certaines sont capables de survivre. La thermo résistance des spores peut être décrite à l'aide de modèles mathématiques. Ces modèles permettent d'estimer la thermo résistance d'une population, noté D ou Σ , et sa thermo sensibilité, notée z_T (Table 1) (Mafart and Leguérinel, 1997). Bien sûr, l'intensité du traitement thermique a une grande importance notamment sur la diversité des contaminants : un traitement thermique élevé (comme l'appertisation dans l'industrie de la conserve) va inactiver les *Bacillus* peu thermorésistants et psychrotrophes alors que ces derniers peuvent présenter un risque pour les produits chauffés modérément et stockés à basse température (Augustin, 2011). En effet, il existe un lien entre les températures cardinales de croissance et la thermorésistance des spores bactériennes. Cela peut venir de l'adaptation des cellules végétatives à la température : les protéines entrant dans la composition des spores des espèces les plus thermophiles sont moins thermolabiles et donc les spores sont plus thermorésistantes (Luu-Thi *et al.*, 2014; Warth, 1980, 1978). Ainsi, dans le produit fini, ou traité thermiquement, subsistent en majorité des spores bactériennes. On peut aussi noter que certains procédés comme le séchage vont concentrer le produit, comme le lait en poudre par exemple. Ainsi, les contaminants thermorésistants vont être aussi concentrés

dans le produit fini (Scott *et al.*, 2007, p. 7). Au cours du stockage des produits finis, si les conditions sont favorables, les spores sont capables de germer et de se développer dans l'aliment. Les conditions dans lesquelles se retrouvent les spores après le traitement thermique, la température ou le pH du produit par exemple (qui sera désigné dans ce travail comme « milieu de récupération »), impactent fortement sur leur capacité à retrouver une activité métabolique et donc à se multiplier (Leguérinel *et al.*, 2006; Leguerinel *et al.*, 2000; Daelman *et al.*, 2013; Gaillard *et al.*, 2005). De plus, lorsque la température n'est pas suffisamment élevée pour entraîner une perte de viabilité des spores (inactivation), elle est susceptible d'accroître la vitesse de germination des spores par un phénomène d'activation (Abee *et al.*, 2011)(Ter Beek and Brul, 2010). La germination et la reprise de croissance jouent un rôle très important car ces processus sont préliminaires à la formation des cellules végétatives capables de se multiplier et de coloniser les aliments. Les molécules retrouvées dans la matrice alimentaire, comme les sucres, les nucléotides ou les protéines peuvent initier la germination, en plus des traitements thermiques pouvant agir dans l'activation des spores (Figure 1).

La forme cellulaire la plus résistante aux traitements thermiques est donc la spore. Nous nous intéressons donc plus précisément à cette structure capable de résister à de nombreux traitements, persister dans des environnements extrêmes et se revivifier malgré tous les stress subis.

1.1.2 Le cycle de vie des bactéries sporulées

La particularité des *Bacilliales* est de former des spores leur permettant de résister à de nombreux stress. Au cours de leur développement, les cellules végétatives peuvent rencontrer des conditions défavorables comme un manque de nutriments après une croissance. Les cellules végétatives font face à cette carence nutritionnelle en entamant un processus de sporulation qui leur permettra de survivre dans un environnement qui ne permet plus leur développement (Errington, 2003; Tan and Ramamurthi, 2014).

Le processus de sporulation est irréversible et conduit à la formation d'une spore dormante et résistante. Les conditions dans lesquelles sont produites ces spores influent sur les caractéristiques des spores elles-mêmes (Atrih and Foster, 2002, p. 199; Baril *et al.*, 2011). En

effet, la température ou le pH de sporulation influent sur i) la structure-même des spores, ii) sur leurs résistances à différents stress et iii) sur leur capacité à germer et se multiplier de nouveau (Baril *et al.*, 2012, 2011; Garcia *et al.*, 2010; Planchon *et al.*, 2011). Ces trois phénomènes seront donc présentés dans les différentes parties de cette étude bibliographique.

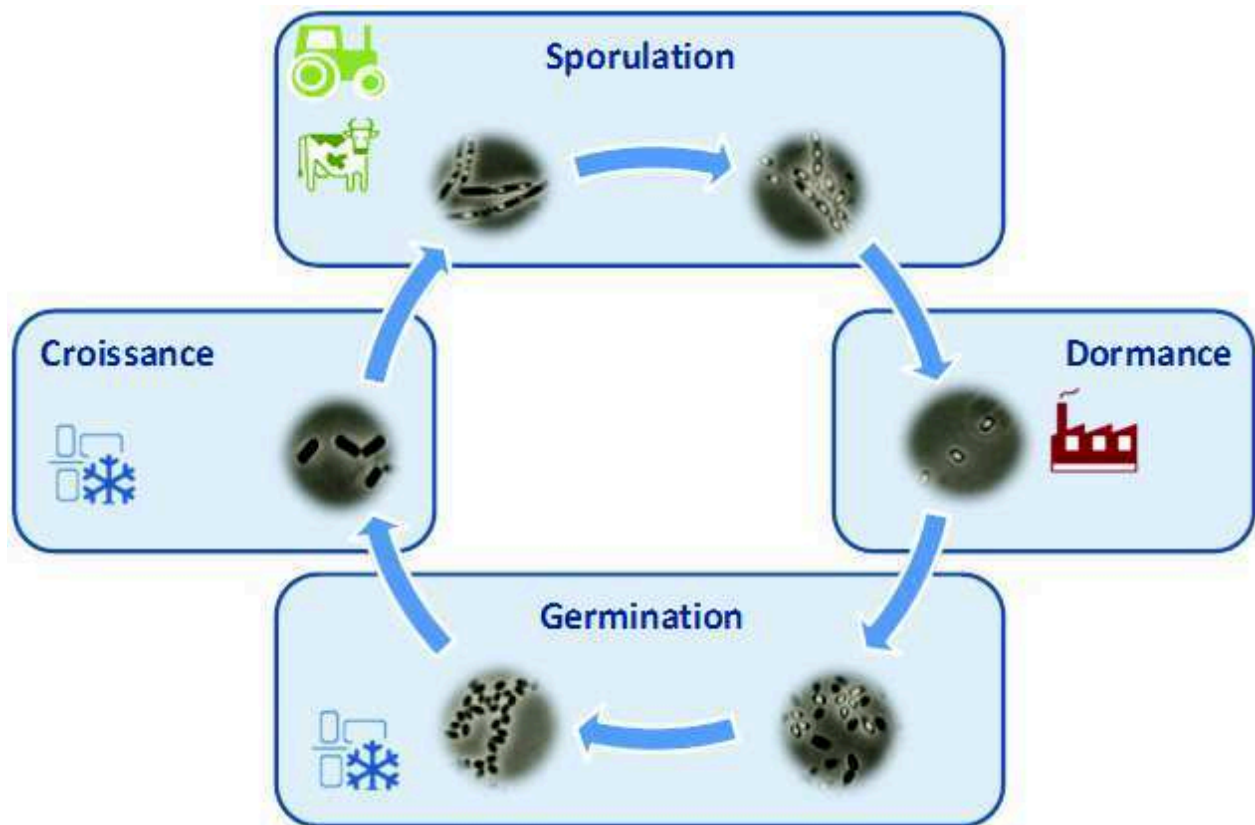






Figure 1 : Mise en parallèle des étapes du cycle de vie de *Bacillus* et des étapes de procédé industriel (d'après Errington 2006, Postollec 2012, Carlin 2011).

 représente l'environnement agricole. La flore sporulée est largement retrouvée dans les sols, et va donc pouvoir être retrouvée sur les matières premières issues des champs.

 représente les réservoirs animaux. On peut retrouver des spores bactériennes dans le tractus gastro-intestinal de certains mammifères ainsi que chez certains arthropodes et vers. On les retrouve donc dans les fèces d'animaux. On peut donc retrouver dans les matières premières des cellules végétatives et des spores. On cherche à limiter la contamination des matières premières par les bactéries sporulées.

 représente les procédés industriels pendant lesquels les spores et les cellules végétatives vont subir des traitements thermiques. A l'issue de ce traitement, seuls les cellules les plus résistantes subsistent, c'est-à-dire les spores. Les traitements thermiques doivent être optimisés pour éliminer ces spores.

 représente le produit fini. Durant le stockage, les spores peuvent germer et reprendre une croissance si les conditions leur permettent. Ici, la formulation du produit ou la température de stockage vont impacter la capacité des spores à germer et reprendre une croissance.

Structure d'une cellule bien particulière, la spore bactérienne

La spore bactérienne a une structure complexe. Chaque élément joue un rôle dans sa résistance aux stress ou est lié à sa capacité de germination et de reprise de croissance (Figure 2). Ces différentes structures sont mises en place durant la sporulation. Durant les premiers temps de la germination, seules les structures et les enzymes mises en place au sein de la spore pendant la sporulation vont jouer un rôle. Ce qui rappelle à nouveau l'important lien qui existe entre les conditions de sporulation et les caractéristiques intrinsèques de la spore.

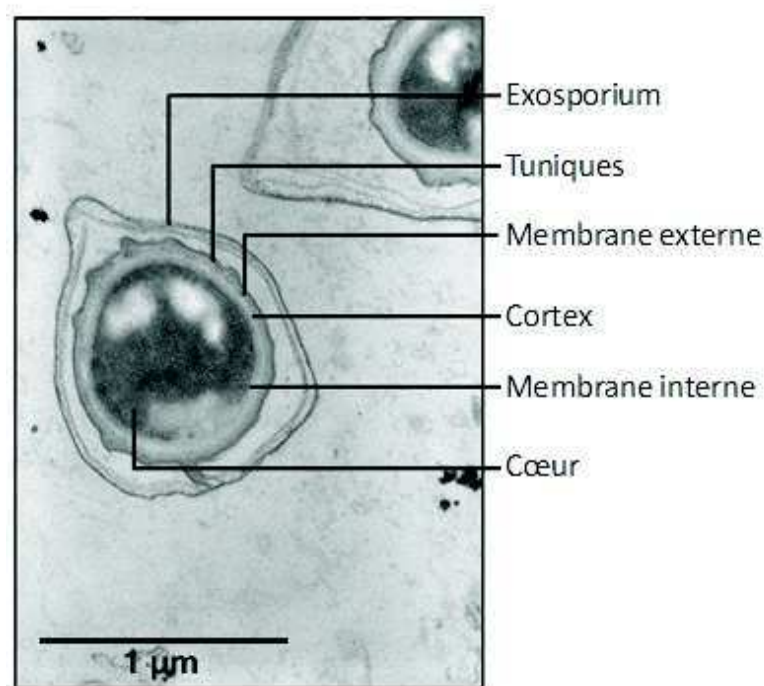


Figure 2 : Coupe transversale d'une spore de *Bacillus weihenstephanensis* KBAB4 montrant les structures concentriques la spore. Observation réalisée au microscope électronique à transmissions (x35000). Photographie : I. Bornard, E. Baril INRA Avignon, 2009.

1.2.1 L'exosporium

L'exosporium est la partie la plus externe des spores bactériennes, principalement chez le groupe *Bacillus cereus* (Todd *et al.*, 2003). Certaines espèces en sont dépourvues ou en possèdent de taille réduite, comme *B. subtilis* (Waller *et al.*, 2004). L'exosporium de *B. cereus* est principalement composé de protéines (43-52% du poids sec). Il est également composé de sucres (20-22%), comme le glucose (9.1%), le rhamnose (6.4%), le glucosamine (3.8%) et le

ribose (0.7%). Les lipides constituent aussi une part importante de cette structure (18%). Enfin, quelques éléments peuvent être retrouvés à l'état de trace dans l'exosporium comme le calcium, le phosphate, le magnésium (Ball *et al.*, 2008; Kailas *et al.*, 2011; Matz *et al.*, 1970). La composition exacte et la taille de l'exosporium varie chez les différentes espèces du groupe *B. cereus sensu lato* (Faille *et al.*, 2010). Cette structure bulbeuse (*balloon-like*) est composée de deux couches : la partie basale paracrystalline (état intermédiaire entre liquide et cristallin) et la partie externe filamenteuse (*hair-like*) (Henriques and Moran, Jr., 2007). Cette dernière semble être formée de molécules proches du collagène, les protéines BclA, chez *B. anthracis*, ou des protéines homologues chez *B. cereus*. (Boydston *et al.*, 2005). Cette structure filamenteuse est impliquée dans la résistance de la spore aux traitements chimiques et enzymatiques, et dans ses capacités d'adhérence aux surfaces dues à ses propriétés d'hydrophobicité (Kozuka and Tochikubo, 1985).

Un exosporium endommagé ne semble pas affecter la germination de *B. cereus*. Cependant, l'exosporium pourrait contenir une enzyme, l'alanine racémase (Alr) qui permet la conversion de L-alanine en D-alanine qui entraîne une auto-inhibition de la germination chez différentes espèces de *Bacillus* (Todd *et al.*, 2003; Venir *et al.*, 2014).

1.2.2 Les tuniques

Les tuniques sont une structure multiprotéique, composées de plusieurs couches minces, sous-jacentes à l'exosporium. (Little and Driks, 2001) (Qin and Driks, 2013). Deux couches principales peuvent être différenciées : la tunique interne (*inner layer*) et la tunique externe (*outer layer*). La formation des tuniques fait intervenir, lors de sporulation, de nombreuses protéines de structure qui permettent la mise en place des couches des tuniques, telles que CotT, CotD ou encore CotE. La synthèse de ces protéines est affectée par les conditions de sporulation, notamment la température (Zheng *et al.*, 1988)(Bourne *et al.*, 1991; Henriques and Moran, Jr., 2007)(Planchon *et al.*, 2011). La composition et la structure des tuniques peuvent varier selon les espèces ou les souches de *Bacillus* étudiées (Chada *et al.*, 2003; Giorno *et al.*, 2007). Le nombre de couches composant les tuniques varie et leur composition protéique peut varier selon les espèces. Ainsi, la protéine CotE est retrouvée chez la plupart des espèces de *Bacillus*

mais CotC ou encore CotT ne sont retrouvées que chez *B. subtilis* (Henriques and Moran, Jr., 2007; Little and Driks, 2001).

Les tuniques sont principalement composées de protéines (environ 30% des protéines totales de la spore) (Henriques *et al.* 2000). Ces protéines sont liées par des ponts disulfures et forment un réseau (*cross linking* proteins) (Leggett *et al.*, 2012). Cette structure permet de limiter le passage de grosses molécules, tel que le lysozyme qui pourrait entraîner la lyse du cortex (Nicholson *et al.*, 2000). Les tuniques permettent à la spore de résister à différents traitements chimiques ou physiques. Par exemple, elles protègent la spore contre les agents oxydants (peroxyde d'hydrogène, peroxinitrite, hypochlorite de sodium) en les détoxifiant (Setlow, 2006). La délétion d'une seule protéine des tuniques, dans la plupart des cas, n'entraîne pas de diminution significative de la résistance ou de la capacité à germer. Cela laisse penser qu'un certain nombre de protéines des tuniques ont des rôles redondants, exception faite de cinq protéines morphogénétiques qui sont SpoIVA, SPOIVD, SafA, CotE et CotH (Caroll, 2008). Par exemple, des mutants dépourvus du gène *cotE* ou des spores dépourvues de tuniques par un traitement chimique sont moins résistantes au peroxinitrite que les souches sauvages (Genest *et al.*, 2002). Les tuniques permettent la résistance à certains rayonnements, UV-A et UV-B, et la comparaison de spores de type sauvage et de mutants ayant des tuniques altérées suggèrent que la structure responsable de cette résistance est la couche interne des tuniques (Riesenman and Nicholson, 2000). Des spores de *Bacillus subtilis* ayant des tuniques immatures ou endommagées par traitement chimique germent spontanément dès qu'elles sont placées dans un milieu favorable à leur germination. La structure même des tuniques joue donc un rôle de barrière vis-à-vis des signaux de nature chimique et permet d'éviter une germination spontanée ou non désirée menant alors à l'apparition d'une nouvelle cellule dans des conditions ne permettant pas son développement (Jenkinson *et al.* 1980).

Certaines enzymes ayant un rôle important dans la germination sont enchâssées dans les tuniques. Ainsi, CwlJ, une enzyme responsable de l'hydrolyse du cortex, se trouve dans les tuniques. Elle est activée par le complexe acide dipicolinique-Ca²⁺ (CaDPA) qui est un composant essentiel de la spore, retrouvé dans le cœur. Le CaDPA est libéré au cours de la germination, comme décrit dans la partie 1.4.1. *La germination*. SleB est aussi une enzyme de lyse du cortex

retrouvée dans les tuniques. Ces enzymes font partie des CLE, pour Cortex Lytic Enzyme (Heffron *et al.*, 2009; Paredes-Sabja *et al.*, 2011; Setlow, 2003). Les protéines GerP, jouant un rôle dans les premiers stades de la germination semblent être retrouvées également dans les tuniques (Behravan *et al.*, 2000).

Les tuniques sont séparées du cortex par la membrane externe qui ne semble pas jouer de rôle dans l'imperméabilité de la spore ni contribuer à la résistance aux traitements physiques ou chimiques (Nicholson *et al.*, 2000). Cependant, des études sont toujours menées pour préciser son possible rôle dans la perméabilité aux germinants. On retrouve, sous cette membrane, une couche de peptidoglycane représentant 10% de la masse sèche de la spore. Il est le composant principal de deux couches : le cortex et la paroi cellulaire interne primordiale (*inner primordial cell wall*).

1.2.3 Le cortex

Le cortex est composé essentiellement de peptidoglycane qui est proche de celui retrouvé chez les cellules végétatives. Plus précisément, le cortex est composé de glycanes alternant acide N-acetyl-glucosamine et acide N-acetyl-muramique (Popham, 2002). Il possède quelques spécificités notables, par rapport au peptidoglycane de la cellule végétative, comme l'absence totale d'acide teichoïque (Atrih *et al.*, 1998). Il contient aussi du D-lactame qui a une importance notable pour la germination puisqu'il semble être reconnu par les enzymes lytiques du cortex (SleB et CwlJ notamment) (Chen *et al.*, 2000; Popham, 2002; Popham *et al.*, 1996). Il participe à l'imperméabilité de la spore, et joue donc un rôle dans le contrôle de la germination spontanée, c'est-à-dire une germination n'impliquant pas d'activation physique ou chimique. Le cortex participe aussi au maintien de la thermorésistance de la spore (Atrih and Foster, 2001).

1.2.4 La membrane interne

Séparant le cortex du cœur, la membrane interne joue un rôle important à la fois dans le maintien de la dormance de la spore et dans la germination. En effet, elle participe à l'imperméabilité de la spore en rendant le cœur inaccessible à de nombreuses molécules,

incluant l'eau (Leggett *et al.*, 2012; Setlow, 2003). Elle contient certains récepteurs de la germination comme GerA, GerB, GerR ou GerK qui permettent le déclenchement de la germination (Setlow, 2003) (*cf* 3. Germination et reprise de croissance). Les récepteurs aux germinants sont regroupés en une formation appelée le germinosome et l'assemblage de cette structure nécessite la présence de GerD (Griffiths *et al.*, 2011; Setlow, 2014). La membrane interne comporte également des protéines canaux, SpoVA, dont il existe plusieurs types (Setlow, 2013). Ces protéines sont synthétisées uniquement lors de la sporulation et ont un rôle important lors de la germination en permettant la libération du CaDPA et l'entrée d'eau dans le cœur.

1.2.5 Le cœur

Au centre de la spore, le cœur contient le matériel génétique ainsi que le matériel cellulaire nécessaire à l'activité métabolique lors de la reprise d'activité qui suit la germination. Ces éléments essentiels sont protégés du stress thermique par la déshydratation de ce compartiment (Atrih and Foster, 2002). Le cœur contient aussi des protéines appelées *Small Acid Soluble Proteins* (ou SASPs) qui représentent 5 à 10 % des protéines totales de la spore (Moeller *et al.*, 2014). Ces protéines sont produites lors de la sporulation et forment une source d'acides aminés lors de la reprise de croissance (Setlow and Setlow, 1995). Les α/β SASPs protègent de l'ADN contre les dommages causés par un traitement UV. En effet, ils préviennent les dommages mais réparent aussi tout dommage de l'ADN au cours de l'émergence (Setlow and Setlow, 1995). La prévention des dommages est possible car ces protéines se fixent directement à l'ADN et le saturent, ce qui est un élément important de la résistance aux stress qui ciblent directement l'ADN (Leggett *et al.*, 2012). Des mutants sous-exprimant les α/β SASPs sont moins résistants aux traitements thermiques, ce qui illustre bien leur rôle dans la résistance aux stress que les spores subissent (Nicholson *et al.*, 2000; Raju *et al.*, 2006) (Tableau 2).

Tableau 2 : Importance de différents facteurs et différentes structures dans la résistance des spores de *Bacillus* à différents traitements (d'après Nicholson *et al.* 2000).

Traitement	Effet sur la résistance					
	Présence α/β SASP	Hydratation du cœur	Minéralisation du cœur	Mécanisme de réparation De l'ADN	Structure des Tuniques	Conditions de sporulation
Chaleur humide	++	++	+	-	+/-	+
UV	++	-	?	++	-	-
Peroxydes	++	++	?	-	+	?

La spore et l'inactivation thermique

Le traitement thermique est largement utilisé en industrie agro-alimentaire. Les spores sont formées dans différentes conditions environnementales qui restent généralement inconnues. Elles se retrouvent donc sous forme dormante dans les ingrédients qui vont subir un traitement thermique. Or, la structure même des spores leur confère une thermorésistance élevée. Le faible taux d'hydratation du cœur de la spore en est la raison principale. Cette déshydratation associée à la présence de protéines particulières dans le cœur de la spore, les *Small Acids Soluble Proteins* (SASPs), permettent de stabiliser l'ADN lors d'un traitement thermique (Nicholson *et al.*, 2000). Il est intéressant de noter que les micro-organismes sont moins résistants aux traitements thermiques en conditions humides qu'en conditions sèches, ces deux traitements n'affectant pas les spores de la même manière (Smelt and Brul, 2014). Le traitement thermique en condition sèche semble inactiver les spores par oxydation alors que le traitement en conditions humides agit sur les structures protéiques de la spore ou sur les membranes (Coleman *et al.*, 2007; Setlow and Setlow, 1998; Zhang *et al.*, 2010). Coleman *et al.* 2010 proposent un modèle mécaniste pour décrire les effets du traitement thermique en

conditions humides. Ce modèle identifie différentes cibles, dans la spore, affectées par le traitement thermique et entraînant l'inactivation des spores par interruption de la germination et de la reprise de croissance à différents stades.

Tout d'abord, le traitement thermique aurait un impact sur les protéines, qui semblent être dénaturées par la chaleur. La dénaturation des protéines au sein des spores est observable notamment par spectrométrie de Raman (Zhang *et al.*, 2009). Ainsi, les protéines canaux (SpoVA) ou les récepteurs de la germination pourraient être dénaturés. Les spores traitées thermiquement ne pourraient alors pas libérer leur CaDPA. Ces spores sont donc incapables de germer, malgré des conditions favorables de récupération, car elles ne sont plus capables de se réhydrater. Ainsi, par des observations en microscopie à contraste de phase, de nombreuses spores traitées thermiquement ne perdent pas leur réfringence. Ceci est aussi observable, par spectrométrie de Raman (*cf.* encart 2), par la rétention de CaDPA par certaines spores après traitement thermique. Les spores seraient alors « bloquées » dans un état de dormance (Coleman and Setlow, 2009; Coleman *et al.*, 2007).

Sur la base de ce modèle, ces spores qui restent réfringentes peuvent être également considérées comme des spores « super dormantes ». Ces dernières peuvent germer et retrouver une activité métabolique longtemps après l'application du traitement d'inactivation (Ghosh and Setlow, 2009). La germination et la reprise de croissance de ces spores sont donc difficilement observables. On comprend l'importance de prendre en compte ce type de comportement de germination dans l'inactivation des spores bactériennes. En effet, ces spores peuvent être considérées comme inactivées alors qu'elles ont encore le potentiel de germer et de se multiplier, longtemps après le traitement. Ce comportement pourrait être dû à un faible nombre de récepteurs de germination ou à la structure du cortex dont la structure fortement réticulée (*cross linking*) influencerait la libération du CaDPA (Ghosh *et al.*, 2012; Zhang *et al.*, 2012). Cela peut être une stratégie de survie supplémentaire. En effet, ces spores restent en dormance même si la majorité des spores germent. Donc si le milieu n'est pas assez favorable à cet instant, les spores « super-dormantes » vont germer plus tard et potentiellement dans un milieu plus favorable. Enfin, le faible taux de spores « super-dormantes » dans une population

est un exemple de variation phénotypique au sein d'une même population clonale (Ghosh and Setlow, 2009).

L'inactivation thermique des spores peut aussi se traduire par un « blocage » de celles-ci après la germination. Coleman *et al.* 2010 ont également observé, par suivi microscopique, des spores inactivées thermiquement mais dont le DPA a été libéré. Ces spores n'atteignent pas le stade de l'émergence ou très lentement (*cf.* 3. Germination et reprise de croissance). Des molécules essentielles au passage de la spore germée à la cellule en émergence, comme des enzymes lytiques (CLEs), auraient donc été inactivées. Une deuxième hypothèse peut-être proposée : seules les enzymes de lyse du cortex seraient dénaturées. Le DPA serait libéré et le cœur partiellement hydraté mais le cortex ne serait pas hydrolysé. La réhydratation et le gonflement du cœur seraient alors partiels et insuffisants pour permettre l'émergence de la cellule.

Les travaux de Coleman *et al.* 2010, permettent de mieux comprendre les événements qui affectent des spores lors d'un traitement thermique. Cependant, il est nécessaire de pouvoir quantifier l'effet des conditions de traitement thermique sur les capacités des spores à germer et reprendre une croissance en vue d'une application pour l'industrie. Il existe des modèles mathématiques, des outils de microbiologie prévisionnelle, permettant de quantifier l'impact de plusieurs paramètres tels que les conditions de sporulation, de température ou de pH de traitement sur le recouvrement des spores (Baril *et al.*, 2012; Coroller *et al.*, 2001; Gaillard *et al.*, 2005; Mañas *et al.*, 2001) (*cf.* encart 1). Ces travaux mettent en évidence que la thermorésistance est elle-même impactée, en apparence, par les conditions de récupération des cellules (Daelman *et al.*, 2013a, 2013b; Leguérinel *et al.*, 2006). Le modèle de Daelman *et al.* 2013 prend en compte, comme réponse biologique, le temps nécessaire à une spore individuelle pour germer, émerger et reprendre une croissance. La quantification de la proportion de spores capables de germer et reprendre une croissance en fonction de la température de récupération est décrite par le modèle de Leguérinel *et al.* 2006. Ces modèles décrivent le recouvrement de spores après un traitement thermique. Plus précisément, ils quantifient l'impact de différents facteurs environnementaux sur la capacité des spores à germer et reprendre une croissance. Dans certains travaux, des modèles décrivant plus

particulièrement l'étape de la germination ont été développés (Smelt *et al.*, 2008; Stringer *et al.*, 2011).

Encart 1 : Modéliser mathématiquement l'inactivation thermique

La quantification de l'inactivation thermique d'une population est rendue possible par l'utilisation de modèles empiriques, ou phénoménologique. Un modèle phénoménologique établit des liens constants, entre des phénomènes observés, sous forme de règles statistiques ou mathématiques. Les cinétiques d'inactivation décrivent, par exemple, la diminution de la population en fonction du temps de traitement (Bigelow, 1921). De nombreux modèles descriptif permettant de quantifier l'inactivation ont été développés (Peleg and Cole, 1998 ; Augustin *et al.*, 1998; Coroller *et al.*, 2006; Mafart *et al.*, 2002; Sapru *et al.*, 1992). Le modèle de Weibull, ou les modèles qui en sont inspirés, prennent en compte l'hétérogénéité des individus au sein d'une population qui s'exprime par des courbes de survie de concavité ou convexité variable (Mafart, 2000). Ce modèle apparait comme un moyen simple de décrire des courbes non linéaires observées lors d'une inactivation thermique (Collado *et al.*, 2006; Mafart *et al.*, 2002) (Equation 1).

$$\log N = \log N_0 - \left(\frac{t}{\delta}\right)^p \quad \text{équation 1}$$

Classiquement lorsqu'on étudie l'inactivation thermique deux paramètres sont estimés. Le premier paramètre est la valeur δ ou D , correspondant au temps nécessaire pour réduire la population initiale de 90%. Le second paramètre est la valeur z , correspondant à la sensibilité thermique, c'est-à-dire la différence de température –pour z_T - ou de pH – pour z_{pH} - nécessaire pour augmenter ou réduire la valeur δ d'un facteur 10. De très nombreux travaux ont donné un nombre conséquent de ces valeurs pour un grand nombre de souches de bactéries sporulées ou non, pour des spores ou des cellules végétatives. La valeur de z_T peut varier selon les souches. Pour *Bacillus cereus* la valeur moyenne de z_T est comprise entre 8,3°C et 12,8°C (Afchain *et al.*, 2008; Luu-Thi *et al.*, 2014; Vanasselt and Zwietering, 2006). L'effet du pH du milieu de traitement est un facteur important impactant l'inactivation thermique (Akterian *et al.*, 1999). Des outils de microbiologie prévisionnelle développés dans de nombreux travaux permettent de quantifier l'impact des conditions de sporulation, de traitement thermique ou de recouvrement sur l'inactivation des spores (Baril *et al.*, 2012; Couvert *et al.*, 1999; Daelman *et al.*, 2013b; Gaillard *et al.*, 1998; Graham *et al.*, 1996; Leguerinel and Mafart, 2001; Mafart, 2000; Mafart and Leguerinel, 1998; Smelt *et al.*, 2008; Warth, 1980). Ces modèles permettent de quantifier l'impact des conditions de sporulation, de traitement thermique ou de recouvrement sur les valeurs de δ et z .

La germination et la reprise de croissance

La germination et la reprise de croissance représente un ensemble de phénomènes au cours desquelles les spores vont passer par différents stades physiologiques. Ces différents stades peuvent être observés grâce à différentes techniques et méthodes reposant sur les propriétés structurales et physiologiques des spores. La première étape est l'activation des spores et l'engagement. Suivent la germination et l'émergence d'une nouvelle cellule végétative (Figure 3).



Figure 3 : Observation de la germination et la de reprise de croissance de spores de *Bacillus weihenstephanensis* KBAB4 au microscope à contraste de phase. Les différents stades présentés sont l'activation (0), la germination (1), au cours de laquelle les spores perdent leur réfringence par l'hydratation du cœur, l'émergence (2), durant laquelle les spores quittent leurs enveloppes sporales et retrouvent une activité métabolique, et enfin la multiplication végétative (3) (D'après Abee *et al.* 2011 ; Crédit photo : Trunet, LUBEM).

1.4.1 La germination

La germination peut être induite par différents agents comme des nutriments spécifiques ou des acides nucléiques, le CaDPA. Les traitements haute-pression peuvent aussi induire la germination. Si la germination est induite par nutriments, les acides aminés ou les sucres sont reconnus par les récepteurs des germinants (notés GRs pour *Germinant Receptors*) situés dans la membrane interne de la spore. Les germinants doivent donc diffuser à travers les tuniques, qui permettent le passage de petites molécules de moins de 2 à 8 kDa (Driks, 1999; Henriques and Moran, Jr., 2007), comme les acides aminés, par exemple, dont la taille est de l'ordre d'une centaine de Da. Chronologiquement, les premiers récepteurs entrant en jeu lors de la germination chez *Bacillus subtilis* et *Bacillus cereus* sont les récepteurs de type GerP. Ce sont des structures multiprotéiques composées d'un groupe de protéines synthétisées uniquement au cours de la sporulation et se trouvent vraisemblablement dans les tuniques (Behravan *et al.*,

2000; Setlow, 2014). GerP permettrait de faciliter le passage des germinants jusqu'aux récepteurs de germination situés dans la membrane interne. Une deuxième hypothèse suggère que GerP serait associé à d'autres protéines des tuniques qui formeraient alors des canaux permettant le passage des germinants (Behravan *et al.*, 2000). Les différents GRs décrits sont spécifiques d'un ligand et donc d'un germinant. Par exemple, la L-alanine interagit avec GerR chez *B. cereus* alors que la combinaison L-asparagine, D-glucose, D-fructose et K⁺ (AGFK) interagit avec GerB et GerK chez *Bacillus subtilis* (Setlow, 2003)(Hornstra *et al.*, 2006). La L-alanine est connue pour induire la germination des spores de *Bacillus weihenstephanensis* et *B. licheniformis* (Garcia *et al.*, 2010; Madslie *et al.*, 2014). Certaines molécules sont connues pour inhiber la germination. Ainsi, la D-Alanine, l'acide sorbique ou d'autres acides organiques, le carvacrol ou d'autres huiles essentielles inhibent la germination chez *B. cereus* (Luu-Thi *et al.*, 2015; van Melis *et al.*, 2012, 2011).

La liaison d'un germinant à son récepteur engage la spore dans la germination. Le phénomène d'engagement est irréversible (Yi and Setlow, 2010). Cet engagement (ou *commitment*) est étudié essentiellement sur *B. subtilis* et *B. cereus*. Il apparaît que cette étape d'engagement est influencée par plusieurs facteurs comme l'activation thermique, la concentration en nutriments et le taux de GRs par spore (Yi and Setlow, 2010). Il apparaît que le facteur impactant le plus significativement l'engagement est le taux de GRs par spore quand la germination est induite par des nutriments. En effet, il semble que les spores possédant le plus de GRs sont celles qui ont les temps d'engagement les plus courts. Ces phénomènes restent peu clairs et, là aussi, certaines questions restent en suspens. Par exemple, il existe un délai entre le moment où les spores sont mises en contact avec les germinants et leur engagement dans le processus de germination. De plus, ce délai est observé pour une germination induite par des germinants mais aussi pour une germination induite par haute pression (Kong *et al.*, 2014).

Afin de suivre cette étape d'engagement, le CaDPA peut être marqué par du Terbium, formant ainsi le complexe Tb³⁺-DPA. La libération du Tb³⁺-DPA peut être suivie au cours du temps par des mesures de fluorescence à 545 nm (avec une excitation à 270 nm) correspondant à la longueur d'onde d'émission et d'excitation du complexe Tb³⁺-DPA (Yang and Ponce, 2009; Yung and Ponce, 2008). La phase d'engagement peut être spécifiquement étudiée en utilisant des

molécules, comme la D-alanine, qui ont la propriété de bloquer la fixation des germinants aux récepteurs mais sans bloquer la germination des spores déjà engagées. En présence de ces molécules, les spores ne sont donc plus capables d'initier l'engagement. Au moment de l'ajout de D-alanine dans le milieu, seules les spores dont le processus de germination est déjà engagé vont libérer leur CaDPA (Yi and Setlow, 2010; Zhang *et al.*, 2014).

La germination est l'étape suivant l'engagement. La fixation des germinants à leurs récepteurs, après avoir traversé les différentes structures de la spore, entraîne l'ouverture des canaux SpoVA. Le CaDPA, contenu dans le cœur est alors libéré et l'eau peut pénétrer dans le cœur. Il apparaît que la libération des molécules lors de cette étape est sélective, c'est-à-dire que seul le CaDPA semble pouvoir être libéré du cœur, et non les autres constituants comme les cations (Setlow, 2008; Vepachedu and Setlow, 2007, 2005). La libération du CaDPA et l'hydratation partielle du cœur termine la Phase I de la germination et de la reprise de croissance (Figure 5). Cette première phase de la germination est qualifiée de Stade I (Setlow *et al.*, 2001). Lors de cette phase, des cations monovalents sont aussi libérés (K^+ , Na^+ , H^+) et le cœur est partiellement hydraté (Setlow, 2003).

La deuxième Phase de la germination (Stade II) consiste principalement en l'hydrolyse du cortex par des enzymes appelées *Cortex Lytic Enzyme* (CLEs) et notamment SleB et CwlJ chez *Bacillus cereus* et *Bacillus subtilis*. L'hydrolyse du cortex permet le relâchement de la structure de la spore, et l'hydratation du cœur de la spore favorise son expansion. La membrane interne entourant le cœur s'agrandit elle aussi mais sans qu'il y ait de synthèse lipidique (Cowan *et al.*, 2004). A la fin de cette étape, le cœur contient environ 80% d'eau (au lieu de 50 % dans la spore en dormance (Atrih and Foster, 2002) et les enzymes qu'il contient, SleB et CwlJ, sont activées, ce qui conduit aussi à la dégradation des SASPs (Paidhungat *et al.*, 2002).

Ces étapes de libération de CaDPA et d'hydratation de la spore peuvent être suivies de différentes par différentes méthodes. La réfringence de la spore est due à son haut niveau de déshydratation. Le suivi de germination peut se faire en quantifiant la perte de Densité Optique à 600 nm (DO_{600}) au court du temps. En effet, la réduction de la DO_{600} reflète la germination au sein d'une population par la perte de réfringence des spores (Hornstra *et al.*, 2006). Ainsi, une réduction de DO_{600} d'une suspension de spores de 62 % environ indique une germination de

plus de 95% des spores de *Bacillus weihenstephanensis* KBAB4 (Garcia *et al.*, 2010). La perte de réfringence peut aussi être observée par microscopie optique à contraste de phase. En effet, lorsque les spores sont déshydratées, et donc réfringentes, elles apparaissent blanches en contraste de phase (*phase bright spores*). Une fois hydratée, les spores apparaissent sombre (*phase dark spores*), tout comme les cellules végétatives (Figure 3). Ces méthodes permettent de mettre en évidence l'impact des conditions environnementales, des conditions de sporulation, de l'intensité du traitement thermique ou encore de la densité cellulaire sur le temps et le taux de germination (Garcia *et al.*, 2010; Pandey *et al.*, 2013; Stringer *et al.*, 2011). L'inconvénient de ces méthodes est la nécessité d'avoir une grande population de spores pour procéder aux observations. Dans le cas d'une observation microscopique, une suspension concentrée, de 10^6 spores.mL⁻¹ au minimum, est nécessaire mais un très faible volume suffit (moins de 10µL). En revanche, pour l'observation de la perte de DO₆₀₀, une suspension concentrée, également de 10^6 spores.mL⁻¹ ce qui correspond à une DO₆₀₀ entre 0.5 et 1, et un volume de 1mL, au minimum, sont nécessaires. La libération de CaDPA lors de la germination peut aussi être quantifiée au cours du temps pour une population de spores, comme décrit précédemment, mais il peut être suivi au niveau individuel. Pour cela la libération de CaDPA peut être suivie grâce à un spectromètre de Raman (Zhang *et al.*, 2010, 2009; Zhou *et al.*, 2013), *cf Encart 2*.

La germination consiste donc en ces deux premières phases : i) la libération de CaDPA et l'hydratation partielle du cœur et ii) la lyse du cortex et l'hydratation totale du cœur. Des phénomènes non liés aux germinants peuvent aussi induire une germination, comme les traitements haute pression ou la présence de CaDPA exogène (Reineke *et al.*, 2013; Setlow, 2013). Les traitements haute pression agissent sur les récepteurs des germinants ou sur les protéines canaux induisant alors la germination (Kong *et al.*, 2014; Paidhungat *et al.*, 2002).

La germination des spores de *Bacillus* peut être activée par traitement thermique. Cette activation entraîne un taux significativement plus important de germination. Des hypothèses ont été proposées pour tenter d'expliquer ce type d'activation mais le processus exact reste obscur. Les traitements thermiques entraîneraient un changement de conformation des récepteurs aux germinants et ainsi la germination de la spore ou bien le traitement thermique

rendrait ces récepteurs plus accessible ou plus réceptifs (Setlow, 2014). L'activation est réversible et il a été observé que les protéines peuvent reprendre leurs conformations initiales, dans le cas d'une activation thermique, lorsque la température après le traitement est abaissée. Ces dénaturations protéiques à la suite d'un traitement thermique ont été observées par spectroscopie de Raman (cf. encart 2). De plus, si les spores sont activées par traitement thermique et ne sont pas incubées de suite dans des conditions favorables, l'effet de l'activation n'est plus observé (Zhou *et al.*, 2015). Ceci permet de poser l'hypothèse que l'activation thermique entraîne un changement de conformation des GRs qui, s'ils ne sont pas liés par la suite à des germinants vont reprendre leur conformation initiale (Setlow, 2014; Zhang *et al.*, 2009). Cependant, l'effet de l'activation de la germination par traitement thermique dépend largement de l'espèce, voire de la souche étudiée.

Encart 2 : La spectroscopie de Raman permet de suivre individuellement la germination des spores

Entre 1950 et 1970, avec le développement des technologies LASER, la spectroscopie de Raman a connu de nombreux domaines d'applications. Dans les années 80, les premières études de compositions chimiques de micro-organismes par cette technique ont été publiées (Maquelin *et al.*, 2002).

L'analyse par spectroscopie Raman repose sur l'excitation d'un mélange moléculaire par un laser. Les molécules absorbent une partie du rayonnement. La radiation réémise par l'échantillon est collectée par un détecteur et la variation entre l'énergie émise et l'énergie renvoyée renseigne alors sur les niveaux énergétiques des molécules concernées (Figure 4).

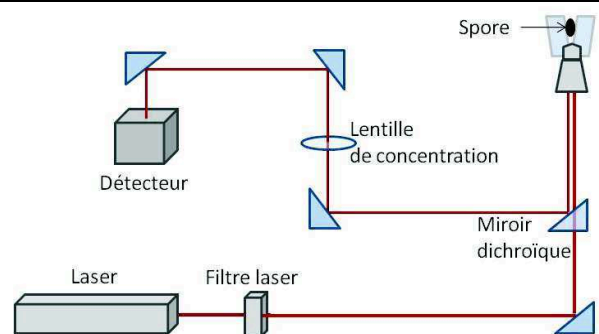


Figure 4 : Schéma simplifié d'un spectromètre de Raman (d'après Zhang *et al.* 2009)

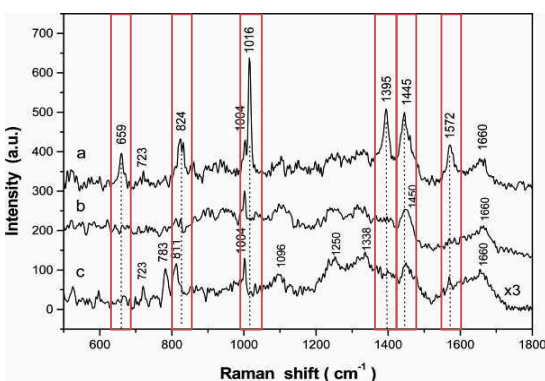
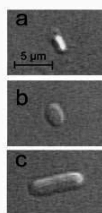


Figure 5 : Spectre Raman d'une spore réfringente (courbe a), d'une spore germée (courbe b) et d'une cellule végétative (courbe c) de *B. thuringiensis*. Les bandes correspondant au CaDPA sont encadrées en rouge et on observe la diminution de ceratins pics (d'après Chen *et al.*, 2006).

Il s'agit de la méthode spectroscopique dotée de la meilleure résolution spatiale (un micron carré) pour l'identification et la caractérisation de composés (Makiabadi, 2010).

Ainsi, les micro-organismes sont considérés comme un mélange moléculaire et cette technique permet de déterminer leur composition moléculaire, en s'appuyant sur les spectres connus des molécules recherchées. Ceci est utilisé pour observer la libération de CaDPA lors de la germination des spores de *Bacillus*. Les pics correspondant au CaDPA sont reconnus au sein du spectre du mélange moléculaire que constitue la spore bactérienne. La disparition ou la baisse d'intensité de ces pics correspondent donc à la libération du CaDPA signifiant que la spore vient de germer (G. Wang *et al.*, 2011; Zhang *et al.*, 2011, 2009).



L'émergence

L'émergence suit la germination dans le processus de recouvrement. C'est durant cette étape que le métabolisme cellulaire est rétabli et la que synthèse de macromolécules reprend. La spore gonfle et devient une cellule végétative (Setlow, 2003). Au cours de ce gonflement, ou *burst*, la nouvelle cellule va sortir de ses enveloppes extérieures, sa membrane interne devenant la nouvelle membrane cytoplasmique. La sortie de cette nouvelle cellule se passe selon le modèle « *bottle cap* » (cf Encadré 3).

L'hydratation du cœur lors de la phase 2 de la germination permet la restauration de l'activité enzymatique. Les SASPs notamment sont dégradées en acides aminés. L'ADN est alors libéré des SASPs et la synthèse d'ARNm est rapidement suivie par la synthèse de protéines. L'acide 3-phosphoglycérique (3PGA) stocké dans la spore est phosphorylé et l'ATP ainsi formé est utilisé comme source d'énergie. Le temps d'apparition d'une cellule végétative capable de se multiplier variera en fonction des conditions environnementales (Pandey *et al.*, 2013).

Afin d'étudier cette nouvelle étape de la vie de la spore, différentes méthodes peuvent être utilisées. La méthode la plus classique est la méthode culturale. Les spores sont mises en culture en milieu nutritif et une cinétique de croissance permet de déterminer le temps de germination et de reprise de croissance. Le suivi de croissance à partir d'une population de spores, ou de spores individuelles, peut aussi être réalisé par suivi de DO₆₀₀. Ainsi on peut, avec ces deux méthodes observer l'impact des conditions environnementales sur le temps ou le taux de germination et de reprise de croissance (Daelman *et al.*, 2013; Garcia *et al.*, 2010).

Encart 3 : Le « Bottle Cap model » et la naissance d'une nouvelle cellule végétative

Lors des premiers stades de la germination et la reprise de croissance, les spores bactériennes se réhydratent. Ceci entraîne une augmentation de leur volume. La membrane interne devenant la nouvelle membrane plasmique de la cellule végétative néoformée, la spore va devoir se débarrasser des enveloppes les plus superficielles que sont les tuniques et l'exosporium.

L'assemblage de l'exosporium des *Bacillus* ou des *Clostridium* n'est pas uniforme. En effet, il a été montré que l'exosporium de *Bacillus anthracis*, qui fait partie du groupe *B. cereus sensu lato*, est divisé en deux parties distinctes. Ceci proviendrait d'un changement de mode d'assemblage de l'exosporium lors de la sporulation (Steichen *et al.*, 2007). Cette structure non-uniforme

crée une zone moins résistante mécaniquement que le reste de l'exosporium. Donc, quand la spore gonfle, elle va pouvoir sortir par cette zone qui fonctionne comme une capsule de bouteille. La cellule en émergence va alors être libérée par cette région. En revanche, l'affaiblissement des tuniques lors de ce phénomène n'est pas bien décrit.

