Microbial ecology of the cocoa chain

Quality aspects and insight into heat-resistant bacterial spores

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Abstract

Cocoa beans (*Theobroma cacao* L.) are the basis for chocolate and cocoa powder production. The first step in the production of these food products consists of a spontaneous fermentation of the beans in the tropical producing countries, in order to allow the formation of the essential precursor compounds of the cocoa flavour. Following this stage, cocoa beans are industrially transformed into a range of different products.

In the first part of this thesis, the state of literature of cocoa bean fermentation was revisited with the aim of interlinking post-harvest processing practices and characteristics of the fermenting microbiota, with the quality of commercial cocoa beans obtained. This literature survey resulted in the identification of a number of research needs that can be used to design agricultural and operational measures, towards improving the quality of commercial cocoa beans. The second part of this thesis addressed the occurrence, levels and diversity of bacterial Thermoresistant Spores (ThrS) in commercial cocoa powder and in cocoa being transformed to cocoa powder in an industrial setting. Thermoresistant Spores were defined as spores which survive a heat-treatment of at least 100°C for 10 min, as opposed to the standard treatment at 80°C for 10 min for Total Spores (TS) determination. It was found that strains of *Bacillus licheniformis* and of the *B. subtilis* complex were the predominant species in the ThrS library and that strains of *B. subtilis* complex, including *B. subtilis* subsp. *subtilis* formed the most heat-resistant spores *in situ* and *in vitro*. Interestingly, a high heat-resistant spore phenotype was associated with diverse genotypes. In addition, a strategy was devised in order to identify biomarkers of spore heat-resistance. The comparison of physicochemical and ultrastructural properties among spores of different heat-resistance allowed the identification of manganese as a potential physiological biomarker for *B. subtilis* subsp. *subtilis* spores heat-resistance. On the other hand, a study of various genomic features conducted across strains of the phylum of *Firmicutes*, did not yield genomic biomarkers related to spore high heat-resistant phenotype, suggesting the primary role of spore physicochemical composition and ultrastructural characteristics as determinant factors of spore differential heat-resistance.

In conclusion, this study presents suggestions to delve into the functionality of the microbiota involved in cocoa bean fermentation and to improve the quality of commercial cocoa beans. Furthermore, it contributes towards the understanding of the ecology of high heat-resistant aerobic bacterial spores in the cocoa chain and provides knowledge regarding their heat-resistance. The data gathered in this thesis are useful input for predictive modelling and to assist in the production of safe and high quality food.

Abbreviations

AA	Acetic Acid
AAB	Acetic Acid Bacteria
AF	Aflatoxin
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
B	Basionym
BCCCA	The Biscuit Cake Chocolate and Confectionary Alliance
CA	Citric Acid
CBS	Centraalbureau voor Schimmelcultures
CCP	Critical Control Point
CFU	Colony Forming Unit
CMAA	Cocoa Merchants' Association of America
COG	Cluster of Orthologous Groups
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DPA	Pyridine-2,6-dicarboxylic acid (Dipicolinic acid)
E	Ethanol
EU	European Union
F	Fructose
FAO	Food and Agriculture Organization
FCC	Federation of Cocoa Commerce
FDA	Food and Drug Administration
FFA	Free Fatty Acid
G	Glucose
GC	Gas Chromatography
GC-content	Guanine plus Cytosine content
GTM	Gene-Trait Matching
HACCP	Hazard Analysis and Critical Control Points
ICA	International Confectionary Association
ICCO	International Cocoa Organization
ICE	Intercontinental Exchange
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectrometry
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
HPLC	High Performance Liquid Chromatography
KD	Kyte and Doolittle

LA	Lactic Acid
LAB	Lactic Acid Bacteria
LC	Liquid Chromatography
M	Mannitol
MDS	Metadata set
MLR	Multiple Linear Regression
MS	Mass Spectrometry
NGS	Next Generation Sequencing
NIRS	Near Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
NOG	Non Supervised Orthologous Groups
OG	Orthologous Group
OMCL	Orthologs Markov Cluster
ORF	Open Reading Frame
OTA	Ochratoxin
PAH	Polycyclic Aromatic Hydrocarbons
PCR	Polymerase Chain Reaction
RDP	Ribosomal Database Project
RNA	Ribonucleic Acid
S	Sucrose
SA	Succinic Acid
SASP	Small Acid Soluble Protein
SFC	Solid Fat Content
SOO	Saturated fatty acid, Oleic acid, Oleic acid
SOS	Saturated fatty acid, Oleic acid, Saturated fatty acid
TAG	Triacylglycerol
TAM	Total Aerobic Microorganisms
ThrS	Thermoresistant Spores
TS	Total Spores
UHT	Ultra-High Temperature
UNCTAD	United Nations Conference on Trade and Development
UV	Ultra Violet
WW	Wimley and White

Chapter 1

Introduction and thesis outline

The cocoa chain

Introduction

Cocoa beans, the fruit seeds from the tropical tree *Theobroma cacao* L. (family *Sterculiaceae*) are the principal raw material for chocolate production (Figure 1). However, before being traded in the countries of origin, the beans must undergo post-harvest processing comprising opening of the fruit (botanically a pod), natural fermentation and drying (Schwan and Wheals, 2004) (Figure 2). After the post-harvest processing, the beans are transported to industrial plants, for chocolate and cocoa powder production as well as cocoa butter extraction. Thus, the cocoa bean chain can be considered to be divided into two stages: the post-harvest and the industrial processing.

Figure 1. The cocoa pod and the cocoa bean. (**A**) Cocoa plantation in São Tomé and Príncipe. (**B**) Cocoa pods with different degrees of ripening. (**C**) Detail of the inside of a cocoa seed, where the white mucilaginous pulp surrounding the seed is visible. (**D**) Fermented and dried cocoa beans. Source: L. Lima. (See back cover for colour version)

Cocoa bean fermentation is the process whereby cocoa beans are converted into a commodity which is suitable to produce chocolate and cocoa powder. Without this process raw and dried cocoa beans do not develop the typical cocoa flavour upon roasting (Afoakwa et al., 2008). The process of fermentation typically involves piling of cocoa beans, mainly by forming heaps or in a cascade of boxes, and allowing microorganisms naturally occurring in the environment to develop on the mucilaginous pulp surrounding the bean during a number of days. The primary

goal of cocoa bean fermentation is to bring about changes in the parenchyma cells of the seeds, in order to form the precursor compounds of the flavour (namely free amino acids, reducing sugars and peptides) (Afoakwa et al., 2008). Other (desirable) roles fulfilled by the fermentation consist of reduction of the astringency and bitterness of the bean, and removal of the pulp, thus facilitating transport and ensuring bean stability.

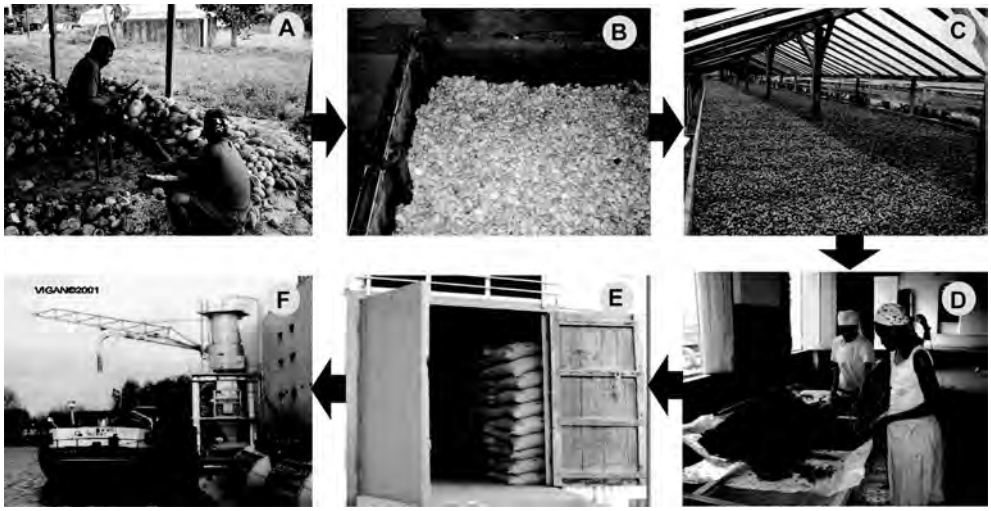

Figure 2. Schematic representation of cocoa bean post-harvest processing. Pod-opening (**A**); cocoa bean fermentation in boxes (**B**); cocoa bean sun-drying (**C**); Cocoa bean sorting (**D**); Cocoa bean storage in jute bags in a farmer's cooperative (**E**); and cocoa bean bulk transportation (**F**). Source: L. Lima (images A-E) and Cargill Cocoa (image F). (See back cover for colour version)

Although the term "cocoa bean fermentation" is widely used, it does not designate the fermentation of cocoa beans themselves, but it refers to the combined activity of microorganisms on the pulp surrounding the beans and the subsequent transformations in the seeds (Ferrão, 2002). The microbiological and biochemical transformations associated with cocoa bean fermentation have been extensively investigated. The studies on the microbiota composition of cocoa bean fermentation have shown that the fermentation is attained by the combined activity of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB). In addition, spore-forming bacteria from the genus *Bacillus* and relatives, other minor bacterial groups as well as moulds may occasionally develop (Ardhana and Fleet, 2003; Nielsen et al., 2008; Schwan and Wheals, 2004). Since this process still takes place in a largely uncontrolled way, the most recent research lines have attempted to steer the fermentation by use of defined starter cultures (Dzogbefia et al., 1999; Lefeber et al., 2011a).

The fermentation process does not proceed homogeneously, due to differences in the distribution of microorganisms and concomitant gradients of metabolites along the cocoa bean mass (Jespersen et al., 2005; Ostovar and Keeney, 1973). For this reason, fermented and dried cocoa beans are always a mixture of well and poorly fermented beans, with the fraction of the latter varying from batch to batch, depending on the fermentation process and practices. Sorting of cocoa beans before trading is encouraged by rewarding systems to farmers which account for absence of defects and visibly unfermented cocoa beans (Fowler, 2009). Fully fermented, dried and sorted cocoa beans receive the status of 'commercial cocoa beans'.

The cocoa bean world production in 2009/2010 was above 3.6 million tonnes, and forecasts for the 2010/2011 season estimated an increase by 17% to 4.25 million tonnes (ICCO, 2011). Africa is the largest producing continent, with over 60% of the world supply originating from Ivory Coast and Ghana (ICCO, 2011).

In the past, commercial cocoa beans would be shipped and transformed outside the producing countries (e.g. in Europe or North America). Presently, cocoa bean processing companies have started to locate manufacturing plants in the producing countries (Kamphuis, 2009), and their cocoa processing activity, as measured by "grindings", corresponds already to 41% of the total world "grindings" (ICCO, 2011). Still, the Netherlands is the largest cocoa bean processor in the world accounting nearly to 14% of the world "grindings" output (ICCO, 2011).

At the industrial plants, cocoa beans are stored in large silos and subsequently they undergo a series of well-defined operations aimed at producing cocoa liquor, cocoa powder and cocoa butter (Kamphuis, 2009). The general principle of production (ICMSF, 2002; Kamphuis, 2009) consists of blending cocoa beans from different origins, after which cocoa beans are cleaned from impurities by sieving, destoning and metal removal. Next, cocoa beans are subjected to surface heat treatments to facilitate the removal of the shell (a hard tegument surrounding the bean) by using infra-red driers or a moistening/pre-drying system. Subsequently, cocoa beans are broken and the shell is removed by winnowing. The resulting nibs (shelled and broken cocoa beans) may undergo alkalising ('dutching'), an operation consisting of nib immersion in an alkaline solution, such as potassium carbonate, at temperatures up to 100°C for a certain amount of time, to induce colour and taste changes (specifically darkening, decrease of acidity and introduction of specific flavours). Alkalising is predominantly performed in cocoa powder processing lines and less often in those aimed at chocolate manufacturing. Next, cocoa beans are roasted in a continuous drum roaster (nib final temperature is typically between 105°C and 140°C). Roasting has a crucial role for the quality of cocoa derived products, since it is through roasting that the precursor compounds formed during the fermentation react together to originate the typical cocoa flavour, by Maillard and Strecker degradation reactions (Afoakwa et al., 2008). Furthermore, this operation is the most important critical control point (CCP) in the process for the inactivation of pathogens (Burndred, 2009; Mazigh, 1999). After roasting, the nibs are cooled down and finely ground into a fluid mass, called liquor. Processing continues with cocoa liquor being pressed in a hydraulic press at pressures up to 540 bar for partial

removal of cocoa bean fat (minimal fat levels in the cake are 10%). The resulting cake is then pulverised and packed as commercial cocoa powder. The pressed cocoa butter is conducted through a separate line from that of cocoa powder and receives very little processing, which may include filtering and deodorising (Meursing and Zijderveld, 1999).

Nowadays, the large majority of chocolate industries outsource cocoa bean processing operations to specialised industrial processors part of the so-called cocoa press industry (Kamphuis, 2009) and they specialise in the transformation of the purchased liquor, powder and butter into a variety of products which reach the retail market.

Microbial ecology along the cocoa chain

Cocoa beans inside undamaged pods are essentially sterile (Schwan, 1998). After pod opening, the cocoa bean pulp becomes inoculated with a variety of microorganisms. However, the persistence and colonisation of the pulp by a given microorganism or microbial group is determined by the capacity to cope with the high acidity of the cocoa bean pulp and an array of metabolites and imposed stresses, such as ethanolic and acid stress as well as increasing temperatures (maximum temperatures in the piled beans are typically in the range 45°C to 50°C (Fowler, 2009; Schwan and Wheals, 2004)).

Yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB), endospore-forming bacteria and moulds can be recovered from commercial cocoa beans (Copetti et al. (2011a); Lima et al. (2012) and L. Lima unpublished results). In addition, during cocoa bean transport in the chain, these are likely to pick up additional undesirable contaminants (Kamphuis, 2009). This issue has important consequences for the design of food safety management strategies along the chain, where the potential occurrence of *Salmonella* and mycotoxins in cocoa derived products is of top concern, as implemented in Hazard Analysis and Critical Control Point (HACCP) plans and in microbiological quality guidelines applying to all cocoa derived products being traded (Kamphuis, 2009; Mazigh, 1999).

Endospore-forming bacteria of the genus *Bacillus* and relatives were shown to constitute the predominant microbiota of commercial cocoa beans (Barrile et al., 1971; Lima et al., 2012). Some spores of the genus *Bacillus* and relatives form highly heat-resistant spores which may survive roasting (Barrile et al., 1971) and, in this way, persist in final cocoa derived products (Gabis et al., 1970). The occurrence of these highly heat-resistant spores in cocoa derived products is currently accounted for by quantification of 'Total Aerobic Microorganisms' (Kamphuis, 2009).

The bacterial spore

What are bacterial spores and what makes them so special?

The bacterial endospore is a metabolically dormant cell type, exhibiting marked morphological differences when compared to the vegetative cells from which they originate (Sadoff, 1973; Setlow and Johnson, 2007) (Figure 3). Spores are formed inside the cells and, thus, are called endospores (hereafter referred to as bacterial spores).

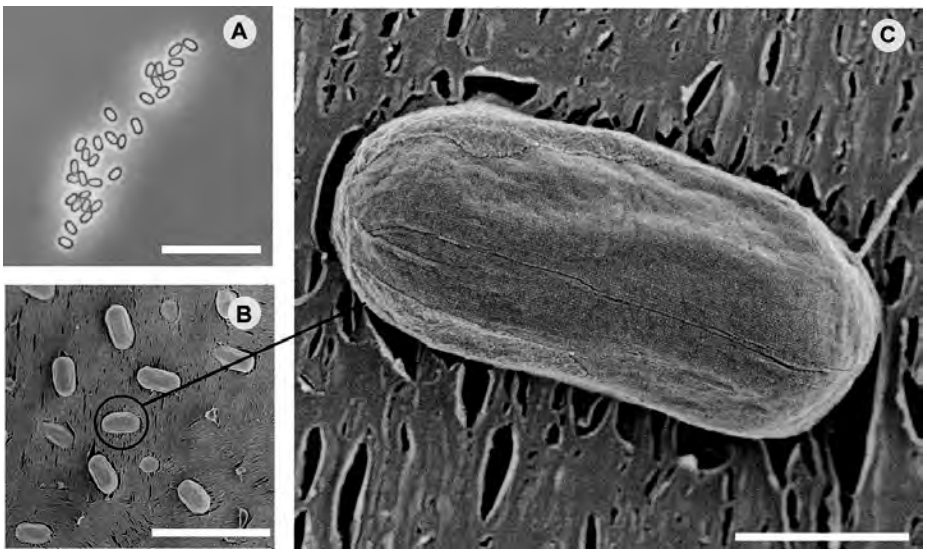

Figure 3. The bacterial spore. (**A**) Dormant *B. subtilis* spores as seen by phase-contrast microscopy. (**B**) Scanning Electron Microscopy (SEM) image of spores of a *B. subtilis* strain studied in this work. (**C**) Enhanced detail of the SEM image. The scale bars indicate 10 µm, 4 µm and 0.5 µm, respectively in figures A, B and C. Source: L. Lima (A) and Adriaan van Aelst (B and C).

