Universidad Politécnica de Cartagena

DEPARTAMENTO DE INGENIERÍA DE ALIMENTOS Y DEL EQUIPAMIENTO AGRÍCOLA

Effect of relevant environmental factors for food preservation and molecular identification (High Resolution Melting) of *Bacillus cereus* group.

Vera Antolinos López

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**EFFECT OF RELEVANT ENVIRONMENTAL FACTORS
FOR FOOD PRESERVATION AND MOLECULAR
IDENTIFICATION (HIGH RESOLUTION MELTING) OF
Bacillus cereus GROUP.**

Tesis Doctoral

Trabajo presentado por **VERA ANTOLINOS LÓPEZ** para
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Cartagena

Dirigida por

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A mis padres y mi hermano

A mi abuela Paca

A Fernando

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Bacillus cereus group species range high in economical and medical importance. Among all of them, *Bacillus cereus sensu stricto* is the causative agent of gastrointestinal diseases and *Bacillus weihenstephanensis* is a psychrotrophic species able to grow in the range of 4-7°C. Both species are also recognized as spoilage bacteria causing economic losses.

Due to their ability to form highly resistant spores, these microorganisms may persist in the food environment and contaminate mildly heat treated products. Moreover, psychrotrophic and some mesophilic strains have been isolated from chilled foodstuffs.

On this ground, to contribute to improve food preservation systems that ensure microbiological quality, the major aim of the present thesis was to study the impact of relevant environmental factors, commonly referred to as hurdles, on the ability to survive and grow of different species of *B. cereus* group and to assess novel methods for fast and reliable identification of these microorganisms as well as to test the feasibility of techniques used in viability studies at single cell level.

Ready to eat vegetable foods are widely consumed in industrialized countries due to their tasty, healthy and wholesome attributes. However, *B. cereus* strains have been previously isolated from these mildly preserved foods. To contribute to the optimization of ready to eat vegetable foods processing, in Chapter 3 the effect of heating medium on *B. cereus* spores heat resistance was assessed in sterile distilled water and different vegetable substrates. It was shown that at higher (105°C) and lower temperatures (90°C) the heating medium had a stronger influence on *B. cereus* heat resistance, indicating that there was not a clear trend in this respect. This effect appeared to be more correlated with the pH of the menstruum than with food composition. Besides, the presence and extension of shoulders, and their impact on pasteurization processes were analyzed by fitting isothermal survival curves to two

different non log-linear models (Weibull and Geeraerd) to compare them with the classical log-linear regression. Root mean square error values revealed that heat inactivation of *B. cereus* spores was more accurately characterized by Weibulls' and Geeraerds' methods. Therefore, we proposed that non log-linear models should be implemented to prevent overlay conservative heat treatments and subsequent decrease of food quality, as well as to reduce energy requirements.

After characterization of *B. cereus* spores heat resistance, the effect of modified atmospheres in combination with mild and severe heat treatments was also studied in Chapter 3. For this purpose, additionally to anaerobic environments, oxygen limitation conditions, obtained by using two different packaging films with different oxygen transfer rates ($35\text{cm}^3/\text{m}^2/24\text{h}$ and $10^{-3}\text{ cm}^3/\text{m}^2/24\text{h}$), were used to assess the growth of *B. cereus* spores in broth and in a vegetable substrate. We observed that although this microorganism efficiently adapts to oxygen limitation, the extent of lag phase increased. Besides, a faster growth was observed in broth than in vegetable products. Likewise, samples exposed to low oxygen restriction ($35\text{cm}^3/\text{m}^2/24\text{h}$) also showed a faster growth. Notably, no significant differences on the time to reach 10^6 CFU/mL were found between food samples subjected to anaerobic environment and to the lowest oxygen transfer rates ($10^{-3}\text{ cm}^3/\text{m}^2/24\text{h}$).

Chapter 4 focused on the relevance of *B. cereus* in the egg industry. Spores and vegetative cells of this microorganism have been isolated from eggs and their products. To avoid coagulation, liquid egg undergoes mild heat treatments and subsequently is stored under refrigeration. Recent studies pointed to mildly treated products stored at low temperature as an appropriate niche for *B. cereus* psychrotrophic and mesophilic strains able to grow below 10°C . With the major aim to contribute to liquid egg preservation and risk management levels, the effect of antimicrobial compounds, nisin and lysozyme, combined with mild heat treatments on growth of *B. cereus* vegetative cells was evaluated in culture medium and liquid

egg, at different incubation temperatures. Growth of *B. cereus* cells subjected to these conditions was measured using an individual-based approach of growth through optical density measurements. From growth curves obtained, lag phase and maximum specific growth rate (μ_{max}) were determined, histograms of the lag phase were generated and distributions were fitted. Normal and Weibull distributions were ranked as the best fit distributions in experiments performed at 25 and 16°C. Finally, a Monte Carlo simulation was carried out to predict the time of growth to a certain microbial concentration in order to generate probabilistic information that allows to compare the growth curves obtained with the food model. These probabilistic predictions can be considered as an initial indication to establish the level of risk associated to liquid egg. Nevertheless, as with any predictive model, we need further tests in real conditions before transferring these results to the industry.

Another relevant environmental factor for food preservation is water activity. In Chapter 5 of this thesis the effects of temperature and water activity downshifts on the lag time of *B. weihenstephanensis* and the dependence of μ_{max} on the growth conditions (temperature and water activity) were modelled through the dependence of the parameter h_0 (“work to be done” prior to growth), on the magnitude of the shift and the stringency of the new environmental conditions evaluated. We observed that temperature shifts were able to induce considerable lag times (up to 20 days) near boundary growth. Meanwhile, water activity shifts within the range studied had less significant effects. Furthermore, the predictive ability of the combined model (h_0 and μ_{max}) was assessed in carrot soup and ready meal products and was found to correctly predict *B. weihenstephanensis* growth in broth and creamed pasta (rich substrates). On the grounds of our results, we suggest that this model can be used to improve predictions of *B. weihenstephanensis* growth in dynamic conditions, which can be useful for Hazard Analysis and Critical Control Points (HACCP) and Microbial Risk assessment purposes.

Chapter 6 addresses the effect of acid stress on cell viability of *B. cereus* and *B. weihenstephanensis* using flow cytometry combined with fluorescent labelling. Flow cytometry is a cell analysis technology which provides multi-information about cell cultures at single cell level by multiparametric analysis of intrinsic and/or extrinsic cell parameters based on light-scattering and fluorescent signals. Suitability of differential staining of both strains with propidium iodide, for membrane evaluation, and carboxyfluorescein diacetate, for esterase activity detection, was assessed. The pH values, selected on the basis of previous screening tests, were in the boundary of growth and in the range of acid levels in gastrointestinal tract. Flow cytometry results showed it clearly discriminates between different populations, viable and damaged cells, leading to successful assessment of acid stress effect on *B. cereus* and *B. weihenstephanensis* vegetative cells viability. Feasibility of flow cytometry analysis for detection of *B. cereus* and *B. weihenstephanensis* vegetative cells was compared with viable plate count. We showed that results obtained were not comparable and classical analytical methods showed higher reliability. However, it is important to note that flow cytometry analysis provides information of individual microorganisms at real time accomplishing the need for development of valid methods that allow the integration of physiological and molecular data at the single-cell level to predict, accurately, the behavior of microorganisms.

A multivariable approach was performed in Chapter 7 to classify further *B. cereus* group species and strains. Previous studies, based on phenotypic and genetic characteristics such as the ribosomal genes sequences and the pantothenate β -alanine ligase (*panC*) gene sequence, have reported the existence of an ecotypic structure within *B. cereus* group populations. This structure, which consists of seven major phylogenetic groups (I-VII) that show specific “thermotypes” and virulence potentials, presents practical interest for food industry as a valid tool for process optimization through establishing appropriate environmental conditions along production lines depending on the risk level associated to contamination with specific

strains of this taxonomic cluster. For this purpose different *B. cereus sensu stricto* strains related to food poisoning from the Spanish Type Culture Collection (Valencia) were selected. Homology studies of *panC* gene sequence revealed that all microorganisms tested belonged to the same phylogenetic cluster, group IV, which includes mesophilic bacteria considered cytotoxic or highly cytotoxic. To assess the ability of this classification as a valuable tool to manage and classify *B. cereus* group isolates in food processing environments, heat resistance experiments were performed. Results showed variability among all the *B. cereus* strains tested.

On the basis of these results, and given that there is a need for a reliable method to successfully identify *B. cereus* group isolates, sensitive and fast techniques to differentiate these microorganisms at species or even strain level were assessed. In this sense, single nucleotide polymorphisms (SNP), one of the most common genetic variations, may be useful. Thus, from *B. cereus* genomic sequences previously reported by different authors and using bioinformatics tools, three different polymorphic regions related with the RNAr operon were identified. The feasibility of genotyping by single nucleotide polymorphisms detection via high resolution melting analysis was examined. High resolution melting analysis of amplified polymorphic 16S-23 intergenic spacer region proved to be discriminatory for *B. cereus sensu stricto* strain typing, while two other polymorphic regions within the bacterial rRNA operon allowed differentiation between *B. cereus*, *B. weihenstephanensis* and *Bacillus thuringiensis*, demonstrating its applicability for discrimination on the species and strain level within *B. cereus* group.

Finally, in Chapter 8, the involvement of the YhcYZ two-component system during growth at low temperature in various *B. cereus* group isolates was studied. As we commented previously, the adaptation of *B. cereus* group species to cold temperatures nowadays is a major concern in order to prevent spoilage and health risk for consumers. On the basis of previous transcriptomics studies of *B. cereus* ATCC

14579 grown at different temperatures, BC2216 and BC2217 genes encoding the sensor and the regulatory domain of YhcYZ two-component system were found to be overexpressed at low temperature and therefore have been related with *B. cereus* cold phenotypes. This chapter provides a preliminary approach by performing isogenic mutants of the BC2216 and BC2217 genes of *B. cereus* ATCC 14579, *B. cereus* AH 187 and *B. thuringiensis* Bt407. Phenotypic characterization revealed that at 12°C *B. cereus* ATCC 14579 BC2216-17 knockout strains showed a slight but highly reproducible impaired growth, meanwhile *B. thuringiensis* Bt407 and *B. cereus* AH 187 BC2216-17 null mutants did not show a clear cold phenotype. This heterogeneous response to BC2216-17 mutation may suggest that cold response varies among *B. cereus* group isolates. Nevertheless, more strains should be tested before obtaining a reliable conclusion. In parallel, different protocols to improve transformation efficiency of *B. cereus* group species were tested. Previously, it has been proposed that strain-specific *in vitro* methylation is a valid method to protect plasmidic DNA from bacterial DNA restriction and modification mechanisms. Unlike previous studies, *in vitro* method for strain-specific methylation of plasmid DNA did not enhance transformation of these microorganisms. However, it is noteworthy that, besides different *B. cereus* and *B. thuringiensis* mesophilic strains, two psychrotrophic strains, *Bacillus weihenstephanensis* WSBC 10688 and *Bacillus mycoides* DSM 2048, were successfully transformed.

Los microorganismos pertenecientes al grupo *Bacillus cereus* poseen importancia económica y para la salud pública. De entre todos ellos, *Bacillus cereus sensu stricto* es el agente causal de enfermedades gastrointestinales y *Bacillus weihenstephanensis* es una especie psicrótrofa capaz de crecer a temperaturas entre 4 y 7°C. Además, ambos son responsables de pérdidas económicas relevantes derivadas del deterioro de alimentos.

Debido a su habilidad para formar endosporos altamente resistentes, estas especies son capaces de persistir en ambientes de producción de alimentos, contaminando aquellos productos que han sufrido un tratamiento térmico moderado. Además, se han aislado cepas psicrótrofas y algunas mesófilas a partir de alimentos refrigerados.

En este contexto y con el fin de contribuir a la mejora de los sistemas de conservación de alimentos, lo que asegura la calidad microbiológica de los mismos, el objetivo principal de la presente tesis ha sido el estudio del impacto de factores ambientales relevantes, comúnmente denominados como barreras, en la supervivencia y el crecimiento de diferentes especies del grupo *B. cereus*. Así mismo, se han realizado estudios centrados en la evaluación de la idoneidad de métodos de identificación rápida de dichos microorganismos y de la fiabilidad de técnicas que permitan estudios de viabilidad bacteriana a nivel de células individuales.

Los alimentos vegetales listos para consumir son altamente demandados en los países industrializados ya que, debido a su conservación mediante tratamientos térmicos moderados, mantienen una alta calidad sensorial y nutricional. Sin embargo, este procesado facilita la persistencia de los microorganismos esporulados y se han aislado diversas cepas de *B. cereus* a partir de estos productos. Con el fin de contribuir a la optimización de los métodos de conservación de dichos alimentos vegetales, en el Capítulo 3 se evaluó el efecto del medio de calentamiento en la

termorresistencia de los esporos de *B. cereus* en agua destilada y en diferentes sustratos vegetales. Se observó que a altas (105°C) y bajas (90°C) temperaturas, el medio de calentamiento poseía una mayor influencia en la resistencia térmica de *B. cereus*. Este efecto parecía estar más correlacionado con el pH del medio que con la composición del alimento. Además, se analizó la presencia y longitud de los hombros de activación, así como su impacto en la evaluación del proceso de pasteurización mediante el ajuste de las curvas isotérmicas de supervivencia a dos tipos de modelos no lineales (Weibull y Geeraerd) para, posteriormente compararlo con el modelo de regresión lineal clásico. Los valores de la raíz cuadrada del error cuadrático medio (RSME) revelaron que la inactivación de esporos de *B. cereus* se caracterizó de un modo más exacto mediante los modelos de Weibull y Geeraerd. En virtud de estos resultados, proponemos que dichos modelos no lineales debieran ser implementados en la industria con el fin de prevenir tratamientos térmicos excesivamente conservativos que disminuyen la calidad de los alimentos y generan un gasto innecesario de energía.

Seguidamente, como parte concluyente del Capítulo 3, se estudió el efecto de las atmósferas modificadas en combinación con tratamientos térmicos (moderados y severos) con el fin de evaluar su impacto en la conservación de alimentos de origen vegetal. Para tal fin, además de investigar el efecto de ambientes anaeróbicos en el crecimiento de *B. cereus* en medio de cultivo y en sustrato vegetal, los microorganismos se expusieron a condiciones con presencia de oxígeno controlada mediante el uso de dos films de embalaje que presentaban diferentes tasas de transferencia de oxígeno ($35\text{cm}^3/\text{m}^2/24\text{h}$ y $10^{-3}\text{ cm}^3/\text{m}^2/24\text{h}$). Los resultados mostraron que, aunque los esporos bacterianos se adaptaban eficientemente a ambientes con presencia de oxígeno limitada, la longitud de la fase de latencia aumentaba en algunos casos. Además, se observó que *B. cereus* crecía más rápido en caldo de cultivo que en el sustrato vegetal. Finalmente, los microorganismos expuestos a menores restricciones de oxígeno ($35\text{cm}^3/\text{m}^2/24\text{h}$) mostraron un

crecimiento más rápido. Es de interés resaltar que no se encontraron diferencias en el tiempo necesario para alcanzar 10^6 CFU/mL entre las muestras de alimentos sujetas a ambientes anaeróbicos y aquellas expuestas a la tasa de transferencias de oxígeno más baja (10^{-3} cm³/m²/24h).

Por otra parte, la presencia de células vegetativas y esporos de *B. cereus* en el huevo y sus derivados es un hecho conocido. Para evitar que coagule durante su procesado, el huevo líquido es sometido a tratamientos térmicos moderados y, posteriormente, se almacena y distribuye en refrigeración. Estudios recientes han señalado a los productos tratados con calor moderado y conservados a bajas temperaturas como un posible nicho para cepas psicrótrofas y mesófilas del grupo *B. cereus* capaces de crecer por debajo de 10°C. Con el fin de contribuir a la mejora de los sistemas de conservación de estos alimentos y a la Evaluación de Riesgos asociada a su producción, en el Capítulo 4 se estudió en medio de cultivo y en huevo líquido el efecto de dos compuestos antimicrobianos (nisina y lisozima) combinados con tratamientos térmicos moderados sobre el crecimiento de células vegetativas de *B. cereus* incubadas a diferentes temperaturas. El crecimiento bacteriano se evaluó a nivel de células individuales mediante métodos basados en diluciones seriadas y mediciones automatizadas por densidad óptica. A partir de las curvas de crecimiento obtenidas se determinó la fase de latencia (λ) y la máxima tasa específica de crecimiento (μ_{max}). Así mismo, se realizaron histogramas de la fase de latencia para el estudio de su distribución de frecuencias utilizando distintos modelos. Los resultados mostraron que las distribuciones de Weibull y Normal presentaron un mejor ajuste para los datos obtenidos a 25 y 16°C. Finalmente, con el fin de generar información probabilística que permitiera comparar las curvas de crecimiento obtenidas con el modelo en alimento, se realizó una simulación de Monte Carlo capaz de predecir el tiempo necesario para alcanzar una determinada concentración microbiana. Estas predicciones resultaron ser orientativas a la hora de establecer el nivel de riesgo asociado al consumo de huevo líquido. No obstante, y como sucede con cualquier

modelo predictivo, es necesario realizar nuevos ensayos en condiciones reales antes de trasladar los resultados a la industria.

Otro factor ambiental relevante para la conservación de alimentos es la actividad de agua. En el Capítulo 5 de la presente tesis, se modelizaron los efectos de los cambios de temperatura y de la actividad de agua en la duración de la fase de latencia de *B. weihenstephanensis*, así como la dependencia de μ_{max} de las condiciones de crecimiento (temperatura y actividad de agua) a partir del parámetro h_0 (“trabajo por hacer”) inducido por la magnitud del cambio y el rigor de las nuevas condiciones ambientales. Los resultados de los experimentos llevados a cabo en medio de cultivo indicaron que cuando los cambios de temperatura sucedían cerca del límite de crecimiento, la duración de la fase de latencia aumentaba considerablemente (más de 20 días). Mientras tanto, modificaciones de los valores de actividad de agua del medio de crecimiento presentaron efectos menos significativos en el crecimiento de *B. weihenstephanensis*. Así mismo, se propuso un modelo dinámico capaz de realizar una adecuada descripción de los datos experimentales. Finalmente, la habilidad predictiva del modelo en condiciones reales (h_0 and μ_{max}) se evaluó en puré de zanahoria y en productos listos para consumir. Se observó que el modelo predecía correctamente el crecimiento de *B. weihenstephanensis* en caldo de cultivo y en salsa realizada a base de nata (sustratos ricos en nutrientes), mientras que dio lugar a una predicción errónea segura (fail safe) en zanahoria, un sustrato más pobre. A partir de estas observaciones, se sugirió que dicho modelo podía emplearse para mejorar las predicciones sobre el crecimiento de *B. weihenstephanensis* en condiciones dinámicas, y en consecuencia ser de utilidad en la implantación del Análisis de Peligros y Puntos de Control Crítico (APPCC) así como para su integración en la Evaluación de Riesgos microbianos (Risk assessment).

Por otro lado, el Capítulo 6 se ocupó del estudio del efecto de medios ácidos en la viabilidad de células vegetativas de *B. cereus* y *B. weihenstephanensis* mediante

el uso de citometría de flujo combinada con marcaje celular fluorescente. La citometría de flujo es una tecnología ampliamente conocida que permite obtener información a nivel de células individuales a partir de cultivos celulares mediante un análisis multiparamétrico basado en la dispersión de señales luminosas y fluorescentes. Para tal fin, y basándose en estudios previos, se realizó una selección de diversos valores de pH que revelaron ser capaces de alterar e incluso inhibir el crecimiento de ambas bacterias. Posteriormente, las células microbianas expuestas a pHs ácidos fueron teñidas diferencialmente con yoduro de propidio, un agente intercalante que permite evaluar daños en la membrana plasmática, y diacetato de carboxifluoresceína, un compuesto capaz de difundir hasta el interior celular y emitir fluorescencia verde indicando la existencia de actividad esterasa inespecífica en células viables. Los resultados mostraron que esta metodología era adecuada para discriminar entre poblaciones de células dañadas y viables de *B. cereus* y *B. weihenstephanensis*, permitiendo el estudio del efecto del estrés ácido en la viabilidad de células vegetativas de estas especies. Adicionalmente, se analizó en profundidad la robustez de dicha metodología realizando paralelamente recuentos en placa de células viables. En concordancia con estudios previos se observó que los resultados obtenidos mediante ambas técnicas no eran comparables y que pueden proporcionar información complementaria. Así mismo, el método clásico mostró una mayor exactitud y fiabilidad. Sin embargo, es importante tener en cuenta que la citometría de flujo permite obtener información en tiempo real y a nivel de células individuales, dando respuesta a la necesidad de desarrollar nuevos métodos válidos para la integración de datos fisiológicos y moleculares que permitan predecir de un modo más exacto el comportamiento de los microorganismos.

Seguidamente, en el Capítulo 7 se realizó un enfoque multivariable cuyo principal objetivo consistió en evaluar la idoneidad y robustez de diversos métodos para la tipificación de microorganismos pertenecientes al grupo *B. cereus*. Estudios

previos han demostrado la existencia de una estructura ecotípica en las poblaciones del grupo *B. cereus* en base a características fenotípicas y genéticas tales como la secuencia de los genes ribosomales y la secuencia del gen pantotenato β -alanina ligasa (*panC*). Dicha estructura se compone de siete grupos filogenéticos (I-VII) principales. Los microorganismos pertenecientes a cada uno de ellos poseen un termotipo y virulencia común. En consecuencia, resulta de gran interés evaluar la aplicabilidad de dicha clasificación filogenética como una herramienta válida para la optimización de procesos en la industria alimentaria. Para tal fin, se seleccionó un conjunto de cepas de *B. cereus sensu stricto* pertenecientes a la Colección Española de Cultivos Tipo (Valencia) y se realizaron estudios de homología de la secuencia del gen *panC*. Los resultados revelaron que todos los microorganismos analizados pertenecían al clúster filogenético IV, el cual incluye a bacterias mesófilas capaces de producir brotes alimentarios que cursan principalmente con diarrea. La termorresistencia de dichos microorganismos fue evaluada, y los resultados mostraron variabilidad entre las cepas.

A partir de estas observaciones y teniendo en cuenta la carencia de métodos efectivos para la correcta identificación de las cepas y especies pertenecientes al grupo *B. cereus*, se evidencia la necesidad de estudiar nuevas técnicas rápidas y robustas que permitan tipificar dichos microorganismos. En este sentido, los polimorfismos de nucleótido simple (SNP), que constituyen una de los tipos de variaciones genéticas más comunes, pueden ser de gran utilidad. Así pues, mediante herramientas informáticas y a partir de la secuencia genómica de diversas cepas de *B. cereus*, se identificaron zonas polimórficas en el operón de ARNr susceptibles de ser utilizadas para el genotipado de los microorganismos de interés. Finalmente, se evaluó la idoneidad de la detección de dichos SNPs mediante análisis de curvas de fusión de alta resolución (HRM). El análisis de HRM de los productos de amplificación de las zonas polimórficas localizadas en la región intergénica 16S-23S ARNr resultó ser capaz de discriminar entre cepas de *B. cereus sensu stricto*.

Mientras tanto, el análisis de los productos de amplificación de otras dos regiones polimórficas localizadas en el operon de ARNr permitieron la diferenciación entre *B. cereus*, *B. weihenstephanensis* and *Bacillus thuringiensis*, demostrando la aplicabilidad de esta técnica para la discriminación entre especies y cepas dentro del grupo *B. cereus*.

Como ya hemos mencionado con anterioridad en diversos capítulos de esta tesis, la adaptación al frío de los microorganismos pertenecientes al grupo *B. cereus* es de gran interés. Recientemente, análisis transcriptómicos de células de *B. cereus* ATCC 14579 sometidas a diferentes temperaturas han permitido identificar genes involucrados en la adaptación de esta bacteria al frío. Los resultados indicaron que el sistema de dos componentes YhcYZ era sobreexpresado a bajas temperaturas. Dicho sistema, compuesto por una proteína sensora (YhcY) y un regulador de la transcripción (YhcZ), está codificado por los genes BC2216 y BC2217, respectivamente. Por ello, finalmente, en el Capítulo 8 se realizó un estudio preliminar de la participación del sistema de dos componentes YhcYZ en la capacidad de crecimiento a bajas temperaturas de varias cepas del grupo *B. cereus*. Así pues, se llevó a cabo la construcción de mutantes isogénicos para los genes BC2216 y BC2217 de *B. cereus* ATCC 14579, *B. cereus* AH 187 y *B. thuringiensis* Bt407. Su caracterización fenotípica reveló que a 12°C los mutantes de *B. cereus* ATCC 14579 mostraban un leve retraso en su crecimiento respecto a la cepa salvaje, que resultó ser altamente reproducible, mientras que los mutantes BC2216-17 de *B. thuringiensis* Bt407 y *B. cereus* AH 187 no presentaron un fenotipo claramente afectado por las bajas temperaturas. Esta heterogénea respuesta a la mutación de los genes BC2216 y BC2217 sugiere que la respuesta al frío varía entre las cepas pertenecientes al grupo *B. cereus*. En cualquier caso, es necesario estudiar un número mayor de cepas para obtener resultados concluyentes. Además y en paralelo, en el Capítulo 8 se llevó a cabo la evaluación de distintos protocolos destinados a mejorar la eficiencia de la transformación de especies pertenecientes al grupo *B. cereus*. La

metilación *in vitro* específica de cepa ha demostrado proteger el DNA plasmídico de la acción de los mecanismos bacterianos de restricción y modificación del DNA. A diferencia de estudios previos, los métodos de metilación *in vitro* de ADN plasmídico no mostraron potenciar las tasas de transformación de estos microorganismos. Sin embargo, es interesante destacar que, aparte de diversas cepas mesofílicas (*B. cereus* y *B. thuringiensis*), dos cepas psicrófilas, *Bacillus weihenstephanensis* WSBC 10688 y *Bacillus mycoides* DSM 2048, pudieron ser transformadas exitosamente.

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CHAPTER 1

Aim and impact of the thesis

Abstract

Bacteria are present in almost all foodstuffs determining their safety and shelf life. Depending on several factors such as processing, preservation techniques and storage conditions different microorganisms can be present in food. Among all of them, *Bacillus cereus* group species, which may cause spoilage and gastrointestinal diseases, are commonly isolated from different products due to their metabolic diversity and their ability to form highly resistant endospores. Members of this group have been reported to contaminate a large number of sources including raw, manufactured, refrigerated and dehydrated foods as well as from hot dairy equipment and industrial surfaces. In recent years, the development and implementation of consumer driven mild preservation techniques have become more popular in order to minimize the damage of the sensorial and nutritional properties of the food. However, this may further enhance the spore survival, and thus the persistence of spore formers in food production environments. Besides, recent studies pointed to mildly treated products stored at low temperature as an appropriate niche for *B. cereus* psychrotrophic strains and mesophilic strains able to grow below 10°C. On this ground, and with the major aim to contribute to improve food preservation systems that ensure microbiological quality and avoid economical losses derived from spoilage, the present thesis deals with the study of the impact of relevant environmental factors, commonly referred as hurdles, on the survival and grow of different species of *B. cereus* group, as well as with the assessment of feasibility of novel methods for genotyping and for viability studies at single cell level.

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1.1. Relevance and appropriateness of the research

Microorganisms belonging to *B. cereus* group show a great metabolic diversity and are able to form adhesive endospores highly resistant to heat, dehydration, chemicals and other environmental stresses. In consequence, these bacteria can colonize a wide variety of habitats with growth temperatures ranging from 4° to 50°C (Guinebretière et al., 2008).

Due to their physiological and ecological characteristics, *B. cereus* group strains are commonly present in food production environments causing food-borne diseases and food spoilage (Kotiranta et al., 2000; Stenfors Arnesen et al., 2008; Jan et al., 2011). Notably, two species of the group are of main concern in this thesis due to their particular relevance in the food microbiology field. On one hand, *Bacillus cereus sensu stricto*, which has been identified as causative agent of two foodborne diseases, the emetic and the diarrheal syndromes (Kotiranta et al., 2000), and on the other hand, *Bacillus weihenstephanensis*, a common and ubiquitous spoilage microorganism characterized by its ability to grow below 7°C but not at 43°C and whose pathogenic potential remains uncertain (Lechner et al., 1998; Stenfors Arnesen et al., 2008).

In the last decades, in order to overcome changing needs and consumer preferences, ready to eat foods as well as those processed or cooked chilled or refrigerated processed foods of extended durability (REPFEDs) have been incorporated to our daily life (Nissen et al., 2002) because, despite to be safe, these products show high organoleptic and nutritional quality. A rapid rise in the demand of these products has been observed in industrialized countries. These modern trends in food consumption have been linked to social changes such as the consumers' perception of food quality and nutrition (Rajkovic et al., 2010).

To minimize degradation of the sensorial, textural and nutritional quality, REPFEDs undergo relatively mild heat processing (typically pasteurization) (Samapundo et al., 2011). These treatments can lead to the persistence of bacterial spores in food environments due to incomplete inactivation and sub-lethal damage of the target microorganisms (Kotiranta et al., 2000). In addition, “modern large-scale food production technology that relies on refrigeration as a means of conservation, has

selected bacteria that are not very competitive, but that can survive heat treatment and also grow at low temperatures” (Stenfors Arnesen et al., 2008).

The adaptive faculty of *B. cereus* group together with the creation of new ecologic niches derived from changing environment should be taken into account since new evolutionary lines of these species might develop and contribute to the survival and pathogenicity of these microorganisms. In fact, changing lifestyles and eating habits have been associated to the increasing number of foodborne illness caused by *B. cereus* in industrial world (Ehling-Schulz et al., 2004). In the reports of food-borne outbreaks in the European Union (EU) of 2007 and 2009 (EFSA, 2009; EFSA, 2011), the European Food Safety Authority (EFSA) and European Centre for Disease Control and Prevention (ECDC) indicated that during this period the total number of outbreaks and hospitalizations caused by bacterial toxins produced by *Bacillus* spp had notably increased.

In addition to health risks, the deterioration of food due to contamination by microorganisms belonging to the *B. cereus* group shortens the life of the products and generates economic losses (Te Giffel, 2001).

In this context it is a major objective for the food industry, and therefore for this thesis, to achieve a deep knowledge of relevant environmental factors affecting *B. cereus* group growth, either alone or in combination, in order to contribute to the development and improvement of preservation systems. This will allow to cope with consumer demands without compromising the microbiological safety and quality of the food.

Thus, the effect of temperature, oxygen limited environment, natural antimicrobial compounds, water activity and pH on *B. cereus* group stress response was investigated. Likewise, mechanistic studies were performed to determine the role of the two-component signal transduction systems on adaptation of these microorganisms to low temperatures.

Besides, the integration of physiological data through mathematical models able to be implemented in Hazard Analysis and Critical Control Points (HACCP) and risk

assessment analysis may be of benefit for scientists, competent authorities and industry since it will allow to better estimate bacterial behavior in food environments and implement it in food safety policies.

An essential part of this thesis was to contribute to the development of novel detection methods for simple, fast, and robust identification of *B. cereus* group species, avoiding classical culture techniques which are tedious and time consuming. For identification purposes it is important to note that *B. cereus* group typing at strain and species level remains being a challenge due to their high genetic similarity (Schmidt et al., 2011). To address this point highly sensitive techniques able to detect significant genomic polymorphisms for typing might be used. For this purpose, reliability assessment of single nucleotide polymorphisms (SNP) genotyping via high-resolution melting analysis (HRM), a novel technique successfully used for specific species identification of clinically relevant bacterial species (Yang et al., 2009) appears to be interesting for implementation in food microbiology.

Finally, flow cytometry analysis (FCM) was considered in this work as a technique of great interest to accomplish physiological studies of *B. cereus* group species. As major advantages, FCM provides real time acquisition of multiparametric data from thousands of individual cells within a sample (Veal et al., 2000). On this basis, this technique could address the need of development of valid methods that allow the integration of the physiological and molecular data at the single-cell level for predictive modelling-based approaches of practical use for the food industry (Hornstra et al., 2009; Ter Beek and Brul, 2010).

1.2. Objectives

The objective of this thesis is to deepen in the understanding of relevant environmental factors that affect the growth of microorganisms belonging to *B. cereus* group, either alone or in combination, in order to contribute to the development of new preservation strategies that ensure an adequate microbiological quality of foods and to develop predictive models that can be useful for HACCP programs and for microbial risk assessment purposes. Likewise, another major goal of this work was to evaluate novel methods for rapid identification of these microorganisms as well as to test reliable

techniques for viability studies at single cell level so that subpopulations of, for example, high heat resistant or psychrotrophic spores of *B. cereus* could be identified. From a global perspective, we aim to contribute to the improvement of food processing with the purpose of preventing economic losses and health risks caused by members of the *B. cereus* group. To this end, we have performed a multidisciplinary study that includes the following partial objectives:

- 1) To contribute to the development of novel preservation methods for ready to eat vegetable foods:
 - By characterizing *B. cereus* spores heat resistance in sterile distilled water and vegetable substrate and the influence of heating medium on it.
 - Studying the presence and extension of shoulders and their impact on evaluation of sterilization processes.
 - By assessing modified atmospheres in combination with mild and severe heat treatments for preservation of foods based on vegetables.
- 2) To develop novel preservation methods for liquid egg employing stochastic models useful to predict the risk of foodborne illness associated to this food:
 - By investigating the effect of lysozyme and nisin combined with mild heat treatment at different incubation temperatures and analyzing this effect on the distributions of individual lag times.
 - Performing a Monte Carlo simulation to estimate the time to a certain growth of heat treated *B. cereus* cells in liquid egg with antimicrobial compounds added.
- 3) To improve predictions of growth of *B. weihenstephanensis* in dynamic conditions:
 - By modelling the effects of temperature and water activity (a_w) downshifts on the lag time of *B. weihenstephanensis* and the dependency of specific growth rate (μ_{max}) on the growth conditions (temperature and a_w).

- 4) To test FCM feasibility for viability studies of acid stressed *B. cereus* and *B. weihenstephanensis* cells at single level:
 - By testing differential fluorescent labelling to discriminate vegetative cell viability.
 - By comparing classical techniques, viable plate counts (VPC), and FCM analysis for evaluation of acid stress effects on vegetative cells.

- 5) To contribute to a reliability assessment of major phylogenetic groups (I-VII) affiliation for practical applications in heat processing:
 - By *panC* gene sequence affiliation of strains tested.
 - By characterizing heat resistance in sterile distilled water of *B. cereus* spores affiliated to group IV.

- 6) To identify *B. cereus* group isolates at species and strain level:
 - Through identification of highly polymorphic genomic regions and subsequent primer design.
 - By assessing feasibility of genotyping by SNPs detection via HRM analysis.

- 7) To characterize the mechanistic basis of *B. cereus* group cold adaptation:
 - By testing *in vitro* methods feasibility for strain specific methylation of plasmid DNA to improve transformation efficiency of *B. cereus* group species.
 - By studying the effect of the deletion of BC2216 and BC2217 genes on the ability of various *B. cereus* group strains to grow at low temperatures

1.3. Thesis outline

General principles about *B. cereus* group, bacterial stress response, predictive microbiology, food preservation, bacterial identification and flow cytometry are described in Chapter 2, the introduction of this thesis.

The experimental results obtained during the development of this work are organized in Chapters 3, 4, 5, 6, 7 and 8 of this document. Each is written in the form of a scientific paper and consists of the following sections: introduction, materials and methods, and results and discussion.

Additionally, Chapter 9 includes the main scientific conclusions of the work and Chapter 10 lists the references used in this thesis.

The background of the page is a light blue image showing a rack of test tubes. Some tubes contain a blue liquid, while others are empty. The tubes are arranged in rows and columns, creating a sense of depth. On the right side of the page, there is a vertical blue bar with large white numbers 10, 9, 8, 7, 6, 5, 4, 3, 2, and 1 from top to bottom.

CHAPTER 2

Introduction

Abstract

To cover relevant background aspects for the work present in this thesis, this introduction deals with *Bacillus cereus* group taxonomy, ecology, pathogenicity and epidemiology, as well as with its importance in food processing environments. On the other hand, main characteristics of general stress response, bacterial SOS response and two component signal systems are detailed in this chapter to understand, from a molecular point of view, *B. cereus* group behavior during food processing, preservation and storage. Likewise, a general description of bacterial life cycle is given here to understand predictive microbiology basis. Besides, a brief description of predictive microbiology development and its importance in risk assessment and HACCP are discussed. As an essential part of this introduction, hurdle technology, gamma hypothesis and relevant factors for food preservation are detailed. Finally, we summarized importance, requirements and methods for *B. cereus* group identification and revised flow cytometry principles and applications.

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2.1. *Bacillus cereus* group

The *Bacillus cereus* group, also known as *Bacillus cereus sensu lato*, is a widely used term describing a genetically highly homogeneous subdivision of the genus *Bacillus* comprising six recognized species: *Bacillus cereus sensu stricto*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis* and *Bacillus anthracis*.

These species range high in economic, medical and biodefense importance. Spore and crystal toxins preparations from *B. thuringiensis* are used as biological insecticides to control a variety of foliage lepidopterans such as moths and budworms, dipterans such as mosquitoes and blackflies and coleopterans such as Colorado potato beetles (Bourque et al., 1995). *B. anthracis* is the causative agent of the fatal animal and human disease anthrax, whose use as biological weapon has been discussed in the latest years (Jernigan et al., 2002; Read et al., 2003). *B. cereus sensu stricto* strains are commonly present in food causing spoilage and food-borne diseases, generally mild and of short duration. This microorganism is also an opportunistic pathogen able to contaminate mammalian tissues resulting in systemic and local infections (Kotiranta et al., 2000) and is used as animal probiotics (Klein, 2011) or as a plant symbiont (Stabb et al., 1994). *B. mycoides*, *B. pseudomycoides* and the psychrotolerant species *B. weihenstephanensis*, are common and ubiquitous soil organisms whose pathogenic potential remains uncertain (Guinebretière et al., 2008). *B. mycoides*, *B. pseudomycoides* are differentiated from *B. cereus sensu stricto* by rhizoidal colony shape and fatty acid composition (Stenfors Arnesen et al., 2008), meanwhile *B. weihenstephanensis* is characterized by its ability to grow below 7°C but not at 43°C (Lechner et al., 1998).

Due to their metabolic diversity and their ability to form endospores highly resistant to heat, chemical and other environmental stresses (Carlin et al., 2010), these ubiquitous microorganisms are able to colonize a wide variety of habitats in a very broad thermal range of growth temperature ranging from 4°C to 50°C (Guinebretière et al., 2008). In consequence, these species have important implications in Food Industry leading to spoilage on raw and processed foods as well as human pathogens involved in food-borne outbreaks.

In recent years, the development and implementation of consumer driven mild preservation techniques have become more popular in order to minimize the damage of the organoleptic and nutritional properties of the food. However, this may further enhance the spore survival, and thus the persistence of spore formers in food production environments. In addition, mesophilic strains subjected to suboptimal refrigeration conditions as well as psychrotrophic strains present in chilled food products can grow at refrigerated temperatures and cause food-borne outbreaks. It is extensively reported that strains of *B. cereus* group have been isolated from raw and processed foodstuff, food stored at refrigerator temperatures, dehydrated foods, hot dairy equipment, industrial surfaces, infected humans, dead animals or insect bodies and soil (Becker et al., 1994; Dufrenne et al., 1995; Helgason et al., 1998; Lechner et al., 1998; Margulis et al., 1998; Kotiranta et al., 2000; Svensson et al., 2000; Guinebretière and Nguyen-The, 2003).

2.1.1. Taxonomy

Previous studies have reported high similarity in *B. cereus sensu lato* DNA sequences, up to 95% of the basic chromosomal genes composition was identical (Periago et al., 2002b). *B. anthracis*, *B. cereus* and *B. thuringiensis* genome sequences are closely related in gene content, and their 16S rRNA gene sequences share more than 99% similarity (Ash et al., 1991; Helgason et al., 2000b).

Phylogenetic studies based on chromosomal markers showed the absence of taxonomic basis to assign to *B. cereus* and *B. thuringiensis* the separate species status, and considered *B. anthracis* as a clone of *B. cereus* (Stenfors Arnesen et al., 2008). However, the organisms belonging to this group show different functional properties which are thought to be caused by genes carried on plasmids or by altered gene expression among strains (Jensen et al., 2003; Read et al., 2003; Helgason et al., 2000b, 2004). In fact, *B. thuringiensis* is defined by the presence of *cry* genes encoding δ -endotoxins located in plasmids, *B. anthracis* contains two large plasmids (pXO1 and pXO2) encoding the two main virulence factors of this species, and *B. cereus* also presents a large plasmid containing *ces* gene which encodes the emetic toxin (Hotton et al., 2005; Leppla, 2006; Stenfors Arnesen et al., 2008).

However, although virulence plasmids pXO1 and pXO2 have significant impact on the pathogenic phenotype of *B. anthracis* and represent 52% of the unique coding capacity found in the genome of this species, this plasmid gene content comprises only 176 genes, representing a small fraction of the total coding capacity of the *B. anthracis* genome (Schmidt et al., 2011). To attempt understand the genomic similarity of these phenotypically diverse microbes, Schmidt et al. (2011) suggested that “subtler genome alterations within the *B. cereus* group isolates, such as gene duplication, divergence and point mutations probably have contributed as much or more than horizontal gene transfer and genome reduction”.

It is important to note that the most evolutionarily flexible portions of the bacterial genome are regulatory sequences and transcriptional networks (Huynen & Bork, 1998; Lozada-Chavez et al., 2006, 2008). In this context, Schmidt et al. (2011) reported that major differences between *B. cereus* group microorganisms reside in the regulation of gene expression rather than gene content. This divergence is evidenced by the fact that *papR* locus, which encodes a quorum-sensing signal (a secreted peptide) that is internalized and binds to PlcR, a transcriptional activator that controls gene expression and is important for *B. cereus* group virulence, presents point mutations that differentiate group members from one another (Mignot et al., 2001; Slamti & Lereclus, 2005).

In addition, Martin et al. (2010) reported that genome reduction has played a moderate role in divergence of the *B. cereus* group, likely being responsible for the reduced genome size of *Bacillus cereus* subsp. *cytotoxis* NVH391-98, a new cluster involving strains able to grow at temperatures 6-8°C higher than mesophilic *B. cereus* strains (Guinebretière et al., 2008). These microorganisms are considered as a new species informally termed *Bacillus cytotoxicus* (Lapidus et al., 2008). Few data are available from these rarely isolated microorganisms whose ecological niche remains uncertain. The first isolate was NVH391/98 strain, causative agent of fatal cases of necrotic diarrhea (Lund et al., 2000). Subsequent studies have revealed that these strains have the σ^B RNA polymerase subunit absent (Lapidus et al., 2008).