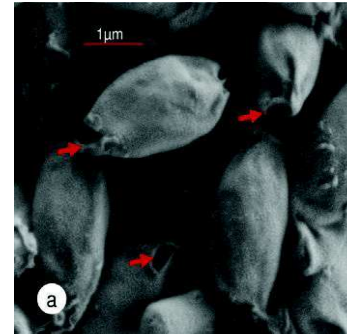


Figure 6 : Observation par microscopie électronique à balayage de spores de *C. sporogenes*. Les flèches rouges indiquent de petites ouvertures dans l'exosporium (0.3-0.4µm) (d'après Brunt *et al.*, 2015)

Cependant, les méthodes culturales ou par DO_{600} présentent une limite importante : les étapes entre la germination et la multiplication végétatives ne sont pas observables. Afin d'observer l'émergence au sein d'une population de spores il faut utiliser des techniques permettant l'observation de ce stade. La microscopie permet d'observer les individus et de les suivre depuis la germination jusqu'à la multiplication. En combinant un microscope avec une chambre d'incubation, l'effet des conditions environnementales sur l'émergence peuvent être observées. Le logiciel, SporeTracker® associé à un microscope à contraste de phase permet de suivre les spores individuellement au cours du temps depuis la germination jusqu'à la reprise de croissance (Pandey *et al.*, 2013). Cette méthode permet même d'observer le « burst » qui correspond à la sortie de la cellule végétative des enveloppes superficielles de la spore. Cela se traduit par une augmentation soudaine de la taille de la cellule. Une autre méthode de microscopie fait intervenir des cassettes contenant du milieu semi gélosé ou des membranes sur lesquels sont déposées les spores (Anopore). On est capable ainsi de suivre le passage de la spore réfringente jusqu'à la cellule végétative et l'influence de plusieurs facteurs sur ce passage

(Besten *et al.*, 2010; Webb *et al.*, 2012). Une autre méthode permettant d'observer la germination et l'émergence est la cytométrie en flux. Plusieurs études ont montré que la discrimination des différents stades physiologiques traversés par la spore lors du recouvrement était possible par cette technique (Cronin and Wilkinson, 2008, 2007; van Melis *et al.*, 2011). Les différents stades peuvent être distingués par plusieurs critères. La taille des cellules permet de différencier les spores des cellules végétatives, les marqueurs fluorescents permettent de mettre en évidence la perméabilité membranaire retrouvée lors de la germination ou encore une activité métabolique lors de l'émergence. Parmi les marqueurs d'activité métaboliques utilisés, nous pouvons citer le Carboxyfluorescein diacetate (CFDA), marquant en vert les cellules ayant une activité estérase (Cronin and Wilkinson, 2007), ou le 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) qui est réduit chez les cellules ayant une activité métabolique qui sont alors marquées en rouge (Laflamme *et al.*, 2004).

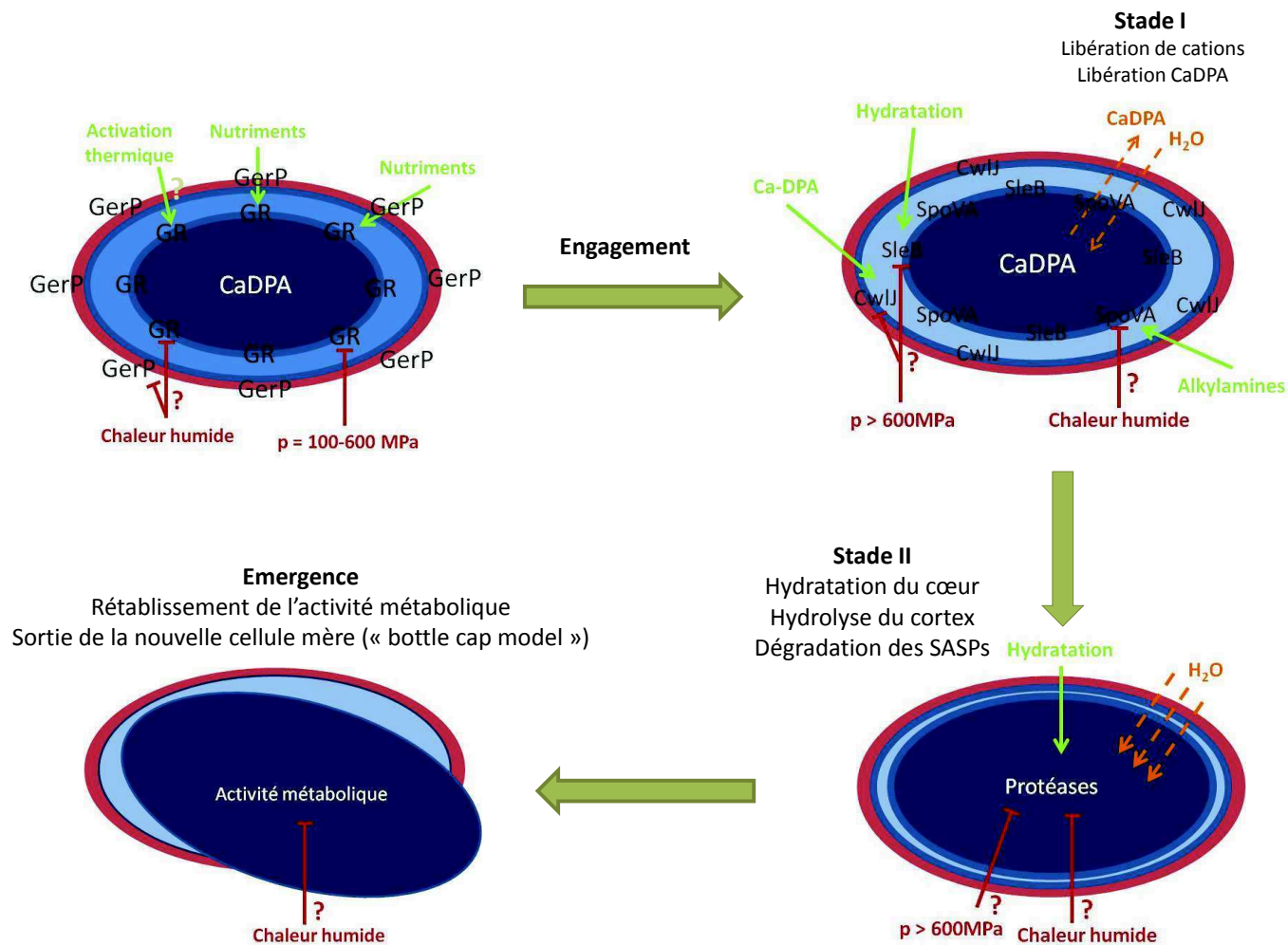


Figure 7 : Représentation schématique du processus de reprise de croissance des spores de plusieurs *Bacillus*. En vert sont présentés les facteurs favorisant la germination et l'émergence et en rouge les facteurs inactivant ces phénomènes. En orange, sont présentés les flux se produisant lors des différentes étapes. Au Stade I, le CaDPA contenu dans le cœur est libéré. Au Stade II, le cœur est réhydraté et les protéases sont activées. Enfin, lors de l'émergence, la nouvelle cellule végétative est expulsée et se débarrasse donc des tuniques et de l'exosporium (d'après Reineke *et al.*, 2013; Setlow, 2013, 2014; Zhang *et al.*, 2009)

Le temps et le taux d'émergence peuvent être impactés par les conditions de traitement thermique ou les conditions de recouvrement : (Broussolle *et al.*, 2008; Coleman *et al.*, 2010; Garcia *et al.*, 2010; Hornstra *et al.*, 2006; van der Voort *et al.*, 2010; Zhang *et al.*, 2009) ; (Abel-Santos, 2012) (Guiwen Wang *et al.*, 2011). De nombreux travaux mettent en évidence ces variabilités de comportements lors de la germination et la reprise de croissance des spores bactériennes : le temps d'émergence augmente, et le taux d'émergence diminue, après un traitement thermique ou lorsque les conditions environnementales sont défavorables.

Connaissant les mécanismes et les effets des différentes conditions de germination et de reprise de croissance, plusieurs hypothèses peuvent être avancées. La température et le pH auxquels sont exposées les spores après un traitement thermique ont une influence sur la germination et la reprise de croissance chez *Bacillus* (Pandey *et al.*, 2013; Smelt *et al.*, 2008). Une température suboptimale allonge le temps d'émergence (Pandey *et al.*, 2015). Ces conditions environnementales pourraient affecter l'activité de certaines enzymes, notamment les CLEs, la fluidité membranaire ou encore sur la vitesse de réhydratation totale de la spore. De plus, le pH interne de la spore, change au cours de la germination. En effet, il y a une libération de H⁺ lors de l'hydratation du cœur, faisant passer le pH de celui-ci de 6,50 à 7,70 chez, *Bacillus subtilis*, en conditions optimales de croissance (Setlow, 2003).

1.4.3. Hétérogénéité du comportement de germination et de reprise de croissance

En étudiant le phénomène de germination et de reprise de croissance au niveau individuel, on observe une très grande hétérogénéité de comportement à chaque stade : la germination, l'émergence et la reprise de croissance (Stringer *et al.*, 2011). Elle peut s'expliquer d'une part par le passé de la spore, sa composition, et par l'impact des conditions environnementales que la spore va rencontrer lors de sa germination et de sa reprise de croissance.

L'hétérogénéité au sein d'une population est donc observable au niveau de la germination, de l'émergence et de la formation d'une nouvelle cellule végétative. Cette variabilité au sein d'une même population clonale peut provenir d'une répartition inégale de protéines au sein de la cellule. Ce phénomène est observable en marquant ces protéines inégalement réparties

(comme SinI et SinR chez *Bacillus subtilis*) et en suivant le taux de copie dans les cellules en divisions ou en sporulation par microscopie (Jahn *et al.*, 2015). Ainsi, les spores pourraient avoir des compositions différentes à l'issue de leur formation (taux d'hydratation, taux de minéralisation, nombre de récepteurs aux germinants...) et donc des capacités de germination et de reprise de croissance différentes. Les conditions dans lesquelles sont produites les spores ont une large influence sur les propriétés des spores elles-mêmes (Baril, 2011; Baril *et al.*, 2012, 2011). De plus, des spores produites dans des conditions suboptimales de température montrent des taux de germination plus faibles (Garcia *et al.*, 2010). Il existe bien un lien direct entre la formation des spores, les conditions dans lesquelles elles sont formées, et leur capacité de résistance ou à germer et reprendre une croissance. Les enzymes, les protéines de structure ou les SASPs sont mises en place uniquement lors de la sporulation. Le degré de minéralisation et le taux de déshydratation dépendent aussi des conditions de sporulation. Ainsi, il peut exister une hétérogénéité de formation des spores entraînant une hétérogénéité de propriété des spores (thermorésistance, taux de germination). En plus de l'histoire de la spore, d'autres paramètres peuvent expliquer l'hétérogénéité de comportement. La germination présente une grande hétérogénéité au sein d'une population (Kong *et al.*, 2014; Zhang *et al.*, 2009; Zhou *et al.*, 2013). Chez *B. cereus* et *B. subtilis* le déclenchement de la germination dépend de la concentration en nutriment et du nombre de récepteurs de la germination. L'hétérogénéité du temps de germination semble donc provenir de la variabilité du temps nécessaire aux nutriments pour accéder aux récepteurs de la germination (Yi and Setlow, 2010).

Objectifs de la thèse

En mettant en parallèle le cycle de vie des bactéries sporulées et les procédés industriels, nous pouvons mettre en évidence deux leviers permettant de maîtriser le développement de cette communauté microbienne dans les aliments. Le premier levier se situe lors de la phase de dormance. A ce stade, la population bactérienne est sous forme de spores et les éliminer est difficile. Les traitements thermiques sont classiquement utilisés pour cela mais l'augmentation des barèmes de stérilisation se fait au détriment des qualités organoleptiques et nutritionnelles des produits alimentaires. Les facteurs importants lors de cette phase sont donc le temps de traitement et l'intensité de celui-ci (en termes de température et de temps pour un traitement thermique). Nous parlerons alors de conditions *per*-traitement.

Le deuxième niveau est le stade de germination et de reprise de croissance, qui nous intéresse plus particulièrement dans ces travaux. Cette étape est fortement influencée par les facteurs environnementaux, comme la température et le pH, du milieu dans lequel vont se retrouver les spores après le traitement thermique. Ces facteurs *post*-traitement vont avoir un effet sur les différents stades du recouvrement. Quel(s) stade(s) est (sont) le(s) plus influencé(s) par les conditions de recouvrement ? La quantification de l'impact des conditions de germination et de reprise de croissance sont établies pour l'heure à l'aide de méthodes culturales principalement. Analyser les évolutions d'une population de spores dans ces différents stades physiologiques nécessite la mise en œuvre de techniques d'analyses plus fines.

Le processus de germination et de reprise de croissance est un processus complexe. Certaines étapes comme l'engagement qui suit l'activation ou la manière dont les nutriments atteignent les récepteurs aux germinants restent peu clairs. Certains mécanismes sont encore mal connus et l'effet de différents facteurs comme la température ou le pH sur les stades de germination ou encore l'impact du traitement thermique sur ces stades restent mal connus. L'utilisation de nombreuses techniques et méthodes permettant d'affiner de plus en plus les observations au cours de la germination et de l'émergence des spores facilitent néanmoins ces études.

Les études sur les effets d'un traitement thermique associé à des conditions suboptimales de recouvrement font défaut, notamment sur la plage de croissance complète. Il serait donc

intéressant de décrire et quantifier l'impact des conditions rencontrées par les spores avant, pendant et après le traitement thermique sur leur capacité à former des colonies.

On se propose donc de développer un modèle mathématique permettant de décrire le comportement de germination et de reprise de croissance en prenant en compte les conditions *pre*, *per* et *post*-traitement thermique. Ce modèle aura pour paramètres les valeurs cardinales de croissance, c'est-à-dire les températures et pH limites et optimaux de croissance. Ainsi, le comportement de germination et de reprise de croissance pourra être décrit à l'aide de paramètres physiologiques, et non plus empiriques (Leguérinel *et al.*, 2006). Même s'il est difficile d'imaginer qu'un modèle mécanistique remplace un modèle descriptif, des éléments mécanistiques peuvent être incorporés grâce aux avancées en physiologie microbienne (Brul *et al.*, 2011). En industrie agro-alimentaire, les modèles simples sont préférés car plus faciles d'utilisation. Mais ajouter des éléments mécanistiques dans de tels modèles permettrait d'améliorer la robustesse des modèles et les prévisions particulièrement aux limites des domaines de croissance ou le comportement est plus variable. Ceci paraît nécessaire notamment pour les modèles basés sur les concepts d'effets barrières (McMeekin, 1993; McMeekin *et al.*, 2010; Zwietering *et al.*, 1992).

Afin d'élargir les observations et les conclusions faites pour des spores traitées thermiquement, la description et la quantification de la germination et de la reprise de croissance a été réalisée pour des spores traitées par un agent chimique (l'acide péracétique) et un autre agent physique (la lumière pulsée). Ces expériences nous permettront de déterminer si le comportement de recouvrement de spores traitées par acide péracétique et lumière pulsée est le même que des spores traitées thermiquement et si ce comportement peut être décrit par le modèle mathématique de prévision de la résistance apparente au traitement en fonction des conditions de reprise (température et pH)

Les travaux décrits dans les derniers paragraphes nécessitent des méthodes culturales classiques permettant uniquement de quantifier les spores capables de former des colonies. Cependant, comme nous l'avons vu, le passage d'une spore réfringente à une cellule végétative est un processus complexe et comportant plusieurs stades. Une méthode de cytométrie en flux a donc été mise au point pour suivre les différents stades physiologiques traversés par les

spores au cours de la germination et de la reprise de croissance. L'impact des conditions pré, per et post-traitement thermique seront donc quantifiés pour chaque état physiologique. De plus, une étude du protéome de spores de *Bacillus weihenstephanensis* KBAB4 nous permettra de mettre en évidence des différences de compositions protéiques en fonction des conditions de sporulation. Ces observations nous permettront de corrélérer la modulation de certains éléments intrinsèques à la spore, et plus précisément son protéome, et ses variations de comportements.

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Chapitre 2

Modeling the recovery of heat-treated *Bacillus licheniformis* and *Bacillus weihenstephanensis* spores at suboptimal temperature and pH using growth limits

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Abstract

The apparent heat-resistance of spores of *Bacillus weihenstephanensis* and *Bacillus licheniformis* was measured and expressed as the time to first decimal reduction (δ value) at a given recovery temperature and pH. Spores of *B. weihenstephanensis* were produced at 30°C and 12°C, and spores of *B. licheniformis* at 45°C and 20°C. *B. weihenstephanensis* spores were then heat-treated at 85°C, 90°C and 95°C and *B. licheniformis* spores at 95°C, 100°C and 105°C. Heat-treated spores were grown on nutrient agar at a range of temperatures (4°C to 40°C for *B. weihenstephanensis*, 15°C to 60°C for *B. licheniformis* or a range of pH (between pH 4.5 and pH 9.5 for both strains). The recovery temperature had a low effect on the apparent heat-resistance, except very near recovery boundaries. In contrast a decrease in the recovery pH had a progressive impact on apparent heat-resistance. A model describing the heat resistance and the ability to recover according to the sporulation temperature, temperature of treatment and recovery temperature and pH was proposed. This model derived from secondary mathematical models for growth prediction. Previously published cardinal temperature and pH values were used as input parameters. The fitting of the model with apparent heat resistance data obtained for a wide range of spore treatment and recovery conditions, was highly satisfactory.

2.1 Introduction

The multiplication of spore-forming bacteria in foods can cause poisoning and/or spoilage. The heat process applied to foods (from mild in cooked and refrigerated foods to very intense in canned or UHT foods) creates a positive selection of spore-forming species of bacteria because of the high resistance of their spores (Postollec *et al.*, 2012). Therefore, control of spore-forming bacteria in foods first of all requires inactivation of dormant spores by heat (or by any other appropriate inactivation treatment). The extent of inactivation depends on a number of factors, naturally including the inactivation process intensity and more importantly the spore resistance properties at the time of treatment, which may vary with the conditions and environment of sporulation (Carlin, 2011). Respect for the organoleptic quality of food may limit the intensity of the process, and therefore the extent of spore inactivation. Controlling the recovery of surviving spores in processed food strengthens the safety and stability level achieved after the inactivation process. Recovery is a complex phenomenon, comprising germination of spores, restoration of metabolic activity in suboptimal or favorable conditions and emergence of the first vegetative cell able to multiply. The incubation temperature during storage and food pH are among factors that will deeply influence the recovery of surviving spores (Abee *et al.*, 2011).

Spore recovery is influenced by multiple physical and (bio)chemical factors such as temperature, pH or water activity (a_w), the presence of germinants (such as amino-acids, ribosides, minerals) or enzymes like lysozyme (Leguérinel *et al.*, 2006; Periago *et al.*, 1998; Setlow, 2013; Suzuki and Rode, 1969). These previous works are mainly descriptive and modeling attempts are rare and moreover rather unsatisfactory (Augustin, 2011). For instance, the model of Leguérinel (Leguérinel *et al.*, 2006) assumed a linear effect of temperature on recovery of heat treated spores while most works describe a maximal recovery at optimal recovery conditions. In contrast, many mathematical models predict the sole impact of heat treatment on micro-organisms (Mafart *et al.*, 2002; Mafart and Leguérinel, 1997; Periago *et al.*, 1998). This work proposes a model describing spores recovery after heat treatment as a function of incubation temperature and pH of the recovery medium and accounting the variations due to sporulation conditions. This model integrates conditions encountered by the spores in many food industries: spores are formed in diverse environmental conditions remaining unknown for most of the

time, transfer to foods, and are inactivated by heat to a certain degree during food processing. Survivors tend to multiply during food storage. The overall impact of sporulation temperature, heat treatment intensity and temperature and pH of recovery is generally quantified by the ability of surviving spores to form a colony on an agar plate, which results from the germination and growth restoration of the heat-treated spores. The experimental work was performed on two strains with different behaviors regarding temperature: *Bacillus weihenstephanensis*, a psychrotrophic species, and *Bacillus licheniformis*, a thermotrophic species.

2.2. Material and Methods

2.2.1. Bacterial strains

The *Bacillus weihenstephanensis* strain KBAB4 (INRA, Avignon, France) isolated from a forest soil (Guinebretière *et al.*, 2008) and *Bacillus licheniformis* strain Ad978 (ADRIA Développement, Quimper, France) isolated from dairy ingredients were used in this work. The strains were stored at -80°C in 1 mL aliquots of Brain Heart Infusion (BHI, Biokar Diagnostics, Beauvais, France) mixed with 50% glycerol (v/v) at a concentration of approximately 2×10^6 CFU.mL⁻¹. A 100 mL volume of BHI was inoculated with 1 mL of the stock suspensions and incubated for 8 h at optimal growth temperature (30°C for *B. weihenstephanensis* KBAB4 and 45°C for *B. licheniformis* Ad978), then 1 mL volume was transferred into 100 mL of BHI for 16 h of incubation at the same temperatures. Finally, 0.1 mL of *B. weihenstephanensis* suspension and 0.01 mL of *B. licheniformis* suspension were added to 100 mL of BHI and were incubated for 6 h. For both strains, the final cell concentration in the pre-culture was approximately 10^8 CFU.mL⁻¹; the number of spores estimated by the number of cells surviving a 5 min, 70°C heat treatment was lower than 100 spores.mL⁻¹.

2.2.2. Sporulation

Spores were produced through a two-step sporulation process (Baril *et al.*, 2011). Volumes of 100 ml of the previously described pre-culture were centrifuged (6000 x *g*, 10 min, 12°C) and suspended in 100 mL of sporulation mineral buffer (SMB) made of K₂HPO₄ at 4.5 g.L⁻¹, KH₂PO₄ at 1.8 g.L⁻¹, CaCl₂, H₂O at 8.0 mg.L⁻¹ and MnSO₄, H₂O at 1.5mg.L⁻¹, filter-sterilized using 0.2 µm pore size filters (Baril *et al.*, 2011). These suspensions were incubated with shaking at 30°C and 12°C for *B. weihenstephanensis* and 45°C and 20°C for *B. licheniformis* Ad978. Spores in SMB were harvested when free spores represented more than 95 % of cells under X1000 magnification in phase-contrast microscopy (Olympus BX50, Olympus Optical Co., Ltd, Hamburg, Germany), *i.e.* after 1-2 days at optimal growth temperature for both strains and up to 10 days at suboptimal temperature for both strains. The spore suspensions were centrifuged (6000 x *g*, 10 min, 12°C). Spore pellets were suspended in 5 mL of sterile distilled water. The 5 mL suspensions were divided into 1 mL aliquots and stored for one month at 4°C before use. Laboratory observations consistently shows that spore heat resistance does not change for at least a six months storage time (unpublished data). The final concentrations of the stock suspensions were 10⁸ spores.mL⁻¹ for *B. weihenstephanensis* and 10⁹ spores.mL⁻¹ for *B. licheniformis*.

2.2.3. Heat treatment

The spores were diluted in buffered peptone water (casein peptone at 10 g.L⁻¹, NaCl at 5 g.L⁻¹, K₂HPO₄ at 4.5 g.L⁻¹, KH₂PO₄ at 1.8 g.L⁻¹, pH 7.00) to a final concentration of around 10⁷ spores.mL⁻¹. Capillary tubes (200 µL volumes) were filled with 100 µL of spore suspension and sealed, then immersed into a water/glycerol bath maintained at 85°C, 90°C and 95°C for *B. weihenstephanensis* and 95°C, 100°C and 105°C for *B. licheniformis* (11, 12). Capillary tubes were removed from the bath at appropriate time intervals and immediately cooled in a water/ice bath for 30 s. The tips were broken and the heat-treated spore suspensions were diluted in tryptone salt broth (Biokar Diagnostics, Beauvais, France). To estimate the spore concentration at the initial time (*t*₀), the spore suspensions were treated in a water bath at 70°C for 5 min using the same capillary tube method.

2.2.4. Recovery

Volumes of 100 μL of the appropriate decimal dilutions of heat-treated spores were spread on Brain Heart Agar (BHA, Biokar Diagnostics, Beauvais) at pH values ranging from 4.5 to 9.5 or incubated at temperatures ranging from 4°C to 40°C for *B. weihenstephanensis* and from 15°C to 60°C for *B. licheniformis*. BHA at a range of pH values was obtained as follows: 2X BHI broth was prepared and adjusted by HCl 1 M addition to the desired pH, measured with a PHM210 pH meter (Meterlab, Villeurbanne, France) and a Tuff Tip® electrode (Fisher Bioblock Scientific, Illkirch-Graffenstaden, France) previously calibrated using pH 4.00, pH 7.00 and pH 10.00 standard solutions. Then, the 2X BHI broth was filtered on a 0.2 μm filter (Steritop system; Millipore Corporation, Billerica, MA), and mixed to an equal volume of 2X molten agar (Agar30 g. L⁻¹). After mixing the BHI broth and the agar, the pH of the solidified and cooled medium was checked using the Tuff Tip® electrode introduced into the top 1 cm layer of the agar. This pH value was recorded as the “recovery pH” for further experiments. Inactivation at a range of temperatures, and recovery at optimum or sub-optimal growth temperatures (8°C, 30°C and 37°C for *B. weihenstephanensis*, 18°C, 45°C and 58°C for *B. licheniformis*) and pH values (pH 5.20, 7.40 and 8.00 for both strains) were performed in triplicate, each replication being performed with an independently prepared spore suspension. The full experimental design is presented in supplementary material Table S1. Colony counts were recorded when they remained unchanged despite increasing incubation time. To ensure that BHA was sufficient for full spore germination, recovery after heat treatment on BHA supplemented with a 25 mM L-Alanine / Inosine mix triggering germination in *Bacillus* sp. strains (Garcia *et al.*, 2010; Madslie *et al.*, 2014) or with 12.5 mg.L⁻¹ lysozyme was evaluated with spores of both strains, at optimal and one suboptimal temperature. Dehydration of recovery agar was monitored by weighing Petri dishes for 20 days at 45°C. In this extreme condition, water loss was about 15% of the agar, resulting in a similar increase in the nutrient concentrations. In most tested conditions the incubation time was shorter and/or the temperature was lower. The media dehydration effects on recovery were therefore assumed to be minor. Spores of both strains remained phase-bright during incubation at room temperature for 15 min, which exceeds the time necessary for inclusion in molten agar and incubation at the target temperatures. Germination between the

end of the heat treatment and incubation under test conditions was therefore considered as negligible.

To ensure that spores density did not impact the recovery ability in our conditions, Petri dishes of different sizes (4.5 cm, 9.0 cm and 15 cm diameter) have been inoculated with suspensions of heat treated spores at 10^6 spores.mL⁻¹, 10^7 spores.mL⁻¹ and 10^8 spores.mL⁻¹ similarly to a previous work evaluating the influence of spore density on *C. botulinum* germination (Webb *et al.*, 2012). For each condition, the spores were incubated at 30°C and 8°C for *B. weihenstephanensis* and 45°C and 20°C for *B. licheniformis*. There was in our conditions no significant effect of spore density on recovery ability (data not shown).

2.2.5. Modeling

2.2.5.1. Primary model

Heat inactivation curves were fitted with the model presented in Equation 2 (Mafart *et al.*, 2002).

$$\log N = \log N_0 - \left(\frac{t}{\delta}\right)^p \quad (2)$$

where N is the surviving population; N_0 is the initial spore population, δ the time to the first decimal reduction and p is a shape parameter. $\log(N)$ will designate the decimal logarithm of N throughout the paper.

2.2.5.2. Secondary recovery model

The developed recovery model is derived from the Gamma concept (Zwietering *et al.*, 1992) (Equation 3).

$$\frac{1}{\delta_{(T_{HT}) @ (T', pH')}} = \frac{1}{\delta_{max}^*} \lambda_{T_{HT}}(T_{HT}) \lambda_{T'}(T') \lambda_{pH'}(pH') \quad (3)$$

where $\delta_{(T_{HT}) @ (T', pH')}$ is the apparent heat resistance of spores heat-treated at temperature T_{HT} then recovered at incubation temperature T' in agar medium at pH' ; δ_{max}^* is the time to the first decimal reduction observed for the heat treatment temperature T^* and at the optimal

recovery temperature, T'_{opt} , and the optimal recovery pH, pH'_{opt} ; $\lambda_T(T')$ and $\lambda_{pH'}(pH')$ are functions describing the effect of incubation temperature T' and pH pH' during recovery (Equations 3 and 4, respectively) and $\lambda_{T_{HT}}(T_{HT})$ is the lethal rate (Equation 5) (Valdramidis and Impe, 2012) (Fellows, 2009). At the optimal recovery temperature and pH, $\lambda_T(T')$ and $\lambda_{pH'}(pH')$ are equal to 1. At the reference temperature and pH, $\lambda_{T_{HT}}(T_{HT})$ is equal to 1. When recovery conditions become adverse, $\lambda_T(T')$ and $\lambda_{pH'}(pH')$ take increasing values and tend towards the infinite when T' and pH' become close to the limit of their domain of definition. The $\lambda_T(T')$ and $\lambda_{pH'}(pH')$ functions were derived from the inverted Rosso function (Rosso *et al.*, 1995) where growth limits or cardinal values are input parameters (Equation 2). The equations (4) and (5) of the $\lambda_T(T')$ and $\lambda_{pH'}(pH')$ are:

$$\lambda(T') = 1 / \frac{(T' - T'_{max})(T' - T'_{min})^{0.1}}{(T'_{opt} - T'_{min})^{-0.9} [(T'_{opt} - T'_{min})(T' - T'_{opt}) - (T'_{opt} - T'_{max})(-0.9 T'_{opt} + T'_{min} - 0.1 T')]}$$
 (4)

on the recovery temperature range and,

$$\lambda(pH') = 1 / \frac{(pH' - pH'_{max})(pH' - pH'_{min})^2}{(pH'_{opt} - pH'_{min}) [(pH'_{opt} - pH'_{min})(pH' - pH'_{opt}) - (pH'_{opt} - pH'_{max})(pH'_{opt} + pH'_{min} - 2pH')]}$$

(5)

on the recovery pH range.

where T'_{min} , T'_{opt} , T'_{max} , pH'_{min} , pH'_{opt} and pH'_{max} are the minimal (min), optimal (opt), maximal (max) conditions of temperature or pH for recovery.

The effect of temperature used for the heat treatment was quantified by the Bigelow model (Equation 6) (Bigelow, 1921):

$$\lambda(T_{HT}) = 10^{\frac{T_{HT} - T_{HT}^*}{z_T}}$$
 (6)

where T_{HT} is the heat treatment temperature, T_{HT}^* is the reference heat-treatment temperature. T_{HT}^* was fixed at 90°C for *B. weihenstephanensis* and at 100°C for *B. licheniformis*. Finally, z_T is heat sensitivity, *i.e.* the change in heat-treatment temperature leading to a tenfold reduction of the decimal reduction time.

The model was fitted on the observations ($\log \delta$ or $\log N$) by minimizing the sum of squared errors (SSE) using *lsqcurvefit* function from *MatlabR2012b* (The Math-works, Natick, USA). The goodness of fit of the model was checked by the Akaike Information Criterion (AICc) and the RMSE (Root Mean Square Error) (Huet *et al.*, 2010; Scherrer *et al.*, 2009). The smaller the AICc, and the RMSE, were, the better the model was fitted on the data. The 95% confidence intervals were calculated using the *nlparci* function from *MatlabR2012b*. The fitting performance of the model was statistically evaluated by the *F* test, comparing the mean square error of the model to the mean square error of the data. The computed *f* value was compared to the *F* table value (0.05 significance level). If the *f* value was lower than the *F* value from the table, the *F* test was accepted indicating that the model fitting was statistically acceptable.

The fitting of models was compared with a likelihood ratio test and a test statistic S_L computed as follows (Equation 7):

$$S_L = n \log RSS_C - n \log RSS_U \quad (7)$$

where n is the number of data, RSS_C is the residual square sum for the constrained (C) model and RSS_U is the residual square sum for the unconstrained (U) model. In this work the “constrained” models were (i) using a unique p (Equation 1) or a unique z_T value (Equation 5), or (ii) using pre-determined cardinal temperatures and pH for each strain (Equations 3 and 4). The “unconstrained” models were (i) using one p -value for each inactivation curve or one z_T value for each of the $\log N = f(T')$ or $\log \delta = f(pH')$ curve, or (ii) using the cardinal parameters estimated by the model. S_L is low when the selection of the model has no significant incidence on the quality of fitting. When n tends towards infinity the limiting distribution of S_L is χ^2 distributed with $p_u - p_c$ degrees of freedom, where p_u is the number of parameters in the unconstrained model and p_c the number of parameters in the constrained model. If S_L is lower than χ^2 ($\alpha=0.05$), the difference in the fitting of both models was considered as not significant.

2.3. Results

A total of 115 inactivation curves of *B. weihenstephanensis* spores and 78 of *B. licheniformis* spores were performed, each with at least six counts of survivors and 4 log reductions (Supplementary material, Table S1 to Table S5). The inactivation curves (Figure 8) were fitted with the equation 1 (Mafart, 2000). No change in the inactivation curve shape (p value) was noted according to sporulation, heat-treatment and recovery conditions. Instead of a different p value for each inactivation curve, a single p value was estimated for all inactivation curves. This p value was estimated at 0.68 ± 0.03 for *B. weihenstephanensis* and 1.96 ± 0.13 for *B. licheniformis* as the curve shape was concave upward and concave downward, respectively. No significant difference could be pointed out between the p values estimated on each inactivation curve and the p value estimated on all the inactivation curves (likelihood ratio test, $\alpha=0.05$).

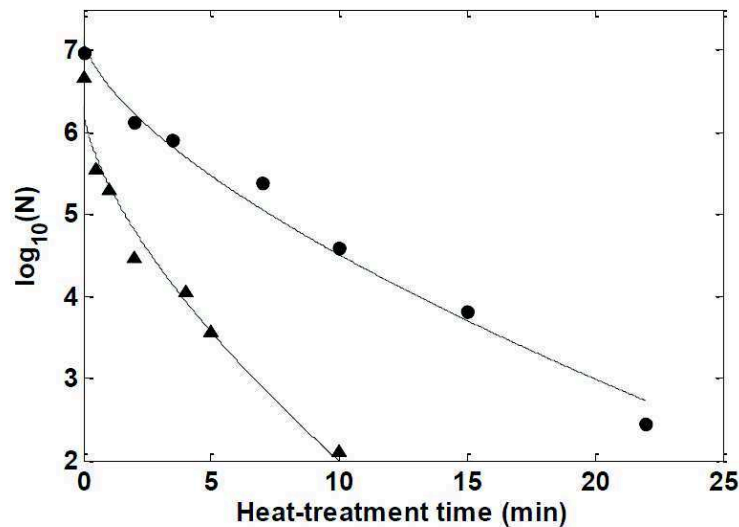


Figure 8: Inactivation curves of *B. weihenstephanensis* spores heat-treated at 90°C and incubated at pH5.20 (▲) and pH7.40 (●) at 30°C. The lines correspond to the fitting of the data to the non-linear Mafart model (Mafart, 2000).

The impact of recovery temperature was significant only at temperatures closed to the recovery boundaries. The extreme temperatures at which recovery was observed were 6°C and 39°C for *B. weihenstephanensis* and 17°C and 59°C for *B. licheniformis*. For *B. weihenstephanensis* the mean \log values of δ , the apparent heat resistance, were $0.31 \pm 0.06 \log$ min at 8°C, $0.39 \pm 0.07 \log$ min at 30°C, $0.39 \pm 0.07 \log$ min at 37°C; for *B. licheniformis* the mean $\log \delta$ -values were $0.98 \pm 0.06 \log$ min at 18°C, $1.14 \pm 0.01 \log$ min at 45°C, $1.00 \pm 0.06 \log$ min at 58°C, for the reference heat treatment temperature. No significant changes in the δ value were observed from 8°C to 37°C for *B. weihenstephanensis* and from 18°C to 58°C for *B. licheniformis* (ANOVA test, $\alpha=0.05$). The time required for survivors to form a colony was unsurprisingly slower at suboptimal temperatures than at optimal growth temperatures. For example, the time to colony counting for *B. weihenstephanensis* KBAB4 was 24 hours at 30°C and pH 7.40, and 20 days at 7°C and pH 7.40.

There was a progressive decrease in δ values as the recovery pH came close to the pH recovery limits. The estimated optimal recovery pH was 7.80 ± 0.23 for *B. weihenstephanensis* and 7.73 ± 0.13 for *B. licheniformis*. A decrease in the recovery pH from 7.00 to pH 5.50 caused a threefold decrease in δ values of both *B. weihenstephanensis* and *B. licheniformis*. Neither strain formed any colonies at a recovery pH lower than 4.70. The δ value replicates for each strain was determined at the optimal recovery pH and at two suboptimal recovery pH values. For *B. weihenstephanensis* the mean $\log\delta$ values were $-0.04 \pm 0.10 \log$ min at pH 5.40, $0.34 \pm 0.13 \log$ min at pH 7.40 and $0.37 \pm 0.11 \log$ min at pH 8.00; for *B. licheniformis* the mean $\log\delta$ values were $0.41 \pm 0.07 \log$ min at pH 5.40, $1.09 \pm 0.03 \log$ min at pH 7.40 and $0.96 \pm 0.21 \log$ min at pH 8.00. Similarly, spore recovery was slower at suboptimal pH than at optimal pH. For example, the time to counting for *B. weihenstephanensis* was 24 hours at pH 7.40, and at 30°C, and 15 days at pH 5.10 and 30°C.

The spores produced at a suboptimal sporulation temperature behaved similarly to those formed at the optimal sporulation temperature (*i.e.* same trend in terms of the influence of recovery temperature and pH). The major difference was in the δ_{max} value, lower at suboptimal sporulation temperature (Figure 9). The spores produced in optimal conditions were treated at

three different temperatures in order to estimate their heat sensitivity (z_T value). From each recovery condition, it was possible to estimate a z value. Whatever the recovery temperature and pH, the z value was comprised between 7.3°C and 8.8°C for *B. weihenstephanensis* (19 z values) and between 7.0°C and 8.0°C for *B. licheniformis* (10 z values). No significant difference could be pointed out between each z value estimated on each recovery conditions and the single estimated z value (likelihood ratio test, $\alpha=0.05$). The heat sensitivity (z value) was therefore assumed to be constant in the range of tested conditions for both strains and equal to 8.02°C \pm 0.26°C for *B. weihenstephanensis* and 7.67°C \pm 0.27°C for *B. licheniformis*. The heat treatment temperature only has an impact on spore heat resistance (Figure 9). There was no interaction between heat treatment temperature and recovery conditions: the z_T value was not impacted by the recovery conditions.

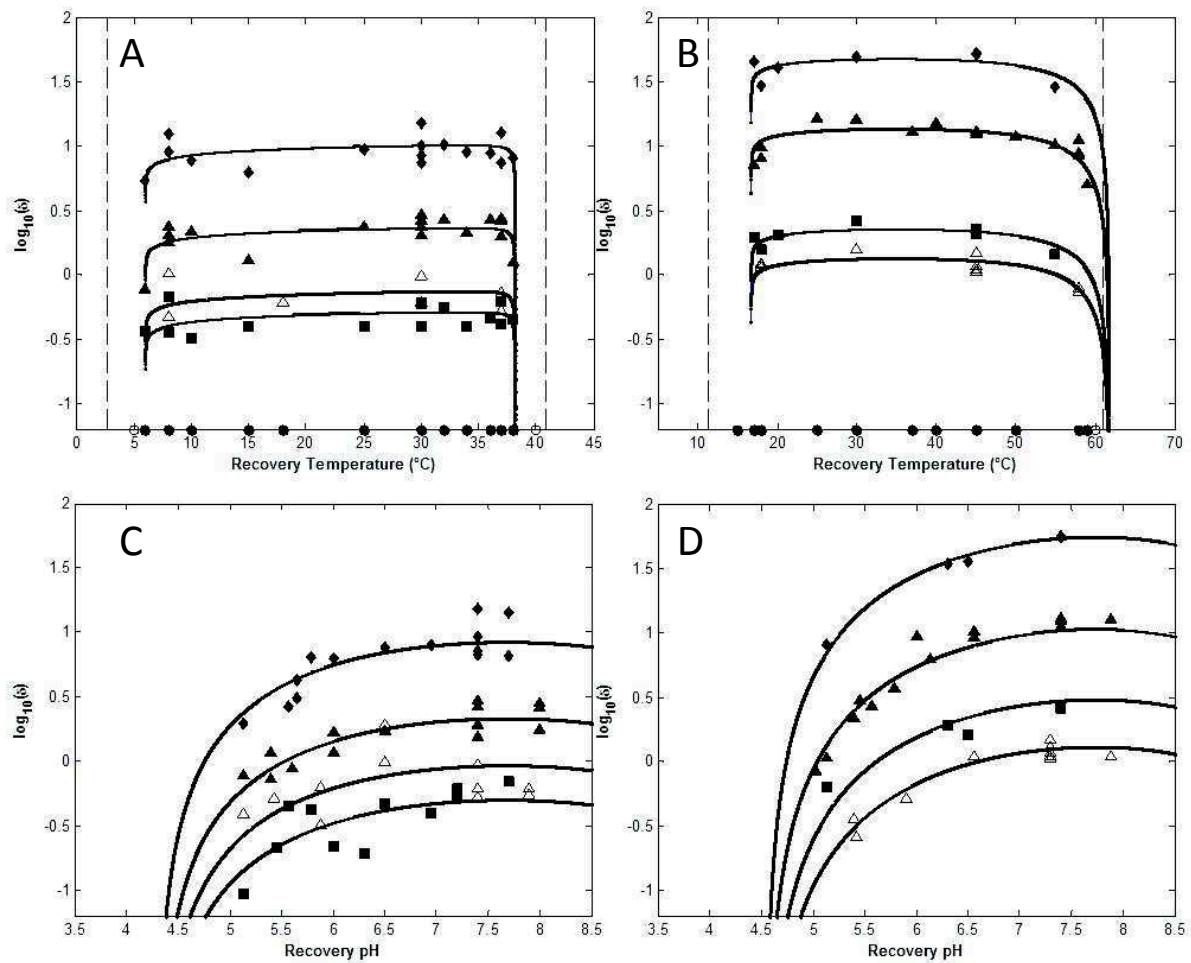


Figure 9: Effect of recovery temperature and pH on spores of *B. weihenstephanensis* (A, C) produced at 30°C (optimal temperature) (solid symbols) and 12°C (suboptimal temperature) (open symbols), treated at 95°C (squares), 90°C (circles) and 85°C (diamonds), and on spores of *B. licheniformis* (B, D) produced at 45°C (optimal temperature) (solid symbols) and 20°C (suboptimal temperature) (open symbols), treated at 105°C (squares), 100°C (circles) and 95°C (diamonds), on apparent heat resistance ($\log \delta$). The vertical dashed lines represent the boundaries of temperature or pH beyond which no recovery was observed. The full lines correspond to the fitting of the model to the data developed in the present work (see Material and Methods for details).

Modeling the effect of recovery temperature and pH conditions on the spores heat-resistance

Equation 2 was used to model the spores' apparent heat resistance according to the heat treatment temperature and the recovery temperature and pH. The z value, corresponding to heat sensitivity, was considered constant for both strains (see above). The model was fitted on 115 $\log\delta$ -values for *B. weihenstephanensis* and on 78 $\log\delta$ -values for *B. licheniformis*. T'_{min} , T'_{opt} , T'_{max} , pH'_{min} , pH'_{opt} and pH'_{max} were estimated for each strain. The RMSE values were 0.15 and 0.11 for *B. weihenstephanensis* and *B. licheniformis* respectively (Table 1). These RMSE values are low compared to the standard deviation on $\log\delta$ values from replicated inactivation curves. Moreover, the fitting performance of the model was statistically accepted by the F test (with $\alpha = 0.05$). Consequently, the model derived from Equation 2 satisfactorily describes the recovery behavior of heat-treated spores of both strains.

Recovery after heat treatment is linked to incubation temperature and pH. Temperature and pH growth limits are among the most commonly available characteristics of bacteria. One option of the model was to use previously published growth limits (Baril, 2011), and therefore to fix the recovery parameters. No significant difference could be pointed out between the fitting with all estimated parameters and the fitting with fixed cardinal values for both strains (likelihood ratio test, $\alpha=0.05$).

Table 3: Estimated values and confidence intervals ($\alpha=0.05$) of cardinal recovery parameters and growth cardinal values of *B. weihenstephanensis* KBAB4 and *B. licheniformis* Ad978 estimated with two models: one with fixed growth parameters, one with an estimation of all parameters

Model fitting	Model parameters:			
		<i>B. weihenstephanensis</i> KBAB4	<i>B. licheniformis</i> Ad978	
with temperature and pH growth limits as fixed parameters	Estimated heat-treatment parameters	$\log\delta^*_{opt}{}^a$ (log min)	0.36 [0.34 ; 0.38] ^c	1.10 [1.08 ; 1.12] ^c
		$\log\delta^*_{opt}{}^b$ (log min)	-0.07 [-0.10 ; -0.04] ^c	0.15 [0.12 ; 0.18] ^c
		z_T (°C)	8.05 [7.79 ; 8.31] ^c	7.66 [7.32 ; 8.00] ^c
	Predetermined cardinal temperature and pH ^d	$Tmin^d$ (°C)	2.72 [0.38 ; 5.60] ^c	11.30 [6.12 ; 17.66] ^c
		$Topt^d$ (°C)	31.91 [30.93 ; 32.60] ^c	49.01 [47.52 ; 50.34] ^c
		$Tmax^d$ (°C)	40.91 [40.41 ; 41.84] ^c	57.87 [56.27 ; 65.83] ^c
		$pHmin^d$	4.35 [4.16 ; 4.51] ^c	4.63 [4.43 ; 4.85] ^c
	$pHopt^d$	7.71 [7.55 ; 7.95] ^c	8.17 [7.86 ; 8.72] ^c	
	Number of data	115	78	
	RMSE	0.15	0.14	
with heat-treatment parameters and recovery limits as estimated parameters	Estimated heat-treatment parameters	$\log\delta^*_{opt}{}^a$ (log min)	0.38 [0.34 ; 0.42] ^c	1.11 [1.08 ; 1.14] ^c
		$\log\delta^*_{opt}{}^b$ (log min)	-0.04 [-0.09 ; - 0.01] ^c	0.16 [0.12 ; 0.20] ^c
		z_T (°C)	8.06 [7.80 ; 8.32] ^c	7.67 [7.40 ; 7.94] ^c
	Estimated recovery limits	$T'min$ (°C)	5.94 [5.82 ; 6.06] ^c	16.76 [15.79 ; 17.73] ^c
		$T'opt$ (°C)	36.37 [24.63 ; 48.12] ^c	31.79 [28.40 ; 35.18] ^c
		$T'max$ (°C)	38.03 [37.63 ; 38.42] ^c	65.97 [56.94 ; 75.01] ^c
		$pH'min$	3.79 [3.01 ; 4.58] ^c	4.54 [4.41; 4.68] ^c
		$pH'opt$	7.80 [7.53 ; 8.07] ^c	7.73 [7.61 ; 7.86] ^c
		$pH'max$	10.34 [9.93 ; 10.75] ^c	9.80 [9.69 ; 9.90] ^c
		Number of data	115	78
		RMSE	0.15	0.11

^a optimal heat resistance, at the reference temperature, for spores produced at optimal temperature

^b optimal heat resistance, at the reference temperature, for spores produced at sub-optimal temperature

^c estimated value [confidence interval at $\alpha=0.05$]

^d Baril, 2011 (Baril, 2011)

2.4. Discussion

The spore heat resistance of many *Bacillus* sp. is highly impacted by the sporulation temperature (Carlin, 2011). As shown in previous work (Baril *et al.*, 2012), the sporulation temperature has mainly an impact on the spore heat resistance (expressed with δ values in this work), but does not impact heat sensitivity (expressed with z_T values in this work). The specific effect of recovery conditions is the same whatever the sporulation and heat treatment conditions. Heat treatment leads to the inactivation of spores but some can be sub-lethally injured and are able to germinate, multiply and form a colony (Mafart and Leguérinel, 1997). Supplementation of the recovery media with an alanine and inosine mix, which is known to trigger germination on *B. weihenstephanensis* and *B. licheniformis* (Garcia *et al.*, 2010; Madslie *et al.*, 2014), or with lysozyme known to restore the germination of damaged spores (Setlow, 2013), had no effect on recovery (*i.e.* similar counts after heat treatment on recovery agar, supplemented or not). Consequently the observed effect is likely due to impaired germination subsequent to damage and, also, a reduced ability of the germinated cells to adapt to suboptimal temperature conditions to form colonies. The developed model satisfactorily describes the recovery behavior of heat-treated *Bacillus* sp. spores, in accounting for pre-, per- and post-treatment conditions. The range of pH and temperature allowing the recovery of spores of the tested *B. weihenstephanensis* and *B. licheniformis* strains was within the range of temperature and pH allowing growth. The domain of growth temperatures and the domain of recovery temperatures have very close boundaries. Consequently the current model used predetermined cardinal temperature and pH values of each strain as control parameters, because these values have a real biological meaning and are reliable estimators of growth limits (McMeekin, 1993). The cardinal temperatures T_{min} and T_{max} are respectively the temperature below which and the temperature above which growth cannot theoretically be observed (Bajard *et al.*, 1996). The minimal temperature for growth estimated by a cardinal temperature model (T_{min}) is always a few °C lower than the observed minimum temperature allowing growth (Ross *et al.*, 2011). As we demonstrated, these values can be used as input parameters to estimate the apparent heat resistance at given recovery temperatures and pH values. The recovery behavior of bacterial spores after heat treatment can therefore be modeled with parameters having a

biological meaning and of relatively easy access to the scientific community through literature review for instance.