The first morphological accounts of bacterial spores were given independently by Robert Koch and Ferdinand Cohn in 1876 for *B. anthracis* and *B. subtilis* spores, respectively (Gould, 2006). Bacterial spores have since then been recognized as the most resilient and the longest living forms of life on Earth, and they have been investigated extensively for the molecular mechanisms responsible for their morphogenesis and resistance to an array of environmental insults (Errington, 2003; Higgins and Dworkin, 2012; Setlow and Johnson, 2007).

Within the phylum of *Firmicutes*, which encompasses the classes of *Bacilli*, *Clostridia*, *Negativicutes*, *Erisipelotrichia* and *Thermolitobacteria*, endospore formation has been observed to take place in bacteria belonging to the first three classes (De Vos et al., 2009; Euzéby, 2012; Marchandin et al., 2010; Sokolova et al., 2007). The best known bacterial spore-formers include

members of the family *Bacillaceae* (specifically species from the genus *Bacillus* and *Geobacillus*) and *Clostridiaceae* (specifically species from the genus *Clostridium*), due to their impact on public health and for the microbiological quality of food and pharmaceutical products (De Clerck et al., 2004c; Jensen et al., 2003; Pflug, 2010; van Zuijlen, 2011). Furthermore, members of *Bacillaceae* and *Clostridiaceae* families are of paramount importance for biotechnological applications in agriculture as well as in the chemical and medical/pharmaceutical industries (Johnson and McGaughey, 1996; Mazza, 1994; Nölling et al., 2001; Outtrup and Jørgensen, 2002; Verschuere et al., 2000).

The bacterial spore consists of a unique structure which has been shown to be largely conserved across members of *Bacilli* and *Clostridia* classes (Atrih and Foster, 1999; Beaman et al., 1982; Orsburn et al., 2008; Paredes-Sabja et al., 2011). Such structure is responsible for spore dormancy and longevity up to thousands of years (Kennedy et al., 1994) as well as its resistance to heat, radiation, toxic chemicals and extreme pH, which are lethal to the vegetative cell (Setlow, 2006; Setlow and Johnson, 2007).

The importance of spores from a public health, safety and spoilage point of view is exacerbated by the fact that spores are ubiquitous in the environment, that is, they can be readily isolated from a large number of ecological niches, including the soil and the gut of animals (Boone et al., 1995; Carlin, 2011), which together with their resistance properties contributes to difficulties in their eradication

In the following sections the physiological and genetic processes involved in the formation of spores, their structure and composition will be introduced, and the features which allow spores to "stretch the limits of life" will be presented. The description of these processes will be mainly focused on *B. subtilis*, which is the model Gram-positive bacterium to study cell differentiation and gene/protein regulation, and which is also a species of importance in the context of this work.

Bacterial spore formation, structure and life-cycle

Spore formation and structure

When actively growing *B. subtilis* cells encounter stress situations, such as starvation for a critical carbon, nitrogen or phosphorus source, a complex and sophisticated signalling cascade leading to the differentiation of the cell into a spore might be activated. This process is designated as sporulation and can last about 8 h (Errington, 2003; Piggot and Hilbert, 2004). Sporulation typically occurs at high cell density and involves cellular communication by accumulation of extracellular peptide factors (Grossman, 1995). For cells growing in a laboratory medium, this process can be observed in cells which have entered the stationary growth phase. Several authors have provided excellent reviews about the process of sporulation, including details of morphological, biochemical and physiological transformations (Eichenberger, 2007; Errington, 2003; Higgins and Dworkin, 2012; Piggot and Hilbert, 2004). Sporulation is briefly addressed here to provide a sufficient background for subsequent sections and chapters in this thesis.

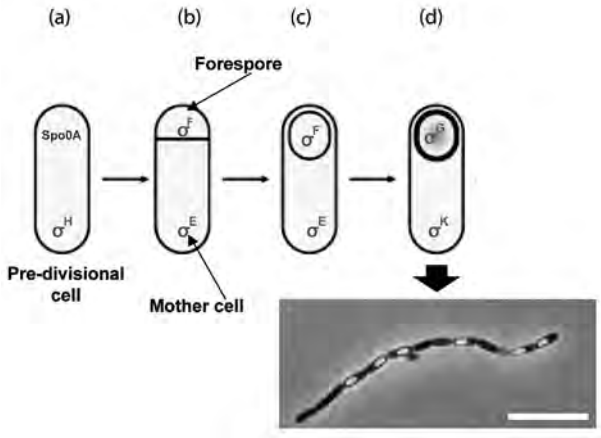

Figure 4. Schematic representation of sporulation and gene regulation in *Bacillus subtilis*. The sequential images represent the activation of protein Spo0A and the sigma factor σ^H in the pre-divisional cell (**a**), which leads to a polar asymmetrical cell division or septation, resulting in the mother cell and the forespore (**b**). Following septation, the forespore is engulfed and released as a protoplast in the mother cell (**c**, **d**). Different sigma factors are involved in the regulation of transcriptional activity in the mother cell and in the forespore, and these determine the compositional and structural components of the spore. Adapted from Piggot and Hilbert (2004) and Errington (2010). The insert shows sporulated cells of *B. subtilis* observed by phase contrast microscopy (Scale bar indicates 10 µm). Source: L. Lima.

When the aforementioned environmental stress signals are sensed by the cell, a master transcriptional regulator protein called Spo0A (Stage 0 sporulation protein A) becomes activated by phosphorylation. Spo0A activation initiates the transcription regulation of a plethora of genes (at least 10-15% of all *B. subtilis* genes), first, in the mother cell, and, secondly, concomitantly in the mother cell and in the spore being formed (Eichenberger, 2007; Errington, 2003; Piggot and Hilbert, 2004). Spo0A in conjunction with the sigma factor σ^H (sigma factors are dissociable subunits in the RNA polymerase involved in promotor region recognition) trigger an asymmetrical cell division (Figure 4a). This cell division, or septation, occurs near one of the poles of the cell, resulting in the formation of a smaller cell, the forespore, and a larger cell, the mother cell (Figure 4b). After septation, the next important events in the sporulation are the forespore engulfment by the mother cell, by a phagocytosis like process (Figure 4c), the development of the complete spore structure (Figure 4d) and the mother cell lysis by a mechanism of programmed cell death, which subsequently results in the release of the spore into the environment (Higgins and Dworkin, 2012; Piggot and Hilbert, 2004). In the mother cell and in the forespore, different programs of gene expression are initiated by activation of sequential sigma factors in each cell compartment. The sigma factor σ^E, followed by the sigma factor σ^K become active in the mother cell, while the sigma factors σ^F, followed by the sigma

factor σ^G become active in the forespore (Higgins and Dworkin, 2012; Piggot and Hilbert, 2004). Taken together, the tightly regulated program of sporulation determines that the forespore receives an identical copy of the mother cell chromosome and that the unique spore structure is formed before lysis of the mother cell takes place (Eichenberger, 2007; Errington, 2003).

The fully formed bacterial spore is composed of a central compartment called the core and successive layers of different biochemical nature, designated by cortex and coat (Henriques and Moran, 2007) (Figure 5).

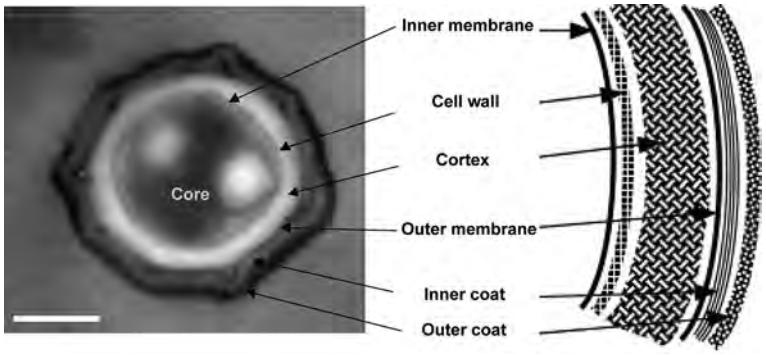

Figure 5. *B. subtilis* spore ultrastructure. The figure shows a thin Transmission Electron Microscopy (TEM) image of a spore cross-section and a schematic representation of the bacterial spore layers. Adapted from Popham (2002). The scale bar indicates 0.2 µm. TEM image source: L. Lima.

The core is a highly dehydrated and mineralized compartment, due to the presence of large amounts of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]), a substance exclusive to spores, which is chelated with divalent cations, mainly calcium (Setlow and Johnson, 2007; Sunde et al., 2009). It is in the core that the chromosomal DNA, ribosomes and enzymes are located. The spore DNA, although identical to that of the mother cell is compacted by low molecular weight proteins which are unique to the spore. These proteins are called Small Acid Soluble Proteins (SASPs) (Setlow, 1995). The dehydrated state of the spore and the protection of the spore DNA by SASPs have an essential role for spore dormancy, longevity and resistance to numerous agents, namely wet heat, Ultra Violet (UV) radiation, freeze-drying and oxidizing agents (Paidhungat et al., 2000; Setlow and Johnson, 2007).

The spore core is surrounded by the inner membrane (Figure 5), which offers low permeability to the passage of a number of chemicals, followed by the cell wall. The inner membrane plays an important role in spore life-cycle, since it is the place where proteins involved in spore germination, the so called germination receptors, are located. Around the inner membrane and cell wall lays the cortex, a tight layer of modified peptidoglycan, which has a fundamental role for the maintenance of the spore core dehydration (Popham et al.,

1996; Popham, 2002). The cortex is in turn, enveloped by an outer membrane and a structured protein layer called the coat. The spore coat plays also an important role in spore resistance, since being the place where peptidoglycan-lytic enzymes are located, it prevents the hydrolysis of the cortex. Furthermore, it is a barrier against protozoan predation and various chemicals, such as hydrogen peroxide, alkylating agents and certain UV wavelengths (Henriques and Moran, 2007; Klobutcher et al., 2006; Moeller et al., 2010). The spore coat is also important for the spore life-cycle, as it harbours proteins which facilitate the passage of specific germinant molecules from the environment into the spore (Henriques and Moran, 2007).

In addition to the coat, a further glycoprotein layer named crust, surrounding the coat, has been recently described in *B. subtilis* and it is speculated that this structure might be conserved across *Bacillus* species (McKenney et al., 2010).

Finally in some species, e.g. in *B. cereus* and *B. anthrax*, but not in *B. subtilis*, the coat is further surrounded by a loose structure called the exosporium. This structure was shown to be important for spore hydrophobicity and adherence properties, and it has been also speculated to be involved in spore germination (Faille et al., 2002; Koshikawa et al., 1984; Setlow and Johnson, 2007).

Spore life-cycle

Although metabolically dormant, the bacterial spore has a monitoring system which responds to environmental stimuli, such as the presence of nutrients, allowing the spore to resume an active cell stage when the conditions are supportive of cell growth (Figure 6).

Nutrient recognition occurs via specific receptors located in the spore inner membrane (Hudson et al., 2001; Paidhungat and Setlow, 2001). Such recognition leads to a series of irreversible degradative reactions, which result in the loss of the spore unique dormancy, refractability and resistance properties. This process is called germination (Moir and Smith, 1990; Moir et al., 2002; Paredes-Sabja et al., 2011). Spore germination can also be initiated involuntarily by chemical compounds, namely dodecylamine and calcium-dipicolinic acid complex as well as by physical treatments, such as high-hydrostatic pressure (Setlow, 2003). It was shown that non-nutrient induced germination in *B. subtilis* occurs independently of the germinant receptors pathway (Paidhungat and Setlow, 2000). However, the exact fashion by which chemical germinants activate spore germination has not been yet fully elucidated. Nonetheless, while calcium-dipicolinic acid induced germination requires the activation of cortex lytic enzymes, this is not the case with respect to dodecylamine-induced germination (Setlow et al., 2009). Recent studies showed that induction of germination in dormant *B. subtilis* spores could also be achieved by peptidoglycan fragments released by growing cells in the same medium, both from the same strain and from other species in the *Firmicutes* phylum (Shah et al., 2008), and bryostatin, a compound with modulating effect over the Serine/Threonine protein kinase (Wei et al., 2010).

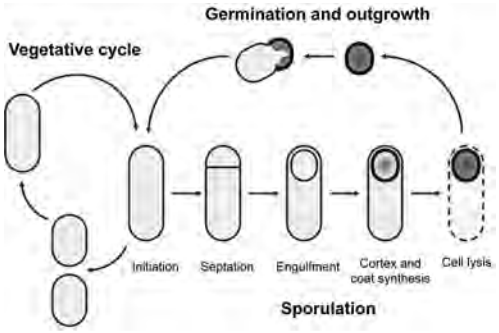

Figure 6. Schematic representation of *B. subtilis* life-cycle. It features the vegetative cycle of the bacterium and the stages of sporulation and germination. Adapted from Errington (2010).

Spore germination by nutrients can be divided into two stages. The stage I corresponds to the binding of nutrients to the germinant receptors, triggering the release of spore depot of cations and dipicolinic acid. This is accompanied by DPA replacement by water and loss of spore resistance. The stage II corresponds to the hydrolysis of spore peptidoglycan by the action of the cortex lytic enzymes. Cortex degradation results in the cell wall expansion and further core hydration (Setlow, 2003; Setlow and Johnson, 2007). Spore germination can be easily followed by phase-contrast microscopy, where typically the initial phase bright spore turns phase dark, as DPA is released from the core and water penetrates the spore (Figure 7).

The germination process is then followed by spore outgrowth, a step which comprises cell enlargement, initiation of catabolic and anabolic reactions, including degradation of SASPs and reutilisation of the derived amino acids, emergence of the new cell from the disintegrated coat and the first cell division (Setlow, 2003) (Figure 6). Spore germination and outgrowth differ with respect to their energy requirements, as germination occurs without detectable energy metabolism, as evidenced by the finding that the process could take place in the presence of DNA, RNA and protein synthesis inhibitors (Johnstone, 1994).

Interestingly, the processes of sporulation and germination do not occur synchronously for all cells or spores within a population, suggesting that growing cells and spores may have different fates in the environment (see insert in Figure 4 and Figure 7).

The generation of heterogeneity within isogenic bacterial populations during sporulation and germination has been proposed to constitute a survival strategy, whereby the cell maximises the adaptation to changing environments (Kearns and Losick, 2005; Losick and Desplan, 2008).

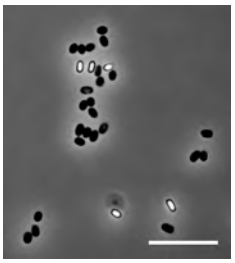

Figure 7. Heterogeneity in spore germination of *B. subtilis* incubated in the presence of L-alanine 10 mM. Both phase bright and phase dark spores can be distinguished. The scale bar indicates 10 µm. Source: L Lima

This heterogeneity appears to be also reflected in spore properties, that is, some spores may have a higher or lower resistance than the average spore (Coleman et al., 2007; Eijlander et al., 2011; Wang et al., 2011; Zhang et al., 2011). Such individual differences have also a direct impact for spore control in the food industry (Hornstra et al., 2009).

The bacterial spore wet heat-resistance

In the food industry, the use of wet heat is one of the most important preservation methods. The processes may vary from pasteurisation to sterilisation, depending on the intended microbial inactivation. Pasteurisation only eliminates heat-sensitive microorganisms from a liquid, as temperature-time treatments are generally in the order of 71°C for 15 s, a process also known as High Temperature Short Time (HTST) or 'flash pasteurisation' (Grant et al., 1998). The term sterilisation refers to the complete inactivation of microorganisms, including spore-formers (Anon., 1992). The food industry uses the term "commercial sterility", meaning that the product is not necessarily free from all living microorganisms, but that the ones surviving sterilisation are unlikely to grow out during the storage period and cause spoilage (UK Department of Health, 2004). Milk can be made commercially sterile by subjecting it to temperatures of 135°C for at least 1 s, followed by packaging in containers, a process called Ultra-High Temperature treatment (UHT) (Anon., 1992). The efficacy of a given commercial sterilisation treatment against bacterial spores depends on the initial level of spore contamination and their properties, specifically their heat-resistance (Stumbo, 1973).