Finally, it is noteworthy that gene-duplication and gene-loss process are the main responsible ones of the wide heterogeneity of the sigma factor content in *B. cereus*

group species (Schmidt et al., 2011). Indeed, it has been suggested that, as consequence of sigma factor expansion and divergence among sigma factor regulons, these microorganisms display a wide variety of phenotypic characteristics that allow to adaptation to different ecological niches (Anderson et al., 2005).

While both the gene content and extent of divergence suggest that *B. cereus* subsp. cytotoxis and perhaps *B. weihenstephanensis* may warrant specific recognition, the lack of correlation between phylogenetic studies and virulence factor which distinguish *B. anthracis*, *B. cereus* and *B. thuringiensis* species generates a discussion about taxonomic classification of these *B. cereus* group strains, but also concerning public health (Stenfors Arnesen et al., 2008). Recently, Schmidt et al. (2011) have reported phylogenetic assessment consistent with other suggestions which point that *B. cereus* group exhibits sufficiently high genetic similarity that these organisms could be members of a single species.

2.1.2. Foodborne disease and nongastrointestinal infections

Besides *B. anthracis*, which is the causative agent of anthrax disease, microorganisms belonging to this group can induce two types of foodborne syndromes, emetic and diarrheal. Both gastrointestinal diseases are normally mild, symptoms do last not more than 24 hours and are very similar to others food-borne illnesses, because of these the true incidence of this disease may be underestimated (Table 1). In contrast, non-gastrointestinal disease has been reported as local and systemic infections frequently associated to immunologically compromised patients (Kotiranta et al., 2000).

Among all *B. cereus* members, *B. cereus sensu stricto* is the main causative agent of gastrointestinal infection through the production of a range of virulence factors. Generally, emetic and diarrheal processes are mild and shelf-limiting, but few severe or even fatal cases have been described, specially related to the emetic type (Table 1). Besides, *B. cereus* is responsible of local and system infection, including keratitis, endophthalmitis, panophthalmitis, periodontitis, meningitis, pneumonia, urinary tract infections and fatal fulminate liver failure (Beecher et al., 2000; Kotiranta et al., 2000) which are specially associated to immunologically compromised patients, neonates, drug addicts and traumatic or surgical wounds and catheters (Drobniewski, 1993; Kotiranta et al., 2000; Hilliard et al., 2003;).

Table 1. Characteristics of the two types of *B. cereus* foodborne disease (Stenfors Arnesen et al., 2008).

Characteristics	Diarrhoeal disease	Emetic disease
Type of toxin	Protein; enterotoxin(s): Hbl, Nhe, CytK	Cyclic peptide; emetic toxin (cereulide)
Location of toxin production	In the small intestine of the host	Preformed in foods
Infective dose	10 ⁵ -10 ⁸ CFU (total) The total number required is lower for spores compared to vegetative cells*	10 ⁵ -10 ⁸ cells /g is often found in implicated foods, but live cells are not required for intoxication. Cereulide: 8-10 µg kg ⁻¹ body weight (animal models) [†]
Incubation time	8-16 h (occasionally > 24 h)	0.5-6 h
Duration of illness	12-24 h (occasionally several days)	6-24 h
Symptoms	Abdominal pain, watery diarrhoea and occasionally nausea. Lethality has occurred [‡]	Nausea, vomiting and malaise. A few lethal cases (possibly due to liver damage) [§]
Foods most frequently implicated	Proteinaceous foods; meat products, soups, vegetables, puddings, sauces, milk and milk products	Starch-rich foods; fried and cooked rice, pasta, pastry and noodles

*Clavel et al. (2004); †Agata et al. (1994, 1995); ‡Shinagawa et al. (1995); ‡Lund et al. (2000); §Mahler et al. (1997); Dierick et al.(2005)

On the other hand, *B. thuringiensis* has been reported to cause food poisoning outbreaks similar to those caused by *B. cereus* (Jackson et al., 1995), and also has been responsible of non-gastrointestinal infections affecting cornea, burn wounds and lungs (Samples & Buettner, 1983; Damgaard et al., 1997; Ghelardi et al., 2007).

2.1.3. Pathogenicity factors

The pathogenic potential of a particular *B. cereus* group strains appear to be highly determined by the level of toxin gene expression, which is temporally controlled in response to cell density, nutrient availability, metabolic state of the cell and environmental conditions such as pH, temperature, glucose concentration and oxygen tension in addition to being coordinately regulated with motility genes.

2.1.3.1. Cytotoxins associated to diarrheal syndrome

The diarrheal type is currently considered to be caused by a single protein, cytotoxin K (CytK1 or CytK2) and by two enterotoxin complexes, haemolysin BL (Hbl) and non-haemolytic enterotoxin (Nhe) (Beecher and MacMillan, 1991; Lund and Granum, 1996; Lund et al., 2000). These heat labile cytotoxins are produced during vegetative growth of the microorganism in the small intestine. The highest specific production of Nhe occurs early during exponential growth, while Hbl was produced later, early in the stationary phase of growth (Zigha et al., 2006).

Besides, others virulence factors have been also investigated including cytotoxins, haemolysins and degradative enzymes such as cereolysin O (Kreft et al., 1983), haemolysin II (HlyII) (Baida et al., 1999), haemolysin III (Baida & Kuzmin, 1995), InhA2 (Fedhila et al., 2003), phosphatidylinositol and phosphatidylcholine specific phospholipases C (PI-PCL and PC-PCL) (Kuppe et al., 1989), sphingomyelinase (SMase) and enterotoxin FM (EntFM) (Granum, 2002).

Hbl and Nhe are related three-component toxins, while the single-component CytK belongs to the family of β -barrel pore-forming toxins. Proteins L2, L1 and B compose Hbl and are encoded by hblC, hblD and hblA genes, respectively, which are cotranscribed from the *hblCDAB* operon (Beecher & MacMillan, 1991; Heinrichs et al.,

1993; Ryan et al., 1997; Lindbäck et al., 1999). Two different types of this chromosomally encoded operon with a conserved genomic location appear to exist in *B. cereus* group members (Stenfors Arnesen et al., 2008). On the other hand, *Nhe* consists of the proteins *NheA*, *NheB* and *NheC*, encoded by the *nheABC* operon (Granum et al., 1999). A single copy of this operon is present in all *B. cereus* group strains. Exceptionally, *B. weihenstephanensis* KBAB4 contains two copies, one chromosomally encoded and the other located in the megaplasmid pBWB401 (Lapidus et al., 2008). Besides, *B. cereus* NVH 391/98 *nhe* operon shows low identity towards *nhe* operons in *B. cereus* group (Lapidus et al., 2008). *CytK* could have two different forms either *CytK-1* or *CytK-2*, according to the *B. cereus* group strain, which are encoded by *cytK-1* and *cytK-2* genes (Fagerlund et al., 2004).

The *nhe* operon has been found to be present in all known *B. cereus* group strains to the date, suggesting that could be also involved in cell viability roles (Stenfors Arnesen et al., 2008). Otherwise, *hbl* and *cytK* genes are present in less than 50% of randomly sampled strains (Ehling-Schulz et al., 2005a, 2006a; Moravek et al., 2006). Notably, this percentage increases in food associated isolates (Guinebretière et al., 2002; Swiecicka et al., 2006). Similarities of *nhe* and *hbl* genes suggest that they have originated from a common gene (Stenfors Arnesen et al., 2008).

In *B. cereus* group isolates, the transcriptional regulator Phospholipase C Regulator (*PlcR*) controls most known virulence factors such as several enterotoxins (*Nhe*, *Hbl* and *CytK*), haemolysins, phospholipases and proteases by integrating at least two classes of signals: cell growth state through *Spo0A* and self cell density through *PapR* (Gohar et al., 2008). *plcR* transcription starts short time before the onset of the stationary phase and reaches a plateau two hours later (Lereclus et al., 1996).

It has been shown that *PlcR* transcription is autoinduced by its binding to DNA on a specific sequence called the 'PlcR box' meanwhile is repressed by the sporulation factor *Spo0A* (Lereclus et al., 1996, 2000). Furthermore, to be active, *PlcR* needs the pentapeptide *PapR* which is expressed as a propeptide under the control of *PlcR*, transformed in active in the extracellular medium and reintroduced in the cell by the oligopeptide permease system *OppABCDF* (Gohar et al., 2008). Together, *PlcR*, *OppABCDF* and *PapR* function as a quorum-sensing system which is present in all *B.*

cereus group organisms and harbors mutations points that result in four distinct phylogenetic groups of the PlcR peptide which exclusively bind to its cognate PlcR sensor indicating a specific activation of *plcR* transcription within *B. cereus* group strains (Slamti and Lereclus, 2005).

Phylogenetic analyses based on PlcR peptide sequences clearly show that strains belonging to a given species (e.g., *B. thuringiensis*) may be more related to strains belonging to another species (e.g., *B. cereus* or *B. weihenstephanensis*) (Slamti and Lereclus, 2005). In addition, *B. anthracis* and a low percentage of *B. cereus* strains, shown a unique nonsense mutation in PlcR that inactivates the quorum-sensing function entirely, what appears to be necessary for full virulence of these microorganisms.

Besides to PlcR regulation, expression of *Hbl* and *Nhe* is also regulated by the redox-sensitive two-component regulatory system ResDE and the redox regulator Fnr (Duport et al., 2006; Esbelin et al., 2008, 2009).

Additionally, *B. cereus* synthesized HlyII, a single-component protein toxin member of the family of β -barrel poreforming toxin (Baida et al., 1999) with independent expression of PlcR, shows haemolytic and cytotoxic activity towards human cell lines (Andreeva et al., 2006) but it has never been involved in diarrheal syndrome.

Hbl and Nhe are spore-forming toxins which produce diarrhea by disrupting the integrity of the plasma membrane of epithelial cells during growth in the small intestine. However, it remains uncertain how the three components in each complex interact or to what extent they oligomerize in the process of forming a transmembrane pore. For both Nhe and Hbl, all three components are necessary for maximal biological activity (Beecher & MacMillan, 1991; Beecher et al., 1995; Lindbäck et al., 2004). To date, no host cell receptor for Nhe or Hbl has been identified. Besides, Cytk and HlyII are also involved in transmembrane pore formation by association into oligomeric prepores at the target cell surface (Bhakdi and Tranum-Jensen, 1991). Notably, Nhe, Hbl and Cytk have haemolytic activity and Hbl and Cytk necrotic activity too (Beecher and Wong, 1994, 2000; Lund et al., 2000; Fagerlund et al., 2008).

To cause disease the most important toxin may vary between *B. cereus* strains. However, meanwhile different authors have suggested Nhe as the major cytotoxic membrane-damaging factor secreted by most *B. cereus* strains, others have proposed that foodborne diseases are caused by multiple toxins that act together, probably in a strain-dependent manner, or even synergistically (Stenfors Arnesen et al., 2008).

2.1.3.2. Emetic toxin

The toxin termed cereulide is the causative agent of the emetic disease. This cyclic dodecadepsipeptide is synthesized by a nonribosomal peptide synthetases (NRPSs) encoded by the cereulide synthetase (*ces*) gene cluster which is present in clonal lineage of *B. cereus* strains that carry a pXO1-like megaplasmid, termed pBCE (Ehling-Schulz et al., 2005b, 2006b). The *ces* gene cluster is polycistronically transcribed from a central promoter in a strict temporally regulated way (Frenzel et al., 2011). Notably, Thorsen et al. (2006) reported that psychrotolerant species *B. weihenstephanensis* was able to produce cereulide at 8°C.

This emetic toxin is synthesized during vegetative growth, at the end of logarithmic phase, usually in improperly refrigerated foods and reheating foods that have been stored at room temperature after a first heating. Production levels of cereulide vary among strains and differences in *ces* genes regulation have been pointed as responsible of this phenomenon (Häggblom et al., 2002; Ehling-Schulz et al., 2005b; Rajkovic et al., 2006b). Likewise, oxygen levels, pH, temperature, the presence of specific amino acids and food composition affect toxin production (Stenfors Arnesen et al., 2008).

Upon ingestion, cereulide moves through gastrointestinal tract until it reaches small intestine without being destroyed by gastric acid and proteolytic enzymes due to its resistant nature to acid environments, proteolysis and heat (Johnson, 1984; Agata et al., 1994; Shinagawa et al., 1996). As has been shown in *Suncus murinus*, an animal model, when toxin reaches duodenum binds to the 5-HT₃ receptor and stimulates the vagus nerve causing vomiting (Agata et al., 1995).

In addition, this toxin can inhibit mitochondrial activity by acting as a cation ionopore, like valinomycin (Mikkola et al., 1999). Likewise, has been reported that cereulide is responsible of cellular damage (Shinagawa et al., 1996) and inhibit human natural killer cells of the immune system (Paananen et al., 2002).

2.1.4. Ecological diversification of *B. cereus* group

Temperature is one of the most important environmental factors to which microorganisms have to respond. Guinebretière et al. (2008) have reported within *B. cereus sensu lato* distinct genetic groups which possess their own growth ranges specificities showing an “ecotypic” structure of populations (Table 2 and 3). Seven major phylogenetic groups were identified (I to VII) by using phenotypic and genetic criteria including AFLP patterns, partial *panC* sequence, ribosomal gene sequences as well as “psychrotolerant” DNA sequences signatures such as *rrs* signature (Pruss et al., 1999) and *cspA* signature (Francis et al., 1998).

Every group (I to VII) shows a specific “thermotype” and different virulence potentials. Phylogenetic branching order of the differential thermal lineages indicates an evolutionary transition towards psychrotolerance and show how *B. cereus* group evolution seems to be strongly determined by ecological adaptations (Guinebretière et al., 2008). Notably, temperature tolerance limits may act as an important mechanism by which these microorganisms have adapted to new or different environments. In fact, relative abundance of the “psychrotolerant” *rrs* signature and the *cspA* signature in *B. cereus* group strains (Table 3) reflect different strategies set up by all groups to adapt to a wide variety of ecological niches with diverse thermal conditions (Carlin et al., 2010). This rich adaptive ability supports the development of new evolutionary lines and long-term persistence of *B. cereus* group (Guinebretière et al., 2008). Nowadays, global warming may be linked to changes in *B. cereus sensu lato* strains ecology towards a thermotolerant status as indicate the fact that many strains isolated from food poisoning cases have a tendency rather to belong to more thermotolerant phylogenetic groups (groups III, IV and VII) (Carlin et al., 2010). Notably, group VII illustrates the relation between ability to grow at elevated temperatures and the pathogenic potential (Carlin et al., 2010).

Recently, Guinebretière et al. (2010) have reported that *nhe* genes are constant part of the *B. cereus* group strains, whilst *cytK-1*, *cytK-2* and *hbl* genes distribution was disrupted. Percentage of strains carrying *hbl* gene varied from 40% to 97% between phylogenetic groups I, II, IV, V and VI, and this operon was seldom carried by strains of phylogenetic group III. *cytK-1* form was specific for group VII, and *cytK-2* was particularly frequent in mesophilic groups III and IV, except for *B. anthracis* strains tested, and was rare or absent in the psychrotolerant or moderately psychrotolerant groups (II, V and VI). To get a global measure of the activity of *B. cereus* group enterotoxins cytotoxic activity assays were performed in Caco2 cells (Guinebretière et al., 2010). Results revealed that food poisoning risk should be highest for group III which include emetic strains previously related with fatal foodborne cases (Mahler et al., 1997; Dierick et al., 2005) and the dangerous pathogen *B. anthracis*. This food poisoning risk is high for groups VII, IV and II, decreased with group V and is very low for group VI isolates.

Augustin (2011) has stated that three different clusters of *B. cereus* group isolates linked to food poisoning can be defined:

- i) diarrheal, psychrotrophic, heat sensitive strains belonging to the genetic group II,
- ii) diarrhoeal, mesophilic, heat resistant strains belonging to the genetic groups III, IV, V, and VII,
- iii) emetic, mesophilic, heat resistant strains belonging to the genetic group III.

Table 2. Characteristic of major phylogenetic groups (I-VII)

Phylogenetic group	Species	Growth temperature range (°C)	Thermal niche	Heat resistance of spores ^a	Temperature range compatible with spore germination (°C) ^c
I	<i>B. pseudomycoides</i>	10-43	Mesophilic	?	?
II	<i>B. cereus</i> II <i>B. thuringiensis</i> II	7-40	25% intermediate to mesophilic 75% of psychrotolerant	+ +	<42
III	<i>B. cereus</i> III (emetic strains included) <i>B. thuringiensis</i> III <i>B. anthracis</i>	15-45	Mesophilic	+ + +	<45
IV	<i>B. cereus</i> IV <i>B. thuringiensis</i> IV	10-45	Mesophilic	+ +	<45
V	<i>B. cereus</i> V <i>B. thuringiensis</i> V	8-40	Intermediate	+ +	<45
VI	<i>B. weihenstephanensis</i> <i>B. mycoides</i> <i>B. thuringiensis</i> VI	5-37	Psychrotolerant	+	<37
VII	<i>B. cytotoxicus</i> ^b	20-50	Thermotolerant	+ + +	<45

a

Ranking obtained from Carlin et al., 2010 ^bNovel species *B. cytotoxicus* (Fagerlund et al., 2007; Lapidus et al., 2008) ^c Ranges stated in Afchain et al., 2008.

Table 3. Phenotypic features for strain identification according with phylogenetic classification established in Guinebretière et al. (2008)

Phylogenetic group	Species	Phenotypic features for strain identification
I	<i>B. pseudomycoides</i>	Rhizoidal colony ^a
II	<i>B. cereus</i> II <i>B. thuringiensis</i> II	Psychrotolerant strains “psychrotolerant” <i>cspA</i> signature absent Parasporal crystal ^b
III	<i>B. cereus</i> III <i>B. thuringiensis</i> III <i>B. anthracis</i>	<i>ces</i> gene for emetic strains identification
IV	<i>B. cereus</i> IV <i>B. thuringiensis</i> IV	- ^c
V	<i>B. cereus</i> V <i>B. thuringiensis</i> V	-
VI	<i>B. weihenstephanensis</i> <i>B. mycoides</i> <i>B. thuringiensis</i> VI	“psychrotolerant” <i>cspA</i> signature present “psychrotolerant” <i>rrs</i> signature present Rhizoidal colony Parasporal crystal
VII	<i>B. cytotoxicus</i>	Growth in minimal médium without tryptophan and growth at 50°C

^aRhizoidal colonies are formed by *B. pseudomycoides* and *B. mycoides* growing cells

^bParasporal crystal is typical of *B. thuringiensis* strains

^cThere are not yet phenotypic features identified

This novel phylogenetic classification can be used in food industry as a practical tool since it allows manufacturers to establish processing and storage conditions able to ensure microbiological quality of products and to assess the risk level associated to food contamination with specific strains of the different taxonomic clusters. To this end, it has been reported that prevalence in food of *B. cereus* group members varies among different phylogenetic groups. Thus, Afchain et al. (2008) estimated that the genetic

group II strains are more frequently isolated of contaminate raw vegetables, meanwhile genetic group III was prevailing in milk proteins, and group IV prevailed in milk proteins and starch. Besides, lightly heat-treated foods with extended refrigerated storage are a favorable environment for *B. cereus* group species, especially for those belonging to groups II and VI.

2.1.5. B. cereus group importance in food processing environments

Soil is the natural reservoir of spores of *B. cereus* group. When this spores come in contact with organic matter or an insect or animal host germination and growth occur (Stenfors Arnesen et al., 2008).

Among *B. cereus* group, *B. cereus sensu stricto* can be found in different soils, plants, sediments and dust, and then spores are passively spread to new environments (Stenfors Arnesen et al., 2008). From this state different life cycles can take place. It has been reported a saprophytic cycle in which spores can germinate, grow and sporulate in soil (Vilain et al., 2006). Margulis et al. (1998) described a symbiont cycle when *B. cereus* colonizes insects gut. Besides, there is a pathogenic life cycle in which microorganisms are present in mammalian intestine, and to a lesser extent, in other tissues, causing disease (Drobniewski, 1993).

B. cereus sensu stricto spores and vegetative cells have been isolated of different food products: spaghetti, pasta, rice, dairy and dried milk products, spices and other dried foodstuff, meat, chicken, vegetables, fruits, grain and seafood (Kotiranta et al., 2000), as well as eggs (Kramer and Gilbert, 1989) and infant formulas (Becker et al., 1994). Besides, previous studies appear to indicate that *B. cereus* could be present in water sources (Østensvik et al., 2004).

This microorganism is implicated in an increasingly number of food-borne outbreaks, limiting products shelf life and generating economic losses. Many factors are responsible of the great incidence of *B. cereus* in food and processing environments on the basis of the absence of complex nutrient requirements and the production of highly resistant spores to physic and chemical treatments with special ability to adhere to

surfaces. Spores hydrophobicity, and the presence of appendages are strongly associated to *B. cereus* adherence and may enhance their attachment to epithelial cells of the small intestine and resistance to regular cleaning of industrial surfaces (Anderson et al., 1995; Kotiranta et al., 2000). Furthermore, this microorganism forms thick biofilms at the air-liquid interface and also weak biofilms on immersed glass or steel surfaces in static culture conditions (Oosthuizen et al., 2001; Auger et al., 2006; Wijman et al., 2007). Ryu and Beuchat (2005), reported that biofilms may protect vegetative cells against inactivation by sanitizers.

This microorganism is able to colonize industrial environments and even food-processing equipment from spores and cells which remain in plant material after harvest, or contaminating dairy products through contact of udders with grass and bedding material or feed (Stenfors Arnesen et al., 2008). Moreover, cross-contamination elicits *B. cereus* to be present in different food such as meat (Stenfors Arnesen et al., 2008). In addition, *B. cereus* can be easily isolated from food stored at ambient temperature or subjected to long heat-keeping below 60°C as well as from food with pH higher than 4.8 (Gilbert and Kramer, 1986; Stenfors Arnesen et al., 2008).

Notably, most of the *B. cereus* strains are mesophilic, but some of them can grow at low temperatures, between 4 and 7°C (Kotiranta et al., 2000). Therefore, processed chilled foods of extended durability, which are increasingly being produced and consumed in Europe, are susceptible to represent a risk of foods spoilage and foodborne illness. Nauta et al. (2003) and others authors (Membré et al., 2006; Afchain et al., 2008) reported that refrigerated processed foods of extended durability at the end of domestic storage were colonized by an heterogeneous population of *B. cereus* conformed by mesophilic heat resistant strains mainly dependent on the initial contamination of raw ingredients, and by psychrotrophic heat sensitive strains dependent on the storage conditions.

In 1998, on the basis of sequence differences of 16S rRNA gene and cold shock proteins, Lechner et al. proposed *Bacillus weihenstephanensis* sp. nov. as a new species comprising psychrotolerant *B. cereus* strains which were able to grow at 4-7°C but not at 43°C. Further studies (Stenfors and Granum, 2001) showed that intermediate forms

between the two species exists and not all the psychrotolerant *B. cereus* strains were necessary *B. weihenstephanensis*.

At present, this new specie is a well know spoilage microorganism previously isolated from spoiled egg products (Heyndrickx and Scheldeman, 2002; Svensson et al., 2004; Baron et al., 2007; Jan et al., 2011;) dairy products (Bartosewicz et al., 2008), and vegetable products (Valero et al., 2002).

Different authors have reported the presence in *B. weihenstephanensis* strains of the genetic makeup for producing essential pathogenicity factors. Stenfors et al. (2002) reported that out of 50 *B. weihenstephanensis* strains studied, the majority of them (72%) were not cytotoxic in a Vero cell assay, although all of the strains had part of at least one of the *B. cereus* enterotoxins Hbl, Nhe or CytK. Besides, one psychrotolerant emetic *B. cereus* strain (Altayar and Sutherland, 2006) and several *B. weihenstephanensis* strains were identified as being cereulide producers (Thorsen et al., 2006; Hoton et al., 2009). In 2011, Stenfors Arnesen et al. reported for first time virulence in an *in vivo* model (*Galleria mellonella*) at low temperature. Since cytotoxins produced by *B. weihenstephanensis* might not be very relevant due to higher mammalian body temperature, the possibility of cereulide formation at refrigeration temperatures in food products before consumption may pose a risk of food poisoning (Stenfors Arnesen et al., 2011). Notably, on the basis of previous studies a high level of genetic exchange has been suggested among *B. weihenstephanensis* strains compared with other species of the *B. cereus* group (Sorokin et al., 2006). This fact is remarkable since may facilitate horizontal transmission of plasmid encoding virulence factors.

On the other hand, it should be take into account that *B. thuringiensis* strains, widely used as bioinsecticides in agriculture, are able to cause foodborne illness (Jackson et al., 1995). Thus, this species, which is closely related to *B. cereus*, deserves to be taken into account by microbiologist and food producers.

2.1.6. Epidemiology

Recent changes on eating habits have enhanced consumption of refrigerated processed foods increasing the probability of spoilage incidents and outbreaks caused

by *B. cereus* group strains. In fact, the latest Community Summary Report on Food-borne Outbreaks in the European Union of 2009 (EFSA, 2011), the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) reported that *Bacillus* species, among which all of them the main etiological agent is *B. cereus*, registered an upward trend in the number of outbreaks, as well as in the number of affecting people and of hospitalization cases (Table 4)

Table 4. Relevant epidemiological parameters of *Bacillus* spp outbreaks reported by EFSA (2009 and 2011).

EFSA report	Total outbreak number	Number of verified outbreaks	Number of people affected	Number of hospitalizations
2007	105	102	1,062	29
2009	124	59	1,132	93

Notably, the total number of outbreaks in 2007 was reported to be increased in 41.9% over 2006 (EFSA, 2009). Although to a lesser extent (approximately 18%), this growing trend, has been also observed in the period between 2007 and 2009 years (EFSA, 2011). However, it is important to take in account that the number of hospitalizations was considerably higher in 2009, 8.2 %, compared to 3.6 % in 2008, and noteworthy, three time bigger compared with hospitalizations (29 cases) declared by EFSA report in 2007 (EFSA, 2009, 2011).

Among European Union countries, France presents the major incidence of *Bacillus* outbreaks (approximately 67%), followed by Netherlands (approximately 11%) and Spain (approximately 5%) (Table 5). However, verified cases in France were just the third part of the total of reported outbreaks.

Its widely known that *B. cereus* foodborne illness are underreported. In fact, emetic and diarrheal syndromes are normally mild, self-limiting and of short duration, and because of this medical care is often not needed. Besides, differential diagnosis is problematic since emetic syndrome symptoms are similar to those observed in *Staphylococcus aureus* intoxications (Seo and Bohach, 2007), and diarrheal syndrome symptoms are similar to those observed in *Clostridium perfringens* foodborne illness (Granum, 1990).

Table 5. Total outbreaks and human cases in possible and verified food-borne outbreaks caused by *Bacillus* toxins in 2009 (EFSA, 2011).

Country	Total outbreaks		Verified outbreaks				Possible outbreaks			
	N	Reporting rate per 100,000	N	Human cases			N	Human cases		
				Cases	Hospitalised	Deaths		Cases	Hospitalised	Deaths
Belgium	4	0.04	4	53	0	0	0	-	-	-
Denmark	2	0.04	2	61	8	0	0	-	-	-
France	83	0.13	26	437	12	0	57	507	16	0
Germany	4	<0.01	4	6	0	0	0	0	0	0
Italy	4	0.01	-	-	-	-	4	118	-	-
Netherlands	14	0.08	14	45	-	-	0	-	-	-
Poland	3	0.01	3	129	5	0	0	0	0	0
Portugal	1	0.01	1	120	50	0	0	-	-	-
Slovakia	1	0.02	1	16	0	0	0	-	-	-
Spain	6	0.01	4	62	1	0	2	39	0	0
United Kingdom	2	<0.01	-	-	-	-	2	26	0	0
EU Total	124	0.03	59	929	76	0	65	690	16	0
Norway	3	0.06	1	2	0	0	2	9	0	0

It is noteworthy that Portugal, which reported a single outbreak, showed the higher number of hospitalization cases (Table 5). On the grounds of data showed in table 5, a highly variable virulence is observed. This is not strange since *B. cereus* group virulence level is mainly associated to gene expression which in turns is dependent on environmental factors.

Moreover, although no deaths were caused by *Bacillus* species during 2009 (EFSA, 2011), different fatal cases of *B. cereus* gastrointestinal illness have been previously reported (Mahler et al., 1997; Lund et al., 2000; Dierick et al., 2005). Recently, a 20-year-old man died in Brussels (Belgium) following the ingestion of pasta contaminated with emetic *B. cereus* strains (Naranjo et al., 2011).

Figure 1. Distribution of food vehicles in verified outbreaks caused by *Bacillus* toxins in European Union during 2009 (EFSA, 2011). Other food vehicles¹ include: other foods (13 outbreaks), ovine meat and products thereof (one outbreak), cheese (one outbreak), fish and fish products (one outbreak), turkey meat and products thereof (one outbreak).

On the other hand, attending to the distribution of food vehicle involved in *Bacillus* species outbreaks (Figure 1), it was shown that out of 59 verified outbreaks, 28.8% were caused by the consumption of ovine and turkey meat, fish and cheese, 27.1% by mixed or buffet meals and 11.9% by cereal products including rice. Agata et al. (2002), reported that boiled rice and farinaceous food could sustain production of high levels of cereulide, although this toxin have been also detected in infant formulas and at low level in egg, meat and liquid foods such as milk and soy milk (Stenfors Arnesen et al., 2008). Otherwise, diarrheal disease is often associated with protein-rich foods, such as meat, vegetables, puddings and milk products (Abee et al., 2011). Additionally, depending on habits of consumption, food vehicles appear to be associated with the global distribution of the *B. cereus* foodborne disease. Thus, in Japan and UK the emetic type is prevalent, whilst in North Europe and North America the diarrheal syndrome is more frequent (Kotiranta et al., 2000; Stenfors Arnesen et al., 2008).

2.2. Stress response in *B. cereus* group microorganisms

2.2.1. General stress response

Bacteria are exposed to changing environmental stresses such as temperature, pH osmolarity and nutrient availability fluctuations. To cope with these adversities, bacteria can use flagellum to move to more favorable locations or adapt by changes in the pattern of gene expression whose products are required to combat the deleterious nature of stress (Marles-Wright and Lewis, 2007). The transcription of these genes is tightly controlled through activation of transcription factors which interact with RNA polymerase (RNAP).

Sigma factors (σ) are a family of transcription factors with a role in stress resistance. Upon exposure to stress, sigma factor binds to the core of RNAP (composed of five subunits ($\alpha_2\beta\beta'\omega$)) forming the RNAP holoenzyme able to recognize promoters and melt DNA (van Schaik and Abee, 2005). In exponentially growing cells transcription is mediated by RNAP holoenzyme which carries a “house-keeping” sigma factor that is similar to σ^{70} in *Escherichia coli* and σ^A in *Bacillus subtilis* (van Schaik and Abee, 2005). In addition, during stress conditions and cellular differentiation in

most of bacteria other sigma factors, termed alternative sigma factors, can be present in the cell. Among Gram-positive bacteria with low GC content, genera *Bacilli*, *Staphylococcus* and *Listeria* have been shown to increase their resistance to stress through the activation of the alternative sigma factor σ^B .

The stress that activates σ^B and the organization of the σ^B operon are different for *B. cereus* group in comparison to other Gram-positive species (van Schaik et al., 2004). In *B. cereus* ATCC 14579 a gene cluster encodes σ^B consisted of five open reading frames which encodes regulators of σ^B activity, the structural σ^B gene, and a protein stress-Inducible Dps-Like Bacterioferritin (Ivanova et al., 2003; Wang et al., 2009). The products of all this open reading frames exhibit high amino acid identity with homologues encoded in *B. anthracis* Ames e, *B. cereus* ATCC 10897 and *B. thuringiensis* subsp. *Israelensis* ATCC 35646 genome, highlighting the close relationship among the different members of the *B. cereus* group (van Schaik et al., 2004).

σ^B operon is transcribed as a 2.1-Kb transcript including *rsbV*, *rsbW*, *sigB* and *orf4*. *orf4* is under control of an additional σ^B -dependent promoter. Transcription start upstream *rsbV* and ends downstream *orf4* where is a loop that was suggested to acts as a terminator. *rsbY* gene is located downstream of the σ^B operon encoding a protein with a C-terminal PP2C phosphatase (van Schaik et al., 2004).

σ^B activation involves RsbV and RsbW regulators which are conserved in all the Gram-positive bacteria that have σ^B . However, RsbY regulator is only crucial for σ^B activity in *B. cereus* (van Schaik et al., 2005a). Figure 2 shows the proposed model for regulation of σ^B activity in *B. cereus*. Under unstressed conditions σ^B is bound to its anti-sigma factor, RsbW, in this form σ^B cannot bind to RNAP and remains in inactive state. Under stress conditions the sigma factor antagonist, RsbV, binds RsbW forming the RsbV-RsbW complex, released σ^B alternative factor that now can bind to RNAP and start transcription of σ^B -dependent genes. RsbW can also acts as a kinase and phosphorylate a serine residue of RsbV making impossible complex formation in non-stressed cells. Under environmental stress, RsbV is desphosphorylated by the action of RsbY phosphatase and then RsbV can bind to RsbW (van Schaik et al., 2005a). Recently, de Been et al. (2010) have proposed that PP2C-type phosphatase RsbY

receives input signal from the multi-sensor hybrid kinase RsbK, and both together constitute one functional module for the control of σ^B activity in members of the *B. cereus* group (Figure 2). Besides, an additional fine-tuning system proposed by de Been et al. (2011) may include CheR-mediated methylation processes of RsbK.

Figure 2. Model for σ^B activation in *B. cereus* (de Been et al., 2011). Upon different stresses, two different routes have been proposed for σ^B activation, most likely RsbK ‘auto’-phosphorylates a conserved histidine residue within its H-box, (1) the phosphoryl group is then either transferred directly to a conserved aspartate within the REC domain of RsbY (2) or shuttled to RsbY indirectly via RsbK’s own REC domain and a potential phosphotransferase protein (de Been et al., 2010). In the former scenario, the RsbK REC may play a role in fine-tuning RsbK kinase activity.

σ^B deletion mutants of *B. cereus* ATCC14579 had minor importance during exponential growth, but from early stationary phase, which is the moment of the glucose depletion, may be important for metabolism (de Vries et al., 2004). In addition, deletion of σ^B did not cause delay in sporulation but spores had slightly lower heat resistance than parental. Likewise, it has been shown that σ^B is essential for optimal morphogenesis of the spore. Besides, the germination in response to inosine and to a

combination of inosine and L-alanine was delayed after a σ^B deletion (de Vries et al., 2004) suggesting that probably, important metabolic pathways that contribute to the process of sporulation are affected in the σ^B null mutant.

On the other hand, was not unexpected that σ^B does not play a role in the production of virulence factors and enterotoxin Nhe since in the *B. cereus* group the production of these factors is governed by the pleiotropic regulator PlcR and σ^B did not seem to be involved in transcription of the *plcR* gene (van Schaik et al., 2004). However, *B. anthracis* σ^B deletion mutants showed 1 log difference in the 50% lethal dose (LD₅₀) with the parental strains (Fouet et al., 2000).

Besides, σ^B is activated under several stress conditions, particularly during heat shock but also during other stresses such as high osmolarity, high ethanol concentrations, high and low pH and oxidizing agents (van Schaik et al., 2005b). σ^B deletion mutants of *B. cereus* ATCC14579 subjected to heat shock from 30 to 42°C leads to 20.1-fold activation of σ^B and is by far the most powerful trigger leading to σ^B activation in *B. cereus* (van Schaik et al., 2004). Addition of 4% of ethanol or 2.5% NaCl or an acid shock at pH 5.2 led to 4.6-4.2 and 1.8-fold induction of σ^B respectively (van Schaik et al., 2004).

Conversely to expected results, deletion of σ^B in *B. cereus* ATCC 14579 leads to hyperresistance to hydrogen peroxide, in fact, catalase activity is threefold higher during the mid-exponential growth phase than in parental strain (van Schaik et al., 2005b).

Finally, σ^B shows to be related with growth and survival under low temperatures of *L. monocytogenes* and *B. subtilis*, in resistance against antibiotics of *B. subtilis* and *Staphylococcus. Aureus* and has a role in biofilm formation of *Staphylococcus epidermidis* (Becker et al., 2000; Bandow et al., 2002; Brigulla et al., 2003; Singh and Moskovitz, 2003; Knobloch et al., 2004).

On this ground, fundamental knowledge about σ^B activation is essential to understand the behavior of stressed bacteria and therefore to successfully design sequential preservation steps.

2.2.2. Two component systems

Two-component systems (TCS) are involved in signal transduction across the cell membrane. This signaling pathways are the most widely used by bacteria (including archaeobacteria) to sense and transduce environmental signals in order to overcome external stress and adapt to changing conditions (Stock et al., 1989; Abee et al., 2011). Indeed, TCS are linked to cell response, nutrient deprivation, cold and heat shock, antimicrobial compounds, osmotic stress, low pH and many other factors (Sun et al., 1996; Bearson et al., 1998; Aguilar et al., 2001; Jung and Altendorf, 2002; Jordan et al., 2008). Besides, they participate in the control of motility, chemotaxis, sporulation, biofilm formation and quorum sensing (Jiang et al., 2000; Lyon & Novick, 2004; Szurmant and Ordal, 2004; Lopez et al., 2009).

TCS consist of an input-sensing domain composed of a trans-membrane sensor histidine kinase (HK) and an output effect or domain, a response regulator (RR) which generally functions as a transcription factor, able to modify expression of genes involved in stress responses (Hoch, 2000). For signal transduction by TCS, a phosphate is transferred from a conserved histidine located in the HK to an aspartate residue in the phosphotransferase and RR receiver domain (Hoch, 2000).

Ubiquitous *B. cereus* and close species display a wide variety of TCS that allow rapid and robust responses which contribute to the problematic presence of these microorganisms in Food Industry (Abee et al., 2011). Various studies (de Been et al., 2006, 2008, 2010) have reported that TCS of *B. cereus* strains are involved in numerous cellular functions such as sporulation, biofilm formation, host-microbe interactions, chemotaxis, nutrient uptake and antibiotic resistance (Figure 3).

Probably due to a major genome size, *B. cereus* group isolates possess a relatively large number of TCS in comparison with other low-GC Gram-positives bacteria (Abee et al., 2011). In each member of this taxonomic cluster, around 40 HK-RR gene pairs and many HKs and RRs genes not encoded in pairs ('orphans') were detected by applying a bidirectional best-hits method combined with gene neighbourhood analysis to a set of HKs and RRS recovered through *in silico* techniques from eight sequences of *B. cereus* group genomes (Figure 3) (de Been et al., 2006).

Besides, *B. anthracis* lacks specific HKs and RRs and contains a major number of truncated and HK and RR genes, possibly because of a reduced exposition to environmental stimuli as consequence of the pathogenic specialization of this microorganism (de Been et al., 2006).

Figure 3. Overview of TCSs in *B. cereus*. HKs are indicated in red (sensory) and ecru (phosphotransferase) and RRs are indicated in grey (receiver) and green (DNA-binding domain). Blue domains indicate protein-protein interaction domains. White and light (red, green, blue) domains indicate the absence/truncation of the respective domain in at least two of the *B. cereus* group members analyzed. Incoming arrows indicate predicted or established HK-specific stimuli. Inside the cell, black and grey arrows represent predicted phosphotransfers between paired and “orphan” HKs and RRs, respectively. RRs for which specific operators have been predicted are “connected” to the DNA. Other connection lines illustrate protein-protein interactions. Specific stimuli are numbered as follows: 1, general stress response (heat and NaCl), 2, antibiotics, 3, antimicrobials, 4, heme, 5, calcium, 6, metals, 7, oxidoreduction potential, 8, phosphate limitation, 9, chemotactic signals, 10, amino acids (glutamine), 11, antimicrobial (cationic antimicrobial peptide), 12, tricarboxylic acid cycle intermediates, 13, ComX-like auto-inducing peptide, 14, antimicrobials, 15, antibiotics, 16, ComC- or ArgD-like auto-inducing peptide, 17, general stress response (proton motive force), 18, antimicrobials. A corresponds to protein CheA, B to protein CheV, C to protein CheY, all participating in *B. cereus* chemotactic response. Besides, D corresponds to Spo0F, E to Spo0B, F to Spo0A which are involved in *B. cereus* sporulation. Furthermore, G corresponds to RsbK, H to RsbY and I to alternative sigma factor σ^B . Finally, J corresponds to LytS which is a sensor histidine kinase and K to the transcriptional regulator LytR. Each TCS depicted has been numbered by de Been et al. (2006) (Modified from Abee et al., 2011).

2.2.3. SOS response and adaptive mutagenesis

SOS is widespread bacterial response induced after exposure to various stresses and agents that cause damage to DNA or the collapse of the replication forks (van der Veen and Abee, 2011). This conserved adaptive mechanism, with an active role in stress resistance and in the induction of genetic diversity, is regulated by the repressor LexA (Butala et al., 2009) and the activator RecA (Cox, 2007) and generally consists of proteins involved in DNA repair and in translesion DNA systems (Lusetti and Cox, 2002). Recent studies have shown alternative genetic pathways to activate SOS response in *E. coli* cells subjected to high pressure, to acid environments and to high salt concentrations (DiCapua et al, 1990; Aertsen and Michiels, 2005; Sousa et al., 2006)

As a natural consequence of the SOS response, mutagenesis processes can occur. In fact, translesion DNA polymerase due to its low specificity is able to introduce mutations giving rise to “adaptive mutagenesis”. “Adaptive mutagenesis” is defined as a process that produces advantageous mutations during exposure to nonlethal stress, even when non-beneficial mutations may occur simultaneously (van der Veen and Abee, 2011). It has been shown that food preservation stresses, starvation periods, stationary phase, aging colonies, resting organisms and exposure to antibiotics may induce genetic changes and subsequent “adaptive mutagenesis” through mechanisms which require one or more factors of the SOS response (Galhardo et al., 2007; van der Veen and Abee, 2011). Otherwise, factors and mechanism involved in various stress responses independent of SOS response are also involved in “adaptive mutagenesis” (Goldfless et al., 2006).