The impact of recovery temperature on the spore colony forming ability is low in the recovery range. This has also been observed for different species such as *B. cereus* CNRZ 110, *A. acidoterrestris* ATCC 49025 and several strains of *B. stearothersophilus* (Leguérinel *et al.*, 2006), 29, 30). Only the time taken to form a colony was significantly influenced by the recovery temperature. Recently, a model describing the effect of different factors on the lag time of *B. cereus* spores has been developed (Daelman *et al.*, 2013). In this study, the observed biological response is the estimated lag time corresponding to the time taken for spores to germinate, outgrow and grow, taking into account only the time required to detect the germination and growth of at least one spore. The effect of recovery temperature could be explained by a prolongation of the germination and outgrowth duration as the temperature approaches the growth boundaries, as demonstrated for several *Bacillus* and *Clostridium* sp. (Knaysi, 1964; Levinson and Hyatt, 1970; Stringer *et al.*, 2009; Vary and Halvorson, 1965), and by the decrease in growth rates at temperatures lower or higher than the optimal temperature. Many foods, such as REFREDS or cooked chilled foods, are processed with mild heat treatments and rely on refrigeration for preservation and/or combining suboptimal pH to low temperature as additional hurdles to prevent growth of surviving pathogenic or spoilage spore forming bacteria (Peck, 2006). In these foods, our results suggest that storage at low temperature will mainly delay the growth of spore-forming bacteria, not prevent the growth of surviving spores and that recovery pH could actually affect the recovery ability of surviving spores. The recovery pH has a more progressive effect on the colony-forming ability, mathematically described with an exponent value of 2.0 in equation (4). pH values near the optimal growth pH offer the highest colony formation ability for both strains. Germination rates at low pH values may be lower and/or colony formation slower, as previously observed for *B. cereus* for instance (Broussolle *et al.*, 2008; Leguerinel *et al.*, 2000) or *C. botulinum* (Blocher and Busta, 1985). As with temperature, the domain of growth pH values and the domain of recovery pH values have very close boundaries. Prolonged outgrowth caused by low pH could be due to the H⁺ effect on cytoplasmic pH and, although highly strain/species-dependent, to significant inner pH

modifications during germination (Setlow, 2003). A slight change of temperature or pH near to the boundaries caused a dramatic decrease in apparent heat resistance values. This can be explained by the growth behavior of bacteria cells in conditions close to the growth/no growth boundaries, where the probability of cells forming a colony is lower than in optimal conditions (Koutsoumanis, 2008). This phenomenon could be strengthened by a decrease in the probability of germination of surviving spores. Moreover, an effect of spore density on spore germination has been shown, on *Clostridium* sp. for instance (Webb *et al.*, 2012). Again, the phenomenon is complicated by the release of dipicolinic acid, triggering germination, during spore germination. Interestingly, the number of inactivated cells can be described by cumulating the heat inactivation effect and the inhibitory effect due to suboptimal recovery conditions (Equation 8).

$$n = n_{HT} + n' \quad (8)$$

where n is the apparent total \log reduction, n_{HT} is the \log reduction due to heat-treatment and n' is the virtual decimal decrease due to recovery conditions.

Equation 1 can therefore be written as follows:

$$n = \log \frac{N_0}{N} = \left(\frac{t}{\frac{\delta_{max}}{\lambda_{HT} \lambda_T' \lambda_{pH}'}} \right)^p \quad (9)$$

This equation is equivalent to equation 9:

$$n = \left(\frac{1}{\delta_{max}} t \cdot \lambda_{HT} \right)^p \cdot (\lambda_X')^p \quad (10)$$

where the effect of heat treatment could be expressed by:

$$n_{HT} = \left(\frac{1}{\delta_{max}} t \cdot \lambda_{HT} \right)^p$$

And the effect of recovery by:

$$n' = n_{HT} [(\lambda_X')^p - 1] \quad (11)$$

The impact of recovery can be calculated knowing the impact of the heat treatment (n_{HT}) and of the recovery medium formulation ($\lambda_{X'}^D$). There is no effect of the recovery environment ($n'=0$) when $\lambda(X')$ is equal to 1, *i.e.* when the recovery conditions are optimal. On the contrary, when the recovery conditions are beyond the recovery limits ($\lambda(X')$ tends to $-\infty$), colony formation on the recovery medium is fully inhibited (n' tends to $-\infty$). This can also be linked to the germination rate where the influence of heat treatment intensity and recovery temperature and pH are taken into account.

In conclusion, using a proper set of parameters for each strain and a model based on generic mathematical functions, the recovery of *B. weihenstephanensis* and *B. licheniformis* spores after heat treatments at diverse temperatures and as a function of the incubation temperature and the pH of the recovery medium was quantified. A similar approach can be used to quantify the impact of pH in addition to temperature during heat treatment on the recovery of spores. In this new model, only the heat resistance at optimal recovery temperature and pH has to be estimated, since the other parameters – cardinal growth temperature and pH – are obtained from independent experiments/sources. The spore population considered here is the population able to germinate and recover physiological activity in order to form a colony on a nutrient agar. It remains undetermined whether the germination or the adaptation of the germinated cell is affected by sub-lethal heat treatment. The biological process leading to the formation of a colony from a stressed spore is likely stochastic and further research is needed to quantify the relative part of these two steps in the spore recovery process.

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Chapitre 3

Recovery at suboptimal temperature and pH of *Bacillus weihenstephanensis* KBAB4 spores treated by pulsed light and peracetic acid.

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Abstract

Heat treatment is widely used to inactivate spores and vegetative cells in food industry. However, non-thermal techniques are also used to inactivate spores such as chemicals treatment or pulsed light. The apparent resistance to peracetic acid and pulsed light of spores of *Bacillus weihenstephanensis* KBAB4 was estimated in different conditions of temperature and pH of recovery. Spores of *B. weihenstephanensis* were produced at 30°C, pH 7.00, at 30°C pH 5.50 and at 12°C, pH 7.00. The spores were treated with 80 mM peracetic acid at 18°C or at a range of fluences (J.m^{-2}) for pulsed light treatment. After each treatment, the spores were incubated on nutrient agar at three different temperatures (12°C, 30°C and 37°C) or at three different pH (pH 5.10, pH 6.00 and pH 7.40). The recover temperature had a significant impact only near the recovery limits. In contrast a decrease in the recovery pH had a progressive impact on apparent resistance to peracetic acid and pulsed light treatments. These impacts of recovery conditions on spore ability to form colonies after these two treatments were similar to the impacts of recovery conditions after heat treatment. Despite the difference of spore inner targets impacted by the different treatments, the recovery conditions had the same impact on the spore ability to germinate, outgrow and recover growth. Finally, the sporulation conditions had no impact on the spore resistance to peracetic acid or pulsed light treatments.

3.1. Introduction

Spore forming bacteria can develop in food products, leading to poisoning or spoilage (Durand *et al.*, 2015; Postollec *et al.*, 2012). Many treatments are available to inactivate spores and vegetative cells. Heat treatments are widely used in food industry but non-thermal techniques, such as chemical treatment or irradiation, are also used to inactivate spores. A classical non-thermal technique used in food industry for surface or material decontamination is the use of chemicals, such as such as hypochlorite solutions, organic acids, hydrogen peroxide... There are particular interests in peroxyacetic acid (also named peracetic acid) because of its antimicrobial activity at low concentration (30 ppm). Peracetic acid combines the effect of active oxygen within an acetic acid molecule and belongs to the class of organic peroxides (Kitis, 2004). The target of this kind of molecule within microorganism inactivation can be DNA, causing mutations in vegetative cells, or the inactivation of functional enzymes. In *Bacillus* spores the main target of chemicals seems to be the inner membrane (Setlow, 2006). The inner membrane becomes, after germination and outgrowth, the membrane of the newly formed vegetative cell. Damages of the inner membrane cause the inactivation of the spore (Genest *et al.*, 2002; Young and Setlow, 2003). Strong acids treatment leads to spores pop up (Leggett *et al.*, 2012). The permeabilization of this inner membrane can also allow the chemicals to enter the core and reach the DNA. But the spore DNA is still protected by its saturation in α/β SASPs (Small Acid Soluble Proteins) (Leggett *et al.*, 2012; Moeller *et al.*, 2014). FDA has approved its use in various fields, including food and beverage industries, notably thanks to its easy decomposition into non-toxic compounds like acetic acid and oxygen (Kitis, 2004).

Pulsed light is a non thermal technology consisting in a short and intense pulse of a spectrum (Elnmanasser *et al.* 2007). In the pulsed light technologies the UV-C (200-290 nm), corresponding to approximately 40% of the emitted light, are the main frequencies causing inactivation (Aguirre *et al.*, 2015; Artíguez *et al.*, 2011). The inactivation efficacy depends on the dose, expressed in $\text{J}\cdot\text{cm}^{-2}$, and the number of flashes (Elnmanasser *et al.* 2007). The inactivation of micro-organisms by pulsed light is mainly due to DNA damages by the formation of pyrimidine dimers and other photo products (Takeshita, 2003). Pulsed light is a good candidate to be used in food industry since it is well adapted to transparent liquids, air disinfection,

surface decontamination and food contact materials (Aguirre *et al.*, 2015; Levy *et al.*, 2012). Pulsed light is used to decontaminate surfaces in many food industries such as bakeries, cheese and meat plants as complement to regular cleaning plans. It is also used for decontamination of boxes, caps, bottles or other packaging types. Efficacy of pulsed light depends on surface materials characteristics (Koutchma, 2008). The decontamination of different food products has been investigated such as vegetable, honey or dairy products (Gomezlopez *et al.*, 2005; Hillegas and Demirci, 2003).

Heat treatments are widely used to inactivate micro-organisms in foods. To optimize heat processes the bacterial spores are used as target micro-organisms. Many works have showed the impact of heat treatment conditions on spore heat resistance (Augustin, 2011; Collado *et al.*, 2003; Mafart, 2000; Mafart *et al.*, 2002). Moreover, the impact of recovery conditions on spore behavior has been demonstrated (Gaillard *et al.*, 2005; Leguérinel *et al.*, 2006; Mtimet *et al.*, 2015; Trunet *et al.*, 2015). Nevertheless, not only the heat treatment is used in food industry to eliminate the bacterial spores. Non-thermal techniques such as chemical treatment or radiation treatments are used to inactivate spores. The recovery behavior and the impact of recovery conditions on spores subjected to non thermal treatments are not known

The impact of recovery temperature and pH on heat treated spore recovery behavior has been described and modeled in previous works (Mtimet *et al.*, 2015; Trunet *et al.*, 2015). Indeed, the recovery temperature has an impact on spore recovery only near the recovery limit and recovery pH has a more progressive effect on the pH recovery range. The aim of this study was to quantify the impact of recovery temperature and pH on spores treated by non thermal stresses, namely peracetic acid treatment and pulsed light treatment.

3.2. Material and methods

3.2.1. Bacterial strains

The *Bacillus weihenstephanensis* strain KBAB4 (INRA, Avignon, France) isolated from a forest soil (Guinebretière *et al.*, 2008). The strain was stored at -80°C in 1.5 mL aliquots of Brain Heart Infusion (BHI, Biokar Diagnostics, Beauvais, France) mixed with 50% glycerol (v/v) at a concentration of approximately 2×10^6 CFU.mL⁻¹. A flask of BHI (100 mL) was inoculated with 1 mL of the stock suspension and incubated for 8 h at 30°C, then 1 mL volume was transferred into 100 mL of BHI for 16 h at 30°C. Finally, 0.1 mL of *B. weihenstephanensis* suspension was added to 100 mL BHI and was incubated for 6 h at 30°C. The final cell concentration in the culture was approximately 10^8 CFU.mL⁻¹; the number of spores estimated by the number of cells surviving a 5 min, 70°C heat treatment was lower than 100 spores.mL⁻¹.

3.2.2. Spore preparation

Spores were produced through a two-step sporulation process (Baril *et al.*, 2011). One hundred mL of the previously described culture, were centrifuged (6000 x *g*, 10 min, 12°C) and the vegetative cells were resuspended in 100 mL of sporulation mineral buffer (SMB) at pH 7.00 (4.5 g.L⁻¹K₂HPO₄ at, 1.8 g.L⁻¹KH₂PO₄ at, 8.0 mg.L⁻¹ CaCl₂, H₂O and 1.5mg.L⁻¹MnSO₄, H₂O at) or in 100 mL of SMB at pH 5.50 made of K₂HPO₄ at 5.04 g.L⁻¹, KH₂PO₄ at 0.35 g.L⁻¹, CaCl₂, H₂O at 8.0 mg.L⁻¹ and MnSO₄, H₂O at 1.5mg.L⁻¹, filter-sterilized using 0.2 µm pore size filters (Baril *et al.*, 2011). The suspensions in SMB at pH 7.00 were incubated at 30°C or 12°C, and the ones at pH 5.50 at 30°C. All cultures in SMB were maintained under agitation at xx rpm. Spores in SMB were harvested when free spores represented more than 95 % of cells under X1000 magnification in phase-contrast microscopy (Olympus BX50, Olympus Optical Co., Ltd, Hamburg, Germany), *i.e.* after 1-2 days of incubation at 30°C and up to 10 days at 12°C. The spore suspensions were centrifuged (6000 x *g*, 10 min, 12°C). Spore pellets were suspended in 5 mL of sterile distilled water. The 5 mL suspensions were divided into 1 mL aliquots and stored for one month at 4°C before use. Spore heat resistance does not change for at least a six months storage time (data not shown). The final concentrations of the stock suspensions were 10^8 spores.mL⁻¹ for

B. weihenstephanensis in SMB at 30°C and pH 7.00, 10^7 spores.mL⁻¹ in SMB at 30°C and pH 5.50, 10^7 spores.mL⁻¹ in SMB at 12°C and pH 7.00 (Trunet *et al.*, 2015).

3.2.3. Peracetic acid treatment

The spores from the stock were diluted to 10^7 spores.mL⁻¹ in 10 mL of peracetic acid solution at 80 mM at 18°C and maintained under agitation at 100 rpm (Oxyanios 5, Anios Laboratoire, Lille, France). At regular time interval, 0.5 mL of the suspension were sampled and then mixed to 0.5 mL of 2.5% (wt/vol) sodium thiosulfate neutralizing solution (Na₂S₂O₃, Sigma Aldrich, Saint Louis, France). To estimate the initial concentration 0.5 mL of suspension was instantly sampled after addition of peracetic acid solution with spores and diluted with 0.5 mL of sodium thiosulfate. The toxicity and efficiency of the neutralizer solution was verified as recommended in NF EN 1276/2010 norm. Sodium thiosulfate solution at 25 g.L⁻¹ had no significant inactivation effect on *Bacillus weihenstephanensis* KBAB4 spores (data not shown).

3.2.4. Pulsed light treatment

The resistance to radiations delivered by pulsed light was performed by using pulsed-light (PL) equipment (Claranor SA, Avignon, France) delivering 250 μs pulses of broad-spectrum white light (100 to 1,100 nm) containing 18.5% of fluence corresponding to UV (wavelengths between 200 nm and 400 nm) emitted by xenon flash lamps under an input voltage of 2,500 V and at fluences ranging up to 5.28 J.cm⁻² (Levy *et al.*, 2012). Fluence was measured using a joulemeter Gentec Solo 2 connected to a 1cm² probe UP17 65H5 DO (Gentec Electro Optics Inc., Québec, Canada). The *Bacillus weihenstephanensis* KBAB4 spores were diluted in 20 mL of sterile demineralized water to a final concentration of 10^6 spores.mL⁻¹. The suspensions were heat treated at 70°C during 10 min to eliminate vegetative cells (Levy *et al.*, 2011). The suspensions were kept on ice throughout the experiment. For each tested fluence, 3 mL of suspension were poured into 30 mm diameter polystyrene Petri dish and placed under the optical cavity. PL fluences (PL doses) varied from 0.397 J.cm⁻² to 2.330 J.cm⁻² according to the number of delivered flashes (one or two at 1 s interval) and the distance to the lamp (between 8 cm and 22

cm). The tested fluences were 0.397 J.cm^{-2} (1 flash at a distance of 22 cm), 0.615 J.cm^{-2} (1 flash at 15.5 cm), 0.966 J.cm^{-2} (1 flash at 10.5 cm), 1.532 J.cm^{-2} (2 flashes at 12.5 cm) and 2.330 J.cm^{-2} (2 flashes at 8 cm). Untreated samples were used as controls.

3.2.5. Recovery

Volumes of 1 mL of the appropriate decimal dilution in sterile demineralized water of spores exposed to peracetic acid or to PL were introduced into BHA at pH 5.00, 6.00 or 7.40. Acidified BHA was prepared as follows. The pH of twice-concentrated (2X) BHI was adjusted by HCl 1M addition and then, filtered-sterilized with $0.22 \mu\text{m}$ pore-size filters. Before use, BHI was mixed with sterile molten agar at 30 g/L (Biokar Diagnosis, Beauvais, France) maintained at 55°C and poured into Petri dishes (Trunet *et al.*, 2015). Colony counts were recorded when they remained unchanged despite increasing incubation time, i.e. from 48 h at 30°C and on BHA at pH 7.40 to 11 days at 10°C and on/or BHA at pH 5.10. Spores were recovered on BHA at pH 7.40 at 10°C , 30°C and 37°C on BHA 8.00 at pH and 5.10 at 30°C . These recovery temperature and pH were the same as the ones tested in a previous work (Trunet *et al.*, 2015).

3.2.6. Modelling

3.2.6.1. Primary model

Inactivation curves of spores treated with peracetic acid were fitted with the model presented in equation 12 (Mafart *et al.*, 2002).

$$\log N = \log N_0 - \left(\frac{t}{\delta}\right)^p \quad (12)$$

and curves of spores treated with pulsed light were fitted with the model presented in equation 13.

$$\log N = \log N_0 - \left(\frac{F}{F_1}\right)^p \quad (13)$$

where N is the surviving population; N_0 is the initial spore population, F was the fluence, δ and F_1 were respectively the time to the first decimal reduction and the fluence to the first decimal reduction, and p was a shape parameter specific to peracetic acid or pulsed light inactivation curves. $\log N$ designated the decimal logarithm of N throughout the paper.

3.2.6.2. Secondary model

The model used to fit the δ values and F_1 values is presented in Equation 14 et 15 (Trunet *et al.*, 2015) :

$$\frac{1}{\delta_{(T,[PAA])@ (T', pH')}} = \frac{1}{\delta_{max}^*} \lambda_{T'}(T') \lambda_{pH'}(pH') \quad (14)$$

$$\frac{1}{F_{1@ (T', pH')}} = \frac{1}{F_{1max}^*} \lambda_{T'}(T') \lambda_{pH'}(pH') \quad (15)$$

where $\delta_{(T,[PAA])@ (T', pH')}$ ($F_{1@ (T', pH')}$) is the time to first decimal reduction at a treatment temperature T and peracetic acid concentration $[PAA]$ (fluence to first decimal reduction for a PL treatment) then incubated at a recovery temperature T' in BHA at pH' ; δ_{max}^* (F_{1max}^*) is the time (the fluence) to the first decimal reduction observed for the PAA treatment (PL treatment) at the optimal recovery temperature T'_{opt} and the optimal recovery pH, pH'_{opt} ; $\lambda_{T'}(T')$ and $\lambda_{pH'}(pH')$ are functions describing the effect of recovery temperature T' and pH pH' (Trunet *et al.*, 2015). At the optimal recovery temperature and pH, $\lambda_{T'}(T')$ and $\lambda_{pH'}(pH')$ are equal to 1. When recovery conditions become adverse, $\lambda_{T'}(T')$ and $\lambda_{pH'}(pH')$ take increasing values and tend towards the infinite when T' and pH' become close to the limit of their domain of definition. The $\lambda_{T'}(T')$ and $\lambda_{pH'}(pH')$ functions were derived from the inverted Rosso function (Rosso *et al.*, 1995) where growth limits or cardinal values are input parameters.

3.2.6.3. Statistics

Each experiment (one survival curve and recovery at a given temperature and pH) was at least triplicated. An independently-prepared spore suspension was used in each replicated experiment.

The model was fitted on the observations ($\log \delta$, $\log F_1$ or $\log N$) by minimizing the sum of squared errors (SSE) using *lsqcurvefit* function (*MatlabR2012b*, The Math-works, Natick, USA). The goodness of fit of the model was checked by the Akaike Information Criterion (AICc) and the RMSE (Root Mean Square Error) (Huet *et al.*, 2010; Scherrer *et al.*, 2009). The smaller the AICc, and the RMSE, were, the better the model was fitted on the data. The 95% confidence intervals were calculated using the *nlparci* function from *MatlabR2012b*. The fitting performance of the model was statistically evaluated by the *F* test, comparing the mean square error of the model to the mean square error of the data. The computed *f* value was compared to the *F* table value (0.05 significance level). If the *f* value was lower than the *F* value from the table, the *F* test was accepted indicating that the model fitting was statistically acceptable.

The fitting of models was compared with a likelihood ratio test. Then, a test statistic S_L computed as follows (Equation 16):

$$S_L = n \log RSS_C - n \log RSS_U \quad (16)$$

where n is the number of data, RSS_C is the residual square sum for the constrained (C) model and RSS_U is the residual square sum for the unconstrained (U) model. In this work the model was “constrained” by using pre-determined cardinal temperatures and pH (Huet *et al.*, 2010). The “unconstrained” condition led to estimation of the recovery limit parameters on the data generated by this study. S_L is low when the constraint has no significant incidence on the model quality of fit. When n tends towards infinity the limiting distribution of S_L is χ^2 distributed with $p_u - p_c$ degrees of freedom, where p_u is the number of parameters in the unconstrained model and p_c the number of parameters in the constrained model. If S_L is lower than χ^2 ($\alpha=0.05$), the difference in the fitting of both models was considered as not significant.

3.3. Results

3.3.1. Impact of recovery temperature and pH on δ and F_1 values

A total of 65 inactivation curves has been obtained for peracetic treatment and 54 for pulsed light treatment, with an average of 7 and 5 counts per curve respectively. For each inactivation curve a minimum of 4 log reductions were obtained. The p value estimated was 0.55 ± 0.11 for the spores treated with peracetic acid and 1.13 ± 0.06 for the spores treated by pulsed light, the curves being respectively concave upward and slightly concave downward (Figure 10).

The $\log \delta$ values, corresponding to the time for 1 log reduction with peracetic acid treatment, were 1.58 ± 0.16 minutes at 10°C , 1.47 ± 0.15 minutes at 30°C and 1.40 ± 0.37 minutes at 37°C for the spores produced in optimal conditions, namely 30°C , pH 7.00. No significant changes in the δ values were observed at 10°C , 30°C and 37°C (ANOVA test, $\alpha=0.05$). The $\log F_1$ values, the fluence ($\text{J}\cdot\text{cm}^{-2}$) for 1 log reduction by pulsed light treatment, were $-0.41 \pm 0.08 \text{ J}\cdot\text{cm}^{-2}$ at 10°C , $-0.34 \pm 0.06 \text{ J}\cdot\text{cm}^{-2}$ at 30°C and $-0.41 \pm 0.08 \text{ J}\cdot\text{cm}^{-2}$ at 37°C , for the spores produced in optimal conditions. No significant changes in the F_1 values were observed at 10°C , 30°C and 37°C (ANOVA test, $\alpha=0.05$). The time required for survivors to form a colony was slower at suboptimal temperatures than at optimal growth temperatures. For example, the time to colony counting 24 hours at 30°C and pH 7.40, and 11 (average) days at 10°C and pH 7.40 for both treatments.

The \log values of δ , were 0.71 ± 0.69 minutes at recovery pH, $pH' = 5.10$, 1.41 ± 0.21 minutes at $pH' 6.00$ and 1.47 ± 0.15 minutes at $pH' 7.40$ for the spores produced at the optimal conditions. A significant difference has been observed between the $\log \delta$ values at $pH' 5.10$ and $pH' 6.00$ or $pH' 7.40$ for the spores produced in optimal conditions (ANOVA, $\alpha=0.05$). The \log values of F_1 , were -0.39 ± 0.05 at $pH' 5.10$, -0.23 ± 0.06 at $pH' 6.00$ and -0.34 ± 0.06 at $pH' 7.40$ for the spores produced at the optimal conditions. Again, a significant difference has been observed between the $\log F_1$ values at $pH' 5.10$ and $pH' 6.00$ for the spores produced in optimal conditions (ANOVA, $\alpha=0.05$). The time for counting the colonies was also impacted by the recovery pH. For example, the time for counting the colonies for *B. weihenstephanensis* was 24 hours at $pH' 7.40$ and 30°C , and 15 days at $pH' 5.10$ and 30°C .

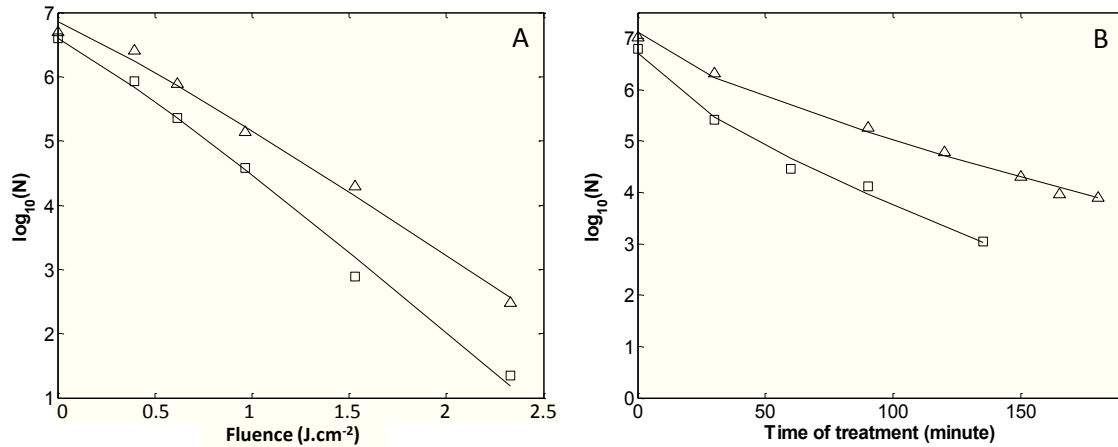


Figure 10: Inactivation curves of *B. weihenstephanensis* KBAB4 spores treated by pulsed light (A) or peracetic acid (B) and incubated at pH7.40 (□)and pH5.20 (△)at 30°C. The lines correspond to the fitting of the data to the non-linear Mafart model (Mafart, 2000).

Table 4 : Time (δ associated to a single value of the parameter p estimated at 1.13 ± 0.06) and fluence (F_1 associated with a value of the parameter p estimated at 0.55 ± 0.11) to the first decimal reduction of *B. weihenstephanensis* spore exposed to PAA and PL as a function of sporulation temperature and pH, and recovery temperature, T' , and recovery pH, pH' .

^a Values significantly impacted by the recovery conditions, compared to values at optimal conditions (ANOVA, $\alpha=0.05$).

Sporulation conditions	Recovery Temperature (°C)	Recovery pH	$\log \delta \pm SD$	RMSE	$\log F_1 \pm SD$	RMSE
30°C, pH 7.00	30	7.40	1.47±0.15		-0.34±0.06	
	30	6.00	1.41±0.21		-0.23±0.06	
	30	5.10	0.71±0.69 ^a		-0.49±0.05 ^a	
	37	7.40	1.40±0.37		-0.38±0.08	
	10	7.40	1.58±0.16		-0.39±0.08	
30°C, pH 5.50	30	7.40	1.64±0.12		-0.46±0.07	
	30	6.00	1.57±0.09		-0.34±0.03	
	30	5.10	1.01±0.14 ^a		-0.52±0.09 ^a	
	37	7.40	1.20±0.14		-0.40±0.02	
	10	7.40	1.24±0.15		-0.54±0.05	
12°C, pH 7.00	30	7.40	1.53±0.09		-0.41±0.03	
	30	6.00	1.47±0.20		-0.36±0.02	
	30	5.10	0.93±0.07 ^a		-0.53±0.02 ^a	
	37	7.40	1.26±0.07		-0.38±0.04	
	10	7.40	1.39±0.06	0.12	-0.50±0.12	0.14

3.3.2. Impact of sporulation conditions on δ and F_1 values

The sporulation conditions seemed to have no effect on the recovery behavior of *B. weihenstephanensis* KBAB4 after peracetic acid or pulsed light treatments. Indeed, no significant difference has been observed between the $\log \delta$ values of spores produced in adverse conditions and $\log \delta$ values of spores produced in optimal conditions (ANOVA $\alpha=0.05$). The spores produced in suboptimal conditions have the same resistance to the tested stresses than the spores produced in optimal conditions. Moreover, the evolution of the $\log \delta$ values and $\log F_1$ values have the same trend regarding the recovery temperature and pH for spores produced in different conditions (Table 4).

3.3.3. Modeling the δ and F_1 values regarding the recovery and sporulation conditions

Equation 3 was used to model the time (minutes) to first decimal reduction –for a peracetic acid treatment- according to the recovery temperature and pH. Firstly, the 65 $\log \delta$ values allowed T'_{min} , T'_{opt} , T'_{max} , pH'_{min} , pH'_{opt} and pH'_{max} estimation. The RMSE value was 0.24 and was low compared to the standard deviation on $\log \delta$ values from the replicated inactivation curves. Moreover, the fitting performance of the model was statistically accepted by the F test (with $\alpha = 0.05$). Then, the 65 δ values were fitted using equation 3 using growth cardinal parameters previously estimated by (Baril, 2011) (Figure 11). The RMSE value was 0.25 and the fitting performance was statistically accepted by the F test ($\alpha=0.05$). The growth cardinal values could be used as input parameters in recovery model, allowing describing the recovery behavior of spores treated by peracetic acid (likelihood ratio test, 5%).

Equation 4 was used to model the spore fluence ($J.cm^{-2}$) to first decimal reduction –for a peracetic acid treatment- according to the recovery temperature and pH. F_1 values ($n=54$) were fitted with equation (2) and T'_{min} , T'_{opt} , T'_{max} , pH'_{min} , pH'_{opt} and pH'_{max} were estimated in a first time. The RMSE value was 0.10 and was low compared to the standard deviation on $\log \delta$ values from the replicated inactivation curves. Moreover, the fitting performance of the model was statistically accepted by the F test (with $\alpha = 0.05$). Then, the 54 F_1 values were fitted with equation 2 using fixed cardinal parameters (Baril, 2011) (Figure 11). The fitting performance was not satisfactory because of the pH'_{opt} value. Indeed, the first fitting estimated the pH'_{opt} at

6.53±0.49 and the pH_{opt} obtained from the literature was 7.71±0.24. Inputting the estimated value of 6.53±0.49 as fixed pH'_{opt} in equation 3 the fitting was statistically accepted by the F test ($\alpha=0.05$) and the likelihood ratio test was accepted. The model allowed describing the recovery behavior of spores treated by pulsed light. No significant difference was pointed out between the $\log \delta$ values of the spores produced in different conditions of sporulation, no matter the recovery temperature or pH. The same observation was made for $\log F_1$ values. So, only one fitting was needed with equation 4 or 5 to describe the spore recovery behavior no matter the sporulation condition.

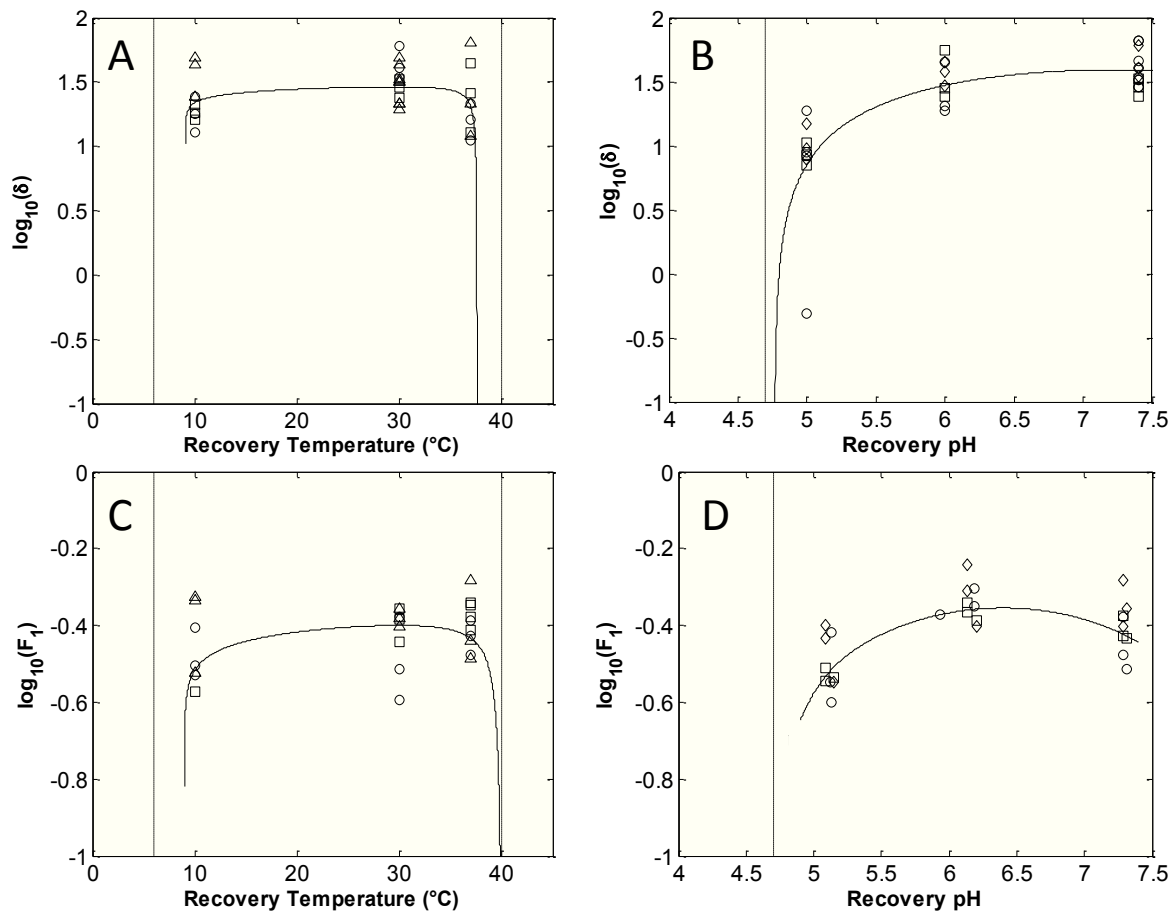


Figure 11: Effect of recovery temperature and pH on the resistance of spores of *B. weihenstephanensis* KBAB4 to peracetic acid (A, B) or to pulsed light (C, D). The spores were produced at pH 7.00 and 30°C (optimal temperature) (□), at pH 5.50 and 30°C (△) or at pH 7.00 and 12°C (○). δ (F_1) is the time (Fluence) to first decimal reduction of spores exposed to PAA (Pulsed light). The full lines correspond to the fitting of the data to the model (equations 4 and 5) with cardinal values as parameters: $T_{min}=5.94$, $T_{opt}=36.37$, $T_{max}=38.03$, $pH_{min}=3.79$, $pH_{opt}=7.80$ except for pulsed light treatment (D) where $pH_{opt}=6.53$ was used.

3.4. Discussion

The recovery temperature had no impact on the apparent resistance of *B. weihenstephanensis* spores to PAA treatment and PL treatment. It was also described for heat treated *B. weihenstephanensis* spores: the impact of recovery temperature was observed only near the recovery boundaries for different strains (Mtimet *et al.*, 2015; Trunet *et al.*, 2015). In this work, the extreme tested temperatures were close enough to limit conditions to confirm that the impact of recovery temperature must be significant only for temperatures near the boundaries for recovery after treatment with peracetic acid or pulsed light. The recovery conditions had the same impact on *Bacillus weihenstephanensis* spore recovery after wet heat treatment, peracetic acid treatment, or pulsed light treatment. The temperature had no impact on the numbers of treated spores able to grow on agar media but impacted the time of colony apparition. This can be explained by the prolongation of the germination, the outgrowth and the growth as the temperature approaches the growth limits (Garcia *et al.*, 2010; Knaysi, 1964; Stringer *et al.*, 2009). The recovery pH seemed to have a more progressive impact on recovery after peracetic acid treatment. A significant difference of $\log \delta$ was observed between pH 6.00 and pH 5.20. The time for the first \log reduction decreased of 0.5 \log with variation of 1 recovery pH unit as it was observed for heat treated spores. The impact of recovery pH can be explained by the effect of external pH on spore germination and outgrowth. Indeed, during those phenomena, the inner pH knows important modifications, passing from 6.5 to 7.7. So, if the external pH is low that can have an impact on germination and outgrow (Setlow, 2003). The fitting of the data with the model developed by Trunet *et al.* 2015, using cardinal growth temperatures and pH, showed satisfactory results for recovery temperature impact on spores treated with PAA.

For the spores treated by pulsed light, the optimal pH of recovery was significantly lower than the optimal growth pH value. Nevertheless, the fitting was statistically satisfactory using the optimal recovery pH estimated in a first time and was equal to 6.53 ± 0.49 . The estimated minimal recovery pH (pH'_{min}) was not significantly different of minimal growth pH. This may be a simple shift of the optimal recovery pH and a plateau may exist around pH 6.50 meaning that the recovery pH had still the same impact on spore recovery after pulsed light treatment than

after heat treatment. The difference may also result from the difference in the elements targeted by this kind of treatment.

In vegetative cells, the resistance to chemicals depends on inactivation of toxic agents by protoplast enzymes such as catalase or super oxide dismutase. In dormant spores such enzyme activity plays no role in resistance even if such enzymes are found in the spore core. The metabolic activity is possible only when the spores are rehydrated (Casillas-Martinez and Setlow, 1997). In dormant spores, the first factor that allows resistance to chemicals is the spore coat which plays a role of an impermeable layer (Setlow, 2006). The second important resistance factor is the inner membrane structuration as it is also highly impermeable to many kinds of molecules (Leggett *et al.*, 2012; Setlow, 2006).

The main target of pulsed light treatment is the DNA. The spores are 10 to 50 fold more resistant to UV radiation than vegetative cells (Nicholson *et al.*, 2000). The spore resistance to pulsed light depends on several elements like DNA repair, DNA protection by α/β SASPs and high level of CaDPA (Dipicolinic acid- Ca^{2+}) in the core. The resistance to UV can be due to the presence of pigments in the spore coats absorbing a part of the energy (these pigments do not exist in all *Bacillus* species) (Nicholson *et al.*, 2005, 2000; Setlow, 2006).

Despite that elements targeted by the three types of treatment (wet heat, peracetic acid, pulsed light) are different, the impact of recovery conditions seemed to be the same. The spores that resist to these treatments are sub-lethally injured and the recovery conditions that they will encounter impact their germination, outgrowth and growth abilities. Thus, their colony forming ability will be also impacted by the encountered conditions. It is impossible to say if the recovery conditions have the same physiological impact on the spores treated by different techniques but the rate of spores able to germinate, outgrow and form a colony is the same whatever the treatment.

The main difference between the heat treated spores and the spores treated by the methods in this work was the absence of impact of sporulation conditions on resistance to treatment. Indeed, the spores produced at sub optimal temperature have a lower heat resistance than spores produced in optimal conditions. In this study, the spores produced in suboptimal

conditions had the same resistance than spores produced in optimal conditions. It can be explained by the target of the different treatments and the impact of different factors, proper to bacterial spores, on the resistance to these stresses. The main factor impacting the resistance to wet heat is core hydration, the presence of α/β SASP and the sporulation conditions. The main factor impacting the resistance to UV light is the α/β SASP but also the DNA repair process. The sporulation conditions do not impact the resistance to UV as it was already demonstrated (Nicholson *et al.*, 2000). Resistance to chemicals (like peroxide) is impacted mainly by spore impermeability to hydrophilic molecules (Nicholson *et al.*, 2000; Setlow, 2006). Proteomic analysis of spores produced at pH 5.50 did not show differences in structure protein, or SASPs, compared to spores produced at pH 7.00 (data not shown) but only in functional protein (ribosomal proteins or enzymes involving in germination process). In contrast, several works have shown that protein involving in coat structure was differently synthesized (Bourne *et al.*, 1991; Henriques and Moran, Jr., 2007; Zheng *et al.*, 1988). The results obtained in this work seemed to show that sporulation conditions did not impact the resistance to chemicals. Thus the chemical resistance may be rather due to the inner membrane integrity than spore coat integrity. Finally, the resistance to pulsed light did not seem to be linked to coat integrity, which is consistent with previous results (Nicholson *et al.*, 2000). Moreover, our results showed that spores produced at 12°C or pH 5.50 may have the same SASPs content as no significant was pointed out between detection of SASPs in spores produced at pH 5.50 and spores produced at pH 7.00 (data not shown).

3.5. Conclusion

In conclusion, the recovery behavior of spores seemed to be the same for spores treated by wet heat treatment, acetic acid or pulsed light. So, despite the fact that the targets are different for the different treatments, the impact of recovery conditions on treated spore colony forming ability was the same. The recovery behavior of *Bacillus weihenstephanensis* KBAB4 spores after treatment with peracetic acid or pulsed light can be described by the mathematical model developed by Trunet *et al.* 2015. This model was only configured with physiological parameters: the cardinal growth values confirming again the importance of these parameters in the bacteria physiology.

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Chapitre 4

Germination and outgrowth of bacterial spores: several scales of investigation for better understanding the spore physiology and better control it through the food chain

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Article in preparation for submission.

Abstract

Germination, outgrowth and growth recovery make up a complex and highly heterogeneous process. Various techniques and methods allow monitoring this process and/or the quantification of the impact of environmental conditions on this process. These techniques can be classified by different criteria: the level of information given by each methods about the cellular or molecular analysis, the number of analyzed cells and the potential of the method to describe and/or quantify the impact of lethal (or sub lethal) treatments or environmental conditions. This review present the current knowledge about the different techniques and methods allowing to investigate all or part of the germination, outgrowth and growth recovery process. These techniques are applied on total spore population to single spores and take into account parameters at cellular level (growth capacity, morphological properties) to molecular level (proteomics, transcriptomics, spore molecular composition). It appears that, for a deeper comprehension of germination, outgrowth and growth recovery process, the use of several complementary methods is necessary.

4.1. Introduction

Spore forming bacteria are largely studied as model micro-organism in experimental science and industrial contexts. Indeed, spores of bacilli or clostridia have are able to stay in dormancy stage for years, to resist to many treatments and finally to germinate and they can be responsible of food poisoning and food spoilage (Mallozzi et al., 2010; Setlow, 2014). Recovery is a complex phenomenon, comprising germination of spores, restoration of metabolic activity in suboptimal or favorable conditions and emergence of the first vegetative cell able to multiply (Setlow, 2013). This germination and growth restore, also named recovery, process is widely studied throughout its complexity and its importance food industry as it can initiate *Bacillus* development in foods (Abee et al., 2011; Setlow, 2013). Germination and growth recovery are characterized by a high variability in the individual behavior of cells, which is moreover highly influenced by environmental conditions. This variability can be consecutive to differences in the expression of genes governing sporulation and/or environmental conditions among the population of cells during sporulation (Chastanet et al., 2010). This variability comes out as spore dehydration rate, enzymatic material and the number of germinant receptors which are put in place during sporulation (Setlow, 2014). This variability then impacts heat resistance and germination and growth restore ability (Hornstra et al., 2009; Setlow, 2013). Monitoring the germination and growth recovery of bacteria spores meets two major objectives: a better understanding of a major physiological process of cell differentiation in the microbial world and a quantitative evaluation of the impacts of physical and chemical treatments and/or of environmental conditions on the ability of spores to germinate and grow, and then causing problems in human activities.

This review provides a description of the different techniques and methods allowing the monitoring of spore germination and growth restore. The discussion will focus on the level of information given by each methods about the cellular or molecular analysis, the number of analyzed cells and the potential of the method to describe and/or quantify the impact of treatment or environmental conditions.

4.2. Overview of spore germination and evolution of spore properties

Germination encompasses deep morphological, physiological and modifications of dormant spores towards vegetative cells. The physiological stages observed during germination and growth restore can be defined by different criteria linked to the spore properties changes. Indeed, morphological changes, such as refractivity loss due to rehydration or increasing cell size can be observed during recovery process; physiological stages can be discriminated by respiratory activity restoration, membrane activity or heat resistance loss; finally, evolution can be observed during recovery at molecular level with CaDPA release, molecule synthesis or RNA expression. Monitoring germination will mainly consists in following the kinetics of representative changes among a spore population over time.

Spore characteristics evolve during germination, outgrowth and vegetative multiplication. These characteristics allow discriminating dormant spores, germinated spores, outgrowing cells and vegetative cells (Figure 12).