Spore heat-resistance is most frequently expressed in thermobacteriology by the concept of spore *D*-value (Stumbo, 1948). The *D*-value refers to the decimal reduction time and it is defined as the time needed to inactivate 90% of the microorganisms (or by 1 log CFU/ml) at a specified temperature in a specific medium. A semi-log plot of *D*-values against the temperature often yields a linear relationship, from which the so called *z*-value can be calculated. The *z*-value corresponds to the increase in the temperature required to achieve a ten-fold reduction in the *D*-value (Stumbo, 1973). Both *D*- and *z*-values, are parameters

characteristic of an individual strain and are assumed to be constant for a given strain at a given heating condition. The *D*- and *z*-values are related by the equation of Bigelow (Bigelow, 1921), which allows obtaining an unknown *D*-value at a given temperature, knowing the spore D_T-value at a reference temperature *T* and the spore *z*-value.

In the canning industry, the concept of a 12-D process has been developed. The aim of a 12-D process is to reduce the initial population by 12-logs. This safety target was established primarily to control the presence of spores of *Clostridium botulinum*. Stumbo (1973) calculated the time to achieve a 12-log reduction for *C. botulinum* spores from the *D*-value at 121.1°C as equivalent to 2.52 min. In practice, this value also corresponds to a sterilisation value (F_0-value) at 121.1°C of 3 min, which is the minimum "*botulinum cook*".

Deviations from a linear relationship between the logarithm of the number of surviving spores and the heating time (spore survival curve) are often seen (Geeraerd et al., 2005; van Boekel, 2002). These deviations, which may include the presence of a shoulder prior to the occurrence of inactivation, a tail, or combinations of both, are indications of heterogeneity within a spore population, that is, the occurrence of subpopulations of spores with different heat-resistance.

Spore heat-resistance varies widely, not only among species but also among strains. For instance, in the case of *B. subtilis* spores a *D*-value of 1.4 min at 105°C was reported for strain 168 (model strain of *B. subtilis*), while for strain A163 a *D*-value of 42 s was measured at 120°C (Kort et al., 2005), a temperature which would not yield any survivors for spores of strain 168. The *D*-values for the important food-borne pathogen *Clostridium botulinum* may vary between 0.055 min and 1.43 min at 121°C (Brown et al., 2012). On the other hand, for spores of *Desulfotomaculum kuznetsovii* DSM 6115, a thermophilic anaerobe of the *Clostridia* class, a *D*-value of 19.8 min at 140°C was reported, making it one of the highest heat-resistant spore-formers described so far (Goorissen, 2002).

Although some ascospores of filamentous fungi, such as those belonging to the genera *Talaromyces* and *Byssochlamys* (Dijksterhuis, 2007) associated with food spoilage are as heat-resistant as bacterial spores, the latter have proven to be the most resilient spore types and they still challenge the implemented thermal processes within the food industry (Sevenier et al., 2012; Smelt et al., 2008).

A number of physicochemical and ultrastructural characteristics of bacterial spore were unveiled as important factors for the mechanism of spore wet heat-resistance. Different studies showed an important role for spore core low water content and protection of essential biomolecules in a dehydrated core, for instance spore DNA protection by SASPs, for spore heat-resistance (Beaman et al., 1982; Beaman and Gerhardt, 1986; Bender and Marquis, 1985; Fairhead et al., 1993; Marquis and Bender, 1989; Nakashio and Gerhardt, 1985; Paidhungat et al., 2000). In addition, the intrinsic thermostability of spore proteins and a reduced ratio between the core and the sporoplast (compartment comprising the core and the cortex layer) have been found to be correlated with the spore high heat-resistance (Beaman et al., 1982; Orsburn et al., 2008).

The observation that several other factors which contribute to spore wet heat-resistance contribute to a decreased core water content, such as the formation of cation-DPA complexes (Paidhungat et al., 2000), points to the importance of spore water content as a major determinant of spore wet heat-resistance (Setlow, 2006). Although it is not yet established how a low water content contributes to an increase in wet heat-resistance, it has been hypothesised that reduced core water content decreases the amount of water associated with spore protein, and, in this way, immobilising them and thereby preventing irreversible protein aggregation (Setlow and Johnson, 2007; Sunde et al., 2009).

Spore resistance to wet heat is substantially lower than that to dry-heat. For instance in *B. subtilis* PS832, a *D*-value of 18 min at 90°C was quantified for wet heat, while for dry-heat the temperature giving the same reduction within approximately the same time was 120°C (Setlow, 2006). This suggests different primary targets of spore killing. Indeed, studies in the laboratory of Professor Peter Setlow, with the objective of mapping the targets of spore killing by wet heat and dry-heat, showed that, while killing by means of dry-heat was accompanied by accumulation of DNA damage, the reason why spores treated by wet heat were inactivated was rather due to the denaturation or inactivation of one or more critical protein in the spore (Coleman et al., 2007; Setlow and Setlow, 1985; Setlow, 2006).

Spore heat-resistance is not an absolute property of the spore, since although largely determined by the strain genetic make-up, it depends on the cell sporulation environment (type of medium, temperature, pH, presence of stresses), the physiological state of the cell as well as the heating and recovery conditions.

Cazemier et al. (2001) and Oomes and Brul (2004) showed that spores of *B. subtilis* prepared on nutrient agar supplemented with a mixture of mineral cations (calcium, potassium, magnesium, manganese and iron) were considerably more heat-resistant than spores obtained in nutrient agar with only manganese. Likewise, spores produced in a medium without mineral fortification are in general more heat-sensitive than spores produced in medium supplemented with one or more divalent cations (Beaman and Gerhardt, 1986). However, a species dependence in relation to this factor has been reported, since the heat-resistance of *Alicyclobacillus acidoterrestris* spores was not affected by the presence of individual divalent cations in the sporulation medium (Yamazaki et al., 1997).

In general, spores produced at higher temperature have higher heat-resistance (Palop et al., 1999a), but it has been recently shown that spore heat-resistance does not increase indefinitely with this variable, but instead it shows a plateau, after which the heat-resistance of the spore decreases (Baril et al., 2012b). The natural thermal adaptation of a strain has been assumed to be an inherent or intrinsic molecular component of the spore heat-resistant phenotype which is genetically determined. It is often seen that thermophilic species produce spores of higher heat-resistance than that of mesophilic or psychrophilic species (Beaman and Gerhardt, 1986), but more and more data is being generated showing that heat-resistance is a spore property independent of the temperature growth ability of the strain (André et al., 2012; Huemer et al., 1998; Kort et al., 2005).

The *D*-value of *B. subtilis* spores PS533 which had been produced in liquid media was three-fold lower than that of spores produced in the same medium but in a solidified form (Rose et al., 2007). Mazas et al. (1997) showed that the $D_{100°C}$ for various strains of *B. cereus* decreased with decreasing pH of the medium.

In addition to the influence of the sporulation conditions, the general physiological state of the cell and stresses that the cell is subjected to during sporulation may modulate spore heat-resistance. For instance, it is known that different spore batches may result in differences in heat-resistance. At the same time, a decrease in the *D*-value for *B. sporothermodurans* spores was reported after multiple passages under laboratory conditions (Huemer et al., 1998). Several authors reported a 'heat-induced resistance' of spores after sublethal treatment of growing vegetative cells (Lee et al., 2003; Lee and Sim, 2006). Several heat shock proteins could be detected in the cytoplasm of these cells. However, according to Melly and Setlow (2001), heat shock proteins do not directly influence the wet heat-resistance of *B. subtilis* spores, since deletion mutants in genes encoding heat shock proteins did not exhibit decreased spore heat-resistance when compared to the wild-type.

Moreover, the precise method of heat-resistance determination (Brown, 1994) and composition of the heating and recovery medium can lead to differences in heat-resistance data. The influence of the pH of the heating medium on the heat-resistance has long been known. Most authors have reported that spores have their maximum heat-resistance at pH values close to neutrality (Mazas et al., 1998; Sala et al., 1995).

The influence of the recovery conditions on the perceived spore heat-resistance have been discussed extensively (Condón et al., 1996; Fernandez et al., 1994; Palop et al., 1999a). It was demonstrated over a temperature range from 30°C to 51°C that the *D*-values of spores of *B. subtilis* 4524 were negatively affected by increasing recovery temperature (Condón et al., 1996). Acidification of the recovery medium decreased the *D*-value of spores of *G. stearothermophilus* 12980 and the extent dependend on the nature of the acidulant (Fernandez et al., 1994). The addition of NaCl decreased the recovery of potentially viable *B. subtilis* spores, while the presence of calcium-DPA and lysozyme increased spore recovery (Cazemier et al., 2001). The oxygen tension is also an important variable. For *G. stearothermophilus* strain 12980, spore survival decreased for recoveries under anaerobic conditions when compared to spore recovery in the same medium but under aerobic conditions.

All these observations indicate the difficulties in obtaining comparable heat-resistance data for spores and their interpretation. As a consequence spore heat-resistance should be evaluated carefully for each specific (food) situation.

The genus *Bacillus* and relatives

The genus *Bacillus* is the type genus within the family *Bacillaceae*, a family which presently comprises 46 other genera (Euzéby, 2012). Many newly described genera include species which

in the past were grouped together within the genus *Bacillus*, such as the formerly known *B. stearothermophilus* and *B. sphaericus*, which now belong to the *Geobacillus* and *Lysinibacillus* genera, respectively (Ahmed et al., 2007; Nazina et al., 2001).

As mentioned before, the genus *Bacillus* includes one of the most studied spore-formers, *B. subtilis*. *B. subtilis* is also the type strain for the genus *Bacillus*.

The genus *Bacillus* includes members which are characterised as Gram-positive, rod-shaped, with aerobic to facultative anaerobic metabolism, but a few species are also described as strictly anaerobic. They are classified as catalase-positive and oxidase-positive to negative (De Vos et al., 2009). Members of the genus *Bacillus* exhibit a large physiological capacity, ranging from psychrophilic to thermophilic, and acidophilic to alkaliphilic. Others are salt-tolerant or salt-loving (halophilic). They are also notable for the diversity in colony and cell morphology. In sporulating cells not more than one spore per cell is formed. The most common spore shape is ellipsoidal or oval, but other shapes have also been observed and for this reason, spore shape, together with their location in the cell, has an important role in the taxonomic identification of species (De Vos et al., 2009; Fritze, 2002).

The GC-content of *Bacillus* is listed to range from 32 to 66%, illustrating the wide genomic heterogeneity of the genus. Variations in the GC-content are observed both from species to species and among strains within a species (De Vos et al., 2009).

In total, 259 species have been described so far within the genus *Bacillus* (Euzéby, 2012), but given the number of new species discovered every year and the advancements in techniques for species discrimination this number is likely to increase in the future.

With regard to species relevance within this group it is important to highlight *B. subtilis* and *B. licheniformis*. Both *B. subtilis* and *B. licheniformis* are commonly found in foods and are well known causes of food spoilage incidents (Heyndrickx and Scheldeman, 2002; Pepe et al., 2003; van Zuijlen, 2011). Although they have been implicated in food-borne illness (Brown, 2000; Gilbert et al., 1981), they are not widely recognised as pathogenic. At the same time, both species have also industrial importance for the production of fermented foods (Parkouda et al., 2009) and as source of enzymes (Outtrup and Jørgensen, 2002).

Other notable species within the genus *Bacillus* consist of members of the *B. cereus* group, specifically *B. anthrax*, which is the etiological agent of anthrax disease; *B. cereus*, which is a known cause of food-poisoning and toxico-infection as well as spoilage of dairy products; and *B. thuringiensis*, which is used as a biopesticide or biological control agent (Greig and Ravel, 2009; Heyndrickx and Scheldeman, 2002; Jensen et al., 2003; Nicholson, 2002).

Spores of different species of *Bacillus* (*B. clausii*, *B. cereus*, *B. pumilus and B. subtilis*) are currently being used as commercial probiotics for prophylactic and therapeutic treatment in animals and humans as well as growth promotors in livestock (Duc et al., 2004; Mazza, 1994). The use of spores as probiotics over *Lactobacillus*-type products has the advantage of allowing longer shelf-life, as they can be stored in a desiccated form for extended period of time (Duc et al., 2004). The use of *Bacillus* spores as probiotics is possible due to the capacity of spores to germinate in the intestinal tract (Casula and Cutting, 2002), to initiate immunogenic reaction

in the host and to compete with the gastrointestinal pathogens by competitive exclusion and production of antimicrobial compounds (Duc et al., 2004). Furthermore, spores of *Bacillus* species are considered promising agents for vaccine delivery in humans (Amuguni et al., 2012; Cutting et al., 2009). Indeed, spores of attenuated strains of *B. anthracis* were already used since the nineteenth century in livestock vaccination against anthrax disease (Oggioni et al., 2003).

Approaches to study microbial ecology

The discipline of microbial ecology addresses the diversity and activity of microorganisms in the environment, and therefore it is concerned with the role of microbial interactions and their contribution to biogeochemical cycles (Merchant and Helmann, 2012; Xu, 2006). Its study relies on a number of methods, techniques and approaches aimed at identifying, monitoring and assessing the structure and functionality of microorganisms and their microbial communities.

Bacteria comprise two of the three Domains of life, the prokaryotic Bacteria and Archaea (Woese et al., 1990). The first step to characterise individual strains, consists of their isolation from (environmental or food) samples, for instance, by making use of selective cultivation media, with or without enrichment, followed by appropriate medium incubation under temperature and oxygen availability conditions of relevance for the microorganism. Spores can be isolated by suspending the sample in water or in a suitable isotonic medium, and heating at 80°C for 10 min, a treatment which kills the vegetative cells but not spores (Dijk et al., 2007; Roberts and Greenwood, 2003). The next step is the identification of the axenic isolate. Traditional identification methods were performed solely based on phenotypical tests, comprising morphological, biochemical (e.g. ability to use different carbon and nitrogen sources) and physiological (e.g. growth at various temperatures and pH values) characteristics. Although phenotypic data are still the basis for the formal description of taxa, different genotypic methods are now used to allocate taxa on a phylogenetic tree and to delimit the classification systems (De Vos et al., 2009; Vandamme et al., 1996).

The most prominent genotypic taxonomic approach for the classification of strains at species or genus level, consists of sequence comparison of the ribosomal RNA gene (Woese et al., 1990), typically the 16S ribosomal RNA gene. This is due to the presence of ribosomes in all bacteria, their constant function and the presence of highly conserved and variable domains, which make them a biological chronometer (Xu, 2006). According to the recommendations for species delineation, microorganisms with less than 97% 16S rRNA gene sequence similarity (this was shown to be equivalent to less than 70% DNA-DNA relatedness) should be considered as belonging to different species (Stackebrandt and Goebel, 1994; Stackebrandt et al., 2002).

Despite the usefulness of the 16S rRNA gene sequencing for strain classification this gene may show limited resolution to distinguish closely related strains, due to limited sequence variation. This is the case for strains belonging to the species of *B. subtilis* and *B. cereus*

(Manzano et al., 2009; Rooney et al., 2009). In that case, other techniques should be employed to unveil diversity. Nowadays, the most widely used techniques to assess strain diversity consist of comparison of genomic DNA characteristics, such as the sequence of selected genes (e.g. gyrase A and B subunits), or comparison of fragment distribution in a polyacrylamide gel of whole or partial genome digests, as incorporated into the so-called molecular typing techniques (Chun and Bae, 2000; De Vos, 2002). Pulsed Field Gel Electrophoresis (PFGE) is the example of a technique that visualises the entire genome, while Amplified Fragment Length Polymorphism (AFLP) or ribotyping only visualise randomly selected parts of the genome (De Vos, 2002; Tindall et al., 2010).

Obviously, in addition to strain identification and genotypic characterisation, the study of phenotypic properties is essential to understand their functional role in the environment and their survival. In particular, the use of microbial modelling approaches to quantify microbial behaviour assumes a central place within certain disciples, namely food microbiology, since it allows an objective assessment of microbial physiological capacity and comparison of microbial performances among strains or species (Den Besten et al., 2006; van Asselt and Zwietering, 2006; van Boeijen et al., 2008; Zwietering et al., 1990). Phenotypical data are important inputs for predictive microbiology, a branch within food microbiology which makes use of mathematical models and various statistical approaches to describe microbial responses to variables of relevance to the food environment (McKellar and Lu, 2004).