During food preservation, especially in minimally processed foods, to reduce negative influence of overprocessing and maintain nutritional and microbiological quality, different mild simultaneous stresses or successive exposure to mild treatment are widely used. However, this food preservation technology together with factors such as the specific composition of the raw materials, the storage conditions and the cleaning regimes may trigger SOS response (van der Veen and Abee, 2011). As previously reported, contaminated food exposed to nonlethal doses of ultraviolet light (Clerch et al., 1996), to industrial oxidative disinfectants (Goerlich et al., 1989), to heat treatments (van der Veen et al., 2007), to high pressures (Aertsen and Michiels, 2005), to

preservative compounds such as acids (Sousa et al., 2006) and to high salt concentration (DiCapua et al., 1990), result in the activation of the SOS response which may subsequently lead to adapted pathogenic and spoilage bacteria that are more difficult to eradicate from food production chain (Figure 4). In addition, SOS response is specifically associated to the production of phenotypic variants generally associated with biofilms and even in the formation of some types of this structured communities (Boles et al., 2004; Boles and Singh, 2008; Nuryastuti et al., 2008; van der Veen and Abee, 2010).

Figure 4. Overview of environmental stresses involved in SOS response trigger and mechanisms of SOS response in bacteria. At different steps of the food chain different stresses such as composition of raw materials, storage conditions of final products, processing steps, addition of specific preservatives and/or cleaning regimes (heat, high pressure, UV-radiation, oxidative compounds, acids, and/or antimicrobial compounds) may trigger SOS response through DNA damage (D), replication fork inhibition (referred as I), blockage of origin of replication (referred as B) and activation of Mrr (referred as Mrr), a restriction endonuclease which digest DNA resulting in double-stranded DNA fragments.(van der Veen and Abee, 2011).

Is it noteworthy that this mechanism may induce diversity, stress resistant subpopulations and cross-protection phenomena, as for instance *E. coli recA* knockout mutants, which are more sensitive to heat inactivation (Duwat et al., 1995; van der Veen et al., 2010). Therefore, SOS response should be taken into account for successful design of novel preservative methods based on combined treatments.

2.3. Predictive microbiology

As it is widely known, predictive microbiology is the field of study that combines microbiology elements, mathematics and statistics, to develop models that mathematically describe and predict the growth or death of the microorganism when they undergo some specific environmental conditions (Whiting, 1995).

In 1949, Monod reported that "...the growth of the bacterial cultures, despite the immense complexity of the phenomena to which it testifies, generally obeys relatively simple laws...The accuracy, the ease, the reproducibility of bacterial growth constant determinations is remarkable and probably unparalleled, so far as biological quantitative characteristics are concerned". Few decades later, and on the premise that the responses of population of microorganisms to environmental factors are reproducible, modelling techniques are fully implemented in Food Sciences for estimating the shelf life and safety of many food products allowing reliable, fast and accurate predictions of microbiological food quality.

2.3.1. Lag phase and exponential growth

Viable cells of different bacterial species have a comparable life cycle (Figure 5). When microorganisms are subjected to growth promoting environments there is a time elapsed until the onset of growth which is the so-called lag phase. During this period, cells adjust their metabolism to the new environment. The duration of this phase depends on the magnitude of the change between previous and current environmental conditions.

When lag phase ends, microorganisms start to grow exponentially giving place to the exponential growth phase. The speed of growth is expressed as the maximum specific growth rate. Métris et al. (2005) reported that the time to first division is

notably longer and more variable than the second and third generations as an obvious consequence of the lag time needed to resume growth. From second a third generation, time and variance decrease. Few generations are needed until reach a maximum division rate.

Besides, the generation time is the time needed for doubling the initial bacterial population during the exponential growth phase. However, in asynchronous cultures, doubling time is not equivalent to generation time, in consequence, generation time is estimated on the basis of the Poisson process considering that cell division can be approximately a random birth process (Rubinow, 1984). In 1998, Baranyi proposed that the time to the first division is the sum of the lag time and the generation time on the grounds that exponential period begins even when after lag period cell necessary do no divide.

When a microbial population reaches an approximate level of 10^9 colony forming units (CFU) per mL or per g of food, generally enters in stationary phase. From this moment the number of microorganisms remains constant and depending on environmental conditions such as oxygen availability and nutrient depletion, microorganisms reach starvation phase, in which the level of microorganisms decrease.

Figure 5. Bacterial life cycle with indication of initial population density (n_0), maximum population density ($n_{\max}$), the maximum specific growth rate ($\mu_{\max}$) and the lag parameter (λ). (Swinnen et al., 2004)

As is depicted in Figure 5, maximum specific growth rate, denoted by $\mu_{\max}$, is estimated from the slope of the tangent drawn to the inflexion of the sigmoid curve which is fitted to the data representing the natural logarithm of the cell concentration against time. On the other hand, the lag period (λ) is usually interpreted as the time from the inoculation to the intercept of the tangent with the level of inoculum (Baranyi and Pin, 1999).

In food microbiology, lag phase occurs after food contamination with external sources or when contaminated food products undergo environmental fluctuations (Delignette-Muller et al., 2005). Despite genotypic and phenotypic characteristics and the physiological history of the population, other factors such as inoculum size and changes in physicochemical environments pose a great impact in lag phase duration. Thus, modification of pH, water activity, nutrient availability, temperature and gaseous content of food products are a common tool to obtain longer shelf-life by increasing lag phase extent. Likewise, environmental conditions inside and outside a food product have a major impact in the maximum specific growth rate of microorganisms.

Finally, it is remarkable that lag and stationary phases are regions of zero growth rates with important physiological and practical implications since present a minimal metabolism and therefore are extremely difficult to inactivate. This trait is termed “Persisters” cells and has been described for *E. coli* isolates (Shah et al., 2006).

2.3.2. Modelling

Predictive microbiology began over 1920, when Bigelow and Esty published a work which enabled to calculate the time of thermal death (Rodrigo et al., 2005). Thus, from survivor curves, the value of the primary parameter D_T (decimal reduction time) was calculated using a first order model based on the assumption that survivor rate of microorganisms subjected to heat treatment at a constant temperature undergoes an exponential decrease depending on time. At a given temperature, population decreases in the same proportion for a given time interval. Later, the great incidence of non-linear inactivation kinetics promoted the creation of alternative primary models to describe survival curves (Rodrigo et al., 2005). Thus, Logistic and Weibull models (Peleg and Cole, 1998) were proposed. Besides, during the 80's and linked to computational

improvements, the attention on application of mathematical modelling to the growth and survival of microorganisms in food increased (Ross and McMeekin, 1994; Rodrigo et al., 2005). To overcome this new needs Logistic, Gompertz (Zwietering et al., 1990) and Baranyi (Baranyi and Roberts, 1994) primary models were developed.

On the other hand, changes in food formulation spurred the development of secondary models. These models describe the effect of some environmental factors such as temperature and pH on growth parameters (Delignette-Muller, 1998). The first model of secondary level was the curve of thermal die that provides the change of D_T within the heating temperature. The inverse negative of the slope of the regression line is the parameter z (Rodrigo et al., 2005). Subsequently, different secondary predictive models were enounced to describe inactivation rate and model growth. Thus, Arrhenius-type models, developed on the basis of the “classical Arrhenius equation, and Square-root type models, including Ratkowsky model (Ratkowsky et al., 1982) and other derived from this one, were developed to describe the rate of microbial growth as a function of temperature (Davey et al., 1978 ; Ross and McMeekin, 1994). Otherwise, Modified Arrhenius models, introduced by Davey (1989), were used to model effects of temperature and water activity on microbial growth kinetic parameters (Rodrigo et al., 2005). Likewise, other authors have developed models based on Bigelow equation (Mafart and Legerinel, 1998) to describe the effect of temperature and pH on D value. In addition, Polynomial or Response surface models, which consist in regression equations that are fitted by using standard of regression techniques and can contain quadratic, cubic or reciprocal terms including also the interactions among the considered factors (Buchanan and Bagi, 1994), were proposed as an empirical approach to the problem of summarizing growth rate responses (Ross and McMeekin, 1994) and are among the most used secondary predictive models.

However, secondary models do not always take lag phase into account. Polynomial-type models more commonly are independent of generation time and lag time. Conversely, square root-type models are generally developed assuming that lag time is proportional to the generation time (Delignette-Muller, 1998). Furthermore, it has been shown that unlike generation time, lag time depend not only on current growth conditions, but also on previous conditions.

Most of deterministic models traditionally described lag phase based on a geometrical interpretation (Metris et al., 2005). To accurately calculate lag time, new models were developed taking into account inoculum history. Thus, Baranyi et al. (1993), Baranyi and Roberts (1994) and Hills et al. (1994, 1995) proposed the first mechanistic models to estimate the lag phase. In the model of Baranyi and Roberts (1994) “work to be done” parameter (h_0) was proposed, and corresponds with the time required for cells to adapt to a new environment, and is defined as the product of the exponential growth rate and the lag time when the rate at which the work is carried out is equal to the maximum specific growth rate. Meanwhile, Hills et al. proposed that lag phase extent depends on the time required to reach a maximum value of cell biomass for a particular environment.

Subsequent studies analyzed relationship between lag phase duration and growth rate. Robison et al. (1998) reported that environmental factors differently affect this relationship. Numerous authors have studied the dependence of h_0 on the inoculum history (Le marc et al., 2010; Muñoz-Cuevas et al., 2010). Mellefont and Ross (2003) defined the relative lag time (RLT) as the ratio of the lag time to the doubling time, to quantify the effect of the environment on h_0 and the rate at which this work is done during lag phase.

One step further, different authors studied the dependence of both parameters on the previous and current conditions in fluctuating environments. Indeed, microbial quality and safety may be characterized by a high level of variation (Poschet et al., 2004). Notably, dynamic approaches are of great interest since foodstuffs are subjected to changing conditions during their shelf-life. Temperature, pH, gaseous atmospheres and water activity fluctuations affect bacterial life-cycle and growth parameters and should be take into account for modelling purposes. On the basis of the dynamic model for growth of bacterial population described by Baranyi and Roberts (1994), Muñoz et al., (2010) proposed that a population of cells in lag phase which are subjected to environmental fluctuations restart a new lag phase and therefore, the work to be done in the new lag phase also include the uncompleted workload from the previous lag phase.

On the other hand, taking into account that some foodborne pathogens can be infective at very low concentrations and deterministic models are not suitable for

situations when the bacterial population consists of less than 10^2 CFU/mL (Metris et al., 2005), stochastic models have been developed to avoid inaccurate results that could be obtained from the extrapolation of population growth parameters to low-level contaminated food (D'Arrigo et al., 2006).

Stochastic models have been applied for long time in biotechnology and medical studies, and besides allow predicting probabilities of survival and growth at low cell numbers, are able to accomplish the increasing demand of robust predictions for quantitative risk assessment application (Baranyi et al., 2002).

Unlike deterministic models which are able to predict a single value for the microbial load at a certain time instant, stochastic models use random variables to obtain uncertain results. To perform stochastic models numerical methods are needed. Numerical methods are algorithms that rely on random number generators, for each trial the sequence of these numbers will be different so the result will also be different, and to draw conclusions is needed to perform statistics of many trials. Thus, stochastic predictive microbiology is a key element that encompasses model type incorporating uncertainty at the level of measurements and models parameters and predicts the microbial load by a probability mass function (Nicolăi and Van Impe, 1996).

In 2002, Baranyi described a model to accurately estimate probability of survival and growth based on the analysis of the distribution of the individual cell lag times. It has been shown that changes in substrate or sublethal injury results in different distributions of the individual lag times indicating a certain history effect. Therefore, this information could be of major interest for optimization of food processing procedures (Baranyi et al., 2000).

Novel predictive models, classified as Tertiary models, are algorithms incorporated into devices that record and integrate the effect of environmental variables. Microbial Resources Viewer (MRV) (Koseki, 2009), a web-tool for application of these predictive models, provides information concerning growth/no growth boundary conditions and the specific growth rates of required microorganisms allowing the identification of minimum processing condition for a required log reduction to successfully determine appropriate food design and processing.

Future challenges for predictive microbiology is to generate mechanistic models able to describe cellular heterogeneity in genetically homogeneous populations on the basis of molecular physiology studies performed at single cell/spore level. Likewise, development of mathematical models able to describe bi-stable signaling networks might be relevant for food microbiology, however, previous studies under relevant physiological (food) conditions are needed as much as possible using the relevant food isolates (Havelaar et al., 2010).

Finally, it is essential to bear in mind that to accurately understand the reliability of models it is not sufficient to observe that a particular method or model appears to work, but is essential to know why it works to understand and judge their results and improve them (Bratchell et al., 1989).

2.3.3. HACCP and Risk assessment

Hazard analysis and critical control points (HACCP) were developed as an alternative method for time-consuming and laborious analysis used to determine microbiological quality of the food product. This safety analysis system is focused on “the identification and real-time monitoring of physical and chemical attributes at critical control points as a means of controlling foodborne pathogens” (Buchanan, 1995). Together with good manufacturing practice (GMP), HACCP has been extensively applied showing effective results (Van Der Spiegel et al., 2004).

To reach public health objectives, microbial risk assessment (MRA) plays a key role in HACCP. MRA aims to evaluate the probabilities and severities for consumers’ health derived from the exposure to pathogenic microorganisms present in foods for further implementation of appropriate management options. Usually, these evaluations consist in quantitative estimations obtained from mathematical models which describe the evolution of bacterial populations (Augustin, 2011). Thus, predictive microbiology is essential for HACCP implementation in Food Industries (Elliot, 1996). Indeed, stochastic models are used to characterize microbial quality and safety taking into account microbial variability.

Appropriate implementation of microbial risk management requires a deep understanding of the whole food production chain (Havelaar et al., 2010) and is composed of the following four steps (Augustin, 2011):

- Hazard identification, which consists of identifying and describing the pathogenic microorganisms. Notably, correct identification of sporulated bacteria remains being a challenge because of their diversity and the absence of specific markers.
- Exposure assessment, which consists in evaluating the quantity of foodborne pathogens or toxins ingested by consumers. In this regard, it has been shown that final contamination of spore-forming microorganisms is hardly affected by bacterial biodiversity and physiological variability.
- Hazard characterization, generally consisting of dose-response relationships allowing estimating the probability and severity of adverse effects knowing the ingested dose. At this point, is it remarkable that pathogenicity of sporulated bacteria is greatly affected by biodiversity resulting in highly variable responses to an ingested dose of these microorganisms.
- Risk characterization, allowing the estimation of risk from exposure and dose-response modelling.

As consequence, many difficulties have been described to successfully implement quantitative microbial risk assessment for spore-forming bacteria such as *B. cereus* group (Carlin et al., 2000). As described above, this is due to the extreme intra-species biodiversity of these microorganisms, but also because of the lack of an appropriate model to predict spore germination and outgrowth processes (Augustin, 2011). Notably, intra-species biodiversity of sporulated bacteria cause an extremely variable individual poisoning risk for consumers and therefore, has a great impact on hazard identification, exposure assessment and hazard characterization. However, risk assessment still constitutes a valuable tool to justify the implementation of management options (Augustin, 2011).

Finally, mildly preserved foods, which are based on the principle of hurdle technology, represent a great challenge for risk managers and producers since they require a well controlled food processing through adequate product and process design and proper implementation and monitoring through HACCP (Havelaar et al., 2010).

2.4. Food preservation

Food preservation techniques depend largely on increasing the lag phase, decreasing the rate of growth in the exponential phase and reducing the maximum population density.

In the latest decades, consumers' perception of food quality and nutrition has changed. Great number of alternative methods and technologies are replacing traditional heat based treatments to satisfy modern trends in food consumption and obtain fresh-looking food, ready to eat, with high nutritional and organoleptical quality. To ensure microbiology safety minimizing impacts on food quality, is required from industry to implement different techniques such as mild heat treatments, non-thermal treatments and natural preservatives that allow avoiding classical sterilization and pasteurization methods (Havelaar et al., 2010). Usually, these techniques are used in combination, known as "hurdle technology", to achieve an overall microbiological food safety (Leistner and Gorris, 1995).

In 2005, Raso et al. defined the attributes of ideal processing technologies for the food producers as follows:

- Improvement of the shelf life and safety by inactivating enzymes, spoilage and pathogenic microorganisms
- No changes in organoleptic and nutritional attributes
- No residues left on food
- Convenient to apply
- Cheap
- No objections from consumers and legislators

Nowadays, many mildly technologies have shown to ensure food quality and safety within the extended shelf-life. However, to control all parameters involved in food preservation such as relevant environmental factors, food product and microorganism, new investigation are needed, maximum when *B. cereus* group isolates are ubiquitous and endospore forming bacteria with a high adaptability.

2.4.1. Hurdle technology and gamma hypothesis

In 1995, Leistner and Gorris proposed to reduce the negative influence of severe heat treatments, the combination of various antimicrobial factors, hurdles, to guarantee microbial safety with an acceptable shelf-life. This combined process for food preservation is commonly known as “hurdle technology” and it is especially important for those microorganisms and bacterial spores which are highly resistant to inactivation technologies and are a limiting factor for the application of mild treatments (Rajkovic et al., 2010).

This preservation technology is based in the biological fact that bacterial cells exposed to combined methods undergo different homeostasis injuries and to resume growth should active complex and varied stress responses (Raso et al., 2005). Aertsen and Michiels (2004) reported that under mildly lethal stress, the ultimate cause of bacterial inactivation is the extra effort involved in cellular response to overcome environmental adversities. Results showed that inactivation levels and the percentage of sub-lethal injured cells obtained depended on strain and type preservation technique used (Rajkovic et al., 2010).

Nowadays, the concept of hurdle technology is well established. However, the quantification of the combined impact of hurdles is still being under study Besides, there are opposite views about how antimicrobial factors combine when are used together in a combined approach. On one hand, one view states that synergy phenomena occur when several antimicrobial factors are used together. Synergy can be defined as the effect observed when hurdles are applied together and give a significantly greater protection. An opposite view considers that combined effects are complex but there are no interactive effects resulting in just an apparent synergy (Biesta-Peters, 2011). The

alternative option, called gamma hypothesis, states that inhibitory environmental factors combine in a multiplicative manner to produce the observed overall microbial inhibition (Zwietering et al., 1993).

Gamma hypothesis is based on gamma factor (γ) parameter which is the ratio between the growth rate of a microorganism when growing with one hurdle ($\mu_{\max}$) and the optimal growth rate when no hurdles are present (μ_{opt}). “When multiple hurdles have been applied, the growth rate of a microorganism can be calculated by multiplying all gamma factors for the single hurdles and multiplying this again with μ_{opt} ” (Biesta-Peters, 2011).

It is noteworthy that, most of treatments that do not cause complete inactivation of microorganisms induce sub-lethal injury to the present bacterial cells, and depending on environmental conditions microorganisms are able to readjust their metabolism and resume growth (Smigic et al., 2009; Rajkovic et al., 2010;). Sub-lethal injury recovery is determined by food intrinsic factors (water activity, pH and nutrients) and by extrinsic factors (modified atmosphere, temperature and humidity). In addition, food is a dynamic system and in consequence, intrinsic factors change during the period extending from processing to consumption playing a role of a different magnitude depending on the time (Raso et al., 2005).

2.4.2. Relevant factors for food preservation

Several environmental factors such as pH, mild and severe heat treatment, water activity, atmosphere packaging and chilling can be used to ensure food safety and stability by limiting bacterial growth. The application of these factors, the hurdles, results in slowing down or blocking growth rather than killing microorganisms present, and their effectiveness, mainly depends on the intensity of the hurdle and on the possible resistance of the particular microorganism to this hurdle.

It is noteworthy that, to apply principles of food preservation by combined processes correctly, is needed a thorough knowledge of the antimicrobial activity of these environmental factors alone and in combination.

2.4.2.1. Temperature

Heat as a food preservation method has been used by mankind for ages. When heat treatments are properly applied can eliminate spoilage or pathogenic biological agents present on food ensuring microbiological quality. However, sensory and nutritional attributed of the food are also affected. Undesirable odors, flavors and textures cause consumers' rejection of these foods. Besides, nutritional components of great importance for health or even essential, such as vitamins, may be destroyed or modified by heating.

Depending on time of exposure and temperature regime, microbial load decrease or even is eliminated obtaining a complete sterilization. Microbial inactivation by means of heat treatments is commonly characterized using a D_T value (decimal reduction time) which is defined as the time of the heat treatment in minutes which gives an expected microbial inactivation of 90%. From D_T data a z value, which is the temperature change required for a 10-fold change in D value, is derived. Otherwise, F value is a classical parameter used in sterilization processes which consists in time in minutes at the reference temperature of 121 °C to obtain desired sterilization effect.

Vegetative cells of *B. cereus* group isolates growth in a temperature range between 5 and 50°C (Guinebretière et al., 2008). In bacterial cells heat causes damage to macromolecular cell components such as proteins and DNA. Denatured proteins lose their specific structure and in consequence are unable to perform their function. To cope with these adverse conditions, response to heat stress involves protein chaperones that assist in folding and assembly of heat damaged proteins (Yousef and Courtney, 2003). Besides, some microorganisms modify their cell membrane composition by increasing the ratio of trans to cis fatty acids, decreasing fluidity caused by high temperatures (Cronan, 2002).

Bacterial spores are characterized by higher heat resistance in comparison to vegetative cells, e.g. vegetative cells of *B. cereus* will be destroyed by a heat treatment of 20 min at 70°C, while spores will mainly remain unharmed (Faille et al., 1997).

Among the most important spores in terms of public health are psychrotrophic and mesophilic strains of *B. cereus* group.

On the other hand, low temperatures are commonly used for raw and processed food preservation. Cells subjected to cold environment undergo a reduction in biochemical reaction rates (Brillard et al., 2010). Besides, membrane fluidity decrease and inefficient folding of proteins and secondary structures of RNA and DNA occur (Schumann, 2009).

B. cereus group strains, mesophilic and psychrotrophic, are able to grow below 10°C. This represents a major challenge for microbiologists since low levels of *B. cereus* in foods can easily increase under commonly reported suboptimal refrigeration products (EFSA, 2007). Additionally, these strains are able to resist heat treatment and resume growth in refrigerated processed foods of extended durability (REPFED) which are processed at a lower temperature, with maximum within the range of 65–95 °C (Peck, 1997), to maximize their nutritional and sensory qualities

2.4.2.2. pH

The pH is a measure for the acidity or the basicity of a solution, and is defined as the negative logarithm (p) of the molar concentration of the hydrogen ion ($[H^+]$) (Atkins and de Paula, 2002). It has been reported that *B. cereus*, depending on the acidulant used, is able to grow at pH values as low as 5 or 6 (Adams and Moss, 2000).

Strong acids added to non-saturated solution are completely dissociates into the hydrogen ion and the anion part. A drop in the pH of medium or a food product may cause changes in microorganisms' internal pH or in its membrane fatty acid profile (Biesta-Peters, 2011). On the other hand, molecules of weak acids in solution are in equilibrium between the dissociated and the undissociated form. In low pH environment this equilibrium is shifted to the undissociated state. In this state and depending on the lipophilic nature of these compounds, neutral acids are able to dissolve and diffuse across plasma membrane (Ter Beek and Brul, 2010). Once inside the cell, due to the higher cytoplasm pH, equilibrium is shifted to dissociate state releasing protons and anions.

These charged molecules are unable to back across plasma membrane generating a cytoplasmic pH decline and concentration of toxic anions that should be metabolized or transported outside the cell expending ATP (Mols and Abee, 2011). In this conditions growth declines and lag phase increases. Mols and Abee (2011) reported that after acid shock, general stress response and secondary oxidative response are triggered in *B. cereus* isolates. Likewise, pH homeostasis mechanisms and metabolic rearrangements occur therefore eventually eliminating growth.

2.4.2.3. Water activity

The definition of water activity (a_w) is the ratio of the vapor pressure (fugacity) of water in the system at a given temperature and the vapor pressure of pure liquid water at the same temperature. From a practical point of view, this parameter, which measures the tendency of the water to escape from the food product, is important during food processing and storage not only at microbiological level, but also for chemical and physical stability of the product (Reid, 2007).

Water is present in all foods and food ingredients at different levels and depending on its content and mobility prevents or limits microbial growth. Previous studies indicate that *B. cereus* can grow at a_w values as low as 0.95 (Adams and Moss, 2000). Besides, Tapia et al. (2007) reported that spore production and toxin formation of bacteria also depend on a_w .

Salt and sugar are widely used as additives in order to control bacterial growth by decreasing a_w of foods. Thus, less “free” water is available to accomplish metabolic needs of microorganisms. Besides, hiperosmolar solutions triggers in bacterial cells water efflux. To recover and maintain cellular turgor, *B. cereus* cell uptake ions using different osmoprotectant transporters (den Besten et al., 2009). Moreover, transcriptomic analysis of mildly and severely salt-stressed *B. cereus* showed upregulation of *sigB* and σ^B -dependent genes and also of genes encoding enzymes of central metabolism and others involved in chemotaxis and motility (den Besten et al., 2009).

2.4.2.4. Oxygen availability

B. cereus group are facultative anaerobic microorganisms. Therefore, in presence of oxygen these bacteria obtain energy from nutrient through cellular respiration. The energy obtained is used to synthesize adenosine triphosphate (ATP). Most of the ATP (approximately 90%) is produced by the oxidative phosphorylation. This metabolic pathway comprises the electron transport chain that establishes across the membrane a chemiosmotic gradient able to drive the phosphorylation of ADP. Finally, exogenous oxygen acts as acceptor of electrons and together with two protons, water is formed. In oxygen deprived environment, *B. cereus* isolates use nitrate as an electron acceptor (ammonification). In the absence of electron acceptors, these microorganisms perform a mixed-acid fermentation (Rosenfeld et al., 2005) to synthesize ATP. In this case, electrons are transferred from reduced equivalent to an internal electron acceptor such as pyruvate or acetyl coenzyme (Carlin et al., 2010).

Notably, it has been shown that *B. cereus* subjected to anaerobic atmospheres needs for proper growth carbohydrates in addition to amino acids. Besides, many authors have reported that nutritional adaptation to oxygen deprived environments is reflected in the expression of the pathogenicity of bacteria. Indeed, a lower Hbl enterotoxin production was observed during anaerobic growth on glucose than on fructose or sucrose (Carlin et al., 2010).

Samapundo et al. (2011), reported that in general, at lower initial dissolved O₂ levels slower maximum growth rate, longer lag phase duration and smaller maximum population density. Atmosphere packaging consists in obtain lowered amount of oxygen to limit bacterial growth of aerobic organism and to decrease oxidative reaction rates. Removed oxygen is commonly replaced by different gases such as nitrogen, an inert gas, or carbon dioxide. On this basis, this technology appears like an interesting option for food preservation in combination with other environmental factors such as heat treatments and refrigeration. Nevertheless, the application of limited oxygen atmospheres to control *B. cereus* group growth emerges a challenge due to the ability of these bacteria to adapt even to free-oxygen environments.

2.5. Methods for *B. cereus* group identification

“Reliable methods to detect pathogens in foods are required by government authorities, industry and researchers for trade, and for public health protection and surveillance ...In all cases the test methods must produce accurate, repeatable and reproducible (or at least consistent) and reliable results” (McMeekin et al., 2010).

Betts (2002) considered that requirements for detecting a target organism should be:

- Provision of a result with an acceptable time frame, perhaps even in real-time.
- Ease of use characteristics that allow routine application in industry, including being amenable to automation.
- To have undergone rigorous validation to allow comparison with “standard” methods.
- To meet the requirements of laboratory accreditation authorities and standards agencies.
- Cost-effectiveness in terms of both capital outlay and running costs.
- To provide a quantitative estimate of levels of the organism of concern.

B. cereus group identification usually include traditional culture-based techniques by using selective media, biochemical confirmations and other parameters such as spore morphology or presence of crystal protein. Nowadays, two international standards, ISO 7932:2004 and ISO 21871:2006, are recommended for the isolation, identification and enumeration of *B. cereus* in food. Both methods are based on polymyxin B resistance, on the inability to ferment mannitol and on the lack of lecithinase activity (Martínez-Blanch et al., 2009). The identification process is usually completed by using API strips, which are not sufficiently discriminative among the members of group *B. cereus*

These classical techniques are expensive, time consuming and may lead to misidentifications (Martínez-Blanch et al., 2009). On the other hand, microorganisms from complex food matrices, such as cheese or processed meat, are more difficult to

detect or quantify, even if enrichments are used. Likewise, still being a challenge direct extraction of low numbers of bacteria from food matrices (McMeekin et al., 2010).

Since the latest years, molecular techniques are being developed as an alternative to these traditional methods. Methods for phylogenetic analysis and isolate typing include multi-locus variable number of tandem repeat analysis (MLVA) (Keim et al., 2000; Ciammaruconi et al., 2008), microarrays (Doran et al., 2007; Zwick et al., 2008), whole-genome sequencing (Read et al., 2002), multilocus enzyme electrophoresis (MLEE) (Carlson et al., 1994; Helgason et al., 1998; Helgason et al., 2000a; Helgason et al., 2000b; Vilas-Boas et al., 2002), AFLP (Keim et al., 1997; Jackson et al., 1999; Ticknor et al., 2001; Radnedge et al., 2003; Hill et al., 2004; Guinebretiere et al., 2008) and multilocus sequence typing (MLST) (Helgason et al., 2004; Ko et al., 2004; Priest et al., 2004; Candelon et al., 2004; Sorokin et al., 2006; Tourasse et al., 2006; Tourasse and Kolstø, 2008; Cardazzo et al., 2008; Dielot et al., 2009; Hoffmaster et al., 2008) have been used. Recently, Tourasse et al. (2011) have developed an updated global snapshot of the *B. cereus* group population based on data of MLST and/or AFLP of closely related species profiles compiled on HyperCAT data base (Tourasse et al., 2010). Besides, Polymerase chain reaction-based fingerprinting methods have been applied including restrictive fragment length polymorphism (PCR-RFLP), repetitive sequence-based PCR (REP-PCR), enterobacterial repetitive intergenic consensus-PCR analysis (ERIC-PCR), randomly amplified fragment length polymorphism (RAPD) and PCR analysis of internal spacer region between 16S-23S rRNA genes (PCR-ISR) (Martinez Blanch et al., 2010).

These techniques have not proved to adequately identify all *B. cereus* group species due to the lack of species specific markers (Martinez Blanch et al., 2010). In addition, phylogenetic analysis highlights taxonomical complexity of *B. cereus* group species revealing high intraespecific diversity, especially in *B. cereus* and *B. thuringiensis*. Therefore, as discussed previously, on the basis of gene content and phylogenetic studies, identification of *B. cereus* species and strains remains a challenge and new techniques should be assessed.

2.6. Flow cytometry analysis

“The term ecophysiology suggests that a natural connection exists between microbial ecology and microbial physiology, the former being concerned with the responses of microbial populations to environmental influences, and the latter with activities within individual cells” (McMeekin et al., 2010).

In the latest decades, many data of microbial ecology have been obtained through the use of predictive models and have been stored in enormous databases. Nowadays, ecophysiology of food pathogens is considered as an essential knowledge to improve safety, and therefore there is a needed of link this quantitative data to those obtained at single-cell level (McMeekin et al., 2010).

“Besides, rapid detection of microorganisms in samples is one of the key questions to obtain real-time data for the development of more accurate quality control programs, more efficient and less time-consuming methods. Likewise, cell viability evaluation constitutes an issue of great importance in industrial microbiology since often, the industrial activity involves decision making of high economical impact, which is normally determined by the results obtained from cell viability tests” (Díaz et al., 2010).

In this regard, flow cytometry appears as an interesting technique since allow both the measurement of heterogeneity within a population and the analysis of individual microorganisms of the cell. Besides, it has proved to be more rapid than growth based methods providing real-time information on physiological status.

FCM quantitatively measures the optical characteristics of cells (or other particles) as they are presented in single file in front of a focused light (Veal et al., 2000). Many thousands of cells per second can be analyzed whilst are transported in the centre of the sheath flow where is a laminar flow of cells moving in single file. When samples are illuminated using high-pressure mercury vapour lamp or laser devices the light beam passes through the cell stream and at this point different detectors, one on line with the light beam, several perpendicular to it and one or more fluorescent detectors are used to measure three parameters: forward scatter (FSC), side scatter

(SSC) and fluorescence. Depending on the light scattered by cells it is possible to know cell size. Likewise, cell refractability is related with surface proteins and internal structure of cells given information about cell shape and cytoplasmic content (Gunasekera et al., 2003). Intrinsic fluorescent or fluorescent staining may be used to obtain information about specific component or physiological states, e.g. DNA content, protein content, cell membrane integrity or cytoplasmic esterase activity (Shapiro, 2003).

Figure 6. Scheme of a typical flow cytometer. Shortly, the hydrodynamic focusing gives rise to the formation of a single stream of particles in the flow cell (1). These cells pass through a laser beam and impact with the laser in a confined site, emitting different signals related to diverse cell parameters (2). Scattered and fluorescence emissions of each particle are separated by a group of filters and mirrors (optical system) according to certain wavelengths (3). Signals are collected by the detection system which is formed by a collection of photodiodes, two scattering (FS and SS) and three fluorescence (FL1, FL2, FL3) detectors, in this case. Finally, signals are sent to a computer obtaining a representation of the distribution of the population with respect to the different parameters. Abbreviations: FS, forward scatter, SS, side scatter (Diaz et al., 2010).

FCM has been used as a powerful, reliable and fast tool to assess the resistance and survival of *B. cereus* vegetative cells to acid and osmotic stress, antibiotics, food processing treatments, process equipment sanitation regimes (Cronin and Wilkinson, 2010), supercritical extracts (Muñoz et al., 2009) and essential oils (Paparella et al., 2008). Moreover, previous studies reveal this technique as an effective method of verifying the viability of microorganisms present in milk, wine and water (Porter et al., 1995; Gunasekera et al., 2000; Malacrinò et al., 2001).

CHAPTER 3

Survival of heat treated *Bacillus cereus* spores in vegetable puree under oxygen limitation conditions.

Abstract

Bacillus cereus spores may survive pasteurization and heat resulting in contaminated food production or processing systems, therefore causing a potential risk for foodborne disease. To increase knowledge of *B. cereus* spores heat resistance, the effect of heating medium was tested in sterile distilled water and vegetable purees at 90, 95, 100 and 105°C. Results showed that the heating medium tested had higher influence on bacterial heat resistance at higher temperatures and at lower ones. This effect appeared to be more correlated with pH of the menstruum than with food composition. As previously described, the presence of shoulders appeared frequently in the survival curves obtained. Shoulder length increased in vegetable purees. To study the presence and extension of shoulders and its impact on evaluation of sterilization processes, survival curves were fitted to two different non-log linear models (Weibull and Geeraerd) in order to compare them with the classical, log-linear regression. Root Mean Sum of Squared Errors (RSME) values revealed that heat inactivation of *B. cereus* spores was better characterized by Weibulls' and Geeraerds'. Finally, the effect of modified atmospheres in combination with mild (1D) and severe (4D) heat treatments was studied in broth and vegetable substrates. Besides anaerobic environment, two different packaging films with different oxygen transfer rates (OTR) ($35\text{cm}^3/\text{m}^2/24\text{h}$ and $10^{-3}\text{cm}^3/\text{m}^2/24\text{h}$) were used to obtain oxygen limitation conditions. Results revealed that *B. cereus* vegetative cells efficiently adapt to oxygen limitation although a faster growth was observed in broth. Likewise, *B. cereus* spores exposed to air or film with higher OTR showed major growth rate. Notably, no significant differences on the time to reach 10^6CFU/mL were found between food samples subjected to anaerobic environment and to lowest OTR ($10^{-3}\text{cm}^3/\text{m}^2/24\text{h}$).

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3.1. Introduction

“At present, thermal processing is still the most widely used method to preserve food” (van Zuijlen et al., 2010). Traditionally, it is supposed that during heat treatments, such as pasteurization, the rate of survivors decreases exponentially as a function of heating time when a bacterial population is subjected to the action of heat at a stable temperature. Based on this assumption, heat inactivation obeys a log linear law, from which decimal reduction time (D_T) can be derived. Official methods to calculate sterility use D_T values as measures of the organisms’ or the spores’ heat resistance at a given temperature (Periago et al., 2004).

Frequently, survival curves show deviations from the log linear behavior, resulting in shoulder and tail phenomena (Fernandez et al., 2001; van Zuijlen et al., 2010). Different factors have been pointed out as causative agents of this phenomenon. In 1990, Teixeira and Rodríguez reported that cell heat damage after treatments can restore their structures and metabolism under favorable conditions causing variations on survival curves. Fernandez and Peck (1999) observed that a subpopulation of non proteolytic *Clostridium botulinum* with a higher heat resistance in the presence of lysozyme caused curves with tailing. Recently, mechanistic details of bacterial spore resistance are being investigated as a major key to understand heat resistance heterogeneity. Coleman et al. (2007) reported that shoulder length seemed to be associated with a lethal damage to one or more key spore proteins.

Due to the presence of these “non log linear phenomena” the classical concept of inactivation modelling fails to assess accurately most of these survival curves (Geeraerd et al., 2005). In this context, overprocessing is often applied to products that have a prolonged shelf life to ensure food safety and prevent spoilage. This, in one hand, is unfavorable for the product quality, and in the other hand, increases energy consumption and CO₂ emission (van Zuijlen et al., 2010).

To model non-linear survival curves different mathematical solutions have been proposed. The “y intercept” concept proposed by Pflug (1982), takes into account only the linear portion of the survival curve, whilst Moats (1971) fitted survival curves using a multi-parameter description. Later, mathematical models based on frequency

distributions have been used to describe concave and convex survival curves based on the fact that survival graph is the result of the cumulative mortality over time and the shape of the curve depend on how heat resistance is distributed in the population (Mafart et al., 2002; Van Boekel, 2002).

B. cereus strains have important implications for the food industry causing spoilage and foodborne outbreaks. Due to their metabolic diversity and their ability to form highly resistant endospores these microorganisms are able to colonize a wide variety of habitats at a very broad range of growth temperatures. Members of this specie have been isolated from a large number of sources including ready to eat food products, such as vegetable purees (Carlin et al., 2000; Rajkovic et al., 2006a; Turner et al., 2006), causing emetic and diarrheal foodborne illnesses after consumption (Samapundo et al., 2011).

The molecular genetics of heat stress response of *B. cereus* involve a wide range of heat shock proteins (HSPs) among which are molecular chaperones such as DnaK, GroEL, CSpB and CspE and ATP-dependent proteases (ClpP) (Periago et al., 2002a). These HSPs mediate in folding, assembly, and repair and prevention of aggregation under stress and nonstress conditions maintaining quality control of cellular proteins (Periago et al., 2002a). Likewise, anti-sigma factor antagonist RsbV is also induced by heat shock indicating the activation of σ^B in *B. cereus* under these conditions (Periago et al., 2002a; van Schaik et al., 2004). In addition, HSPs homolog to stress proteins and other related with sporulation as well as proteins involved in metabolic process and rod shape-determining protein MreB are induced upon heat stress (Periago et al., 2002a).

Thus, bacterial heat resistance is partially determined by their genetic makeup but also by environmental factors (Stumbo, 1973). A relevant parameter for process evaluation is food composition, since it may influence microbial heat resistance (Condon and Sala, 1992). Mineral salts, pH, presence of organic acids, carbohydrates, proteins and fats should be taken into account to obtain realistic data of thermal inactivation in the food substrate.

On the other hand, ready-to-eat, cooked chilled or refrigerated processed foods of extended durability (REPFEDs) (Nissen et al., 2002) are subjected to mild heat

processing in order to minimize the damage of the organoleptic and nutritional properties of the food. However, this may further enhance the spore survival, and thus the persistence of spore formers in food production environments.

Combining hurdles can be used to achieve an overall level of protection in food stability and safety while minimizing impacts in food quality (Leistner and Gorris, 1995). In this regard, it is largely known that bacteria are more demanding with environmental conditions after heat treatment (Adams, 1978) and therefore, recovery of damaged cells is strongly influenced by physico-chemical factors such as pH, water activity or anaerobic atmospheres (Feeherry et al., 1987).

Modified atmospheres are extensively utilized in the food industry to ensure quality and extend the shelf-stability of food products on the basis that oxygen availability affects redox-dependent bacterial functions such as growth, metabolic pathways, radical formation and virulence gene transcription (Duport et al., 2004, 2006; Rosenfeld et al., 2005; Zigha et al., 2006, 2007; Esbelin et al., 2008, 2009; Mols et al., 2009). Nevertheless, Carlin et al. (2000) reported that growth of both obligate and facultative anaerobes may occur in presence of residual oxygen in vacuum packages or modified atmospheres. In fact, *B. cereus* is a facultative anaerobic organism which grows in oxygen-deprived environments by two different pathways, respiration using nitrate as an electron acceptor (ammonification) and mixed-acid fermentation in the absence of electron acceptors with lactate, acetate, formate, ethanol, succinate, pyruvate and traces of 2,3-butanediol as major products (Rosenfeld et al., 2005; Zigha et al., 2007). Anaerobic growth under nitrate respiratory conditions with either glucose or glycerol as carbon source is preferred over fermentation in *B. cereus* (Zigha et al., 2007).

Regulatory metabolic mechanisms in response to oxygen limitation involve the two-component system ResDE, composed of a membrane-bound histidine sensor kinase (ResE) and a cytoplasmic response regulator (ResD), and the conserved Fnr protein among *B. cereus* group microorganisms, member of the Crp/Fnr (cyclic AMP-binding protein/fumarate nitrate reduction regulatory protein) family of helix-turn-helix transcriptional regulators. *B. cereus* Fnr plays a moderate role in anaerobic nitrate respiration meanwhile is essential for anaerobic fermentation conditions (Zigha et al.,

2007). Besides, *fnr* expression is also essential for Hbl and Nhe production with or without presence of oxygen (Zigha et al., 2007). For its part, ResDE is not essential for enterotoxin expression (Esbelin et al., 2008) and was found to play a major role in *B. cereus* growth under low oxidoreduction conditions (Duport et al., 2006). Recent studies (Esbelin et al., 2009) have reported that ResDE acts as a sentinel system capable of sensing redox changes and coordinate a response that modulates *B. cereus* virulence through a signaling-transduction process in which phosphorylated ResD may act as a coactivator of Fnr forming transcriptionally active complexes (ResD~P-Fnr-*pnhe-phbl*), meanwhile unphosphorylated ResD could interact with Fnr to form inactive complexes (ResD-Fnr-*pnhe-phbl*). This finding suggests that the regulatory pathway controlling the catabolic genes expression under anaerobic conditions in *B. cereus* could be similar to this one due to the interplay between fermentative growth and virulence in *B. cereus* with Fnr as major regulatory protein.

Most of the previous studies on stress response have been conducted in the presence of oxygen, and up to date, the lack of data on *B. cereus* growth under oxygen limited conditions, such as modified atmospheres, highlights the need of novel studies on this matter, maximum if they are performed on real foods and taking into account that such conditions enhance bacterial virulence.

In this paper, we carried out a dual work that focused on *B. cereus* heat resistance studies and assessment of the combined effect of heat treatment and modified atmosphere on *B. cereus* growth. In order to establish the effect of food substrate on the heat resistance of spores, two commercial purees with different nutritional composition, courgette and vegetable purees, were tested as heating media.