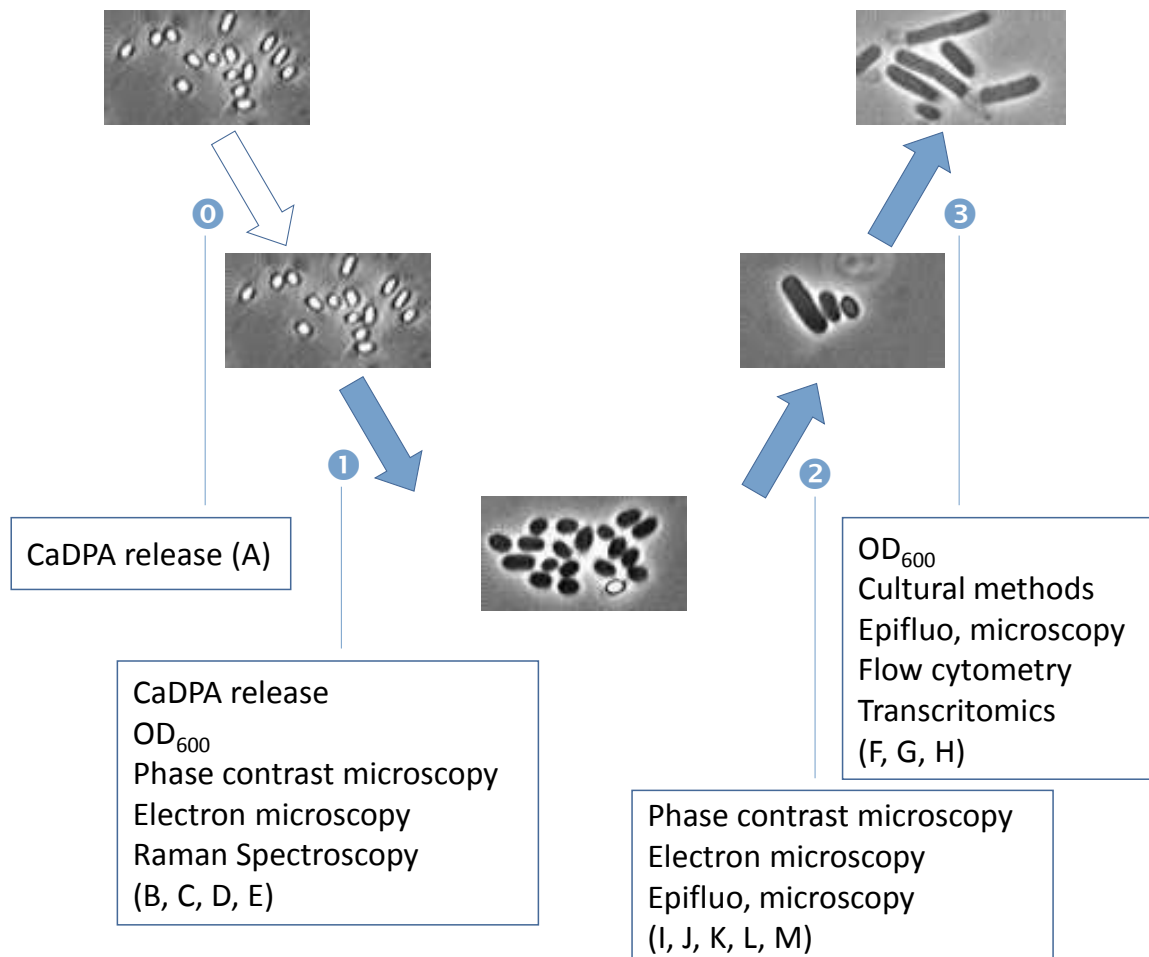


Figure 12 : Transfer through successive stages during spore recovery and results obtained with various methods to monitor each step. ❶ Commitments step can be monitored following the release of CaDPA (A) (Zhang et al., 2014). ❷ Germination step can be monitored by phase contrast microscopy, on spore populations or on individual spores (B, D) (Pandey et al., 2013; Tychinsky et al., 2007); by Raman spectroscopy (C) (Chen et al., 2006); by following the CaDPA release on culture medium (E) (Zhang et al., 2012). ❸ Outgrowth can be monitored using phase contrast microscopy associated with time lapse image system (F) (Pandey et al., 2013); by electronic microscopy (G) (Saif Zaman et al., 2005); by epifluorescence microscopy (H) (van Melis et al., 2014). ❹ The growth restoration can be monitored by following the OD_{600} evolution (I) or by cultural methods (K) (Roubos-van den Hil et al., 2010); by epifluorescence microscopy (J) (van Melis et al., 2014); by transcriptomic methods (L) (Segev et al., 2013); by flow cytometry (M) (van Melis et al., 2011).

The term “germinant” designates factors triggering spore germination and are classically classified as nutrient and non-nutrient germinants (See Setlow 2003 for an extensive review of the germination mechanism of bacterial spores). Nutrient germinants are low molecular weight compounds including, but not exclusively, amino acids, sugars or purine nucleosides possibly acting in combination and whose effect can be reinforced by co-germinants such as cations. Non-nutrient germinants include the dipicolinic acid-calcium complex (CaDPA, a specific compound of spore-forming bacteria) or high-pressure. The germination leads to the rehydration of the spore and release of CaDPA concentrated in the spore core. Then, the cortex is hydrolyzed by Cortex Lytic Enzymes (CLEs), such as SleB and CwlJ the two major CLEs of *Bacillus* sp., allowing the core to extend and complete rehydration to approximately 80% of spore weight. Spores lose their high resistance to moist-heat and chemicals early after core hydration. The spore is now permeable as the inner-membrane surrounding the core extended. In dormancy stage, the low inner-membrane permeability prevents the small molecules and even water to penetrate the core (Sunde et al., 2009). Finally, the hydration of the spore and the release of CaDPA leads to the loss of spore refractivity, easily detected (observed) by phase contrast microscopy (figure 12).

Metabolic activity and macromolecule synthesis is restored with spore hydration. The spores swell up and escape from the outermost layers, the proteinaceous coats and exosporium. This step corresponds to the outgrowth of the germinated spores and leads to a new vegetative cell able to multiply. The passage from a germinated spore to a vegetative cell is distinguished by morphological changes, the cell size increases and the new cell comes out from coats and exosporium, and by physiological changes as the metabolic activity is restored.

Monitoring germination will mainly consists in following the kinetics of representative changes with time among a spore population. The observed previously described changes are all quantifiable criteria for discrimination of the successive physiological stages the spores pass through during germination and growth restore. These changes can be observed by different techniques and methods giving various levels information, from population to individual spore or from cellular behavior to molecular properties (figure 13).

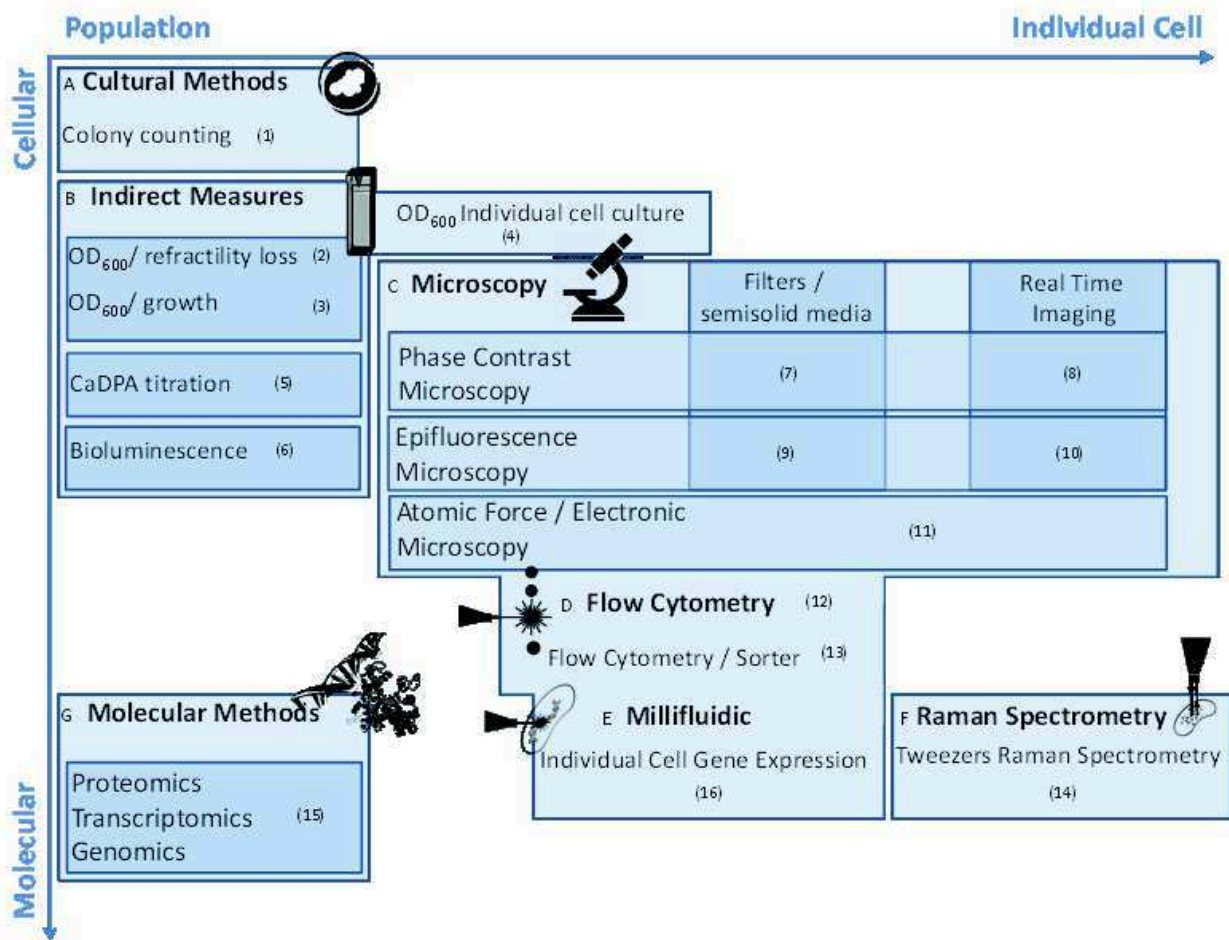


Figure 13: A synthetic view of the potential of different techniques to monitor germination of bacterial and growth recovery at different levels of investigation. On the X-axis, they are distinguished by the number of cells on which the method is applied. On the Y-axis they are distinguished by the level of investigation, from the cell (cellular level) to specific cells components (molecular level). A) Cultural methods, on solid media, allow the enumeration of spores able to germinate, outgrow and give a vegetative cell able to form a colony. The detection threshold for these methods depends on the applied dilution procedure and can be 10 or 10^2 CFU.mL⁻¹ (1) (Collado et al., 2003; Leguérinel et al., 2006). B) OD₆₀₀ measurement permits to monitor the spore refractility loss (OD₆₀₀ decreases when spore lose their refractility). The initial spore concentration must be high, $>10^7$ spore.mL⁻¹, in order to observe a significant OD₆₀₀ decrease (2) (Garcia et al., 2010). The increase of OD₆₀₀ allows also monitoring the growth restoration of a spore population. The spore concentration must be $>10^6$ spore.mL⁻¹ in order to obtain significant DO₆₀₀ values (3) (Daelman et al., 2013a). Combining OD₆₀₀ measurement to microplate which wells are inoculated with a single spore, this technique permits to monitor the germination and growth restoration of single spores (4) (Smelt et al., 2008). CaDPA titration, in the culture medium, released by the spores allows monitoring spore germination (5) (Zhang et al., 2014). Bioluminescence methods consist in observing bioluminescence of *lux*-containing engineered spores. The needed concentration is high, $>10^9$ spore.mL⁻¹ (Ciarciagliani et al., 2000) (6). C) Phase contrast microscopy de permits to discriminate refractive spores, phase bright spores, from germinated spores and vegetative cells which are not refractive anymore. The observation by Phase contrast microscopy of spores on semi solid media or on stripes (Anapore) allows monitoring spores into micro colonies during their germination, outgrowth and growth restoration (7) (Webb et al., 2012). This technique associated with live imaging and a dedicated software (SporeTracker) permits to follow germination, outgrowth and growth restoration of single spores (8) (Pandey et al., 2013). Epifluorescence microscopy allows monitoring germination, outgrowth and growth restoration giving supplementary information about membrane permeability or metabolic activity thanks to fluorochromes use (9) (Bruno, 2014). This technique associated with live imaging or the use of semi solid media or stripes would allow monitoring over time the metabolic activity restore or the membrane permeabilization during germination (10) Generally, a concentration $>10^6$ spore.mL⁻¹ is necessary to observe spore by microscopic methods. Atomic Force Microscopy (AFM) has been used to highlight morphology evolution of spores during germination (11) (Pinzón-Arango et al., 2009a). D) Flow cytometry allows monitoring germination, outgrowth and growth restoration of spores taking into account morphological criteria (size, refractility) associated to physiological criteria (membrane permeability, metabolic activity...). A High number of spores can be analyzed one by one by this method ($>10^5$ spore.mL⁻¹) (12) (van Melis et al., 2011). The use of a sorter would allow to retrieve single spores, according to their physiological stages (13). F) Raman spectroscopy allows monitoring single spore germination following CaDPA release. This technique also allows the observation of proteins denaturation during germination or after heat treatment (14) (Zhang et al., 2009). G) Genomic, transcriptomic and proteomic methods allow the observation of gene expression or protein synthesis into a spore population (15) (Abhyankar et al., 2013). E) Techniques of transcriptomic associated to millifluidic techniques will allow the observation of gene expression during germination of single spores (16).

4.3. Cultural methods and indirect measurements

In this type of method, the spores are not directly observed and the biochemistry measurements, such as CaDPA assay, are not performed within the spore but in the medium. Cultural methods require nutritive media containing the necessary elements to provide spore germination, outgrowth and cell multiplication. Rich media are used under broth or solid agar form. Cells forming a colony on agar plates have definitely experienced germination, outgrowth and multiplication but this method does not allow distinguishing cells at different stages. On one hand, the detection threshold is 10 to 10^2 CFU.mL⁻¹ depending on the dilution procedure. On the other hand, the sensibility of this method is high as spores can be detected among a large population after heat treatment. Indeed, this type of method allows the enumeration of 10 spores among a cell population of several thousand or millions of total cells. This method has been used in order to estimate the impact of sporulation conditions, heat treatment conditions and environmental conditions on spore colony forming ability on agar plates after heat treatment (Baril et al., 2012; Leguérinel et al., 2006; Mafart, 2000; Mafart and Leguérinel, 1998; Mtimet et al., 2015; Trunet et al., 2015). The proportion of spores able to germinate, outgrow, multiply and form a colony can be estimated by this type of method. Mathematical models have been developed to quantify the impact of sporulation, heat treatment and recovery conditions on spore recovery using cultural methods in order to acquire the data. These types of methods are used in order to evaluate the growth potential or the survival potential of spore forming bacteria in foods allowing a better food quality and security.

The optical density at 600 nm (OD_{600}) can be used to monitor specifically the germination step. This step is characterized by the rehydration of the spore revealed by the transfer from phase-bright spores to dark-phase spores. This transfer leads to a decrease of the OD_{600} in a dense inoculum. This property of germination has been used to observe the impact of sub-optimal sporulation and recovery conditions on the germination step (Garcia et al., 2010; van Melis et al., 2012). A drop of 62% of initial OD_{600} for *Bacillus weihenstephanensis* KBAB4 or a drop of 45% for *Bacillus cereus* strain ATCC 14579 correspond to 99.9% of germination. The limit of this method is the amount of spores used to detect germination since the concentration is up to $\sim 10^8$ spore.mL⁻¹. Moreover, the spores are not directly observed and variability within the spore population cannot be taken into account.

Another widely applied method, using OD_{600} , allows the study of the differentiation from a dormant spore to a vegetative cell able to. Indeed, the spores germinate, outgrow and multiply. The vegetative cell multiplication leads to an increase of the OD_{600} with the increase of turbidity. Monitoring the evolution of OD_{600} allows to estimate the lag time of germination and growth, corresponding to the time

to first cell division (Collado et al., 2006; Daelman et al., 2013a, 2013b; Gaillard et al., 2005; Roubos-van den Hil et al., 2010). This method allowed observing a longest spore germination lag time and a lower germination level for spores produced in sub-optimal sporulation conditions, after a heat treatment or for spores incubated at sub-optimal recovery conditions. The limit of this method is that only one spore germinating and giving a new vegetative cell able to multiply, leads to an increase of OD₆₀₀. The observations can thus be due to only few spores and the variability of spore behavior during germination, outgrowth and growth is not taken into account. To take into account the variability between the spores of a population, the same technique was applied to single spores, isolated in micro plate wells by dilution or using sorting flow cytometry (Smelt et al., 2008; Stringer et al., 2011). The estimation of spore germination and first cell division lag times from single spores revealed a shorter lag time and a lower variability of lag time when spores were heat activated. By the way, the tremendous variability in those lag time depends on sporulation conditions, on the heat treatment conditions (heat activation or heat inactivation) and on the recovery conditions (Garcia et al., 2010; Smelt et al., 2008; Stringer et al., 2011).

Another way to indirectly observe the germination is to measure the concentration of CaDPA in the recovery medium. Indeed, while the spores germinate, they release the CaDPA contained in the core which is then rehydrated. So, observing the apparition of CaDPA in the recovery medium is linked to the spore germination. The spores are incubated in germination medium containing TbCl₃. Spore germination is thus observed by Tb-DPA fluorescence. This complex is excited at 270 nm and the emitted fluorescence wavelength is 545 nm. Relative Fluorescence Units (RFU) are obtained and are linear with the DPA release. When the whole spore population complete the germination, the DPA released is considered as its maximum level. The DPA release at time t is calculated as follow: $RFU \text{ at } t / \text{maximum } RFU \text{ reached}$ (Yi and Setlow, 2010; Zhang et al., 2012). This method allows the study of early events in recovery process such as commitment and germination. The commitment step follows the spore exposure to nutrient germinants, committing spores into germination even if germinants are removed (Paidhungat et al., 2002). This step can be specifically studied using molecules blocking the germination such as D-alanine or by reducing the germination medium pH.

Finally, a last method consists in the observation of bioluminescence of *lux*-containing engineered spores. Germinating, the spores emit a bioluminescence and Relative Light Units can be calculated (Hill et al., 1994). This method was notably used to quantify the impact of mild acidity, lactic acid and pasteurization on *B. subtilis* spores and to establish first steps of germination process (Ciarciaglini et al., 2000; Setlow et al., 2001).

4.4. Microscopy methods

These methods discriminate of the different physiological stages by morphological criteria (size, refractivity) and/or physiological criteria and markers (enzyme activity, membrane permeability or motility). The spores are directly observed or detected.

The phase contrast microscopy discriminate refractive spores from germinated spores and the direct observation of the passage from spore to vegetative cell (figure 13). Phase contrast microscope facilitates the observation of various structures with different refractive indexes changing these elements into different contrast levels. The germination is observable by phase contrast microscopy thanks to the refractive properties of dormant spores. Indeed, the very low hydration of spore leads to spore high refractive index. Germinating, the spore is rehydrated and the refractive index is low. The discrimination between dormant spores and germinated spores can thus be done by the difference between phase-bright spores (dormant, refractive) and phase-dark spores (germinated) (Chen et al., 2006; Hornstra et al., 2009; Tychinsky et al., 2007). The discrimination between germinated spores and outgrowing cell is, by (phase contrast) microscopy, possible by the increasing cell size. This observation can be quantified using specific software as explain in the following paragraph.

The spore preparation can be realized in different ways in order to optimize the visualization of the germination and outgrowth. The spores can be set on a microscope slide and then dried. The spore inoculum is then covered with a solution containing the germinant (such as L-alanine or inosine) (Kong et al., 2014). Another way to prepare the spore for such observations is to set the spores on a semi-solid medium previously prepared on a microscope slide (Pandey et al., 2015; Stringer et al., 2005; Webb et al., 2012). Pandey *et al.* also added a closed air containing chamber by attaching GeneFrame[®] to the slide. A last way to optimize the observation of spores is to set the spores on Anapore[®] strips (Besten et al., 2010).

In order to monitor the germination and outgrowth processes, the microscopes are associated with Time lapse image system. Using spore fixation methods, described in the previous paragraph, the spores can be followed individually.

The crucial part of this method is the treatment of the image. Indeed, several methods have been used in order to monitor the apparition of each recovery step. As previously described, the germination is characterized by the loss of refractivity, traduced on images by the passage from phase-bright to phase-dark spores. This passage from bright to dark phase is analyzed as the decreased of pixel intensity on spore images (Pandey et al., 2015, 2013; Stringer et al., 2009; Ter Beek et al., 2011; Webb et al., 2012).

The software SporeTracker[®], used by Stanley Brul's team allows the quantification of different parameters: the decreasing pixel intensity during spore germination, as previously cited, and the increasing area as the outgrowing cell size increases. It is also possible to observe, via this method, the "burst" corresponding to the new cell popping out of the external spore layers (coats and exosporium) (Figure 13). To do so, SporeTracker first detects all bright-phase spores which are, then, followed over time. When the pixel intensity falls under a pre-set threshold, the spores are considered as germinated. Afterwards, the contour of the germinated spore is determined by the program allowing the observation of the increasing area as the cell outgrows (Pandey et al., 2013).

This type of method permitted to estimate the time of germination, outgrowth and first cell division of individual cells and the impact of environmental conditions on these times. This allowed observing that the first germinated spores were not necessarily the first spores to give a new vegetative cell. Moreover, the impact of environmental condition (recovery temperature or pH), the impact of lethal or sub-lethal heat treatment, or the impact of some other agents (tea compounds, sorbic acid). The impact of cell concentration has been also estimated on the different stages: the number of spores and the proximity of the spores impacts the germination of *Clostridium* spores (Ragkousi et al., 2000). The proportion of spores entering each recovery step among populations generally comprising several hundred to several thousands of individuals can be determined.

Tychinsky *et al.* 2007 used, in addition to Transmission Electronic Microscopy (TEM) and phase contrast microscopy, the Dynamic Phase Microscopy (DPM) and estimated another parameter called Phase Thickness (noted PT) that is the difference of optical path (nm) in order to study *B. licheniformis* spore germination (Tichinsky et al., 2006; Tychinsky et al., 2007). DPM is performed using a laser phase microscope with He-Ne-laser, 633 nm, as light source. The Phase Thickness (PT) of spores was defined as the spatial distribution of optical path difference between interfering beams, reflecting the state of an object. Thus, they showed that the dormant spores (refractive) germinated spores had significantly different PT fluctuation values. Moreover, heat inactivated spores had a lower PT value than dormant spores. Finally, they observed that the PT values of heat activated spores incubated in unfavorable conditions for germination (without any nutrient) was reversible: the PT value return to initial value when the heat activation was stopped. So, this method seems to allow the observation of spore heat activation.

Epifluorescence microscopy can also provide good opportunities to study spore recovery in detecting and interpreting the fluorescence signal emitted by fluorescent markers. The dormant spores are impermeable to many molecules. The outer layers permit the passage of small molecules (from 2 kDa to

8 kDa) (Driks, 1999; Henriques and Moran, Jr., 2007). The first problem of staining spores is this impermeability property. Staining spores with Syto9 is widely used. This molecule is a DNA intercalate and is excited at 488 nm and emits 540 nm light. The observation of dormant spores stained with Syto9 show hardly stained spores. Actually, a green halo can be observed, at the surface of the spore. This can be explained by the presence of mother cell DNA on the spore surface (Cronin and Wilkinson, 2007). This can be also explained by the probable penetration of Syto9 in the spore outer layers but the impossibility to reach spore DNA in the core that is its normal target. So, with core hydration, the spore DNA is reachable and Syto9 staining leads to green fluorescent spores (Cronin and Wilkinson, 2007; Reineke et al., 2013). Thus, the spore germination can be observed by epifluorescent microscopy. In addition to DNA intercalate probes, like Syto9 or Propidium Iodide (PI), markers can be used to observe metabolic activity within cells (Fuller et al., 2000; Hoefel et al., 2003). And this method can be specifically applied to spore metabolic activity restore during recovery (Cronin and Wilkinson, 2007). Another study using epifluorescence microscopy in order to monitor *Bacillus* spore germination and outgrowth provide another example of method. Ragkousi et al., 2000 used the analysis of nucleoid morphology by epifluorescence microscopy to show an evolution of this structure over germination. The staining of nucleoid was not possible in dormant spores due to impermeability of the structure (Ragkousi et al., 2000).

Other microscopy methods can also be used in order to observe spore germination and outgrowth processes. Transmission Electronic Microscopy (TEM) has been used to observe the evolution of the structures during germination and outgrowth. Indeed, TEM is based on variation of electron transmission through different structures. The bacterial spore, being a multilayer structure, presents major interest to be observed by this method. Moreover, during germination, events like the core hydration or the cortex lyses lead to change in the spore structures, so in the spore aspect by TEM (Figure 13) (Dey et al., 2012; Saif Zaman et al., 2005). This method showed the phenomenon called « bottle cap » model that consists in the opening of a precise region of spore exosporium leading to the escape of the new vegetative cell. This observation can be related to the “burst” observed by phase contrast microscopy. This structure observation has been done for *Clostridium sporogenes* spores (Brunt et al., 2015). Scanning Electron Microscopy (SEM) has been also used to observe the spore structural evolution during germination and notably, here again, the aperture allowing the cell to escape from external spore layers (Bassi et al., 2009; Brunt et al., 2015; Steichen et al., 2007). This methods, in contrast with TEM allows the observation of entire spores (versus cross-section with TEM). These two methods are complementary for the analysis of spore structure evolution during recovery.

Atomic Force Microscopy is another method used to observe steps of spore recovery. It allows the observation of surface topology of a spore. This means that this method can be used only to study changes in external layers of the spores, namely the exosporium and the coats. This method led to interesting observations of alteration of coats architecture and topology during germination or after heat treatments (Pinzón-Arango et al., 2009b; Plomp et al., 2007; Tabit and Buys, 2010). The AFM can, in addition of topology measurements, provide information about forces, capacity and other parameters at the molecular level. This method leads to examine other methods focusing on the molecular level of study of spore recovery.

Flow Cytometry can be assimilated to microscopy. Indeed, the spores or cells pass through a laser beam and optical parameters are collected by detectors (instead of camera in microscopy methods). Forward Scatter (FSC) give information about cell size and Side Scatter gives information about cell inner structure or complexity. Associated with fluorescent staining, supplementary information about cell permeability, viability or metabolic activity can be obtained (as described about epifluorescent microscopy). This method allows the monitoring of germination, outgrowth and cell division, namely the whole recovery process. As Van Melis *et al.* 2011 have shown the discrimination between dormant spores and germinated spores can be done by the increase of fluorescence when spore spores germinate. Indeed, when spores germinate, they turn permeable and the fluorescent markers can reach the inner spore structures. Then, the discrimination between germinated spores and vegetative cells is done by size criteria. The vegetative cells size is more important than germinated spores. This method provides interesting aspects as it allows the observation of several characteristics at the same time (size and fluorescence corresponding to morphological or physiological stages) and allows the discrimination of individual spores within a population.

Nevertheless, in contrast to phase contrast microscopy methods, flow cytometry does not allow the monitoring of single cells over time but it permits the analysis of a high number of cells. This method was used to estimate the impact of High Pressure (HP) or heat treatments, the impact of sorbic acid on spore germination and outgrowth (Bunthof and Abee, 2002; Cronin and Wilkinson, 2008, 2007; Mathys et al., 2007; Reineke et al., 2013; van Melis et al., 2011).

4.5. Molecular methods

Molecular methods of monitoring spore germination and recovery can be presented as biochemical analysis, destructive or not, performed on spore population or spore populations extracts.

Remarkable details of spore germination at individual cell levels have been obtained with. This method is based on the excitation of a molecular mix by a laser (the spore or cell is then considered as a molecular mix). The emitted radiation is collected by a detector and the variation of emitted energy gives information about the energetic levels of the molecules composing the spore or cell. This method has been used to monitor germination of single spores, combining to a Raman spectrometer a laser tweezers. The CaDPA bands are known and can be identified in the Raman spectra of spores of different genus. Thus, the disappearance of the CaDPA band in the spore reveals the CaDPA release from the spore core corresponding to its germination. The spore germination monitoring has been realized for several spore forming bacteria, such as *B. subtilis* or *G. stearothermophilus* (Chen et al., 2006; Vepachedu and Setlow, 2004; Wang et al., 2011, 2015; Zhang et al., 2009; Zhou et al., 2013). Moreover, comparing the spectra of dormant spores, germinated spores and heat treated spores lead showed that the spore proteins are denaturated by wet heat treatment leading to inhibition of the germination (Coleman et al., 2010, 2007; Coleman and Setlow, 2009; Wang et al., 2011, p. 20)..

The same kind of results are be obtained by X Ray analysis of the spores during their germination. This technique is based on the electrons from an external source striking the atoms in a spore, leading to energy emitting under the form of an X Ray photon, giving the X Ray characteristics of the element (Bassi et al., 2009). Thus, in this method again, the disappearance of CaDPA from the spore core can be observed in order to monitor spore germination. The CaDPA can also be detected by mass spectrometry or Fourier Transform Infrared Spectroscopy (FTIR) and can even be a biomarker of the presence of bacterial spores. Moreover, Goodacre *et al.* 2000 used mass spectrometry on individual spores to detect CaDPA.

Transcriptomic methods have been used in order to study bacterial spore recovery process. This type of method consists in the study of gene expression all along the germination and outgrowth. The RNA is extracted from spores or outgrowing cells over time during recovery using microarrays (Keijsers et al., 2007; Moeller et al., 2006). The gene expression data can be performed using specific pipelines (Vaquerizas et al., 2005) and the transcripts quantification can be realized by quantitative reverse PCR. This type of molecular approach gives a view of physiological processes that occur during recovery process. For example, spore transcripts decrease during germination and early outgrowth until they are

undetectable as the RNA stored in the spores are used as source for de novo RNA and an initial source of nucleotides (Keijser et al., 2007). The impact of different treatment, such as heat treatment or filtration, lead to inhibition or activation of some germination and/or sporulation genes of *B. anthracis* spores (Liu and Ream, 2009). To well understand these gene expression data, it is important to take into account the spore composition. To study this composition, proteomics analysis can be performed.

Indeed, proteomic methods can be applied in order to study germination and outgrowth. These methods can be related to Scanning Electron Microscopy, Atomic Force Microscopy or Transmission Electronic Microscopy as they provide information about structural proteins but they also can give information about functional proteins like enzymes or germinant receptors for example. The extracted proteins are digested and analyzed by liquid chromatography associated with mass spectrometry (Abhyankar et al., 2014, 2011; Brul et al., 2011). Several proteins are essential to early germination steps: the germinant receptors, the DPA channel proteins, the Cortex Lytic Enzymes (CLEs) and lipoproteins that are potentially implicated in the transduction of the germinant binding signal (Setlow, 2014, 2003). Analyzing dormant spores, germinated spores and outgrowing cell reveal an evolution of their proteome during recovery. For example, it has been observed that YpeB, which is required for SleB (one of the Cortex Lytic Enzymes) incorporation in spore, is degraded during germination and is thus 14-fold less detected in germinated spores than in dormant spores (Chen et al., 2014). The changes in proteome can be monitored using iTRAQ method (Ross et al., 2004). Jagtap *et al.* 2006 used this method in order to monitor the changes in coat proteins during recovery process. Here again a set of proteins appear to significantly reduced during the germination step (Jagtap et al., 2006).

Associating methods could lead to perspectives on obtaining results at cellular and molecular levels like the use of mass spectrometry analysis of individual spores during germination, and more precisely by mass cytometry. This method is currently used only for human cells, but that was the case for many other techniques like flow cytometry for example and an extension to micro-organisms and more specifically bacterial spores could be imagined, targeting specific spore molecules such as CaDPA. Moreover, the advance in microfluidic methods, like Droplet PCR, can give us novel approaches and new opportunities to develop methods allowing the gene expression analysis or the proteome study of single spores.

4.6. Conclusion

Many techniques and methods allow monitoring the recovery process. They permit this study at different scales (from cellular to molecular) and for different number of studied cell (from population to single spores). It is important to note that these different methods are complementary on different aspects. Firstly, experimentally the recovery process needs to be observed via multiple methods to validate the results. For example, microscopy is necessary to validate the state of the spores which the proteins, or RNA, will be extracted from. Another example is that TEM or SEM gives interesting observations on spore structure and its evolution during recovery. Then, proteomics analysis comes and completes these observations by determining the nature of these structures.

Secondly, some of these methods, like transcriptomics or proteomics, permit a better knowledge of the recovery process but do not allow the quantification of environmental impact or heterogeneity of this process. This quantification is possible via other methods like cultural methods, phase contrast microscopy or Raman spectroscopy. So the combination of several methods can lead to a better understanding of the recovery, its heterogeneity and the impact of environmental conditions.

An ideal method should give information at single level and at population level and it should be based on quantitative data acquisition such as germination biomarkers. In this perspective, mass cytometry should be an interesting technique since it should allow the quantification of specific biomarkers on individual spores within a larger population.

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Chapitre 5

Germination and outgrowth kinetics of *Bacillus weihenstephanensis* and *Bacillus licheniformis* spores: impact of incubation conditions, heat treatment and sporulation conditions

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Article in preparation

Abstract

Suboptimal incubation conditions (temperature and pH) lead to a lower proportion of spores able to germinate, recover growth and form a colony on nutrient agar media (Trunet *et al.* 2015). However, the state of spores which growth is inactivated by suboptimal incubation conditions remains unknown. A flow cytometry method has been developed in order to monitor the successive physiological stages the spores pass through during germination, outgrowth and growth recovery: dormant spores, germinated spores and vegetative cells. This method allowed monitoring the evolution of each physiological stage over time in optimal and suboptimal conditions. A primary model was developed to describe the evolution of each stage. This model integrated the time of germination, the heterogeneity of the time of germination, the proportion of germinated spores, the time of outgrowth, the heterogeneity of the time of outgrowth and the proportion of outgrowing cells. All parameters were estimated for kinetics performed from 10°C to 37°C for *Bacillus weihenstephanensis* KBAB4 and from 18°C to 59°C for *Bacillus licheniformis* AD978, and from pH 4.70 to pH 7.40 for both strains. Lower temperatures or pH led to extended times of germination, lower proportions of dormant spores able to germinate, extended times of outgrowth, a higher heterogeneity of outgrowth time, and lower proportion of germinated spores able to outgrow. A secondary model, based on the cardinal model, was proposed to quantify the impact of incubation temperature and pH on the evolution of each physiological stage over time. The impact of heat treatment varied regarding the temperature and time of treatment. Indeed, a treatment at 85°C during 12 minutes or at 95°C during 2 minutes did not have the same impact on germination and outgrowth kinetics of *Bacillus weihenstephanensis* despite they both lead to a tenfold reduction. Finally, a suboptimal sporulation pH impacts the time of outgrowth and the proportion of spores able to germinate and outgrow. A proteomic analysis of spores produced at pH 7.00 and pH 5.50 showed that proteins involved in germination and outgrowth were under detected in spores produced at pH 5.50. This observation can be correlated to the germination and outgrowth behavior of spores produced at suboptimal pH.

5.1. Method development: monitoring germination, outgrowth and growth by flow cytometry

5.1.1. Introduction

The germination and growth recovery process is a succession of phenomena bringing a dormant spore to become a vegetative cell. During this process, the spores pass through different physiological stages including, in chronological order, the dormant spores, the germinated spores, and the vegetative cells (Moir, 2006; Setlow, 2003). Many techniques and methods have been developed to study those different steps such as cultural methods (Collado *et al.*, 2006), optical density measurement at 600 nm (OD_{600}) (Daelman *et al.*, 2013; van Melis *et al.*, 2012), phase contrast microscopy (Pandey *et al.*, 2013), epifluorescence microscopy (Ragkousi *et al.*, 2000) or electronic microscopy (Dey *et al.*, 2012), or analysis at molecular scale such as Raman spectrometry (Zhou *et al.*, 2013). The microscopy methods allow the observation of each stage but on a limited number of cells (a few thousand of cells at maximum) (Kong *et al.*, 2014; Pengfei Zhang *et al.*, 2010). In contrast, flow cytometry allows the monitoring of all the different physiological stages on hundreds of thousands spores. Passing through a laser, the cells are detected and different information are collected. The characteristics of each event, cells or spores, are based on light dispersion and/or fluorescent intensities. Several detectors are placed in different positions, and filters and/or dichromic mirrors allow the selection of the collected wavelengths. In line with the laser beam, the forward scatter (FSC) detector provides information about the size of the cell or of its external area. Perpendicularly to the laser beam, the side scatter (SSC) provides information about cell granularity or “internal complexity”. In addition, the cells are excited by the laser and the emitted light, at different wavelength, is collected by the fluorescence detector (FL). Thus, flow cytometry allows the simultaneous morphological and physiological analysis of cells. In addition, those parameters, FSC, SSC and FL, have already been used to discriminate the successive physiological stages: dormant spores, germinated spores and vegetative cells of *Bacillus cereus* 14579 (Mathys *et al.*, 2007; van Melis *et al.*, 2011).

The aim of this study was to consider the successive physiological stages (dormant spores, the germinated spores and the vegetative cells) by flow cytometry for *Bacillus weihenstephanensis*

KBAB4, a psychrotrophic strain, and *Bacillus licheniformis* Ad978, a thermotrophic strain. Then, the dynamics of germination and growth recovery of *Bacillus weihenstephanensis* KBAB4 and *Bacillus licheniformis* Ad978 were characterized by the quantification of each physiological stage over time, in optimal conditions and a sub-optimal condition.

5.1.2. Material and Methods

5.1.2.1. Bacterial strains

The *Bacillus weihenstephanensis* strain KBAB4 (INRA, Avignon, France) isolated from a forest soil (Guinebretière *et al.*, 2008) and *Bacillus licheniformis* strain Ad978 (ADRIA Développement, Quimper, France) isolated from dairy ingredients were used. The strains were stored at -80°C in 1 mL aliquots of Brain Heart Infusion (BHI, Biokar Diagnostics, Beauvais, France) mixed with 50% glycerol (v/v) at a concentration of 2.0×10^6 CFU/mL. A 100 mL volume of BHI was inoculated with 1 mL of the stock suspensions and incubated for 8 h at optimal growth temperature (30°C for *B. weihenstephanensis* KBAB4 and 45°C for *B. licheniformis* Ad978), then 1 mL volume was transferred into 100 mL of BHI for 16 h of incubation at the same temperatures. Finally, 0.1 mL of *B. weihenstephanensis* suspension and 0.01 mL of *B. licheniformis* suspension were added to 100 mL of BHI and were incubated for 6 h. For both strains, the final cell concentration in the pre-culture was approximately 10^8 CFU/mL; the number of spores estimated by the number of cells surviving a 5 min-70°C heat treatment was lower than 100 spores/mL.

5.1.2.2. Spore preparation

Spores were produced through a two-step sporulation process (Baril *et al.*, 2011). Volumes of 100 mL of the previously described pre-culture were centrifuged (6000 x g, 10 min, 12°C) and suspended in 100 mL of sporulation mineral buffer (SMB) at pH 7.00 made of K₂HPO₄ at 4.5 g/L, KH₂PO₄ at 1.8 g/L, CaCl₂, H₂O at 8.0 mg/L and MnSO₄, H₂O at 1.5mg/L, and pH 5.5 made of K₂HPO₄ at 4.5 g/L, KH₂PO₄ at 1.8 g/L, CaCl₂, H₂O at 8.0 mg/L and MnSO₄, H₂O at 1.5mg/L, filter-sterilized using 0.2 µm pore size filters (Baril *et al.*, 2011). These suspensions were incubated

with shaking at 30°C, pH 7.00 and pH 5.50 for *B. weihenstephanensis* KBAB4 and 45°C for *B. licheniformis* Ad978. Spores in SMB were harvested when free spores represented more than 95 % of cells observed under X1000 magnification by phase-contrast microscopy (Olympus BX50, Olympus Optical Co., Ltd, Hamburg, Germany), *i.e.* for both strains after 1-2 days at optimal growth temperature and up to 10 days at suboptimal temperature.

The spore suspensions were centrifuged (6000 x *g*, 10 min, 12°C). Spore pellets were suspended in 5 mL of sterile distilled water. The 5 mL suspensions were divided into 1 mL aliquots and stored for one month at 4°C before use. Laboratory observations consistently showed that spore heat resistance did not change for at least a six months storage time (unpublished data). The final concentrations of the stock suspensions were 10⁸ spores/mL for *B. weihenstephanensis* and 10⁹ spores/mL for *B. licheniformis*.

5.1.2.3. Sub-populations gating on flow cytometry

Dormant or refractive spores, germinated spores and vegetative cells were used to precisely define the region of each sub-population regarding Forward Scatter (FSC), Side Scatter (SSC) and fluorescence intensity (Syto9). Control samples of each sub-population were performed as follows using spores from independent stock suspensions, stored at 4°C.

Refractivity of dormant spores from was verified by phase contrast microscopy. The dormant spores are named refractive spores in the rest of the paper.

The germination time has been determined by monitoring the Optical Density loss at 600 nm (OD₆₀₀) after inoculation of 10⁹ spores/mL of Brain Heart Infusion broth (BHI, Biokar Diagnostics, Beauvais, France). This result also was validated by phase contrast microscopy as spores lose their refractivity as they germinate and turned from phase bright to phase dark.

A volume of 10 mL of BHI was inoculated with spores at a final concentration of 10³ spores/mL in optimal conditions (30°C for *Bacillus weihenstephanensis* KBAB4 and 45°C for *Bacillus licheniformis* Ad978). A 100 µL volume of suspension was sampled at regular time intervals,

diluted in Tryptone Salt (TS, Biokar Diagnostics, Beauvais, France) and then spread onto Brain Heart Agar (BHA, Biokar Diagnostics, Beauvais, France). The time to germination and growth was defined as the growth lag time. This result was validated by phase contrast microscopy in order to confirm that the population was only composed of vegetative cells.

Control samples of dormant (or refractive) spores, germinated spores and vegetative cells prepared as previously described, were analyzed on a flow cytometer (Cyflow space, Sysmex Europe GmbH, Norderstedt, Germany) equipped with a 488 nm laser as excitement light source and four detectors: FSC, SSC (detecting light emission at 488 nm), Fluorescence Light detector with a 536/40 nm filter, to detect green fluorescence (FL1) and Fluorescence Light detector with a 610 nm filter (high pass), to detect red fluorescence (FL2). The software used to analyze the flow cytometry data was *Flomax 2.3* (Cyflow space, Sysmex Europe GmbH, Norderstedt, Germany).

Bacillus weihenstephanensis KBAB4 and *Bacillus licheniformis* Ad978 spores from three independently prepared spore stocks were diluted in 1mL of PBS made of K_2HPO_4 at 4.5 g.L^{-1} , KH_2PO_4 at 1.8 g.L^{-1} and NaCl at 8 g.L^{-1} , at 4°C to obtain a suspension of 10^5 spores/mL. The spores were stained with $10 \mu\text{L}$ of 5mM Syto9 (Molecular Probes, Life Technologies, Saint Aubin, France) immediately before flow cytometry assay. Previous tests have shown that staining intensity was independent of Syto9 concentration or contact times (data not shown). A volume of $850 \mu\text{L}$ of the suspension in PBS was used for flow cytometry analysis. The suspension was always maintained on melting ice during the sample preparation. In parallel to the flow cytometer analysis, a volume of $150 \mu\text{L}$ of the suspension in PBS was used for observation by phase contrast microscopy and epifluorescence microscopy using a 470-490 nm excitation wavelengths (Microscope Olympus BX50, Olympus Optical Co., Ltd, Hamburg, Germany).

To validate the method ability to identify each physiological stage and to quantify the events by flow cytometry, different mixes were prepared: refractive and germinated spores in a 1:1 proportion; refractive spores and vegetative cells 1:1; germinated spores and vegetative cells 1:1. Each cell suspension was stained with 5mM Syto9 (1%, vol/vol as previously described) and then was analyzed by flow cytometry at $1.0 \mu\text{L.s}^{-1}$.

5.1.2.4. Germination and growth recovery dynamics

BHI was inoculated with 10^6 spores/mL in optimal and one suboptimal conditions: at 30°C and 15°C for *Bacillus weihenstephanensis*, and at 45°C and 30°C for *Bacillus licheniformis*. At different times of incubation, 100µL of the suspension were sampled and transferred into 900µL of PBS at 4°C and the diluted suspension was kept on ice. Suspensions were stained with 5mM Syto9 immediately before analyzes by flow cytometry at 1.0 µL spores suspension/s.

The number of events per mL was estimated by the CyFlow Space cytometer based on the number of events in 200 µL. Each event was analyzed via FSC, SSC, FL1 (green fluorescence) parameters. For each time, the count of each sub-population was plotted regarding incubation time.

5.1.3. Results

5.1.3.1. Flow cytometry discriminates dormant spores, germinated spores and vegetative cells

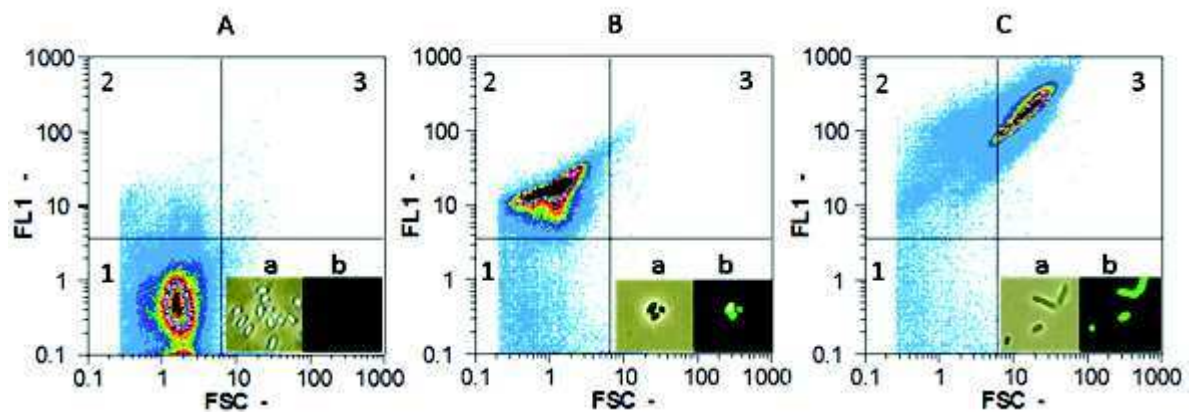


Figure 14: Flow cytometry dot plots for Identification of the populations successively observed during germination and growth recovery of *Bacillus weihenstephanensis* spores in optimal conditions. These results were obtained with one culture, at different times of incubation. (A) At initial time the population is made of dormant spores; spores appeared small and hardly fluorescent and refractive (Left bottom Panel A1) as confirmed with phase contrast microscopy and epifluorescent microscopy (phase bright refractive spores (Aa), a green fluorescent halo was hardly observed (Ab)). (B) After 10 minutes of incubation, the germinated spores had the same size than refractive spores but were highly fluorescent (B2). Phase contrast microscopy and epifluorescence microscopy confirmed this observation: phase dark spores (Ba), highly fluorescent (Bb). To verify the population was properly gated, the same experiment was performed passing ten-fold diluted suspensions (data not shown). (C) After 120 minutes of incubation, the vegetative cells had a larger size (higher FSC values) and a higher fluorescence than germinated spores. Phase contrast microscopy and epifluorescent microscopy confirmed this observation: vegetative cells (Ca), highly fluorescent (Cb). To verify the population was properly gated, the same experiment was performed passing ten-fold diluted suspensions (data not shown).