From the community point of view, it is of importance to characterise the structure, by having acess to the occurrence of different groups of microorganisms and their taxonomic diversity. Again, the combination of selective media with appropriate incubation approaches is of great relevance. With regard to the community/population structure it might be of interest to quantify the abundance of the different groups and this can be performed based on well-established culturable media and approaches in microbiology (Roberts and Greenwood, 2003).

Besides culture-dependent methods, culture-independent approaches are the hallmark for the characterisation of complex microbial communities or species of difficult cultivation (Su et al., 2012). Culture-independent approaches combine the use of DNA or RNA extraction techniques, followed by polymerase chain reaction (PCR) of selected genes and their identification by sequencing, or alternatively, by their direct visualisation in appropriate gel systems (Xu, 2006). Popular culture-independent techniques consist of cloning of environmental DNA (Pace et al., 1985)1985 and the application of Denaturing Gradient Gel Electrophoresis (DGGE), or its variant Temperature Gradient Gel Electrophoresis (TGGE) (Muyzer et al., 1993; Muyzer and Smalla, 1998). Gradient gel electrophoresis techniques allow for the separation of DNA fragment mixtures of the same size, but which are resolved due to differences in their base-pair sequence (Muyzer et al., 1993). These techniques are expedite techniques to compare microbial communities from different environments and to monitor changes in abundance of specific members over time or in different samples (Spiegelman et al., 2005). A more elaborated stage of culture-independent methods is the so called Metagenomics approach (Handelsman et al., 1998). Metagenomics consist of the study of genomic material

and it seeks to clone larger genomic fragments than in the traditional cloning approach where phylogenetic markers are targeted. The cloned DNA is then screened via high-throughput platforms for specific sequences or functions of interest (Chen and Pachter, 2005; Handelsman et al., 1998)2005; Handelsman et al., 1998.

Advances in conventional Sanger DNA-sequencing technology by development of Next Generation Sequencing (NGS) techniques has brought a new dimension to the field of microbial ecology, since NGS techniques are faster, cheaper and produce a large amount of sequence data (Novais and Thorstenson, 2011; Scholz et al., 2012). One of these NGS techniques called 454 pyrosequencing has been applied to characterise complex microbial ecosystems in the medical field as well as in food-related ecosystems (Claesson et al., 2009; Sakamoto et al., 2011), using the same phylogenetic marker as used in the strategies of cloning and PCR-DGGE. Pyrosequencing of the 16S rRNA gene to access microbial diversity has the advantage that it provides greater sampling depth than cloning and PCR-DGGE (Henry et al., 2011; Shokralla et al., 2012). However, the choice of the technique or approach in research depends on the research question being answered and the level of detail required (Hamady and Knight, 2009).

NGS also constitutes a milestone in the field of genomics since the genome sequence of individual microorganisms can now be decoded in a matter of a few days at a very affordable price (Henry et al., 2011). The availability of an increasing number of genomes per species had a direct impact for a new definition of species diversity, which can now be described in the so-called concept of the “Pan-genome” (Medini et al., 2005). The “Pan-genome” corresponds to the sum of the core genome (genes shared by all the strains) and the dispensable genome (genes only present in some strains) and can exceed the size of an individual genome by several orders of magnitude (Medini et al., 2005).

Finally, an efficient analysis of all genomic data would not be possible without the use of diverse bioinformatics tools and approaches. The discipline of Bioinformatics seek for patterns in the observed biological data and attempt to build a mechanism for those patterns (Xu, 2006). It also offers options for the organisation of the data-set and facilitates data interpretation.

Thesis outline

The research presented in this thesis was initiated by the interest in the role of cocoa bean post-harvest processing in the quality of commercial cocoa beans. For some countries, such as Ivory Coast, Ghana and São Tomé and Príncipe, cocoa bean exportation is an important source of revenues and it contributes to a significant fraction of the country's Gross Domestic Product. Therefore, measures undertaken to improve the quality of exported beans are expected to have an impact on cocoa bean demand and reputation of the country. These measures need to be guided by a thorough scientific understanding of the factors affecting the quality of commercial cocoa beans. Thus, the first aim of this thesis was to critically evaluate the state of the art about cocoa post-harvest processing science, with special focus on cocoa bean fermentation, and commercial cocoa bean quality benchmarks, to help prioritise targets for quality-oriented interventions.

A second motivation resulted from the interest of the cocoa processing industry to acquire knowledge about the occurrence of heat-resistant spores in commercial cocoa products, namely cocoa powder, and the importance of gaining insight into the wet heat-resistance and germination properties of such spores. Strains of *B. subtilis* have been implicated in spoilage incidents of UHT treated chocolate drinks manufactured with cocoa powder (H. Kamphuis, personal communication), but *B. subtilis* is not particularly widely recognised as forming highly heat-resistant spores, which made this a very intriguing problem. Published data documenting these incidents and possible causes were lacking.

For this reason, the second aim of this thesis was to gain insight into the occurrence, levels, diversity and properties of heat-resistant spores in the cocoa chain, in order to generate data to allow for their control in the food industry and provide the basis for further quantitative predictive modelling initiatives in downstream preservation technologies. With respect to spore properties, attention was given to putative mechanistic factors involved in spore heat-resistance and to the characterisation of germination responses for spores of highly heat-resistant strains.

Chapter 2 presents a comprehensive review about the biochemical transformations which take place during cocoa bean post-harvest processing and the way through which they lead to the formation of the essential precursor compounds of the cocoa flavour. The information from microbial ecology studies conducted in different countries was critically compared and research needs, which may help to improve the quality of commercial cocoa beans, were identified.

The progression of commercial cocoa beans in the chain following the post-harvest processing, depends on grading of commercial cocoa bean batches, based on different standards and criteria. This topic, which had been introduced in the previous chapter, is extensively addressed in **chapter 3.** In this chapter, the production environment of cocoa beans and the implications of this environment from a quality and safety perspective are

analysed. Subsequently, the methods presently used to ensure cocoa bean quality and safety are discussed. Attention is given to regulatory bodies and legislative aspects.

With respect to the second objective in the project, **chapter 4** presents the result of the investigation concerning the prevalence of heat-resistant spores in cocoa powder. Commercial cocoa powder samples collected from industrial and retail origins were first assessed for their compliance with the microbiological quality guidelines. Next, spore heat-resistance was characterised *in situ* and *in vitro*. Several strains were isolated for phenotypical and genotypical analyses.

In **chapter 5**, the microbiota during cocoa bean processing to cocoa powder was studied. In addition, it was investigated whether ThrS detected in commercial cocoa powder originated from commercial cocoa beans, thereby constituting roasting survivors, or whether they originated during processing. To address these questions, culture-dependent and culture-independent methods and approaches were used to study the microbiota composition of cocoa beans being processed into cocoa powder.

In the course of the research summarised in chapters 4 and 5, a number of strains of *B. subtilis* with considerably different heat-resistance were isolated. A selection of these strains, representing the diversity of high and low heat-resistant spores was compared in detail for important spore characteristics known to be involved in spore heat-resistance. Such characteristics consisted of spore physicochemical composition and ultrastructure (**chapter 6**).

In **chapter 7**, spore germination response to various nutrient-germinants, including ingredients of relevance for the manufacturing of chocolate milk drinks, was determined with focus on highly heat-resistant spores.

So far, genomic factors involved in spore heat-resistance have only been questioned to a very limited extent in the literature, and it is still not known what strains able to produce spores of high heat-resistance share in common at the genomic level. **Chapter 8** describes an approach aimed at identifying genomic biomarkers for spore heat-resistant phenotype focused on members of the phylum *Firmicutes*.

Finally, in **chapter 9**, the results obtained in this thesis are discussed and suggestions for future research are presented.

Chapter 2

Theobroma cacao L., "The food of the gods": Quality determinants of commercial cocoa beans, with particular reference to the impact of the fermentation

Lima, L. J. R., Almeida, M. H., Nout, M. J. R. and Zwietering, M. H.

The quality of commercial cocoa beans, the principal raw material for chocolate production, relies on the combination of factors that include the type of planting material, the agricultural practices and the post-harvest processing. Among these, cocoa bean fermentation is still the most relevant, since it is the process whereby precursors of the cocoa flavour arise. The formation of these precursors depends on the activity of different microbial groups on the bean pulp. The comparison of fermentations in different countries showed that a well-defined microbial succession does not always take place and that the role of *Bacillus* spp. in this process remains unclear.

Considering the overriding importance of the fermentation to achieve high quality commercial cocoa beans, we discuss the need of addressing in future research the impact of the farming system, the ripeness state of the pods and the role of microbial interactions on the fermentation. In addition, the problem of high cocoa beans acidification, aspects dealing with the volatile fraction of the flavour and the cocoa butter properties were identified as critical aspects that need further investigation.

The standardisation of the microbiological methods and the application of metagenomic approaches would magnify the knowledge in this domain.

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Introduction

From the twenty-two species that constitute the genus *Theobroma* (family *Sterculiaceae)*, *Theobroma cacao* L., is commercially the most important, due to the value of its seeds (Bartley, 2005; Wood, 1975). The seeds, commonly known as cocoa beans, are the principal raw material for chocolate production. Not only is it not possible to make chocolate without cocoa beans, but also the distinctive flavour of chocolate is due to the presence of these beans. Other products derived from cocoa beans are cocoa powder, widely used in the food industry, and cocoa butter that in addition to its confectionary use has also cosmetic and pharmaceutical applications. However, before cocoa beans can be traded and processed into final industrial products they have to undergo post-harvest processing on farms and plantations comprising the steps of pod opening and beans removal from the pod, beans fermentation and drying. In this sequence, the fermentation constitutes an essential critical step for the development of flavour quality attributes of commercial cocoa beans (Rohan, 1964). This is due to the fact that during the fermentation, biochemical transformations are induced within the beans leading to the formation of important precursors of the cocoa flavour, some of its highly volatile compounds as well as causing browning, and reduction of bitterness and astringency of the beans (Almeida, 1999; Cros and Jeanjean, 1998). The full cocoa flavour is developed later upon roasting, through complex reactions, mainly of Maillard type (Afoakwa et al., 2008).

Two important milestones achieved in the scientific area of cocoa bean fermentation consisted of the understanding of the basic mechanisms leading to the formation of cocoa flavour precursors (see for instance, the work by Forsyth and Quesnel (1963)); and the conduction of the first controlled fermentation using a defined microbial inoculum as starter culture (Schwan, 1998). This contributed to a significant improvement of cocoa post-harvest processing. However, we have identified relevant aspects linked to the fermentation that are still not very well understood and whose comprehension could bring important breakthroughs for the future of cocoa fermentation science and practice. Therefore, this article will focus on the impact of the fermentation on the quality of commercial cocoa beans, although it is known that this is a combination of factors ranging from the planting material to the drying method (Figure 1) (Almeida, 1999; Clapperton et al., 1994; Jeanjean, 1995; Kattenberg and Kemmink, 1993).

After an introduction on cocoa botany, production and methods of post-harvest processing, the current state of knowledge about the microbiota of cocoa bean fermentation and the mechanisms leading to the formation of the cocoa flavour precursors are presented. Next, research needs in the field of cocoa fermentation are discussed. These deal with the influence of the farming system and pod ripeness on the fermentation, and the subsequent way through which the fermentation affects bean acidification, the volatile fraction of the flavour and the cocoa butter properties. Finally, the quality requirements of cocoa beans are addressed and final considerations presented.

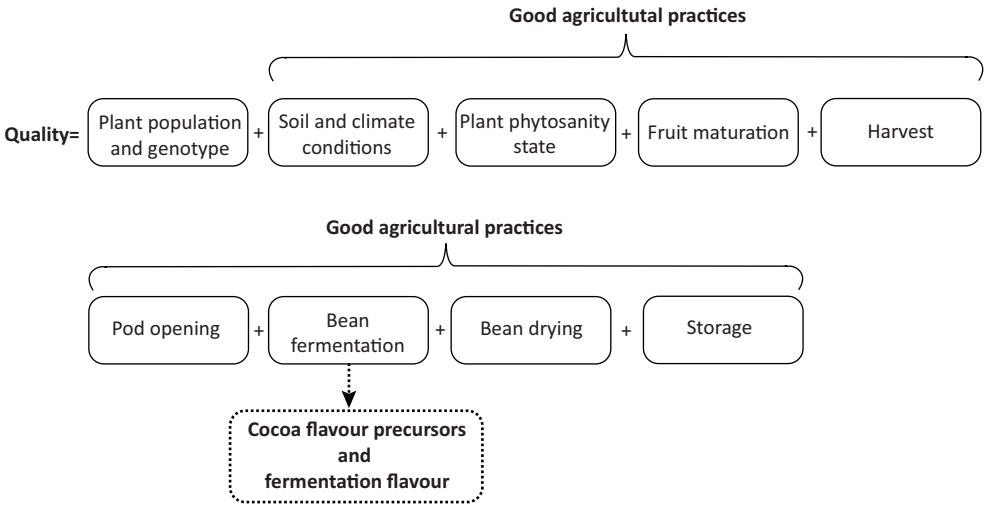

Figure 1. Stages that contribute to the quality of commercial cocoa beans, where the fermentation has a core importance.

Botany, production and post-harvest processing

The cocoa tree is a perennial tree, 8 to 15 m in height, which under a more intensive cultivation is limited to 2.5 to 3 m by pruning, for better phytosanitary control (Fowler, 1999; Wood, 1975). Its natural habitat is the lower storey of the evergreen rain forest in the Amazon basin and other tropical areas of South and Central America (Fowler, 1999; Lass, 1999). Although there is still controversy concerning the origin and domestication of wild populations of cocoa, recent investigations suggest a centre of origin in South America (Motamayor et al., 2002).

It was the Swedish botanist Linnaeus who named the genus "*Theobroma*", from the Greek "Theos" (meaning God) and "broma" (meaning food), prompted by the recognized legendary Mayan and Aztec popular belief of cocoa tree deific origin (Cook, 1982). After the encounter of Hernando Cortés with the Aztecs in the actual Mexico city in 1520, cocoa drinks were introduced in the Spanish court (Coe and Coe, 1996). Their popularity would later lead to the spread of cocoa tree rootstocks to other European colonies bearing their growth (Lass, 1999; Thompson et al., 2007).

The fruit of the cocoa tree is botanically an indehiscent drupe, usually called pod (Lass, 1999; Wood, 1975). These are oval in shape, measure between 12 and 30 cm and contain 30 to 40 beans embedded in a mucilaginous pulp, which comprises approximately 40% of the bean fresh weight (Schwan and Wheals, 2004). The pulp is characterised by a sugar content around 11 to 13% (w/w), high acidity, conferred by the presence of diverse organic acids, but mainly

citric acid, and a protein content in the range of 0.4 to 0.6% (w/w) (Table 1, where the amount of fat, minerals and vitamins is also presented).

Table 1. Composition of cocoa pulp (g.100 g^{-1} fresh weight pulp)

	Ivory Coast[a]	Nigeria[a]	Malaysia[a]	Indonesia[b]		Ghana[c]
				F1	F2	
Water	82.60	82.50	85.90	-	-	-
Glucose and fructose	6.80	11.13	10.25	10.3	6.6	10.77
Sucrose	4.35	1.92	1.35	3.2	2.1	0
Plant and cell wall polymers	2.81	-	1.48	-	-	-
Citrate	1.31	0.79	0.29	2.4	2.1	0.93
Protein/ peptides	0.57	0.51	0.43	-	-	-
Free amino acids	0.15	0.11	0.21			
Fat	0.45	0.75	0.35	-	-	-
Metals	0.24	0.22	-	-	-	-
Vitamins (composite sample)	0.05	0.05	-	-	-	-

[a](Pettipher, 1986a); [b](Ardhana and Fleet, 2003); [c](Camu et al., 2007).
F1, F2- Fermentary 1 and 2; - Not determined.