3.2. Materials and methods

3.2.1. Microorganism

The strain used in the experiment was *B. cereus* INRA-AVZ421, kindly provided by the National Institute of Agronomic Research (INRA, Avignon, France). This microorganism was isolated from refrigerated processed foods of extended durability based on vegetables.

B. cereus spores were activated at 80°C during 10 minutes (Fernández et al., 2001) to ensure that subsequent vegetative cells grown would have the same physiological state. Next, an overnight culture was performed in Brain Heart Infusion broth (BHI; Scharlau, Barcelona, Spain) at 30°C. Aliquots of this culture were plated onto Brain Heart Infusion agar (BHIA; Scharlau, Barcelona, Spain) to test the absence of bacterial contaminations.

Following the procedure described by Mazas et al. (1995), sporulation was carried out in Fortified Nutrient agar (FNA) containing (per liter) 20 g of agar, 13 g of nutrient broth, 3 g of NaCl, 0.1g of glucose, 0.08 g of (NH₄)₂SO₄, 0.06 g of CaCl₂, 0.05 g of MnSO₄.H₂O, 0.008 g MnCl₂.4H₂O, 0.005 g of CuSO₄.5H₂O and 0.005 g ZnSO₄.7H₂O; pH 7.

Four *B. cereus* INRA-AVZ421 colonies were selected and resuspended in 100 mL of sterile saline solution 0.85%. Aliquots of 2 mL of this dilution were spread on FNA plates and incubated face up at 37°C for 3-5 days.

After that time, 90% of sporulation was obtained as assessed by phase contrast microscopy (Leica DMLS, Mc Bain instruments, Wetzlar). Spores were collected by flooding the surface of the plate with sterile distilled water. After harvesting, spores were washed three times by centrifugation and resuspended in sterile distilled water.

The spore suspension (10⁸-10⁹ spores/mL, assessed by microscopic count with a Neubauer counting chamber) was stored at -20°C in sterile distilled water. Heat resistance of the stored spore suspension remained unchanged during the time that the experiment was performed.

3.2.2. Food substrates for heat resistance studies

To study the effect of heating medium on *B. cereus* heat resistance two commercial vegetable purees were selected on the basis of their composition (Table 1).

Courgette puree (CP) had the following composition: water, courgette 17%, potato, onion, extra virgin olive oil, corn starch, milk protein, salt and aromas.

Mixed vegetables puree (MVP) had the following composition: water, vegetables 45% (potato, celery, leek, broccoli, carrots, spinach, onion, green beans, watercress, peas, chervil and dock), butter, modified starch, salt, sugar, yeast extract, aroma and vitamins (C, B₆ y B₉).

The products were stored refrigerated at 4°C until use.

The average pH values of these products was measured in triplicate using a pH and ion-Meter GLP22 equipment (Crison, Barcelona, Spain), at room temperature.

3.2.3. Heat treatments

Heat resistance of sporulated *B. cereus* cells was determined with a thermoresistometer Mastia (Universidad Politécnica de Cartagena, Cartagena, Spain) (Figure 1A.) (Conesa et al., 2009), both in sterile distilled water and in the food matrices tested, in isothermal conditions.

This equipment consists of a main vessel made of stainless steel in which heat treatments are applied (Figure 1B.). The caliber of this container is 400 mL and its external measurements are 8.5 cm in diameter × 12 cm height, with a screw cap, closed through an o-ring seal. The cap features a tube that guides the stirring shaft, which is equipped with a propeller at its lower end and is driven by a motor. The lid also houses seven ports: two are for the arms of the electrical resistance, two for the arms of the cooling coil, one for a Pt-100 temperature sensor which determines the temperature during treatment, one for injection of microorganisms and a final for the sampling tube. Finally, the lid is equipped with a rapid pressure connector through which the instrument is connected to the external pressure source (cylinder of dry nitrogen).

The motor speed is regulated by a variable-frequency drive (VFD) through a programmable logic controller (PLC). The homogeneity is favored by the presence inside of the instrument of a baffle screen that enhances turbulence. The sampling tube is stainless steel, and is prolonged in its end by a silicone tube. The latter is closed by a pinch solenoid valve. The opening can be operated via a switch on the main control unit. The solenoid valve, normally closed, held closed extraction tube, even when the

main vessel is pressurized, and only opens when it is operated from the main unit for sampling. The opening time can be regulated by a timer via PLC. The PLC also allows control of temperature. For this purpose has a proportional-integral-derivative controller (PID) connected to an electrical resistance of 2100W, to a valve that regulates the flow of cooling water through the coil, and to the Pt-100 temperature sensor. The PID and the sampling valve and motor agitation are controlled by a PLC which allows heating ramps and complex temperature profiles. The controller can be connected to a computer and allows recording data of temperature and treatment duration.

Figure 1. Thermoresistometer Mastia (UPCT) (A) Equipment overview (B) Detailed view of the main vessel.

The instrument can be kept under pressure due to the existence of boards of Teflon (PTFE) on all ports, including the shaft. The micro-injection port is closed by a chromatography septum. External pressure is provided by a cylinder of dry nitrogen and is regulated by a pressure regulator. This pressure allows the extraction of samples at low temperatures (below 100°C) or when the heating medium is too viscous. Maintaining a constant pressure of work throughout an experiment and an opening time

of the solenoid valve also constant, allows to obtain samples of the same volume throughout the experiment. The sample injection can be performed using sterile medical syringe or using a Hamilton syringe (Hamilton Company, Nevada, USA) when the instrument has been under pressure.

The heating of micro-organisms is almost instantaneous and substrates can be liquid, or viscous fluids, or liquids containing particles. Its design allows working at temperatures up to 140°C, simulating heating and cooling ramps and sterilized within the substrate used.

Heat resistance studies were performed in distilled sterile water and vegetable purees. Once the medium was sterilized inside the main vessel and operating temperature was stabilized, 0.2 mL of *B. cereus* spores suspension (10^8 - 10^9 spores/mL) activated at 80°C during 10 min were injected by using Hamilton syringe. At specific time intervals, which depended on the treatment temperature (90, 95, 100 and 105°C), samples were collected into sterile tubes, immediately diluted and plated onto BHIA.

3.2.4. Incubation and enumeration of survivors after heat treatments

Incubation of plates for survivor counting was carried out at 37°C for 24 h. Previous experiments showed that longer incubation times did not modify significantly the profile of survival curves (Palop et al., 1999).

After incubation, Petri dishes with a microbial concentration in the range of 30 and 300 colonies were selected for survival counting.

3.2.5. Data analysis

To obtain an accurate assessment which allows us to design safety processing treatments avoiding those which are overly conservative, survival curves were fitted using Geeraerd and Van Impe Inactivation Model Fitting Tool (GInaFIT; Geeraerd et al., 2005). Through traditional log-linear model (Bigelow and Esty, 1920), log-linear model with shoulder (Geeraerd et al., 2000) and Weibull model (Mafart et al., 2002) *B. cereus* spore heat resistance was described.

In static conditions log-linear model can be written as follows (Geeraerd et al., 2005):

$$\log_{10}(N) = \log_{10}(N(0)) - \frac{k_{\max} t}{\ln(10)} \quad (1)$$

where N represents the microbial cell density, expressed in, for example, CFU/mL, $N(0)$ the initial microbial cell density (CFU/mL), $k_{\max}$ (1/time unit) the first order inactivation constant and t is time (time unit).

This model is based on the assumptions that all cells in a population have equal heat sensitivity and that the random chance of death of a microorganism depends on the heat that receives a key molecule or “target” within it (Cole et al., 1993).

On the other hand, log-linear model with shoulder has been successfully applied to survival data previously (Geeraerd et al., 2000). For the parameter estimation by GinaFiT, a model derived from the following format was used by setting N_{res} equal to zero (Geeraerd et al., 2005):

$$\begin{aligned} \log_{10}(N) = \log_{10} & \left[\left(10^{\log_{10}(N(0))} - 10^{\log_{10}(N_{\text{res}})} \right) \times e^{-k_{\max} t} \right. \\ & \left. \times \left(\frac{e^{k_{\max} S_l}}{1 + (e^{k_{\max} S_l} - 1) \times e^{-k_{\max} t}} \right) + 10^{\log_{10}(N_{\text{res}})} \right] \end{aligned} \quad (2)$$

where N is the microbial cell density (CFU/mL), $N(0)$ is the initial microbial cell density (CFU/mL), N_{res} is the residual population density (CFU/mL) which corresponds to a more heat resistant subpopulation, $k_{\max}$ is the specific inactivation rate (1/time unit), S_l (time unit) a parameter representing the shoulder and t is time (time unit).

Finally, the Weibull model, which is applied to describe microbial inactivation, is based on “the cumulative form of the asymmetric Weibull probability density function for the heat resistances of individual microbial cells” (Geeraerd et al., 2005). Unlike log-linear model with shoulder, in this model the shoulder is not included a priori, but emerges “due to the fact that the distribution reflecting the spectrum of resistances has a large mean relative to its variance” (Peleg, 2000).

In GInaFiT, the version selected was the one proposed by Mafart et al. (2002) which can be formulated as follows:

$$\log_{10}(N) = \log_{10}(N(0)) - \left(\frac{t}{\delta}\right)^p \quad (3)$$

where δ (time unit) is a scale parameter and can be denoted as the time for the first decimal reduction if $p=1$, and p is a shape parameter. For $p>1$, convex curves are obtained, while for $p<1$, concave curves are described. Besides, N is the microbial cell density (CFU/mL), $N(0)$ is the initial microbial cell density (CFU/mL) and t is time (time unit).

Maximum inactivation rate (K_{max}) was obtained from the log-linear model. Besides, from log-linear model with shoulder, the specific inactivation rate (K_{max}) and the shoulder length were determined. From Weibull model δ and p parameters were obtained.

RMSSE (Ratkowsky, 2003) was selected as a measure of goodness-of-fit. The magnitude of RMSSE should be comparable to the standard deviation (as a measure of the precision of replicates) of the experimental data (Geeraerd et al., 2000).

3.2.6. *Heat treatment before exposure to modified atmosphere*

All heat treatments were conducted in sterile distilled water at 100°C in a thermoresistometer Mastia. As described above, once the heat treatment temperature had reached 100°C, a 0.2 mL volume of the spore suspension was injected into the medium. Samples were exposed to heat temperature, 100°C, for 3 minutes to obtain a 1D reduction (mild treatment) and for 14 minutes to obtain a 4D reduction.

3.2.7. *Anaerobic packaging*

Packaging was carried out inside of an anaerobic cabinet Whitley VA500 Workstation (D.W. Scientific, West Yorkshire, UK). To obtain atmospheres of 0% oxygen, air was withdrawn and replaced by pure N₂. Anoxicity was checked periodically using a Check Point PBI Dansensor (PBI Dansensor, Ringsted, Denmark). Besides, resazurin strips were used as an additional method to test atmosphere inside cabinet.

Two packaging films, laminated aluminium oxide coated PET (AlOx) and a four layer PA/PE-foil with an EVOH barrier (EVOH) (Figure 2), were used to make plastic bags with different oxygen permeability. The combination of hybrid polymer coatings with thin inorganic oxidic layers made of AlOx (Figure 2A) was very effective to achieve extremely low OTR values below $10^{-3} \text{ cm}^3/\text{m}^2/24\text{h}$ (Amberg-Schwab et al., 2006). To obtain higher oxygen permeation, approximately $35 \text{ cm}^3/\text{m}^2/24\text{h}$ (Vicik, 1995), four layer PA/PE-foil with an EVOH barrier was used (Figure 2B).

The two packaging films evaluated were commercially available multilayer laminates (Figure 2) currently used in different heat treated products. The film depicted in Figure 2A is used for packaging of low and high acid ambient stable s/sauces. The film shown in Figure 2B is used for packaging different products such as pasteurised sausages.

Figure 2. Schematic representation of multilayer structure of packaging materials evaluated in this study **(A)** Laminated aluminium oxide coated PET **(B)** Four layer PA/PE-foil with an EVOH barrier. AlOx corresponds to aluminium oxide, PET corresponds to polyethylene terephthalate, OPA corresponds to biaxially oriented polyamide, cPP corresponds to cast polypropylene, PA corresponds to polyamide, EVOH corresponds to ethylene-vinyl alcohol and PE corresponds to polyethylene.

Polymers were sealed to obtain bags with the following measures: 8 cm wide x 6.5 cm high (Figure 3).

Figure 3. Example of EVOH plastic bag used to obtain oxygen limited conditions. Black arrows indicate bag measures.

Depending on atmosphere composition and packaging, three different limited oxygen conditions were tested:

- Anaerobiosis: ALOx bag incubated into an anaerobic jar.
- Impermeable: ALOx bag.
- Permeable: EVOH bag

Besides, two controls were established, one in the absence of heat treatment (indicated as control) and a positive control for the atmosphere (indicated as air), which consists in an aerobic culture incubated in a flask sealed with leaky parafilm.

B. cereus growth in all conditions (anaerobiosis, impermeable, permeable and air) was tested in culture medium and food. Aliquots of 15 mL of BHI and courgette puree were transferred into plastic bags and flask, depending on the condition tested, inoculated with 0.1 mL of spore suspension 1D and 4D and sealed. Impermeable condition samples were introduced in an anaerobic jar during incubation period and resazurine strips were used to test reduced atmosphere.

3.2.8. Assessment of oxygen availability impact during incubation

BHI and CP samples were incubated at 30°C. At appropriate time intervals viable counts and pH were measured.

Modifications on pH values during incubation period were checked using a pH Meter Basic 20 (Crison, Barcelona, Spain). Measurements were performed in triplicate.

To estimate bacterial growth viable plate count method (VPC) was applied. Periodically, aliquots of samples were plated onto BHIA from 10-fold dilutions in buffered peptone water (Scharlau, Barcelona, Spain). After incubation at 37°C for 24 hours, colonies were counted. Given that *B. cereus* minimum infective dose is 10^5 UFC/mL (Samapundo et al., 2011), samples were spread at appropriate intervals until counts reached 10^5 - 10^6 UFC/mL.

3.3. Results and discussion

3.3.1. Effect of heating medium on heat resistance of *B. cereus* under isothermal conditions

To study the effect of the food composition on heat resistance of *B. cereus*, different isothermal treatments at 90, 95, 100 and 105°C were tested in sterile distilled water (SDW), CP and MVP. Heat treatments were performed at least in triplicate. The isothermal curves are presented in Figure 4. Each value in plot is the arithmetic mean of three counts.

Table 1. Nutritional information of vegetable food substrates (values per 100 g of products) and pH values.

Food substrates	Food energy (Kcal)	Proteins (g)	Carbohydrates (g)	Fats (g)	pH
Courgette puree	42	1.5	3.5	2.5	5.85
Mixed vegetables puree	32	1.0	5	0.9	5.3

Figure 4. Survival curves at 90 (violet), 95 (blue), 100 (orange) and 105°C (green) of *B. cereus*. (A) Survival curves in sterile distilled water (B) Survival curves in courgette puree (C) Survival curves in mixed vegetables puree.

As shown in figure 4, for the same temperature heating medium had bigger influence on spores heat resistance at higher (105°C) and lower (90°C) temperatures. At 90°C food samples showed lower heat resistance in comparison with SDW, especially samples tested in MVP. However, at 105°C heat resistance of *B. cereus* in CP and MVP was approximately 2 folds higher related to SDW samples. At 95 and 100°C there were no significant differences on *B. cereus* spores heat resistance in the substrates tested.

During heat treatments at 90°C there is a long exposition period (1 hour) of bacterial spores to the physicochemical factors of the heating medium. Different authors have reported that the pH of the heating substrate has a strong influence on *Bacillus* spp survival subjected to thermal treatments causing a decrease on heat resistance the more acid the pH (Palop et al., 1996). As was described above (Table 1) CP and MVP showed lower pH values compared to SDW (5.85 and 5.3, respectively). According to this, pH effect may explain this effect at 90°C. However, at 105°C, heat treatment duration is too short (2.5 minutes) to allow the heating medium with pH values in a range between 5 and 6 cause significant effects on *B. cereus* spores. Accordingly, Palop et al. (1996) reported that the decrease in *Bacillus licheniformis* heat resistance at acid pHs became smaller the higher the temperature of treatment. In this context, the results suggest that differences in resistance may be due to heating medium properties other than pH. In 1992, Condón and Sala reported that the composition of the heating medium can influence the heat resistance of microorganisms. They found that heat resistance of *Bacillus subtilis* in food at its natural pH was somewhat different than that in buffer of the same pH. Likewise, other properties such as viscosity affect heat transfer within the heating medium and can exercise a protector effect of bacterial spores when viscosity increases, especially in short treatments. On the other hand, no significant differences between courgette and mixed vegetables purees were observed. So we cannot conclude that composition (proteins, carbohydrates and fats) affects heat resistance.

Survival curves corresponding to heat treatments at 90, 100 and 105°C showed shoulders in every heating medium (Tables 2, 3 and 4). At 95°C only in mixed vegetables puree shoulder were showed (Tables 2, 3 and 4). Shoulder length increase when *B. cereus* spore heat resistance was tested on vegetable purees (particularly upon heating spores in courgette pure). In 2010, van Zuijlen et al. reported that when spores

were heated in different commercial s as food substrates the effect of heating menstruum on the extent of shoulder length was evident.

3.3.2. Comparison of log-linear vs. non-log-linear kinetics to describe survivor curves

In this study, the phenomenon of the presence of shoulders in the thermal inactivation of *B. cereus* spores was described. Different models were used to fit survivor curves. Both the non-log-linear models adapted from Weibulls' and Geeraerds' models describe data points satisfactory in most cases (Figure 4).

Table 2. Comparison of log linear vs. non-log-linear kinetics to describe survivor curves obtained in sterile distilled water.

Model	Parameter	Temperature (°C)			
		90	95	100	105
Log linear	Kmax	0.06	0.14	0.69	4.07
	RMSE	0.1	0.066	0.18	0.27
Geeraerd	Kmax	0.07	- ^a	1.19	4.61
	SL	7.42		2.09	0.38
	RMSE	0.097		0.14	0.22
Weibull	δ	39	16.69	3.97	0.74
	p	1.16	0.93	1.86	1.22
	RMSE	0.097	0.07	0.13	0.247

-^a No shoulder present

Table 3. Comparison of log linear vs. non-log-linear kinetics to describe survivor curves obtained in courgette puree.

Model	Parameter	Temperature (°C)			
		90	95	100	105
Log linear	Kmax	0.07	0.16	0.69	4.18
	RMSE	0.18	0.05	0.17	0.12
Geeraerd	Kmax	0.10	- ^a	1.27	5.03
	SL	18.57		2.25	0.22
	RMSE	0.1100		0.09	0.08
Weibull	δ	41.40	13.61	4.01	0.66
	p	1.5	0.86	2.01	1.31
	RMSE	0.12	0.04	0.09	0.08

-^a No shoulder present

Table 4. Comparison of log linear vs. non-log-linear kinetics to describe survivor curves obtained in mixed vegetable puree.

Model	Parameter	Temperature (°C)			
		90	95	100	105
Log linear	Kmax	0.07	0.23	0.84	3.05
	RMSE	0.133	0.12	0.18	0.2848
Geeraerd	Kmax	0.08	0.29	1.23	4.29
	SL	11.5	3.33	1.65	0.77
	RMSE	0.1151	0.117	0.16	0.14
Weibull	δ	39.02	11.18	3.52	1.22
	p	1.27	1.28	1.74	1.7
	RMSE	0.1151	0.12	0.15	0.15

In sterile distilled water and in both vegetable purees, Geeraerds' and Weibulls' models gave consistently slightly lower RSME values when compared with the log-linear model (Tables 2, 3 and 4). The models of Weibull and Geeraerd better characterized the heat inactivation of spores of *B. cereus* than the conventional log-linear model. This is in agreement with van Zuijlen et al. (2010). On the basis of these results and in accordance with Geeraerd et al. (2005) it is clear that the log-linear inactivation models fails to asses accurately the majority of these survival curves. In this regard non-log-linear models should be implemented to prevent overlay conservative heat treatments which affect food quality, and to reduce energy requirements and subsequent emission of contaminants such as CO₂. In this context, as previously suggest van Zuijlen et al. (2010), we propose to that take into account systematically shoulder effect to establish safe.

$K_{\max}$ values did not show significance differences between different heating media and between different models.

3.3.3. Combined effect of heat treatment and modified atmosphere on B. cereus growth

Here, the *B. cereus* growth response to different oxygen limitation conditions combined with mild (1D) and severe (4D) heat treatments was studied in BHI and courgette pure. As described above, samples were packaged after heat treatments and

incubated at 30°C. Two controls were established, one in the absence of heat treatment (indicated as control) and a positive control for the atmosphere, recovering in the presence of air (indicated as air). Aliquots of samples were spread at appropriate intervals onto BHIA plates.

When heat treated samples were recovered in air, maximum growth occurred within 24 h, independently of the severity of the heat treatment applied (Table 5). In the more permeable plastic tested (EVOH) maximum growth took place within 24 h for the unheated control and the mild inactivation treatment (1D) whereas it was slightly delayed for the severe heat inactivation (4D), where the maximum was reached between 24 and 48 h (Table 5). In the more impermeable bag tested (AlOx), both heat treatments showed a delay in the onset of growth. That effect was more evident for the severe heat treatment, where the maximum growth was reached after 5 days (Table 6). In anaerobic conditions, growth was delayed for both heat treatments similarly, where the maximum was reached after 5 days of incubation (Table 6).

Table 5. Effect of incubation in air or permeable flexible bag on log counts (CFU/mL) of heat-treated *B. cereus* spores in BHI.

Time (h)	Air			Permeable (EVOH bag)		
	Control	1D	4D	Control	1D	4D
0	5.26	2.75	2.18	5.25	2.75	2.18
24	7.75	7.57	7.32	7.23	6.72	6.58
48	6.55	6.46	6.64	6.59	4.90	7.26
120	- ^a	6.29	6.36	4.95	4.26	3.5

^aNot determined

Table 6. Effect of incubation in rigid impermeable bag and anaerobiosis on log counts (CFU/mL) of heat-treated *B. cereus* spores in BHI.

Time (h)	Impermeable (AlOx bag)			Anaerobiosis		
	Control	1D	4D	Control	1D	4D
0	5.26	2.75	2.18	5.26	2.75	2.18
24	6.88	6.40	5.85	7.01	5.04	5.59
48	5.86	7.87	6.58	6.19	6.78	7.2
120	- ^a	-	7.45	-	7.42	7.22

^aNot determined

When *B. cereus* spores were inoculated in courgette puree, slower growth was observed in all cases. In the EVOH flexible bag, maximum growth occurred after 48 h (after a heat treatment of 1D) or 5 days (for a heat treatment of 4D) (Table 7). In the AlOx film (the more impermeable one), growth did not exceed 6 log cycles after 5 days, when it was observed after 48 h (for 1D) or 5 days for a 4D kill (Table 7). And in the anaerobic conditions, similar growth was observed for heat treatments equivalent to 1 and 4 log kills, with slow growth not exceeding 6 log cycles after 5 days (Table 8).

Table 7. Effect of incubation in air or permeable flexible bag on log counts (CFU/mL) of heat-treated *B. cereus* spores in courgette puree.

Time (h)	Permeable (EVOH bag)			Impermeable (AlOx bag)		
	Control	1D	4D	Control	1D	4D
0	5.26	2.75	2.18	5.26	2.75	2.18
24	5.73	4.36	4.13	5.65	3.53	3.75
48	6.85	6.18	5.38	6.48	5.15	4.65
120	5.60	5.24	6.83	5.97	5.95	5.60

Table 8. Effect of incubation in air or permeable flexible bag on log counts (CFU/mL) of heat-treated *B. cereus* spores in courgette puree.

Time (h)	Anaerobiosis		
	Control	1D	4D
0	5.26	2.75	2.18
24	4.52	3.67	3.08
48	6.53	5.07	4.71
120	6.13	5.46	5.32

Under limited oxygen conditions *B. cereus* activates a large number of alternative metabolic pathways and alternative respiratory chains, as shown for instance by the induction of mixed acid fermentation, the arginine deiminase pathway and alternative respiration (van de Voort and Abee, 2009). It has been shown that in general, the lower the initial dissolved O₂ level the slower the maximum growth rate, the longer the lag phase duration and the smaller the maximum population density (Samapundo et al., 2011). Van de Voort and Abee (2009) reported that *B. cereus* ATCC 14579 cultured in BHI at 30°C during approximately 10 hours showed specific growth rates of 0.22 and 0.011 (min⁻¹), resulting in generations times of 31.9 and 61.9 minutes for aerobic and anaerobic cultures, respectively. In our study heat untreated samples inoculated in BHI reached 10⁶-10⁷ CFU/mL after 24 hours of incubation at 30°C for all the conditions tested. These results indicate that *B. cereus* vegetative cells efficiently adapt to different oxygen limitation environments in vegetable puree resuming rapidly their growth and without affect final biomass yield. However, studies performed by Rosenfeld et al. (2005) and Mols et al. (2009) reported that growth rate and yield were lower for *B. cereus* cells subjected to anaerobic and microaerobic conditions in comparison to those grown in aerobic cultures. As previous studies suggest, further experiment are required to assess the existence of differences on lag phase duration and maximum specific growth rate.

The dissolved O₂ level combined with severe heat treatments can play a very important role in the growth of *B. cereus* on vegetable puree products packaged under modified atmospheres. As we expected, more restrictive atmospheres combined with severe heat treatments showed the greatest delay on the time to reach 10⁵-10⁶ CFU/mL. When samples were incubated in bags made with EVOH polymer which has a high OTR (35cm³/m²/24h), faster growth was observed in comparison with more oxygen limiting conditions. This implies that heat treated products packaged in atmospheres which allow high dissolved O₂ level have shorter shelf-stabilities as *B. cereus* growth would take a shorter time to reach the minimum infective dose. Moreover, bacterial growth observed in AlOx bag which has a very low OTR (below 10⁻³ cm³/m²/24h) did not show significant differences with growth observed under anaerobic conditions, which is in agreement with different studies (Nakano et al., 1996; Reents et al., 2006; Mol et al., 2009b). Mols et al. (2009) reported that the growth of *B. cereus* in microaerobic conditions was shown not to be significantly different from anaerobic culturing possibly due to the rapid depletion of oxygen by the metabolism of the bacteria and the absence of oxygen influx during growth. On the basis of these results, film packaging containing AlOx coating may be used as an alternative to anaerobic packaging in order to prevent *B. cereus* growth in heat treated vegetable products avoiding health risks associated to anaerobic microflora although other risks should be also considered.

The observation that *B. cereus* growth in courgette puree was slower than in BHI was not unexpected since high viscosity of the vegetables product limit the mobility of the O₂ at gas-puree interface and in the purees themselves (Samapundo et al., 2011). Notably, food samples packaged by using EVOH film showed a significant delay on the time to reach 10⁵-10⁶ CFU/mL respect to BHI samples incubated in the same conditions.

Despite this study shows how combination of oxygen limitation and deprivation and heat treatments delay *B. cereus* growth at 30°C up to five days, further experiments are needed to assess aluminium oxide coated PET and four layer PA/PE-foil with an EVOH barrier as packaging films able to generate appropriate conditions to extent vegetable puree shelf-life. In addition, to get and overall overview of this preservative technique, cross-protection phenomena and induction of virulent status concomitant to

limited oxygen environments should take into account. Recently, Mols et al. (2009) have reported that *B. cereus* vegetative cells become more resistant to heat and acid when grown under these conditions. Besides, a previous study performed by George et al. (1998) using *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enteritidis* showed how oxygen limitation have a positive effect on the recovery of sub-lethally injured cells.

CHAPTER 4

Combined effect of lysozyme and nisin at different incubation temperatures and mild heat treatment on the probability of time to growth of *Bacillus cereus*

Abstract

Stochastic models can be useful to predict the risk of foodborne illness. The presence of *Bacillus cereus* in liquid egg can pose a serious hazard to the food industry, since a mild heat treatment cannot guarantee its complete inactivation. However, most of the information available in the scientific literature is deterministic, including growth of *B. cereus*. In this chapter, a stochastic approach to evaluate growth of *B. cereus* cells influenced by different stresses (presence of nisin and lysozyme separately or in combination) was performed, using an individual-based approach of growth through optical density measurements. Lag phase duration was derived from the growth curves obtained. From results obtained, histograms of the lag phase were generated and distributions were fitted. Normal and Weibull distributions were ranked as the best fit distribution in experiments performed at 25°C. At 16°C, lag values (obtained in presence of combinations of both antimicrobials) were also fitted by Gamma distribution. Predictions were compared with growth curves obtained in liquid egg exposed to mild heat, nisin and/or lysozyme to assess their validity.

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4.1. Introduction

Among *Bacillus cereus* group, *B. cereus* is an ubiquitous sporulated bacteria able to grow at 7°C or below (Carlin et al., 2006). Besides, spores of this microorganism are strongly adhesive and may survive to sanitizing and heat treatments such as gamma-ray and pasteurization (Kotiranta et al., 2000). Previous studies reported that *B. cereus* spores were highly resistant to temperatures in the range of 90-100°C with $D_{90^{\circ}\text{C}}$ values of, *f. e.g.* 3-5 minutes (Fernández et al., 2001). As consequence, after food processing, germination can occur resulting in spoilage and foodborne outbreaks even in refrigerated products (Valero et al., 2003). Indeed, *B. cereus* has been easily isolated from many types of foods, especially of plant origin, but also from meat, eggs and dairy products (Kramer and Gilbert, 1989).

A recent study reveals that whole egg appears to be a good medium for the development of psychrotrophic strains of the *B. cereus* group (Jan et al., 2011). Unlike in the dairy industry, only a limited number of studies have been focused on the relevance of these microorganisms in the egg product industry (Baron et al., 2007; Pina-Pérez et al., 2009; Jan et al., 2011) even when are commonly consumed by food manufacturers and caterings due to a wide variety of advantages among which are bacteriological safety, versatility, easy to use and dosing, saving time and labor, easy storage and control of sell-by date, as well as easy distribution and international trade (Instituto de Estudios del Huevo, 2006).

Because of the higher thermo-sensitivity of constitutive proteins, heat treatment for liquid egg preservation should be mild. Spanish egg product industry, following European regulations, exposes egg products to treatments comprised between 64-65°C for 120-240 seconds or 70°C for 90 seconds (Instituto de Estudios del Huevo, 2006). However, these sanitizing treatments that ensure the destruction of major vegetative cells, including microorganisms of public health importance such as *Salmonella*, may enhance the spore forming bacteria survival, and thus their persistence in food production environments. Besides, the storage period at refrigeration temperatures may lead to the selection of a psychrotrophic flora able to survive to thermal processing giving rise to vegetative cells that can grow at low temperatures (Jan et al., 2011).

In this context, egg product industry requires suitable food preservative systems to prevent health risks and economical problems derived from food spoilage. A combination of factors can be efficient to ensure microbial safety and maintain sensorial, functional and technological properties of this mildly processed food. It is widely known that hurdle technology advocates the combination of various antibacterial techniques in order to drastically limit the growth of spoilage and pathogenic bacteria (Leistner, 1999). Some food preservation systems can be used in combination with heat treatments to reduce the risk of outbreaks of *B. cereus* food poisoning. However, these systems may have undesired effects which are against manufacturers' and consumers' demands, who prefer additive-free, fresher and more natural tasty food products (Dillon and Board, 1994) while maintaining microbiological safety (Periago and Moezelaar, 2001). On this ground, the use of natural antimicrobial compounds, such as hen egg white lysozyme (HEWL) and nisin, can answer technological needs for egg products preservation.

HEWL is a natural protein present in egg (30 g/kg albumen). This antimicrobial compound, with specificity for bacterial cell walls, is used in food and pharmaceutical industries and is Generally Recognised As Safe (GRAS). According to the general norm of Codex Alimentarius for Food Additives (Codex General Standard for Food Additives, 2011) established by the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO), HEWL can be used as preservative agent in wine and cider and pear cider with a maximum dose of 500 mg/L as well as in ripened cheese following good manufacturing practices. Different studies have reported that HEWL is effective as a preservative of cheeses, cow's milk, beer, fresh fruits, vegetables, fish, meat and wine (Makki and Durance, 1996; Gill and Holley, 2000; Nattress et al., 2001; Nattress and Baker, 2003; Tribst et al., 2008).

“Lysozymes (EC 3.2.1.17) are 1,4- β -N-acetylmuramidases, cleaving the glycosidic bond between the C-1 of N-acetylmuramic acid (NAM) and the C-4 of N-acetylglucosamine (NAG) in the bacterial peptidoglycan (PG)” (Nakimbugwe et al., 2006). As PG of Gram-positive bacteria are directly exposed, most of them are sensitive to HEWL. However, it has been reported that some modifications of PG structure, such as N-acetylation of glucosamine (Zipperle et al., 1984) and O-acetylation of the C-6 hydroxyl group of NAM residues (Clarke and Dupont, 1992) may occur driving to

resistant bacteria. Besides, different authors have proposed a mechanism of action independent of muramidase activity and reported the existence of antimicrobial peptides derived from HEWL with a wide-spectrum of microbicidal activity (Abdou et al., 2007). Notably, most of Gram-negative bacteria are resistant to lysozymes due to the presence of the outer membrane which acts as a physical barrier protecting PG. Nevertheless, some compounds and treatments can damage their outer membranes and extend the range of action of lysozyme to Gram-negative bacteria (Nakimbugwe et al., 2006).

On the other hand, nisin, classified as GRAS since over 20 years (US Food and Drug Administration, 1998), is a well known heat-stable bacteriocin produced by *Lactococcus lactis* ssp. *Lactis* which is used as a natural food preservative in processed cheese, milk, dairy products, canned foods, hot baked flour products (crumpets) and pasteurized liquid egg (Ray, 1992; Delves-Broughton and Gasson, 1994; Delves-Broughton et al., 1996). The general norm of Codex Alimentarius for Food Additives (Codex General Standard for Food Additives, 2011), established by the WHO and the FAO, allow utilization of nisin in cream and curd (10 mg/Kg), ripened cheese (12.5 mg/Kg), cheese analogues (12.5 mg/Kg), cheese made of whey protein (12.5 mg/Kg) and dessert based on cereal and starch (3 mg/Kg).

Numerous studies have proved nisin antimicrobial activity against many Gram-positive bacteria such as *Listeria monocytogenes* and *Staphylococcus aureus*. Likewise, this compound has been shown to prevent the outgrowth of sporeformers like *Clostridium* and *Bacillus* spp. (Ray, 1992; Delves-Broughton and Gasson, 1994; Jack et al., 1995). Antibacterial effectiveness of nisin depends on growth and environmental conditions (Abee et al., 1994; Thomas and Wimpenny, 1996; Beuchat et al., 1997; Jaquette and Beuchat, 1998; Pol and Smid, 1999). Thus, it has been shown that nisin is usually more active when is exposed to acid pH whilst temperature influence is still controversial (Periago and Moezelaar, 2001). Mechanism of action of this bacteriocin is based on the fact that nisin binds to the cytoplasmic membrane of vegetative cells through its C-terminal region and penetrates into the lipid bilayer (Breukink et al., 1997). As consequence, pores that allow the efflux of potassium ions, ATP, and amino acids are formed (Ray, 1992; Abee et al., 1994; Delves-Broughton and Gasson, 1994; Jack et al., 1995; Crandall and Montville, 1998) causing proton motive force dissipation

and eventually cell death (Gao et al., 1991; Bruno and Montville, 1993; Breukink et al., 1999).

It is noteworthy that synergistic antibacterial activity between nisin and lysozyme has been observed by various researchers (Chung and Hancock, 2000; Gill and Holley, 2000; Nattress et al., 2001; Nattress and Baker, 2003).

Predictive microbiology is applied to estimate the growth of microorganisms under a given set of conditions. Microbiologists classically use four main parameters to characterize the bacterial growth curve: the initial cell density (N_0), the maximum cell density ($N_{\max}$), the specific growth rate (μ) and the lag phase (λ). As long as exponential growth can be predicted with high accuracy as a response to current growth environmental conditions, lag is inherently more difficult to predict than growth rate because it depends on the previous and current environmental conditions of the bacterial cells. In order to improve lag estimations, some authors have described lag phase as an adjustment process whose duration correspond with the time required for cells to carry out the work necessary to get ready to divide (Baranyi and Roberts, 1994; Hills and Wright, 1994; Hills and Mackey, 1995).

Deterministic growth models describe the behaviour of bacterial populations at a certain time instant while ignoring measurement uncertainty, inoculum history, variation among strains and individual cell variability (Poschet et al., 2004). In 1998, Baranyi reported that the mean of the lag times of the individual cells can be much bigger than the commonly perceived lag time of the whole culture. Besides, Renshaw (1991) pointed that deterministic models are not appropriate to predict population dynamics at low counts. Different authors have shown that duration and variability of lag time increases with lower inoculum size, specially, when severe stress conditions are applied (Robinson et al., 2001; Metris et al., 2002). In consequence, it is becoming clear that to develop a more complete understanding of the lag phase process, the behaviour of single cells has to be taken into account through the development of stochastic models (Baranyi and Pin, 2003). Such models are used to establish the connection between the traditional food microbiology concept of bacterial lag period, before the exponential growth, and the distribution of the lag times of individual cells (Baranyi and Pin, 2001) and are able to predict probabilities of survival and growth at

low cell number. To deal with stochastic models, Monte Carlo analysis is a general method previously employed in food microbiology which can be used to calculate the probability mass function for the microbial load at a certain time instant (Poschet et al., 2004).

However, it is well known that lag time from single cells can not be deduced from a population growth curve. To estimate growth parameters of bacteria automated turbidity measurements are used as an alternative (McMeekin et al., 1993) since they are able to produce a large quantity of replicate detection time measurements. In 2003, Metris et al. assuming that the specific growth rate is the same and constant for each population engendered by a single cell, proposed that the distribution of the detection times (time for a cell population to reach a detectable level of turbidity) should be close to the distribution of the lag times of the initial individual cells. Therefore lag times of individual cells can be measured by the distribution of the times the respective single-cells-generated subpopulations need to reach a certain (constant) detectable level (Figure 1).

Figure 1. For a subpopulation generated by a single cell ($N_0 = 1$), the distribution of the detection times is the same as the distribution of the lag times of the individual cells, assuming the same growth rate. N_{det} correspond to the bacterial number at detection time and N_0 refers to the initial bacterial number (Metris et al., 2003).

Based on the fact that “it is expected that the changes in the substrate, or sublethal injury result in a different distribution of the individual lag times, and this information could be used to optimize food processing procedures, in the interest of minimally processed foods” (Baranyi and Pin, 2001), the aim of this study was to

compare the inhibitory effects of combined treatments of heat, nisin and lysozyme concentrations on the distributions of the lag times of *B. cereus*. Using the distributions adjusted, predictions of time to growth (10^6 CFU mL⁻¹) of *B. cereus* were established by a Monte Carlo simulation and they were compared with deterministic predictions and observations in liquid egg.

4.2. Materials and methods

4.2.1. Bacterial strain

The strain used in the experiment was *B. cereus* INRA-AVZ421, kindly provided by the National Institute of Agronomic Research (INRA, Avignon, France). *B. cereus* was isolated from refrigerated processed foods of extended durability based on vegetables. The microorganism was sporulated in Fortified Nutrient agar (FNA) at 37°C (Mazas et al., 1995) and stored at 4°C until use (Fernández et al., 2001).

A 100 µL volume of spores were diluted in 0.5 mL of sterile distilled water suspension and heated at 80°C for 10 min to ensure that subsequent vegetative cells grown would have the same physiological state. A 100 µL volume of this culture was grown at 30°C in 100 mL Brain Heart Infusion broth (BHI; Scharlau, Barcelona, Spain) overnight. After incubation, the culture was centrifugated at 2,500 g for 15 min. Pellets were resuspended in 2.5 mL of peptone water (Scharlau, Barcelona, Spain) and these vegetative cells were used as inoculum. The proportion of spores was found to be lower than 0.001% and it was not taken into consideration further.

4.2.2. Food product preparation

Fresh eggs, stored at refrigeration temperature until use, were used for the experiments. Liquid egg was made in our laboratory in a domestic blender (Turbomix Plus 300, Moulinex, Lyon, France). Homogenized liquid egg samples were heated at 60°C for 10 min to inactivate background microflora before inoculation studies.

4.2.3. Chemicals

Lysozyme was obtained from Sigma-Aldrich Chemie, (Steinheim, Germany). It was dissolved in sterile bidistilled water and sterilized by filtration (0.45 μm Millex-HV, Millipore, Bedford, USA). Lysozyme working solution was prepared to give a final concentration of 100 mg/mL and stored refrigerated until use.

Nisin was obtained from Sigma-Aldrich Chemie, (Steinheim, Germany). It was dissolved in ethanol 50% (v/v) (Panreac, Barcelona, Spain). Nisin working solution was prepared to give a final concentration of 0.13 μM and stored refrigerated until use.

4.2.4. Optical density calibration curves

From an overnight culture of *B. cereus* in order to obtain vegetative cells, dilution series were made in BHI and BHI supplemented with different amounts of antimicrobial compounds: lysozyme (0.34 mmol/L), nisin (0.13 $\mu\text{mol/L}$), or combination of both compounds (lysozyme 0.34 mmol/L + nisin 0.13 $\mu\text{mol/L}$ and lysozyme 0.34 mmol/L + nisin 0.065 $\mu\text{mol/L}$). Microwell plates were filled with 400 μL of diluted culture per well and incubated in the Bioscreen C automatic reader (Labsystems, Helsinki, Finland) at 16 and 25°C and the optical density (OD) was measured at 600 nm. At time intervals, samples were removed from the wells, diluted and plated onto BHI agar (BHIA; Scharlau, Barcelona, Spain) for viable counts.

The OD of the well was recorded immediately before the sample was taken. OD values were plotted against viable counts.

4.2.5. Growth curves and determination of the lag phase

Growth curves were made in BHI with the addition of lysozyme and nisin concentrations described above and combinations of both of them. The dilution procedure of Francois et al. (2003) was applied to obtain single *B. cereus* cells in the wells of the microwell plate. Serial ten and two fold dilutions of control or stressed cells were made in the corresponding modified medium and aliquots (400 μL) were added into the wells of a microwell plate to achieve a cell concentration of 1×10^3 to 1×10^0 . The

plates were incubated in the Bioscreen C automatic reader at 16 and 25°C and OD was determined at 15 minutes intervals.