The first step was to discriminate the refractive spores, the germinated spores and the vegetative cells. To do so, the selected flow cytometry parameters were FSC and green fluorescence (FL1), corresponding to Syto9 fluorescence (Figure 14).

Mean values (and standard deviation) were calculated for FSC, corresponding to spore or cell size, SSC, corresponding to granularity, and FL1 values, corresponding to green fluorescence intensity. The refractive spores appeared at low FSC mean values, 2.65 ± 0.06 a.u. (arbitrary units) for *Bacillus weihenstephanensis* and 1.67 ± 0.07 a.u. for *Bacillus licheniformis*. Low Syto9 fluorescence mean values were observed also, 0.65 ± 0.14 a.u. for *Bacillus weihenstephanensis* and 0.88 ± 0.11 a.u. for *Bacillus licheniformis*. The observation by flow cytometry was completed for both strains with phase contrast microscopy observation and the spores were confirmed to be dormant as they appeared as phase bright spores.

The FSC mean values of germinated spores were equivalent to refractive spores FSC mean values: 1.72 ± 0.21 a.u. for *Bacillus weihenstephanensis* and 1.33 ± 0.17 a.u. for *Bacillus licheniformis*. Moreover, the SSC mean values of germinated spores were lower than for refractive spores. The Syto9 fluorescence mean values were significantly higher for germinated spores than for refractive spores: 8.91 ± 0.15 a.u. for *Bacillus weihenstephanensis* and 4.81 ± 0.13 a.u. for *Bacillus licheniformis*. The germinated stage of the spores was confirmed by phase contrast microscopy as the spores appeared as phase dark spores. Moreover, the germinated stage of the spores was confirmed by the OD_{600} observation for both strains.

The vegetative cells appeared with a high FSC mean values: 12.66 ± 2.12 for *Bacillus weihenstephanensis* and 7.42 ± 1.23 a.u. for *Bacillus licheniformis*. The Syto9 fluorescence mean values were also significantly higher for vegetative cells: 55.46 ± 4.56 a.u. for *Bacillus weihenstephanensis* and 82.39 ± 5.64 a.u. for *Bacillus licheniformis*.

The regions were confirmed analyzing mixes of refractive spores and germinated spores, refractive spores and vegetative cells, germinated spores and vegetative cells, refractive spores germinated spores and vegetative cells (Table 5). The gates were definitely set when a slight shift of the gates did not lead to a significant change in the count of dormant spores, germinated spores and vegetative cells.

Table 5: Expected and observed proportions of refractive spores, germinated spores and vegetative in mixes analyzed by phase contrast microscopy and by flow cytometry (Relative Scale).

	Proportions		
	Expected	Phase contrast microscopy	Flow cytometry
Refractive spores	0.5	0.51±0.07	0.48±0.06
Germinated spores	0.5	0.48±0.08	0.55±0.08
Refractive spores	0.5	0.52±0.02	0.51±0.03
Vegetative cells	0.5	0.51±0.05	0.52±0.04
Germinated spores	0.5	0.49±0.03	0.51±0.04
Vegetative cells	0.5	0.48±0.06	0.48±0.03

*Mean values and Standard Deviation obtained with triplicates

The last confirmation test for the gate definition consisted in the recovery of physiological activity of inoculated spores in BHI, and then the passage of events from a sub-population to another. The passage from refractive spore stage to germinated spore stage occurred in 10.10 ± 0.15 minutes. Germination was essentially observed by an increase in Syto9 fluorescence intensity (from 0.27 a.u. to 14.18 a.u. [mean FL1 value]) and more than 95% of the population transferred from dormant spore population to germinated spore population.

The passage from germinated spore stage to vegetative cell stage was more progressive and appeared as an increase in size (from 1.03 a.u. to 16.89 a.u. [mean FSC value]) and an increase of fluorescence (from 14.18 a.u. to 185.19 a.u. [mean FL1 value]) between 10 minutes and 120 minutes in optimal conditions and more than 95% of the population transferred from germinated spore population to vegetative cell population.

Two important observations were notable: (i) the total number of events, in the three sub-populations, was constant over the incubation time, meaning that neither loss nor vegetative cell multiplication occurred during this time and (ii) the transformation from a physiological stage to another was only from refractive to germinated spores and from germinated spores to vegetative cells. When vegetative cell multiplication occurred, the total number of events increased and the experiments was stopped.

5.1.3.2. Conversion of flow cytometry assays into transfer kinetics of dormant spores, germinated spores and vegetative cells

The germination and outgrowth kinetics were performed by sampling, at regular times a spore suspension incubated at two temperatures.. The flow cytometry assays were realized as previously described and, for each time, the absolute number of spores (or cells) was counted in each sub-population. The three sub-populations (refractive spores, germinated spores and vegetative cells) could be monitored this way at optimal condition and one sub-optimal condition (30°C and 15°C for *Bacillus weihenstephanensis*) (Figure 15).

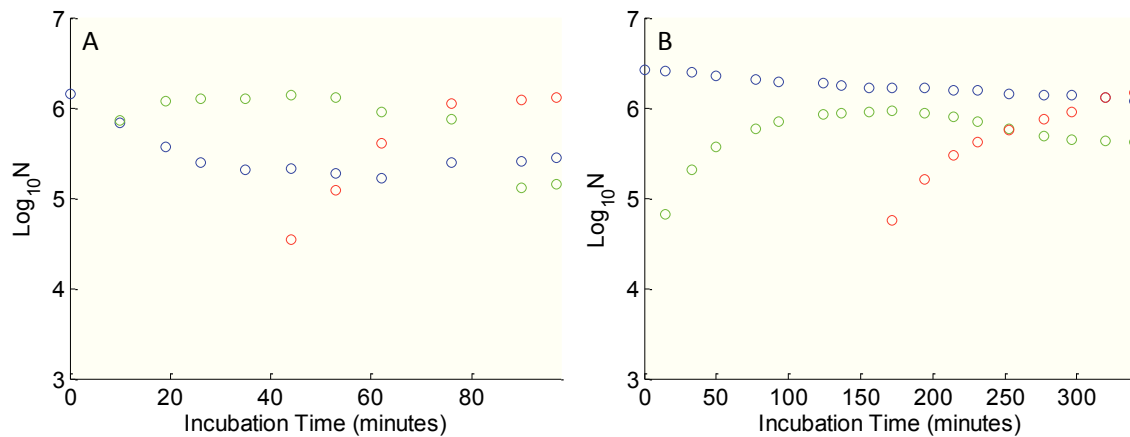


Figure 15: Evolution of the number (N) of refractive *Bacillus weihenstephanensis* KBAB4 spore (○), the germinated spores (○) and of vegetative cell (○) over time of incubation, at 30°C (A) and 15°C (B). Cell counts were performed by flow cytometry (see Materials and methods for technical details). At 30°C >90% of dormant spores germinated in 20 minutes and >90% of germinated spores outgrew in 75 minutes. At 15°C <20% of dormant spores germinated in 120 minutes and <40% of germinated spores outgrew in 300 minutes.

5.1.4. Discussion

The physiological stages of germination and growth recovery could be determined by flow cytometry thanks to particular characteristics of spores during germination, outgrowth and growth process. Indeed, the refractive spores remained fluorescent in presence of Syto9. However, the spores seemed to be stained only on the outer layer of the spores. The first reason for this observation could be the presence of remaining mother cell DNA on the surface of the spore (Cronin and Wilkinson, 2007). Another hypothesis was that the spore coat allows small molecules to pass through (2-8 kDa) (Driks, 1999; Henriques and Moran, Jr., 2007). Syto9 may pass through the spore coat thanks to its small size (approximately 2kDa). Nevertheless, Syto9 could not reach the spore DNA which is protected by mineralization, mainly Dipicolinic Acid Calcium complex (CaDPA), and the inner membrane which is highly impermeable (Dittmann *et al.*, 2015; Leggett *et al.*, 2012; Setlow, 2007).

The first change during incubation in BHI, observed by flow cytometry, was the sudden increase of green fluorescence when the spores germinated. This must be due to permeabilization that occurs during the first phase of germination (Setlow, 2014, 2003, p. 201). The FSC value did not significantly change meaning that the size of the spores did not significantly change but their DNA was accessible to Syto9. Indeed, when spores germinate, CaDPA is released from the core which is partially hydrated (Moir, 2006; Setlow, 2003). Thus, Syto9 could reach DNA of hydrated spores. The first, and sudden, increase in fluorescence could be defined as the first step of germination, which is triggered by the binding of germination molecules on germinant receptors (Behravan *et al.*, 2000; Setlow, 2014). This binding leads to the partial rehydration of the core and the syto9 can reach the spore DNA as soon as this step is completed.

Then the germination process continues and the cortex is hydrolyzed and the core is totally hydrated. It results in a swelling of the spore until it comes out of the outermost layers (exosporium and coats) (Pandey *et al.*, 2013; Setlow, 2003; Steichen *et al.*, 2007). The flow cytometry assays showed that the size and the fluorescence progressively increased from germinated spores to vegetative cell stage.

The germination, the outgrowth and the growth can be observed by flow cytometry as it had been observed in previous work (van Melis *et al.*, 2011). The definition of the gates was

confirmed for *Bacillus weihenstephanensis* KBAB4 and *Bacillus licheniformis* Ad978. Even if other germination and growth recovery stages have been described, the three subpopulations we chose allowed a satisfactory description of the germination and growth recovery process (Setlow, 2013).

The germination and outgrowth kinetics could be monitored in an optimal condition and a sub-optimal condition for both strains. Moreover, the used equipment allowed the absolute counting of events in each sub-population and, thus, the absolute composition of the suspension can be known at each time. Nevertheless, the observation was validated until the vegetative cell multiplication. At this point, the number of vegetative cell was too high to satisfactorily observe the evolution of the other populations. Moreover, the culture medium must be significantly changed due to the cell multiplication leading to a change in nutrient availability, pH, etc.

The correspondence between the gates, set on flow cytometry dataset, and the physiological stages was established. The detection threshold of the method was 10^3 cells/mL among a total population of 10^6 cells/mL. The relatively high threshold was due to the background noise which was observed in the culture medium. Nevertheless, small changes in the events count could be observed as a change of 5% of the event number within a sub-population was observable.

5.2. Mathematical modeling of germination, outgrowth and growth kinetics

5.2.1 Introduction

Predictive microbiology is a tool allowing the description of the microbial behavior thanks to mathematical models. Those models allow quantifying the impact of environmental factors (temperature, pH, a_w) on the behavior of bacteria (Membre *et al.*, 2005; Ter Beek *et al.*, 2011). Primary models describe the evolution of a population regarding the time. Numerous primary models have been developed to describe growth behavior, thermal or non-thermal inactivation or other types of kinetics. Those models can provide the bacterial number over time and parameters such as lag times or growth rates (Lebert and Lebert, 2006).

Previous works have described the evolution of a spore population containing dormant spores, germinated spores and outgrowing cells. In those works several parameters have been quantified such as the time of germination and the time of outgrowth (Stringer *et al.*, 2009; Zhang *et al.*, 2011). Some models have been developed in order to describe the evolution of these different sub-populations, or one of these sub-populations, over time. Thus, Mathys *et al.* 2007 have developed a three step model describing the evolution of dormant spores, germinated spores and inactivated spores over time, based on a differential equation system (Mathys *et al.*, 2007). Other models described the evolution of germinated spore population over time. One model was based on the probability of spores to germinate over time, under dynamic conditions of temperature (Peleg and Normand, 2013). Another model was based on the OD₆₀₀ loss observed during germination. This OD₆₀₀ decrease is linked to the proportion of spores able to germinate. Thus, a model was developed describing the evolution of the proportion of germinated spores over time. In this work, the germination also was observed by flow cytometry (Smelt *et al.*, 2008).

The germination and outgrowth curves were obtained by flow cytometry, as described previously. The used method provided absolute counts of each sub-population over time: the refractive spores, the germinated spores and the vegetative cells.

The aim of this part was to develop a single model describing of the evolution of dormant spores, germinated spores and vegetative over time for *Bacillus weihenstephanensis* KBAB4 and

Bacillus licheniformis Ad978. This model must describe the evolution of the three populations over time in optimal condition and a suboptimal condition.

5.2.2. Material and Methods

Changes in dormant spores, germinated spores and vegetative cells over time have been obtained as previously described in section 5.1. The germination and outgrowth kinetics were obtained at optimal condition, namely 30°C for *Bacillus weihenstephanensis* and 45°C for *Bacillus licheniformis*, and a suboptimal condition, namely 15°C for *Bacillus weihenstephanensis* and 30°C for *Bacillus licheniformis*.

5.2.2.1. Theory

The whole population is made of three sub-populations: the refractive spores, the germinated spores and the vegetative cells. This hypothesis leads to write this situation under the form of a simple equation:

$$N_T = N_{RS} + N_{GS} + N_{VC} \quad (17)$$

where, N_S is the total number of spores and/or vegetative cells, N_{RS} is the number of dormant (refractive) spores, N_{GS} is the number of germinated spores and N_{VC} is the number of vegetative cells.

At initial time, the total number is equal to the number of refractive spores initially inoculated in the nutrient medium. This initial number was noted N_0 . The three stages occur in a succession (ie refractive spores to germinated spores and from germinated spore to vegetative cells), with no possible reverse transformation or direct transformation of spores to vegetative cells.

Over time, the refractive spore population (N_{RS}) decreased as the germinated spore population (N_{GS}) increased. The number of refractive spores losing their refractivity is the same that the number of spores entering the germinated spore sub-population. The maximal proportion of germinated spores was noted τ_{germ} . As defined here, the time needed to decrease the number of refractive spores of 90% of its initial number is therefore the same as the time needed to reach 90% of the maximal germinated spore number and was noted t_{germ} .

The same hypothesis was set for the link between the germinated spore population and the vegetative cell population. Thus, the number of germinated spores (N_{GS}) able to outgrow is the same that the maximal number of vegetative cells (N_{VC}). The maximal proportion of outgrowing cells was noted τ_{outgr} . In the same way than previously described, the time needed to lose 90% of the maximal germinated spore number is the same than the time to reach 90% of the maximal vegetative cell number and was noted t_{outgr} .

5.2.2.2. Primary model

In the model we developed in this work, we assumed that variability of the times of transfer from one stage to the next one can be described by a Weibull distribution. Consequently the germination and outgrowth kinetics are described as the Weibull cumulative distribution.

At each time, the evolution curves of refractive spore population were fitted using the model presented in Equation 18.

$$\log_{10} (N_R) = \log_{10} (N_{R_0}) + \log_{10} (1 - \tau_{germ} \cdot P_{W_{germ}}) \quad (18)$$

where $\log_{10} (N_R)$ is the log value of refractive spore number, N_{R_0} is the number of refractive spores at the initial time, τ_{germ} is the proportion of refractive spores able to germinate and $P_{W_{germ}}$ is the cumulative distribution of Weibull parameterized by t_{germ} , a scale parameter corresponding to the time to reach 90% of the number of refractive spores able to germinate, and S_{germ} , the Weibull shape parameter displaying the scattering of the germination times.

The evolution curves of germinated spore population were fitted using the model presented in Equation 19.

$$\log_{10} (N_G) = \log_{10} (N_{R_0} - N_R) + \log_{10} (1 - \tau_{outgr} \cdot P_{W_{outgr}}) \quad (19)$$

where $\log_{10} (N_G)$ is the log value of germinated spore number, N_{R_0} is the number of refractive spores at the initial time, N_R is the number of refractive spores, τ_{outgr} is the proportion of germinated spores able to outgrow and $P_{W_{outgr}}$ is the cumulative distribution of Weibull parameterized by t_{outgr} , a scale parameter corresponding to the time to reach 90% of the

number of germinated spores able to outgrow, and S_{outgr} , the Weibull shape parameter displaying the scattering of the outgrowth times.

The evolution curves of germinated spore population were fitted using the model presented in Equation 20.

$$\log_{10} (N_{VC}) = \log_{10} (N_{VC_{max}}) + \log_{10} (1 - \tau_{outgr} \cdot P_{w_{outgr}}) \quad (20)$$

where $\log_{10} (N_{VC})$ is the log value of vegetative cell number, $N_{VC_{max}}$ is the maximal number of vegetative cells, τ_{outgr} is the proportion of germinated spores able to outgrow and $P_{w_{outgr}}$ is the cumulative distribution of Weibull parametrized by t_{outgr} , a scale parameter corresponding to the time to reach 90% of the number of germinated spores able to outgrow, and S_{outgr} , the Weibull shape parameter displaying the scattering of the outgrowth times.

The model was fitted on the observations ($\log N_{RS}$ or $\log N_{GS}$ or $\log N_{VC}$) by minimizing the sum of squared errors (SSE) using *lsqcurvefit* function from *MatlabR2012b* (The Math-works, Natick, USA). The goodness of fit of the model was checked by the RMSE (Root Mean Square Error) (Huet *et al.*, 2010; Scherrer *et al.*, 2009). The smaller the RMSE, was, the better the model was fitted on the data. The 95% confidence intervals were calculated using the *nlparci* function from *MatlabR2012b*. The fitting performance of the model was statistically evaluated by the *F* test, comparing the mean square error of the model to the mean square error of the data. The computed *f* value was compared to the *F* table value (0.05 significance level). If the *f* value was lower than the *F* value from the table, the *F* test was accepted indicating that the model fitting was statistically acceptable.

5.2.3. Results

5.2.3.1. Fitting quality of the model

The time to germinate or to outgrow of individual spores is highly heterogeneous within a spore population. To take this heterogeneity into account, the model included a distribution function of the time of germination and outgrowth. Several distributions have been tested to fit the germination and outgrowth kinetics curves: Normal distribution, Lognormal distribution, Gamma distribution and Weibull distributions. The best fitting was obtained with the Weibull distribution as it provided the lower RMSE and AIC values (data not shown).

The Weibull distribution of germination times was parameterized by a parameter describing the speed of the phenomenon (time of 90% germination), the scattering of this time (shape parameter) and the proportion of refractive spores which are able to germinate. The Weibull distribution of time of outgrowth was parameterized by the time of 90% outgrowth, the scattering of this time and the proportion of germinated spores which are able to outgrow.

5.2.3.2. Biological interpretation of the model parameters

The t_{germ} value corresponded to the time needed to reach 90% of the maximal germinated spore number. This parameter also corresponded to the time needed to reduce the initial refractive spore population of 90%. The t_{germ} was significantly higher in suboptimal conditions than at sub-optimal condition for both strains (ANOVA, $\alpha = 0.05$) (Figure 16C, D and 17C, D).

The shape parameter S_{germ} was linked to the heterogeneity of spore germination time. The higher was S_{germ} the more homogeneous was the time to germinate within the spore population. Nevertheless, no significant difference of S_{germ} value was observed between optimal and suboptimal conditions. S_{germ} values were considered equal to 1.00 minute in optimal and suboptimal conditions for both strains. No significant difference could be pointed out between the fitting with estimated S_{germ} and the fitting with $S_{germ}=1.00$ minute for both strains (likelihood ratio test, $\alpha=0.05$).

The τ_{germ} value corresponded to the proportion of refractive spores which were able to germinate. This parameter was significantly higher at optimal conditions than in suboptimal conditions for both strains (ANOVA, $\alpha = 0.05$).

The t_{outgr} value corresponded to the time needed to reach 90% of the maximal vegetative cell number. This parameter also corresponded to the time needed to reduce the germinated spore population of 90%. The t_{outgr} was significantly higher in suboptimal conditions than at suboptimal condition for both strains (ANOVA, $\alpha = 0.05$) (Figure 16E,F and 17E,F).

The shape parameter S_{outgr} was linked to the heterogeneity of outgrowth time. The higher was S_{outgr} the more homogeneous was the time to germinate within the spore population. This parameter was higher at optimal condition than at suboptimal conditions (ANOVA, $\alpha = 0.05$) (Figure 16E,F and 17E,F).

Finally, the τ_{outgr} value corresponded to the proportion of germinated spores which were able to outgrow. This parameter was significantly higher at optimal conditions than in suboptimal conditions for both strains (ANOVA, $\alpha = 0.05$).

Using this set of six parameters (t_{germ} , τ_{germ} , S_{germ} , t_{outgr} , τ_{ougr} and S_{outgr}), this primary model described the evolution each sub-population over time: refractive spores, germinated spores and vegetative cells, for both strains. This observation also confirmed that (i) all refractive spores losing their refractivity were counted as germinated spores and (ii) all spores leaving germinated spores were counted as vegetative cells. Moreover, his model allowed the representation of the repartition of the germination time and its scattering as well as the distribution of the outgrowth time and its scattering in optimal and suboptimal conditions (Figure 16 and 17).

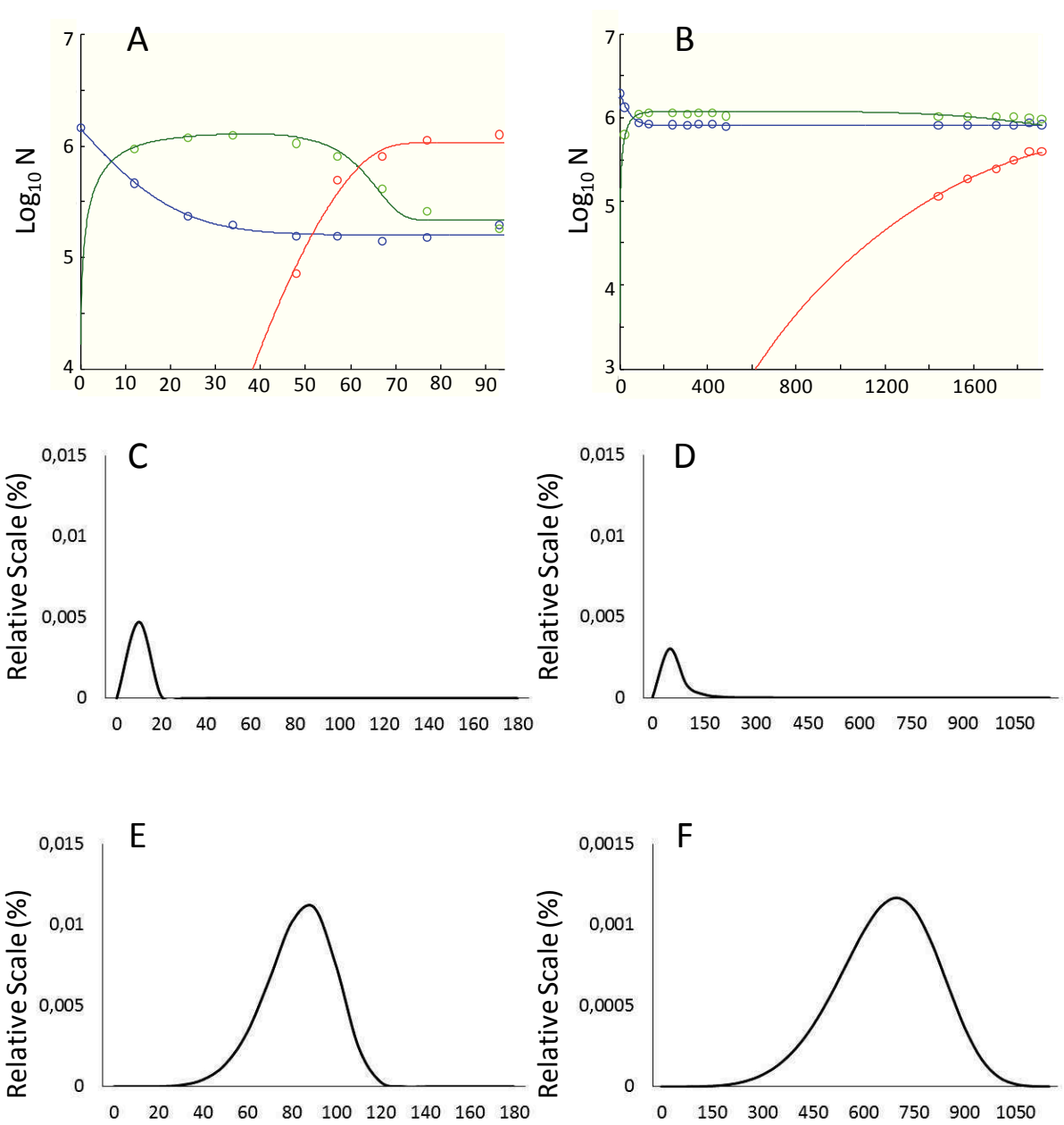


Figure 16: Cumulative function representing the evolution of *Bacillus weihenstephanensis* KBAB4 refractive spore population (○), the germinated spore population (○) and vegetative cell population (○) over time at 30°C (A) and 15°C (B). The lines represent fitting of the curves with the model developed in this work. The distribution function of germination time at 30°C (C) and 15°C (D) and the distribution of outgrowth time at 30°C (E) and 15°C (D) are presented.

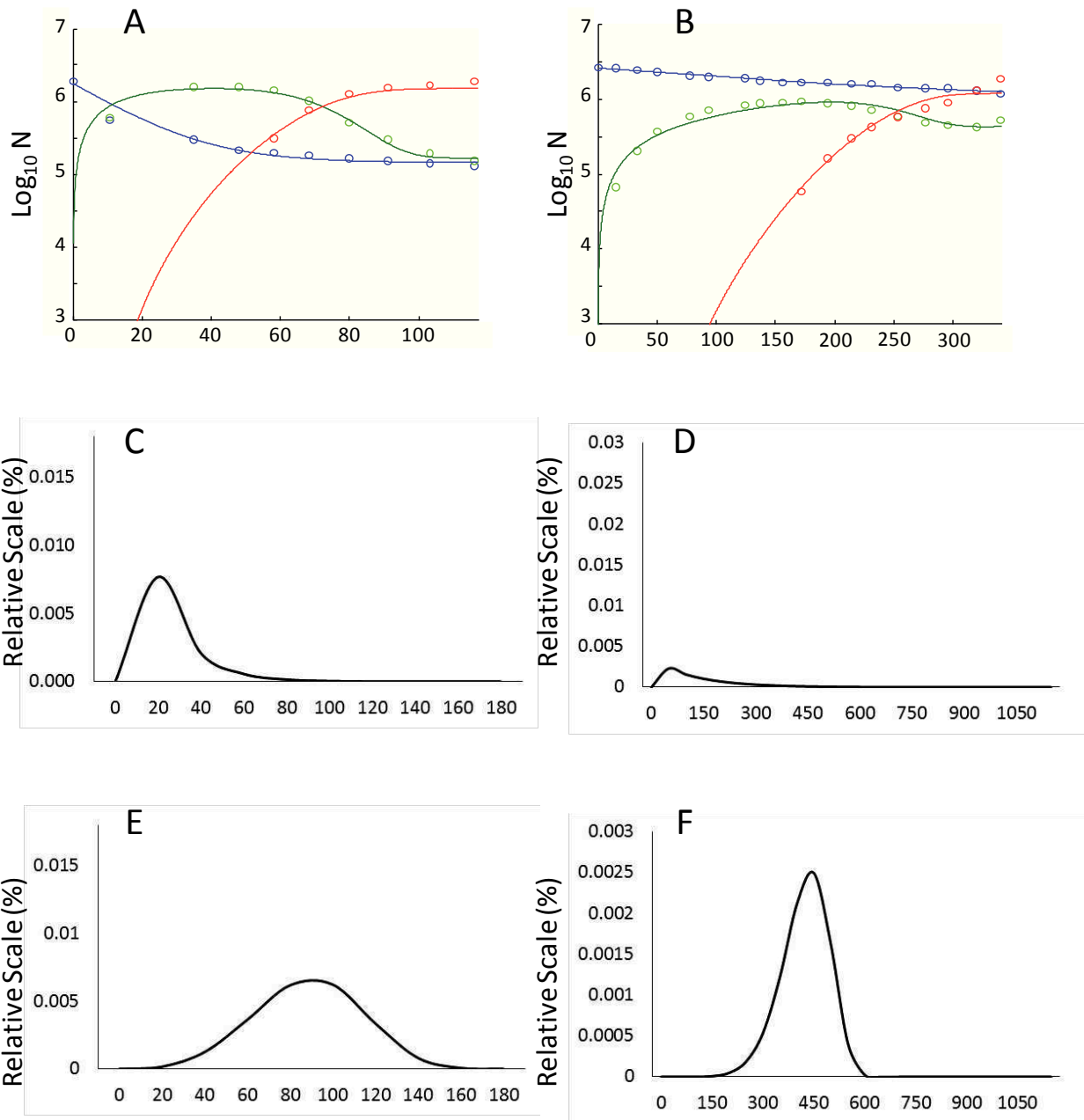


Figure 17: Cumulative function representing the evolution of *Bacillus licheniformis* Ad978 refractive spore population (○), the germinated spore population (◐) and vegetative cell population (◑) over time at 45°C (A) and 30°C (B). The lines represent fitting of the model developed in this work. The distribution function of germination time at 45°C (C) and 30°C (D) and the distribution of outgrowth time at 45°C (E) and 30°C (D) are presented.

5.2.4. Discussion

Firstly, the developed model allowed a satisfactory fitting on data acquired in optimal condition and suboptimal incubation conditions for both *Bacillus weihenstephanensis* KBAB4 and *Bacillus licheniformis* Ad978. The agreement of the model to describe the data means that (i) all the spores that lose refractivity, thus disappearing from the refractive spore population, were counted as germinated spores, (ii) all the germinated spores able to outgrow, thus disappearing from germinated spore population, were counted as vegetative cells, thus appearing in vegetative cell population and (iii) the number of stained spores and cells was constant over time for all tested conditions.

The parameters describing flow cytometry kinetics, t_{germ} , S_{germ} , τ_{germ} , t_{outgr} , S_{outgr} and τ_{outgr} , are similar to parameters used to describe germination and outgrowth kinetics with diverse methods in previous works. Stringer *et al.* 2009 described the germination and outgrowth thanks to median germination and outgrowth time values and the percentage of germinated spores and outgrowing cells. The lag time of germination and outgrowth can be also used as parameter to describe how fast is the germination and outgrowth process and what is the impact of environmental conditions on this process. The scattering of the lag times also describes the homogeneity of the lag time within a spore population (Stringer *et al.*, 2011).

Previous works have used different parameters quantifying the germination and growth restore and various primary models were designed to describe these parameters. In order to challenge a correlation between the germination and the distance between the spores, a model describing the difference of time of germination within a spore pair, as the time for the spores to turn from phase bright to phase dark, and the distance between the spores within the pair was developed (Zhang *et al.*, 2011). In this work, the time of germination corresponded to the loss refractivity at the single spore level. The germination, observed by OD₆₀₀ decrease, was modeled using a sigmoid model (Geeraerd *et al.*, 2000; Smelt *et al.*, 2008). The parameters of this model was the initial OD, the maximum germination rate -corresponding to the maximum rate of fall in OD-, the incubation time and a shape parameter traducing the curve shoulder length (Smelt *et al.*, 2008). In the same work, this model was used to describe the germination observed by flow cytometry. The germination also was described by a stochastic model using an

asymptotic germination level – the percentage of germinated spores-, a characteristic time and a shape parameter. These parameters were used to build a model describing the probability of germination of bacterial (and fungal) spores (Peleg and Normand, 2013). The model developed in this work was built only on biological parameters.

The Weibull cumulative distribution has been configured with the time of germination (time needed to obtain 90% of the maximal germinated spore number) and the scattering of this time which corresponded to the heterogeneity of the germination time distribution. The same configuration was done for the outgrowth modeling. In previous work, the germination time, outgrowth time and first doubling time was modeled using a composite model based on lognormal distribution for *C. botulinum* (Smelt *et al.*, 2013). In our case, the Weibull distribution gave the more satisfactory results as it was shown for *Bacillus subtilis* (Collado *et al.*, 2006; Smelt *et al.*, 2008). Whatever the case, the germination and outgrowth times are described using unimodal and asymmetric distributions. This observation is consistent with the relative homogeneity of the germination and outgrowth process and the existence of a minor part of the population which needs a longer times of incubation for germination and for the outgrowth. Thus, the changes in counts of dormant spores, germinated spores and vegetative cells occurring in a whole germination and outgrowth process were described using this new mathematical model. Previously, a three step model was developed to describe the simultaneous evolution of dormant (refractive) spores, inactivated spores and germinated spores after high pressure treatment (Mathys *et al.*, 2007). This model is based on differential equation describing the three populations. This type of model allows obtaining velocities of passage from a stage to another. This velocity is calculated between two times due to the differential nature of the model. The model we developed in this work described the evolution of the refractive spores, the germinated spores and the vegetative cells with time distributions. This allowed obtaining the composition of the population at any time of incubation. Such results were verified at optimal and suboptimal conditions for both strains.

The hypothesis we postulated, on the conservation of the number of spores and/or cells during the transfer along successive sub-populations, were verified and this simplified the model. Indeed, a single set of six parameters allowed the description of the evolution of refractive

spore population and the first part of the evolution of germinated spore population and a second single set of parameters allowed the description of the evolution of vegetative cell population and the second part of the evolution of the germinated spore population. Nevertheless, the description of this evolution was possible until the vegetative multiplication. It would be interesting to add to our model a module describing the cell multiplication in introducing the growth rate as an additional parameter.

In conclusion, the model we developed is only based on physiological meaning parameters and allows the description of the successive physiological stages encountered during spore germination and outgrowth. The results were validated in optimal and suboptimal conditions for both *Bacillus weihenstephanensis* KBAB4 and *Bacillus licheniformis* Ad978. This model allowed obtaining the composition of a population containing refractive spores, germinated spores and vegetative cells at any time.

5.3. Impact of incubation temperature and pH on germination, outgrowth and growth kinetics

5.3.1. Introduction

The germination and outgrowth are influenced by the sporulation conditions, the heat treatment and the environmental conditions like temperature, pH or a_w (Abee *et al.*, 2011; Leguerinel *et al.*, 2005; Mtimet *et al.*, 2015; Stringer *et al.*, 2011, 2009; Trunet *et al.*, 2015). Some works have highlighted the impact of incubation conditions on germination and/or outgrowth, generally focusing on the impact of temperature (Pandey *et al.*, 2013; Stringer *et al.*, 2011, 2005). The impact of heat treatment on germination and/or outgrowth has been also investigated (Smelt and Brul, 2014; Stringer *et al.*, 2009; Wang *et al.*, 2011). Several methods have been used to quantify the impact of environmental conditions and inactivating treatments (heat treatment or high pressure treatment) on the germination and/or outgrowth such as phase contrast microscopy, Raman spectroscopy or flow cytometry (Mathys *et al.*, 2007; Stringer *et al.*, 2011; Webb *et al.*, 2012). As shown in the previous part, a set of parameters can be estimated in order to describe the germination and outgrowth kinetics. A primary model based on these parameters has been developed. These parameters have physiological meaning as they corresponded to germination time, the scattering of germination time, the germinated spore proportion, the outgrowth time, the scattering of outgrowth time and the proportion of vegetative cells. Diverse secondary models have been developed to describe and quantify the impact of incubation condition of spores without heat treatment or after a heat treatment (Collado *et al.*, 2003; Leguérinel *et al.*, 2006; Smelt *et al.*, 2008). Nevertheless, few models describe the impact of incubation conditions, heat treatment or sporulation conditions on each stage of germination and outgrowth process.

The aim of this part was, firstly, to quantify the impact of incubation temperature and pH on each spore physiological stage, namely the dormant (refractive) spores, the germinated spores and the vegetative cells for *Bacillus weihenstephanensis* and *Bacillus licheniformis*. Secondly, the aim of this part was to develop a model, based on physiological parameters, describing and quantifying the impact of incubation conditions (temperature and pH) on each stage the spores pass through during germination and outgrowth process. To evaluate the robustness of our observations, the impacts of a suboptimal sporulation condition and the impact of heat treatment on each physiological stage were investigated for *Bacillus weihenstephanensis* spores. A proteomic analysis was performed on *Bacillus weihenstephanensis* KBAB4 spores, produced in different conditions, in order to correlate the variations in germination and outgrowth behavior with an intrinsic protein component of the spores.

5.3.2. Material and Methods

5.3.2.1. Germination and outgrowth kinetics

A final concentration of 10^6 spores/mL was inoculated in BHI at pH values ranging from 4.70 to 7.40 (seven levels for both strains) or incubated at temperatures ranging from 10°C to 37°C for *B. weihenstephanensis* (six levels) and from 15°C to 59°C for *B. licheniformis* (seven levels). Germination and outgrowth kinetics at optimum or suboptimal growth temperatures (10°C, 30°C and 37°C for *B. weihenstephanensis*, 18°C, 45°C and 58°C for *B. licheniformis*) and pH values (pH 5.00, 6.00 and 7.40 for both strains) were performed in, at least, three replicates, each replication being performed with an independently prepared spore suspension.

At different times, 100µL of the suspension were sampled and transferred into 1mL of PBS at 4°C and the diluted suspension was kept on melting ice to the end of the flow cytometry assay. Suspensions were stained with 5mM Syto9 and analyzed by flow cytometry at $1.0 \mu\text{L}\cdot\text{s}^{-1}$. At each time, the number of events in each gated sub-population was counted (as previously described in section 5.2).

5.3.2.2. Heat treatment and recovery conditions

All the performed heat treatment were standardized in order to obtain the inactivation of $90\pm 2\%$ of spores, based on previous works (Baril *et al.*, 2011, p. 2; Trunet *et al.*, 2015). The spores of *Bacillus weihenstephanensis* KBAB4 were diluted in PBS to a final concentration of around 10^7 spores/mL. Capillary tubes of 200 µL were filled with 100 µL of spore suspension and sealed, then immersed into a water/glycerol bath maintained at 85°C during 12 minutes or at 95°C during 2 minutes (Baril *et al.*, 2011; Trunet *et al.*, 2015). Capillary tubes were removed from the bath at appropriate time intervals and immediately cooled in a water/ice bath for 30 s. The tips were broken and were transferred into BHI medium (at different conditions of temperature or pH). The incubation of heat treated spores was performed at pH 7.40 and pH 6.00, at 30°C, and the evolution of dormant spores, germinated spores and vegetative cells was followed by flow cytometry as previously described. When the vegetative cells appearance occurred, a staining with 5 mM 5-Cyano-2,3-ditoyl tetrazolium chloride CTC was performed, at

30°C during 20 minutes in dark, to evaluate the number of cells and/or spores with metabolic activity.

In order to verify the 90% inactivation after heat treatment at 85°C or 95°C, capillary tubes were removed from the heat treatment bath at appropriate times and immediately cooled in a water/ice bath for 30 s. The tips were broken and the heat-treated spore suspensions were diluted in tryptone salt broth (Biokar Diagnostics, Beauvais, France). Volumes of 1mL of the appropriate decimal dilutions of heat-treated spores were mixed into molten Brain Heart Agar (BHA, Biokar Diagnostics, Beauvais) at pH 7.40 and pH 6.00, and incubated at 30°C. To estimate the spore concentration at the initial time (t_0), the spore suspensions were treated in a water bath at 70°C for 5 min using the same capillary tube method.

5.3.2.3. Secondary model

The set of five estimated parameters was composed of the time to reach 90% of the maximal germinated spore number (t_{germ}), the proportion of refractive spores which were able to germinate (τ_{germ}), the time needed to reach 90% of the maximal vegetative cell number (t_{outgr}), the scattering of this time (S_{outgr}), and the proportion of germinated spores which were able to outgrow (τ_{outgr}) (defined in section 5.2). The developed models, describing the evolution of each parameters regarding temperature or pH, were derived from the Gamma concept (Zwietering *et al.*, 1992) (equations 21 to 24).

$$\frac{1}{t_{germ}} = \frac{1}{t_{germ}^*} \cdot \gamma_X(X) \quad (21)$$

where $\frac{1}{t_{germ}^*}$ was the $\frac{1}{t_{germ}}$ in optimal conditions temperature and pH, and $\gamma_X(X)$ was a function describing the impact of temperature (equation 25) or pH (equation 27).

$$\frac{1}{t_{outgr}} = \frac{1}{t_{outgr}^*} \cdot \gamma_X(X) \quad (22)$$

where $\frac{1}{t_{outgr}^*}$ was $\frac{1}{t_{outgr}}$ in optimal conditions temperature and pH, and $\gamma_X(X)$ was a function describing the impact of temperature (equation 26) or pH (equation 27).

$$S_{outgr} = S_{outgr}^* \cdot \gamma_X(X) \quad (23)$$

where S_{outgr}^* was S_{outgr} in optimal conditions of temperature and pH, and $\gamma_X(X)$ is a function describing the impact of temperature (equation 11) or pH (equation 13).

$$\tau_i = \tau_i^* \cdot \gamma_X(X) \quad (24)$$

where τ_i^* was the τ_i value in optimal conditions of temperature and pH, and $\gamma_X(X)$ is a function describing the impact of temperature (equation 26) or pH (equation 28).

The function presented in equation 16 was used to model the impact of temperature on $\frac{1}{t_{germ}}$.

$$\gamma_T(T) = \begin{cases} 0 & T < T_{min} \\ \left(\frac{T-T_{min}}{T_{opt}-T_{min}} \right)^2 & T \geq T_{min} \end{cases} \quad (25)$$

where T_{min} , and T_{opt} are the estimated minimal (min), optimal (opt) conditions of temperature of germination and outgrowth.

The function presented in equation 15 was used to model the impact of temperature on $\frac{1}{t_{outgr}}$,

S_{outgr} , t_{germ} and t_{outgr} .

$$\gamma_{T'}(T) = \begin{cases} 0 & T \leq T_{min} \\ \frac{(T-T_{max})(T-T_{min})^2}{(T_{opt}-T_{min})^1[(T_{opt}-T_{min})(T-T_{opt})-(T_{opt}-T_{max})(T_{opt}+T_{min}-2T)]} & T_{min} < T < T_{max} \\ 0 & T \geq T_{max} \end{cases} \quad (26)$$

where T_{min} , T_{opt} and T_{max} are the estimated minimal (min), optimal (opt), maximal (max) conditions of temperature of germination and outgrowth.

The function presented in equation 17 was used to model the impact of pH on $\frac{1}{t_{germ}}$, $\frac{1}{t_{outgr}}$ and

S_{outgr} .

$$\gamma_{pH'}(pH) = \begin{cases} 0 & pH \leq pH_{min} \\ \frac{(pH-pH_{max})(pH-pH_{min})}{[(pH_{opt}-pH_{min})(pH-pH_{opt})-(pH_{opt}-pH_{max})(pH_{min}-pH)]} & pH_{min} < pH < pH_{max} \\ 0 & pH \geq pH_{max} \end{cases} \quad (27)$$

where pH_{min} , pH_{opt} and pH_{max} are the estimated minimal (min), optimal (opt), maximal (max) conditions of pH of germination and outgrowth.

The function presented in equation 18 was used to model the impact of pH on τ_{germ} and τ_{outgr} .

$$\gamma_{pH'}(pH) = \begin{cases} 0 & pH \leq pH_{min} \\ \frac{(pH-pH_{max})(pH-pH_{min})^{0.2}}{(pH_{opt}-pH_{min})^{-0.8}[(pH_{opt}-pH_{min})(pH-pH_{opt})-(pH_{opt}-pH_{max})(-0.8pH_{opt}+pH_{min}-0.2pH)]} & pH_{min} < pH < pH_{max} \\ 0 & pH \geq pH_{max} \end{cases} \quad (28)$$

where, pH_{min} , pH_{opt} and pH_{max} are the estimated minimal (min), optimal (opt), maximal (max) conditions of pH of germination and outgrowth.

Statistics

The model was fitted on the observations by minimizing the sum of squared errors (SSE) using *lsqcurvefit* function from *MatlabR2012b* (The Math-works, Natick, USA). The goodness of fit of the model was checked by the RMSE (Root Mean Square Error) (Huet *et al.*, 2010; Scherrer *et al.*, 2009). The smaller the RMSE were, the better the model was fitted the data. The 95% confidence intervals were calculated using the *nlparci* function from *MatlabR2012b*. The fitting performance of the model was statistically evaluated by the *F* test, comparing the mean square error of the model to the mean square error of the data. The computed *f* value was compared to the *F* table value (0.05 significance level). If the *f* value was lower than the *F* value from the table, the *F* test was accepted indicating that the model fitting was statistically acceptable.

The fitting of models was compared with a likelihood ratio test and a test statistic S_L computed as follows (Equation 29):

$$S_L = n \log RSS_C - n \log RSS_U \quad (29)$$

where n is the number of data, RSS_C is the residual square sum for the constrained (C) model and RSS_U is the residual square sum for the unconstrained (U) model. In this work the “constrained” model was the model using a pre-determined cardinal temperatures and pH for each strain values as input parameters. The “unconstrained” model was the models using estimated recovery cardinal values. When n tends towards infinity the limiting distribution of S_L is χ^2 distributed with $p_u - p_c$ degrees of freedom, where p_u is the number of parameters in the unconstrained model and p_c the number of parameters in the constrained model. If S_L is lower than χ^2 ($\alpha=0.05$), the difference in the fitting of both models was considered as not significant.

5.3.2.3. Proteomics analysis

Total spore proteins extraction

A volume of 1 mL of spore suspensions of 5×10^8 spores/mL from the stock of spores produced at 30°C, pH 7.00 and 30°C, pH 5.50 were centrifuged at 6000 g, 12°C during 10 minutes. The pellet was resuspended in 300 μ L of grinding buffer (pH 7.5 Tris HCl at 50mM, EDTA at 5mM). At the very last time 1X protease inhibitor cocktail is added and the suspension is homogenized. The 300 μ L suspension is transferred into a FastPrep tube containing 0.3 g of zirconium beads. The suspension was subjected to 2 minutes agitation at 1500 rpm using a 1600 MiniG® (SPEX® Sample Prep, Metuchen, United States of America). This agitation was repeated at least 5 times, in order to obtain more than 95% of broken spores. The breakage efficiency was checked by phase contrast microscopy were more than 95% of the spores appeared phase dark and damaged. Then, the suspension containing beads was centrifuged at 4000g, 12°C during 10 minutes. The supernatant was transferred into 1.5 mL tubes. The protein extract was concentrated on ultrafiltration membrane 10 kDa at 14000g, 4°C during 15 minutes, to 200 μ L (Bagyan *et al.*, 1998; Hudson *et al.*, 2001). The protein concentration was evaluated by the Bradford method (Bradford, 1976).