Each cocoa bean consists of two cotyledons (“nibs”) and a small embryo, all enclosed in a skin (“shell”). The cotyledons are constituted by two types of cells: storage or parenchyma cells, containing fat globules, protein bodies and starch granules, and bigger pigmented cells, containing polyphenols and methylxanthines (Biehl et al., 1977; Del Boca, 1962; Ferrão, 2002). In the cotyledons, the fat is the most important nutrient, representing about half the weight of the dry seed (Table 2). The methylxanthines, mainly theobromine and caffeine, may occur at an average level of 1.5% (w/w) in the dried nibs and impart a very bitter taste to the cocoa beans (Table 2). The total amount of polyphenols in dried fresh cocoa beans, may vary between 12 and 20% (w/w) and these are responsible for its high astringency, contributing as well to the bitterness (Forsyth and Quesnel, 1957; Kim and Keeney, 1984). Three main groups of polyphenols are present: anthocyanins, flavan-3-ol (catechins) and proanthocyanidins, corresponding to approximately 4, 37, and 58%, respectively (Kim and Keeney, 1984; Wollgast and Anklam, 2000). Within the catechins group, the (-)-epicatechin is the predominant fraction amounting to 35% of total polyphenol content (Kim and Keeney, 1984).

Notwithstanding the existence of morphological and genotypical differences among cocoa populations, the most prominent classification system is based on flavour quality attributes of the cocoa seeds. Three main botanical populations are distinguished: Forastero, Criollo and Trinitario (Bartley, 2005; Cheesman, 1944; Motamayor et al., 2002). Forastero

populations, probably native to the Amazon basin, supply over 95% of the world's cocoa (ICCO, 2007b). The seeds of this population are flat, astringent and purple in colour (more rarely ivory or pale), due to the presence of anthocyanins. Forastero cocoa trees are very productive and are considered to have a moderate resistance to pests and diseases (Bartley, 2005; Ferrão, 2002). Nowadays, most populations around the world use locally adapted Forastero varieties augmented by specific genotypes (Sounigo et al., 2005). These hybrids are then selected based on their biological resistance and yield (Jonfia-Essien et al., 2008). The commercial beans are called 'bulk cocoa in trade', being used for the manufacture of milk chocolate, cocoa butter and cocoa powder (Fowler, 1999; Fowler, 1994; Thompson et al., 2007). Criollo is the original cultivated population, indigenous to Northern South and Central America. The beans are white to ivory or have a very pale purple colour, due to an anthocyanin inhibitor gene (Ferrão, 2002; Fowler, 1999). The low yields and the susceptibility to many diseases, make them rare in cultivation. Nowadays, its cultivation is limited to Central America and a few regions in Asia (Ferrão, 2002; Fowler, 1999; Thompson et al., 2007). The Trinitario type originated in Trinidad and covers all the products of natural hybridization and recombination of Criollo and Forastero populations (Ferrão, 2002; Fowler, 1999). The beans have variable colour, although rarely white, and the trees show susceptibility to pests and diseases intermediate to Forastero and Trinitario populations (Bartley, 2005; Ferrão, 2002; Fowler, 1999). Both Trinitario and Criollo varieties produce mainly the 'fine' or 'flavour' cocoas, whose share in the total world production is below 5% (ICCO, 2007b). These cocoas are used to make high quality dark chocolate (Fowler, 1999). Nonetheless, some special Forastero types are considered to produce 'fine' cocoa, due to a distinctive aromatic nature. This includes for instances the Nacional type, grown in Ecuador, or some cocoa from São Tomé and Príncipe (Fowler, 1994; UNCTAD, 2001).

Table 2. Average chemical composition of roasted nibs (g.100g^{-1})

Constitutents	**Roasted nibs**	
Water[a]	3.0	3.7
Fat	54.0	54.0
Protein	12.5	-
Starch	6.0	6.0
Fibre	2.5	2.5
Ash	3.0	2.8
Theobromine	1.3	1.3
Caffeine	0.2	0.1
References	(Valiente et al., 1994)	(Minifie, 1980)

[a] Varies according to the degree of drying and roasting.
* Nibs consist of shelled and ground cotyledons of commercial cocoa beans.
- Not determined.

In the producing countries, the post-harvest processing of cocoa beans starts by breaking the fruits during a period of 3 to 4 days to 2 weeks after the harvesting (Lass, 1999; Thompson et al., 2007). Next, the wet beans are removed manually or, on some large estates in West African countries, Mexico and Brazil, by means of mechanical systems (Ferrão, 2002). When care is given to the processing, cocoa pods that are diseased or broken are separated to a different pile or buried in the soil and defected beans are separated from the main fermenting mass (Ferrão, 2002). Subsequently, the wet beans are submitted to the fermentation or curing process. The method for cocoa bean fermentation consists of piling a certain quantity of fresh beans and allowing naturally occurring microorganisms to develop (Fowler, 1999). The growth of these microorganisms is supported by the sugars and other minor components present in the cocoa bean pulp (Ardhana and Fleet, 2003; Nielsen et al., 2007b).

Different methods of cocoa bean fermentation are used worldwide but wooden boxes, heaps, baskets or drying platforms are the most widespread (Cook, 1982; Thompson et al., 2007; Wood, 1975). The fermentation period may take between two to three days in the case of Criollo and five to ten days in the case of Forastero and Trinitario populations (Afoakwa et al., 2008; Cook, 1982; Rohan, 1964; Thompson et al., 2007; Wood, 1975). During this period the beans can be aerated and mixed ('turning' operation), by transfer to another box or heap. When the fermentation is completed, the moisture content of the beans has to be reduced to levels between 6-8% and they are stored at relative humidity of 65 to 70%, in order to avoid mould growth (Cook, 1982; Fowler, 1999; Schwan and Wheals, 2004). The drying process is conducted either by sun drying, during a minimum of 7 days to 3 weeks, or, alternatively, in mechanical driers (Cook, 1982; Thompson et al., 2007). Finally, the beans are cleaned, selected, calibrated and bagged in 60 kg jute sacks or are transported in bulk to the manufacturing plants.

The world cocoa bean production in 2006/2007 was 3.4 million tonnes. Ivory Coast is the world's largest producer (38% of the world production in the 2006/2007 season), followed by Ghana, Indonesia, Nigeria, Cameroon, Brazil and Malaysia. Together these countries contributed for an output of 86% of the total production in 2006/2007 season. Ivory Coast and Ghana supplied alone, 56% of the global fraction (ICCO, 2008). However, in the producing countries, the economic importance of cocoa differs considerably. While in countries like Indonesia or Malaysia it faces competition from other crops such as palm oil, coffee or rubber (Fowler, 1999), elsewhere it is a main source of foreign exchange earnings, as in the case of Ivory Coast and Ghana (FAO, 2007).

It is estimated that about 90 to 95% of all cocoa in the world is produced by small holder farmers, with a typical size of a farm with around 3 hectares (ICCO, 2008).

It is expected that grindings of cocoa beans will continue to rise caused by the consumer demand for chocolate with higher cocoa content, as the perceived health-beneficial effects from polyphenol consumption gain higher attention (Arlorioa et al., 2008; Scalbert et al., 2005). At the same time, consumer concerns about social, ethical, environmental, safety and economic sustainability of the cocoa chain will probably shape the pattern of cocoa production (ICCO, 2008).

Biochemical transformations during the fermentation

Although the term 'cocoa bean fermentation' is widely used, it does not truly designate the fermentation of the cocoa beans themselves, but the totality of the activity of microorganisms in the pulp surrounding the beans and the subsequent transformations in the cotyledons (Ferrão, 2002). It can be divided in external and internal fermentations. The external fermentation refers to the microbial activity in the pulp and to the metabolites whereby formed, while the internal fermentation, refers to enzymatic and other chemical reactions inside the cotyledons (Ferrão, 2002).

This section will be focused on Forastero cocoa, the most cultivated population worldwide.

The microbiology of cocoa bean fermentation

Microbial groups

The results from investigations on the microbiology of cocoa bean fermentation have come from several producing regions but mainly from Trinidad, Ivory Coast, Brazil, Ghana, Indonesia, the Dominican Republic and Nigeria. Table 3 summarises thirteen fermentations, investigated in five of these countries, which were selected for the fact that studies on different microbial groups, as well as physical and chemical determinations on the cocoa bean pulp had been performed. In these countries, the taxonomic studies on the microbial isolates revealed that different types of microorganisms are present during cocoa beans fermentation. These microorganisms belong mainly to the group of yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB) and spore-forming bacteria of the genus *Bacillus* and related genera. In addition, in some fermentations, a number of other bacterial species and moulds were reported.

These data illustrate the diversity of fermentation methods, and how even in a single country, different practices can be adopted. In 6 out of the 13 presented fermentations, wooden boxes of variable capacity and depths were used, while the seven described Ghanaian fermentations took place in heaps of different sizes or in trays. Turning of the cocoa beans mass was carried out in 9 out of the 13 fermentations and the fermentation duration varied between 96 and 168 h.

The microbial activity in the cocoa bean pulp has often been described as a well-defined microbial succession led by yeasts that will dominate the total microbial population during the first hours, after which their level is surpassed by those of LAB that on turn decline after 48h of fermentation in detriment of the vigorous development of AAB.

Towards the end of the fermentation *Bacillus* spp. would predominate over all the other microbial groups (Forsyth and Quesnel, 1963; Schwan and Wheals, 2004; Thompson et al., 2007). While for some fermentations in Table 3, a microbial progression by way of a clear succession takes place, for others this succession was somehow dimmed by the dominance of a certain group, particularly LAB. This is a novel aspect that had never been reported in earlier investigations on the microbial ecology of cocoa beans fermentation. In the former situation fall the fermentations of Trinidad (1-2A), Brazil (3A), Indonesia (4-5A), the Dominican Republic

Table 3. Characteristics of cocoa beans fermentation conducted in different countries

	A	B	C	D	E	F	G	H
	Country; Cultivar; Ref.	**Fermentation Method; Sampling**	**Cocoa mass Aeration**	**Yeasts**	**LAB**	**AAB**	***Bacillus* spp.**	**Other microorganisms**
1	**Trinidad;** Mention to pinkish coloured beans- possibly a Forastero cultivar (Ostovar and Keeney, 1973)	**Centeno Estate** 168h; 4 m^3 wooden boxes (1m depth) covered by plantain leave; Sampling at 45 cm from surface	Beans transfer after 72 and 120h	Initial level 4.8 log CFU/g (0h); maximum level of 5.7 log CFU/g (48h), followed by a decrease to 4.9 log CFU/g (96h); not detected in samples after 96h	Initial level 4.9 log CFU/g (0h); maximum level of log 6.1 CFU/g (48h), followed by a decrease to 4.6 log CFU/g (168h)	Detected at levels of 4.8 log CFU/g (48h); maximum level of 5.1 log CFU/g (72h); not detected in samples taken from 96h	Detected at levels of 5.2 log CFU/g (96h); maximum level of 5.2 log CFU/g (96h) and decrease to 5.1 log CFU/g (168h)	*Micrococcus colpogenes* and *Nocardia* were restricted to 0h, together at levels of 4.4 log CFU/g. *Zymomonas mobilis* was detected at an average level of 5.7 log CFU/g between 24-48h and at 4.4 log CFU/g at 72h; not detected after 72h
2	**Trinidad;** Mention to pinkish coloured beans- possibly a Forastero cultivar (Ostovar and Keeney, 1973)	**San Louis Estate** 168h; 18m^3 wooden boxes (2m depth) covered by plantain leaves; Sampling at 45 cm from surface	Beans transfer after 72 and 120h	Initial level 5.5 log CFU/g (0h); maximum level of 6.5 log CFU/g (24h), followed by a decrease to 4.3 log CFU/g (168h)	Initial level of 5.1 log CFU/g (0h); maximum level of 5.8 log CFU/g (72h), followed by a decrease to 4.6 log CFU/g (144h); not detected in the sample taken at 168h	Initial level of 4.8 log CFU/g (0h); maximum level of log 5.8 CFU/g (24h); not detected in samples taken after 24h	Detected at levels of 5.5 log CFU/g (96h); maximum level of 5.86 log CFU/g (120h), followed by a decrease to 5.6 log CFU/g (168h)	*Micrococcus flavus*, *Cellulomonas cellasea* and *Brevibacterium ammoniagenes* were present together at levels of 4.9 log CFU/g (0h); not detected from 24h

Table 3. Characteristics of cocoa beans fermentation conducted in different countries *(continued)*

	A	B	C	D	E	F	G	H
	Country; Cultivar; Ref.	**Fermentation Method; Sampling**	**Cocoa mass Aeration**	**Yeasts**	**LAB**	**AAB**	***Bacillus* spp.**	**Other microorganisms**
3	**Brazil;** Forastero *Comum* hybrids (Schwan, 1998)	168h; wooden boxes (1m depth), covered by jute sacs; 200 kg cocoa beans; Sampling at 45 cm from surface	Beans transfer daily	Initial level 7.0 log CFU/g (0h); maximum level of log 8.3 CFU/g (36h), followed by a decrease to 1.8 log CFU/g (120h) and then an increase to 4.1 log CFU/g (168h)	Initial level 6.0 log CFU/g (0h); maximum level of 6.5 log CFU/g (36h), followed by a decrease to 0.5 log CFU/g (84h) and then an increase to 2.0 log CFU/g (168h)	Initial level 3.8 log CFU/g (0h); maximum level of 7.0 log CFU/g (72h), followed by a decrease to undetectable levels from samples taken at 132h onwards	Spore-formers levels were initially 3.8 log CFU/g (0-96h); maximum level of 7.0 log CFU/g (150-168h)	Not mentioned
4	**Indonesia;** Forastero (Ardhana and Fleet, 2003)	**Estate A** 144h; wooden boxes, 1.125-2 m^3; 0.75-1m depth; Sampling at 37.5-50 cm from surface	Beans transfer after 12-16h and then every 20-24h	Initial level ~ 5.0 log CFU/g (0h); maximum level of log 8.5 CFU/g (36h), followed by a sharp decrease to ~3.5 log CFU/g after 72h until 120h; not detected after 120h	Initial level of 5.5 log CFU/g (0h); maximum level of log 8.0 CFU/g (48h), followed by a decrease to 5.0 log CFU/g (96-108h); not detected after 108h	Initial level of 3.8 log CFU/g (0h); maximum level of 6.0 log CFU/g (24h), followed by a decrease to 3.0 log CFU/g (84h); not detected after 84h	Initial level of 5.0 log CFU/g (0h); maximum level of 8.0 log CFU/g (84h), followed by a decrease to 7.0 log CFU/g (120h) (no data provided after 120h)	Moulds were present at initial level between 2-3.0 log CFU/g (0h); maximum level of 5.6 log CFU/g (12h); not detected after 36h
5	**Indonesia;** Trinitario (Ardhana and Fleet, 2003)	**Estate B** 96h; wooden boxes 1.125-2 m^3 (0.75-1 m depth); sampling at 37.5-50 cm from surface	Beans transfer after 12-16h and then every 20-24h	Initial level of 4.5 log CFU/g (0h); maximum level of log 8.5 CFU/g (36h), followed by a decrease to 3.0 log CFU/g after 72h until 120h; not detected after 120h	Initial level of 4.8 log CFU/g (0h); maximum level of log 8.1 CFU/g (48h), followed by a decrease to ~ 5.0 log CFU/g (72h); not detected after 72h	Initial level of ~ 3.9 log CFU/g (0h); maximum level of 5.8 log CFU/g (12h), followed by a decrease to 3.0 log CFU/g (48h); not detected after 48h	Initial level of 5.30 log CFU/g (0h); maximum level of 7.8 log CFU/g (60h) (no more information provided after 60h)	Moulds were present at initial level between 2.0-3.3 log CFU/g (0h); maximum level of 6.0 log CFU/g (36h); not detected after 36h

Table 3. Characteristics of cocoa beans fermentation conducted in different countries *(continued)*