The kinetic parameters of the single-cell lag times were estimated by the method described by Metris et al. (2006). The lag times of *B. cereus* cells were obtained through the time of detection (T_d), which is the time required for the microbial population to generate a 0.2 increase in optical density from the start of the incubation. When T_d values are plotted against the corresponding inoculum size (calculated as $\ln$ CFU/mL), μ can be calculated as the negative reciprocal of the slope of the regression line (Cuppers and Smelt, 1993). Assuming an exponential bacterial growth at a constant specific growth rate (μ) until the detection time, T_d is related to the lag time of the culture (λ) by the following formula proposed by Baranyi and Pin (1999):

$$T_d = \lambda + (\ln(N_d) - \ln(N_0))/\mu \quad (1)$$

where N_d is the bacterial number at T_d , obtained by means of calibration curves, and N_0 the number of cells initiating growth in the considered well.

4.2.6. Heat resistance determination of vegetative cells *B. cereus* in liquid egg

Heat resistance determinations of vegetative cells were carried out in capillary tubes (Hirschmann Laboratory, Germany) that were exposed to different temperatures using a thermostated water bath. Capillary tubes were filled with 20 μ L of liquid egg inoculated with an overnight culture of *B. cereus* at a concentration of 10^9 CFU/mL. At time intervals, chosen depending on the temperature of treatment (52.5-57.5°C), capillaries were removed from the water bath and cooled in ice water. Dilutions were made and plated onto BHIA. Plates were incubated at 37°C for 24 h. D_T values (minutes of heat treatment at temperature T for the number of survivors to drop one log cycle) were calculated from the slope of the regression line obtained plotting logarithmic counts versus their corresponding heating times. The z values (degrees of temperature increase for the D_T value to drop a log cycle) were determined from the regression line obtained plotting $\log D_T$ values versus heating temperatures.

4.2.7. Combined effect of heat, nisin and lysozyme on *B. cereus* in liquid egg

Successive individual stresses were applied to cells in early stationary phase of *B. cereus*. Volumes of 5 mL of liquid egg were inoculated with vegetative cells of *B. cereus* (approximately 10^8 - 10^9 CFU/mL) and mild heat treatment was applied (55°C for 3 minutes) in order to achieve between 2 and 3 log reductions. Aliquots were taken (0.1 mL) and they were then inoculated in 2 mL of liquid egg which contained antimicrobial compounds at the following concentrations: lysozyme (0.34 mmol/L), nisin (0.13 µmol/L), or in combination of both compounds (lysozyme 0.34 mmol/L + nisin 0.13 µmol/L and lysozyme 0.34 mmol/L + 0.065 µmol/L). Then, they were incubated at 16 and 25°C. Samples were taken at different time intervals during incubation, dilutions were made and plated into BHIA. Plates were incubated at 37°C for 24 h.

4.2.8. Statistical data processing, distribution fitting and Monte Carlo Analysis

Statistical data processing was performed using Statgraphics Plus 5.1 software (Statistical Graphics Corp., Rockville, MD). Histograms were made from every set of conditions showing the distribution of the lag phases. From each histogram, the most common statistical parameters (mean value, standard deviation, etc.) were determined.

The most appropriate distribution to lag data was investigated using the Best fit tool of @RISK 4.5.2 Professional Edition (Palisade Corporation, Newfield, New York, USA). Distributions were ranked using the χ^2 goodness of fit statistics (BestFit Users Guide, Palisade Corporation, 2002). The test value is expressed as a p value, indicating the probability that a dataset randomly sampled from the hypothetical distribution would be equally or even more extreme than the observed dataset. The lower is p the better is the fitting of the hypothetical distribution to our experimental data (Francois et al., 2005).

Monte Carlo simulation was performed to predict the time to growth to a certain microbial concentration (10^6 CFU/mL, in this case). Eq. (1) was used to analyse the dependence of N_0 and λ on the time to reach 10^6 CFU/mL. The individual cell lag times were assumed to follow a Gamma distribution of shape parameter β and scale parameter

α . The parameters of the Gamma distribution and the growth rate, μ , in each environmental condition were calculated from the detection times. On the other hand, the initial number of cells in a well followed a Poisson distribution with an average of 1 cell per well according to Metris et al. (2006). The simulation was performed using the parameters and calculations presented and the model run for 10,000 iterations.

This generated probabilistic information which was compared with growth curves obtained in liquid egg as model food.

4.3. Results and discussion

4.3.1. Effect of individual and combined stress on the biological parameters

The effect of different stress conditions on the biological parameters, individual cell lag phase (λ) and maximum specific growth rate ($\mu_{\max}$) was investigated for *B. cereus* on BHI (Table 1).

The mean lag phase duration increased when antimicrobial compounds were applied and the increase was higher when the incubation temperature was 16 °C. Nisin (0.13 $\mu\text{mol/L}$) and lysozyme plus nisin (0.13 $\mu\text{mol/L}$) were the most effective conditions, reaching lag values of 15.0 and 15.7 h, respectively at 16°C. Therefore, nisin (alone or in combination) presented a more pronounced antimicrobial effect than lysozyme at the levels tested in this study. This antimicrobial effect was more evident at lower incubation temperatures (16°C) and differences between antimicrobials and their combinations increased.

From results, histograms of the lag phase were generated. Histograms showed that controls presented lower lag times and a relatively narrow distribution. The combination of lysozyme and nisin at high dose delayed the average onset of growth (Figure 2) and distributions were much wider, indicating a dispersion of the lag phase, due to the presence of damage. Cells have to go through a repair and adaptation phase before they can start to grow. Distributions of lag time of *B. cereus* grown in presence of nisin at 25°C were similar to the ones obtained in the presence of both antimicrobials, as it can be observed from standard deviation in Table 1 or from histograms in Figure

2A. More dispersion was observed when cells were exposed to a lower temperature although no differences between both antimicrobials were found (Figure 2B and Table 1). This indicates that in the presence of more stringent conditions (lower temperature and both antimicrobials) cells need longer time to recover. This effect was not evidenced at higher temperatures.

Table 1. Growth parameters of *B. cereus* as a function of lysozyme and nisin concentrations.

Treatment	Incubation temperature (°C)	Growth conditions		Growth parameters	
		Lysozyme (mmol/L)	Nisin (μmol/L)	$\mu_{\max}(\text{h}^{-1})$	$\lambda(\text{h})$
Control	25	-	-	1.34	1.11±0.30
L	25	0.34	-	1.08	1.75±0.47
N	25	-	0.13	0.95	3.03±0.38
L+N	25	0.34	0.065	1.28	4.08±0.33
L+N*	25	0.34	0.13	0.95	4.59±0.47
Control	16	-	-	0.37	3.41±0.8
L	16	0.34	-	0.46	7.71±2.15
N	16	-	0.13	0.20	15.00±1.37
L+N	16	0.34	0.065	0.31	8.67±0.95
L+N*	16	0.34	0.13	0.20	15.67±1.25

L: Lysozyme; N: Nisin; L+N: Low concentration of nisin; L+N*: High concentration of nisin.

Figure 2. Individual cell lag phases expressed in hours of *B. cereus* grown in presence of antimicrobial compounds at different incubation temperatures. **(A)** Samples incubated at 25°C **(B)** Samples incubated at 16°C. Each histogram was based on 50-100 individual observations.

4.3.2. Effect on distribution fitting

18 distributions were fitted to the different datasets of lag time values of *B. cereus* to compare the distributions of the data. Tables 2 and 3 show the fitting of distributions to the pooled data of each treatment. The χ^2 statistic showed that in most cases the listed distributions gave good fits to the data. Probability values (p) were generally >0.05 , but in a few cases significance was low.

Table 2. Best fit distribution of the different datasets of individual cell lag phases by *B. cereus* grown at 25°C incubation temperature.

Treatment	Growth Conditions		Lag distribution parameters			
	Lysozyme (mmol/L)	Nisin (μ mol/L)	Type	α	β	p -value (χ^2)
Control	-	-	Normal			0.651
			Weibull	3.13	1.54	0.020
L	0.34	-	Normal			0.863
			Weibull	2.45	1.03	0.904
N	-	0.13	Normal			0.020
			Weibull	2.89	2.501	0.012
L+N	0.34	0.065	Normal			0.042
			Weibull	5.47	3.55	0.003
L+N*	0.34	0.13	Normal			0.061
			Weibull	6.28	3.85	0.104

L: Lysozyme; N: Nisin; L+N: Low concentration of nisin; L+N*: High concentration of nisin.

Table 3. Best fit distribution of the different datasets of individual cell lag phases by *B. cereus* grown at 16°C of incubation temperature.

Treatment	Growth Conditions		Lag distribution parameters			
	Lysozyme (mmol/L)	Nisin (μ mol/L)	Type	α	β	p -value (χ^2)
Control	-	-	Normal			0.211
			Weibull	1.99	2.74	0.140
L	0.34	-	Gamma	6.78	1.05	0.916
			Weibull	2.15	6.37	0.822
N	-	0.13	Normal			0.029
			Weibull	4.68	12.18	0.007
L+N	0.34	0.065	Normal			0.045
			Gamma	10.03	0.98	0.072
L+N*	0.34	0.13	Logistic	15.50	1.67	0.067
			Gamma	84.18	0.35	0.010

L: Lysozyme; N: Nisin; L+N: Low concentration of nisin; L+N*: High concentration of nisin.

Weibull, Gamma and Normal distributions were selected as they have been described in other studies and they covered the whole range of observed dataset and gave a good description of the experimental data. The Gamma distribution has been extensively described in other studies for exposure to a single factor (Metris et al., 2006; George et al., 2008) or also for moderate stress conditions (Francois et al., 2005). Weibull has been used to fit high levels of stress conditions before (Francois et al., 2005, 2007) and also normal distribution (Wu et al., 2000; Delgado et al., 2004; Francois et al., 2005).

In general, Weibull and Normal distributions gave the best fit to describe individual lag times of *L. monocytogenes* under all condition tested at 25°C. Also, these distributions fitted the great majority of datasets at 16°C, although Gamma distribution fitted the data when combinations of nisin and lysozyme were tested at the same temperature. This is in agreement with the previous observation cited.

4.3.3. Heat resistance of *B. cereus* vegetative cells in liquid egg

The survivor curves of *B. cereus* followed first-order kinetics showing that when the microbial population was heated at a specific temperature, the bacteria were inactivated at a constant rate. This was evidenced by a high correlation coefficient, equal or higher than 0.98 in all cases.

D_T values were found to be between 2.12 min at 52.5°C and 0.40 min at 57.5°C. The z value for *B. cereus* was 6.44. Values obtained in our study can be considered comparable to those described in the scientific literature (Delgado et al., 2004).

4.3.4. Effect of antimicrobial compounds on the viability of *B. cereus* in liquid egg

In order to evaluate the antibacterial activity of lysozyme and nisin, individually or combined, samples of homogenized egg containing concentrations of lysozyme (0.34 mmol/L) or/and nisin (0.13 $\mu\text{mol/L}$, 0.065 $\mu\text{mol/L}$) were inoculated with heat damaged *B. cereus*. The effect of antimicrobials depended on the incubation temperature (Figures 3 and 4). At 25°C, only the combination of lysozyme and the highest concentration of nisin delayed slightly bacterial growth until 10 hours of incubation. The same delay was observed in liquid egg added with nisin or with lysozyme plus nisin (0.13 $\mu\text{mol/L}$) at 16°C. However, the bacterial growth was delayed approximately 30 h when the combination of lysozyme and nisin (0.065 $\mu\text{mol/L}$) was applied at 16°C. When *B. cereus* was exposed to lysozyme no significant delay in the onset of growth was observed (Figures 3 and 4). It was reported that *B. cereus* strains were among the least sensitive *Bacilli* against different antimicrobial including nisin, lysozyme and nisin-monolaurin combination (Mansour and Milliere, 2001; Pirttijarvi et al., 2001). In agreement with our study, López-Pedemonte et al. (2003) reported that the low sensitivity of *B. cereus* would be due to the use of high initial loads (around 6 log CFU/mL) although in our case inoculums size was low (3-4 log CFU/mL).

When *B. cereus* was grown in presence of nisin (0.13 $\mu\text{mol/L}$) and lysozyme (0.34 mmol/L) plus nisin (0.13 $\mu\text{mol/L}$) the antibacterial effect was higher (Figures 2 and 3). At 16°C, the onset of growth was 15.3 and 27.8 h respectively. Periago and

Moezelaar (2001) reported that an exposure to 0.15 $\mu\text{g/mL}$ of nisin resulted in a biphasic reduction of the viable count of cells in different strains of *B. cereus*.

Figure 3. Effect of different lysozyme and nisin concentrations on *B. cereus* grown in liquid egg during incubation at 25°C. **Lysozyme 0.34 mmol/L** (black), **nisin 0.13 $\mu\text{mol/L}$** (orange), **lysozyme 0.34 mmol/L + nisin 0.13 $\mu\text{mol/L}$** (blue) and **lysozyme 0.34 mmol/L + nisin 0.065 $\mu\text{mol/L}$** (pink).

Figure 4. Effect of different lysozyme and nisin concentrations on *B. cereus* grown in liquid egg during incubation at 16°C. **Lysozyme 0.34 mmol/L** (black), **nisin 0.13 $\mu\text{mol/L}$** (orange), **lysozyme 0.34 mmol/L + nisin 0.13 $\mu\text{mol/L}$** (blue) and **lysozyme 0.34 mmol/L + nisin 0.065 $\mu\text{mol/L}$** (pink).

4.3.5. Monte Carlo simulation to estimate time to a certain growth

A probabilistic approach to obtain predictions for individual treatments selected at the most stringent conditions and for controls was used, considering an initial contamination/survival of 1 CFU/mL to achieve a final concentration of 10^6 CFU/mL of *B. cereus*. The growth rate was considered as constant and the lag time was considered to follow the distributions fitted. Results were compared with growth curves obtained in liquid egg (Table 4). At 16°C, predictions were on the lower side, therefore growth observed in egg was faster the predictions, whereas at 25°C the observation were close to the predictions. Since the inoculum level used in egg was higher (in the range of 10^4 - 10^5 CFU/mL) and vegetative cells had been exposed to a mild heat treatment to induce damage, probabilistic predictions can be considered as an initial indication to establish the level of risk associated to liquid egg but test in real conditions are still necessary. Similar validations are necessary in each food group to ensure that probabilistic data are a valid tool to establish food safety limits in relation to *B. cereus* but they give a useful and easy method to interpret information to establish food safety criteria.

Table 4. Predicted time (h) to a 10^6 increase of *B. cereus* exposed to the conditions indicated obtained from a Monte Carlo simulation and observations obtained in liquid egg.

Incubation temperature (°C)	Growth Conditions		Time to reach 10^6 CFU/mL (h)	
	Lysozyme (mmol/L)	Nisin (μmol/L)	Monte Carlo simulation	Liquid egg
25	-	-	11.2 ± 0.4	9.2
25	0.34	0.13	18.8 ± 0.7	24.3
16	-	-	39.9 ± 1.4	23.6
16	0.34	0.13	83.0 ± 2.6	49.0

CHAPTER 5

Modelling the effects of temperature and osmotic shifts on the growth kinetics of *Bacillus weihenstephanensis* in broth and food products

Abstract

This study aims to model the effects of temperature and water activity (a_w) shifts on the lag time of *Bacillus weihenstephanensis* KBAB4 and the dependence of maximum specific growth rate (μ_{max}) on the growth conditions (temperature and a_w). The effects of shifts were modeled through the dependence of the parameter h_0 (“work to be done” prior to growth) induced on the magnitude of the shift and the stringency of the new environmental conditions. Effects of temperature downshifts were studied on 30 experimental conditions (shifts magnitude ranging from 12 to 30°C, temperature after shift from 10 to 20°C and a_w ranging from 0.997 to 0.977). Osmotic shifts were studied for 13 conditions (shift magnitude ranging from 0.997 to 0.989 a_w values, temperature from 10 to 30°C, a_w after shift from 0.989 to 0.977). Experimental results show that temperature downshifts were able to induce considerable lag times (up to 20 days) when occurring near the growth limits. In comparison, osmotic shifts within the range studied have less significant effects. Validation experiments in food showed that the model provide valid predictions in creamed pasta but overestimate bacterial growth in carrot soup (fail safe predictions).

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5.1. Introduction

B. weihenstephanensis strains, proposed as a new species on the basis of sequence differences of 16S rRNA gene and cold shock proteins (Lechner et al., 1998), are psychrotrophic bacteria closely related to *Bacillus cereus*. Due to its ability to grow at low temperature and to form heat-resistant spores, these microorganisms can contaminate chilled and minimally processed foods constituting a potential hazard for consumers' health, limiting products shelf life and generating economic losses (Priest, 1993; Abee and Wouters, 1999; Anderson Borge et al., 2001). Indeed, *B. weihenstephanensis* is a frequent contaminant of dairy products (Christiansson et al., 1999; Heyndrickx and Scheldeman, 2002; Svensson et al., 2004; Bartoszewicz et al., 2008) and can be found in a number of vegetable products (Valero et al., 2002) and spoiled egg products (Baron et al., 2007; Jan et al., 2011).

In order to improve effective control of psychrotrophic bacterial growth, especially in minimally processed foods, combination of physical and chemical environmental factors with low temperatures are widely used. Preservative stresses such as a_w , pH and low temperature are typical examples.

Salt is a common additive widely used in food industry to control bacterial growth (Abee and Wouters, 1999). Bacterial cells exposed to salt stress generate a water efflux. In *B. cereus*, to recover and maintain cellular turgor, there is an induction of osmoprotectant transporters. den Besten et al. (2009) reported that ion-dependent symporters encoded by genes *opuD* and *opuE*, a gene encoding a proton-dependent di- and tripeptide transporter and a gene encoding a proline/betaine transporter, were all highly induced upon exposure to salt stress. Likewise, upregulation of Na^+/H^+ antiporters and other cation transporters were also induced. Besides, upregulation of *sigB* and σ^B -dependent genes was observed after mild and severe salt stresses. However, *sigB* and *rsbY* deletion mutant did not show impaired growth in comparison with parental strains indicating that the role of σ^B and its regulon is limited under these conditions (den Besten et al., 2009). Moreover, Browne and Dowds (2001) reported that glycolytic enzymes were regulated by osmotic shocks. Further studies (den Besten et al., 2009) reveal that severe salt stress increases expression of tricarboxylic acid cycle

proteins and components of electron transport chain after resumption of growth, meanwhile expression of genes involved in nucleotide and amino acid transport were repressed. Likewise, genes involved in chemotaxis and motility were downregulated resulting in filamentous cells with affected chemotaxis behavior after long exposition periods (den Besten et al., 2009). In addition, genes involved in oxidative response were upregulated upon exposure to osmotic shock (den Besten et al., 2009). This increased catalase activity in salt-adapted cells resulting in a cross-protection against H₂O₂. Besides, activation of this general salt stress response has been shown to increase bacterial heat and chilling resistance through the induction of the transcription of osmoprotectant transporters (Holtmann and Bremer, 2004; Wemekamp-Kamphuis et al., 2004).

On the other hand, temperature is one of the most important environmental factors to which bacteria have to respond. Depending on its thermal niche, each microorganism shows an optimal growth temperature. When bacteria are challenged by increased or decreased temperature in their environment they undergo modifications in cellular physiology which show substantial differences. At low temperatures, reduction of biochemical reaction rates is observed resulting in cold shock and acclimation response (for further information see Chapter 7). Moreover, bacteria subjected to high temperatures develop a heat shock response which includes increased synthesis of a set of conserved heat-shock proteins (HSPs) and ATP-dependent proteases such as ClpP (Periago et al., 2002a) (for further information see Chapter 2).

Lag phase corresponds with the delay before exponential growth, during which microbial cells do not divide and adjust their metabolism to environment. In food microbiology, a lag phase might occur just after food product contamination with environment, or when a contaminated food product undergoes important environmental fluctuations (Delignette-Muller et al., 2005). Lag phase duration varies, depending on the phenotype of the bacteria (Buchanan and Cygnarowicz, 1990), physiological history of the population (McMeekin et al., 1993) and changes in the physicochemical environment (Zwietering et al., 1994).

The duration of the lag phase depends on the amount of work to be carried out by cells prior to exponential growth and the rate at which this work is undertaken

(Robinson et al., 1998; Ross, 1999). According to Baranyi and Roberts (1994), the “work to be done” is proportional to h_0 , the product of the lag time (λ) and the μ_{max} at which the work is carried out. Robinson et al. (1998) pointed out that there is no direct way to measure this rate. In the commonly used version of the model of Baranyi and Roberts, it is assumed to be the same as the cells’ specific maximum growth rate. Numerous studies have shown the influence of the magnitude of the change in the environmental factors on the “work to be done” prior to growth (Mellefont et al., 2003; Mellefont and Ross, 2003; Delignette-Muller et al., 2005). Further studies (Le Marc et al., 2010; Muñoz-Cuevas et al., 2010) found that h_0 depends not only on the magnitude of the change between the previous and the current conditions but also on the conditions prevailing after shifts. In most of these studies, the models developed for h_0 were not validated in food products. An attempt to apply a model for the intermediate lag time of *Listeria monocytogenes* developed in culture medium to experiments in milk was made by Le Marc et al. (2010). In their study on the effects of osmotic stress on the lag time of *Salmonella enterica*, Zhou et al. (2011) found that for the same osmotic stress, the parameter h_0 was significantly different in minimum and rich media. Therefore, the direct use of h_0 models developed in rich laboratory medium to describe the bacterial lag time in some food products lacking the essential elements for bacterial adaptation is questionable.

In this study, we developed a model for the effects of water activity (a_w) and temperature on the specific growth rate and lag phase duration of *B. weihenstephanensis*. The lag time was modelled through the effects of magnitude shifts of change between pre-incubatory conditions and the stringency of the new environmental conditions on the parameter h_0 (“work to be done” prior to growth). The model was used to predict both initial and intermediate lag times. The predictive ability of the developed model under fluctuation temperature conditions was tested in culture medium as well as in cream pasta and carrot soup. Those two products are also representative of the product categories in which *B. weihenstephanensis* can be found. Besides, pasta cream is an example of a product rich in nutrients whereas carrot soup composition is not as favorable for bacterial growth (Hernández-Herrero et al., 2008)

5.2. Material and methods

5.2.1. Bacterial strain and inoculum preparation

The bacterial strain used in this study was *B. weihenstephanensis* KBAB4, kindly provided by the National Institute of Agronomic Research (INRA, Avignon, France). This microorganism was sporulated in Fortified Nutrient Agar (FNA) (Mazas et al., 1995) and stored at -20°C until use. Spores were activated 10 minutes at 80°C before every experiment to ensure that subsequent vegetative cells growth would have the same physiological state. Next, two consecutive overnight subcultures were performed in Brain Heart Infusion broth (BHI; Scharlau, Barcelona, Spain) at 30°C for experimental model and at 20°C for validation procedure.

5.2.2. Previous growth conditions and shifts to final growth conditions

To assess the effect of environmental shifts on the amount of work to be done (h_0) bacterial cultures were grown in different previous conditions and transferred to different final growth conditions as is indicated in Tables 1 and 2. Depending on incubation temperature and a_w , 43 combinations of previous and final environments were studied. Growth curves were performed in modified BHI by adding NaCl (Panreac, Barcelona, Spain).

The a_w value was calculated from the concentration of NaCl using the following equation (Resnik and Chirife, 1988):

$$a_w = 1 - NaCl \frac{5.2471 + 0.1221xNaCl}{1,000} \quad (1)$$

where $NaCl$ is the concentration of NaCl (% w/w).

Tubes filled with 9 mL of BHI adjusted to a_w values of 0.997, 0.989 and 0.977 and pre-incubated at the target temperature were inoculated with 10^3 CFU/mL approximately of bacterial culture and incubated at 10, 12, 16, 20 and 30°C depending on the condition tested (Tables 1 and 2). When optical density (OD) of the culture

reached a value between 0.17 and 0.2, exponentially growing population was subject to different final growth conditions (Tables 1 and 2).

5.2.3. Measurement methods of exponential populations growth after a_w and temperature shifts

Estimation of bacterial growth during final conditions was carried out by using either viable plate count (VPC) or OD measurements. To perform VPC estimations, 9 mL of modified BHI (added with NaCl) already at the target temperature were inoculated with 10^3 CFU/mL and incubated at target temperature. At appropriate time intervals, samples were plated onto BHI agar (BHIA; Scharlau, Barcelona, Spain) previous 10-folds dilutions in buffered peptone water (Scharlau, Barcelona, Spain). After incubation at 30°C for 24 hours, colonies were counted. Growth curves were determined in duplicate.

To determine bacterial growth after shift by using OD measurements, calibration curves to relate OD and bacterial concentration were constructed. From a culture growing exponentially in BHI at 30°C, serial two fold dilutions were performed as described by Cuppers and Smelt (1993) in BHI and BHI supplemented with 4% NaCl. Microwell plates were filled with 400 µl of diluted culture per well and incubated at 30°C in the Bioscreen C automatic reader (Labsystems, Helsinki, Finland). At appropriate time intervals, samples were removed from the wells, diluted and plated onto BHI agar for VPC. The plates were incubated at 30°C for 24 h. The OD of the wells was recorded immediately before the sample was taken. OD values were plotted against viable counts.

The relationship between the bacterial concentration and OD values was not found to be affected by temperature and a_w and showed to be linear in the range that is described as follows:

$$CFU/ml = 5.9 \times 10^7 \times OD - 1.0 \times 10^7 \quad (2)$$

To carry out growth curves by using OD measurements, serial ten and two fold dilutions of exponentially growth bacteria were carried out in modified BHI already at the target temperature. Ten dilutions were obtained with concentrations ranging from 10^1 - 10^6 CFU/mL approximately. Aliquots of 400 μ l of each dilution were added into 10 wells of a sterile microwell plate. Depending on the condition tested (Tables 1 and 2) plates were incubated at 16, 20 and 30°C in the Bioescreen C automatic reader and optical density was measured at appropriate time intervals.

5.2.4. Determination of growth parameters during final conditions

The kinetic parameters of the population subjected to temperature and a_w shifts were estimated by the method described by Métris et al. (2006). The detection time (T_d), was determined to be the required time for the microbial population to generate a 0.2 increase in optical density from the start of the incubation in each microwell plate. When T_d values are plotted against the corresponding inoculum size (calculated as $\ln$ CFU/mL), μ_{max} can be calculated as the negative reciprocal of the slope of the regression line (Cuppers and Smelt, 1993). Assuming an exponential bacterial growth at a constant specific growth rate (μ) until the detection time, T_d is related to the lag time of the culture as follows (see e.g. Baranyi and Pin, 1999):

$$Td = \lambda + \frac{\ln(N_d) - \ln(N_0)}{\mu_{max}} \quad (3)$$

where N_d is the bacterial number at T_d , obtained by means of calibration curves, and N_0 the number of cells initiating growth in the well considered.

When growth after shifts was determined by using VPC measurements, growth rates and h_0 values were calculated by fitting the growth curves obtained after shifts with the model of Baranyi and Roberts (1994).

5.2.5. Growth rate and h_0 model

To describe the dependence of μ_{max} on the specific growth rate, we used the square root model proposed by Muñoz-Cuevas et al. (2010):

$$\sqrt{\mu_{max}} = b_0 (T - T_{min}) \sqrt{bw_{max} - bw} \quad (4)$$

where $b_w = 100\sqrt{1 - a_w}$, T_{min} is the theoretical minimum temperature for growth, bw_{max} is the maximum b_w value supporting growth and b_0 another model parameter.

The models for h_0 developed by Muñoz-Cuevas et al. (2010) for *L. monocytogenes* were modified to allow the description of the high values of the parameter h_0 observed after downshifts near growth limiting conditions for *B. weihenstephanensis*. The equations proposed for temperature shifts and osmotic shifts are as follows:

$$\sqrt{h_0} = (T_p - T_c)^{a_1} (T_c - T_{min})^{a_2} bw_c^{a_3} \quad (5)$$

where the subscripts p and c indicate the conditions before and after shifts, respectively, and T_{min} , a_1 , a_2 and a_3 are the model parameters.

$$\sqrt{h_0} = (bw_p - bw_c)^{c_1} (T_c)^{c_2} (bw_{max} - bw_c)^{c_3} \quad (6)$$

where bw_{max} , c_1 , c_2 and c_3 are the model parameters identified by non linear regression.

5.2.6. Validation of the experimental model for bacterial population subjected to sudden temperature shifts

The predictive ability of the combined model (h_0 and μ_{max}) was assessed by comparing model predictions and observed growth curves of *B. weihenstephanensis* in broth medium, carrot soup and creamed pasta under changing conditions of temperature. The BHI medium was prepared according to the manufacturer indications.

To elaborate the soup, carrots were washed and peeled before being homogenized in a commercial blender (Moulinex, Lyon, France) and partially sieved. Creamed pasta was made in our laboratory in a food processor (Thermomix TM 31, Vorwerk, Germany) using olive oil, bacon, cream, cheese, water and macaroni. In order to ensure appropriate homogenization of bacterial population within the food matrix, creamed pasta was diluted ten folds in sterile distilled water for validation experiments. NaCl was added to carrot soup and diluted creamed pasta until reaching a concentration of 0.5% and pH was adjusted to 6.5. Aliquots of 9 mL of homogenized food products were added into tubes and heated at 100°C for 10 min during three consecutive days to inactivate background microflora before inoculation studies. Samples were stored in refrigeration until use.

Inoculum was prepared as described above. After pre-incubation at 20°C, bacterial cultures were inoculated in 9 mL of sterilized food and broth medium to give a final concentration between 10^2 - 10^3 CFU/mL. Temperature shifts were setting resulting in two different profiles. Samples were incubated at target temperatures during 240 hours subjected to the following temperature shifts, Profile 1: 84 hours at 12°C, followed by 84 hours at 10°C and finally 72°C hours at 15°C. Profile 2: 96 hours at 10°C, next 29 hours at 16°C and to conclude, 115 hours at 10°C. At appropriate time intervals, samples were removed from the tubes, diluted and plated onto BHIA for VPC. The plates were incubated at 30°C for 24 h. Growth curves were determined in duplicate.

Reference curves were obtained in BHI and in the studied foods to evaluate the difference between the μ_{max} in food products and in BHI. Predictions were performed by solving the system consisting of the model of Baranyi and Roberts (1994) and equations 4 to 6 by a Runge-Kutta method (ODE23, Matalab 2007b, TheMathWorks). The parameter y_{max} in the model of Baranyi and Roberts was fixed to the maximum concentration observed in BHI: $y_{max} = 10^{10}$ CFU/mL. Two criteria were used to assess model performance: the Mean Deviation (MD) and the Mean Absolute Difference (MAD) which are, respectively, the mean of the difference and the mean of the absolute difference between the observed and the predicted bacterial concentration.

5.3. Results and discussion

5.3.1. Effects of shifts on the growth model parameters

Tables 1 and 2 show the observed maximum specific growth rates, lag times and h_0 values after the temperature and the water activity downshifts applied in this study. Our results support the hypothesis that the maximum specific growth rate depended only on the current growth conditions and was not affected by the previous environment. F-tests ($p=0.05$) performed did not highlight significant differences between growth rates obtained at the same growth conditions but after different previous environment.

The observed maximum growth rates after downshifts were used to fit equation 3. Fitted parameters and their standard errors are proposed in Table 3. The good correspondence between fitted and observed maximum specific growth rates is shown in Figure 1 where all the datapoints were very close to the line of unity, indicating a very good prediction of the observed values. This indicates that once adaptation to the new conditions was reached, maximum growth rate was only dependent on the conditions of the recovery medium as previously reported Muñoz-Cuevas et al. (2010).

The observed values of lag times and h_0 show that *B. weihenstephanensis* is more sensitive to temperature shifts than other microorganisms, such as *L. monocytogenes*. Whereas the h_0 values observed by Muñoz-Cuevas et al. (2010) for *L. monocytogenes* did not exceed ca. 3.0, the maximum h_0 values observed in this study reached a level of ca. 20 (Table 1). The results also show that the parameter h_0 depends not only on the magnitude of the shifts but also on the conditions prevailing after shift. At 0.5% NaCl a downshift of 10°C (from 30 to 20°C) induces a workload $h_0=0.6$ (corresponding to a lag time of 0.9 h) whereas a downshift from the same magnitude applied from 20 to 10°C results in a h_0 value of 4.86 (corresponding to a lag time of 109 h).

Table 1. Growth environments tested with variable temperatures and constant NaCl concentrations

N°	Pre-incubatory conditions		Final conditions		Measurement method ^(a)	Growth parameters		
	T(°C)	NaCl (%)	T(°C)	NaCl (%)		$\mu_{\max}$ (h ⁻¹)	Lag (h)	h_0
1	30	0.5	20	0.5	OD	0.703	0.9	0.63
2	30	2	20	2	OD	0.591	3.3	1.95
3	30	4	20	4	OD	0.479	2.7	1.29
4	30	0.5	16	0.5	OD	0.437	5.5	2.40
5	20	0.5	16	0.5	OD	0.394	3.5	1.38
6	30	2	16	2	OD	0.274	17.2	4.70
7	20	2	16	2	VPC	0.258	23.2	5.98
8	30	4	16	4	OD	0.18	47.2	7.07
9	20	4	16	4	OD	0.165	16.8	2.78
10	30	0.5	12	0.5	VPC	0.149	45.0	6.71
11	20	0.5	12	0.5	VPC	0.097	18.9	1.84
12	16	0.5	12	0.5	VPC	0.169	6.3	1.07
13	30	2	12	2	VPC	0.08	63.7	5.12
14	20	2	12	2	VPC	0.101	48.4	4.88
15	16	2	12	2	VPC	0.095	64.0	6.06
16	30	4	12	4	VPC	0.089	110.0	9.82
17	20	4	12	4	VPC	0.08	71.9	5.79
18	16	4	12	4	VPC	0.077	184.6	14.21
19	30	0.5	10	0.5	VPC	0.035	136.0	4.81
20	20	0.5	10	0.5	VPC	0.045	107.8	4.85
21	16	0.5	10	0.5	VPC	0.034	29.81	1.01
22	12	0.5	10	0.5	VPC	0.054	66.9	3.60
23	30	2	10	2	VPC	0.032	348.8	11.27
24	20	2	10	2	VPC	0.067	165.2	11.08
25	16	2	10	2	VPC	0.031	142.1	4.46
26	12	2	10	2	VPC	0.038	65.4	2.45
27	30	4	10	4	VPC	0.044	456.0	19.93
28	20	4	10	4	VPC	0.057	246.0	13.95
29	16	4	10	4	VPC	0.034	527.1	18.03
30	12	4	10	4	VPC	0.034	366.3	12.31

^a OD, optical density; VPC, viable plate count.

Table 2. Growth environments tested with constant temperatures and variable NaCl concentrations

N°	Pre-incubatory conditions		Final conditions		Measurement method ^(a)	Growth parameters		
		(%)		(%)		$\mu_{\max}$ (h ⁻¹)	Lag (h)	h_0
31	30	0.5	30	2	OD	1.558	1.3	2.03
32	30	0.5	30	4	OD	0.979	1.8	1.76
33	30	2	30	4	OD	1.253	1.6	2.00
34	20	0.5	20	4	OD	0.294	5.8	1.71
35	20	2	20	4	OD	0.321	6.9	2.21
36	16	0.5	16	2	OD	0.259	16.9	4.38
37	16	0.5	16	4	OD	0.136	30.8	4.19
38	12	0.5	12	2	VPC	0.115	25.5	2.95
39	12	2	12	4	VPC	0.060	37.4	2.23
40	12	0.5	12	4	VPC	0.460	0	-
41	10	0.5	10	2	VPC	0.039	99.1	3.86
42	10	0.5	10	4	VPC	0.038	204.5	7.81
43	10	2	10	4	VPC	0.039	97.4	3.75

^a OD, optical density; VPC, viable plate count

The effects of temperature downshifts are usually more pronounced when they occur near growth limiting conditions (i.e. low temperatures and high concentrations of NaCl). For example, shifting temperature from 16 to 10°C in presence of 4% NaCl induced a workload of $h_0=18$ (corresponding to a lag time of ca 530 h). In comparison with temperature, osmotic shifts within the range studied have less significant effects on the lag time of *B. weihenstephanensis*. Out of 13 downshifts tested, only 2 induced a lag time longer than 2 days. As for temperature shifts, workloads induced by osmotic shifts also increased with the stringency of the new environmental conditions. A shift from 0.5% NaCl to 4% NaCl induced a “work to be done” $h_0= 1.76$ at 30°C (corresponding to a lag time of 1.8 h) but the workload observed at 10°C for the same NaCl shift is $h_0=7.81$ (which correspond to a lag time of 204 h). The estimated parameters for the h_0 models are shown in Table 3. As shown in Figure 2, the combination of the models for h_0 and $\mu_{\max}$ provides an accurate description of the observed lag times after temperature and a_w downshifts.

Table 3. Estimated parameters of the maximum specific growth rate and h_0 models

Model	Parameter	Estimate	95% Confidence Interval		R ²
			Inf	Sup	
Sqrt(μ_{max})	b_0	0.0148	0.012	0.016	0.98
	T_{min}	6.10	5.65	6.56	
	bw_{max}	25.3	22.5	28.0	
Sqrt(h_0) (temperature)	a_1	0.13	0.03	0.24	0.76
	a_2	-0.55	-0.74	-0.36	
	a_3	0.66	0.53	0.80	
Sqrt(h_0) (NaCl)	c_1	0.39	0.10	0.69	0.61
	c_2	-0.41	-0.69	-0.14	
	c_3	0.40	0.11	0.68

Figure 1. Square root of the observed and fitted specific growth rates of *B. weihenstephanensis*.

Figure 2. Comparison between lag times observed and predicted with the model (combination of equations 1, 2 and 3). (○) Temperature shifts (●) NaCl shifts.

This indicates that adaptation to new, more stressful conditions affected significantly both lag time and h_0 since they are indicators of the adaptation needed to reach the physiological state where cells can replicate. This is in agreement with the h_0 concept, showing that it provides a good prediction of the time required for the cells to adapt to different conditions (Baranyi and Roberts, 1994; LeMarc et al., 2010; Muñoz-Cuevas et al., 2010)

5.3.2. Model validation

Figures 3 and 4 show the observed experimental curves for profiles 1 and 2 in BHI, creamed pasta and carrot soup. Predictions were performed using the model developed from the experiments conducted in BHI. Predictions are usually close to the observed kinetics in BHI and creamed pasta. MD and MAD values of 0.49 and 0.69 log CFU/ mL were obtained in BHI. In creamed pasta, values of 0.89 and 1.15 log CFU/ mL were observed for MD and MAD respectively. However, the model does not perform as well in carrot soup: in this case, MD and MAD were above 2 log CFU/ mL (2.02 and 2.10 log CFU/mL for MD and MAD respectively).

Figure 3. Growth of *B. weihenstephanensis* subjected to temperature profile 1 (dashed line). Shown is a comparison between the model prediction (straight line) with BHI (●), pasta (□) and carrot (◇).

For profile 1, the predictions are in agreement with the observed bacterial concentrations in BHI until 168 h. The initial lag time induced by the shift from 20°C to 12°C was correctly predicted by the model. The temperature downshift from 12 to 10°C induced the growing population into a lag period. However, the temperature upshift from 10 to 15°C did not resume the bacterial growth as it is predicted by the model. The bacterial growth kinetics in creamed pasta are very close to those observed in BHI. However, in carrot soup the initial lag phase duration of *B. weihenstephanensis* is considerably longer than in BHI and creamed pasta (between 72 and 96 h in carrot soup against ca. 15 h in BHI and cream). The downshift from 12 to 10°C applied when *B. weihenstephanensis* was at concentration of 3.8 log CFU/mL induced an intermediate lag time. Contrarily to the experiments in BHI and creamed pasta, growth resumed after the downshift and the temperature upshift applied accelerated bacterial growth until the bacterial concentration reached ca. 10 log CFU/mL.

Figure 4. Growth of *B. weihenstephanensis* subjected to temperature profile 2 (dashed line). Shown is a comparison between the model prediction (straight line) with BHI (●), pasta (□) and carrot (◇).

For profile 2, the model describes accurately the kinetics observed in BHI. The initial lag phase and the lag phase induced by a shift from 16 to 10°C are both well predicted by the model. Contrarily to profile 1, the initial lag phase was shorter in creamed pasta than in BHI. As for profile 1, the temperature downshift from 16 to 10°C applied when the bacterial concentration was at ca 8 log CFU/mL stopped the growth of *B. weihenstephanensis*. In carrot soup, an initial decline of the number of bacterial cells was observed before the surviving cells can adapt to their environment and start growing. As for profile 1, the initial lag time observed in carrot soup (ca. 96 h) is also longer than in BHI (ca. 48 h). However the downshift applied at 125 h has less effect on the bacterial growth than in BHI and creamed pasta.

Validation results indicated that for a rich food substrate such as creamed pasta, predicted growth was correctly described by the model and compared well to growth in BHI (Figures 3 and 4). In some cases faster growth was recorded in pasta than in BHI. In both substrates, Profile 1 showed that there was no growth after a downshift in temperature, indicating that they both entered a new, longer than expected lag phase

after a change from 12 to 10°C when active growth was taking place. A downshift has been reported to have bigger effects at high cell concentrations (Le Marc et al., 2010) which would be in agreement with our results. For Profile 2, there was a different behavior between both substrates, since a short upshift was correctly predicted in BHI with a slight delay in the growth rate. However in creamed pasta the same behavior as in Profile 1 was found (a delay in the onset of growth after the shift), indicating that *B. weihenstephanensis* adapted faster after the changes in the laboratory medium (BHI) than in the food (pasta).

In the carrot soup, a longer lag time was recorded for both profiles (Figures 3 and 4) as it was expected, since a transfer from a rich medium such as BHI to poorer and new nutrient conditions requires a longer adaptation period (Zhou et al., 2010). In Profile 1, however, since this longer lag time included the first temperature downshift, once cells were adapted and started to grow, there was no second lag phase, as it was observed in BHI and creamed pasta, where higher cell concentrations were registered in the carrot soup. This is in agreement with observations that, once cells have been adapted, their growth rate only depends on the present environmental conditions (Muñoz et al., 2009; Muñoz-Cuevas et al., 2010). For Profile 2 in carrot soup an initial decline was observed, which could correspond to a selection of a subpopulation resistant to low temperature and a poorer substrate in combination. It has also been described an initial decline when current conditions are near the growth limits as would be a temperature of 10°C (Zhou et al., 2010). Again, as in Profile 1, since the temperature shift happened at the early stages of the onset of growth, no delay in growth rate or new lag phase were observed.

These results show that the model proposed can describe correctly growth under dynamic conditions in refrigerated foods of *B. weihenstephanensis*, which can be considered as a worst case scenario for *B. cereus* group. However, the composition of the food can induce deviations from the predictions, more notably for poor growth substrates, therefore it is important to validate predictions in conditions close to the real scenario.