Alkylation and digestion of total spore protein extract

Iodoacetamide was added at a final concentration of 15 mM to the protein extracts and were incubated in the dark at room temperature during one hour. Then, the enzyme Lys-C (Wako, Richmond, United States of America) was added with of ratio 1:50 w/w (enzyme:proteins) and the extract was incubated 3 hours at room temperature. Trypsin (Proméga, Madison, United States of America) was added with ratio of 1:100 w/w (enzyme:proteins) and the extract was incubated at 37°C during 3 hours. Finally, trypsin was again added at the same ratio and the extract was incubated at 37°C during 16 hours. The sample was acidified to pH 2.00 with 1% trifluoroacetic acid (TFA). The volume was reduced down to 200-300 µL by SpeedVac (Abhyankar *et al.*, 2011; Misra *et al.*, 2011).

Extract desalting

A Strata-x 33u polymeric reversed phase 30 mg/1 mL column (Phenomenex, Torrance, United States of America) was activated with 500 µL of pure acetonitrile. Then, the column was rinsed with buffer A (3% acetonitrile, 0.06% glacial acetic acid, in water) three times. The protein extract sample was adjust to 500 µL with buffer A. The diluted sample was charged on the column and the column was rinsed three times with 500 µL of buffer A (flow-through). The column was eluted with 300 µL of buffer B (40% acetonitrile, 0.06% of glacial acetic acid, in water) two times and the total volume (600 µL) was collected in 1.5 mL tube. The sample was totally dried using SpeedVac and stored at -20°C. The extracts were prepared in order to obtain 100 µg of protein in each sample (Wiśniewski and Mann, 2012).

Mass Spectrometry analysis

The dried samples were resuspended in 400 μL of loading buffer (0.05% of TFA, 0.05% of formic acid, in water) and 4 μL were injected on a 50 cm Qexactive column (ThermoScientific, Waltham, United States of America) coupled with Eksigent nano-HPLC (AB-sciex, Framingham, United States of America) was used for the nano-LC-MS/MS analysis. Runs were 4 hours long for each sample. The assays were realized in triplicate for both conditions, issued from three independent spore batches.

Analysis of Mass Spectrometry data

The generated spectra were merged and searched by *X!tandem* 3.3.3 (<http://www.thegpm.org/tandem/>) using *X!TandemPipeline* (v3.3.3) developed by PAPPISO platform (<http://pappso.inra.fr/bioinfo/>). 2013.09.01). The database used to confront the results was *B.W.KBAB4* from *UniprotKB* (<http://www.uniprot.org/> updated on November 2014), containing 5717 entries. The results were also confronted to a contaminant database.

The statistic analysis was performed using *MasschroQ* 0.20 *R package* (*R* 3.1.1) to determine which proteins were more or less detected regarding the sporulation conditions.

5.3.3. Results

5.3.3.1. Impact of temperature and pH on the germination and outgrowth parameters

A total of 45 germination and outgrowth kinetics of *Bacillus weihenstephanensis* spores and 27 of *Bacillus licheniformis* spores were performed, each with at least 10 counts of dormant spores, germinated spores and vegetative cells. The evolution curves of each sub-population were fitted with the model presented in section 5.2. *Mathematical modeling of germination, outgrowth and growth kinetics* and the parameters t_{germ} , τ_{germ} , t_{outgr} , S_{outgr} and τ_{outgr} were estimated for all kinetics. The impacts of incubation pH and temperature were quantified for each parameter.

There was a progressive evolution of all parameters as the incubation pH came close to the germination and outgrowth pH limit. Indeed, a decrease in the incubation pH from 7.40 to 5.00

caused a 4.4 fold increase of the germination time, t_{germ} , for *Bacillus weihenstephanensis*, and a 3.3 fold increase for *Bacillus licheniformis*. The impact of incubation pH was lower on the outgrowth time as a decrease in the incubation pH from 7.40 to 5.00 caused a 2.7 fold increase of t_{outgr} values for *Bacillus weihenstephanensis* and a 2.5 fold increase for *Bacillus licheniformis*. The S_{outgr} values were lower when the incubation pH decreased, meaning that the heterogeneity of the outgrowth time was higher. Indeed, a decrease in the incubation pH from 7.40 to 5.00 caused a 1.5 fold decrease of S_{outgr} values for *Bacillus weihenstephanensis* and a 1.2 fold decrease for *Bacillus licheniformis*. Then, a decrease in the incubation pH from 7.40 to 5.00 caused 2.1 fold decrease of the proportion spores able to germinate τ_{germ} for *Bacillus weihenstephanensis*, and a 1.8 fold decrease for *Bacillus licheniformis*. The incubation pH had the same impact on the proportion of germinated spores able to outgrow, τ_{outgr} , as a decrease in the incubation pH from 7.40 to 5.00 caused 2.15 fold decrease for *Bacillus weihenstephanensis* and 2.03 fold decrease for *Bacillus licheniformis*. Moreover, a significant impact of incubation pH was pointed out each parameter between pH 7.40 and pH 5.00 (ANOVA, $\alpha=0.05$) and optimal germination and outgrowth pH was observed, for all parameters, between pH 6.00 pH 7.40.

The parameters τ_{germ} , t_{outgr} , S_{outgr} and τ_{outgr} followed the same trend regarding temperature as they progressively evolved when the temperature decreased. There was a tremendous impact of incubation temperature on t_{germ} as a decrease of temperature from 30°C to 10°C caused a 13.2 fold increase of the germination time for *Bacillus weihenstephanensis* and as a decrease of temperature from 45°C to 18°C caused a 14.7 fold increase for *Bacillus licheniformis*. The same general impact of incubation temperature was observed on the time of outgrowth as a decrease from 30°C to 10°C caused a 11.3 fold increase of the t_{outgr} values for *Bacillus weihenstephanensis* and a decrease from 45°C to 18°C caused a 13.3 fold increase of t_{outgr} for *Bacillus licheniformis*. As observed for the incubation pH, the heterogeneity of the outgrowth time was higher when the incubation temperature decreased. Indeed, a decrease from 30°C to 10°C caused a 2.8 fold decrease of S_{outgr} values for *Bacillus weihenstephanensis* and a decrease from 45°C to 18°C caused a 2.3 fold decrease of S_{outgr} values for *Bacillus licheniformis*. Finally, the proportion of dormant spores able to germinate, τ_{germ} , and the proportion of geminated spores able to

outgrow, τ_{outgr} , were similarly impacted by the incubation temperature as a decrease from 30°C to 10°C caused a 4.03 fold decrease of τ_{germ} and a 4.11 fold decrease of τ_{outgr} for *Bacillus weihenstephanensis* and a decrease from 45°C to 18°C caused a 5.34 fold decrease of τ_{germ} and a 5.31 fold decrease of τ_{outgr} . Again, a significant impact of incubation temperature was pointed out for the parameters τ_{germ} , t_{outgr} , S_{outgr} and τ_{outgr} between 30°C and 10°C for *Bacillus weihenstephanensis* and between 45°C and 18°C for *Bacillus licheniformis* (ANOVA, $\alpha=0.05$) and optimal germination and outgrowth temperature was observed between around 30°C for *Bacillus weihenstephanensis* and around 45°C for *Bacillus licheniformis*. A notable exception was observed for the evolution of t_{germ} values regarding temperature. Indeed, the germination time decreased when the incubation temperature increased and no optimal germination temperature was observed, for both strains.

5.3.3.2. Modeling the impact of temperature and pH on germination and outgrowth parameters

The evolution of t_{germ} , t_{outgr} , S_{outgr} , and τ_{vc} seemed to have the same general trend regarding the incubation pH. Consequently, the same function with $n=1$ (equation 27) was used to describe the impact of pH. The general trend of the evolution of τ_{germ} and τ_{outgr} were slightly different regarding the incubation pH and a function with $n=0.2$ (equation 28) was used to describe this effect (Figure 19 and 21). The evolution of τ_{germ} , t_{outgr} , S_{outgr} , τ_{outgr} and τ_{vc} seemed to have the same general trend regarding the incubation temperature so the same function with $n=2$ (equation 26) was used to describe this evolution. The evolution of t_{germ} was different and another function, without optimal germination temperature (equation 25), was used to describe this evolution (Figure 18 and 20).

The models presented in equations 21 to 24 were used to fit the set of 5 parameters (t_{germ} , τ_{germ} , t_{outgr} , S_{outgr} and τ_{outgr}) we obtained for each tested condition of temperature and pH (Figure 18 to 21). These models were fitted on t_{germ} , τ_{germ} , t_{outgr} , S_{outgr} and τ_{outgr} for the impact of incubation temperature and on 13 values of t_{germ} , τ_{germ} , t_{outgr} , S_{outgr} and τ_{outgr} for the impact of incubation pH on *Bacillus weihenstephanensis* KBAB4 spores; and on 11 t_{germ} , τ_{germ} , t_{outgr} , S_{outgr} and τ_{outgr} values

for the impact of incubation temperature and 13 t_{germ} , τ_{germ} , t_{outgr} , S_{outgr} and τ_{outgr} values for the impact of incubation pH on *Bacillus licheniformis* spores. In addition, the models were fitted on the maximal number of vegetative cells. T_{min} , T_{opt} , T_{max} , pH_{min} , pH_{opt} and pH_{max} were estimated from the fitting of each t_{germ} , τ_{germ} , t_{outgr} , S_{outgr} , τ_{outgr} and the proportion of vegetative cells (τ_{vc}) for both strains. The fitting performance of the models was statistically accepted by the F test (with $\alpha = 0.05$). Consequently, the model presented in equations 21 to 24 satisfactorily described the impact of temperature and pH on each of the 5 parameters for spores of both strains.

The use of predetermined growth cardinal values as input parameters in these secondary models were verified using likelihood ratio test. No significant difference could be pointed out between the fitting with all estimated cardinal pH values and the fitting with fixed growth cardinal pH values, for both strains (likelihood ratio test, $\alpha=0.05$). No significant difference could be pointed out between the fitting with all estimated cardinal temperatures and the fitting with fixed growth cardinal temperatures, for data between T_{min} and T_{opt} , for both strains (likelihood ratio test, $\alpha=0.05$). Nevertheless, the use of predetermined growth cardinal temperatures was not verified for data between T_{opt} and T_{max} .

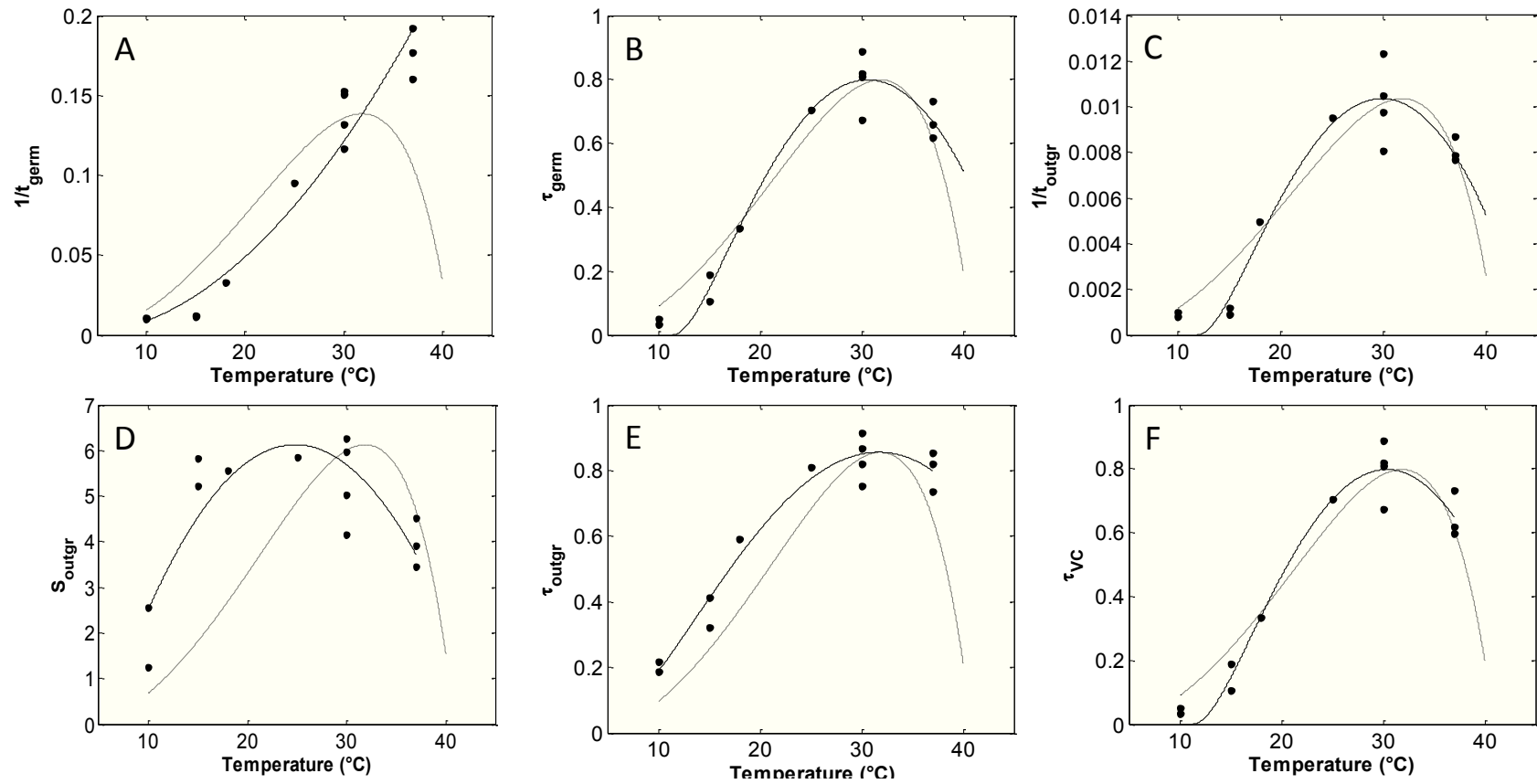


Figure 18: Impact of temperature on t_{germ} value (A), τ_{germ} value (B), t_{outgr} value (C), S_{outgr} value (D), τ_{outgr} value (E) and vegetative cell proportion (F) for *Bacillus weihenstephanensis* KBAB4 spores. The full lines represent the fitting of the data with equations 21 to 24. The dotted lines represent the fitting of the data with equations 5 to 9 with the cardinal growth values as input parameters.

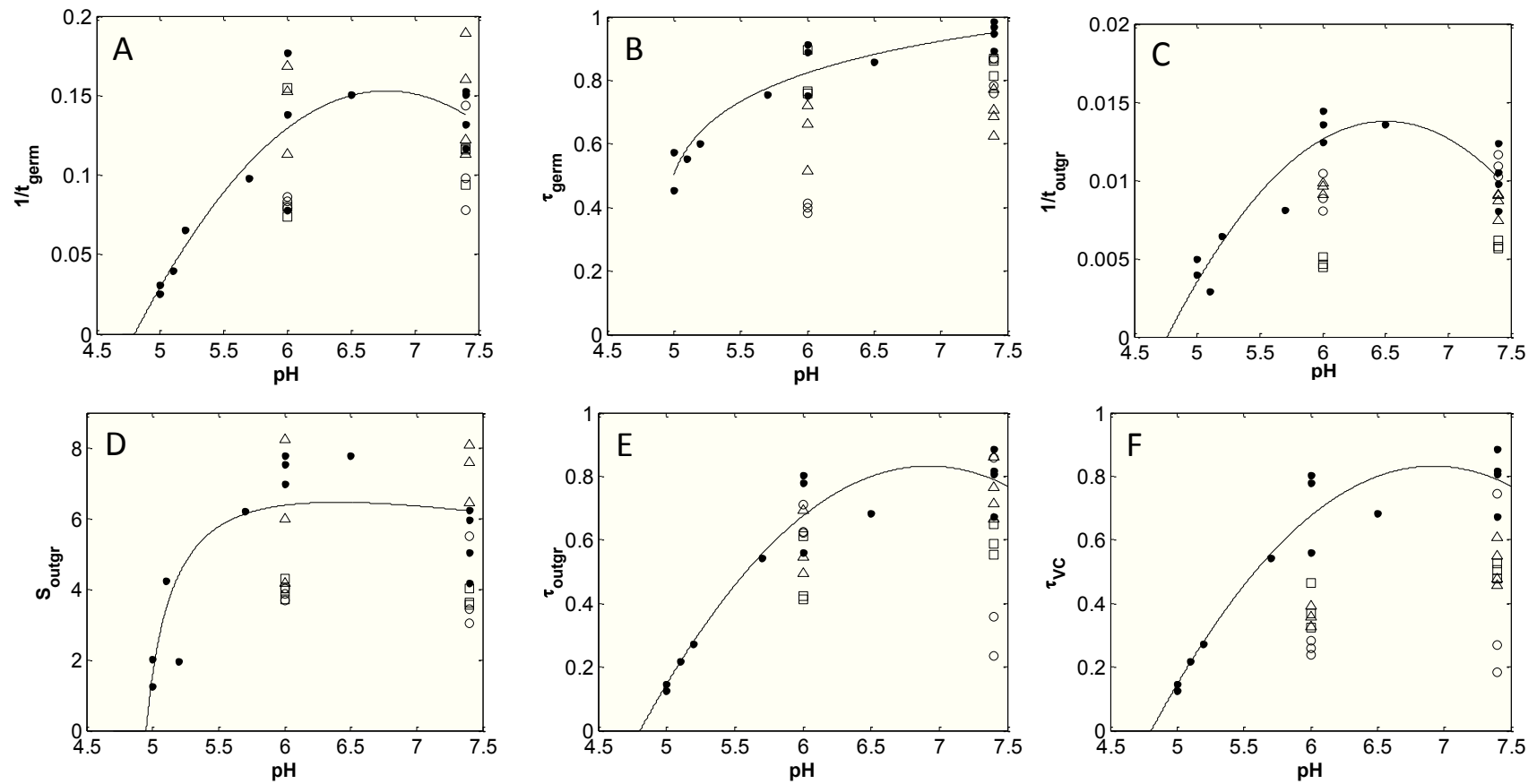


Figure 19: Impact of pH on t_{germ} value (A), τ_{germ} value (B), t_{outgr} value (C), S_{outgr} value (D), τ_{outgr} value (E) and vegetative cell proportion (F) for *Bacillus weihenstephanensis* KBAB4 spores produced in optimal conditions (●), for spores produced at pH 5.50 (△), for spores treated at 85°C (□) and for spores treated at 95°C (○). The full lines represent the fitting of the data with equations 21 to 24.

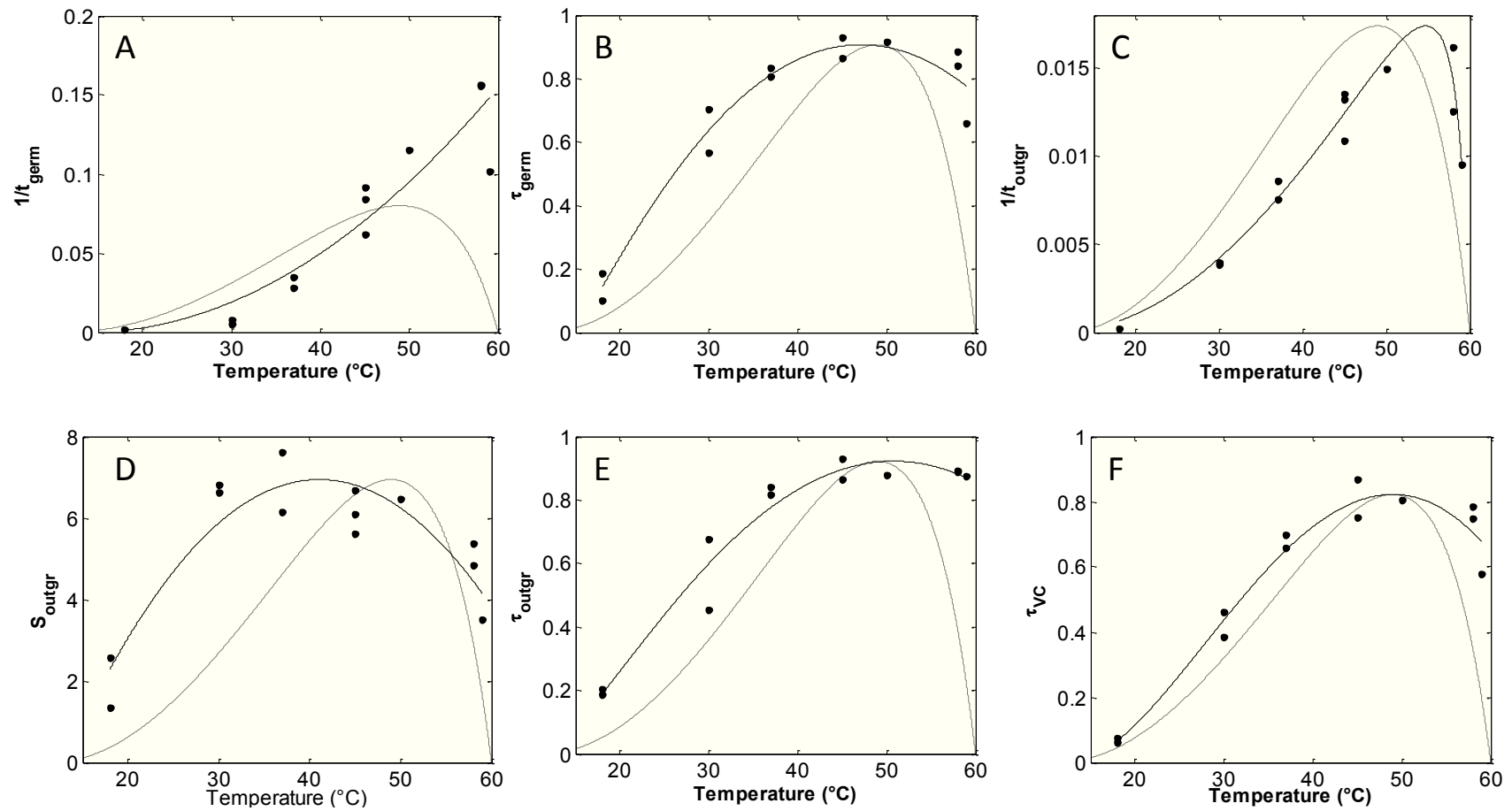


Figure 20: Impact of temperature on t_{germ} value (A), τ_{germ} value (B), t_{outgr} value (C), S_{outgr} value (D), τ_{outgr} value (E) and vegetative cell proportion (F) for *Bacillus licheniformis* Ad978 spores. The full lines represent the fitting of the data with equations 21 to 24. The dotted lines represent the fitting of the data with equations 5 to 9 with the cardinal growth values as input parameters.

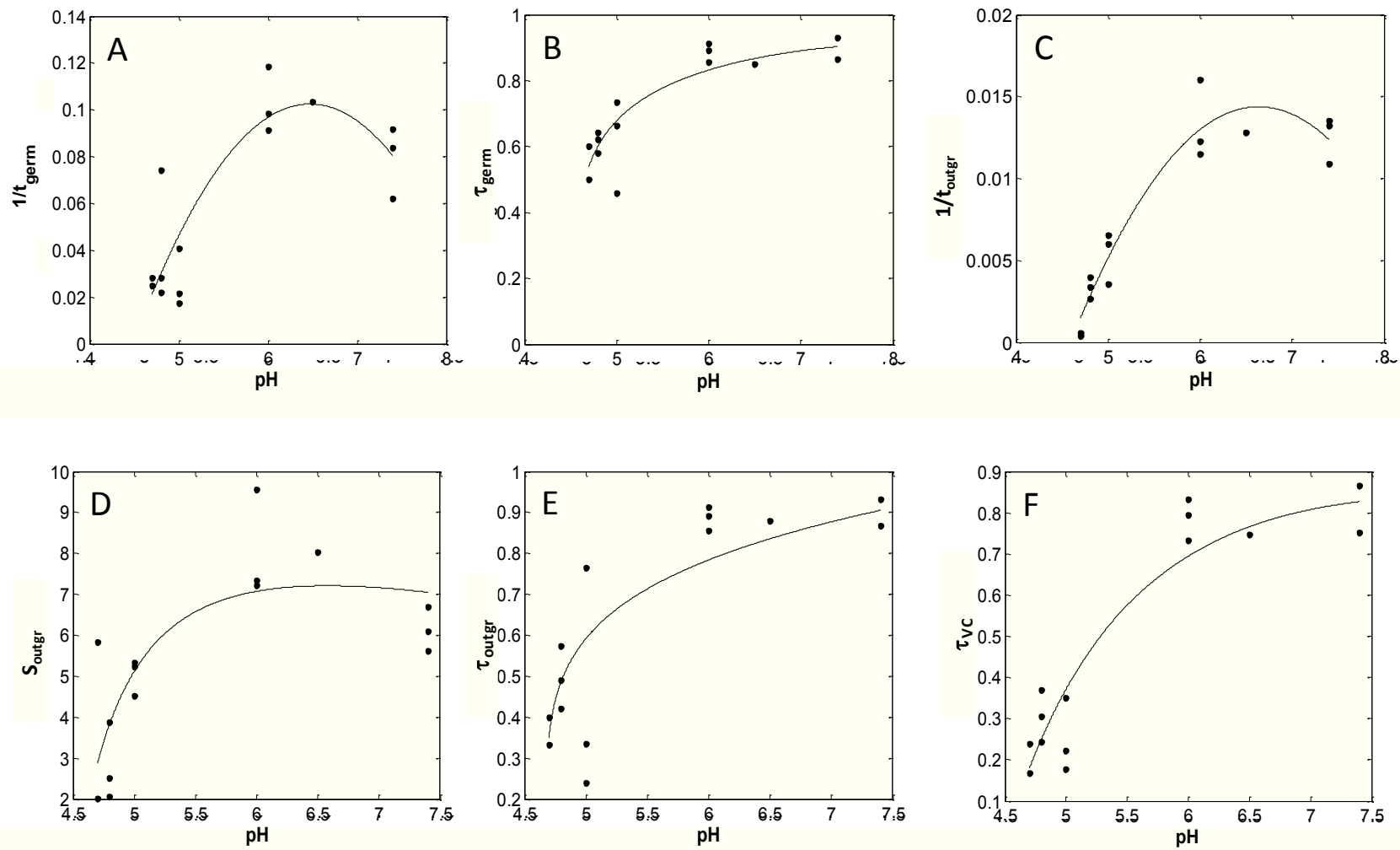


Figure 21: Impact of pH on t_{germ} value (A), τ_{germ} value (B), t_{outgr} value (C), S_{outgr} value (D), τ_{outgr} value (E) and vegetative cell proportion (F) for *Bacillus licheniformis* Ad978 spores. The full lines represent the fitting of the data with equations 21 to 24.

5.3.3.3. Recovery dynamics of spores produced at pH 5.50

The pH of sporulation seemed to have a significant impact on the outgrowth time. Indeed, the t_{outgr} of spores produced at pH 5.50 was significantly higher than for spores produced in optimal conditions. The outgrowth time was 117.7 ± 7.7 minutes at pH 7.40 for spores produced at pH 5.50 and 100.8 ± 5.3 minutes for spores produced in optimal conditions. The proportion of dormant spores able to germinate and the proportion of germinated spores able to outgrow was significantly lower for spores produced at pH 5.50 than for spores produced in optimal conditions. The time of germination, t_{germ} values, and the heterogeneity of outgrowth time, S_{outgr} , values, were not significantly impacted by the pH of sporulation for *Bacillus weihenstephanensis* KBAB4 (Figure 19).

5.3.3.4. Recovery dynamics after heat treatments

Spores of *Bacillus weihenstephanensis* were heat treated at 85°C during 12 minutes and at 95°C during 2 minutes, leading to a reduction of 90% of the initial population, and incubated at pH 6.00 and pH 7.00. This reduction has been verified by plate counting and the reduction of 90% of the initial population was validated for all experiments.

The heat treatment at 85°C did not significantly impact the germination time, t_{germ} value, but lead to a higher outgrowth time, showed by a significantly extended t_{outgr} value (ANOVA, $\alpha = 0.05$). The outgrowth time was 171.7 ± 7.7 minutes at pH 7.40 for the heat treated spores and 100.8 ± 5.3 minutes for untreated spores. The treatment at 85°C led to a higher heterogeneity of the outgrowth time as S_{outgr} value was significantly lower for heat treated spores, 3.73 ± 0.26 minutes at pH 7.40, than for untreated spores, 6.10 ± 0.88 minutes at pH 7.40. The proportion of spores able to germinate, τ_{germ} , was not significantly impacted by this treatment at the contrary of the proportion of germinated spores able to outgrow, τ_{outgr} , as this value equal to 0.48 ± 0.03 for pH 7.40 for heat treated spores and equal to 0.83 ± 0.07 for untreated spores. Those results showed that *Bacillus weihenstephanensis* spores were affected by the heat treatment at 85°C at different levels: the proportion of spores able to germinate was not impacted but the proportion of spores able to outgrow was significantly lower and presented significantly longer, and more heterogeneous, times of outgrowth.

The heat treatment at 95°C led to significantly more heterogeneous times of outgrowth. Indeed, S_{outgr} value was 3.85 ± 0.16 minutes at pH 7.40 for heat treated spores and 6.10 ± 0.88 minutes at pH 7.40 minutes for untreated spores. The proportion of spores able to germinate, τ_{germ} , was significantly impacted by this heat treatment as 0.48 ± 0.08 of heat treated spores were able to germinate at pH 7.40 compared to 0.95 ± 0.04 of untreated spores were able to germinate. Finally, the proportion of germinated spores able to outgrowth was significantly lower for heat treated spores, 0.48 ± 0.08 for pH 7.40, than for untreated spores, 0.83 ± 0.07 .

Interestingly, the impact was different for heat treatments at different temperatures but still leading to the same inactivation. Indeed, those results showed that *Bacillus weihenstephanensis* spores were affected by the heat treatment at 85°C as follow: the proportion of spores able to germinate was not impacted but the proportion of spores able to outgrow was significantly lower and presented significantly longer, and more heterogeneous, times of outgrowth. In contrast, *Bacillus weihenstephanensis* spores treated at 95°C presented significantly lower proportion of spores able to germinate and lower proportion of germinated spores able to outgrow. Moreover, the outgrowth times were significantly more heterogeneous but the time of germination and outgrowth were not significantly impacted.

5.3.3.5. Proteomics analysis

Proteomic analysis revealed significant differences of protein detection between spores produced at 30°C, pH_{spo} 7.00 and spores produced at 30°C, pH_{spo} 5.50. First, 788 proteins were detected and 187 were significantly differently detected between the spores produced in different conditions. 166 proteins were over-detected for pH_{spo} 7.00 compared to pH_{spo} 5.50. The large majority of the proteins differently detected were function proteins, as ribosomal proteins or glycolyse proteins (data not shown). Interestingly, proteins linked to germination process have been over detected for spores produced at pH_{spo} 7.00 compared to pH_{spo} 5.50. Indeed, SpoV proteins –canal protein having a role during germination- were significantly more detected for pH_{spo} 7.00 spores, and the same observation was made for SleB, which is a cortex lytic enzyme (also named CLE) which is highly important in the germination process. Finally, 21 proteins were over-detected for spores produced at pH_{spo}

5.50 compared to pH_{spo} 7.00. Notably peroxiredoxin, a protein evolving in oxidative stress response was significantly over-detected at pH_{spo} 5.50 (Table 6).

Table 6: Identification of proteins linked to germination behavior variation, for *Bacillus weihenstephanensis* KBAB4 spores produced at 30°C, pH_{spo} 7.00 and spores produced at 30°C, pH_{spo} 5.50.

Code*	Protein	Description	Number of detected spectra	
			pHspo 7.00	pHspo 5.50
A9VFH1	Stage V AD	SpoVAD localizes to the spore inner membrane and appears to interact with the GerAB and GerAC nutrient germinant receptor proteins	67.7 ¹	10.0
A9VUD3	SpoV AE	Transketolase central region, canal protein located in spore inner membrane	70.7 ¹	29.7
A9VSI2	SleB	Hydrolase, Cortex peptidoglycan hydrolysis	14.3 ¹	4.3
A9VUD2	GerA (predicted protein)	Germination protein, germinant receptor	43.0 ¹	14.0
A9VRK1	Peroxiredoxin	Peroxidase activity, oxidative stress response	27.7	41.3 ¹

¹ Significant difference in protein detection

* From database: UniprotKB_Bacillus_weihenstephanensisKBAB4-dec2014

5.3.4. Discussion

5.3.4.1. Impact of incubation temperature and pH on germination and outgrowth

A set of five parameters has been defined to describe the evolution of the physiological stages of spores during germination and outgrowth process. The impact of incubation temperature and pH has been quantified on each parameter: the germination time, t_{germ} , the proportion of spores able to germinate, τ_{germ} , the outgrowth time, t_{outgr} , the scattering of the outgrowth time, S_{outgr} and the proportion of spores able to outgrow, τ_{outgr} .

First, S_{germ} , corresponding to the heterogeneity of germination time, was not impacted by the incubation temperature or pH. This may be caused by the high speed of germination within *Bacillus weihenstephanensis* and *Bacillus licheniformis* populations in the tested conditions leading to a lack of data in the early germination ($t < 5$ minutes). Taking into account these observations and in order to simplify the model, the S_{germ} value was considered as equal to 1 as explained in section 5.2 *Mathematical modeling of germination, outgrowth and growth*. No significant difference was pointed out between the fitting with estimated S_{germ} for each kinetic curves or $S_{germ} = 1$ minute for all kinetic curves (likelihood ratio test, $\alpha = 0.05$).

The other parameters tend to evolve in the same trend regarding the incubation temperature. Indeed, t_{germ} and t_{outgr} decreased, and τ_{germ} , S_{outgr} and τ_{outgr} increased as incubation temperature decreased. Moreover, an optimal temperature has been observed for those parameters. In contrast no optimal recovery temperature was observed for the germination time for both strains. The first step of germination is triggered by germinant molecules accessing to the inner membrane, binding to the germinant receptors and leading to a partial hydration of the core (Behravan *et al.*, 2000; Setlow, 2014). Molecule agitation increases as reaction temperature increases. We can therefore assume that the germinant molecules are more agitated when the incubation temperature increases and they are able to diffuse more easily to their receptors within the spore surface layers. It may facilitate the germination initiation and decrease the germination time. The parameters describing the behavior of outgrowth, namely t_{outgr} and S_{outgr} , were impacted by incubation temperature the same way. Indeed, these parameters increased when incubation increased until the reach of an optimal temperature. The steps following the early germination involve enzymatic activity, such as the Cortex Lytic Enzymes (or CLEs).

Moreover, during outgrowth, the metabolic activity is restored (Hornstra *et al.*, 2009; van Melis *et al.*, 2011). Thus, the incubation temperature may impact these enzymatic activities and the outgrowth time and heterogeneity.

The incubation pH had the same impact on t_{germ} , t_{outgr} , and S_{outgr} . Indeed, t_{germ} and t_{outgr} progressively increased when the incubation pH came close to the germination and outgrowth pH limit and an optimal pH was observed between pH 6.00 and pH 7.40. The spore inner pH changes from 6.5 to 7.7 during germination So, adverse external pH may have an impact on germination kinetics as observed in the current work (Setlow, 2003). Interestingly, the recovery pH had a low impact on the proportion of spores able to germinate with a lowest value at 0.41 ± 0.09 at pH 5.00 for *Bacillus weihenstephanensis* and 0.55 ± 0.05 at pH 4.80 for *Bacillus licheniformis*. That may due to the mechanisms of the early germination. Indeed, the binding of germinant on the germinant receptor may be faintly impacted by the recovery pH, by contrast with recovery temperature. The same impact was observed on the proportion of dormant spores able to germinate, as previously explained, and the proportion of germinated spores able to outgrow, τ_{outgr} .

5.3.4.2. Modeling the impact of incubation temperature and pH on germination and outgrowth

All the parameters, with exception of the germination time, tended to evolve with the same trend regarding the incubation temperature. Two models were used to describe these evolutions. The model used to describe the impact of temperature on t_{germ} value was based on the modified Radkowski model (Zwietering *et al.*, 1992). This model allowed the use only of T_{min} and a reference temperature, which was consistent with the observation of absence of optimal temperature for this parameter. All the other secondary models were based on the Rosso function (Rosso *et al.*, 1995). All these models have only physiological parameters: minimal temperature, optimal temperature and maximal temperature; and minimal pH, optimal pH and maximal pH. The use of a single model to satisfactorily describe different steps of the process may imply that the variation of the temperature was the same on all the parameters.

Moreover, the minimal, optimal and maximal temperature of germination and outgrowth were estimated for each parameter (except for maximal temperature with germination time model). On one hand, the germination minimal and optimal temperatures of germination were close to predetermined minimal (T_{min}) and optimal (T_{opt}) growth temperatures (Baril, 2011). On the other hand, the estimated maximal temperature (T_{max}) of germination was higher than maximal temperature of growth (Baril, 2011).

To simplify the model, the predetermined growth T_{min} , T_{opt} and T_{max} were used as input parameters in the models we designed. As it was expected, the estimated maximal temperature of germination was significantly higher than predetermined growth T_{max} . Nevertheless, no significant difference could be pointed out between the fittings of the data with estimated minimal, maximal and optimal germination temperature and the fittings with predetermined growth T_{min} and T_{opt} (likelihood test, $\alpha=0.05$). Thus, the evolution of each parameter could be described with the models we developed, using predetermined growth cardinal values, between the minimal and optimal germination temperature.

The estimation of a higher maximal temperature of germination and outgrowth (than maximal growth temperature) may be linked to the capacity of spores to germinate beyond the growth limits as it had been previously shown (Knaysi, 1964a, 1964b). This capacity could be due to nutrient and energy reserve that can be found in the spore itself, and can be used during outgrowth in addition to the available nutrient in the medium. Thus, the spore would be less impacted by the adverse temperatures during germination and outgrowth than during growth (Segev *et al.*, 2013; Setlow, 2003). Nevertheless, additional experiments should be performed beyond the growth limits in order to confirm that maximal germination and outgrowth limit is significantly higher.

All parameters tended to evolve in the same trend regarding the incubation temperature. The impact of recovery pH was modeled for all parameters by the Rosso function with $n=1$ except for the τ_{germ} and τ_{outgr} values, where $n=0.2$, meaning that the impact of incubation pH was less progressive on τ_{germ} and τ_{outgr} . Nevertheless, the proportion of vegetative cells, obtained by multiplication of τ_{germ} and τ_{outgr} , could be described with the Rosso function with $n=1$. As it was done for the temperature, the predetermined minimal and optimal growth pH (pH_{min} and pH_{opt})

were used as input parameters in the models (Baril, 2011). No significant difference were pointed out between the fittings with estimated minimal and optimal pH of germination and with the fittings with predetermined growth pH_{min} and pH_{opt} (likelihood ratio test, $\alpha=0.05$). Thus, the model we developed allowed the description of the impact of incubation pH on germination and outgrowth using predetermined growth pH_{min} and pH_{opt} .

These models allowed obtaining the value of each parameter regarding the recovery temperature and pH. Thanks to the models developed in this section and the primary model developed in section 5.2, it is possible to determine the proportions of refractive spores, germinated spores and vegetative cells regarding the recovery temperature or pH and the time of incubation using a single set of parameters (the cardinal growth values).

5.3.4.3. Recovery Dynamic of Spores Produced at pH 5.50

The spores of *Bacillus weihenstephanensis* produced at 30°C, pH_{spo} 5.50 presented some significant differences in recovery kinetics compared to spores produced at 30°C, pH_{spo} 7.00.

The germination time was not significantly different for the spores produced in optimal and sub optimal conditions. Again, this observation could be linked to the high speed of germination and the lack of data in the early germination ($t < 5$ minutes of incubation). Nevertheless, the proportion of refractive spores able to germinate was significantly lower for spores produced at pH 5.50 (ANOVA, $\alpha = 0.05$). That observation may come from a lower number of germinant receptors set during sporulation at low pH as the sporulation conditions affect the germinant synthesis (Abee *et al.*, 2011). Moreover, our proteomics results suggested that proteins involved in the germination process, SpoV family proteins, were under detected in spores produced at pH 5.50. These proteins are involved in the release of CaDPA during germination (Setlow, 2014). A lack of these proteins may lead to a dysfunctional release of CaDPA and thus a lower proportion of spores able to germinate.

The time of outgrowth was significantly impacted by the sporulation pH. This may be due to a lower metabolic activity or due to a lower amount of necessary molecules “stock” in the spore leading to an extension of the time of metabolic activity restoration. It was interesting that

proteomic analysis we performed showed a lower detection of cortex lytic enzyme SleB for spores produced at pH 5.50. It is one of the essential enzymes to cortex hydrolysis, occurring during the second phase of germination (Black *et al.*, 2005; Heffron *et al.*, 2009; Li *et al.*, 2013). So, if this enzyme is less detected in spores produced at pH 5.50, we can think that the hydrolysis of cortex will be less efficient than for spores produced at pH 7.00. The time for total spore hydration and then swell up and outgrowth may be longer because of a lower amount of essential enzymes like SleB. It would have been interesting to add lysozyme to spores produced at pH 5.50 after germination to confirm if the observed effect on outgrowth time was due to a deficient cortex hydrolysis (Setlow, 2014; Suzuki and Rode, 1969). Nevertheless, S_{outgr} was not impacted by the pH of sporulation, meaning that the homogeneity within the spores able to outgrow was not impacted by this condition of sporulation.

5.3.4.4. Recovery Dynamic of Heat Treated Spores

The heat treatment at two different temperatures, 85°C and 95°C, led to different germination and outgrowth kinetics despite the inactivation rate was the same (90% of the initial population). In the present work, the t_{germ} value was not significantly impacted by the heat treatment (ANOVA $\alpha=0.05$). The impact of heat treatment on germination time has been investigated in several previous work, by different methods and showed a small extension of germination time for *Bacillus subtilis* (10% fold increase) (Pandey *et al.*, 2015), and a drastic extension of germination time for *Geobacillus stearothermophilus* and *Clostridium botulinum* (threefold increase) (Stringer *et al.*, 2011, 2009; P. Zhang *et al.*, 2010). The impact of heat treatment on germination time seems to depend on the bacteria genera or species. Thus, the heat treatment may have an impact at molecular level since *Bacillus*, *Geobacillus* and *Clostridium* present different spore structure and composition (Nicholson *et al.*, 2000)

Surprisingly the heat treatment at 85°C did not significantly impact the proportion of spores able to germinate, τ_{germ} value, compared to the heat treatment at 95°C which lead to a lower τ_{germ} value. That could be explained by the impact of heat treatment on germinant receptors or CaDPA canals (Coleman *et al.*, 2010). This observation means that a proportion of spores were

blocked in refractive spore stage after an intense heat treatment, which is consistent with other works (Coleman and Setlow, 2009; Coleman *et al.*, 2007). However, this proportion of spores remaining refractive after an intense heat treatment may also be considered as hyper dormant spores (Ghosh and Setlow, 2009).

The time of outgrowth was affected by heat treatment at 85°C, leading to longer time of outgrowth as it was observed for *Clostridium botulinum* (Stringer *et al.*, 2011, 2009). The extension of outgrowth time may be due the impact of the treatment on enzymes or functional proteins within the spores (Coleman *et al.*, 2010). The heat treatment at 95°C led to a lower proportion of spores able to outgrow, τ_{outgr} , but the time to outgrow was not impacted. The damages due to heat treatment may be repaired before or during the outgrowth of spores (Pandey *et al.*, 2013). This hypothesis makes sense because metabolic activity is, at least, partially restored when the spores are rehydrated, allowing the repair of damages caused by heat treatment, mainly on proteins and enzymes (Coleman *et al.*, 2010, 2007; Warth, 1980). After both heat treatments, a higher heterogeneity within spore population was observed during outgrow. This observation was consistent with other results obtained with *C. botulinum* for example (Stringer *et al.*, 2011, 2009). The results were not impacted by recovery temperature and pH after heat treatment. Those results are consistent with other works for germination time values (Wang *et al.*, 2011; Zhou *et al.*, 2013) and confirmed that germination is impacted only by heat treatment temperature.

The CTC staining of heat treated spores, revealed a proportion of active cells (with positive respiratory activity) always higher than results given by count on agar medium (data not shown). This suggests that a part of vegetative cells obtained after heat treatment are active but not cultivable, classically called viable but non cultivable (VBNC) (Oliver, 2005). Thus, heat treatment can lead to spores inactivated at refractive spore stage, germinated spore stage or give non cultivable cells. Moreover, the intensity of the heat treatment seemed to have different impacts on the spore population, affecting different stages: the heat treatment at 85°C seemed to have a more important impact on outgrowth time and proportion of spores able to outgrow and the heat treatment at 95°C seemed to have an impact on the proportion of refractive spores to germinate and the proportion of spores able to outgrow. Finally, a non-

negligible number of germinated spores were positively stained with CTC meaning that they were metabolically active. However, after the vegetative cells outbreak, the vegetative multiplication occurred rapidly giving a number of vegetative cells avoiding further study of the process.

5.4. Conclusion

In conclusion, the flow cytometry allowed the study of the evolution of dormant spores, germinated spores and vegetative cells over time, for *Bacillus weihenstephanensis* and *Bacillus licheniformis*. Moreover, the method we developed in this work provided the quantification of the impact of incubation temperature and pH, heat treatment conditions and sporulation conditions on the evolution of each physiological stages of germination, outgrowth and growth process.

Mainly five parameters were chosen to quantify these impacts: the germination time, the proportion of refractive spores able to germinate, the outgrowth time, the heterogeneity of spore population during outgrowth and the proportion of refractive spores able to germinate. It appeared that incubation conditions impacted all these parameters. Sporulation pH had an impact mainly on germination proportion and outgrowth time, which could be a result of a lower amount of lytic enzymes in spores produced at low pH. This was consistent with our proteomics analysis which indicated a lower amount of cortex lytic enzyme SleB in spores produced in suboptimal conditions. Finally, the heat treatment impacted the recovery dynamics and the intensity of the treatment led to different impacts. The heat treatment had also a tremendous impact on the spore population heterogeneity. Moreover, it appeared that, after heat treatment, spores could be inactivated as refractive spores, germinated spores or vegetative cells. Those observations were done before the multiplication of the newly formed vegetative cells. After the first cell doubling, the vegetative cell population was too important to allow the study of the behavior of the spores blocked as refractive or germinated spores. To go further on these particular spores, sorting the cells should be a good way in future work.

The models we developed allow describing the recovery dynamics of *Bacillus weihenstephanensis* KBAB4 and *Bacillus licheniformis* Ad978 using a single set of parameters: the cardinal temperature and pH of growth. So, knowing the cardinal growth values, we are able to determine the proportions of refractive spores, germinated spores and vegetative cells regarding the recovery temperature or pH and the time of incubation. These models could be an interesting tool to improve the control of the development of spore forming bacteria in foods. Indeed, knowing the heat treatment conditions of a process, the formulation of the product (pH) and the conservation temperature, our model can provide the probability to spores to germinate, outgrow and grow in the product.