	A	B	C	D	E	F	G	H
	Country; Cultivar; Ref.	**Fermentation Method; Sampling**	**Cocoa mass Aeration**	**Yeasts**	**LAB**	**AAB**	***Bacillus* spp.**	**Other microorganisms**
6	**The Dominican Republic;** Trinitario and Trinitario hybrid trees (Gálvez et al., 2007)	144h; wooden boxes 0.216 m^3 (0.6m depth); 100 kg cocoa beans); Composite sample from different zones	Beans transfer after 24, 48 and 96h	Initial level of 5.8 log CFU/g (0h); maximum level of 6.2 log CFU/g (24h), followed by a decrease to 5.8 log CFU/g (96h); no data provided after 96h	Initial level of 3.8 log CFU/g (12h); maximum level of 7.2 log CFU/g (48h), followed by a decrease to 6.4 log CFU/g (144h)	Initial level of 5.4 log CFU/g (0h); maximum level of 7.4 log CFU/g (48h), followed by a decrease to 5.9 log CFU/g (144h)	Not mentioned	Not mentioned
7	**Ghana**; Mixed hybrids (Nielsen et al., 2007b)	96h; heap fermentation; ~ 50 kg cocoa beans covered by plantain leaves; Sampling at 15cm from surface	Not performed	Initial level of 7.8 log CFU/g (0h); maximum level of 8.0 log CFU/g (12h), followed by a decrease to 4.3 log CFU/g (96h)	Initial level of 6.3 log CFU/g (0h); maximum level of 9.2 log CFU/g (48h), followed by a decrease to 6.4 log CFU/g (84h); not detected at 96h	Initial level of 6.2 log CFU/g (12h); maximum level of 7.9 log CFU/g (36h), followed by a decrease to 6.6 log CFU/g (84h); not detected at 96h	Detected at levels of 4.1 log CFU/g (48h); maximum level of 5.8 log CFU/g (84-96h)	Not mentioned
8	**Ghana**; Mixed hybrids (Nielsen et al., 2007b)	144h; heap fermentation; 500-750 kg cocoa beans covered by plantain leaves; Sampling at 15 cm from surface	Beans transfer after 48 and 96h	Initial level of 7 log CFU/g (0h); maximum level of 7.3 log CFU/g (96h), followed by a decrease to 5.6 log CFU/g (144h)	Initial level of 6.0 log CFU/g (0h); maximum level of 9.2-9.3 log CFU/g (84-96h), followed by a decrease to 9.0 log CFU/g (144h)	Initial level of 5.6 log CFU/g (0h); maximum level of 7.8 log CFU/g (48h), followed by a decrease to 6.0 log CFU/g (144h)	Detected at levels of 5.5 log CFU/g (48h); maximum level of 9.1 log CFU/g (96h) followed by a decrease to levels of 7.8 log CFU/g (144h)	Not mentioned

Table 3. Characteristics of cocoa beans fermentation conducted in different countries *(continued)*

	A	B	C	D	E	F	G	H
	Country; Cultivar; Ref.	**Fermentation Method; Sampling**	**Cocoa mass Aeration**	**Yeasts**	**LAB**	**AAB**	***Bacillus* spp.**	**Other microorganisms**
9	**Ghana**; Mixed hybrids (Nielsen et al., 2007b)	144h; heap fermentation; 500-750 kg cocoa beans covered by plantain leaves; Sampling at centre of the fermenting mass	Beans transfer after 48 and 96h	Initial level of 7.2 log CFU/g (0h); maximum level of 7.5 log CFU/g (12h), followed by a decrease to 4.3 log CFU/g (144h)	Initial level of 5.5 log CFU/g (0h); maximum level of 9.4 log CFU/g (48h), followed by a decrease to 5.6 log CFU/g (144h)	Initial level of 5.6 log CFU/g (24h); maximum level of 7.1 log CFU/g (60h), followed by a decrease to 3.7 log CFU/g (120h), not detected in samples taken from 132 h	Detected at levels of 7.8 log CFU/g (60h); maximum level of 7.8 log CFU/g (60h); followed by a decrease to 6.8 log CFU/g (144h)	Not mentioned
10	**Ghana**; Mixed hybrids (Nielsen et al., 2007b)	96h; tray fermentation; 0.108 m^3 (0.1m depth); 100 kg; Sampling at centre from the top tray	Not performed	Initial level of 7.5 Log CFU/g (0h); maximum level of 7.6 log CFU/g (12h), followed by a decrease to 4.3 log CFU/g (96h)	Initial level of 7.2 log CFU/g (0h); maximum level of 9.7 log CFU/g (24h), followed by a decrease to 8.6 log CFU/g (96h)	Detected at levels of 7.0 log CFU/g (24h); maximum level of 7.7 log CFU/g (36h); followed by a decrease to 4.0 log CFU/g (84h); not detected at 96h	Not found	Not mentioned
11	**Ghana**; Mixed hybrids (Criollo and Forastero) (Camu et al., 2007)	144h; heap fermentation; 250-1000 kg cocoa beans covered by plantain leaves; Sampling at 30 cm from surface in different points	Not performed	Initial level of 6.3 log CFU/g (0h); maximum level of 7.8 log CFU/g (12h), followed by a decrease to 4.0 log CFU/g (144h)	Initial level of 7.0 log CFU/g (0h); maximum level of ~ 8.8 log CFU/g (42h), followed by a decrease to 6.4 log CFU/g (144h)*	Initial level of 4.0 log CFU/g (0h); maximum level of ~ 7.0 log CFU/g (66h), followed by a decrease to 5.0 log CFU/g (144h)	Not mentioned	

Table 3. Characteristics of cocoa beans fermentation conducted in different countries *(continued)*

	A	B	C	D	E	F	G	H
	Country; Cultivar; Ref.	**Fermentation Method; Sampling**	**Cocoa mass Aeration**	**Yeasts**	**LAB**	**AAB**	***Bacillus* spp.**	**Other microorganisms**
12	**Ghana**; Mixed hybrids (Criollo and Forastero) (Camu et al., 2007)	144h; heap fermentation (heap 12); ~150 kg cocoa beans covered by plantain leaves; Sampling at 30cm from surface in different points	Beans transfer after 48 and 96h	Initial level of ~ 6.0 log CFU/g (0h); maximum level of 7.5 log CFU/g (18h), followed by a decrease to 4.0 log CFU/g (120h); not detected at 144h	Initial level of ~ 6.0 log CFU/g (0h); maximum level of ~ 8.9 log CFU/g (18h), followed by a decrease to 6.5 log CFU/g (144h)	Initial level of 5.5 log CFU/g (0h); maximum level of 7.8 log CFU/g (24h), followed by a decrease to ~6.6 log CFU/g (144h)**	Not mentioned	In 12 and 13A reference is made to *Pantoea* sp.and other spp. that constituted between 0.9-8% and 39-72% of the total population, respectively, depending on the type of medium used
13	**Ghana**; Mixed hybrids (Criollo and Forastero) (Camu et al., 2007)	144h; heap fermentation (heap 13); ~150 kg cocoa beans covered by plantain leaves; sampling at 30cm from surface in different points (placenta removed from beans)	Not performed	Initial level of 6.3 log CFU/g (0h); maximum level of 7.2 log CFU/g (18h), followed by a decrease to 4.0 log CFU/g (120h); not detected at 144h	Initial level of 6.6 log CFU/g (0h); maximum level of 7.3 log CFU/g (18h), followed by a decrease to 6.3 log CFU/g (144h)	Initial level of 6.5 log CFU/g (0h); varying between 6.5 log CFU/g and values below 7.5 log CFU/g until before 96h; maximum level of 7.5 log CFU/g (96h), followed by a decrease to 5.8 log CFU/g (144h)**	Not mentioned	

Abbreviations: LAB- Lactic Acid Bacteria; AAB- Acetic Acid Bacteria.

* The data retrieved from this article for LAB refers to growth in MRS medium (de Man-Rogosa-Sharpe); ** The data retrieved from this article for AAB refers to growth in DMS medium (Deoxycholate-mannitol-sorbitol agar).

Note: This table is the result of data conversion from graphs from the original article, with exception for the work by Nielsen et al. (2007b) of which data was directly collected from the tables. The microbiological levels were rounded to one digit after the decimal point.

(6A) and Ghana (9A). The remaining fermentations epitomize the latter situation, all reported in recent studies in Ghana.

A common pattern of microbial development in the fermentations (Table 3) consisted in the dominant colonization of the cocoa pulp by yeasts at the start of the process. However, in 11 out of 13 of the fermentations, LAB were also present at the onset of the process. In fact, in 7 out of the 11 fermentations LAB number was at the same level or significantly surpassed those of yeasts (considering a difference of 0.5 log CFU/g as significant). This was the case of the fermentations in Trinidad (1-2DE), Indonesia (4-5 DE) and Ghana (11-13DE). Maximum levels of yeasts were reached between 12 and 96 h (for example in Ghana 7D and Ghana 8D), whereas for LAB, peak levels were generally observed at later stages, the earliest maximum at 18 h (Ghana 12-13E) and the latest at 96 h (Ghana 8E). Towards the end of the process, both levels of yeasts and LAB decreased and they did not persist until the end for some fermentations. This was the case, for instance, of Indonesia fermentation 4-5DE.

Another general pattern of these fermentations is the early appearance of AAB. In 9 out of the 13 fermentations, they were detected in samples at the start of the process, in some fermentations at levels of the same order of magnitude as yeasts and LAB (e.g. Ghana 12-13DEF), while in others, they constituted a fraction of the population of 10% or lower (e.g. Ghana 7DEF and Brazil 3DEF). This shows that AAB can also have an important role from the start of the fermentation, in addition to yeasts and LAB. However, in contrast with yeasts and LAB, the activity of AAB was chiefly arrested ahead of the former and this seemed to be independent of the oxygen tension in the cocoa beans mass, since, for example in Trinidad (2F) and Indonesia (5F) fermentations, aeration was practiced but AAB were not detected after 24 and 48 h, respectively. In contrast, a striking exception occurred at Ghana fermentation (13F), where while yeasts were not detected at samples taken at 144 h, AAB were detected until the end of the fermentation at approximately the same levels as LAB.

The occurrence of *Bacillus* spp. during cocoa beans fermentation is less predictable in comparison to the other mentioned microbial groups. In the fermentations in the Dominican Republic (6G) and Ghana (11-13G), no reference is made to this group and it is not clear from the publications whether the authors did not investigate their presence or whether *Bacillus* spp. simply did not occur. Only in Ghana (10G) the authors clearly reported the absence of *Bacillus* spp. during tray fermentation, while in Ghana (11G) the authors stated that in addition to yeasts, LAB and AAB no other major group participated in the process. The fermentations conducted in Trinidad (1-2G) and Ghana (7-9G) were characterised by a late appearance of *Bacillus* spp., typically between 60 and 96h. On the contrary, in the Indonesia fermentations (4-5G), *Bacillus* spp. were detected at the start of the process at approximately the same level as yeasts and LAB, becoming the dominant group throughout the fermentation. In Ghana fermentations (7-8G), the pattern of occurrence of *Bacillus* spp. was intermediate between that of Indonesia (4-5G) and Trinidad (1-2G) fermentations, since this group was detected in samples taken from 48 h. In Brazil fermentation (3G), spore-formers rather than total viable counts were determined. Interestingly, the fact that spore counts remained constant until 72

h correlates well with the results of fermentations of Trinidad (1-2G), Indonesia (5-6G) and Ghana (7-9G), which might indicate unfavourable conditions for the development of this group of microorganisms during the first days. With regard to the fate of *Bacillus* spp. towards the end of the fermentation, this constitutes a microbial group that persists until the end, as shown in fermentation in Trinidad (1-2G), Brazil (3G) and Ghana (7-9G).

A range of microbial groups other than yeasts, LAB, AAB and *Bacillus* spp. have been isolated and identified in cocoa bean fermentations. In Trinidad fermentation (1-2H), a diversity of Gram-positive bacteria like *Micrococcus* spp., *Nocardia*, *Cellulomonas cellasea*, *Brevibacterium ammoniagenes* and the Gram-negative bacterium *Zymomonas mobilis* were reported. In both Trinidad Estates, Gram-positive bacteria were isolated just at the start of the fermentation, at levels that were of the same order of magnitude as of yeasts, LAB and AAB (Table 3: 1-2H). In Centeno Estate (1H), the bacterium *Zymomonas mobilis* was detected at 24 h, constituting 50% of the total microbiota, after which its level decreased and was not detected after 72 h. In San Louis Estate reference was also made to *Zymomonas mobilis*, but deeper in the cocoa beans mass (at 90 cm), where it was present in samples taken at 24 h and 48 h, constituting, respectively, 18 and 24% of the total population (Ostovar and Keeney, 1973). In Indonesia fermentation (4-5H), moulds were observed at initial levels between 2.0-3.2 log CFU/g and their level increased to 5.6-6.0 log CFU/g at 12 h and 36 h, respectively at Estates A and B. Nevertheless, in both Estates, this group was not detected after 36 h. In addition, in both Estates *Micrococcus kristinae*, several *Staphylococcus* spp. (Staph. *capitis*, Staph. *aureus* and Staph. *hominii*) together with *Pseudomonas cepacea* (Estate B) were detected at levels between 5-6 log CFU/g, but their growth was restricted to the first 24 h-36 h (Ardhana and Fleet, 2003).

The divergent microbial dynamics observed during the reported cocoa bean fermentations (Table 3) indicate that more research is required on the properties and ecophysiology of the microbial species.

Microbial properties and functional roles

At the onset of the fermentation, the pulp is characterised by ambient temperatures, low oxygen availability, due to the tightly packed structure of the cocoa bean mass, low pH and high sugar levels (Table 4).

With respect to pH, levels as low as 3.3-3.5 were reported in the Trinidad (1-2J), Brazil (3J) and Ghana fermentations (11-12J). Higher values for the pulp pH were observed in Indonesia (5J) and in Ghana (8 and 10J), where the pH varied between 4.1 and 4.8. In most cases, sucrose levels were very low indicating the complete conversion of sucrose into glucose and fructose (Table 4: 6-13K).

Yeasts and LAB are the microbial groups that are physiologically better adapted to thrive under the conditions of cocoa bean fermentations, since both groups display a remarkable tolerance to low pH values (Axelsson, 2004; Deak, 2006). Additionally, yeasts can withstand high sugar concentrations (Barnett et al., 2000).

Table 4. Pulp conditions during cocoa bean fermentation

	A	I	J	K	L
	Country/Ref.	**T (°C)**	**pH**	**Sugars (mg g^{-1})**	**Pulp metabolites (mg g^{-1})**
1	Trinidad- Centeno Estate (Ostovar and Keeney, 1973)	T_{0h}:28.5, T_{48h}:42.5, T_{72h}: 56=T_{Max}, T_f:45	pH_{0h}: 3.3, pH_{48h}: 4.05, pH_{72h}: 4.15 pH_{Max}: 4.6 (156h), pH_f: 4.5		
2	Trinidad- San Louis Estate (Ostovar and Keeney, 1973)	T_{0h}:36, T_{48h}:39, T_{72h}: 49= T_{Max}, T_f: 42	pH_{0h}: 3.5, pH_{48h}: 4.0, pH_{72h}: 4.5 pH_{Max}:~ 4.53 (156h), pH_f: 4.4		
3	Brazil (Schwan, 1998)	T_{0h}:26, T_{48h}:36.5, T_{72h}:46, T_{Max}:52.5 (96h), T_f: 45	pH_{0h}: 3.5, pH_{48h}: 4.0, pH_{72h}: 4.25 pH_{Max} = pH_f: 5.9		E_i: 0; E_{Max}: 7.4 (24h); E_f: 0 LA_i: 0; LA_{Max}: 1.9 (48h); LA_f: 0.7 AA_i: 0; AA_{Max}: 5.5 (72h); AA_f: 0.75
4	Indonesia- Estate A (Ardhana and Fleet, 2003)	Indonesia 4-5A T_{0h}:20-25ºC, T_f:48-50ºC	pH_{0h}: 3.7; pH_{120h}: 3.9	F_{0h}: 62; F_{120h}: 11 G_{0h}: 41; G_{120h}: 7 S_{0h}: 32; S_{120h}: 0	CA_i: 24; CA_{120h}: 11 E_i: 0.5; E_{24h}: 65; E_{120h}: 0.1 LA_i: 0.3; LA_{120h}: 6.0 AA_i: 0.4; AA_{120h}: 12
5	Indonesia- Estate B (Ardhana and Fleet, 2003)		pH_{0h}: 4.8; pH_{72h}: 4.9	F_{0h}: 42; F_{72h}: 9 G_{0h}: 24; G_{72h}: 5 S_{0h}: 21; S_{72h}: 0	CA_i: 21; CA_{72h}: 9 E_i: 0.23; E_{24h}: 52; E_{72h}: 1.6 LA_i: 0.3; LA_{72h}: 5.0 AA_i: 0.4; AA_{72h}: 10
6	The Dominican Republic (Gálvez et al., 2007)	T_{0h}:24, T_{48h}:44, T_{72h}:49, T_{Max}:50 (78h), T_f: 47	pH_{0h}: 4, pH_{48h}: 4.4, pH_{72h}: 4.3; pH_{Max}= pH_f: ~ 4.5 (96h)	F_{0h}: 21; F_f: 3.75 G_{0h}: 50; G_f: 0.6	CA_i: 6; CA_{72h}: 0.75 E_i: 0; E_{Max}: 2.2 (24h); E_{72h}: 0 LA_i: 0; LA_{Max}: 0.76 (48h); LA_f: 0 AA_i: 0; AA_{Max}: 5.18 (72h); AA_f: 0.75