Predictions from the dynamic model can be useful to simulate the behavior of the microbial evolution in the real conditions of the refrigeration chain and with shifts in

a_w that can happen when an ingredient is used to prepare a final product. They can be an useful tool for risk assessment purposes in foods where *B. weihenstephanensis* can be considered as a suitable indicator.

CHAPTER 6

Determination of the effect of acid stress on growth and viability of *Bacillus cereus* and *Bacillus weihenstephanensis* using flow cytometry.

Abstract

The present work studies the effect of acid stress on cell viability and growth of *Bacillus cereus* and *Bacillus weihenstephanensis*. Experiments were performed by means of flow cytometry (FCM) combined with fluorescent labeling. Propidium iodide, for membrane evaluation, and carboxyfluorescein diacetate, for esterase activity assessment were used to obtain differential staining of both strains. pH values of 3.4, 3.8 and 4.2 were selected on the basis of previous screening tests. FCM analysis showed to clearly discriminate between different populations, viable and damaged cells, leading to successfully study acid stress effect on *B. cereus* and *B. weihenstephanensis* vegetative cells' viability. Reliability of FCM analysis for detection of *B. cereus* and *B. weihenstephanensis* vegetative cells was compared with classical viable plate count techniques (VPC). FCM and VPC results were not comparable and classical analytical methods showed higher reliability.

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6.1. Introduction

It is well known that acid resistance is a key parameter to control bacterial growth. Low-pH foods are widely used to ensure microbiological quality and stability by adding organics acids. Besides, the risk of *B. cereus* food poisoning is strongly determined by their resistance to low pH since the diarrheal syndrome is caused by vegetative cells ingested with food which subsequently produce enterotoxins in the small intestine after surviving to the acid environment of human stomach (Kotiranta et al., 2000).

It has been shown that *B. cereus* response to acid stress involves general stress response, metabolic rearrangements, pH omeostasis and secondary oxidative response (Figure 1) (Mols and Abee, 2011). Besides, *Bacilli* exposed to low pH undergo a cell envelopes adaptation which involves elongation, branching and desaturation of membrane as well as changes in the lipid composition and charge of the membrane (Ter Beek and Brul, 2010). Likewise, Hornbaek et al. (2004) reported that *Bacillus licheniformis* tries to control the permeability of the cell membrane to protons by changing the fatty acid structure corresponding to a more rigid membrane at low external pH values.

Protein repair chaperones Dnak and GroELS (Mols and Abee, 2011) and transcriptional regulator alternative σ^B factor (van Schaik et al., 2004) mediate general stress response upon acid stress. The *clp* genes showed to be upregulated at low pH as *ctsR* and *hrcA* genes (van de Guchte et al., 2002) which encode heat stress regulators indicating cross-protection of *B. cereus* exposed to various stress conditions.

On the other hand, metabolic rearrangements conducing to consume intracellular protons or to restore NAD^+/NADH balance are induced by *B. cereus* upon acid stress. In 2010(a), Mols et al. reported that metabolic rearrangements were found specifically correlated with lactic acid or acetic acid stress. In fact, after acid shock there is an induction of genes encoding enzymes involved in fermentative pathways such as acetoin production, alcohol and lactate dehydrogenases and rerouting of pyruvate metabolism (Mols and Abee, 2011).

To achieve pH homeostasis upon acid stress, cells should avoid proton inward flux and favor their transport out of the cell. For this purpose under mild acid stress F_1F_0 -ATPase genes are repressed (Mols et al., 2010a) and proton-dependant transporters genes such as *napA* and *nhaC* are downregulated. However, at lethal pH values *napA* and *nhaC* are highly induced and F_1F_0 -ATPase protein can be synthesized (Mols and Abee, 2011). Likewise, to contribute to pH homeostasis alkaline compounds are produced in order to counteract cytoplasmic acidification through the arginine deiminase pathway which is moderately upregulated upon exposure to acid in aerobic conditions and highly upregulated under mild acid anaerobic conditions (van der Voort and Abee, 2009). In addition, arginase was induced under low pH by *B. cereus* cells (Mols and Abee, 2011).

Finally, after exposure to low pH a secondary oxidative stress response has been described. In 2010(a), through comparative phenotype and transcriptome analyses of *B. cereus* strains subject to acid conditions, Mols et al. reported growth rate reduction, growth arrest and loss of viability due to metabolic shifts and to the induction of general stress response mechanisms with a major oxidative component including upregulation of catalases and superoxide dismutases associated to reactive oxygen species formation. Further studies showed the induction of oxidative stress associated genes encoding thioredoxins, catalases, superoxide dismutase and the major stress regulator PerR upon acid shock (Mols and Abee, 2011).

Besides, acid shock may affect electron transfer chain (ETC) activity because alternative ETC associated genes are induced. Notably, the most prominent induction by *B. cereus* ATCC 14579 upon acid shock was nitrate and nitrite reductases (Nar/Nas) (Mols et al., 2010a) may be because they form an alternative ETC and are involved in proton consuming reactions. In addition, cytochrome *bd* oxidase genes such as cytochrome *d* ubiquinol oxidase (CydAB), were highly induced upon organic acid shock and are part of alternative ECT together with NAD(P)H-dependent dehydrogenases such as lactate and alcohol dehydrogenase (Mols and Abee, 2011). Expression of cytochrome *bd* oxidase and lactate dehydrogenase genes is coordinated and controlled by other genes which are part of the anaerobic Fnr regulon (Reents et al., 2006). In fact, in 2009 Mols reported that *B. cereus* is more resistant to acid stress when is grown under oxygen limitation conditions.

Figure 1. Overview of acid stress-associated mechanisms in *B. cereus* divided in four different groups: (i) general stress response, (ii) metabolic rearrangements, (iii) pH homeostasis and (iv) oxidative response. Abbreviations used: S, substrate; Arg, arginine; Orn, ornithine; ADI, arginine deiminase pathway; PT, proton-dependant transporters; ETC, electron transfer chain; AlsDS, acetoin production; AdhA, alcohol dehydrogenases; Ldh, lactate dehydrogenases; SodA, superoxide dismutase; KatA, catalase; CydAB, cytochrome *d* ubiquinol oxidase; Nar/Nas, nitrate/nitrite reductase (Mols and Abee, 2011).

The efficacy of low pH has been generally assessed using classical VPC which may underestimate the numbers of viable bacteria due to biases introduced by selected incubation conditions as well as by different environment factors (Paparella et al., 2008). In this regard, FCM offers many potential advantages over conventional techniques such as real time information on the physiological status of an organism. Different authors have proposed FCM as a powerful, reliable and fast tool to assess the responses of individual cells to antimicrobial compounds or treatments (Alvarez-Barrientos et al., 2000; Steen, 2000). Indeed, Biesta-Peters et al. (2011) used FCM in combination with fluorescent probes to study relevant physiological parameters of *B. cereus* at the end of acid-induced lag phases.

B. weihenstephanensis and *B. cereus* adaptation and survival in low pH environments are factors of particular interest in food safety and health since these microorganisms encounter such conditions in acidified foods and during transit through the stomach (Jobin et al., 2002). Besides, knowledge of acid stress resistance can be useful to design probiotic *Bacilli* (Ter Beek and Brul, 2010). In addition, development of new methods is needed “for resolution at single cell level to make the data applicable for predictive modelling based approaches of practical use to the food industry” (Ter Beek and Brul, 2010). On this basis, the present research aims to characterize the physiological behavior of *B. weihenstephanensis* vegetative cells compared to *B. cereus* strain type populations after exposure to low pH environments by using FCM analysis in combination with fluorescent techniques. To discriminate cells in different physiological states, two specific labels, propidium iodide (PI) and carboxyfluorescein diacetate (cFDA), were successfully used in concordance with previous studies of Paparella et al. (2008) and Biesta-Peters et al. (2011). Likewise, reliability of FCM analysis for detection of *B. cereus* and *B. weihenstephanensis* vegetative cells was assessed in comparison with classical techniques (VPC).

6.2. Materials and methods

6.2.1. Bacterial strains

Two bacterial strains were used in this study, *B. weihenstephanensis* KBAB4 kindly provided by the National Institute of Agronomical Research (INRA, Avignon, France) and *B. cereus* CECT 148 (which corresponds to the type strain *B. cereus* ATCC 14579) selected from Spanish Type Culture Collection (CECT, Valencia, Spain). Aliquots of each microorganism were resuspended in sterile NaCl 0.85%, then spread onto plates of Fortified Nutrient Agar (FNA) (Mazas et al., 1995) and incubated at 30°C for 3-5 days. Plates were checked daily by phase contrast microscopy (Leica DMLS, Mc Bain instruments, Wetzlar, Germany) examination, and sporulations were harvested when at least 90% of the cells had produced spores. The biomass was removed from agar surface by flooding twice with distilled sterile water, washed three times with distilled sterile water and stored at -20°C until use. Immediately before use, spore suspension was heat shocked at 80°C for 10 minutes to ensure that subsequent cells grown would have the same physiological state.

6.2.2. Study of low pH effect on *B. cereus* and *B. weihenstephanensis* grown

To test acid resistance of *B. weihenstephanensis* KBAB4 and *B. cereus* CECT 148, growth curves were made in Brain Heart Infusion broth (BHI, Scharlau, Barcelona, Spain) acidified to pH 3, 3.4, 3.8, 4.2, 4.6 and 5 with HCl 1M (Panreac, Barcelona, Spain). A positive control was performed in BHI pH 7. Two consecutive subcultures of *B. weihenstephanensis* KBAB4 and *B. cereus* CECT 148 were carried out in BHI at 30°C with shaking (200 rpm) up to stationary phase. Bacterial cultures were inoculated in the corresponding modified medium and serial dilutions were done to obtain approximately a population of 10^3 CFU/mL. Microwells plates were filled with 400 μ L of diluted culture per well and incubated in Bioescreen C automatic reader (LabSystems, Helsinki, Finland) at 30°C. Optical density was measured at 600 nm every 10 minutes during 25 hours and plates were shaken periodically. Each condition was tested by triplicate.

6.2.3. Acid stress treatment

B. weihenstephanensis KBAB4 and *B. cereus* CECT 148 were revitalized by two consecutive subcultures in BHI at 30°C with shaking (200 rpm) up to stationary phase. Next, 50 mL of each bacterial suspension was concentrated by centrifugation at 2,500 rpm for 10 minutes and pellets were resuspended in 10 mL of BHI to obtain a final concentration of 10^8 CFU/mL. To study the effect of pH on viability of cells, 1 mL of concentrated bacterial culture was inoculated in 4 mL of BHI acidified with HCl 1 M to pHs 3.4, 3.8 and 4.2 and incubated for 5 minutes at 30°C. At designed time points (30 minutes, 2 hours and 24 hours), samples were taken for FCM analysis and bacterial growth was measured by VPC.

6.2.4. Culturability evaluation by plate counts

All the conditions tested for *B. weihenstephanensis* and *B. cereus* strains (both controls and pH treatments) were plated onto BHI agar (BHIA, Scharlau, Barcelona, Spain) once 10-folds dilutions were performed in buffered peptone water (Scharlau, Barcelona, Spain). After incubation at 30°C for 24 hours, colonies were counted.

6.2.5. *Fluorescent reported dyes*

cFDA and PI were used to evaluate cell viability. Both of them selectively stain different structures allowing differentiation of viable and damaged cells. As long as PI is a membrane impermeant dye which binds to DNA by intercalating between the bases when membrane integrity is lost (Ananta et al., 2004), cFDA is a lipophilic dye able to diffuse inside the cells where is cleaved by unspecific esterases converting in a membrane-impermeant fluorescent compound, carboxyfluorescein (cF), which is retained in viable cells with intact cytoplasmic membrane (Petit and Ratinaud, 1993).

Viable cells stained with cFDA emitted green fluorescence, whereas DNA of damaged cells labeled with PI emitting red fluorescence. Green fluorescence was collected at 530 nm bandpass filter in the FL1 channel (FL1) and red fluorescence at 580 nm in the channel FL3 (FL3). For this experiment we used a commercial solution of PI 1mM (Molecular Probes, Oregon, USA). A stock solution 1 mM of cFDA (Sigma Aldrich Chemie) was performed in phosphate buffered saline to stain *Bacillus* cells. Both fluorescent dyes were stored at -20°C until use.

6.2.6. *Flow cytometry measurement*

Flow cytometry was performed using a FACSCalibur Flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 15 mW air-cooled 488 nm argon-ion laser. The software used for data acquisition was BD CellQuest Pro (BD Biosciences). The sheath fluid used for all experiments was FacsflowTM (BD Biosciences), filtered through a 0.2 µm Millipore Millex-GN filter (Millipore Corporation, Bedford, USA). Fluorescent beads (CalibriteTM three colors calibration beads, BD Biosciences) were used as an internal standard for scatter and fluorescence. The FCM analyses were performed with the following detector settings: forward scatter (FSC), E00; side scatter (SSC), 400; FL1, 400; FL3, 500 using logarithmic gains. FCM samples were adjusted to an event rate of 200-700 cells per second and a total of 10,000 events were registered per sample.

To study viability and damage of vegetative cells of *B. weihenstephanensis* KBAB4 and *B. cereus* CECT 148 incubated at different pH (3.4, 3.8 and 4.2) 10 µL of

each condition were added to 990 μL of prefiltered FascFlow to obtain an approximate concentration of 10^6 CFU/mL and stained with 1 μL of fluorescent dye, cFDA or PI. Samples were vigorously mixed and incubated in the dark at 30°C temperature for 15 min prior to analysis.

6.2.7. Flow cytometry controls

Positive and negative controls were performed to discriminate *Bacillus* population in dot-plot. To test cFDA and PI as fluorescent dye of viable cells aliquots of 1 mL of bacterial culture were inoculated in 5 mL of BHI and stained independently with 1 μL of each fluorochrome. To assess cFDA and PI as fluorescent dyes of damaged cells, aliquots of 1 mL of bacterial culture were inoculated in 5 mL of BHI and then heated at 80°C during 4 minutes before adding independently 1 μL of fluorochromes to assess loss of cell membrane integrity.

6.2.8. Data analysis of flow cytometry

Data were analyzed with the software package BD CellQuest Pro (BD Biosciences). Fluoresce collected in channels FL1 and FL3 was analyzed using FCM-derived dot plots of green or red fluorescence versus SSC. To discriminate bacteria from artifacts a gate in the dot-plot of FSC vs. SSC was determined. Quantitative assessment of each bacterial subpopulation was performed by counting the number of events included inside the corresponding gate (Paparella et al., 2008). Data were displayed as dual parameter fluorescence density plots.

6.3. Results and discussion

6.3.1. Effect of acid environment on *B. cereus* and *B. weihenstephanensis* growth

The acid responses of *B. cereus* and *B. weihenstephanensis* populations on stationary phase were studied in BHI medium adjusted at different pH values ranging between 3 and 5. Growth experiments were performed with an automated turbidity meter, Bioscreen C microbiology reader, and OD measurements were taken every 10

minutes during 25 hours at 30°C. Both strains were grown in BHI fitted to pH 7 as positive control.

B. weihenstephanensis vegetative cells were able to grow in BHI adjusted to pH from 5.0 to 3.4 during an incubation period of 25 hours (Figure 2). No growth was observed at pH 3.0 whilst growth was slow at pH 3.4. The maximum growth rate was significantly higher for cultures grown at pH 5, especially compared to lower pHs. Samples incubated in BHI pH 3.4 and 4.2 showed a lag phase with a similar duration (approximately 10 hours), significantly longer than pH 4.6 and 5.0 (Fig. 2). The maximum cell concentration was also lower at low pHs.

Figure 2. Growth curves of *B. weihenstephanensis* in acidified BHI. The optical density determined at different time points upon exposure to pH 3.0 (yellow), pH 3.4 (green), pH 4.2 (red), pH 4.6 (violet) and pH 5 (grey) are depicted. As positive control *B. weihenstephanensis* was grown in BHI pH 7 (black).

B. cereus vegetative cells exposed to pH 3.4 were not able to grow after 25 hours of incubation (Figure 3). Similar to *B. weihenstephanensis* experiments, samples with pH values of 3.8 and 4.2 showed approximately a two-fold extension of lag phase compared to samples incubated at pHs 4.6 and 5.0 (Figure 3). Under pH 5.0 and 4.6, *B. cereus* cells showed a multiphase behavior conformed of an increase phase followed by a short steady or decreasing phase and, subsequently, another growth phase, with a higher slope than the first one.

Figure 3. Growth curves of *B. cereus* in acidified BHI. The optical density determined at different time points upon exposure to pH 3.4 (green), pH 3.8 (blue), pH 4.2 (red), pH 4.6 (violet) and pH 5.0 (grey) are depicted.

Results revealed that upon acid stress *B. cereus* CECT 148 and *B. weihenstephanensis* KBAB4 growth was readily affected as it has been reported previously (Biesta-Peters et al., 2010; Mols et al., 2010a,b), showing greater effect at lower pHs. Specifically, in response to acid environment *B. weihenstephanensis* population appeared to be more resistant to low pH than *B. cereus* CECT 148 strain. In fact, *B. weihenstephanensis* was able to grow at lower pH (pH 3.4) and showed a significantly higher growth at all pH values tested in comparison with *B. cereus* microorganisms (Figures 2 and 3).

Furthermore, for both strains growth rate decreased and lag phase increased when the pH was reduced (Figures 2 and 3) in agreement with Biesta-Peters et al. (2010). To what extent these growth parameters are affected depends on initial growth rate, strain and acidulant used (Mols and Abee, 2011). It has been reported that *B. cereus* lag phase increases upon acid exposure because cells are repairing damage, buffering internal pH and increasing ATP concentration (Mols and Abee, 2011).

Notably, pH 4.6 is normally assumed as a lethal pH value for *B. cereus*. However, late stationary cells of strains tested in these experiments were able to grow at pH 4.2 and 3.8. *B. weihenstephanensis* was also able to grow at pH 3.4. In 2002, Jobin et al. reported that acid tolerance response (ATR) in *B. cereus* was clearly induced at

late stationary growth phase possibly due to the exponential phase metabolism which results in medium acidification, oxygen depletion and energy starvation. Thus, “*B. cereus* ATR appears to be a complex system involving both pH-dependent, and/or growth-phase-dependent mechanisms” (Jobin et al., 2002).

6.3.2. Effect of acid environment on *B. cereus* and *B. weihenstephanensis* viability

Flow cytometry analysis based on SSC, FSC and fluorescence detection was used to estimate the impact of acid stress on *B. weihenstephanensis* and *B. cereus* viability. For this purpose vegetative cells were stained with two fluorescent dyes, PI and, cFDA. Green fluorescent emission by cells stained with cFDA was detected by receptor FL1 (530nm $\pm$ 15 nm), and red fluoresce emitted by cells stained with PI by the receptor FL3 (650 nm). Data obtained were used to get scatter graphics by plotting red or green fluorescent signals vs. SSC (Figures 4 to 9).

In order to test PI as *B. cereus* and *B. weihenstephanensis* dye of damaged cells, controls were performed as described in the materials and methods section. To test PI positive control, cells were subjected to heat treatments for 10 min at 80°C. To confirm cell damage samples were plated onto BHIA. Counts revealed a decrease of bacterial population of several log cycles for both *B. cereus* strain and *B. weihenstephanensis* isolate. Comparison of heat treated cells with those untreated showed red fluorescence in treated cells indicating that inactivation of bacterial cells by heat treatment was coincident with physical compromise of the plasmatic membrane and consequently with electron transfer chain activity. For its part, cFDA positive control (non-treated cells) showed green fluorescence, indicating the maintenance of esterase activity and intact cytoplasmic membranes. There is a dependency between pH and cF signal since this compound shows high fluorescence at intracellular pH values of 7 and up (Biesta-Peters et al., 2011). It is noteworthy that induced acid stress response may result in increase of cytosolic pH through activation of pH homeostasis mechanisms (Foster, 1995). In addition, it should be taken into account that a fluorescence decrease at later phases of growth may be due to the active transport of cF out of the cells (Breeuwer et al., 1996).

Based on previous studies (epigraph 3.1), three pH values were selected for *B. weihenstephanensis* viability experiments. Thus, pH 3.4 and 4.2 were selected because both caused lag phases of similar duration meanwhile exponential growth rates were different. To test an intermediate condition pH 3.8 was selected. To perform a comparative study of *B. weihenstephanensis* and *B. cereus* acid resistance, the same pH values were used for both of them. In addition, these pH values are closer to those present in stomach allowing us to evaluate the pathogenic potential of these microorganisms. Effects of acid media were tested at 30 minutes, 2 and 24 hours of exposure.

Samples incubated in BHI acidified to pH 3.4 showed a sharp effect on *B. cereus* and *B. weihenstephanensis* populations (Figures 4 and 7). The percentage of viable cell as measured by cFDA staining after 30 minutes was 1.36% for *B. cereus* (Table 3) and 1.38% for *B. weihenstephanensis* (Table 1), indicating that most of the cells did have damaged membranes and therefore electron transport was suspended (Tables 2 and 4). After 24 hours, results were very similar to those obtained at time 30 minutes, however, at time 2 hours viable cells percentage slightly increased compared with results obtained after (Tables 1 and 3).

At time 30 minutes samples exposed to pH 3.8 showed a decrease of green fluorescence of 30% approximately both for *B. weihenestephanesis* and *B. cereus* populations (Tables 1 and 3). However, the major effect was observed at time 2 hours (Figure 5 and 8). Percentage of damaged cells increased approximately 96% for *B. weihenestephanesis* population and 86% for *B. cereus* (Tables 2 and 4). From 2 to 24 hours of incubation just a slight decrease of viable cells percentage was observed for both species (Tables 1 and 3).

The effect of pH 4.2 showed the lowest overall activity against *B. weihenstephanesis* and *B. cereus* during the first two hours of exposure to acid stress (Tables 1 to 4). At time 2 hours only a slight decrease on viable cell percentage was observed, however after 24 hours most of the population showed damaged membranes (Figures 6 and 9). In fact, after one day of incubation at pH 4.2 damaged cell percentage increased approximately 95% for *B. weihenestephanesis* vegetative cells and 82% for *B.*

cereus population reaching approximately similar values than those observed at this time in cells subjected to pH 3.4 and 3.8 (Tables 2 and 4).

VPC results of samples exposed to pH 3.4 reveal at time 30 minutes that *B. cereus* population had decreased 5 log CFU/mL (from 5.4×10^7 to 9.1×10^2), and after 2 hours of incubation no colonies were counted (Table 3). However *B. weihenstephanensis* population decreased 5 log CFU/mL (from 2.13×10^8 to 2.14×10^2) upon 2 hours of incubation and after 24 hours 10^2 CFU/mL could still be counted (Table 1).

When vegetative cells of microorganisms tested were exposed to pH 3.8 after 2 hours, a sharp decrease was noticed for both species (Tables 1 and 3). From 2 hours to 24 hours, *B. weihenstephanensis* VPC only showed a slight decrease and after 1 day incubation 1.35×10^2 CFU/mL were found. However, *B. cereus* decreased 2 log CFU/mL in this period and after 24 hours of incubation colonies were below detection limit (Tables 1 and 3). At pH 4.2 during the first 2 hours of incubation only a slight decrease was noticed for both species. After 24 hours, a 3 log decrease in CFU/mL for *B. weihenstephanensis* was observed; meanwhile, *B. cereus* decreased 2 log cycles in this period.

Table 1. Viable cells percentage detected by flow cytometry and viable plate counts of *B. weihenstephanensis* KBAB4 strain.

Time (h)	pH					
	3,4		3,8		4,2	
	Viable cells (%)	CFU/mL	Viable cells (%)	CFU/mL	Viable cells (%)	CFU/mL
0	98.83±0.79	2.13E+08	98.83±0.79	2.13E+08	98.83±0.79	2.13E+08
0.5	1.33±0.27	2.30E+05	70.71±5.21	3.26E+07	85.59±0.09	1.43E+08
2	2.42±0.94	2.14E+02	2.89±0.41	3.10E+02	89.02±0.71	1.05E+07
24	1.35±0.08	1.00E+02	1.84±0.34	1.35E+02	1.57±0.18	2.25E+05

Table 2. Damaged cells percentage detected by flow cytometry and viable plate counts of *B. weihenstephanensis* KBAB4 strain.

Time (h)	pH					
	3,4		3,8		4,2	
	Damaged cells (%)	CFU/mL	Damaged cells (%)	CFU/mL	Damaged cells (%)	CFU/mL
0	0.28±0.09	2.13E+08	0.28±0.09	2.13E+08	0.28±0.09	2.13E+08
0.5	94.67±0.44	2.30E+05	35.1±0.66	3.26E+07	1.48±0.06	1.43E+08
2	94.68±0.65	2.14E+02	96.56±0.26	3.10E+02	2.92±0.19	1.05E+07
24	95.91±0.04	1.00E+02	96.1±1.40	1.35E+02	95.41±0.23	2.25E+05

Table 3. Viable cells percentage detected by flow cytometry and viable plate counts of *B. cereus* CECT 148 strain.

Time (h)	pH					
	3,4		3,8		4,2	
	Viable cells (%)	CFU/mL	Viable cells (%)	CFU/mL	Viable cells (%)	CFU/mL
0	91.35±0.51	5.40E+07	91.35±0.51	5.40E+07	91.35±0.51	5.40E+07
0.5	1.26±0.10	9.10E+02	63.35±0.22	7.10E+07	73.07±2.17	7.80E+07
2	3.14±1.46	0.00E+00	8.05±1.01	1.00E+02	84.65±0.55	1.78E+07
24	0.95±0.31	0.00E+00	6.34±0.38	0.00E+00	12.11±0.47	4.40E+05

Table 4. Damaged cells percentage detected by flow cytometry and viable plate counts of *B. cereus* CECT 148 strain.

Time (h)	pH					
	3,4		3,8		4,2	
	Damaged cells (%)	CFU/mL	Damaged cells (%)	CFU/mL	Damaged cells (%)	CFU/mL
0	0.20±0.01	5.40E+07	0.20±0.01	5.40E+07	0.20±0.01	5.40E+07
0.5	75.65±1.13	9.10E+02	24.81±1.75	7.10E+07	1.42±0.19	7.80E+07
2	87.28±0.51	0	86.6±0.74	1.00E+02	23.36±2.51	1.78E+07
24	88.85±1.30	0	91.19±2.02	0.00E+00	82.9±0.67	4.40E+05

Figure 4. Flow cytometry dot plots of FL1 (green fluorescent) vs. SSC and FL3 (Red fluorescence) vs. SSC of *B. cereus* CECT 148 vegetative cells grown in BHI fitted to pH 3.4 (A) at time 0 (B) after 30 minutes of exposition to acid medium (C) after 2 hours of exposition to acid medium (D) after 24 hours of exposition to acid medium.

Figure 5. Flow cytometry dot plots of FL1 (green fluorescent) vs. SSC and FL3 (Red fluorescence) vs. SSC of *B. cereus* CECT 148 vegetative cells grown in BHI fitted to pH 3.8 (A) at time 0 (B) after 30 minutes of exposition to acid medium (C) after 2 hours of exposition to acid medium (D) after 24 hours of exposition to acid medium.

Figure 6. Flow cytometry dot plots of FL1 (green fluorescent) vs. SSC and FL3 (Red fluorescence) vs. SSC of *B. cereus* CECT 148 vegetative cells grown in BHI fitted to pH 4.2 (A) at time 0 (B) after 30 minutes of exposition to acid medium (C) after 2 hours of exposition to acid medium (D) after 24 hours of exposition to acid medium.

Figure 7. Flow cytometry dot plots of FL1 (green fluorescent) vs. SSC and FL3 (Red fluorescence) vs. SSC of *B. weihenstephanensis* KBAB4 vegetative cells grown in BHI fitted to pH 3.4 (A) at time 0 (B) after 30 minutes of exposition to acid medium (C) after 2 hours of exposition to acid medium (D) after 24 hours of exposition to acid medium.

Figure 8. Flow cytometry dot plots of FL1 (green fluorescent) vs. SSC and FL3 (Red fluorescence) vs. SSC of *B. weihenstephanensis* KBAB4 vegetative cells grown in BHI fitted to pH 3.8 (A) at time 0 (B) after 30 minutes of exposition to acid medium (C) after 2 hours of exposition to acid medium (D) after 24 hours of exposition to acid medium.

Figure 9. Flow cytometry dot plots of FL1 (green fluorescent) vs. SSC and FL3 (Red fluorescence) vs. SSC of *B. weihenstephanensis* KBAB4 vegetative cells grown in BHI fitted to pH 4.2 (A) at time 0 (B) after 30 minutes of exposition to acid medium (C) after 2 hours of exposition to acid medium (D) after 24 hours of exposition to acid medium.

As results showed, significant differences were observed in cell responses to the different pH values studied. We showed that cellular viability decreases at lower pH values. As we expected, the effect of pH 3.4 was the most effective one against these microorganisms and pH 4.2 had the lowest overall activity. In any case, at all the pH values tested, *B. cereus* and *B. weihenstephanensis* viable cell percentage decreased when exposure time increases indicating the absence of growth. Both species showed the strongest effect on cell viability at pH 4.2 between 2 and 24 hours, at pH 3.8 in the period between 30 minutes and 2 hours, and at pH 3.4 in the first 30 minutes for *B. cereus* and in the first 2 hours for *B. weihenstephanensis*.

On the other hand, 10^2 log CFU/mL of *B. weihenstephanensis* were counted after 24 hours of incubation at pH 3.8 and 3.4. Likewise, both microorganisms were rather resistant to pH 4.2 and upon 24 hours of exposure to this acid environment VPC showed that 5 log CFU/mL of vegetative cells had survived. A stomach pH higher than 4.5 is frequent in aged people, in patients with achlorhydria or even at the end of a copious meal (Dressman et al., 1990; Russell et al., 1993; Clavel et al., 2004). Besides, infective doses as low as 10^{-2} - 10^{-3} *B. cereus* CFU/g have been found in foods causing disease, even lower numbers of spores compared with vegetative cells can probably cause diarrheal syndrome due to their higher resistance to gastric acids (Gilbert and Kramer, 1986; Granum, 1994; Clavel et al., 2004).

Microorganisms exposed to acid stress develop an ATR able to protect them towards a subsequent exposure to a lethal pH. These mechanisms have been largely reported in various bacteria such as *Salmonella typhimurium*, *Lactococcus lactis* and *Listeria monocytogenes* (Jobin et al., 2002). As we described above, ATR was induced in *B. cereus* cells at late stationary phase under low pH (Jobin et al., 2002). In this work, at every pH tested, acid stress effects on late stationary phase cells were higher at longer exposition periods (Tables 1 to 4). According to plate counts, pH 3.4 and 3.8 had approximately the same effect on *B. weihenstephanensis* populations after 2 and 24 hours of incubation. On the other hand, VPC of *B. cereus* populations incubated at pH 3.4 and 3.8 did not show VPC after a long incubation period (24 h). In this regard, ATR effect on acid resistance of *B. cereus* did not seem to be observed at pH 3.4 and 3.8, meanwhile it may protect *B. weihenstephanensis* cells at similar pHs.

At all pH tested FCM data did not show significant differences between *B. cereus* and *B. weihenstephanensis* vegetative cells acid resistance. However, on the basis of VPC results, it appeared that *B. weihenstephanensis* is more resistant to acid stress or able to recover faster, after 24 hours of exposition at lower pHs (pH 3.4 and pH 3.8) and less than *B. cereus* at pH 4.2. It is important to note that at present, the number of VPC and the percentages of viable and damaged cells presented by the FCM, cannot be related. Mathys et al. (2007) reported that “the comparison of different methods within a 1 log₁₀ (90%) detection range with the highly concentrated cell suspensions will be subjected to sources of error making quantitative comparison of the FCM data and VPC data more difficult”. Furthermore, cells stained with fluorescent dyes could exist in a number of different functional states. Thus, viable cells are those which have intact their metabolic activity and membrane integrity and in consequence are able to divide (Nebe-von-Caron et al., 2000) and to grow on specific routine bacteriological media and develop into colonies. Under exposition to detrimental nutrition conditions, toxic chemicals and sub-optimal physical conditions we can differentiate “sublethally stressed cells” (Neidhardt and VanBogelen, 2000) that present impaired growth due to cellular damage without affecting their survival and “injured cells” which may recover or dye (Yousef and Courtney, 2003). Biesta-Peters et al. (2011) reported that compromised membranes after acid stress decrease during lag phase because cells are able to repair membrane damage at pH 7, 5.3 and 4.9. In addition, some authors (Kell et al., 1998) have reported the existence of physiological states of non-growing microorganisms among which are “active but non culturable cells” (ABNC) and “viable but non culturable cells” (VBNC) (Roszak et al., 1984; Kell et al., 1998). Both of them are cells that do not form colonies on non-selective growth media and can remain in this state for long periods. However, definition of these states is still controversial. On the other hand, we can find dead or inactivated cells which are not able to growth when are inoculated in growth medium. Therefore, in response to stress conditions different states of vegetative cells occur. Thus, “sublethally injured cells” can be stained either with PI or with cFDA depending on plasmatic membrane integrity. After, they can grow on plate or die, which makes VPC and FCM data comparison inaccurate as they are providing close, but different information. Additional factors explaining these differences would help to understand microbial recovery mechanisms (or the interface growth/no growth).

On the basis of our results we can assert that fluorescent labeling by using PI and cFDA in combination with FCM techniques can be used to successfully study physiological response of *B. weihenstephanensis* vegetative cells to acid stress as previously reported for *B. cereus* strains (Biesta-Peters et al., 2011). In addition, FCM analysis was unable to reliably discriminate when population of vegetative cells stained both PI and cFDA were lower than 10^5 CFU/mL as for example it has been shown by *B. weihenstephanensis* vegetative cells exposed to pH 3.4 (Tables 1 and 2). This result is not unexpected since the detection threshold of this technique is fixed in 10^5 CFU/mL. However, although FCM and VPC results were not comparable and classical analytical method shows higher reliability, it is important to note that FCM analysis provides information at real time of damage at single cell level, whereas VPC only gives an indication of cells able to grow at that time. In this context, FCM can respond to the need of development of valid methods that allow the integration of the physiological and molecular data at the single-cell level to predict, with the necessary confidence, the behavior of microorganisms thus greatly improving efficiency of defining key experimental conditions to be tested in food stability validation experiments (Hornstra et al., 2009).

CHAPTER 7

Development of a high resolution melting-based approach for efficient differentiation among *Bacillus cereus* group isolates

Abstract

Strains belonging to *Bacillus cereus* group include six different species among which are *Bacillus thuringiensis*, *Bacillus weihenstephanensis* and *Bacillus cereus sensu stricto*, a causative agent of food poisoning. Sequence of *panC*-housekeeping gene is used for *B. cereus* group affiliation to seven major phylogenetic groups (I-VII) with different ecological niches. Thermal growth range and spore heat resistance of *B. cereus* group microorganisms varies among phylogenetic groups. We assigned a selection of *B. cereus sensu stricto* strains related to food poisoning from the Spanish cultivar Collection (Valencia) to group IV strains based on *panC* gene sequence. To assess the ability of this classification as a valuable tool to manage *B. cereus* group isolates in food industry heat resistance experiments were performed. Thermal inactivation assays revealed variability of spore heat resistance within these group IV strains. Adequate food sanitizing treatments therefore require fast and reliable identification of particular strains. In the present study, feasibility of genotyping by single nucleotide polymorphisms (SNPs) detection via high resolution melting (HRM) analysis was examined. HRM analysis of amplified polymorphic 16S-23 ISR region proved to be discriminatory for *B. cereus sensu stricto* strain typing while two other polymorphic regions within the bacterial rRNA operon allowed differentiation between *Bacillus* species, demonstrating its applicability for discrimination on the species and strain level within *B. cereus* group.

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7.1. Introduction

The classification of *B. cereus* group microorganisms into seven major phylogenetic groups (I to VII) (Guinebretière et al., 2008), which show specific “thermotypes” and different virulence potentials, means a direct practical application for the food industry since it allows manufacturers to establish appropriate environmental conditions for processing, storage and distribution of food products depending on the risk level associated to contamination with specific strains of this taxonomic cluster.

In this regard, this classification may lead on the one hand to improve shelf-life and microbiological quality of refrigerated products by detecting psychrotrophic microorganisms belonging to group II and VI, and on the other, to prevent health hazards through discrimination among strains of different phylogenetic clusters, especially for those belonging to group VII, III and IV which may present highest pathogenic potential (Guinebretière et al., 2008).

For successful affiliation to phylogenetic groups (I to VII) a fast and effective homology study of the pantoate- β -alanine ligase gene (*panC*) sequence has been proposed (Candelon et al., 2004; Guinebretière et al., 2008). It offers a reliable alternative to solve practical problems at industrial scale derived from the lack of typing techniques able to discriminate between *B. cereus* group species and strains, and to avoid the use of expensive and time-consuming classical microbiological and biochemical probes used for bacterial identification.

In the present work, a preliminary study was carried out to characterize the practical application in heat processing of major phylogenetic groups. For this purpose we selected five *B. cereus sensu stricto* strains of diverse isolation origin and virulence/enterotoxin genotype belonging to the Spanish Type Culture Collection (CECT). Origins encompass animals, soil and unspecific contaminations. They were classified by Martínez-Blanch et al. (2011) into three groups based on different toxigenic profiles including a broad range of virulence factors involved in diarrheal syndrome: CytK, Hbl, Nhe, PC-PCL, Clo, SMase, and EntFM. These strains are able to colonize food products at any step during the processing chain from “farm to table”, making thermal niche affiliation a necessity in order to minimize pathogen attack,

improve food processing and thereby ensure consumers health. Accordingly, we performed strain assignation to ecotype niches based on partial *panC* gene sequencing. All strains included in this study belonged to group IV. Characterization of cardinal parameters is a main concern to gain a profound understanding of the relationship between physiology of *B. cereus* group strains and their phylogenetic affiliation. Our microbiological analysis focused on spore heat resistance, thus establishing references for food processing and *B. cereus* risk assessment. Results show that spore heat resistance of *B. cereus* group IV isolates varies among the strains tested.

This variability shown by strains belonging to the same phylogenetic cluster is not unexpected since “the high polyphyletic character of *B. cereus sensu stricto* and *B. thuringiensis* will result in highly different characters for strains inside each species” (Guinebretière et al., 2008). Considering these differences, and attending to the need of an appropriate technique valid for *B. cereus* group strain and species typing, different 16S-23S rRNA intergenic spacer region PCR-based methods (ISR-PCR) were evaluated in the present study.

All members of the *B. cereus* group show a very high similarity level (from 99.5 to 100%) of the 16S and 23S rRNA genes sequences (Daffonchio et al., 1998). However, the intergenic spacer region (ISR) situated between 16S–23S rRNA genes are the most variable regions of the ribosomal operon due to nucleotide substitutions, insertions, deletions and presence of tRNA genes (Cherif et al., 2003). It has been proposed that ISR sequences vary extensively more than conserved ribosomal genes because they are under minimal selective pressure (Barry et al., 1991), leading to species-specific or even strain-specific sequences useful for strain genotyping.

Ribosomal operons are present in multiple copies within prokaryote genomes and most species of the *B. cereus* group possess 13 rRNA operons meanwhile psychrotolerant strains usually contain 14 operons (Candelon et al., 2004). Nevertheless, the number of operon copies is not a tool for phylogenetic studies among *B. cereus* group due to existing exceptions. Studies on *B. cereus* ATCC 14579 strain and *Bacillus anthracis* show that these operons can be divided into two different classes based on polymorphisms present downstream of the 23S rRNA gene (Read et al., 2003; Candelon et al., 2004).

Until now, ISR characterization has allowed to discriminate species within the *Bacillus* genus and *B. cereus* group, while differentiation of closely related species and strains of the *B. cereus* group remains a challenge which could be addressed using high resolution separation techniques.

In contrast to conventional DNA melting analysis, HRM is a sensitive analysis method based on highly controlled temperature transitions and DNA binding dyes with improved saturation properties, able to detect single base sequence variations, which generate a melting behavior characteristic of a particular DNA sample. Furthermore, it does not require laborious and time-consuming gel electrophoresis as in case of strain discrimination based on RAPDs. HRM has been reported to obtain successful results in specific species identification of clinically relevant bacterial species and in medical diagnosis applications (Odell et al., 2005; Cheng et al., 2006; Montgomery et al., 2007; Van Ert et al., 2007; Erali et al., 2008; Yang et al., 2009; Tuohy et al., 2010; Temesvári et al., 2011). Besides, this technology has been used to genotype different *Pseudomonas aeruginosa* strains isolated during an outbreak linked to mineral water bottles in a neonatal intensive care unit (Naze et al., 2010).

HRM analysis begins when polymerase chain reaction (PCR) products, previously stained with specific intercalating fluorescence dyes, are subjected to a thermally-induced DNA dissociation. Under controlled temperature increase, DNA samples are characterized as fluorescence decreases when DNA intercalating dye is released from double-stranded DNA (dsDNA) consequence of its dissociation (melting) into single strands. HRM fluorescent dyes have low toxicity in an amplification reaction and can therefore be used at higher concentrations for greater saturation of the dsDNA sample. “Greater dye saturation means measured fluorescent signals have higher fidelity, apparently because there is less dynamic dye redistribution to non-denatured regions of the nucleic strand during melting and because dyes do not favor higher melting temperature products” (Wittwer et al., 2003). At a characteristic temperature, melting temperature (T_m), fluorescence rapidly drops reflecting dsDNA dissociation. It has been shown that, T_m it is characteristic for each dsDNA and depends on its GC content and length and sequence (Reed et al., 2007). However, T_m reflects only one point of the melting curve and more information is obtained from the complete curve.

Indeed, it has been reported that the shape of the curve contains valuable information for genotyping (Reed et al., 2007).

A rapid and cost efficient discrimination at the strain level is an important tool, also valuable for discrimination on the species level. One of the most common types of genetic variation are single nucleotide polymorphisms (SNP). SNPs occur in a given region of the genome, such as 16S-23S ISR, when there is a single base substitution of one nucleotide (A, T, C, or G) with another. This DNA sequences variants can be efficiently used for genotyping through HRM analysis (White and Potts, 2006). We therefore investigated the feasibility of HRM analysis for SNP genotyping of *B. cereus*, *B. weihenstephanensis* and *B. thuringiensis* strains amplifying a 16S-23S ISR region as well as two other polymorphic areas within rRNA gene operon.

Our study is a multivariable approach that combines strains and species identification with phylogenetic affiliation of isolates and characterization of their spore heat resistance.