5.5. References

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Conclusion générale et perspectives

Le développement des bactéries sporulées dans les aliments peut être responsable d'intoxication alimentaire ou d'altération du produit menant à d'importantes pertes économiques. Les produits concernés sont généralement traités thermiquement, ce qui sélectionne les micro-organismes les plus résistants comme les spores. Les objectifs de cette thèse étaient de décrire et de quantifier la germination et la reprise d'activité des spores après traitement thermique en prenant en compte les conditions de formation des spores, de traitement thermique et d'incubation (température et pH). Basés sur ces nouvelles données à la fois quantitatives et physiologiques sur les capacités de développement des flores sporulées ayant subi un traitement thermique, le comportement de germination et de reprise de croissance a été décrit et quantifié par des modèles mathématiques. Dans un premier temps, un modèle mathématique a été développé afin de décrire et quantifier la germination et la reprise de croissance des spores par leur capacité à reprendre une croissance et former des colonies sur milieu gélosé. Ensuite, un modèle décrivant les cinétiques de germination, d'émergence et de reprise de croissance a permis de quantifier l'évolution des sous-populations rencontrées au cours de la germination et de la reprise de croissance : les spores dormantes, les spores germées et les cellules végétatives. Ces modèles comportent des paramètres physiologiques, les températures et pH cardinaux de croissance, et permettent de quantifier l'impact des conditions de sporulation, des conditions de traitement thermique et des conditions environnementales (température et pH) sur la capacité des spores à germer et reprendre une croissance. Ces modèles peuvent alors être utilisés en vue de mieux maîtriser le développement de la flore sporulée dans les denrées alimentaires.

Dans la première partie de ces travaux, la germination et la reprise de croissance ont été quantifiées par la capacité des spores à former des colonies sur milieu gélosé après un traitement thermique en fonction des conditions de sporulation, de traitement thermique et d'incubation. Ainsi, seulement la population de spores capables de germer, reprendre une croissance et former une colonie a été étudiée. La température de reprise de croissance a un impact uniquement lorsque la température est proche des limites de croissance. Le pH du

milieu de reprise, en revanche, a un effet plus progressif. En effet, une diminution de un point de pH entraîne une diminution d'un facteur 3 de la thermorésistance apparente. La température de conservation va donc retarder la germination puis la croissance des spores mais la probabilité de croissance reste inchangée alors que le pH de reprise a un effet additionnel à l'effet du traitement thermique. Ainsi, il semblerait plus intéressant de diminuer légèrement le pH d'un produit alimentaire. De plus, les limites de germination et de reprise de croissance et les limites de croissance sont très proches. Ainsi, il a été possible de modéliser l'impact de la température et du pH sur la reprise de croissance des spores après un traitement thermique grâce aux températures et pH cardinaux de croissance. Il est donc aisé de connaître quelle température doit être choisie pour conserver un produit, ou quel changement de pH d'un produit peut être envisagé, pour éviter la germination et la reprise de croissance de spores bactériennes.

Un modèle mathématique décrivant le comportement de germination et de reprise de croissance des spores après un traitement thermique a été développé (Trunet *et al.* 2015). Ce modèle intègre des paramètres physiologiques, les valeurs cardinales de croissance, contrairement aux modèles existant qui intègrent des paramètres empiriques. De plus, ce modèle prend en compte les conditions de formation de la spore, l'intensité du traitement thermique subi ainsi que les facteurs environnementaux rencontrés durant la germination et la reprise de croissance (température et pH). Le modèle repose sur les limites de croissance, ce qui lui confère plusieurs avantages. Notamment, l'utilisation des limites de croissance permet de faciliter l'usage du modèle car elles peuvent être obtenues à partir de la littérature, de données d'experts ou à partir d'un plan d'expérience simple le cas échéant. La proportion de spores capables de germer, de reprendre une croissance en fonction de l'intensité du traitement et des conditions de reprise peut être calculée à partir de ce modèle. Il est donc possible de simuler le comportement de germination des spores bactériennes après un traitement thermique et connaissant la formulation du produit et ses conditions de conservation. Cependant, il reste à être validé en matrice laitière. Des expériences préliminaires ont été entamées et ont pour but de quantifier l'impact du pH de la formulation et de la température de stockage sur la reprise de croissance des spores de *B. licheniformis* Ad978 après traitement thermique. Deux produits ont

été sélectionnés en concertation avec les industriels laitiers de Bretagne Biotechnologie Alimentaire (BBA) : une matrice laitière type « crème dessert » et une poudre de lactosérum. Ces produits seront traités à 95°C, à pH 5.5 et pH 6.8 puis incubés à pH 5.5 et pH 6.8 dans deux conditions de températures (18°C et 30°C). Cette validation nous permettra de confirmer si notre modèle peut s'adapter à une matrice laitière et s'il permet de prévoir le développement de la flore sporulée dans un produit en fonction de sa formulation et de sa température de conservation.

Outre le traitement thermique, d'autres techniques, comme les rayonnements ultra-violet ou encore les désinfectants, sont utilisés pour décontaminer plus particulièrement les surfaces. En ayant observé, dans un premier temps, l'impact des conditions de sporulation, de traitement thermique et de reprise sur la capacité des spores à former des colonies, on peut se demander si le comportement de germination et de reprise de croissance des spores est le même, quel que soit le type de traitement subi. Ainsi, nous nous sommes intéressés au comportement de recouvrement de spores de *Bacillus weihenstephanensis* KBAB4 traitées chimiquement (acide péracétique) et par rayonnement (lumière pulsée-UV). Nous avons pu mettre en évidence que l'impact des conditions environnementales (température et pH) sur le recouvrement de spores traitées par l'acide péracétique ou par lumière pulsée était le même que pour des spores traitées thermiquement. Malgré les différentes cibles affectées par ces différents traitements – principalement la membrane interne pour le traitement thermique chimique, l'ADN pour les rayons UV - l'impact des conditions de recouvrement est le même.

Afin de quantifier l'impact des conditions de sporulation, de traitement thermique et de reprise sur la germination et la reprise de croissance des spores bactériennes, nous avons, dans un premier temps, utilisé une méthode culturale. De nombreuses méthodes existent pour étudier la germination et la reprise de croissance, de l'aspect cellulaire à moléculaire, sur des populations de spores ou sur des spores individuelles. La cytométrie en flux permet d'observer les principales étapes de la germination et de la reprise de croissance sur un grand nombre de spores.

Une méthode par cytométrie en flux a donc été développée. Cette méthode a été choisie car elle permet de suivre l'évolution de l'état morphologique et physiologique au cours du temps,

sur un grand nombre de spores/cellules. Elle a permis d'observer la germination et l'émergence en mettant en évidence trois états physiologiques successifs, correspondant à trois sous-populations distinctes : les spores dormantes, les spores germées et les cellules végétatives. Ces trois étapes successives ont été discriminées grâce à des critères morphologiques et physiologiques. Les spores dormantes, réfringentes en microscopie à contraste de phase, sont de petite taille et imperméables aux marqueurs fluorescents. Les spores germées, sont de même taille que les spores dormantes mais sont perméables aux marqueurs et ont donc une fluorescence intense. Enfin, les cellules végétatives ont une taille plus importante que les spores ainsi qu'une fluorescence plus intense. Cette méthode a permis d'évaluer l'évolution de chaque sous-population en fonction des conditions de sporulation, de traitement thermique et de reprise. Un modèle primaire a été développé. Il permet de décrire l'évolution des spores dormantes, des spores germées et des cellules végétatives au cours du temps à l'aide d'un ensemble de cinq paramètres. Ces paramètres sont impactés par la température et le pH d'incubation et les limites de germination et de reprise de croissance semblent proches des limites de croissance. Ainsi, les cinq paramètres décrivant la germination et la reprise de croissance semblent évoluer comme les paramètres de croissance (taux de croissance, temps de latence), en fonction des conditions d'incubation. Les températures et pH minimaux, optimaux et maximaux de germination et d'émergence sont proches des températures et pH minimaux, optimaux et maximaux de croissance. Cependant, l'effet de la température semble légèrement différent. Il apparaît que la vitesse de germination augmente avec la température et qu'il n'existe pas de température optimale de vitesse de germination sur la plage de croissance. La première étape de la germination consiste simplement en la liaison de molécules spécifiques, les germinants, avec des récepteurs situés dans la spore. Ce phénomène est passif vis-à-vis de la spore et la température entrainerait alors simplement une augmentation de l'agitation moléculaire et les germinants diffuseraient plus facilement jusqu'à leurs récepteurs lorsque la température augmente. Cependant, les paramètres de germination et d'émergence n'ont pas été estimés pour des températures supérieures à la température maximale de croissance. Il pourrait être très intéressant de quantifier le comportement de germination et de reprise de croissance au-delà des limites de croissance. Cela pourrait donner de nouveaux leviers pour

inactiver la flore sporulée : le produit pourrait subir un traitement thermique modéré, entre 40°C et 60°C dans le cas de *Bacillus weihenstephanensis* et *Bacillus licheniformis*, entraînant la germination des spores dormantes tout en empêchant leur reprise de croissance (car la température serait au-dessus de leur limite de croissance) ; ensuite, un deuxième traitement, entre 70°C et 80°C, permettrait d'éliminer les spores germées (qui ont perdu leur haute thermorésistance) et les cellules végétatives.

Les paramètres décrivant la germination et la reprise de croissance évoluent également en fonction des conditions de sporulation et de traitement thermique. Nous avons développé un modèle secondaire permettant de décrire l'évolution des paramètres de germination et de reprise de croissance en fonction des conditions de température et de pH d'incubation. Les modèles primaires et secondaires ainsi développés permettent de connaître la composition en spores dormantes, spores germées et cellules végétatives en fonctions des conditions de sporulation, de traitement thermique, des conditions environnementales (température et pH) et du temps d'incubation. Ces modèles pourraient permettre d'améliorer la maîtrise de la flore sporulée en prenant en compte l'aspect cinétique de la germination et de la reprise de croissance ainsi que les proportions de spores capables de reprendre une croissance. En effet, en connaissant les conditions de traitement thermique, la formulation du produit (le pH notamment) et de la température de stockage, les modèles présentés dans cette partie permettent de connaître la probabilité de développement de la flore sporulée dans le produit.

Les résultats obtenus par cytométrie en flux montrent également que le traitement thermique ou les conditions défavorables d'incubation entraînent l'inactivation des spores à différents stades. En effet, une partie de la population peut être bloquée au stade de dormance, une autre au stade de la germination et une dernière sous forme de cellules végétative. Cependant, la méthode de cytométrie développée dans ces travaux ne permettait pas d'analyser la population de spores après multiplication des cellules végétatives. Tous les résultats présentés concernent donc des « temps courts » de germination et de reprise de croissance. Afin de pouvoir étudier les spores qui semblent bloquées par les conditions défavorables, il faudra les récupérer à l'aide d'un trieur. La méthode de cytométrie en flux que nous avons développée dans ces travaux permet le suivi de l'évolution d'un grand nombre de cellules (plus de 200 000) et de quantifier

l'impact des conditions de traitement thermique ou des conditions d'incubation sur cette évolution. Cette méthode est complémentaire avec les méthodes de microscopie, qui permettent de suivre les spores individuellement d'un bout à l'autre du processus de germination et de reprise de croissance, mais sur un nombre de cellules de l'ordre du millier.

En associant les résultats obtenus sur milieu gélosé, en section 2 et 3, et les résultats obtenus en cytométrie en flux, en section 5, nous avons pu mettre en évidence que les conditions de sporulation, de traitement thermique et d'incubation impactaient le nombre de spores capables de germer, de donner une cellule végétative qui sera capable, ou non, de se multiplier, mais aussi les vitesses de transformation des cellules au cours de ce processus. Chaque sous-population – spores dormantes, spores germées et cellules végétatives – est impactée par les conditions environnementales et les modèles que nous avons développés permettent de décrire ces effets. Ces travaux ont permis d'obtenir à la fois une réponse quantitative du comportement des spores après un traitement thermique et à chacune des étapes du processus de germination et de reprise de croissance. Les modèles développés pour décrire et quantifier ces réponses ont un double intérêt. Tout d'abord, ces modèles décrivent bien le comportement des spores à l'aide de paramètres physiologiques, les valeurs cardinales, *i.e.* les températures et pH minimaux, optimaux et maximaux de croissance. Les valeurs cardinales sont largement utilisées pour modéliser la croissance bactérienne et leur utilisation a été étendue à la germination et la reprise de croissance dans ces travaux. Ceci permet de réaffirmer l'intérêt de ces paramètres physiologiques. En effet, leur cycle de vie est complexe, comprenant des formes sporulées et des cellules végétatives, mais il est entièrement régi par les mêmes limites biologiques de croissance. Le deuxième intérêt de ces modèles est industriel. En effet, ils permettent de contrôler plus efficacement la flore sporulée responsable d'altération ou liée à des risques sanitaires. De plus, ils permettent de mieux définir les possibilités de formulation et de conservation qui pourront être utilisées sans développement de la flore sporulée. En connaissant les conditions de traitement thermique, la formulation du produit (son pH notamment) et la température de conservation, les modèles que nous avons développés permettent de connaître la probabilité de développement de la flore sporulée mais aussi la

composition de la population de bactéries sporulées, en terme de spores dormantes, de spores germées et de cellules végétatives.

Nous avons pu quantifier l'impact du traitement thermique sur la capacité des spores à former des colonies ainsi que sur les étapes successives de la germination et la reprise d'activité. Nous avons pu montrer que si l'intensité du traitement thermique variait, son impact sur les processus de germination et de reprise de croissance était différent. Le traitement thermique semble toucher plusieurs cibles au sein de la spore. Des travaux précédents indiquent que ces cibles sont multiples, ce qui peut expliquer que nous ayons observé des spores inactivées au stade dormance, d'autre au stade de spore germée ou encore de cellules en émergence. Cependant, ces cibles ne sont pas clairement identifiées. S'agit-il des protéines de structure, des enzymes impliquées dans la germination ou des protéines du métabolisme général ? Une étude de l'impact du traitement thermique sur le protéome des spores pourrait éclairer cette question en comparant le protéome de spores non traitées et le protéome de spores traitées thermiquement à différentes intensités. Différentes hypothèses peuvent être posées, basées sur des travaux existant (Coleman et al., 2010; Setlow, 2013). En fonction de l'intensité du traitement thermique, ce sont les protéines de structures du manteau qui seront touchées ou les enzymes de lyse du cortex, situées plus en profondeur dans la spore. Nous pourrions quantifier l'impact du traitement thermique, et l'impact de l'intensité du traitement, sur ce protéome et déterminer quelle(s) protéine(s) est (sont) déterminante(s) dans la résistance au traitement thermique (Coleman et al., 2007). Les données ainsi acquises pourront nous aider à mieux comprendre la germination et la reprise de croissance après un traitement thermique. De plus, ces données pourraient être utilisées en microbiologie prévisionnelle. En effet, les données de types « omics » (génomique, transcriptomique, protéomique) sont utilisées depuis quelques années pour décrire et quantifier le comportement bactérien en les intégrant comme paramètres dans des modèles mathématiques. Ce type de données a déjà été utilisé pour quantifier le stress acide sur des cellules végétatives ou pour étudier la germination de spores de *Bacillus* (Desriac et al., 2012). Identifier les biomarqueurs de la capacité à germer et reprendre une croissance après un traitement thermique et intégrer ces données en

microbiologie prévisionnelle serait de grand intérêt, notamment en industrie agro-alimentaire pour améliorer la maîtrise de la flore sporulée.

La méthode de cytométrie en flux qui a été développée dans ces travaux a un potentiel très intéressant à plusieurs niveaux. Tout d'abord, cette méthode nous a permis d'observer l'évolution des spores dormantes, des spores germées et des cellules végétatives au cours du temps. Cependant, le marquage des spores dormantes n'était pas spécifique ce qui menait à un seuil de détection relativement haut. L'utilisation d'autres marqueurs que le Syto9 peut être envisagé afin de résoudre ces problèmes. En effet, nous pourrions marquer les spores dormantes avec des anticorps associés à un marqueur fluorescent en ciblant des antigènes à la surface des spores, ou un autre marqueur se fixant aux spores comme l'OregonGreen qui est un marqueur se liant aux thiols exofaciaux des spores. Cette méthode permettrait alors de mieux détecter les spores dormantes et donc permettrait de mieux analyser le passage de spore dormante à spore germée. En plus de nous permettre de descendre le seuil de détection et ainsi détecter des événements rares, marquer spécifiquement les spores permettrait aussi de détecter les spores dans une matrice alimentaire comme le lait. La cytométrie en flux a un grand potentiel en industrie agro-alimentaire pour détecter les micro-organismes et y est de plus en plus utilisée. La méthode de marquage spécifique des spores que nous développerons permettrait alors de détecter les spores bactériennes qui sont difficilement détectables de par leur taille et la difficulté à les marquer spécifiquement.

Ensuite, la cytométrie en flux peut être associée à un trieur qui permet de récupérer les cellules d'intérêt après analyse. Ainsi, nous pourrions récupérer les spores qui restent au stade de dormance, ou au stade germé ou au stade de l'émergence, à cause des conditions environnementales défavorables ou après un traitement thermique, et procéder à de nouvelles expérimentations : (i) les incuber dans des conditions de température et de pH favorables afin d'observer si ces spores sont capables de reprendre le processus de germination et de reprise de croissance ou si elles sont réellement inactivées, (ii) procéder à des analyses protéomiques de ces cellules dans différents états et mettre en évidence de potentielles différences dans leur composition protéique qui pourrait être corrélées à leur comportement de germination et de reprise de croissance. L'analyse protéomique devra être adaptée au nombre limité de cellules

(de l'ordre du millier) que nous obtiendrons. Plusieurs méthodes pourront être appliquées comme la méthode *Filtered-Aided Sample Preparation* (FASP) qui permet de concentrer la fraction protéique extraite d'un échantillon biologique de petite taille (Feist and Hummon, 2015). Une méthode émergente en microbiologie, la cytométrie de masse, pourrait aussi être utilisée (Leipold et al., 2011) : elle permet de fournir, au sein de cellules individuelles, la quantité de protéines ciblées, comme les protéines liées à la thermorésistance. Cette méthode pourrait alors être d'un grand intérêt pour caractériser et expliquer l'hétérogénéité de résistance au traitement thermique, ou de germination et de reprise de croissance, au sein d'une population. Ces méthodes d'étude au niveau moléculaire nous permettront de mieux décrire et quantifier la germination et la reprise de croissance de spores en conditions défavorable ou après un traitement thermique en apportant des données physiologiques comme, par exemple, quelles sont les protéines clés de la résistance au traitement thermique. Ces futurs travaux permettraient aussi de donner de nouveaux types de données, des biomarqueurs, qui pourront être intégrées dans des modèles mathématiques, des outils qui permettront de mieux appréhender le développement de la flore sporulée en fonction des conditions de sporulation, de traitement thermique et de reprise de croissance.

5.4. References

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a.u.	Arbitrary Units
BHA	Brain Heart Agar
BHI	Brain Heart Infusion
CaDPA	Calcium-Dipicolinic Acid complex
CTC	5-Cyano-2,3-ditoyl tetrazolium chloride
FSC	Forward Scatter
FL (1 or 2)	Fluorescence (1 or 2)
GR	Germinant Receptor
OD ₆₀₀	Optical Density at 600 nm
PAA	Peracetic Acid
PBS	Phosphate Buffer Saline
SASP	Small Acid Soluble Proteins
SMB	Sporulation Mineral Buffer
SSC	Side Scatter

Valorisation des travaux de thèse

4 PUBLICATIONS

- Trunet C.**, Mtimet N., Mathot A.G., Postollec F., Leguerinel I., Sohier D., Couvert O., Carlin F., Coroller L. (2015). Modeling the recovery of heat treated *Bacillus licheniformis* Ad978 and *Bacillus weihenstephanensis* KBAB4 spores at suboptimal temperature and pH using growth limits. *Applied and Environmental Microbiology*, **81**:562-568 (IF2013= 3,952).
- Mtimet N., **Trunet C.**, Mathot AG., Venaille L., Leguérinel I., Coroller L., Couvert O. (2016). Die another day: Fate of heat-treated *Geobacillus stearothermophilus* ATCC 12980 spores during storage under growth-preventing conditions. *Food Microbiology*, **58**:85-96 (IF2013 = 3,320).
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6 COMMUNICATIONS ORALES (presenting author)

- Trunet C.**, Mtimet N, Mathot A-G, Postollec F, Leguerinel I, Sohier D, Couvert O, Carlin F, Coroller L 2016. Quantifying the impact of sub-optimal temperature and pH on the variability of germination and growth recovery among sub-populations of heat-stressed *Bacillus weihenstephanensis* KBAB4 and *Bacillus licheniformis* Ad978 spores. 7th European Spore Conference, Londres, Royaume-Unis, 18-20 avril 2016
- Trunet C.**, Mtimet N, Mathot A-G, Postollec F, Leguerinel I, Sohier D, Couvert O, Carlin F, Coroller L 2015 Modeling the impact of sub-optimal temperature and pH on the variability of germination and growth recovery among sub-populations of heat-stressed *Bacillus weihenstephanensis* KBAB4 and *Bacillus licheniformis* Ad978 spores ICPMF 9th International Conference on Predictive Modelling in Food, Rio de Janeiro, Brésil. 8-1 septembre 2015
- Trunet C.**, Mtimet N, Mathot A-G, Postollec F, Leguerinel I, Sohier D, Couvert O, Carlin F, Coroller L 2015 Hétérogénéité de la reprise d'activité des spores suite à un traitement thermique, Bactéries Sporulées Pathogènes et d'Intérêt Technologique BSPIT, Paris, France, 6-7 juillet 2015
- Trunet C.**, Mtimet N, Mathot AG, Postollec F, Leguerinel I, Sohier D, Couvert O, Carlin F, Coroller L 2015 Bacterial spore heterogeneity of behavior due to sporulation and recovery conditions. International Association for Food Protection IAFP Europe, Cardiff, Pays de Galles, 20-22 avril 2015
- Mtimet N, **Trunet C.**, Mathot A.G, Venaille L, Leguérinel I, Coroller L & Couvert O 2015 Will I survive? International Association for Food Protection IAFP Europe, Cardiff, Pays de Galles, 20-22 avril 2015
- Trunet C.**, Mtimet N, Mathot AG, Postollec F, Leguerinel I, Sohier D, Couvert O, Carlin F, Coroller L 2014 Using Physiological growth parameters to predict spore-forming bacteria behavior all along their life cycle as vegetative cells or spores. International Association for Food Protection IAFP USA Indianapolis, Indiana, USA, 3-6 août 2014

Mtimet N, **Trunet C**, Mathot A.G, Venaille L, Leguérinel I, Coroller L & Couvert O 2013 Cardinal values: a single set to predict growth and heat resistance recovery. International Conference on Predictive Microbiology in Food, ICPMF 8th, Paris, France, 16-20 septembre 2013

10 POSTERS

Trunet C., Mtimet N., Baril E., Mathot A.G., Postollec F., Leguerinel I., Sohier D, Couvert O, Carlin F. & Coroller L. 2014. Prediction of germination and growth recovery of *Bacillus weihenstephanensis* and *Bacillus licheniformis* spores after heat treatment 24th International ICFMH Symposium, Food Micro, Nantes, France, 1-4 septembre 2014

Mtimet N., **Trunet C.**, Mathot A. G., Venaille L, Leguérinel I., Coroller L. & Couvert O. 2014. An overall model to predict heat resistance of *Geobacillus stearothermophilus* taking into account the sporulation history and the recovery conditions. 24th International ICFMH Symposium, Food Micro, Nantes, France, 1-4 septembre 2014

Trunet C., Mtimet N., Baril E., Mathot A.G., Postollec F., Leguerinel I., Sohier D, Couvert O, Carlin F. & Coroller L. 2014. What if a general law could be applied to bacterial growth and spore recovery: the use of physiological growth parameters to predict spore germination and growth after heat treatment. BSFM, Bruxelles, Belgique, 8-10 septembre 2014

Mtimet N., **Trunet C.**, Mathot A.G., Venaille L., Leguérinel I., Coroller L. & Couvert O. 2013. Cardinal values: a single set to predict growth and heat resistance recovery. Belgian Society for Food Microbiology. 18th Conference on Food Microbiology, Brussels, Belgique, 8-10 septembre 2014

Mtimet N., **Trunet C.**, Mathot A. G., Venaille L., Leguérinel I., Coroller L., Couvert O. 2014. Toward a global approach in food preservation by investigating the physiological behavior of *Geobacillus stearothermophilus*. IAFP US Indianapolis, Indiana ,USA, 3-6 août 2014

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Trunet C., Mtimet N., Mathot A-G., Postollec F., Leguerinel I., Couvert O., Coroller L., Carlin F., 2013. Quantifying the *Bacillus weihenstephanensis* and *Bacillus licheniformis* spore recovery considering the sporulation, the heat-treatment and the recovery conditions. ICPMF 8 8th International Conference on Predictive Modelling in Food, Paris, France. 16-20 septembre 2013

Mtimet N., **Trunet C.**, Mathot A.G., Venaille L. , Leguérinel I. , Coroller L. & Couvert O. 2013. Cardinal values: a single set to predict growth and heat resistance recovery. International Congres Microbial Spoilers in Food, Spoilers 2013, Quimper , France, 1-3 juillet 2013

Trunet C., Mtimet N., Mathot A.G., Postollec F., Leguerinel I., Couvert O., Carlin F. & Coroller L. 2013. Quantifying the *Bacillus licheniformis* spore recovery considering the sporulation, the heat-treatment and the recovery conditions. International Congres Microbial Spoilers in Food, Spoilers 2013, Quimper , France, 1-3 juillet 2013



Spoilers In Food
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POSTER

Quantifying the *Bacillus licheniformis* spore recovery considering the sporulation, the heat-treatment and the recovery conditions

Trunet C., Mtimet N., Mathot A-G., Postollec F., Leguerinel I., Couvert O., Coroller L., Carlin F.

Introduction: Spore-forming bacteria development in food is a major cause of food spoilage and food poisoning, leading to economical losses. Empirical models have been developed to predict spores in variable environments after heat-treatment. The aim of this study is to understand and quantify *Bacillus licheniformis* spore recovery taking into account the properties induced by sporulation environments, the heat-treatments intensity and the recovery conditions. A predictive model, based on physiological parameters, will be designed to describe and quantify the spore recovery.

Material and methods: *Bacillus licheniformis* strain AD978 was isolated from raw dairy ingredients and was kindly provided by ADRIA Développement (Quimper, France). The sporulation was performed in sporulation mineral buffer (pH7.00) at two different temperatures: 45°C and 20°C. Spores were treated at 95°C, 100°C and 105°C following the capillary method, in buffered peptoned water (pH 7.00). Then, a method of dilution-inclusion in the recovery media has been used for survivor counts. The spores were incubated in Brain Heart Agar plates at pH ranging from 4.50 to 8.00 and incubation temperature ranging from 15°C to 60°C.

Results: Inactivation kinetics have been obtained for each heat-treatment conditions and recovery temperature and pH. An optimal recovery has been observed at around 45°C. Moreover, stronger are the conditions of heat-treatment, stronger is the impact of the recovery medium. The spore heat-sensitivity (z_T value) was constant regardless to the recovery pH and temperature. Only the apparent heat-resistance (D' value: time for the first decimal reduction) was affected by the temperature and pH. Bigelow-like models, using z'_T values, have been previously used to fit the data but these models led to over-estimations and predict possible recovery at 15°C, while *Bacillus licheniformis* AD978 is not able to grow at this temperature. The new model is based on the growth physiological parameters (minimal, optimal and maximal recovery temperature and pH), which are often available in scientific literature and have a real physiological meaning. It also avoids over-estimation of the predicted heat-resistance.

Significance: The new model would be useful to predict spore recovery after a heat treatment. The parameters used in this model can be obtained by elicitation of expert opinion, allowing a possible extension to other bacterial species of concern for food safety and quality.



International Conference on Predictive
Modelling in Food
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POSTER

Quantifying the *Bacillus weihenstephanensis* and *Bacillus licheniformis* spore recovery considering the sporulation, the heat-treatment and the recovery conditions

Trunet C., Mtimet N., Mathot A-G., Postollec F., Leguerinel I., Couvert O., Carlin F., Coroller L.

OBJECTIVE(S) Spore-forming bacteria development in food is a major cause of food spoilage and food poisoning, leading to economical losses. Empirical models have been developed to predict spores in variable environments after heat-treatment (Mafart *et al.* 2010). The aim of this study is to understand and quantify *Bacillus weihenstephanensis* and *Bacillus licheniformis* spore recovery taking into account the properties induced by sporulation environments, the heat-treatments intensity and the recovery conditions. A predictive model, based on physiological parameters, will be designed to describe and quantify the spore recovery.

METHODS(S) *Bacillus licheniformis* strain AD978 was isolated from raw dairy ingredients and was kindly provided by ADRIA Développement (Quimper, France) and *Bacillus weihenstephanensis* KBAB4 strain was kindly provided by the Institut National de la Recherche Agronomique (INRA, Avignon, France).. The sporulation was performed in sporulation mineral buffer (pH7.00) at two different temperatures: 12°C and 30°C for *Bacillus weihenstephanensis* and 45°C and 20°C for *Bacillus licheniformis*. Spores were treated at 95°C, 100°C and 105°C following the capillary method, in buffered peptoned water (pH 7.00). Then, a method of dilution-inclusion in the recovery media has been used for survivor counts (Baril *et al.*, 2011). The spores were incubated in Brain Heart Agar plates at pH ranging from 4.50 to 8.00 and incubation temperature ranging from 4°C to 40°C for *Bacillus weihenstephanensis* and 15°C to 60°C for *Bacillus licheniformis*.

RESULTS Inactivation kinetics have been obtained for each heat-treatment conditions and recovery temperature and pH. An optimal recovery has been observed at around 30°C for *Bacillus weihenstephanensis* and 45°C for *Bacillus licheniformis*. Moreover, the stronger are the conditions of heat-treatment, the stronger is the impact of the recovery medium. The spore heat-sensitivity (z_T value) was constant regardless to the recovery pH and temperature. Only the apparent heat-resistance (δ value: time for the first decimal reduction) was affected by the temperature and pH. Bigelow-like models, using z'_T values, have been previously used to fit the data but these models led to over-estimations and predict possible recovery at 15°C, while *Bacillus licheniformis* AD978 is not able to grow at this temperature. The model to be developed will be based on the growth physiological parameters (minimal, optimal and maximal recovery temperature and pH), which are often available in scientific literature and have a real physiological meaning. It shall also avoid over-estimation of the predicted heat-resistance out the range of temperature and pH allowing the growth.

CONCLUSIONS AND IMPACT OF THE STUDY The new model would be useful to predict spore recovery after a heat treatment. The parameters used in this model can be obtained by elicitation of expert opinion, allowing a possible extension to other bacterial species of concern for food safety and quality.

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European Spores Conference
(9-11 avril 2014 Londres)

POSTER

Bacillus spores germination and activity recovery monitored by flow cytometry

Trunet C., Mtimet N., Mathot A-G., Postollec F., Leguerinel I., Sohier D., Couvert O., Carlin F., Coroller L.

Spore recovery behavior after a heat-treatment is highly heterogeneous. The heat-treatment process inactivates a part of a spore population but depending on the recovery conditions only a part of the survivors are able to grow. The germination and activity recovery of *Bacillus weihenstephanensis* KBAB4 and *Bacillus licheniformis* Ad978 have been monitored using flow cytometry and classical methods as contrast-phase microscopy, OD at 600nm and colony forming unit plate counting. Thanks to size characteristics and fluorescent markers accessibility criteria (Syto9 and Propidium Iodide (PI)), intact spores, damaged spores, germinated spores and outgrowing cells have been discriminated. CFDA staining allows a discrimination of active spores, since their germination, and active vegetative cells. The staining of intact spores is heterogeneous but always low. Even if heat-inactivated spores do not lose refractivity, they are accessible to markers. The germinated spores are well stained by Syto9 (live cells) or PI (dead cells). Finally, the vegetative cells are stained with Syto9 or PI but are bigger than germinated spores. Monitoring the evolution of each sub-population over time by flow cytometry the transition through each stage has been quantified and appears to be variable in time. The activity recovery (CFDA positive staining), since the germinated spore stage, is variable in time too. This method allows the description of the spore population surviving the heat-treatment but inactivated by the recovery conditions. Classical culture methods allow only the study of spores able to survive, to germinate and to give an active cell forming a colony on a solid medium. The quantification of the behavior of late germinating or hyper-dormant spores would have a significant importance in the control of spore-forming bacteria.



International Association for Food Protection
(7-9 mai 2014 Budapest)

POSTER

Monitoring the kinetics of the germination and activity recovery of *Bacillus* spores after a heat-treatment by flow cytometry

Trunet C., Mtimet N., Mathot A-G., Postollec F., Leguerinel I., Sohier D., Couvert O., Carlin F., Coroller L.

Introduction: Heat-treatment is the major hurdle used to eliminate spores population in food products. However, spores can resist to this treatment and are able to germinate and recover an activity during the storage. Using culture based method, bacterial population is estimated by counting the unit forming colony that account for the population ability to germinate and recover a growth activity without quantifying late germinating or hyper-dormant spores.

Purpose: The aim of this work is to develop a method to monitor the germination and activity recovery of *Bacillus* spores over time and quantify the effect of recovery conditions on germination and activity recovery kinetics.

Methods: Subpopulations of spores of *B. weihenstephanensis* KBAB4 and *B. licheniformis* Ad978 after a heat-treatment have been identified using flow cytometry. Intact spores, damaged spores, germinated spores, outgrowing cells were clustered using fluorescent markers: Syto9 (live cells), PI (dead cells) and CFDA (esterase activity) in conjunction with classic methods (OD 600nm, cultural methods and phase-contrast microscopy). The transition through each sub-population, corresponding to physiological stages, has been observed over time by flow cytometry.

Results: The intact and heat-inactivated spores were not permeable to Syto9 or PI and were dimly fluorescent; germinated spores could be marked by Syto9 or PI, and had the same size than intact spores; the vegetative cells were well marked with Syto9 or PI but were bigger than germinated spores. Finally, CFDA marking allows following the activity recovery since the germination stage. Thanks to a monitoring over time, germination and activity recovery kinetics have been determined.

Significance: This work allows a description of the behavior of hyper-dormant or late germinating spores after a heat-treatment. The germination and activity recovery behavior of individual spores after a heat-treatment could improve the control of spore forming bacteria in food.



International Association for Food Protection
(3-6 août 2014 Indianapolis)

ORAL

Using physiological growth parameters to predict spore forming bacteria behavior all along their life cycle as vegetative cells or spores

Trunet C., Mtimet N., Mathot A-G., Postollec F., Leguerinel I., Sohier D., Couvert O., Carlin F., Coroller L.

Introduction: Spore-forming bacteria in food are a major cause of food poisoning or food spoilage, leading to a heavy burden. Empirical models have been developed to predict vegetative cells and spores behavior but very few integrate parameters with physiological meaning.

Purpose: The aim of this work is to model *Bacillus weihenstephanensis* KBAB4 (*B. cereus* group) and *Bacillus licheniformis* AD978 behavior during sporulation, spores germination and growth after a heat-treatment, using one single set of parameters: T_{min} , T_{opt} , T_{max} and pH_{min} , pH_{opt} , pH_{max} .

Methods: Effects of temperature and pH on growth of vegetative cells, their ability to produce spores and the spores' germination and activity recovery capacity after a heat-treatment have been quantified. For every phenomenon, the effect of temperature was quantified from 4°C to 40°C for *B. weihenstephanensis* and from 15°C to 60°C for *B. licheniformis* and the pH was studied from 4.5 to 9.5 for both strains. For the sporulation and the spores recovery, heat-resistances at 85°C, 90°C, 95°C for *B. weihenstephanensis* and 95°C, 100°C, 105°C for *B. licheniformis*, were estimated using a Bigelow-like model.

Results: Optimal growth temperature was around 31.9°C for *B. weihenstephanensis* and at 49.0°C for *B. licheniformis* and optimal pH was between 7.5 and 8.0 for both strains. Sporulation and recovery conditions range are circumscribed in growth conditions field (temperature and pH). Models for each phenomenon have been developed, based on modular mathematical approach, and only one set of values (minimal, optimal and maximal growth temperature and pH) with biological significance are integrated as parameters.

Significance: These models can be easily used to identify process conditions yielding microbial hazard related to spore contamination and predict spore forming bacterial behavior using physiological parameters, largely available in literature.



Food Micro
(1-4 septembre 2014 Nantes)

POSTER

Prediction of germination and growth recovery of *Bacillus weihenstephanensis* and *Bacillus licheniformis* spores after heat treatment

Trunet C., Mtimet N., Mathot A-G., Postollec F., Leguerinel I., Sohier D., Couvert O., Carlin F., Coroller L.

Spore-forming bacteria development in food is a major cause of food poisoning and food spoilage, leading to economical losses. Models, based on empirical parameters, have been developed to predict spores' behavior in variable environments after heat-treatment.

In the present work, we account for the effect of sporulation temperature, temperature during treatment, and temperature and pH in the recovery environment in a single mathematical model to predict the survival and growth of spores of *Bacillus weihenstephanensis* (a *Bacillus* species belonging to the *B. cereus* group) and *Bacillus licheniformis* recovery after heat-treatment.

B. weihenstephanensis KBAB4 spores were produced at 12°C and 30°C and spores of *B. licheniformis* strain AD978 at 45°C and 20°C. Spores inactivation kinetics were obtained at 85°C, 90°C and 95°C for *B. weihenstephanensis* and 95°C, 100°C and 105°C for *B. licheniformis* using the capillary tube method. Spores treated by heat were sampled at different times of treatment, then incubated on BHI agar at 10 pHs ranging from 4.50 to 9.50 at optimal growth temperature, or at pH 7.40 at 12 temperatures from 4°C to 40°C for *B. weihenstephanensis* and 15°C to 60°C for *B. licheniformis*. The survivors amount was estimated by enumerating colonies on agar medium for each condition of sporulation, treatment and recovery. Recovery temperature has an effect only near the recovery boundaries for both strains. The survivor ability to recover decreased from recovery neutral pH to the limits of recovery. Pre- and per-heat-treatment conditions impact only the heat-resistance and not on the colony forming ability of spores. Based on a modular approach, a model was developed, describing the colony forming ability of spores knowing the sporulation, treatment, and recovery conditions. In this model, only a reduced set of parameters was used to describe the bacterial sensitivity to the environment pre, per and post-treatment, which are the temperature and pH growth limits, easily reachable in literature or by expert elicitations.

The model predicting spore recovery was designed with reduced set of parameters, mainly temperature and pH growth limits. These parameters are advantageously available in the scientific literature or may be obtained by expert elicitations. The fitting of the model to the experimental data evaluated by reduction of sum of square residuals was highly satisfactory. In conclusion we propose a model predicting the extent of recovery of heat-treated spores based only on generic descriptors (e.g. temperature pH grow limits, Heat-resistance parameters in standard conditions) of microbial behavior.



POSTER

Using physiological growth parameters to predict spore forming bacteria behavior all along their life cycle as vegetative cells or spores

Trunet C., Mtimet N., Mathot A-G., Postollec F., Leguerinel I., Sohier D., Couvert O., Carlin F., Coroller L.

Introduction: Spore-forming bacteria in food are a major cause of food poisoning or food spoilage, leading to a heavy burden. Empirical models have been developed to predict vegetative cells and spores behavior but very few integrate parameters with physiological meaning. The aim of this work is to model *Bacillus weihenstephanensis* KBAB4 (*B. cereus* group) and *Bacillus licheniformis* AD978 behavior during sporulation, spores germination and growth after a heat-treatment, using one single set of parameters: T_{min} , T_{opt} , T_{max} and pH_{min} , pH_{opt} , pH_{max} .

Methods: Effects of temperature and pH on growth of vegetative cells, their ability to produce spores and the spores' germination and activity recovery capacity after a heat-treatment have been quantified. For every phenomenon, the effect of temperature was quantified from 4°C to 40°C for *B. weihenstephanensis* and from 15°C to 60°C for *B. licheniformis* and the pH was studied from 4.5 to 9.5 for both strains. For the sporulation and the spores recovery, heat-resistances at 85°C, 90°C, 95°C for *B. weihenstephanensis* and 95°C, 100°C, 105°C for *B. licheniformis*, were estimated using a Bigelow-like model.

Results: Optimal growth temperature was around 31.9°C for *B. weihenstephanensis* and at 49.0°C for *B. licheniformis* and optimal pH was between 7.5 and 8.0 for both strains. Sporulation and recovery conditions range are circumscribed in growth conditions field (temperature and pH). Models for each phenomenon have been developed, based on modular mathematical approach, and only one set of values (minimal, optimal and maximal growth temperature and pH) with biological significance are integrated as parameters.

Discussion: These models can be easily used to identify process conditions yielding microbial hazard related to spore contamination and predict spore forming bacterial behavior using physiological parameters, largely available in literature.



International Association for Food Protection
(20-22 avril 2015 Cardiff)

Oral

Bacterial spore heterogeneity of behavior due to sporulation and recovery conditions

Trunet C., Mtimet N., Mathot A-G., Postollec F., Leguerinel I., Sohier D., Couvert O., Carlin F., Coroller L.

Spore-forming bacteria in food are a major cause of food spoilage or food poisoning, leading to economic burden. Even after a heat treatment, surviving spores could be able to recover. The recovery consists of different physiological stages: germination, outgrowth and the first cell multiplication.

The sporulation conditions, heat treatment intensity and recovery conditions have an effect on spore recovery ability and recovery kinetics. Nevertheless, the physiological stage which is the most impacted by the recovery conditions and the heat treatment intensity remains unclear.

To quantify the impact of sporulation condition, heat treatment intensity and recovery conditions on the different recovery stages, recovery kinetics were performed using flow cytometry. The transfer from a physiological stage to the next one was monitored thanks to size and fluorescence intensity of cells which are labeled with Syto9 (permeability of spores) or 5-Cyano-2,3-ditolyl tetrazolium chloride (CTC, respiratory activity).

Sporulation and recovery conditions could highly impact the yield and time of germination. The heat treatment impact is more complex as spores can be altered by various ways. It could be put in evidence by using cell criteria as size, Syto9 and CTC fluorescence intensity.

Flow cytometry enables the quantification of the impact of sporulation, heat treatment and recovery conditions on the different stages during spore recovery. It allows the analysis of a large number of events over time and to discriminate what stage(s) is (are) the most impacted by each condition.



International Conference on Predictive Modelling in Food
(8-12 septembre 2015 Rio de Janeiro)

ORAL

Modeling the impact of sub-optimal temperature and pH on the variability of germination and growth recovery among sub-populations of heat-stressed *Bacillus weihenstephanensis* KBAB4 and *Bacillus licheniformis* Ad978 spores

Trunet C., Jouhanel E., Mtimet N., Mathot A-G., Postollec F., Leguerinel I., Sohier D., Couvert O., Carlin F., Coroller L.

Heat-treatment is a major hurdle widely applied for inactivation of bacterial spores in foods. The aim of this study is to follow by flow cytometry the fate of spores after specific heat-treatments and recovery in sub-optimal conditions. Especially the acquisition of kinetics and proportion of spores becoming vegetative cells were studied. A mathematical model taking into account the impact of recovery conditions and the dynamic aspect of the process was proposed.

The spores of *Bacillus weihenstephanensis* KBAB4 and *Bacillus licheniformis* AD978 were obtained at optimal and suboptimal growth temperature. After heat-treatments allowing a ten-fold reduction, spore recovery in nutritive broth was investigated for different pHs and temperatures distributed along the growth domain (5-40°C for *B. weihenstephanensis*, 15-60°C for *B. licheniformis* and pH 4.0-8.0 for both). The germination and growth recovery were monitored over time using flow cytometry, taking into account cell size (Syto9 staining) and respiratory activity (CTC staining).

Different physiological stages were efficiently evidenced: refractive spores, germinated spores, outgrowing cells and vegetative cells. In optimal conditions, most cells evolved rapidly towards multiplication. While in suboptimal conditions slower recovery process was observed and a lower proportion of spores successfully emerged into vegetative cells. Nevertheless, a large proportion of spores are subjected to germination and outgrowth after a heat-treatment, even only a few are able to form a colony on agar plates. A primary model was developed to describe this process.

Monitoring spore recovery using flow cytometry is a powerful method, allowing an exploration among cell populations evolution. The number of analysed cells is over 200000, offering an accurate estimation of variability in individual cell development within populations. The developed mathematical model describes the dynamic aspects of heat-treated spore recovery and takes into account the heterogeneous distribution of spores in the different physiological stages observed after a heat-treatment and the impact of environmental conditions.



Bactéries Sporulantes
Pathogènes ou d'Intérêt
Technologique
(6-7 juillet 2015 Paris)

ORAL (Prix de la meilleure présentation)

Hétérogénéité de la reprise d'activité des spores suite à un traitement thermique

Trunet C., Mtimet N., Mathot A-G., Postollec F., Leguerinel I., Sohier D., Couvert O., Carlin F., Coroller L.

Introduction : Les bactéries sporulées sont une cause majeure d'intoxication et d'altération d'aliment. Leur présence représente un danger pour les aliments transformés ou les ingrédients (plats cuisinés, poudre de lait, épices). En effet, elles sont capables de résister aux procédés assainissants comme le séchage, les traitements thermiques ou les traitements chimiques. Certaines spores peuvent ainsi survivre aux traitements et se développer. Afin de quantifier l'impact des conditions de sporulation, de l'intensité du traitement thermique et des conditions de recouvrement (*i.e.* les conditions *pre-*, *per-* et *post-traitement*) sur les différents stades du recouvrement, des cinétiques de germination et de reprise d'activité métabolique ont été réalisées par cytométrie en flux. Les différents stades du recouvrement correspondent aux sous-populations observées au cours de ce processus : spores réfringentes, spores germées, cellules en émergence, cellules végétatives.

Matériel et méthodes : Les spores de *Bacillus weihenstephanensis* KBAB4 et de *Bacillus licheniformis* Ad978 ont été obtenues à la température optimale et une température sous-optimale de croissance. Après un traitement thermique permettant une réduction 90% de la population initiale, le recouvrement des spores en milieu nutritif a été suivi pour différents pH et différentes températures distribuées sur le domaine de croissance (5-40°C pour *B. weihenstephanensis*, 15-60°C pour *B. licheniformis* et pH 4.0-8.0 pour les deux souches). Le passage d'un stade physiologique à un autre a pu être suivi grâce à des critères de taille et de d'intensité de fluorescence des cellules marquées avec du Syto9 (indicateur de la perméabilité des spores) et de 5-Cyano-2,3-ditolyl tetrazolium chloride ou CTC (indicateur de l'activité respiratoire).

Résultats : Le passage par les stades physiologiques est impacté différemment par les conditions de sporulation, l'intensité du traitement thermique et les conditions de recouvrement. Dans les conditions optimales (de sporulation et de recouvrement), la plupart des cellules ont évolué rapidement vers la multiplication. Alors que dans des conditions sub-optimales (de sporulation ou de recouvrement), le processus de recouvrement est plus lent et une plus faible proportion de cellules était capable de parvenir jusqu'au stade de cellule végétative active. Même si une large proportion des spores traitées thermiquement est capable de germer voire de donner une cellule végétative, seulement un faible nombre d'entre elles est capable de retrouver une activité métabolique et de se multiplier. Le traitement thermique impacte donc les spores à différents niveaux et ces dernières peuvent alors être bloquées sous forme de spores réfringentes, de spores germées ou de cellules végétatives incapables de se multiplier.