Table 4. Pulp conditions during cocoa bean fermentation *(continued)*

	A	I	J	K	L
	Country/Ref.	**T (°C)**	**pH**	**Sugars (mg g^{-1})**	**Pulp metabolites (mg g^{-1})**
7	Ghana- Small heap (Nielsen et al., 2007b)	T_{0h}:28.5, T_{48h}:46, T_{Max}:47 (60h), T_{72h}: 45, T_f: 44.5	pH_{0h}: 3.94, pH_{48h}: 4.06, pH_{72h}: 4.12 pH_{Max}: 4.29 (84h), pH_{48h}: 4.06	Ghana 7-10A G_{0h}: 54-66 F_{0h}: 63-74 S_{0h}: 3 80% of sugars consumed in 24h	Ghana 7-10A CA_i: 6-7; CA~0 (12h) E_{Max}: 20 (after 24-36h) ; E_f: 0 LA_i, <2; LA_{Max}: 10 (after 24-48h) AA_i<2; AA_{Max}: 20 (after 60-72h); AA_f: 7-10 Lower production of AA in the centre of the large heap
8	Ghana- Big heap (Nielsen et al., 2007b)	T_{0h}:28, T_{48h}:43, T_{72h}=T_{Max}:48 (60h), T_f:44	pH_{0h}: 4.10, pH_{48h}: 3.98, pH_{72h}: 4.21 pH_{Max} = 4.58 (132h), pH_f: 4.55		
9	Ghana- Big heap (Nielsen et al., 2007b)	T_{0h}:28, T_{48h}:33.5, T_{72h}:44, T_{Max}:46 (84h), T_f:44	pH_{0h}: 3.95, pH_{Max}: 4.69, pH_{48h}: 4.21, pH_{72h}: 4.12 pH_f: 4.41		
10	Ghana- Tray (Nielsen et al., 2007b)	T_{0h}: 28.5, T_{48h}: 45, T_{Max}: 46 (60, 72, 84h), T_f: 45.5	pH_{0h}: 4.12, pH_{48h}: 3.98, pH_{72h}: 4.20 pH_{Max} = 4.29 (84h), pH_f: 4.26		
11	Ghana (Camu et al., 2007)	T_{0h}: 27.5, T_{48h}:40, T_{72h}=T_{Max}=T_f: 45	pH_{0h}: 3.4, pH_{48h}: 3.7, pH_{72h}: ~ 3.8, pH_{Max}= pH_f: 4.3	F_{0h}: 57; F_f: 5 G_{0h}: 54; G_f: 5	CA_i: 9.2; C_f: 2.5 (144h) E_i: 0; E_{Max}: 22.5 (54h); E_f: 7 LA_i: 0; LA_{Max}: 8.6 (60h); LA_f: 8.2 AA_i: 0; AA_{Max}=AA_f: 6 (60h) M_i: 0; $M_{Max=}$ M_f: 15 (48h)
12	Ghana- Heap 12 (Camu et al., 2007)	T_{0h}: 26.3, T_{Max}= 45.9*	pH_{0h}: 3.5; pH_f: 4.4	F_{0h}: 44.5; F_f: 8 G_{0h}: 42; G_f: 2.5	E_i: 0; E_{Max}: 18 (36h); E_f: 0 AA_i: 0; A_{Max}: 12 (120h); A_f: 9.5
13	Ghana- Heap 13 (Camu et al., 2007)	T_{0h}: 27.0, T_{Max}= 42.8*	pH_{0h}: 3.7; pH_f: 4.2	F_{0h}: 52; F_f: 17 G_{0h}: 50; G_f: 10.5	E_i: 0; E_{Max}: 20 (30h); E_f: 2.5 AA_i: 0; AA_{Max}: 11.5 (54h); AA_f: 10

Abbreviations: F- Fructose, G- Glucose, S- Sucrose, CA- Citric acid, E-Ethanol, LA- Lactic acid, AA- Acetic acid, M- Mannitol.
Índices: i- Initial, MAX- Maximum, f- Final; * The time was not indicated.
Note: This table is the result of data conversion from graphs in the original articles, exception for the work by Ardhana and Fleet (2003) and Nielsen et al. (2007b) of which data was directly collected from tables or the text.

The metabolic activity of yeasts in the cocoa bean pulp leads to the production of ethanol, carbon dioxide, acids and volatile compounds, with concomitant increase of the temperature (Table 4 and Table 5). With respect to ethanol, different maximum concentrations have been detected during cocoa bean fermentation. In the case of Ghana fermentations (Table 4: 7-10L), concentrations of around 20 $mg.g^{-1}$ were detected after 24-36 h, with the same level of concentrations being detected in Ghana (11L) after 54 h. On the contrary, in Brazil (3L) and the Dominican Republic (6L), the peaks corresponded to concentration of 7.4 and 2.2 $mg.g^{-1}$, respectively, both registered at 24 h.

Some yeasts are able to metabolize citric acid (CA) (Jespersen, 2003), which contributes to an increase in the pH of the pulp, as reported for instance in fermentations in Trinidad (Table 4: 1-2J) and Brazil (3J). However, this appears to be a variable property among yeasts. Jespersen (2003) reported that while *Pichia kluyveri* had the ability to assimilate CA, *Pichia fermentans* strains exhibited a weak or variable assimilation. Moreover, yeasts have the ability to produce pectinolytic enzymes, including polygalacturonase or pectin methylesterease, but no pectin lyase (Gauthier et al., 1977). Their secretion reduces the pulp viscosity, increasing the oxygen availability and provides a source of supplementary carbohydrates (Gálvez et al., 2007; Schwan et al., 1995; Schwan et al., 1997). The ability to produce these enzymes has been shown to be not only extremely variable among yeasts (Ardhana and Fleet, 2003; Schwan et al., 1997), but also not exclusive of this group (Table 5).

Products of the metabolism of LAB include LA, E, AA and other organic acids as well as glycerol, mannitol, carbon dioxide and volatiles (Table 5). Lactic acid can be produced by a homofermentative metabolism, with a yield higher than 85%, or heterofementative metabolism, with a yield of only 50% (Axelsson, 2004). The production of acids causes a drop in pH of the pulp, which can be detected around 24 h to 48 h (Nielsen et al., 2007a; Ostovar and Keeney, 1973). However, the fact that yeasts can assimilate LA, in conjunction with the fermentative ability of LAB to use CA, partly explains why the overall effect of acids production may not produce such a pronounced drop in pH during the first two days of fermentation (Camu et al., 2007; Ostovar and Keeney, 1973). Another reason for this, is the fact that LA and AA have lower acid-dissociation constants than CA. As observed for ethanol, LA levels varied considerably between fermentation (Table 4). In the Dominican Republic fermentation (Table 4: 6L), a relatively low maximum value of LA (0.76 $mg.g^{-1}$) was reported, when compared to the concentrations between 8.6 and 10 $mg.g^{-1}$ that were detected in Ghana fermentations (7-13L). Final levels of LA varied between concentrations close to zero, in Brazil (3L) and the Dominican Republic (6L), while in Indonesia (4-5L) and Ghana (11L), concentration between 5.0-8.2 $mg.g^{-1}$ were present. This contradicts previous works that mention that mainly AA is present in excess at the end of the fermentation (Schwan, 1998).

The activity of yeasts and LAB reduce the sugars to residual levels, with usually a slightly higher level of fructose being measured than glucose, probably due to the preferential use of glucose by microorganisms (Gancedo, 1998) (Table 4: K).

Table 5. Functional role of microbial groups during cocoa beans fermentation as reported

		Yeasts	LAB	AAB	*Bacillus* spp.	Moulds
Oxygen requirements		**Facultative anaerobic or aerobic**	**Aerotolerant anaerobic**	**Obligate aerobic**	**Aerobic or facultative anaerobic**	**Aerobic**
Assimilation	Ethanol	+		+		+
	Mannitol	-		+		+
	Acetate			+		
	Citrate	+				
	Lactate	+		+		
Fermentation	Ethanol	+				
	Mannitol	-	+			
	Citrate		+			
Production of acids	Acetate	+	+	+	+	*
	Citrate		+			
	Lactate		+	+	+	
	Oxalate	+				
	Phosphorate	+				
	Succinate	+		+		
	Malate	+				
Production of volatiles	Alcohols					
	- Ethanol	+	+			
	- Methanol	+				
	- Mannitol		+			
	- Butanediol	+			+(?)	
	Aldehydes	+				

Table 5. Functional role of microbial groups during cocoa beans fermentation as reported *(continued)*

		Yeasts	LAB	AAB	*Bacillus* spp.	Moulds
Oxygen requirements		**Facultative anaerobic or aerobic**	**Aerotolerant anaerobic**	**Obligate aerobic**	**Aerobic or facultative anaerobic**	**Aerobic**
Production of volatiles	Ketones	+	+			
	Fatty acids				+	
	Esters	+				
	Pyrazines				+	
Excretion of enzymes	Cellulases	+				+
	Pectinases	+			+	+
	Proteases				+	+
	Amylases				+	+
	Lipases				+	+**
	References	(Deak, 2006; Gálvez et al., 2007; Gauthier et al., 1977; Jespersen et al., 2005; Schwan et al., 1997; Schwan and Wheals, 2004)	(Deak, 2006; Gálvez et al., 2007; Gauthier et al., 1977; Jespersen et al., 2005; Schwan et al., 1997; Schwan and Wheals, 2004)	(Axelsson, 2004; Camu et al., 2007; Nielsen et al., 2007a; Passos et al., 1984)	(Bartowsky and Henschke, 2008; Camu et al., 2007; Cleenwerck and De Vos, 2008; Schwan and Wheals, 2004)	(Ardhana and Fleet, 2003; Lopez and Quesnel, 1971; Ouattara et al., 2008; Schwan and Wheals, 2004; Zak and Keeney, 1976)

* A discrimination of acids was not made (Ribeiro et al., 1986). ** Weak reaction (Ardhana and Fleet, 2003); (?) Role not totally understood yet.

The increased access of air in the cocoa bean mass, as result of the enzymatic collapse of the pulp, stimulates the development of AAB. Due to the low levels of glucose and fructose, caused by their fast metabolisation by yeasts and LAB (Nielsen et al., 2007b), the metabolism of AAB is shifted towards the utilization of ethanol as a main carbon source. AAB can oxidize ethanol to AA and species from the genus *Acetobacter* have the additional capacity to oxidize AA to carbon dioxide and water (a property also characteristic of *Gluconoacetobacter* species) (Bartowsky and Henschke, 2008) (Table 5). They are able to perform this activity both at neutral and pH levels until 4.5. This can explain the negligible levels of ethanol in the samples at the end of the fermentations (Table 4: L). The levels of AA may be higher in the end of the fermentation when turning of the cocoa beans is not performed, by direct impact on the activity of AAB or due to a reduced evaporation (Table 4: 11-13L). During the fermentations the maximum concentrations of AA were higher than those of LA, although towards the end they tended to be present at comparable levels (Table 4). Maximum concentrations of AA varied between 5.5 $mg.g^{-1}$ in Brazil (3L) to 20 $mg.g^{-1}$ in Ghana fermentations (7-10L). The acetic acid fermentation is an extremely exothermic process enhancing the rise in temperature initiated by the activity of yeasts. Temperature levels as high as 56ºC were reported in Trinidad (Table 4: 1I) at 72 h and the lower temperature was around 43ºC in Ghana fermentation (13I).

The genus *Bacillus* comprises a group of ubiquitous microorganisms in the environment, aerobic and facultative anaerobic that display an enormous metabolic diversity as manifested by their ability to use a big diversity of carbon sources and to proliferate under very extreme conditions (Nazina et al., 2001). Towards the end of the fermentation, the major sources of carbon are organic acids such as AA and LA, and mannitol. Many *Bacillus* spp. are able to use those compounds as a source of energy, which may explain their growth during the later stages (Sneath, 1986). The role of *Bacillus* spp. during cocoa bean fermentation is still not very well understood. The studies by Zak and Keeney (1972) suggested the involvement of *Bacillus subtilis* in the production of tetramethylpyrazines, while other studies associated the presence of *Bacillus* spp. with the occurrence of off-flavours that are regularly encountered towards the end of the fermentation, such as C3-C5 free fatty acids and 2,3-butanediol (Lopez and Quesnel, 1971; Lopez and Quesnel, 1973; Schwan et al., 1986). More recently, Ouattara et al. (2008) demonstrated the ability of *Bacillus* spp. to produce polygalacturonase and pectin lyase, over a temperature range of 30-50°C and pH levels of 3 to 6 (Table 5). The observation that polygacturonase production correlated positively with the temperature may indicate an important functional role of this microbial group during the fermentation. *Bacillus* spp. constitute the group that persists in commercial cocoa beans and some cocoa derived products, such as cocoa powder, due to their ability to differentiate into spores, some of them of very high heat-resistance (Barrile et al., 1971; Mossel et al., 1974; Ostovar and Keeney, 1973).

Filamentous fungi are reported as not playing an important role during cocoa beans fermentation. When present, they are often noted at the surface of the cocoa beans mass, where the oxygen tension is high and temperature and the concentration of metabolites

like AA is low. Mixing or turning the cocoa mass impairs fungal development (Rohan, 1964). However, in Indonesia fermentations (Table 3: 4-5H) moulds constituted an important part of the microbiota at the start until 36 h, were strong polygalacturase producers and all the isolates were able to produce amylolytic and proteolytic enzymes. Considering that yeasts isolated during those fermentations did not show pectinolytic activity, it is conceivable a possible beneficial effect of moulds during the fermentation. Furthermore, the study of moulds occurring during Brazil fermentation by Ribeiro et al. (1986), showed the ability of the isolates to assimilate both ethanol and mannitol, which might be crucial in the reduction of ethanol induced-stress.

Ethanol, LA and AA, together with oxygen and temperature are key factors shaping the dynamics of the microbial development during cocoa bean fermentation. For instance, the heterogeneity of oxygen distribution throughout the cocoa bean mass has repercussions not only on the dominance of microbial groups, but also on the speed at which a particular group grows, therefore, impacting metabolic processes such as the increase in the temperature. Consequently, it can be expected that the cocoa bean mass is characterised by an inhomogeneous distribution of both microorganisms and their metabolites, an aspect which becomes more important with bigger volumes of fermenting mass.

In a fermentation study conducted in Ghana, Jespersen et al. (2005) observed a slower increase in the yeast population in the outer part that lagged about 48 h in comparison to the development in the inner part. This spatially different development had implications for variables like the pH and the temperature. For example, while in the inner part a pH drop from 4.2 to 3.4 occurred within 24 h, in the outer part this level was still 4.1. Another situation of microbial heterogeneity during cocoa bean fermentation can be found in the study conducted in Trinidad by Ostovar and Keeney (1973), where a considerable variability in the microbial levels and species at 5, 45 and 90 cm of depth was observed. In both Trinidad Estates a downward trend in the level of Total Aerobic Microorganisms (TAM) along the fermentation box occurred, with higher levels registered at the top surface. Towards the end of the fermentation, the total microbial levels did eventually reach approximately the same number. More specifically, with respect to the microbial groups, it is interesting to observe that, for instance, in Centeno Estate at 5 cm depth, AAB comprised 2 and 10% of the population, respectively at 24 and 48 h but were not detected at 45 or 90 cm. This indicates that the extent of the modifications of the cocoa bean mass due to the production of AA and increase in temperature were localized at the top layer. This emphasises the importance of turning and mixing practices during the fermentation.

Cocoa bean fermentation constitutes an (man-made) ecological niche, with a remarkable diversity of microbial species. More than 100 species, displaying different metabolic properties have been reported. Recently, new species of yeasts, LAB and AAB have been detected, due to improved culturing strategies together with the use of molecular biology tools (e.g. Internally Transcribed Spacer-Polymerase chain reaction- ITS-PCR- and DNA-DNA hybridizations) (Cleenwerck et al., 2007; Cleenwerck et al., 2008; Nielsen et al., 2007a; Nielsen et al., 2007b).

Species that appear to be indigenous to cocoa bean fermentation throughout the world are *Hanseniaspora guilliermondii, Issatchenkia orientalis* and *Saccharomyces cerevisiae* in the yeast group; *Lactobacillus fermentum* and *L. plantarum* in the LAB group; *Acetobacter aceti* and *A. pasteurianus* belonging to the AAB group; and *Bacillus subtilis*, *B. licheniformis*, *B. pumilus* and *B. cereus* in the *Bacillaceae* family (Tables 6-9). Many of these species are also found during the fermentation of other food products.