7.2. Materials and methods

7.2.1. Bacterial strains and growth conditions

B. cereus sensu stricto strains used in this study were selected from the Spanish Type Culture Collection (CECT, Valencia, Spain). *B. weihenstephanensis* (KBAB4) and *B. thuringiensis* (Bt407) strains were kindly provided by the National Institute of Agronomy Research (INRA, Avignon, France). All the strains used in this study are listed in Table 1.

Strains were grown on nutrient broth (Scharlau Microbiology, Barcelona, Spain) at 30°C or 37°C for 18 hours and stored in growth liquid medium containing 20% (v/v) glycerol at -80°C. For DNA extraction the cultures were grown overnight in Brain Heart Infusion broth (BHI; Scharlau, Barcelona, Spain) at 30°C for 18 hours with agitation at 200 rpm. Strains were sporulated in Fortified Nutrient Agar at 30°C (Mazas et al., 1995) and stored at -20°C until use. To ensure that vegetative cells grown from spores would have the same physiological state, spores were heated at 80°C for 10 minutes (Fernández et al., 2001).

Table 1. Bacterial strains used in this study

Abbreviation	Strain	Source
CECT 131	<i>Bacillus cereus</i> CECT 131	CECT
CECT 148	<i>Bacillus cereus</i> CECT 148 (type strain)	CECT
CECT 193	<i>Bacillus cereus</i> CECT 193	CECT
CECT 4014	<i>Bacillus cereus</i> CECT 4014	CECT
CECT 4094	<i>Bacillus cereus</i> CECT 4094	CECT
Bt407	<i>Bacillus thuringiensis</i> Bt407	INRA
KBAB4	<i>Bacillus weihenstephanensis</i> KBAB4	INRA

CECT, Spanish Type Culture Collection; INRA, National Institute of Agronomy Research, France.

7.2.2. DNA extraction

Genomic DNA for *panC* gene sequence analysis was extracted from pure cultures of each *B. cereus* strain. Cells were lysed in 400 µL extraction buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8 and 1 mM EDTA pH 8.5) (Manzano et al., 2003), supplemented with lysozyme (0.5 mg/µL final concentration) and proteinase K (0.023 mg/µL final concentration) (Candelon et al., 2004) during 30 min at 37°C. Cell lysis was completed by adding SDS (1% final concentration) to the lysis buffer and an incubation at 65°C for 30 min. DNA purification included phenol-chloroform-isoamyl alcohol (25:24:1) extraction, followed by isopropanol precipitation and DNA was finally resuspended in 40 µL of sterile distilled water. Genomic DNA for RT-PCR and HRM-PCR was isolated using DNeasy Blood and Tissue kit (Quiagen, Germantown, MD, USA). Accurate DNA quantification for PCR assays was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Delaware, USA).

7.2.3. *PanC* amplification and sequencing

Two primers, *panC*for and *panC*rev, were designed with Primer3 (v 0.4.0) software for the amplification of *panC* gene of the *B. cereus* group species on the basis of *panC* gene sequence of *B. cereus* ATCC 14579 (accession number: NP_831320.1) retrieved from the GenBank (Benson et al., 2004). Sequences, expected amplicon size and T_m are listed in Table 3. PCR for *panC* gene amplification of CECT 131, CECT 148, CECT 193 and CECT 4094 strains was carried out in 25 µL reaction mixtures containing 5 µL 5X Colorless GoTaq® Flexi buffer (Promega), 20 µM of each dNTP,

0.4 μM of the forward and the reverse primer, 2 mM MgCl_2 , 0.2 μL *Taq* polymerase (GoTaq[®] DNA Polymerase [5 u/ μL], Promega) and template DNA (50-100 ng/ μL). The amplification conditions were as followed: initial denaturation for 5 min (94°C), 35 cycles of denaturation at 94°C (15 s), annealing at 55°C (30 s) and extension at 72°C (30 s) and final extension at 72°C (7 min). PCR was optimized for CECT 4014 strain using Herculase[®] II Fusion Enzyme kit (Stratagene, La Jolla, CA). The final reaction mixture volume (50 μL) contained 10 μL of 5X Herculase II Reaction Buffer, 250 μM of each dNTP, 0.25 μM of each primer, 0.5 μL of *Taq* polymerase and template DNA (100-300 ng/ μL). The amplification protocol included one cycle at 95 °C for 2 min followed by 35 cycles at 95°C for 20 s, 50°C for 20 s and 72 °C for 30 s, and 10 min of final extension at 72°C. PCR amplifications were performed on Applied Biosystem Thermal Cycler 9700 and aliquots of amplicons were visualized on 1 % agarose gels, stained with ethidiumbromide. Automated sequencing of PCR products was performed by Savia Biotech (Almería, España) using panCfor and panCrev primers.

7.2.4. Phylogenetic analysis and affiliation to *B. cereus* group

Nucleotide sequence alignment and phylogenetic analysis of *panC* gene between microorganisms of the *B. cereus* group was performed with the ClustalX 2.1 program applying Neighbour-Joining method (Saitou and Nei, 1987). The analyzed data set also included *panC* gene sequences of *Bacillus* species and strains available from the GenBank (Benson et al., 2004) with the following NCBI accession numbers: *B. weihenstephanensis* (5841679), *B. cereus* ATCC 10987 (2748449), *B. cereus* G9842 (7186600), *B. cereus* AH187 (7073679), *B. cereus* E33L (3024889), *B. cereus* AH820 (7191073), *B. cereus* Q1 (7376764), *B. cereus* 03BB102 (7689603), *B. cereus* B4264 (7097070), *B. cereus* subsp. cytotoxis NVH 391-98 (5346025), *B. anthracis* str. Ames (1086855), *B. anthracis* str. A0248 (7853564), *B. anthracis* str. 'Ames Ancestor' (2815431), *B. anthracis* str. Sterne (2849787), *B. anthracis* CI (9454079), *B. anthracis* str. CDC 684 (7785245), *B. thuringiensis* str. Al Hakam (4546784), *B. thuringiensis* BMB171 (9192413) and *B. thuringiensis* serovar konkukian str. 97-27 (2858358).

Applying the classification system proposed by Guinebretière et al. (2008), *panC* gene sequences were used to assign the strains to phylogenetic groups I-VII performing the homology search' algorithm offered at

<https://www.tools.symprevius.org/Bcereus/english.php>. Percentages of homology calculation were done by using the following mathematical formula described in this web tool:

$$\% \text{ homology} = (\text{number of matched bases} / \text{alignment length}) \times 100 \quad (1)$$

where, alignment length is the number of bases covering the alignment.

Identification to phylogenetic groups is considered as not significant and rejected when % homology values are minor than 90%. Besides, when the highest % homology is <75%, identification to the *B. cereus* group is considered as not significant and rejected.

7.2.5. Heat resistance determination of *B. cereus sensu stricto* spores

Heat resistance determination of sporulated cells on sterile distilled water was carried out using Mastia thermoresistometer (Conesa et al., 2009). At specific time intervals, which depended on the treatment temperature (90, 95 and 100°C), samples were collected into sterile tubes, diluted, plated onto BHI agar (BHIA; Scharlau, Barcelona, Spain) and incubated at 37°C for 24 h. Survival curves were fitted using Geeraerd and Van Impe Inactivation Model Fitting Tool (GInaFIT; Geeraerd et al., 2005). Through traditional log-linear model (Bigelow and Esty, 1920) and log-linear model with shoulder (Geeraerd et al., 2000), *B. cereus* spore heat resistance was described to obtain an accurate assessment which allows us to design safety processing treatments avoiding those which are overly conservative. Maximum inactivation rate (K_{max}) was obtained from log-linear model. Besides, from the log-linear model with shoulder specific inactivation rate (K_{max}) and shoulder length were determined. Root Mean Sum of Squared Errors (RMSSE) (Ratkowsky, 2003) was selected as a measure of goodness of fit. The magnitude of RMSSE should be comparable to the standard deviation (as a measure of the precision of replicates) of the experimental data (Geeraerd et al., 2000).

7.2.6. Identification of highly polymorphic genomic regions and primer design

Genome sequences of *B. cereus* strains with the following accession numbers were obtained from GenBank database and used for primer design: NC 004722 *B. cereus* ATCC 14579 and NC 0011772 *B. cereus* G98421. Both sequences were aligned using the multiple sequence alignment algorithm MUSCLE, version 3.8 freely available at <http://www.drive5.com/muscle>. Conserved genome fragments flanking hypervariable nucleotide regions were selected between both strains. The selected polymorphic nucleotide sequences (Figures 3 and 4) were analyzed using Blast-N (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Primer3 (v 0.4.0) software was used to design and pre-evaluate primers. Autofolding was studied using Oligo Calculator (v 3.6) and probability of secondary fold using Quikfold tool available at DINAMelt Web server. *In silico* PCR using Ipcress (*In-silico PCR Experiment Simulation System*) was performed in order to assess the number of amplicons generated by the designed primer. Primer names, primer sequences, expected size of amplification products and T_m are presented in Table 3.

7.2.7. Amplification of polymorphic region by conventional and real-time PCR

Highly polymorphic 16S-23S ISR region were amplified by conventional PCR using primers 16S-23SISRfor and 16S-23SISRrev (Table 3) from *B. cereus* CECT 131, CECT 148, CECT 193, CECT 4014, CECT 4094, *B. thuringiensis* Bt407 and *B. weihenstephanensis* KBAB4. The PCR was performed on Applied Biosystem Thermal Cycler 9700 with Herculanase[®] II Fusion Enzyme kit (Stratagene, La Jolla, CA) as described above for *panC* gene amplification. PCR conditions were as followed: one cycle at 95 °C for 2 min, 35 cycles at 95°C for 30 s, 55°C for 30 s and 72 °C for 30 s, and 5 min of final extension at 72°C. PCR amplicons were analyzed on 2 % agarose gels.

Real-time PCR for the region 16S-23S ISR and for polymorphic regions flanking 5S and 16S rRNA genes was performed for the *B. cereus* group strains described above as well as for *B. thuringiensis* Bt407 and *B. weihenstephanensis* KBAB4. The 25 µL PCR reaction contained 12.5 µL of Brilliant II SYBR Green QPCR master mix (Stratagene, La Joya, CA), 0.3 µM of forward and reverse primers 16S-

23SISR, 5S-GT and 16S-ISR (Table 3) and template DNA (50-150 ng). Amplifications were performed in triplicate using Rotor-gene Q thermal cycler (Qiagen, Germantown, MD, USA).

PCR conditions consisted of one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 30 s, 57°C (ISR) or 58°C (5S-GT and 16S-ISR) for 30s and 72°C for 1 min. Fluorescence was measured at the end of each extension step. Melting curve analysis was performed in a temperature range from 57°C to 95°C increasing by 0.1°C each step. Data were analyzed using the software Rotor-Gene 2.02.4.

7.2.8. High resolution melting of polymorphic regions

For the detection of gene mutations and SNPs, we performed HRM analysis of 16S-23S ISR region and of polymorphic regions flanking 5S and 16S rRNA genes from *B. cereus* CECT 131, CECT 148, CECT 193, CECT 4014, CECT 4094, *B. thuringiensis* Bt407 and *B. weihenstephanensis* KBAB4 using Type-it[®] HRM kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. This commercial kit contains EvaGreen DNA binding dye with a 10-fold higher SNP detection sensitivity compared to SYBER Green I. PCR runs prior to HRM analysis were performed in triplicate using Rotor-gene Q thermal cycler and repeated three times. 16S-23S ISR region was amplified using oligonucleotides 16S-23SISRfor and 16S-23SISRrev and polymorphic regions flanking 5S and 16S rRNA genes were amplified using forward and reverse primers 5S-GT and 16S-ISR (Table 3). PCR conditions consisted in one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 30 s, 57°C (ISR) or 58°C (5S-GT and 16S-ISR) for 30 s and 72°C for 1 min. Fluorescence was measured at the end of each extension step. High resolution melting curve analysis was performed in a temperature range from 57°C (ISR) or 58°C (5S-GT and 16S-ISR) to 95°C increasing 0.1°C each step. Rotor-Gene 2.02.4 software was used to run HRM-PCRs and analyze results. Raw data of HRM analysis were normalized by setting the same start and ending fluorescent signal level for all the curves through adjusting two normalization regions, one region for the start (trailing range) and other for the end (leading range). Threshold values for genotyping confidence percentages were set. Genotypes were determined automatically by the software establishing confidence

values as an integrity check of obtained results. Samples bellow 90% threshold value were considered as a genotypic variant.

7.3. Results and discussion

7.3.1. *panC* gene sequence affiliation

Affiliation of *B. cereus* strains CECT 131, CECT 148, CECT 193, CECT 4014 and CECT 4094 to the phylogenetic *B. cereus* groups I-VII was performed by amplification and sequencing of *panC* gene (Candelon et al., 2004; Guinebretière et al., 2008). Obtained sequences were submitted to the web tool link '<https://www.tools.symprevius.org/Bcereus/english.php>' which performs comparison with known sequences compiled in the database of UMR408, INRA-University of Avignon (France). Percentages of homology calculation were done as described above.

Results revealed that *B. cereus* CECT strains tested in this study belong to group IV. Microorganisms of group IV are generally cytotoxic, some of them even highly cytotoxic. Concerning the toxygenic profile, 100% of tested strains form group IV carried the *nhe* gene, whereas the genes *hbl* and *cytK2* did not consistently appear in group IV strains (Guinebretière et al., 2010).

On the other hand, this phylogenetic group, which includes *B. thuringiensis* and *B. cereus* isolates, has a temperature growth range between 10-45°C which selectively determines their prevalence in different food products. Afchain et al. (2008) estimated that the genetic group IV prevailed in milk proteins and starch. Additionally, presence and growth in refrigerated food therefore should not occur, even so growth of strains of this mesophilic group in chilled pasteurized vegetables has been reported (Guinebretière et al., 2008).

7.3.2. Heat inactivation of *B. cereus* strains affiliated to group IV

To broaden the understanding of *B. cereus* strains affiliated to group IV concerning thermal processes, we characterized the heat resistance of strains CECT 131, CECT 148, CECT 193, CECT 4014 and CECT 4094 under isothermal conditions

at 90, 95 and 100°C. Results are summarized in Table 2 and include the parameters shoulder length (to describe shoulder phenomena), $K_{\max}$ values (referred to maximum inactivation rate and specific inactivation rate depending on the mathematical model used) and Root Mean Sum of Squared Errors (RMSSE), the square root of MSE, as a measure of goodness of fit, both for linear and non-linear models (Ratkowsky, 2003). Strain CECT 4094 was the most heat resistant one, followed by CECT 131 and CECT 148, while strains CECT 193 and CECT 4014 are the most heat sensitive ones (Figure 1A).

Shoulder phenomena with length values equal or lower than 0.5 minutes were considered as not significant. Strain CECT 4094 presented a shoulder phenomenon with length values between 23.88 and 31.19 min at 90°C, strain CECT 131 with values between 9.35 and 10.93 min and strain CECT 4014 with values between 2.68 and 3.51min. Shoulder length diminished at higher temperatures in a range of 2.4 to 4 times for strain CECT 4094 at 95 and 100°C, whereas for strains CECT 131 and CECT 4014, shoulder phenomenon at 95 and 100°C was not significant (Figure 1B). Coleman et al. (2007) reported that the length of the shoulder seems to be associated with lethal damage to one or more key spore proteins. Previous studies confirm that shoulders are widely occurring phenomena in heat resistance studies (van Zuijlen et al., 2010).

Augustin (2011) has stated that three different clusters of *B. cereus* group isolates linked to food poisoning can be defined: a) diarrhoeal, psychrotrophic, heat sensitive strains belonging to the genetic group II, b) diarrhoeal, mesophilic, heat resistant strains belonging to the genetic groups III, IV, V, and VII, and c) emetic, mesophilic, heat resistant strains belonging to the genetic group III. Besides, a ranking of spore heat resistance for *B. cereus* strains was established by Carlin et al. (2010). Those bacteria belonging to group III and VII were the most resistant to temperature injury, strains included in groups II, IV and V show an intermediate position and group VI members were the most sensitive ones. Heat resistance values of *B. cereus* strains connected to diarrheal foodborne outbreaks and of *B. cereus* food and environment strains, among which are included *B. cereus* affiliated to group IV, have been reported (Carlin et al., 2006) and were clearly higher than those obtained in this study at 90°C indicating heat resistance variability among group IV strains. Furthermore, $K_{\max}$ and shoulder length values obtained also indicate variations within *B. cereus* CECT strains, especially for *B. cereus* CECT 4094.

Table 2. Effect of heat treatments on *B. cereus sensu stricto* strains

Strain	90°C			95°C			100°C		
	<i>Sl</i> (min)	$k_{\max}$ (1/min)	RMSSE	<i>Sl</i> (min)	$k_{\max}$ (1/min)	RMSSE	<i>Sl</i> (min)	$k_{\max}$ (1/min)	RMSSE
CECT 131	9.35	0.23	0.15	3.34	0.73	0.25	0.75	0.78	0.48
	10.93	0.24	0.11	4.85	0.81	0.14	0.00	0.78	0.33
	10.22	0.23	0.10	4.13	0.73	0.13	0.00	0.74	0.21
CECT 148	0.00	0.30	0.19	0.00	1.30	0.10	0.00	1.99	0.29
	0.00	0.28	0.17	0.00	1.37	0.12	0.19	2.14	0.18
	0.00	0.30	0.27	0.45	1.39	0.08	0.00	2.08	0.11
CECT 193	0.00	0.42	0.18	0.13	1.48	0.29	0.00	2.14	0.32
	0.00	0.47	0.17	0.47	1.51	0.27	0.00	2.05	0.20
	0.00	0.49	0.19	0.51	1.77	0.25	0.00	2.08	0.19
CECT 4014	3.30	0.43	0.12	0.00	2.12	0.26	0.00	1.94	0.31
	3.51	0.45	0.05	0.00	2.02	0.20	0.00	1.45	0.39
	2.68	0.45	0.11	0.00	2.07	0.22	0.46	1.21	0.29
CECT 4094	28.61	0.13	0.26	7.08	0.36	0.19	9.06	0.37	0.31
	23.88	0.09	0.06	7.67	0.37	0.16	9.95	0.38	0.29
	32.19	0.11	0.09	8.66	0.35	0.25	10.19	0.39	0.26

Sl, Shoulder length; $K_{\max}$, Maximum inactivation rate or Specific inactivation rate; RMSSE, Root Mean Sum of Squared

A

B

Figure 1. Survival curves (A) at 90°C of *B. cereus* CECT 131 (orange), *B. cereus* CECT 148 (violet), *B. cereus* CECT 193 (black), *B. cereus* CECT 4014 (red) and *B. cereus* CECT 4094 (blue) and (B) of *B. cereus* CECT 131 at 90°C (yellow), 95°C (green) and 100°C (pink).

Notably, this heterogeneous behavior of *B. cereus* strains heat resistance and the presence of shoulders in their survival curves should be taken into account in order to prevent fail-dangerous processing treatments. Therefore, accurate identification of *B. cereus* strains could be a valuable tool to ensure consumer health and avoid spoilage.

7.3.3. Phylogenetic analysis of *panC* gene

We performed a phylogenetic analysis with tree construction in order to study the phylogenetic relatedness of the *panC* gene sequences obtained in the present work for *B. cereus* CECT strains with those available at the NCBI GenBank (accession numbers are listed above (see 2.4)). We had previously submitted the corresponding *panC* gene sequences to the web tool link mentioned above (see 3.1.1) for affiliation to phylogenetic groups I-VII (Figure 2). Dendrogram construction was carried out using ClustalX 2.1 program by the Neighbor-Joining method (Saitou and Nei, 1987).

Consistent with results of Guinebretière et al. (2008), the phylogenetic tree shows two main branches. One branch clusters members of group III, IV and VI and *B. cereus* subsp. cytotoxis NVH 391-98 of group VII as a separate entity (Figure 2). Besides, strains from group IV and VI are closer related than those affiliated to group III. CECT *B. cereus* strains are closely related to other strains of the same species as well as *B. thuringiensis* and *B. weihenstephanensis* strains. This phylogenetic assessment is consistent with others previously performed. In 2011, Schmidt et al. reported that some *B. cereus* strains were significantly more closely related to *B. anthracis* or *B. thuringiensis* than they are to other strains of *B. cereus*. Thus, these results suggest high genetic similarity among *B. cereus* group species making a challenge differentiation at strain or species level.

Figure 2. Genetic relationship among *B. cereus* group strains based on *panC* gene. Neighbour-Joining method (Saitou and Nei, 1987) was used for inferring tree topology. Bootstrap values (10,000 bootstrap replicates) are shown in the major nodes.

7.3.4. Primer design for single nucleotide polymorphism analysis

SNP analysis in combination with HRM analysis was found to be a rapid, efficient and economic genotyping method for applications in clinical areas like pharmacogenomics (Temesvári et al., 2011) as well as in plant genotyping (De Koeyer et al., 2009). Our objective was to apply this technique in the area of food microbiology for species and strain differentiation within *B. cereus*, which would then allow improve food processing techniques such as thermal treatment procedures and to determine the source of an outbreak and take effective control and preventing measures.

For amplification prior to HRM, we selected the 16S-23S ISR, which is widely used for genotyping bacteria based on its high degree of sequence polymorphism. Sequence alignment of rRNA operons between *B. cereus* G98421 (NC 0011772) and *B. cereus* ATCC 14579 (NC 004722) verified sequence polymorphism in the ISR region. Alignment information was used to design primers 16S-23SISRfor and 16S-23SISRrev flanking the polymorphic 16S-23S ISR (Figure 3).

In order to find additional candidate regions for amplification and HRM genotyping, we screened aligned sequences of *B. cereus* G98421 (NC 0011772) and *B. cereus* ATCC 14579 (NC 004722) to select variable regions flanked by conserved sequences. Candidate regions with a high probability of secondary fold and multiple amplicons based on *in silico* PCR were discarded as well as primer combinations resulting in more than a single peak in melting peak analysis of PCR products. Two pairs of primer, 5S-GTfor and 5S-GTrev and 16S-ISRfor and 16S-ISRrev (Table 3), fulfilled the necessary requirements and were used in our study. The corresponding amplified nucleotide regions are detailed in Figures 4A and 4B. 5S-GT-PCR product spans part of the 5S rRNA gene followed by a sequence highly homologous to glycosyltransferase. 16S-ISR primers result in an amplicon encoding 16S rRNA gene followed by a sequence highly homologous to ISR.

Table 3. PCR primers used in this study

Target	Primer	Primer sequence	Product size (bp)	T_m (°C)
<i>panC</i> gene	panCfor	CGTAAGGCAAGAGAAGAAAA	651	50/55
	panCrev	TCCTTCGACTTTCTCTACCA		
16S rRNA gene-23S rRNA gene ISR	16S-23SISRfor	CGAAGCATATCGGCGTTAGT	560	55/57
	16S-23SISRrev	CCCTTATGACCTGGGCTACA		
5S rRNA gene and glutamiltransferase	5S-GTfor	GAGAGTAGGACGTCGCCAAG	206	58
	5S-GTrev	TGAAACAGATTGGTGGTATGACTT		
16S rRNA gene and 16S-23S ISR	16S-ISRfor	AATAAGCGTCGTCAGGAACG	185	58
	16S-ISRrev	AGCCAGGATCAAACCTCTCCA

Figure 3. Comparative nucleotide sequence analysis of 23S rRNA gene, 16S-23S ISR and 16S rRNA gene from *B. cereus* ATCC 14579 and *B. cereus* G9842. Nucleotide position for the primers 16S-23SISRfor and 16S-23SISRrev are indicated. The polymorphic ISR region is highlighted.

Figure 4. Comparative nucleotide sequence analysis of polymorphic regions ‘5S-GT’ and ‘16S-ISR’om *B. cereus* ATCC 14579 and *B. cereus* G9842. **(A)** Nucleotide position for primers 5S-GTfor and 5S-GTrev **(B)** Nucleotide position for primers 16S-ISRfor and 16S-ISRrev.

7.3.5. 16S-23S intergenic spacer region PCR and *in silico* PCR

PCR-based fingerprinting methods have been extensively used for *B. cereus* group isolates identification. For amplification, we selected the 16S-23S ISR for differentiation of closely related bacterial species, such as *Bacillus* genus isolates, based on its high degree of sequence polymorphism (Jensen et al., 1993; Wunschel et al., 1994; Daffonchio et al., 1998; Nagpal et al., 1998; Shaver et al., 2002; Cherif et al., 2003). Sequence alignment of rRNA operons between *B. cereus* G98421 (NC 0011772) and *B. cereus* ATCC 14579 (NC 004722) verified sequence polymorphism in the ISR.

We amplified the 16S-23S ISR of *B. cereus* CECT 131, CECT 148, CECT 193, CECT 4014, CECT 4094, *B. weihenstephanensis* KBAB4 and *B. thuringiensis* Bt407 by conventional PCR. In accordance with Daffonchio et al. (1998, 2000) we observed identical and reproducible patterns in all strains tested revealing that 16S-23S ISR-PCR was unable to differentiate *B. cereus* CECT strains and those from *B. weihenstephanensis* KBAB4 and *B. thuringiensis* Bt407 (Figure 5A). This may suggest that length of 16S-23S ISR is conserved among *B. cereus* isolates and different techniques should be used to discriminate between closely related isolates that have essentially identical ISR sequences. Recently, Martínez-Blanch et al. (2011) reported that ISR-PCR profiles can be used for rapid assignation of isolates to *B. cereus* group meanwhile for typing at species level RAPD profiles were needed.

In silico PCR with our 16S-23SISRfor and 16S-23SISRrev primers applied to *B. cereus* ATCC 14579 (NC 004722) genomic DNA yielded a total of 13 possible matches, which possibly refer to the 13 rRNA operons mentioned by Candelon et al. (2004). Differences between ISR-PCR band pattern and expected *in silico* profile might partly be due to the presence of heteroduplex products formed during PCR between amplicons from different ribosomal operons (Daffonchio et al., 2003).

Figure 5. Amplification of 16S-23S ISR of *B. cereus* group strains with primers 16S-23SISRfor and 16S-23SISRrev (A) Agarose gel-PCR profiles (B) Major melting peaks obtained by real time PCR.

7.3.6. Evaluation of T_m profiles for strain and species discrimination

Melting peak analysis of PCR products from diverse polymorphic regions has been widely used as a reliable method for fast and sensitive discrimination between *Bacillus* species and strains (De Clerck et al., 2004; Priha et al., 2004; Fricker et al., 2007; Reekmans et al., 2009; Postollec et al., 2010). It was reported that differentiation among *B. cereus* group strains could be achieved by real-time PCR based on SYBER Green I (Martínez-Blanch et al., 2009; Wehrle et al., 2009).

Amplification products of three polymorphic regions, 16S-23S ISR, 5S-GT and 16S-ISR, all related to genes of the rRNA operon in the *B. cereus* group, were analyzed using this technique. Melting peak analysis resulted in two main peaks for 16S-23S ISR PCR product and one peak for 5S-GT and 16S-ISR PCR products, respectively. T_m values were very similar and did not allow consistent differentiation among species and strains. It was reported that T_m variations between amplicons of at least 1°C to 2°C can be differentiated using SYBER Green I, whereas T_m differences of 1°C or less are not valuable for genotyping and bacterial species identification (Odell et al., 2005). Figure 5B exemplarily shows the major melting peak of 16S-23S ISR PCR product for all *B. cereus sensu stricto* strains, *B. thuringiensis* and *B. weihenstephaniensis*.

7.3.7. Evaluation of high resolution melting analysis for species genotyping

Genotyping of bacteria is a powerful tool for identification of bacteria (Towner and Cockayne, 1993) and the detection of SNP can be easily and rapidly performed by HRM, replacing other technologies like sequencing (Tesmevári et al., 2011). According to Liew et al. (2004), 84% of SNPs result in T_m differences of about 1°C, 12% cluster around 0.25°C and 4% are predicted to be identical by nearest neighbour thermodynamics. In general, small insertions and deletions were shown to be more difficult to detect than substitutions (Wittwer, 2009).

In HRM analysis, curve shapes are compared and variations in shapes are usually easier to detect than T_m differences, even so different sequences might generate

similar melt curves (Cheng et al., 2006). The Rotor-Gene 2.02.4 software performs an automatic calling of clusters belonging to different genotypes, recognizing T_m differences as low as 0.1°C between amplicons in addition of curve shape variants among genotypes.

B. cereus CECT 148, *B. thuringiensis* Bt407 and *B. weihenstephanensis* KBAB4 strains were used to test species discrimination by SNP genotyping with HRM analysis, following the amplification of polymorphic regions 16S-23S ISR, 5S-GT and 16S-ISR (Table 3 and figures 3 and 4). 5S-GT and 16S-ISR amplicons gave derivative melting curves with a single peak (Figures 6A and 6B), indicating the presence of one amplification product only. 16S-23S ISR-PCR products showed two melting peaks (data not shown). Normalized plots for genotyping were generated by selecting appropriate areas from the melting curves (Figure 6C and 6D). Figures 6E and 6F show difference plots which were generated by defining representative genotypes followed by subtraction of average plots of the different genotypes from one selected genotype. HRM results indicate that amplicons for regions 5S-GT and 16S-ISR can be applied for discrimination among *Bacillus* species (Figures 6A to 6F), whereas the selected 16S-23S ISR region was not valuable for species differentiation (data not shown).

In summary, we demonstrated that SNPs genotyping by HRM analysis for amplicons 5S-GT and 16S-ISR had a high discriminatory power between *B. cereus* CECT 148, *B. thuringiensis* Bt407 and *B. weihenstephanensis* KBAB4 and an excellent typeability. Amplification of 16S rRNA gene followed by HRM analysis for genotyping of bacterial species was also reported by Cheng et al. (2006), allowing to discriminate between 25 clinically important species among which were *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *E.coli* and *Bacillus* species. Of all the bacterial species tested, 11 were discriminated via 1-step post-PCR HRM analysis and the remaining, including *Bacillus* isolates, by heteroduplex formation between PCR products of the tested and reference bacterial species or by a second real-time PCR targeting a different region of the 16S rRNA gene.

Figure 6. Fluorescence derivative high resolution melting curves of *B. cereus* CECT 148 (violet lines), *B. thuringiensis* Bt407 (blue lines) and *B. weihenstephanensis* KBAB4 (orange lines) are shown in figures (A) for PCR product 5S-GTfor and 5S-GTrev and (B) for PCR product 16S-ISRfor and 16S-ISRrev. Genotyping of the PCR products by HRM analysis was performed after establishing regions of normalization. Normalized regions were determined establishing leading range from 71.23 to 73.13 and trailing range from 78.68±20 to 80.72±20 for 5S-GT primers. For 16S-ISR primers leading range values were 71.23 to 73.13 and trailing range from 79.19 to 81.09. HRM curves of these regions are represented in figures (C) (5S-GT) and (D) (16S-ISR). To obtain difference plot representatives, genotypes were defined (E) (5S-GT) and (F) (16S-ISR).

7.3.8. Evaluation of high resolution melting analysis for strain genotyping

We evaluated the potential to discriminate among *B. cereus* strains based on SNP detection through HRM analysis of amplicons 16S-23S ISR, 5S-GT and 16S-ISR. Melting curves of the 16S-23S ISR for all the strains tested were characterized by two variant domains, corresponding to the two melting peaks observable in the derivative plot (Figure 7A). The appearance of two clearly identifiable melting domains in the ISR region might be related to the relative large amplicon size (Table 3) as was reported already by Reed et al. (2007).

For genotyping with 16S-23S ISR amplicons, a combinatory approach was applied, analyzing the two melting domains separately as well as in combination because successful genotyping for a given domain varied among the biological replicates. Fig. 7B and C exemplarily show the normalized high resolution melting curve and the difference plot generated for the upper domain 1. Our results indicate that HRM analysis detects polymorphisms in both melting domains, but resolution is borderline, thus explaining the variation among biological repetitions.

PCR products 5S-GT and 16S-ISR of the various strains yielded a single peak but HRM analysis did not discriminate between *B. cereus* strains and these polymorphic regions were rejected for strain genotyping by HRM analysis.

Figure 7. (A) Fluorescence derivative high resolution melting curve of *B. cereus* **CECT 131** (green lines), **CECT 148** (violet lines), **CECT 193** (red lines), **CECT 4014** (yellow lines) and **CECT 4094** (black lines) obtained by real time amplification with 16S-23SISRfor and 16S-23SISRrev. Two peak, peak 1 and peak 2, were selected for genotyping of single nucleotide polymorphisms by high resolution melting (B) Normalized high resolution melting curves of *B. cereus* strains obtained from normalization of peak region 1 Normalized regions were determined establishing leading range from 80.75 to 83.6 and trailing range from 88.42 to 90.32. (C) Difference plot minus CECT 148

CHAPTER 8

Involvement of the YhcYZ two-component system during growth at low temperature in various *Bacillus cereus* group strains

Abstract

The high adaptability of *Bacillus cereus* group members to cold niches may be caused by the fact that these species harbor a wide variety of signaling pathways that allow rapid and robust responses. Recently, experiments performed in *Bacillus cereus* ATCC 14579 showed that BC2216 and BC2217 genes, encoding a sensor and a regulatory domain of a two-component system, have been related to resistance to low temperatures. BC2216-17 knockout mutants were performed to develop a preliminary study of *B. cereus* group cold phenotypes. Isogenic mutants of the BC2216 and BC2217 genes of *B. cereus* ATCC 14579, *B. cereus* AH 187 and *Bacillus thuringiensis* Bt407 were obtained. Results revealed that at 12°C *B. cereus* ATCC 14579 knockout strains showed a slight but highly reproducible impaired growth, meanwhile *B. thuringiensis* Bt407 and *B. cereus* AH 187 BC2216-17 knockout mutants did not show a clear cold phenotype. This heterogeneous response to BC2216-17 mutation may suggest that cold response varies among *B. cereus* group strains, although more strains should be tested before obtaining a reliable conclusion. Besides, different protocols were tested in order to improve *B. cereus* group transformation efficiency. Unlike previous studies, *in vitro* method for strain-specific methylation of plasmid DNA did not enhance transformation of *B. cereus* group species. However, it is noteworthy that, besides different *B. cereus* and *B. thuringiensis* mesophilic strains, two psychrotrophic strains, *Bacillus weihenstephanensis* WSBC 10688 and *Bacillus mycoides* DSM 2048, were successfully transformed.

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8.1. Introduction

B. cereus group isolates are ubiquitous microorganisms able to survive and prosper in a wide variety of environmental conditions mainly due to their ability to form spores and to be able to growth in a broad range of temperature defined from 4 to 50°C. In 2008, based on genotypic and phenotypic criteria, Guinebretière et al. established an “ecotypic structure” of *B. cereus* group consisting of seven thermal groups (I-VII). This study provided evidence for a multiemergence of psychrotolerance among these species and showed how modification of temperature tolerance limits has influenced their evolution. Nowadays the adaptation of these microorganisms to numerous environments is a major concern for food industry, especially during storage of refrigerated foods. In fact, psychrotolerant and mesophilic strains able to grow at temperatures below 10°C can contaminate food products stored at refrigeration temperatures or under suboptimal refrigeration conditions and multiply causing spoilage and health risk for consumers (Stenfors Arnesen et al., 2008; Brillard et al., 2010). In this regard, delving into mechanistic details involved in adaptation of *B. cereus* group isolates to low temperatures is a major aim to ensure microbiological quality of chilled food products.

Under cold stress bacterial cells undergo physiological modifications affecting membrane fluidity, protein synthesis and nucleic acid topology (Phadtare, 2004; Pandiani et al., 2011). To cope with these adverse conditions microorganisms use a genetically determined two-phase strategy composed of a cold shock response followed by an acclimatization response (Horn et al., 2007; Carlin et al., 2010).

During conditions of a sudden decrease in temperature (cold shock), mRNA-binding cold shock proteins (Csps) and cold induced RNA helicases (Cshs) appear to be major players in the prevention of secondary mRNA structures which affect transcription and translation mechanisms causing transient inhibition of protein synthesis. Members of both protein families have been shown to interact suggesting a two-step process (El-Sharoud and Graumann, 2007) where Csps mediate transcription elongation and message stability (Brillard et al., 2010) and Cshs remove secondary structures from RNA duplexes stabilized by cold conditions in presence of ATP (Pandiani et al., 2011). In 1996, Mayr et al. reported that *B. cereus* microorganisms harbor a cold stress protein family composed of six Csp with two of them highly cold

induced. The most abundant of these cold-induced proteins was a small polypeptide of 7.5 kDa designated CspA. Further studies (Lechner et al., 1998), revealed that CspA and 16S rRNA sequences display clear differences between psychrotolerant and mesophilic *B. cereus* group strains and may constitute a signature of psychrotolerant character (Francis et al., 1998; Pruss et al., 1999; Carlin et al., 2010). Later, upon acid shock, *Escherichia coli* and *Bacillus subtilis* cells were found to synthesize cold-acclimation proteins (CAPs) which are involved in cell metabolism, DNA and RNA metabolism, in protein folding and degradation, as well as in gene expression or protein synthesis (Carlin et al., 2010). Notably, σ^B factor which is involved in *B. cereus* response to different stresses such as heat-shock and acid exposure did not show to play a role in cold adaptation (van Schaik et al., 2004). In *B. subtilis* isolates, the induction of the general stress response is essential for adaptation to low temperatures during prolonged periods (Brigula et al., 2003) meanwhile cold shock response did not depend on the induction of *sigB* operon suggesting the existence of an alternative pathway (Beckering et al., 2002). Further studies (Wiegeshoff et al., 2006) revealed that σ^L factor forms a complex with BkdR transcriptional regulator activating *bkd* operon which is responsible of the conversion of isoleucine to α -keto acids, precursors molecules for branched chain fatty acids. Additionally, σ^L factor have been shown to interact with YplP transcriptional regulator resulting in the activation of an alternative pathway of unknown function when cells are subjected to cold shock (Wiegeshoff et al., 2006).

Moreover, thermal control of fatty acid synthesis is a universal and conserved regulatory mechanism designed to mitigate the effects of low temperature on the fluidity of membrane through the adjustment of membrane lipid composition (Cronan and Rock, 1996; Aguilar et al., 2001; Mansilla et al., 2004). It can be done, for example, increasing the proportion of branched-chain fatty acids and decreasing their equivalent chain length, and increasing the anteiso-/iso-branched ratio and the proportion of unsaturated fatty acids, as described by Haque and Rusell (2004) for *B. cereus* cells. Thus, when bacteria and most of poikilothermic organisms are subjected to a decrease of ambient growth temperature, proportion of unsaturated fatty acids (UFAs) in the membrane lipids increases (Aguilar et al., 2001). For this purpose *Bacilli* cells introduce double bonds by specific fatty acid desaturase enzymes into pre-existing fatty acids of their membrane phospholipids (Grau and de Mendoza, 1993; Aguilar et al., 1998).

Previous studies revealed that *B. subtilis* contains a unique desaturase, $\Delta 5$ -desaturase, encoded by *des* gene whose transcription is induced upon cold shock (Aguilar et al., 1998, 1999). In 2001, Aguilar et al. reported that temperature regulation of UFAs was controlled in *B. subtilis* by a two-component signal transduction system (TCS) composed of a sensor kinase, DesK, and a transcriptional regulator, DesR, responsible of *des* gene expression at low temperature. Activated by a transmembrane-signal transduction pathway, DesK assumes different signaling states in response to a change in membrane fluidity induced by temperature variations. Upon temperature downshifts proportion of ordered membrane lipids increase and phosphatase-dominant state change to kinase-dominant state and phosphorylates DesR (DesR-P) which binds specifically to the promoter region of *des* gene inducing $\Delta 5$ -desaturase. Newly synthesized UFAs inhibit *des* transcription by favoring DesK dephosphorylation of DesR-P or by causing dissociation of DesR-P from its binding site.

Recently, transcriptome analysis of *B. cereus* ATCC 14579 grown at 37 and 10°C were performed to identify genes involved in *B. cereus* adaptation to low temperature (Brillard personal communication, Nierop-Groot et al., in preparation). Results revealed that a two-component signal transduction system composed of sensor protein YhcY and a transcriptional regulator YhcZ, encoded by BC2216 and BC2217 genes respectively, were overexpressed at low-temperature. Since the genes encoding this two-component system are conserved in most genomes of the *B. cereus* group, including psychrotrophic strains, but are absent in the most thermophilic strains, the study of this TCS during *B. cereus* low-temperature adaptation may be a major key to understand mechanistic basis of cold adaptation in these species.

To study the role of these two-component regulatory proteins during growth at low temperature of *B. cereus* group, BC2216 and BC2217 null mutants were constructed in *B. cereus* ATCC 14579 strain. It is well known that gene knockout strategies depend on the ability to transform *B. cereus* group strains with plasmid DNA. However, there is significant strain-dependent variation in transformation competence and even some strains are completely resistant to transformation (Donahue et al., 2000). To improve transformation efficiency a wide range of protocols have been described for gram-positive bacteria including cell-weakening agents, various washing buffer compositions and a variety of electric pulses without successful results for cold-tolerant

food isolates (Groot et al., 2008). Different authors have proposed as causative factors of variation in plasmid transformation frequency the presence of fortified cell walls that prevent DNA uptake and/or the involvement of DNA restriction and modification (RM) system (Groot et al., 2008). Recent studies developed by Groot et al. (2008) reported that *B. weihenstephanensis* DSM 11821 was resistant to Sau3AI and BamHI digestion due to the methylation of the cytosine residue of the GATC recognition site. This behavior was also observed in other cold-tolerant and mesophilic strains suggesting the existence of a Sau3AI-type RM system which appears to be widespread among *B. cereus* and *B. weihenstephanensis* isolates. On this basis, *in vitro* methylation of plasmid DNA could result in a major improvement of transformation efficiencies as shown by previous studies performed in *B. cereus*, *B. weihenstephanensis* and *Helicobacter pylori* (Donahue et al., 2000; Groot et al., 2008).

In the present work we studied the effect of the deletion of BC2216 and BC2217 on the ability of various *B. cereus* group strains to grow at low temperatures. Previously, an *in vitro* method for strain-specific methylation of plasmid DNA isolated from *B. cereus* ATCC 14579 was tested in order to improve *B. cereus* group transformation efficiency.

8.2. Materials and methods

8.2.1. Strains and plasmids

Different *B. cereus* group strains of the National Institute of Agronomic Research (INRA, Avignon, France) collection listed in Table 1 were compiled for the development and validation of transformation and mutation methods presented in this work. Special care was taken, to include *B. cereus* group strains belonging to different phylogenetic groups and displaying different temperature growth ranges.

All *B. cereus* group strains were characterized in previous studies using molecular data from Amplified Fragment Polymorphisms patterns, ribosomal gene sequences, partial *panC* gene sequences, psychrotolerant DNA sequence signatures and phenotypic and descriptive data from range of temperature, psychrotolerance and thermal niches (Guinebretiere et al., 2008).