Conclusion : La cytométrie en flux permet de quantifier l'impact de la sporulation, du traitement thermique et des conditions de recouvrement sur les différents stades observables durant le recouvrement. Elle permet l'analyse d'un grand nombre d'événements (plus de 200000) au cours du temps ainsi que la discrimination du ou des stade(s) impacté(s) par les conditions environnementales *pre-*, *per-* et *post-traitement*.



European Spores Conference
(18-20 avril 2016 Londres)

ORAL

Quantifying the impact of sub-optimal temperature and pH on the variability of germination and growth recovery among sub-populations of heat-stressed *Bacillus weihenstephanensis* KBAB4 and *Bacillus licheniformis* Ad978 spores

Trunet C., Mtimet N., Mathot A-G., Postollec F., Leguerinel I., Sohier D., Couvert O., Carlin F., Coroller L.

Heat-treatment is a major hurdle widely applied for inactivation of bacterial spores in foods. The aim of this study is to follow by flow cytometry the fate of spores after specific heat-treatments and recovery in sub-optimal conditions. Especially, the dynamic of the physiological states leading the spore to give a vegetative cell was studied.

The spores of *Bacillus weihenstephanensis* KBAB4 and *Bacillus licheniformis* AD978 were obtained at optimal and suboptimal growth temperatures. After heat-treatments allowing a ten-fold reduction, spore recovery in nutritive broth was investigated for different pHs and temperatures distributed along the growth domain (5-40°C for *B. weihenstephanensis*, 15-60°C for *B. licheniformis* and pH 4.0-8.0 for both strains). The germination and growth recovery were monitored over time using flow cytometry, taking into account cell size and permeability (Syto9 staining) and respiratory activity (CTC staining).

Different physiological stages were efficiently evidenced: refractive spores, germinated spores, outgrowing cells and vegetative cells. In optimal conditions, most cells evolved rapidly towards multiplication. Recovery of heat-treated spores was slower at suboptimal pH and temperature than at optimal pH and optimal temperature, and a significantly lower proportion of spores successfully formed vegetative cells. Although large proportion of spores germinated and showed some early signs of potential growth after a heat-treatment, only a few were actually able to form a colony on agar plates.

Monitoring spore recovery using flow cytometry is a powerful method, allowing an exploration of the individual evolution of cells among populations. The number of analysed cells is potentially high (over 200 000 in the present work), offering an accurate estimation of variability in individual cell development within populations. A mathematical probabilistic model has been developed to describe the dynamic aspects of heat-treated spore recovery and takes into account the heterogeneous distribution of spores in the different physiological stages observed after a heat-treatment and the impact of environmental conditions.

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COMPETENCES

- Microbiology** Microbial ecology, bacteriology, mycology
Food safety, microbiology engineering, norms
Spore forming bacteria, flow cytometry, epifluorescence microscopy, predictive microbiology, extraction-purification and analysis by HPLC-MS/MS of spore proteins
- Food science** Industrial process, thermodynamics, fluid mechanics, food quality
Heat treatment, pulsed light, clean-in-place (CIP), norms (ISO9001, ISO22000, ISO14000)
- Computer skills** Microsoft Office (Word, Excel, PowerPoint, Publisher)
Matlab, R (MassChroqR pack)
- Languages** English: scientific et common (read, spoken, written)
Spanish (read, spoken, written)

Education

2012-2015

PhD at Laboratoire Universitaire de Biodiversité et d'Ecologie Microbienne (LUBEM), Université de Bretagne Occidentale and ADRIA Développement : *Germination, outgrowth and growth of bacterial spores after a heat treatment*

- Development of original models describing the germination and growth recovery behavior of bacterial spores after a heat treatment using physiological parameters
- Quantification of the impact of sporulation conditions, heat treatment intensity and incubation conditions on the different physiological stages which can be observed during germination and growth recovery
- Proteomic analysis of spores produced in different conditions and identification of biomarkers of germination and growth recovery ability

2009-2012

Microbiology engineering school : Ecole Supérieure de Microbiologie et de Sécurité Alimentaire de Brest (ESMISAB, now named ESIAB), allowing developing skills in food safety and security, food production and microbiology research.

- **3rd year internship**, Characterisation and optimisation of antifungal activity of *Lactobacillus harbinensis* K.V9.3.1Np and *Lactobacillus coryniformis* K.V9.3.1O in yogurts.
- **2nd year internship** : Effects of invariant chain on endosomal conformation and antibodies presentation in cells infected by *Toxoplasma gondii*.

2011-2012

Master 2 Research in Fundamental and Applied Microbiology at UBO (Université de Bretagne Occidentale)

2007-2009

Preparing class to Grandes Ecoles at Lycée Louis Barthou in Pau (64) BCPST (Biology, Chemistry, Physics, earth sciences)

Teaching activities

- 2015-2016** ***Bioengineering at IUT Quimper***
ATER full employment : Microbiology (192 h TD)
- Hygiene and security at the lab bench
 - Microbiology techniques: Gram coloration, isolation of bacteria
 - Orientation and identification of bacteria
 - Identification of fungi
- 2014-2015** ***Bioengineering at IUT Quimper***
Practical work (40h) : Food Process Engineering
- Heat exchange in heat plate pasteurizer
 - Milk ultrafiltration
 - Filtration by aggradation at constant pression
 - Raw milk skimming
 - Compression refrigerating machine
- 2013-2014** ***Bioengineering at IUT Quimper***
Practical work (48h) tutorials (6h) : Food safety and hygiene
- Microbial analysis of food products
 - Microscopy
 - Identification of bacteria
 - Identification of fungi
 - Counting techniques on PCA medium
 - Microbial analysis of surfaces
 - Efficiency of sanitizer
- 2012-2015** **Internship managing**
- Bioengineering IUT, Quimper (3 étudiants)
 - Licence 3 Biology Environnement, specialty Biotechnologies (1 étudiant)
 - Master 2 Fundamental and applied Microbiology (1 student)
- 2009-2012** **ESMISAB**
Tutoring of students from middle school and college
- 2011-2012** **Preparating class BCPST**
Tutoring for 1st year student from Biology University

Modeling the Recovery of Heat-Treated *Bacillus licheniformis* Ad978 and *Bacillus weihenstephanensis* KBAB4 Spores at Suboptimal Temperature and pH Using Growth Limits

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ADRIA Développement, UMT14.01 SPORE-RISK, Z.A. de Creac'h Gwen, Quimper, France^a; Université de Brest, EA3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, UMT14.01 SPORE-RISK, ScInBioS, Quimper, France^b; Bonduelle, Villeneuve d'Ascq, France^c; INRA, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, Avignon, France^d; Université d'Avignon, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, Avignon, France^e

The apparent heat resistance of spores of *Bacillus weihenstephanensis* and *Bacillus licheniformis* was measured and expressed as the time to first decimal reduction (δ value) at a given recovery temperature and pH. Spores of *B. weihenstephanensis* were produced at 30°C and 12°C, and spores of *B. licheniformis* were produced at 45°C and 20°C. *B. weihenstephanensis* spores were then heat treated at 85°C, 90°C, and 95°C, and *B. licheniformis* spores were heat treated at 95°C, 100°C, and 105°C. Heat-treated spores were grown on nutrient agar at a range of temperatures (4°C to 40°C for *B. weihenstephanensis* and 15°C to 60°C for *B. licheniformis*) or a range of pHs (between pH 4.5 and pH 9.5 for both strains). The recovery temperature had a slight effect on the apparent heat resistance, except very near recovery boundaries. In contrast, a decrease in the recovery pH had a progressive impact on apparent heat resistance. A model describing the heat resistance and the ability to recover according to the sporulation temperature, temperature of treatment, and recovery temperature and pH was proposed. This model derived from secondary mathematical models for growth prediction. Previously published cardinal temperature and pH values were used as input parameters. The fitting of the model with apparent heat resistance data obtained for a wide range of spore treatment and recovery conditions was highly satisfactory.

The multiplication of spore-forming bacteria in foods can cause poisoning and/or spoilage. The heat process applied to foods (from mild in cooked and refrigerated foods to very intense in canned or ultrahigh-temperature foods) creates a positive selection of spore-forming species of bacteria because of the high resistance of their spores (1). Therefore, control of spore-forming bacteria in foods first of all requires inactivation of dormant spores by heat (or by any other appropriate inactivation treatment). The extent of inactivation depends on a number of factors, naturally including the inactivation process intensity and more importantly the spore resistance properties at the time of treatment, which may vary with the conditions and environment of sporulation (2). Respect for the organoleptic quality of food may limit the intensity of the process and therefore the extent of spore inactivation. Controlling the recovery of surviving spores in processed food strengthens the safety and stability level achieved after the inactivation process. Recovery is a complex phenomenon, comprising germination of spores, restoration of metabolic activity in suboptimal or favorable conditions and emergence of the first vegetative cell able to multiply. The incubation temperature during storage and food pH are among factors that will deeply influence the recovery of surviving spores (3).

Spore recovery is influenced by multiple physical and (bio)chemical factors, such as temperature, pH, and water activity (a_w) and by the presence of germinants (such as amino acids, ribosides, and minerals) or enzymes, such as lysozyme (4, 7, 8, 37). The previous works cited here are mainly descriptive, and modeling attempts are rare and moreover rather unsatisfactory (6). For instance, the model of Leguérinel (4) assumed a linear effect of temperature on recovery of heat-treated spores, while most works describe maximal recovery under optimal recovery conditions. In contrast, many mathematical models predict the sole impact of

heat treatment on microorganisms (15, 24, 37). This work proposes a model describing spore recovery after heat treatment as a function of incubation temperature and pH of the recovery medium and accounting for the variations due to sporulation conditions. This model integrates conditions encountered by the spores in many food industries: spores are formed under diverse environmental conditions which remain unknown most of the time, are transferred to foods, and are inactivated by heat to a certain degree during food processing. Survivors tend to multiply during food storage. The overall impact of sporulation temperature, heat treatment intensity, and temperature and pH of recovery is generally quantified by the ability of surviving spores to form a colony on an agar plate, which results from the germination and growth restoration of the heat-treated spores. The experimental work was performed on two strains with different behaviors regarding temperature: *Bacillus weihenstephanensis*, a psychrotrophic species, and *Bacillus licheniformis*, a thermotrophic species.

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(This work is a partial fulfillment of the Ph.D. theses of C. Trunet and N. Mtimet.)

MATERIALS AND METHODS

Bacterial strains. *Bacillus weihenstephanensis* strain KBAB4 (INRA, Avignon, France), isolated from forest soil (9), and *Bacillus licheniformis* strain Ad978 (ADRIA Développement, Quimper, France), isolated from dairy ingredients, were used in this work. The strains were stored at -80°C in 1-ml aliquots of brain heart infusion (BHI; Biokar Diagnostics, Beauvais, France) mixed with 50% glycerol (vol/vol) at a concentration of approximately 2×10^6 CFU ml^{-1} . A 100-ml volume of BHI was inoculated with 1 ml of the stock suspensions and incubated for 8 h at optimal growth temperature (30°C for *B. weihenstephanensis* KBAB4 and 45°C for *B. licheniformis* Ad978); then, a 1-ml volume was transferred into 100 ml of BHI for 16 h of incubation at the same temperatures. Finally, 0.1 ml of the *B. weihenstephanensis* suspension and 0.01 ml of the *B. licheniformis* suspension were added to 100 ml of BHI and were incubated for 6 h. For both strains, the final cell concentration in the preculture was approximately 10^8 CFU ml^{-1} ; the number of spores estimated by the number of cells surviving a 5-min, 70°C heat treatment was lower than 100 spores ml^{-1} .

Sporulation. Spores were produced through a two-step sporulation process (11). Volumes of 100 ml of the previously described preculture were centrifuged ($6,000 \times g$, 10 min, 12°C) and suspended in 100 ml of sporulation mineral buffer (SMB) made of K_2HPO_4 at 4.5 g liter^{-1} , KH_2PO_4 at 1.8 g liter^{-1} , $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ at 8.0 mg liter^{-1} , and $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ at 1.5 mg liter^{-1} and filter sterilized using $0.2\text{-}\mu\text{m}$ -pore-size filters (11). These suspensions were incubated with shaking at 30°C and 12°C for *B. weihenstephanensis* and 45°C and 20°C for *B. licheniformis* Ad978. Spores in SMB were harvested when free spores represented more than 95% of cells at a magnification of $\times 1,000$ in phase-contrast microscopy (Olympus BX50; Olympus Optical Co., Ltd., Hamburg, Germany), i.e., after 1 to 2 days at optimal growth temperature for both strains and up to 10 days at suboptimal temperature for both strains. The spore suspensions were centrifuged ($6,000 \times g$, 10 min, 12°C). Spore pellets were suspended in 5 ml of sterile distilled water. The 5-ml suspensions were divided into 1-ml aliquots and stored for 1 month at 4°C before use. Laboratory observations consistently show that spore heat resistance does not change for at least 6 months of storage (unpublished data). The final concentrations of the stock suspensions were 10^8 spores ml^{-1} for *B. weihenstephanensis* and 10^9 spores ml^{-1} for *B. licheniformis*.

Heat treatment. The spores were diluted in buffered peptone water (casein peptone at 10 g liter^{-1} , NaCl at 5 g liter^{-1} , K_2HPO_4 at 4.5 g liter^{-1} , KH_2PO_4 at 1.8 g liter^{-1} , pH 7.00) to a final concentration of around 10^7 spores ml^{-1} . Capillary tubes (200- μl volumes) were filled with 100 μl of spore suspension, sealed, and then immersed in a water-glycerol bath maintained at 85°C , 90°C , and 95°C for *B. weihenstephanensis* and 95°C , 100°C , and 105°C for *B. licheniformis* (10, 11). Capillary tubes were removed from the bath at appropriate time intervals and immediately cooled in a water-ice bath for 30 s. The tips were broken, and the heat-treated spore suspensions were diluted in tryptone salt broth (Biokar Diagnostics, Beauvais, France). To estimate the spore concentration at the initial time (t_0), the spore suspensions were treated in a water bath at 70°C for 5 min using the same capillary tube method.

Recovery. Volumes of 100 μl of the appropriate decimal dilutions of heat-treated spores were spread on brain heart agar (BHA; Biokar Diagnostics, Beauvais, France) at pH values ranging from 4.5 to 9.5 or incubated at temperatures ranging from 4°C to 40°C for *B. weihenstephanensis* and from 15°C to 60°C for *B. licheniformis*. BHA at a range of pH values was obtained as follows. BHI broth ($2\times$) was prepared and adjusted by addition of 1 M HCl to the desired pH, measured with a PHM210 pH meter (Meterlab, Villeurbanne, France) and a Tuff Tip electrode (Fisher Bioblock Scientific, Illkirch-Graffenstaden, France) previously calibrated using pH 4.00, pH 7.00, and pH 10.00 standard solutions. Then, the $2\times$ BHI broth was filtered on a $0.2\text{-}\mu\text{m}$ filter (Steritop system; Millipore Cor-

poration, Billerica, MA) and mixed with an equal volume of $2\times$ molten agar (30 g liter^{-1}). After the BHI broth and the agar had been mixed, the pH of the solidified and cooled medium was checked using the Tuff Tip electrode introduced into the top 1-cm layer of the agar. This pH value was recorded as the recovery pH for further experiments. Inactivation at a range of temperatures and recovery at optimum or suboptimal growth temperatures (8°C , 30°C , and 37°C for *B. weihenstephanensis* and 18°C , 45°C , and 58°C for *B. licheniformis*) and pH values (pH 5.20, 7.40, and 8.00 for both strains) were performed in triplicate, each replicate being performed with an independently prepared spore suspension. The full experimental design is presented in Table S1 in the supplemental material. Colony counts were recorded when they remained unchanged despite increasing incubation time. To ensure that BHA was sufficient for full spore germination, recovery after heat treatment on BHA supplemented with a 25 mM L-alanine–inosine mix triggering germination in *Bacillus* sp. strains (12, 13) or with 12.5 mg liter^{-1} lysozyme was evaluated with spores of both strains, at optimal and one suboptimal temperature. Dehydration of recovery agar was monitored by weighing petri dishes for 20 days at 45°C . In this extreme condition, water loss was about 15% of the agar, resulting in a similar increase in the nutrient concentrations. Under most conditions tested, the incubation time was shorter and/or the temperature was lower. The medium dehydration effects on recovery were therefore assumed to be minor. Spores of both strains remained phase bright during incubation at room temperature for 15 min, which exceeds the time necessary for inclusion in molten agar and incubation at the target temperatures. Germination between the end of the heat treatment and incubation under test conditions was therefore considered negligible.

To ensure that spores density did not impact the recovery ability under our conditions, petri dishes of different sizes (4.5-cm, 9.0-cm, and 15-cm diameters) were inoculated with suspensions of heat-treated spores at 10^6 spores ml^{-1} , 10^7 spores ml^{-1} , and 10^8 spores ml^{-1} , similar to a previous work evaluating the influence of spore density on *Clostridium botulinum* germination (14). For each condition, the spores were incubated at 30°C and 8°C for *B. weihenstephanensis* and 45°C and 20°C for *B. licheniformis*. Under our conditions, there was no significant effect of spore density on recovery ability (data not shown).

Modeling. (i) Primary model. Heat inactivation curves were fitted with the model presented in equation 1 (15):

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

where N is the surviving population, N_0 is the initial spore population, δ is the time to the first decimal reduction, and p is a shape parameter. $\log(N)$ designates the decimal logarithm of N in this paper.

(ii) Secondary recovery model. The developed recovery model is derived from the gamma concept (22, 38) (equation 2):

$$\frac{1}{\delta_{(T_{\text{HT}})@ (T', \text{pH}')}} = \frac{1}{\delta_{\text{max}}^*} \lambda_{T_{\text{HT}}} (T_{\text{HT}}) \lambda_{T'} (T') \lambda_{\text{pH}} (\text{pH}') \quad (2)$$

where $\delta_{(T_{\text{HT}})@ (T', \text{pH}')}$ is the apparent heat resistance of spores heat treated at temperature T_{HT} and then recovered at incubation temperature T' in agar medium at pH'; δ_{max}^* is the time to the first decimal reduction observed for the heat treatment temperature T^* and at the optimal recovery temperature (T'_{opt}) and the optimal recovery pH (pH'_{opt}); $\lambda_{T'} (T')$ and $\lambda_{\text{pH}} (\text{pH}')$ describe the effect of incubation temperature T' and pH pH' during recovery (equations 3 and 4, respectively), and $\lambda_{T_{\text{HT}}} (T_{\text{HT}})$ is the lethal rate (equation 5) (16, 17). At the optimal recovery temperature and pH, $\lambda_{T'} (T')$ and $\lambda_{\text{pH}} (\text{pH}')$ are equal to 1. At the reference temperature and pH, $\lambda_{T_{\text{HT}}} (T_{\text{HT}})$ is equal to 1. When recovery conditions become adverse, $\lambda_{T'} (T')$ and $\lambda_{\text{pH}} (\text{pH}')$ increase and tend toward the infinite when T' and pH' become close to the limit of their domain of definition. The $\lambda_{T'} (T')$ and $\lambda_{\text{pH}} (\text{pH}')$ functions were derived from the inverted Rosso function (18), where growth limits or cardinal values are input parameters (equation 2). Equations 3 and 4 for $\lambda_{T'} (T')$ and $\lambda_{\text{pH}} (\text{pH}')$ are as follows:

$$\lambda_T(T') = 1 / \frac{(T' - T'_{\max})(T' - T'_{\min})^{0.1}}{(T'_{\text{opt}} - T'_{\min})^{-0.9}[(T'_{\text{opt}} - T'_{\min})(T' - T'_{\text{opt}}) - (T'_{\text{opt}} - T'_{\max})(-0.9T'_{\text{opt}} + T'_{\min} - 0.1T')]}$$
(3)

for the recovery temperature range and

$$\lambda_{pH'}(pH') = 1 / \frac{(pH' - pH'_{\max})(pH' - pH'_{\min})^2}{(pH'_{\text{opt}} - pH'_{\min})[(pH'_{\text{opt}} - pH'_{\min})(pH' - pH'_{\text{opt}}) - (pH'_{\text{opt}} - pH'_{\max})(pH'_{\text{opt}} + pH'_{\min} - 2pH')]}$$
(4)

for the recovery pH range, where T'_{\min} , T'_{opt} , T'_{\max} , pH'_{\min} , pH'_{opt} and pH'_{\max} are the minimal (min), optimal (opt), maximal (max) conditions of temperature or pH for recovery.

The effect of temperature used for the heat treatment was quantified by the Bigelow model (equation 5) (19):

$$\lambda_{T_{\text{HT}}}(T_{\text{HT}}) = 10^{\frac{T_{\text{HT}} - T_{\text{HT}}^*}{z_T}}$$
(5)

where T_{HT} is the heat treatment temperature and T_{HT}^* is the reference heat treatment temperature. T_{HT}^* was fixed at 90°C for *B. weihenstephanensis* and at 100°C for *B. licheniformis*. Finally, z_T is heat sensitivity, i.e., the change in heat treatment temperature leading to a 10-fold reduction of the decimal reduction time.

The model was fitted on the observations [$\log(\delta)$ or $\log(N)$] by minimizing the sum of squared errors (SSE) using lsqcurvefit function in MatlabR2012b (MathWorks, Natick, MA, USA). The goodness of fit of the model was checked by the corrected Akaike information criterion (AICc) and the RMSE (root mean square error) (20, 21). The smaller the AICc and the RMSE were, the better the model was fitted on the data. The 95% confidence intervals were calculated using the nlparci function in MatlabR2012b. The fitting performance of the model was statistically evaluated by the F test, comparing the mean square error of the model to the mean square error of the data. The computed f value was compared to the F table value (0.05 significance level). If the f value was lower than the F value from the table, the F test was accepted, indicating that the model fitting was statistically acceptable.

The fitting of models was compared with a likelihood ratio test and a test statistic, S_L , computed as follows:

$$S_L = n \log \text{RSS}_C - n \log \text{RSS}_U$$
(6)

where n is the number of data, RSS_C is the residual square sum for the constrained (C) model, and RSS_U is the residual square sum for the unconstrained (U) model. In this work the constrained models were those (i) using a unique p value (equation 1) or a unique z_T value (equation 5) or (ii) using predetermined cardinal temperatures and pH for each strain (equations 3 and 4). The unconstrained models were those (i) using one p value for each inactivation curve or one z_T value for each of the $\log(\delta) = f(T')$ and $\log(\delta) = f(pH')$ curves or (ii) using the cardinal parameters estimated by the model. S_L is low when the selection of the model has no significant incidence on the quality of fitting. When n tends toward infinity, the limiting distribution of S_L is χ^2 distributed with $p_U - p_C$ degrees of freedom, where p_U is the number of parameters in the unconstrained model and p_C is the number of parameters in the constrained model. If S_L is lower than χ^2 ($\alpha = 0.05$), the difference in the fitting of both models is considered not significant.

RESULTS

A total of 115 inactivation curves for *B. weihenstephanensis* spores and 78 for *B. licheniformis* spores were performed, each with at least six counts of survivors and 4 log reductions (see Tables S1 to S5 in the supplemental material). The inactivation curves (Fig. 1) were fitted with equation 1 (22). No change in the inactivation curve shape (p value) was noted according to sporulation, heat treatment, and recovery conditions. Instead of a different p value

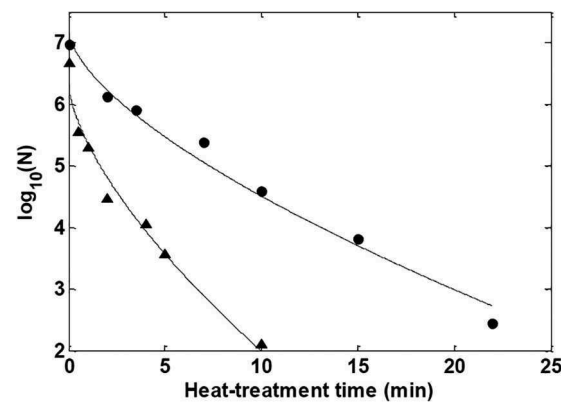


FIG 1 Inactivation curves of *B. weihenstephanensis* spores heat treated at 90°C and incubated at pH 5.20 (▲) and pH 7.40 (●) at 30°C. The lines correspond to the fitting of the data with equation 1 (22).

for each inactivation curve, a single p value was estimated for all inactivation curves. This p value was estimated at 0.68 ± 0.03 for *B. weihenstephanensis* and 1.96 ± 0.13 for *B. licheniformis*, as the curve shapes were concave upward and concave downward, respectively. No significant difference between the p values estimated for each inactivation curve and the p value estimated for all the inactivation curves was detected (likelihood ratio test, $\alpha = 0.05$).

The impact of recovery temperature was significant only at temperatures close to the recovery boundaries. The extreme temperatures at which recovery was observed were 6°C and 39°C for *B. weihenstephanensis* and 17°C and 59°C for *B. licheniformis*. For *B. weihenstephanensis*, the mean log values of δ , the apparent heat resistance, were $\log 0.31 \pm 0.06$ min at 8°C, $\log 0.39 \pm 0.07$ min at 30°C, and $\log 0.39 \pm 0.07$ min at 37°C; for *B. licheniformis*, the mean $\log(\delta)$ values were $\log 0.98 \pm 0.06$ min at 18°C, $\log 1.14 \pm 0.01$ min at 45°C, and $\log 1.00 \pm 0.06$ min at 58°C, for the reference heat treatment temperature. No significant changes in the δ value were observed from 8°C to 37°C for *B. weihenstephanensis* and from 18°C to 58°C for *B. licheniformis* (analysis of variance [ANOVA], $\alpha = 0.05$). The time required for survivors to form a colony was unsurprisingly greater at suboptimal temperatures than at optimal growth temperatures. For example, the time to colony counting for *B. weihenstephanensis* KBAB4 was 24 h at 30°C and pH 7.40 and 20 days at 7°C and pH 7.40.

There was a progressive decrease in δ values as the recovery pH came close to the pH recovery limits. The estimated optimal recovery pH was 7.80 ± 0.23 for *B. weihenstephanensis* and 7.73 ± 0.13 for *B. licheniformis*. A decrease in the recovery pH from 7.00 to pH 5.50 caused a 3-fold decrease in δ values of both *B. weihenstephanensis* and *B. licheniformis*. Neither strain formed any colonies at a recovery pH lower than 4.70. The δ value replicates for each strain was determined at the optimal recovery pH and at two suboptimal recovery pHs. For *B. weihenstephanensis*, the mean $\log(\delta)$ values were $\log -0.04 \pm 0.10$ min at pH 5.40, $\log 0.34 \pm 0.13$ min at pH 7.40, and $\log 0.37 \pm 0.11$ min at pH 8.00; for *B. licheniformis*, the mean $\log(\delta)$ values were $\log 0.41 \pm 0.07$ min at pH 5.40, $\log 1.09 \pm 0.03$ min at pH 7.40, and $\log 0.96 \pm 0.21$ min at pH 8.00. Similarly, spore recovery was slower at suboptimal pH than at optimal pH. For example, the time to counting for *B. weihenstephanensis* was 24 h at pH 7.40 and at 30°C and 15 days at pH 5.10 and 30°C.

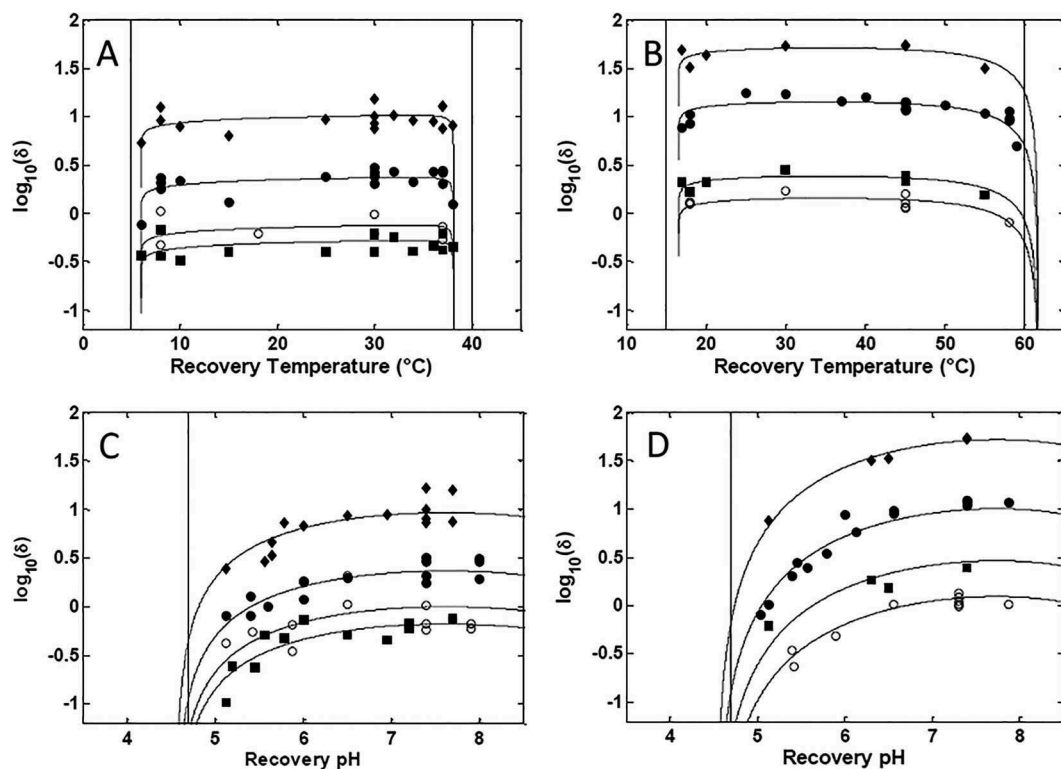


FIG 2 Effect of recovery temperature and pH on spores of *B. weihenstephanensis* (A and C) produced at 30°C (optimal temperature) (solid symbols) and 12°C (suboptimal temperature) (open symbols) and treated at 95°C (squares), 90°C (circles), and 85°C (diamonds) and on spores of *B. licheniformis* (B and D) produced at 45°C (optimal temperature) (solid symbols) and 20°C (suboptimal temperature) (open symbols) and treated at 105°C (squares), 100°C (circles), and 95°C (diamonds) on apparent heat resistance [$\log_{10}(\delta)$]. The vertical dashed lines represent the boundaries of temperature or pH beyond which no recovery was observed. The full lines correspond to the fitting of the data to the model developed in the present work (see Materials and Methods for details).

The spores produced at a suboptimal sporulation temperature behaved similarly to those formed at the optimal sporulation temperature (i.e., they showed same trend in terms of the influence of recovery temperature and pH). The major difference was in the δ_{\max} value, which was lower at suboptimal sporulation temperature (Fig. 2). The spores produced under optimal conditions were treated at three different temperatures in order to estimate their heat sensitivity (z_T value). From each recovery condition, it was possible to estimate a z value. Whatever the recovery temperature and pH, the z value was between 7.3°C and 8.8°C for *B. weihenstephanensis* (19 z values) and between 7.0°C and 8.0°C for *B. licheniformis* (10 z values). No significant difference could be discerned between each z value estimated on each recovery conditions and the single estimated z value (likelihood ratio test, $\alpha = 0.05$). The heat sensitivity (z value) was therefore assumed to be constant in the range of tested conditions for both strains and equal to $8.02^\circ\text{C} \pm 0.26^\circ\text{C}$ for *B. weihenstephanensis* and $7.67^\circ\text{C} \pm 0.27^\circ\text{C}$ for *B. licheniformis*. The heat treatment temperature has an impact only on spore heat resistance (Fig. 2). There was no interaction between heat treatment temperature and recovery conditions: the z_T value was not impacted by the recovery conditions.

Modeling the effect of recovery temperature and pH conditions on the spores' heat resistance. Equation 2 was used to model the spores' apparent heat resistance according to the heat treatment temperature and the recovery temperature and pH. The z value, corresponding to heat sensitivity, was considered constant for both strains (see above). The model was fitted on 115 $\log_{10}(\delta)$

values for *B. weihenstephanensis* and on 78 $\log_{10}(\delta)$ values for *B. licheniformis*. T'_{\min} , T'_{opt} , T'_{\max} , pH'_{\min} , pH'_{opt} , and pH'_{\max} were estimated for each strain. The RMSE values were 0.15 and 0.11 for *B. weihenstephanensis* and *B. licheniformis*, respectively (Table 1). These RMSE values are low compared to the standard deviation of $\log_{10}(\delta)$ values from replicated inactivation curves. Moreover, the fitting performance of the model was statistically accepted by the F test (with an α value of 0.05). Consequently, the model derived from equation 2 satisfactorily describes the recovery behavior of heat-treated spores of both strains.

Recovery after heat treatment is linked to incubation temperature and pH. Temperature and pH growth limits are among the most commonly available characteristics of bacteria. One option of the model was to use previously published growth limits (23), and therefore to fix the recovery parameters. No significant difference could be detected between the fitting with all estimated parameters and the fitting with fixed cardinal values for both strains (likelihood ratio test, $\alpha = 0.05$).

DISCUSSION

The spore heat resistance of many *Bacillus* sp. is highly impacted by the sporulation temperature (2). As shown in previous work (10), the sporulation temperature has an impact mainly on spore heat resistance (expressed with δ values in this work) but does not impact heat sensitivity (expressed with z_T values in this work). The specific effect of recovery conditions is the same whatever the sporulation and heat treatment conditions. Heat treatment leads

TABLE 1 Cardinal recovery parameters and growth cardinal values for *B. weihenstephanensis* KBAB4 and *B. licheniformis* Ad978 estimated with fixed growth parameters and with estimation of all parameters

Model fitting	Parameter	Estimated value (confidence interval ^c) for strain		
		<i>B. weihenstephanensis</i> KBAB4	<i>B. licheniformis</i> Ad978	
Temp and pH growth limits as fixed parameters	Estimated heat treatment parameters			
	Log(δ^*_{opt}) (log min) ^a	0.36 (0.34; 0.38)	1.10 (1.08; 1.12)	
	Log(δ^*_{opt}) (log min) ^b	-0.07 (-0.10; -0.04)	0.15 (0.12; 0.18)	
	z_T (°C)	8.05 (7.79; 8.31)	7.66 (7.32; 8.00)	
	Predetermined cardinal temp and pH ^d			
	T_{min} (°C)	2.72 (0.38; 5.60)	11.30 (6.12; 17.66)	
	T_{opt} (°C)	31.91 (30.93; 32.60)	49.01 (47.52; 50.34)	
	T_{max} (°C)	40.91 (40.41; 41.84)	57.87 (56.27; 65.83)	
	pH _{min}	4.35 (4.16; 4.51)	4.63 (4.43; 4.85)	
	pH _{opt}	7.71 (7.55; 7.95)	8.17 (7.86; 8.72)	
	No. of data	115	78	
	RMSE	0.15	0.14	
	Heat treatment parameters and recovery limits as estimated parameters	Estimated heat treatment parameters		
		Log(δ^*_{opt}) (log min) ^a	0.38 (0.34; 0.42)	1.11 (1.08; 1.14)
Log(δ^*_{opt}) (log min) ^b		-0.04 (-0.09; -0.01)	0.16 (0.12; 0.20)	
z_T (°C)		8.06 (7.80; 8.32)	7.67 (7.40; 7.94)	
Estimated recovery limits				
T'_{min} (°C)		5.94 (5.82; 6.06)	16.76 (15.79; 17.73)	
T'_{opt} (°C)		36.37 (24.63; 48.12)	31.79 (28.40; 35.18)	
T'_{max} (°C)		38.03 (37.63; 38.42)	65.97 (56.94; 75.01)	
pH' _{min}		3.79 (3.01; 4.58)	4.54 (4.41; 4.68)	
pH' _{opt}		7.80 (7.53; 8.07)	7.73 (7.61; 7.86)	
pH' _{max}		10.34 (9.93; 10.75)	9.80 (9.69; 9.90)	
No. of data		115	78	
RMSE		0.15	0.11	

^a Log(δ^*_{opt}), optimal heat resistance, at the reference temperature, for spores produced at the optimal temperature.

^b Optimal heat resistance, at the reference temperature, for spores produced at the suboptimal temperature.

^c $\alpha = 0.05$.

^d Data are from reference 23.

to the inactivation of spores, but some can be sublethally injured and are able to germinate, multiply, and form a colony (24). Supplementation of the recovery medium with an alanine-inosine mix, which is known to trigger germination on *B. weihenstephanensis* and *B. licheniformis* (12, 13), or with lysozyme, which is known to restore the germination of damaged spores (8), had no effect on recovery (i.e., similar counts were obtained after heat treatment on recovery agar, supplemented or not). Consequently the observed effect is likely due to impaired germination subsequent to damage and also to a reduced ability of the germinated cells to adapt to suboptimal temperature conditions to form colonies. The developed model satisfactorily describes the recovery behavior of heat-treated *Bacillus* sp. spores, accounting for pre-treatment, per-treatment, and posttreatment conditions. The range of pH and temperature allowing the recovery of spores of the tested *B. weihenstephanensis* and *B. licheniformis* strains was within the range of temperature and pH allowing growth. The domain of growth temperatures and the domain of recovery temperatures have very close boundaries. Consequently, the current model used predetermined cardinal temperature and pH values for each strain as control parameters, because these values have a real biological meaning and are reliable estimators of growth lim-

its (25). The cardinal temperatures T_{min} and T_{max} are, respectively, the temperature below which and the temperature above which growth cannot theoretically be observed (26). The minimal temperature for growth estimated by a cardinal temperature model (T_{min}) is always a few degrees Celsius lower than the observed minimum temperature allowing growth (27). As we demonstrated, these values can be used as input parameters to estimate the apparent heat resistance at given recovery temperatures and pH values. The recovery behavior of bacterial spores after heat treatment can therefore be modeled with parameters that have a biological meaning and that are relatively easily accessible to the scientific community, for instance, through literature review.

The impact of recovery temperature on the spore colony-forming ability is low in the recovery range. This has also been observed for different species, such as *Bacillus cereus* CNRZ 110, *Alicyclobacillus acidoterrestris* ATCC 49025, and several strains of *Bacillus stearothermophilus* (4, 28, 39). Only the time taken to form a colony was significantly influenced by the recovery temperature. Recently, a model describing the effect of different factors on the lag time of *B. cereus* spores has been developed (29). In this study, the observed biological response is the estimated lag time corresponding to the time taken for spores to germinate, outgrow, and

grow, taking into account only the time required to detect the germination and growth of at least one spore. The effect of recovery temperature could be explained by a prolongation of the germination and outgrowth duration as the temperature approaches the growth boundaries, as demonstrated for several *Bacillus* and *Clostridium* species (30, 31, 33, 40), and by the decrease in growth rates at temperatures lower or higher than the optimal temperature. Many foods, such as refrigerated ready-to-eat foods or cooked chilled foods, are processed with mild heat treatments and rely on refrigeration for preservation and/or combining suboptimal pH with low temperature as additional hurdles to prevent growth of surviving pathogenic or spoilage spore-forming bacteria (32). Our results suggest that in these foods, storage at low temperature will mainly delay the growth of spore-forming bacteria, not prevent the growth of surviving spores, and that recovery pH could actually affect the recovery ability of surviving spores. The recovery pH has a more progressive effect on the colony-forming ability, mathematically described with an exponent value of 2.0 in equation 4. pH values near the optimal growth pH offer the highest colony formation ability for both strains. Germination rates at low pH values may be lower and/or colony formation slower, as previously observed for *B. cereus*, for instance (41, 42), and *C. botulinum* (34). As with temperature, the domain of growth pH values and the domain of recovery pH values have very close boundaries. Prolonged outgrowth caused by low pH could be due to the H^+ effect on cytoplasmic pH and, although this would be highly strain/species dependent, to significant inner pH modifications during germination (35). A slight change of temperature or pH near to the boundaries caused a dramatic decrease in apparent heat resistance values. This can be explained by the growth behavior of bacterial cells under conditions close to the growth/no-growth boundaries, where the probability of cells forming a colony is lower than that under optimal conditions (36). This phenomenon could be strengthened by a decrease in the probability of germination of surviving spores. Moreover, an effect of spore density on spore germination has been shown, with *Clostridium* sp., for instance (14). Again, the phenomenon is complicated by the release of dipicolinic acid, triggering germination, during spore germination.

Interestingly, the number of inactivated cells can be described by cumulating the heat inactivation effect and the inhibitory effect due to suboptimal recovery conditions (equation 7).

$$n = n_{HT} + n' \quad (7)$$

where n is the apparent total log reduction, n_{HT} is the log reduction due to heat treatment, and n' is the virtual decimal decrease due to recovery conditions.

Equation 1 can therefore be written as follows:

$$n = \log \frac{N_0}{N} = \left(\frac{t}{\delta_{max}} \cdot \frac{1}{\lambda_{HT} \lambda_T \lambda_{pH'}} \right)^p \quad (8)$$

This equation is equivalent to equation 9:

$$n = \left(\frac{1}{\delta_{max}} t \cdot \lambda_{HT} \right)^p \cdot (\lambda_{X'})^p \quad (9)$$

where the effect of heat treatment could be expressed by

$$n_{HT} = \left(\frac{1}{\delta_{max}} \cdot t \cdot \lambda_{HT} \right)^p \quad (10)$$

and the effect of recovery by

$$n' = n_{HT} [(\lambda_{X'})^p - 1] \quad (11)$$

The impact of recovery can be calculated knowing the impact of the heat treatment (n_{HT}) and of the recovery medium formulation $[(\lambda_{X'})^p]$. There is no effect of the recovery environment ($n' = 0$) when $\lambda_{X'}$ is equal to 1, i.e., when the recovery conditions are optimal. On the contrary, when the recovery conditions are beyond the recovery limits [$\lambda_{X'}$ tends to $+\infty$], colony formation on the recovery medium is fully inhibited (n' tends to $+\infty$). This can also be linked to the germination rate, where the influence of heat treatment intensity and recovery temperature and pH are taken into account.

In conclusion, using a proper set of parameters for each strain and a model based on generic mathematical functions, the recovery of *B. weihenstephanensis* and *B. licheniformis* spores after heat treatments at diverse temperatures and as a function of the incubation temperature and the pH of the recovery medium was quantified. A similar approach can be used to quantify the impact of pH in addition to temperature during heat treatment on the recovery of spores. In this new model, only the heat resistance at optimal recovery temperature and pH has to be estimated, since the other parameters—cardinal growth temperature and pH—are obtained from independent experiments/sources. The spore population considered here is the population able to germinate and recover physiological activity in order to form a colony on nutrient agar. It remains undetermined whether the germination or the adaptation of the germinated cell is affected by sublethal heat treatment. The biological process leading to the formation of a colony from a stressed spore is likely stochastic, and further research is needed to quantify the relative part of these two steps in the spore recovery process.

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Germination, émergence et reprise de croissance de spores bactériennes après un traitement thermique

Résumé

Le développement des bactéries sporulées dans les aliments peut être responsable d'intoxication alimentaire ou d'altérations des produits. Trois leviers ont été identifiés pour prévenir le développement de ce microbiote : les conditions de sporulation, l'intensité du traitement appliqué pour inactiver les spores et les conditions d'incubation. Ce travail de thèse a pour objectif (i) de quantifier l'impact des conditions de sporulation, de traitement et d'incubation sur la capacité des spores à former des colonies, et (ii) de quantifier l'impact des conditions de sporulation, de traitement et d'incubation sur les cinétiques de germination et de reprise de croissance. Dans un premier temps, un modèle mathématique a été développé pour décrire et quantifier l'impact des conditions d'incubation sur la capacité des spores à former des colonies après un traitement thermique. Ce modèle intègre uniquement des paramètres physiologiques, les limites de croissance des souches étudiées. La germination et la reprise de croissance est un processus complexe au cours duquel les spores passent par plusieurs stades successifs : spores dormantes, spores germées et cellules végétatives. Afin de quantifier l'impact des conditions de sporulation, de traitement thermique et d'incubation sur chacun de ces stades, une méthode par cytométrie en flux a été développée. Elle a permis de suivre l'évolution de chaque stade au cours du temps et un modèle primaire a été proposé afin de décrire l'évolution de chacun de ces stades. A partir de ce modèle il a été possible de décrire l'impact des différentes conditions de sporulation, de traitement thermique et d'incubation sur cette évolution et un modèle secondaire a été développé pour quantifier l'impact de ces facteurs sur les cinétiques de germination et de reprise de croissance. Afin de corréliser les différences de comportement avec la composition protéique des spores, une analyse protéomique a été réalisée sur des spores produites dans différentes conditions. Ces travaux permettent de mieux appréhender le comportement de germination et de reprise de croissance des spores bactériennes. De plus, les résultats apportés ainsi que les modèles mathématiques développés dans cette thèse pourront permettre de mieux contrôler le développement des bactéries sporulées en industrie agro-alimentaire, connaissant l'impact des conditions de stockage et de formulation des produits, comme la température et le pH, sur le comportement des spores.

Mots clés : spores, Bacillus, inactivation, germination, reprise de croissance, cytométrie en flux

Germination, emergence and resumption of growth of bacterial spores after a heat treatment

Abstract

The development of spore forming bacteria in foods can be responsible for food poisoning or food spoilage. Three levers allowing the development of this microbiota were identified: the conditions of sporulation, the conditions of heat treatment and the conditions of incubation. This PhD work objectives were (i) to quantify the impact of sporulation conditions, heat treatment intensity and recovery conditions on the ability of spores to form colonies, and (ii) to quantify the impact of sporulation conditions, heat treatment intensity and recovery conditions on germination and outgrowth kinetics. Firstly, a mathematical model was developed to describe and quantify the impact of recovery conditions on the spore ability to form colonies after a heat treatment. This model integrated only physiological parameters, the growth limits. The germination and outgrowth is a complex process made of successive physiological stages the spores pass through: the dormant spores, the germinated spores and the vegetative cells.

A flow cytometry method was developed in order to quantify the impact of sporulation conditions, the heat treatment intensity and the incubation conditions on each physiological stage.

This method allowed monitoring the evolution of each stage over time and a primary model was proposed to describe these evolutions. Thanks to this model, the impact of sporulation conditions, the heat treatment intensity and the incubation conditions were quantified and a secondary model was developed to quantify the impact of these factors on germination and outgrowth kinetics. In order to correlate the differences of behavior with the proteome of spores, proteomic analysis were performed on spores produced in different conditions. This work allow a better comprehension of germination and outgrowth behavior. Moreover, the results and the mathematical models provided by this work can be applied in food industry to improve the control of spores forming bacteria development knowing the impact of storage conditions and the product formulation, like temperature and pH, on spore behavior.

Key words : spores, Bacillus, inactivation, germination, outgrowth, flow cytometry