Table 1. *B. cereus* group strains used in this work

Strain	Abbreviation	Phylogenetic group ^a
<i>Bacillus cereus</i> ATCC 14579 (type strain)	ATCC 14579	IV
<i>Bacillus cereus</i> ATCC 10987	ATCC 10987	III
<i>Bacillus cereus</i> AH 187	AH 187	III
<i>Bacillus weihenstephanensis</i> WSBC 10688	WSBC 10688	VI
<i>Bacillus thuringiensis</i> Bt407	Bt407	IV
<i>Bacillus weihenstephanensis</i> KBAB4	KBAB4	VI
<i>Bacillus mycoides</i> DSM 2048	DSM 2048	VI

^a Classification established from Guinebretiere et al. (2008)

E. coli DH5 α strain was used for cloning experiments, and *E. coli* SCS110 strain was used to amplify unmethylated pMAD Δ 2216-2217 prior electrotransformation of *B. cereus* group strains.

Table 2. Plasmids and primers used in this study

Plasmid or primers	Description ^a	Source of reference
Plasmid		
pMAD	Ap ^R and Em ^R shuttle vector	Arnaud et al., 2004
pMAD Δ 2216-17	Recombinant pMAD plasmid harboring BC2216-17::Km ^R	Brillard et al. on preparation
pHT304	Ap ^R and Em ^R shuttle vector	Arantes and Lereclus, 1991
Primers		
5M-4814-Eco	GGCGAATTCGTAGTTGGTGATGAGAATGAAC	
RT-BC2215-16_fw	CCATTAAATACGTCGGTGT	
RT-BC2216-17_rv	ATTGATAACCATCTGCTCCA	
5M-2216-Eco	GGCGAATTCGTAGTTGGTGATGAGAATGAAC	
3M-2216-Pst	TGGACTGCAGCAATCCTTCAGGTCTTAATTG	
5V-2216-Sal	TGCAGTCGACGAAAACGAGAGAAGAATTTGA	
3V-2216-Bam	GCGGGATCCTTTATCCTGTAAAAACCGATTA	

^aAp, ampicilin; Em, erythromycin; Km, kanamycin

Plasmids and primers used in the present work are detailed in Table 2. The pMAD plasmid (Arnaud et al., 2004) was selected for generating gene inactivation mutants in *B. cereus* group strains by a homologous recombination between a target gene and homologous sequences carried on the plasmid. This vector contains a thermosensitive pE194 replication vector origin and a constitutively expressed transcriptional fusion with the *bgaB* gene encoding a thermostable β -galactosidase from *Bacillus stearothermophilus*, allowing the easy screening of transformants on X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) plates. In addition, it confers erythromycin resistance to Gram-positive hosts.

8.2.2. Bacterial extract preparation

Cell-free extracts (CFE) of *B. cereus* ATCC 14579, *B. cereus* ATCC 10987, *B. cereus* AH 187, *B. thuringiensis* Bt407, *B. weihenstephanensis* WSBC 10688, *B. weihenstephanensis* KBAB4 and *B. mycoides* DSM 2048 were prepared from bacterial cultures in the exponential phase of growth ($A_{600}=0.6-0.9$), as previously described (Groot et al., 2008). For this purpose two subsequent subcultures in Luria Bertani (LB) broth (Maniatis et al., 1989) at 30°C with shaking at 200 rpm were performed. To recover *Bacillus* cells, inoculum were centrifuged at 9,000 rpm for 7 minutes and bacterial pellets resuspended in five volumes (mL) of extraction buffer (Donahue et al., 2000) composed of 20 mM Tris-acetate (pH 7.9) (Maniatis et al., 1989), 50 mM potassium acetate (Sigma-Aldrich, Steinheim, Germany), 5 mM Na₂EDTA (Sigma-Aldrich, Steinheim, Germany), 1 mM dithiotreitol (DTT) (Amersham Biosciences, Uppsala, Sweden) and buffer protease inhibitor cocktail Set-III EDTA-free, as recommended by the supplier (Calbiochem, USA). Before use, buffer pH was fitted to 7.9 and sterilized by filtration using a 0.2 μ m filter (Millipore, Bedford, USA). Next, cell suspensions were lysed using Bio101 Thermo Savant Fastprep FP120 cell disrupted system (Qbiogene, France) by performing three consecutive runs of 45 seconds between which samples were chilled on ice during one minute. To recover supernatant, bacterial cell suspensions were centrifuged 5 minutes at 15,000 rpm at 4°C. Supernatants were removed and used immediately or after one freeze-thaw cycle (-80°C) for *in vitro* plasmid DNA modification reactions (see below).

Bio-Rad protein assay based on the method of Bradford (Hercules, USA) was used to determine the relative protein concentrations of extracts. Bovine serum albumin was used as standard.

8.2.3. Treatment of plasmid DNA with CFE of the corresponding recipient *B. cereus* group strain

The effect of pMAD Δ BC2216-17 methylation on transformation frequencies of *B. cereus* group strains was assessed in the present work using the CFE of the corresponding recipient microorganism. For this purpose plasmid DNA (12 μ g), prepared from *E. coli* DH5 α was combined with *Bacillus* CFE (400-600 η g of protein) in a 50 μ L reaction containing extraction buffer (see composition above). As a control, plasmid DNA (12 μ g) was mock treated in a reaction without extract. Reactions were incubated at 30 or 37°C for 1 hour and then dialyzed during 15 minutes to remove salts by using MF-membranes filters (0.025 μ m) (Millipore, Ireland). Failure to thoroughly remove the growth media from the cells may result in the sample arcing during electroporation at high fields strengths. Finally plasmid DNA was concentrated by using Microcon YM-30 columns (Millipore, Bedford, USA) used as manufacturer indications to a final concentration of 1.2 μ g/ μ L and incubated at 65°C during 15 minutes to inactivate proteins.

The effect of plasmid size on transformation efficiency of recalcitrant strain *B. weihenstephanensis* KBAB4 was tested. For this purpose pMAD Δ BC2216-17 plasmid (approximately 12,200 bp) was replaced by plasmid pHT304 (approximately 6,500 bp) and then subjected to methylation, dialysis, concentration and protein inactivation as described above.

The effect of concentration and extraction techniques on transformation efficiencies was tested in *B. cereus* ATCC 14579 (type strain). Thus, some samples were extracted with phenol-chloroform as described previously (Maniatis et al., 1989), concentrated by using Microcon YM-30 columns used as manufacturer indications to a final concentration of 1.2 μ g/ μ L, and finally dialyzed during 15 minutes to remove salts by using MF-membranes filters (0.025 μ m) (Millipore, Ireland).

8.2.4. Transformation of *B. cereus* group strains

Two consecutive subcultures of *B. cereus* group strains were performed in Brain Heart Infusion broth (BHI; Scharlau, Barcelona, Spain) at 30 or 37°C (depending on the temperature growth ability of the strain used) with shaking at 200 rpm. To carry out transformation experiments cells were harvested during early exponential phase ($A_{600}=0.3-0.5$). Bacterial cell pellets obtained after centrifugation (at 4,000 rpm during 10 minutes in refrigeration) per triplicate were resuspended in cold polyethylene glycol 6000 (PEG 6000) 40% (Duchefa Biochemie, Haarlem, Netherlands).

Aliquots of 400 μ L of bacterial suspension in PEG were pipetted in electroporation cuvettes and supplemented with 10 to 20 μ g of methylated plasmid DNA prepared as described above. Settings for electroporation were 25 μ F, 1,000 Ω and 2.5 KV.

Additionally, a different transformation protocol was assessed for recalcitrant strain *B. weihenstephanensis* KBAB4 by using an electroporation buffer (EP buffer) composed of 0.5 mM $\text{KH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$, 0.5 mM MgCl_2 and 272 mM sucrose. For this purpose non-treated pMAD Δ BC2216-17 plasmid (between 10 to 20 μ g) was added to 400 μ L of EP buffer. Transformation was performed by using the following electroporation parameters: 25 μ F, 400 Ω and 1.2 KV.

Immediately after electric pulses, bacteria were placed on ice and then resuspended in 1 mL of SOC medium and incubated at 30 or 37°C, depending on the bacterial strain (psychrotrophic and mesophilic, respectively), at 200 rpm during 2 hours.

For selective growth of transformants, suspension of electroporated cells were spread on LB agar plates supplemented with X-Gal (50 μ g/mL) (Duchefa, Haarlem, Netherlands) and with two different antibiotics separately, kanamycin sulfate from *Streptomyces kanamyceticus* 100 mM (100 μ g/mL) (Sigma-Aldrich, St. Louis, USA) or erythromycin (5 μ g/mL) (Duchefa, Haarlem, Netherlands). Plates were incubated at 30 or 37°C depending on bacterial strains (psychrotrophic and mesophilic, respectively) during several days. At regular intervals plates were examined to detect transformant

colonies which were blue, kanamycin resistant (Km^R) and erythromycin resistant (Em^R). Transformation efficiency (TE) was calculated by using the following mathematical expression:

$$TE = \frac{cfu \times transformation\ volume}{plasmid\ DNA\ concentration} \quad (1)$$

where *cfu* is the number of transformant colonies obtained, *transformation volume* correspond with the sum of the volume of bacterial and plasmid suspension introduced into electroporation cuvettes plus the volume of SOC medium used for recovering cell purposes, and finally, *plasmid DNA concentration* is the concentration of plasmid DNA added before electroporation.

8.2.5. DNA manipulation

Plasmid DNA was extracted from *E. coli* DH5 α by a standard alkaline lysis procedure using the Pure Yield plasmid Miniprep System (Promega, Charbonnières, France) and the Plasmid Maxi kit procedure (Qiagen, Germantown, USA). Chromosomal DNA was extracted from *B. cereus* group strains cells harvested in mid-lag phase using the DNeasy Blood and Tissue kit (Qiagen, Germantown, USA). Oligonucleotide primers (Table 2) were synthesized by Eurogentec (Seraing, Belgium). PCR was performed in a GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer, Coutaboeuf, France). Amplified DNA fragments were purified using a PCR purification kit (Roche) and separated on 0.7% agarose gels after digestion. Digested DNA fragments were extracted from agarose gels with a centrifugal filter device (Montage DNA gel extraction kit; Millipore, Molsheim, France). All constructions were confirmed by DNA sequencing by Beckman Coulter Genomics, Essex, UK.

8.2.6. Gene disruption in *B. cereus* group strains using pMAD Δ 2216-17

To assess the role of BC2216 and BC2217 genes on *B. cereus* group adaptation at low temperatures a derivative pMAD vector was constructed (Brillard et al., in preparation) (Figure 2) carrying the upstream region of BC2216 gene (Figure 1), a 1.5-

kb DNA fragment containing the *aphA3* kanamycin resistance gene with its own promoter obtained as previously described (Arnaud et al., 2004), and the downstream region of BC2217 gene (Figure 1). BC2216 and BC2217 DNA fragments, termed M and V region respectively (Figures 1 and 2), were PCR amplified using the oligonucleotide pairs 5M-2216-Eco/3M-2216-Pst and 5V-2216-Sal/3V-2216-Bam (Table 2). Expand high-fidelity DNA polymerase (Roche Applied Science, Meylan, France) and chromosomal DNA from *B. cereus* ATCC 14579 strain as a template. Then PCR products were digested with EcoRI+PstI and SalI+BamHI enzymes, respectively. In parallel, Km^R cassette was amplified as previously described (Brillard et al., 2008) and was restricted with PstI and SalI enzyme. The three digested DNA fragments were purified, ligated by using T4-DNA ligase (Promega, Charbonnières, France). The full length -3kb PCR fragment was then purified on an agarose electrophoresis gel, and cloned in the corresponding restriction site of the pMAD vector.

The recombinant pMAD Δ2216-17 vector was extracted from *E. coli* DH5α, treated by CFE, and prepared for subsequent transformation of *B. cereus* group strains (Table 1) by electroporation. *B. cereus* ATCC 14579, *B. cereus* ATCC 10987, *B. cereus* AH 187, *B. thuringiensis* Bt407, *B. cereus* WSBC 10688 and *B. mycoides* DSM 2048 transformants were then subjected to allelic exchange as described previously Arnaud et al. (2004) (Figure 2). When mutation events occurred through a double-crossover event, chromosomal wild type (WT) fragments of the BC2216 and BC2217 gene were deleted and replaced by the Km^R cassette (Figures 1 to 3A). Then, clones phenotype switched from a Km-resistant, Em-resistant and blue to Km-resistant, Em-sensitive and white. The chromosomal allelic exchange in the mutants was checked by PCR using the appropriate primer couples, 5M4818/RT-2217-Rv for *B. cereus* ATCC 14579 strain and RT-2215-fv/Rt-2217-Rv and RT-2215-fw/RT-2217-rv for *B. cereus* AH 187 and *B. thuringiensis* Bt407 (Figure 3B). Amplification was carried out in 25 μL reaction mixtures containing 5 μL 5X Colorless GoTaq[®] Flexi buffer (Promega), 20 μM of each dNTP, 0.6 μM of the forward and the reverse primer, 2 mM MgCl₂, 0.2 μL *Taq* polymerase (GoTaq[®] DNA Polymerase [5 u/μL], Promega) and template DNA (50-100 ng/μL). The amplification conditions were as follows: initial denaturation for 5 min (94°C), 10 cycles of denaturation at 94 °C (20 s), annealing at 50 °C (25 s) and extension at 72 °C (2 minutes) followed by 23 cycles of denaturation at 94 °C (25 s), annealing at 51 °C (20 s) and extension at 72 °C (2.5 minutes) and final extension at 72 °C (7 min).

Chromosomal position

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2159794 CCATTTAAAT ACGTCGGTGT TACAGACTTA AATCCTAACT TGAAATATGT TGGCTATGAA
2159854 TTTTGTATTA CAGGTTTAAAT AAAGCCAGAA GATTATTTTG AAACCTCTAG ACAAGTAAGA
2159914 TTTGAATTAA TGTCTATGTT CCATAAGAAT CAAGTGCAAA TGCCAGCAGC GAATATGGTT
2159974 GTTACTACTG AAGGTTTACA GCATATCCAT GGTGGACAAT CTTTATCGGA CAGCTAAAGT
2160034 AGAATCGTTT GTTAACAAAA ATTCTGCTTC CAACATTGGA TAGAATAACA ATAATAAAAT
2160094 GATTGATATA AAGAAAGGGC TGAAGGAATT CGGTTCTTTT TTTATTTATA GAATTTAGAA
2160154 ATTTCTTAAT GTTATAATTG TAGTTGGTGA TGAGAATGAA CTTTGAAGAA AAAGAGATGC
2160214 GTCGTTTAGA AGCATTAAAA GAAATTGCTG AATTATTAAA TGAAGCAACA AACTTACAGG
2160274 ATATGTTAGA AAAAGTGTTG CATACATTGT TACAAGTAAT GAATTTACAA ACAGGGTGGG
2160334 TATTCTTTAT TGATGAAAGT GGAAAGCACA GCATGCTTGT AGACGAAAAA TTACCAGAGG
2160394 CTCTTACGTG GCAAAAGAAG AAGCCGATGT GTGAAGGAAA CTGTTGGTGT GTAGAACGTT
2160454 TTGTGAATGG GAGATTGGAA AAAGCGACTA ATATTATTGA ATGTAAGCGA ATAGAAGATG
2160514 CAATTGAATG TAATTGGGGA GAGACAGAAG ATGTTACACA TCATGCGACA ATTCCGCTTA
2160574 GGTCTGGATC AGAAAAATTT GGTCTACTAA ATGTGGCTGC ACCTCATAAG ACTCATTTTT
2160634 CTGAGGAAGA GTTAGCGTTA TTAGAATCAA TTGCATTTC AATTGGAACG ACAATACAAC
2160694 GTATTCAATT AGTTGAGAAA GAACGTAAAT ATGTAGTGGT AGCGGAAAGA AATCGACTTG
2160754 CACGTGATTT ACATGATTCA GTAAACAAT TGTATTTTC TATTATGCTA ACAGCGAAAG
2160814 GTACTCTGAA TATGACGCAA GATAGAGAAT TGCAAGAGAT GTTAAGTTAT ATTGGGGAAT
2160874 TATCACAAGA AGCATTACAA GAGATGAGGC TATTAATTTG GCAATTAAGA CCTGAAGGAT
2160934 TGGAGAAAGG ATTAGCAGAA GCAATTCAAA ATTACGGGAA GTTATTAGGA GTTCAAGTGG
2160994 AAGTTCGAAT TGATGGGATG GTTTCAATTG GGGATGAAAT AGAAGAAGTT TTATGGCGTA
2161054 TTAGCCAAGA GCGCTACAT AATTGTAAAA AGCATGCTTC GTGTGAAAAG GTACATATTC
2161114 TTTTGAAAAT AGAAAATAAC CAGTTACATT TTTACATAGA AGACAATGGA ATAGGATTTA
2161174 TACAAGAACA AGTAAGGGAA TCAGCAGTTG GCCTGAAAAG TATGAAGGAA CGTATTCAGT
2161234 TAATGAAGGG GTCGTTTCAA ATTATAACAG AGTTGAAAAA GGGTACAAA ATAGAAATTC
2161294 AATTGCCGAT TTGAAGGGGA GAATAAGGTT GAAGATTAAA CTGCTACTAG TTGAGGACCA
2161354 TCATATCGTT CGAAGAGGAC TTGTATTCTT TTTGAAAACG AGAGAAGAA TTGAAATTAT
2161414 TGGGGAAGCG GAAAATGGCG AAGAGGCATT ACATTTTCATG CAAAAGAAA GACCAGATGT
2161454 CGTACTAATG GATGTATCGA TGCCGAAAAT GGATGGAATT GAGGCAACAA AACGTCTAAA
2161534 GCAATATGAT GAGACAATAA AGGTACTTAT ATTAAGTAGT TTTTCAGAGC AAGATTATGT
2161594 TATACCAGCA CTTGAAGCTG GAGCAGATGG TTATCAATTA AAAGAAGTAC AACCTGAGCA
2161654 ACTTGTCGCT TCTATTATTG CAGTACATCA AGGAAATGCG AATTTTCACC CGAAAGTAA
2161714 ACCTGCATTA ATGGGGCGTT CTGCAGTAAA GAAGGAAGTA GAAAATCCTT TTTCAATGTT
2161774 AACGAAAAGA GAGCAAGAGG TACTTCGCGA AATTGCGAAA GGAAGAAGCA ACAAGGAGAT
2161834 TGCAGCAGAA CTTTATATTA CAGAACAAAC TGTGAAAACA CACGTTTCAA ACGTTTTAGC
2161894 TAAATTGGAA GTGGATGATC GTACGCAAGC CGCATTATAC GCAGTGAAAC ATGGAGAGAA
2161954 TTATTCATAA GCATGAATAA TTCTGTTATA TCGTATGACA GAATACATAG GAGCAATTAA
2162014 AATTTATAGT AATCCCTAAT ATAGGTTTTT CTTCTTTCGA TAATTAGGTA CAATAAAGTG
2162074 AAACCTAAT CAGTGGGGGA CCATCCCCAC TGATGATTAG TTAACTAAT CGGTTTTTAC
2162134 AGGATAAA

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Figure 1. Nucleotide sequence of BC2216-17 genes region of *B. cereus* ATCC 14579. Nucleotides colored orange correspond to M region, colored green to V region, colored black to the region replaced by Km resistance cassette in BC2216-17 null mutants, and colored grey to 5' region of BC2215 gene (see Figure 2 and 3A). Underlined nucleotides in blue correspond to BC2216 gene and in violet to BC2217 gene (see Figure 3A). Annealing sequence is boxed in yellow for 5M-4814 primer, in pink for RT-BC2215-16-fw primer, and in red for RT-BC2216-17-rv primer (Table 2).

8.2.7. Phenotypic characterization of knockout mutants

To assess BC2216 and BC2217 gene disruption effect on mutant phenotype, *B. cereus* ATCC 14579 and *B. thurigiensis* Bt407 WT and null mutant colonies were grown at 37, 30, and 12°C. *B. thurigiensis* Bt407 WT and null mutant colonies were also grown at 15°C. The temperature of 12°C was used instead of 10°C to avoid cell flocculation (Brillard et al., 2010). *B. cereus* AH 187 WT and null mutants colonies were incubated at 37, 30 and 15°C as this microorganism was not found to be able to growth at temperatures below 15°C (Gunebretière et al., 2008). Experiments performed at 37, 30, 15 and 12°C were carried out by using an automated turbidometer, the Bioscreen C microbiology reader (LabSystems, Uxbridge, United Kindom), in 100-well sterile microplates.

Volumes of 40 µL of overnight cultures at 30°C were inoculated into 4 mL of fresh LB medium and incubated at 30°C with shaking until an optical density (OD) at 600 nm (OD₆₀₀) of 0.8 was obtained. To perform turbidometric experiments, 12 µL of these cultures were inoculated in 300 µL of fresh LB to reach a concentration of approximately 10⁴ CFU per well (depending on experiments). Three replicate wells of the microplate were filled with this dilutions of inoculated medium to a final volume of 312 µL per well. A negative control consisting of uninoculated LB was prepared. The cultures were incubated with vigorous constant shaking, and the OD was measured at 15-min intervals at 37 and 30°C and at 30-min intervals at 12 and 15°C. At least three experiments of each growth condition were performed.

8.3. Results and discussion

8.3.1. Effect of *in vitro* plasmid methylation on the efficiency of *B. cereus* group strains transformation

In vitro methylation of pMAD ΔBC2216-17 plasmid with cell extract of the corresponding recipient strains of *B. cereus* group did not result in an improvement of their transformation efficiency (Table 3). Unmethylated plasmids transformation efficiency increased between log 1 and log 4 transformants per µg plasmid DNA in comparison with treated samples for all the strains tested except for *B. cereus* ATCC

14579. Strain type transformation efficiency reveals only slight differences between methylated and unmethylated samples. Notably, psychrotrophic strain *B. weihenstephanensis* WSBC 10688 showed the highest transformation efficiency meanwhile attempts to transform *B. weihenstephanensis* KBAB4 always were completely unsuccessful.

Table 3. Efficiency of *B. cereus* group transformation with pMAD Δ 2216-17 (12,200 bp)

Recipient strain	Transformation efficiency (no. of CFU/ μ g)	
	Nontreated cells ^a	CFE ^b
<i>Mesophilic</i>		
ATCC 14579	3.19×10^2	3.82×10^2
ATCC 10987	1.29×10^3	7.1×10
AH 187	2.7×10^2	9.8×10
Bt407	1.46×10^2	4.69×10
<i>Psychrotrophic</i>		
WSBC 10688	1.3×10^4	- ^c
KBAB4	-	-
DSM 2048	1×10^3	1.9×10^2

^aNon-methylated plasmid with CFE of the recipient strain. ^b Methylated plasmid with CFE of the recipient strain. Each value was derived from independent experiments. ^c Below the detection threshold (< 0.1 CFU/ μ g).

Previous studies (Donahue et al., 2000; Groot et al., 2008) reported that *in vitro* methylation of plasmid DNA resulted in a major improvement of the transformation efficiencies of *H. pylori*, *B. cereus* and *B. weihenstephanensis*. However, our results reveal the opposite effect in accordance with Macaluso and Mettus (1991). They observed that non-methylated plasmid enhances plasmid transformation efficiency of *B. thuringiensis* isolates.

On the other hand, Donahue et al. (2000) have suggested that other factors in addition to restriction-modification mechanisms affect transformation frequency. As *in vitro* methylation with cell extract of the corresponding recipient strains did not result in an improvement of transformation efficiency, a new protocol based on the use of an EP buffer composed of 5 mM KH_2PO_4 - K_2HPO_4 , 0.5 mM MgCl_2 and 272 mM sucrose (Groot et al., 2008) was assessed for *B. weihenstephanensis* KBAB4 recalcitrant strain

and *B. thuringiensis* Bt407. MgCl_2 (approximately 1 mM) increases the transformation efficiency since in some bacteria is needed to maintain structural integrity of the cell membrane. Besides, sucrose was added to improve electroporation efficiency as increase osmolarity media by protecting cells from osmotic lysis and facilitating the influx of DNA into the cell (Holo and Nes, 1989; Xue et al., 1999). Once more, no *B. weihenstephanensis* KBAB4 transformants were obtained. Besides, *B. thuringiensis* Bt407 isolates showed a lower number of transformants, 3.1 CFU/ μg , in comparison with plasmid DNA mock treated in a reaction without extract cells (Table 3).

It has been shown that transformation efficiencies declines with an increase in plasmid size as previously reported Somkuti and Steinberg (1988). In order to determine if plasmid size was a causative factor of unsuccessful transformation of *B. weihenstephanensis* KBAB4, pTH304 plasmid (6,528 kb), approximately 5,600 pb smaller than pMAD $\Delta\text{BC2216-17}$, was selected to perform *in vitro* methylation protocol. However no transformant were obtained indicating no observable difference in transformation efficiencies as previously described in *Lactococcus lactis* isolates (McIntyre and Harlander, 1989; Powell et al., 1989)

Finally, effects of concentration techniques on transformation efficiency of strain type *B. cereus* ATCC 14579 were studied. For this purpose we compared phenol-chloroform extraction method with Microcon YM-30 columns used as manufacturer indications. Results showed that transformation efficiency was improved more than 1 log per μg when columns were used. Specifically, when plasmid was concentrated by using commercial columns a transformation efficiency of 6.1×10 CFU/ μg was observed, meanwhile phenol-chloroform extraction yielded transformation efficiencies values of 4.7 CFU/ μg . Furthermore, alternative techniques to those in whose ethanol is used, decrease the presence of ionic compounds, and in consequence significantly reduce arcing phenomena.

On the basis of our results, new methods should be assessed to improve *B. cereus* group transformation efficiencies, with special interest in the case of *B. weihenstephanensis* KBAB4 isolate. Recently, novel transformation protocols have been described. Cao et al. (2011) reported a modified electroporation method using

trehalose for the transformation of *B. subtilis* which was found to increase transformation efficiency nearly 2,000-fold compared with the usual method. Likewise, Mironczuk et al. (2008) revealed that expressing the *B. subtilis* ComK protein using an IPTG-inducible system in *B. cereus* ATCC 14579, allow cells grown in minimal medium displaying natural competence, as either genomic DNA or plasmid DNA was shown to be taken up by the cells and integrated into the genome or stably maintained respectively. Besides, Zhang et al. (2011) described a new method to enhance transformation of *Bacillus amyloliquefaciens* strains combining cell weakening by adding glycine and DL-threonine and cell-membrane fluidity disturbing by supplementing Tween 80.

8.3.2. Construction and characterization of BC2216 and BC2217 knockout mutants

Expression of BC2216 and BC2217 genes of the two-component signal transduction system was found to be enhanced at low temperature (Brillard et al., in preparation). To investigate the contribution of both genes to *B. cereus* group cold resistance, mutagenesis experiments were performed. For this purpose, transformed strains (Table 3) with pMAD Δ 2216-17 (Table 2) were subjected to selective growth in plates added with X-Gal and antibiotics. Isogenic mutants of the BC2216 and BC2217 genes of *B. cereus* ATCC 14579 (Δ BC2216-17-M₁-ATCC14579 and Δ BC2216-17-M₂-ATCC14579), *B. cereus* AH 187 (Δ BC2216-17-M₁-AH187) and *B. thuringiensis* Bt407 (Δ BC2216-17-M₁-Bt407 and Δ BC2216-17-M₂-Bt407)) were obtained. Through insertional inactivation, via double-crossover integration (Figure 2), mutation events occurred switching clones phenotype from a Km-resistant, Em-resistant and blue to Km-resistant, Em-sensitive and white. This variation in color and antibiotic resistance allowed identification and isolation of null mutant bacteria. PCR was performed to confirm that selected clones were real mutants (Figure 3B). Primers used are detailed in Table 2 and depicted in Figure 1.

Figure 2. Schematic diagram showing how BC2216-17 null mutants were obtained by allelic exchange. Target fragments, M and V region (see figure 1), and Km resistance cassette were amplified by PCR and cloned in pMAD. *B. cereus* group strains were transformed with pMAD Δ BC2216-17. Then, transformed strains (see Table 3) were subjected to mutation by double cross-over event in which recombination occurs twice. During cell division, homologous recombination (single recombinant event) occurs first (1) at one place, e.g. 5' region, and it leads to the insertion of a mutated copy, in addition to the wild copy, on the chromosome. In the following cell division a second homologous recombination (2) occurs in cointegrates, e.g. 3' region, resulting in gene disruption by Km resistance cassette insertion and excision of pMAD plasmid containing deleted region of target genes. During bacterial multiplication at high temperature, the plasmid is lost (diluted in the population) because of its thermosensitive origin of replication. BC2216 and BC2217 correspond to BC2216 and BC2217 genes (see Figures 1 and 3A), Km correspond to Km resistance gene, V and M correspond to V and M regions (see figure 1) and DR corresponded to deleted region of BC2216 and BC2217 genes (see figure 1). Cross-over events are depicted by orange arrows and lines.

Figure 3. (A) Map of the *B. cereus* ATCC 14579 BC2216 and BC2217 chromosomal region. Blue arrows represent ORFs. Yellow arrows represent Km resistance gene. The position of the Km resistance gene integrated into the chromosomes of the mutant to disrupt BC2216 and BC2217 is indicated. Functions or putative functions of gene products are indicated above the ORFs. Dashed lines with small black arrows annotated by letters in parentheses correspond to PCR amplicons. Wild type PCR products are designed as (a) and (b), and mutant PCR products as (a)* and (b)*. (a) and (a)* correspond to the PCR product amplified by using 5M-4814 and RT-BC2215-16-fw primers, and (b) and (b)* to the PCR product amplified by using RT-BC2215-16-fw and RT-BC2216-17-rv primers (see figure 1) (B) PCR detection of BC2216-17 null mutants. Approximate size of major bands is annotated by numbers. Letters in parenthesis refer to PCR amplicons as described in Figure 3A. WT, refers to wild type strain and NC to negative control. M1 correspond to Δ BC2216-17-M₁-ATCC14579, M2 to Δ BC2216-17-M₂-ATCC14579, M3 to Δ BC2216-17-M₁-Bt407, M4 to Δ BC2216-17-M₂-Bt407 and M5 to Δ BC2216-17-M₁-Bt407.

Agarose gel electrophoresis revealed the existence of longer PCR amplified fragments for two *B. cereus* ATCC 14579 isolates and two *B. thuringiensis* Bt407 isolates that displayed a Km^R, Em^S and white phenotype, confirming that they were genuine mutants (Figure 3B). However, PCR performed on DNA extracted from a *B. cereus* AH 187 displaying a Km^R, Em^S and white phenotype (a probable mutant) did not show an amplified DNA fragment of the expected size (2,800 bp). In fact, no amplification was observed, despite a correct amplification obtained by PCR performed on the wild type (WT) strain (Figure 3B). Because of the Km^R phenotype, we could rule out the possibility that this isolate was a WT isolate. Although we could not rule out that the Km cassette was introduced elsewhere in the genome of this isolate, the fact that no amplification was observed (neither "b" or "b*", figure 3A) suggested a problem with the PCR conditions or with the quality of the DNA of this isolate. Despite several attempts to optimize PCR conditions, by using different primers, varying MgCl₂ concentration and testing various amplification conditions, no amplification could be obtained. Therefore, we decided to consider this isolate as a probable mutant and characterize its phenotype during growth at various temperatures.

8.3.3. Phenotypic characterization of the BC2216 and BC2217 knockout mutants

B. cereus ATCC 14579, *B. cereus* AH 187 and *B. thuringiensis* Bt407 knockout and WT strains were analyzed for the ability to grow at low temperatures, 12 or 15°C, in comparison with growth at 30 and 37°C.

At 12°C, both *B. cereus* ATCC 14579 knockout strains showed a slight but highly reproducible delay on growth in comparison with WT strain (Figure 4C). Besides, at 30 and 37°C, no significance differences were observed (Figures 4B and 4A). This results are in accordance with previous studies (Brillard et al., on preparation) in which *B. cereus* ATCC 14579 BC2216-17 null mutants were unable to grow at 10°C after 21 days whilst WT clones grown and reached stationary phase in 7 days. These results suggest that TCS mutation is responsible of impaired cold growth.

Figure 4. Growth of *B. cereus* ATCC 14579 **wild type** (blue) and BC2216-17 knockout mutants, Δ BC2216-17-M₁-ATCC14579 (red) and Δ BC2216-17-M₂-ATCC14579 (green) (A) at 37°C (B) at 30°C and (C) 12°C.

On the other hand, no delay on growth of *B. cereus* AH 187 knockout mutant at all temperatures tested was clearly evidenced (Figure 5). Growth curves showed slight but no reproducible differences at 15°C and a biphasic shape at 30 and 37°C. In addition, intermediate lag occurs at OD levels of approximately 0.5-0.7. These values were in the range to those at which maximum cell density level was reached at 15°C. When cultures undergo (sudden) environmental variations during growth intermediate lag can occur (Swinnen et al., 2004). Francois et al. (2007) reported that *Listeria monocytogenes* cultures containing glucose result in a higher pH decrease at higher temperatures because major maximum cell density levels were reached. As long as cultures were subjected to a constant temperature, it appears like at given conditions and from a certain maximum cell density level, *B. cereus* AH 187 populations must readjust its metabolism to resume the growth, and at low temperatures this adaptation to new environmental conditions is non-viable. Besides, upon intermediate lag, BC2216-17 mutant showed minor maximum cell density level than WT strain at 37 and 30°C (Figures 5A and 5B). On the basis of these results different hypothesis may be addressed. On the one hand we suggest that BC2216 and BC2217 genes of *B. cereus* AH 187 encode TCS involved in adaptive response different to low temperature such as acid shock, oxygen limitation or nutrient depletion, on the other hand, the absence of cold phenotype indicates the existence of alternative mechanisms more efficient and specific for low temperature adaptation of this strain. In this context, complementation experiment could be very useful to investigate the function of BC2216 and BC2217 transcript. Likewise, growth curves at temperatures below 15°C may contribute to a better understanding of *B. cereus* AH 187 phenotype upon BC2216 and BC2217 disruption.

Finally, at 37 and 30°C, growth of *B. thuringiensis* Bt407 knockout mutant and WT did not show significant differences (Figures 6A and 6B). At 12°C, a heterogeneous behavior of BC2216-17 mutant was observed (Figure 7B). This heterogeneity was also evident at 15°C (Figure 7A). Notably, when temperature decrease, differences in lag phase and in the beginning of exponential phase appear to be less significant (Figures 7A and 7B). In this context, further studies at lower temperature (10°C) might be of interest.

Figure 5. Growth of *B. cereus* AH 187 **wild type** (blue) and BC2216-17 knockout mutants, **Δ BC2216-17-M₁AH187** (red) (A) at 37°C (B) at 30°C. and (C) 15°C.

Figure 6. Growth of *B. thuringiensis* Bt407 **wild type** (blue) and BC2216-17 knockout mutants, $\Delta BC2216-17-M_1-Bt407$ (red) and $\Delta BC2216-17-M_2-Bt407$ (green) (A) at 37°C and (B) at 30°C.

Figure 7. Growth of *B. thuringiensis* Bt407 **wild type** (blue) and BC2216-17 knockout mutants, **Δ BC2216-17-M₁-Bt407** (red) and **Δ BC2216-17-M₂-Bt407** (green) (**A**) at 15°C and (**B**) at 12°C.

Is it noteworthy that populations subjected to environmental stress can show diversity in their responses, even in apparently homogeneous conditions as previously reported den Besten et al. (2007). Likewise, single-cells studies have shown an increasing variability of bacterial growth under restrictive conditions such as low

temperatures. Indeed, den Besten et al. (2010) have reported that low temperatures induced heterogeneity in growth of *B. cereus* populations, possibly due to the loss of membrane integrity of individual cells.

Besides, lag phase is affected by current environment but also by previous conditions. Thus, lag phase duration is readily affected by previous conditions such as temperature and pH causing variability (Francois et al., 2007). Although in this study previous and current conditions were tightly controlled, cell physiological states and individual genetic makeup are a diversity source during bacterial growth (Buchanan and Cygnarowicz, 1990; Muñoz-Cuevas et al., 2010).

On the other hand, turbidity measures follows Beer's law, in consequence, valid data are obtained at high cells density (between 10^6 and $10^{7.5}$ CFU/mL), moment in which turbidity is proportional to cell concentration. Furthermore, the range of proportionality depends on shape and size of the bacteria, which can be affected by environmental conditions (Métris et al., 2003). den Besten et al.(2010) have reported that *B. cereus* colonies at 12°C showed variance of size. Therefore, at low temperature indirect measurements can lead inaccurate results which can be easily confused with heterogeneous growth performance.

Results of this preliminary approach reveal that cold phenotype varies among BC2216-17 knockout mutant tested. As long as *B. cereus* AH 187 null mutant did not show impaired growth at low temperatures, growth curves of *B. cereus* ATCC 14759 and *B. thuringiensis* Bt407 mutants revealed significant differences with the WT strain. However, only *B. cereus* ATCC 14759 knockout strains showed a clear and reliable delay on growth at 12°C. The lack of a homogeneous cold phenotype among strains tested and the certain that TCS is found in these species and in most of the *B. cereus* group members, suggest that TCS participation during growth at low temperature may be restricted to a limited number of strains. Anyway, we have tested the effect of BC2216 and BC2217 deletion just in three strains, so before drawing a clear conclusion, similar experiments should be repeated on various strains, including psychrotrophic bacteria, and determining growth kinetic parameters.

The background of the page is a light blue gradient with a faint, repeating pattern of test tubes in a rack. The test tubes are arranged in rows and columns, creating a sense of depth and perspective. The text is overlaid on this background.

CHAPTER 9

Conclusion summary

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This chapter provides a detailed list of conclusions of all the research topics addressed in this thesis:

- Ready to eat vegetable substrates (courgette puree and mixed vegetables puree) tested did not have a clear trend on heat resistance of *Bacillus cereus* at the temperatures tested (from 90 to 105°C). Survivor curves that presented tailing were better described by non linear models (Weibulls' and Geeraerds').
- At 30°C, heat-treated *B. cereus* efficiently adapted to oxygen limitation and to anaerobiosis without significantly affecting biomass production after 24 hours of incubation, either in an anaerobic environment or packaged in containers of laminated aluminium oxide coated PET ($10^{-3} \text{ cm}^3/\text{m}^2/24\text{h}$).
- Combination of lysozime and nisin at the combinations tested was found to be the most effective preservation method for liquid egg. The effect of both antimicrobials was shown to increase at the lower temperature tested (16°C).
- Normal and Weibull distributions were ranked as the ones that best described lag phase distribution in experiments performed at 25°C and 16°C in presence of both antimicrobials combined. At 25°C, stochastic predictions observed in egg were close to the actual data, which indicates that probabilistic predictions can be considered as an effective indication to establish the level of risk associated to liquid egg, although performing tests in real conditions is still necessary.
- A dynamic model has been obtained to describe the effect of water activity and temperature shifts on growth of *B. weihenstephanensis*. Growth was correctly predicted in broth and creamed pasta (rich substrates), whereas significant differences were observed between predictions and observations in a poor nutrient substrate, carrot soup. This model can be used to predict growth of *B. weihenstephanensis* in dynamic conditions, which can be useful for HACCP and microbial risk assessment purposes.
- Viability studies of acid stress showed that *B. cereus* CECT 148 and *B. weihenstephanensis* KBAB4 growth was readily affected in brain heart infusion

broth acidified at pH values of 3.0, 3.4, 3.8, 4.2, 4.6 and 5.0, showing greater effect at the lower pHs. Meanwhile *B. cereus* was unable to grow at pH 3.4, *B. weihenstephanensis* could grow at the pH level and showed significantly higher growth at all the pH values tested. Cellular viability of both species tested was found to decrease with lower pH values and longer exposure times to acid stress.

- Flow cytometry (FCM) studies using as fluorescent labelling propidium iodide and cFDA identified damaged *B. cereus* and *B. weihenstephanensis* vegetative cells by exposure to acid, although they did not show significant differences between both microorganisms. Results showed that this technique can be used to successfully study their physiological response.
- The assessment of major phylogenetic groups (I-VII) affiliation for practical applications in heat processing showed that *B. cereus* CECT 131, CECT 148, CECT 193, CECT 4014 and CECT 4094 strains belong to group (IV) on the basis of *panC* gene homologies studies. Thermal inactivation assays revealed variability in spore heat resistance within these group IV strains indicating the needed of further studies to state the feasibility of this classification for practical application in food processing.
- The identification of *B. cereus* group isolates at species and strain level by Single Nucleotide Polymorphisms (SNP) genotyping via High Resolution Melting (HRM) analysis of amplified polymorphic intergenic spacer region between 16S and 23S rRNA genes (IRS) proved to be discriminatory for *B. cereus sensu stricto* strain typing. The analysis with this technique of polymorphic regions within the bacterial rRNA operon, corresponding to 5S rRNA gene followed by a sequence highly homologous to glycosyltransferase, and 16S rRNA gene followed by a sequence highly homologous to ISR region, allowed a robust differentiation between *B. cereus* ATCC 14579, *B. thuringiensis* Bt407 and *B. weihenstephanensis* KBAB4. Therefore, SNP genotyping via HRM analysis emerges as a powerful tool for discrimination at species and strain level within *B. cereus* group members.

- To characterize the mechanistic basis of *B. cereus* group cold adaptation, mesophilic strains (*B. cereus* ATCC 14579, *B. cereus* ATCC 10987, *B. cereus* AH 187 and *B. thuringiensis* Bt407) and psychrotrophic strains (*Bacillus weihenstephanensis* WSBC 10688 and *Bacillus mycoides* DSM 2048) were successfully transformed with pMAD Δ 2216-17. Conversely, *B. weihenstephanensis* KBAB4 showed to be recalcitrant. *In vitro* method for strain-specific methylation of pMAD Δ 2216-17 did not enhance transformation of *B. cereus* group species. Besides, phenol-chloroform extraction for plasmid concentration was found to decrease transformation efficiencies in comparison with commercial columns used for DNA concentration.
- Cold response changed among *B. cereus* group species. *B. cereus* ATCC 14759 BC2216-17 knockout strains showed a clear and reliable impaired growth at 12°C. Growth of *B. cereus* AH 187 BC2216-17 knockout mutant and wild type showed no significant differences at 15°C and a biphasic shape at 30°C and 37°C. *B. thuringiensis* Bt407 BC2216-17 knockout mutants and wild type showed a heterogeneous behavior at 12°C and 15°C. These mutants can contribute to understand the mechanisms of psychrotolerance in *B. cereus* group, which presents important processing implications.

CHAPTER 10

References

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