During cocoa bean fermentation, shifts in species dominance take place. These are a result of the fermentation practices, combined with the idiosyncratic properties of the microbial strains. Regarding the yeast population, a wider diversity of fermentative yeasts than respiratory yeasts has been found to occur (Table 6). However, studies referring to the dominance of respiratory yeasts are not scarce. In Indonesia, *Candida inconspicua* was the most prominent yeast throughout the fermentation, having been detected in samples taken in all time-points, while fermentative yeasts like *H. guillermondii* or *P. fermentans* were restricted to the first 72 h (Ardhana and Fleet, 2003). Likewise, in the investigations carried out in Ghana (Nielsen et al., 2007b), *P. membranifaciens*, another respiratory yeast, had the most notable occurrence during all fermentation systems, a role that was shared with *S. cerevisiae* towards the end, in the small heap fermentation, and *C. ethanolica*, in the big heap sampled in the centre (Table 3). In these fermentations, yeasts like *H. guilliermondii*, *C. diversa*, *C. zemplinina* and *C. silvae* were the predominant species but confined to the first 24-48 h. In general, it has been observed that characteristics like the ability to assimilate LA, to grow at 40°C or in the range 40-50°C, and to tolerate high ethanol concentrations are related to the ability of yeasts to persist until the later stages of cocoa bean fermentations. Moreover, the fact that the majority of AAB occurring during cocoa bean fermentation belong to the *Acetobacter* genus may constitute an important factor supporting the prevalence of yeasts until later stages of the fermentation, since an accumulation of ethanol to high toxic levels is precluded. Ardhana and Fleet (2003) reported the capacity of the *Candida* spp. and *Kloeckera* spp. to grow well in the presence of 10% and 5% ethanol, respectively, and Gálvez et al. (2007) described the ability of *C. inconspicua* to assimilate LA, while other yeasts that were abundant at the start did not have this capacity.

Acetic acid is the type of acid that is usually present at higher concentrations at the end of the fermentation and known to exert higher inhibitory effect on yeast growth than LA, propionic acid or CA (Deak, 2006). This might partly explain the reduction of yeast levels toward the end of the fermentation, e.g. decrease in 4 log CFU/g in Ghana fermentations (Table 3: 12-13B) after 120 h, although appreciable levels of sugars were still present (Table 4: 12-13K).

Heterofermentative LAB constitute the major group occurring during cocoa bean fermentations. In Indonesian fermentations (Table 3: 4-5E) *Lactobacillus fermentum* was the most predominant species between 36-48 h (60-80%) together with *L. plantarum*. A very similar pattern of occurrence was found in Ghanaian fermentations where, *L. fermentum* was the most important species throughout the fermentation, accompanied by *L. plantarum* either

Table 6. Yeast species reported in cocoa beans fermentation

	1	2	3	4	5	6	7	8	9	10	11	12	13
Fermentative yeasts													
Candida bombi	N.P.	N.P.	+								N.P.	N.P.	N.P.
Candida diversa							+	+	+	+			
Candida glabrata (T)/ Cryptococcus glabratus (B)													
Candida michaelii								+					
Candida pelliculosa			+	+	+	+							
Candida quercitrusa (A)/ Candida parapsilosis var. querci (T)							+						
Candida rugopelliculosa			+										
Candida tropicalis				+	+								
Candida zemplinina							+	+	+	+			
Hanseniaspora valbyensis						+							
Issatchenkia occidentalis									+				
Issatchenkia orientalis/Candida krusei(A)						+	+	+	+	+			
Kloeckera Africana					+								
Kloeckera apis (A)/ Hanseniaspora guilliermondii			+	+	+	+	+	+	+	+			
Kloeckera javanica					+								
Kluyveromyces marxianus			+										
Kluyveromyces thermotolerans			+										
Lodderomyces elongisporus			+										
Pichia kluyveri									+	+			
Pichia pijperi							+			+			
Saccharomyces cerevisiae var. chevalieri			+										
Saccharomyces cerevisiae			+	+	+		+	+	+	+			
Schizosaccharomyces pombe								+	+	+			
Torulaspora delbreuckii (T)/ Torula colliculosa (A)									+				
Torulaspora pretoriensis			+										

Table 6. Yeast species reported in cocoa beans fermentation *(continued)*

	1	2	3	4	5	6	7	8	9	10	11	12	13
Weak fermentative yeasts													
Candida cylindracea	N.P.	N.P.					+			+	N.P.	N.P.	N.P.
Candida humicola				+	+								
Candida silvae										+			
Candida sorboxylosa							+			+			
Issatchenkia hanoiensis								+		+			
Pichia fermentans			+			+							
Saccharomycopsis crataegensis									+				
Respiratory yeasts													
Candida ethanolica								+	+				
Candida inconspicua (T)/						+							
Candida rugosa			+										
Candida zeylanoides						+							
Pichia membranifaciens (A)/ Candida valida (T)							+	+	+	+			
Rhodotorula glutinis(T)/ Cryptococcus glutinis (A)					+								
Rhodotorula rubra				+									
Saccharomycopsis crataegensis								+					
Yarrowia lipolytica						+							

Numbers correspond to the fermentations sites in table 3 with the respective references: **1-** Trinidad (Centeno Estate), **2**-Trinidad (San Louis Estate), **3**- Brazil (Schwan et al., 1995; Schwan, 1998)., **4**- Indonésia (Estate A), **5**- Indonesia (Estate B), **6**- The Dominican Republic, **7**- Ghana (Heap 7B), **8**- Ghana (Heap 8B), **9**- Ghana (Heap 9B); **10**- Ghana (Tray 10B), **11**- Ghana (Heap 11B), **12**- Ghana (Heap 12B), **13**- Ghana (Heap 13B).
N.P.- Not performed; T- Teleomorph (the sexual reproductive stage of a fungi); A-Anamorph (an assexual reproductive stage of a fungi, often mould-like; B-Basionym (CBS, 2003a).

during the first 48 hours of fermentation, or between 132 h to 144 h (Camu et al., 2007; Camu et al., 2008b; Nielsen et al., 2007b). In the Dominican Republic fermentation, *L. fermentum* was not reported (Table 7). *L. plantarum* or *L. paraplantarum*, in conjunction with *L. paracasei* and *L. pentosus* had rather an important role. With respect to the properties of the LAB species, Camu et al. (Camu et al., 2007) emphasised the aciduric and ethanol-tolerant character of *L. fermentum* as factors explaining the persistence of this LAB until the end of the fermentation, in comparison to *Leuconostoc pseudomesenteroides* and some *Enterococcus casseliflavus* strains that played a small role. In the homofermentative group *Pediococcus acidilactici* was reported to occur in all fermentation systems studied by Nielsen et al. (2007b), while none was found in the investigation by Camu and co-workers (Camu et al., 2007; Camu et al., 2008b). However,

the proliferation of this species was always very restricted and constituted a minority of the total LAB population. On the contrary, in Trinidad fermentation (Table 3: 1E) the detection of LAB until the end of the fermentation was due to the presence of *Streptococcus thermophilus* (a thermotolerant species) that constituted 32% of TAM at 120 h, 27% at 144 h and 32.5% at 168 h, being the only species of LAB from 144 h.

Table 7. Lactic acid bacteria species isolated in cocoa bean fermentation

	1	2	3	4	5	6	7	8	9	10	11	12	13
Homofermentative													
Lactobacillus acidophilus	+												
Lactococcus lactis (B. Streptococcus lactis)	+	+	+				+						
Pediococcus acidilactici		+					+	+	+	+			
Pediococcus damnosus (B. Pediococcus cerevisiae)	+												
Pediococcus dextrinicus (B. Lactococcus fermenti)	+												
Streptococcus thermophilus	+	+											
Heterofermentative													
Lactobacillus bulgaricus	+	+											
Lactobacillus casei		+											
Lactobacillus fermentum (B. L. cellobiosus; L. fermenti)	+	+		+	+		+	+	+	+	+	+	+
*Lactobacillus ghanaensis**								+	+				
Lactobacillus mali											+		
Lactobacillus paracasei						+							
Lactobacillus paraplantarum						+							
Lactobacillus pentosus						+							
Lactobacillus plantarum	+		+	+	+	+	+	+	+	+	+		+
Lactobacillus pseudoficulneum							+						
Lactobacillus rossii									+				
Obligate heterofermentative													
Lactobacillus brevis						+				+	+		+
Lactobacillus hilgardii				+				+	+				
Leuconostoc durionis													
Leuconostoc mesenteroides		+								+			
Leuconostoc pseudoficulneum								+	+	+			

Table 7. Lactic acid bacteria species isolated in cocoa bean fermentation *(continued)*

	1	2	3	4	5	6	7	8	9	10	11	12	13
Leuconostoc pseudomesenteroides							+		+	+	+		
Weissella cibaria											+		
Weissella kimchii											+		
Weissella paramesenteroides												+	

Numbers correspond to the fermentations sites in table 3 with the respective references. **1-** Trinidad (Centeno Estate), **2**-Trinidad (San Louis Estate), 3- Brazil (Schwan et al., 1995; Schwan, 1998), **4**- Indonesia (Estate A), **5**- Indonesia (Estate B), **6**- The Dominican Republic, **7**- Ghana (Heap 7B), **8**- Ghana (Heap 8B), **9**- Ghana (Heap 9B); **10**- Ghana (Tray 10B), **11**- Ghana (Heap 11B), **12**- Ghana (Heap 12B), **13**- Ghana (Heap 13B).
* New species discovered in cocoa beans fermentation (Nielsen et al., 2007a).
B.- Basionym (CBS, 2003b).

With respect to AAB, only the *Acetobacter* and *Gluconobacter* genera were reported in cocoa beans fermentation from the 10 existing genera of AAB (Cleenwerck and De Vos, 2008), with the *Acetobacter* genus being the most represented (Table 8). *G. oxydans* and *G. oxydans-like* spp. were the only *Gluconobacter* spp. reported. Indeed, in all fermentations conducted in Ghana, with exception of the big heap (8B), and in the fermentations in Trinidad (San Louis Estate) and Brazil, no *Gluconobacter* species were found. It is interesting to point out that while many studies show a sharp decrease of the AAB population after the first 24 h (Ardhana and Fleet, 2003; Ostovar and Keeney, 1973), others report their persistence throughout fermentations even when turning was not performed (Camu et al., 2007; Camu et al., 2008b; Nielsen et al., 2007b). AAB strains of the species *A. pasteurianus, A. ghanaensis, A. senegalensis* and *A. lovaniensis-like* were detected until the later stages of Ghana fermentation (Table 3: 11 and 13F), which reveals that AAB strains can have a remarkable ability to cope with a low oxyxen tension environment, despite an obligatory aerobic metabolism. *A. aceti* and *A. pasteurianus* strains with this property were also described in the anaerobic environment of wine sediments by Joyeux et al. (1984).

Cleenwerck et al. (2008) have discussed the difficulties associated with the isolation and culturing of AAB, where the use of a single growth medium proved to induce selective isolation of AAB. Therefore, we believe that future studies on AAB populations occurring during cocoa bean fermentation will benefit from the use of different isolation media, in addition to improved molecular strategies (Camu et al., 2007; Camu et al., 2008b).

Finally, with respect to *Bacillus* spp. it is interesting to highlight the isolates found during Indonesia fermentations that persisted throughout the process and were the dominant microbiota after 48-72 h. These included *Bacilus pumilus*, *B. licheniformis, B. subtilis* and *B. cereus*. Their simultaneous growth with that of yeasts, LAB and AAB, as observed in Indonesia (Table 3: 4-5G) and Ghana (table 3: 7-9G) fermentations, may indicate that these strains had an exceptional ability to deal with ethanolic and acidic stresses. Concerning the relation with the

temperature, thermotolerant species occur more frequently than thermophilic species, with *Geobacillus stearothermophilus* being the only thermophilic strain reported so far (Table 9).

Table 8. Acetic acid bacteria species reported in cocoa beans fermentation

	1	2	3	4	5	6	7	8	9	10	11	12	13
Acetobacter													
Acetobacter aceti	+	+	+	+	+								
*Acetobacter ghanaensis**											+	+	+
Acetobacter lovaniensis						+							
*Acetobacter lovaniensis-like***													+
Acetobacter malorum								+	+	+			
Acetobacter pasteurianus				+	+		+	+		+	+	+	+
Acetobacter roseusa	+												
*Acetobacter senegalensis****												+	+
Acetobacter syzygii							+	+	+	+			
Acetobacter tropicalis							+	+	+	+			
Gluconobacter													
Gluconobacter oxydans (B. Acetobacter oxydans)	+		+					+					
Gluconobacter oxydans-like			+										

Numbers correspond to the fermentations sites in table 3 with the respective. **1-** Trinidad (Centeno Estate), **2**-Trinidad (San Louis Estate), **3**- Brazil (Schwan et al., 1995; Schwan, 1998), **4**- Indonesia (Estate A), **5**- Indonesia (Estate B), **6**- The Dominican Republic, **7**- Ghana (Heap 7B), **8**- Ghana (Heap 8B), **9**- Ghana (Heap 9B); **10**- Ghana (Tray 10B), **11**- Ghana (Heap 11B), **12**- Ghana (Heap 12B), **13**- Ghana (Heap 13B).

* New species discovered in cocoa beans fermentation closely related with *A. syzygii* (Cleenwerck et al., 2007).

** Recently described as new species: *Acetobacter fabarum* (Camu et al., 2008b; Cleenwerck et al., 2008).

*** Recently described new species (Ndoye et al., 2007).

[a] Species not validated in current taxonomic description.

B.- Basionym (CBS, 2003b).

With regard to the ecophysiology of these microorganisms, yeast species involved in cocoa bean fermentation have been isolated from workers hands, cutting utensils, fruit flies, surface of sound pods and the interior of diseased pods.

LAB has been associated with leaves and baskets, as well as cutting utensils, workers hands and fruit flies (Camu et al., 2007; Jespersen, 2003). Fruit flies, at the same time, have been implicated as a source of inoculation for both AAB and *Bacillus* spp. groups (Ostovar and Keeney, 1973). From all these sources, the cocoa pod surface appears to be the most important for microbial pulp inoculation (Camu et al., 2008b).

Table 9. *Bacillus* spp. species isolated from cocoa bean fermentation

	1	2	3	4	5	6	7	8	9	10	11	12	13
Bacillus cereus	+	+		+		+		+		N.D	N.R	N.R	N.R
Bacillus cereus var. mycoides	+												
Bacillus coagulans	+	+				+							
Bacillus licheniformis				+	+	+	+	+	+				
Bacillus megaterium	+	+					+	+	+				
Bacillus mycoides			+										
Bacillus pumilus		+		+	+	+		+	+				
Bacillus sphaericus					+	+			+				
Geobacillus stearothermophilus (B. Bacillus stearothermophilus)	+	+	+										
Bacillus subtilis	+	+	+	+		+		+	+				

Numbers correspond to the fermentations sites in table 3 with the respective references. **1-** Trinidad (Centeno Estate), **2**-Trinidad (San Louis Estate), **3**- Brazil (Schwan et al., 1995; Schwan, 1998), **4**- Indonesia (Estate A), **5**- Indonesia (Estate B), **6**- The Dominican Republic, **7**- Ghana (Heap 7B), **8**- Ghana (Heap 8B), **9**- Ghana (Heap 9B); **10**- Ghana (Tray 10B), **11**- Ghana (Heap 11B), **12**- Ghana (Heap 12B), **13**- Ghana (Heap 13B).
B.- Basionym (CBS, 2003b)
N.D.- Not detected; N.R.- Not reported.

Use of culture-independent techniques

This decade has been marked by the use of culture-independent techniques designated by PCR-DGGE (Denaturing Gradient Gel Electrophoresis). The sensitivity of this technique, allowed the detection of species that played a more important role than those indicated by the results of culture-dependent techniques. This situation was reported for instance in studies during tray fermentation in Ghana, where in samples collected after 3 h the yeast *C. stellimalicola* had been only detected by means of DGGE (Jespersen et al., 2005). Nielsen et al. (2007b) found a good correspondence between the DGGE results and cultured-based techniques. However, similarly, the lower detection limit of DGGE revealed that while based on culturing techniques the activity of the yeast *H. guilliermondii* was restricted to the first 24 h in the centre of the large heap fermentation, the DGGE revealed that it was still present until approximately 96 h, at levels equivalent to 1% of the total population of yeasts. In the same way DGGE profiles of LAB showed that *Leuconostoc pseudoficulneum* played a more important role during the fermentation in Ghana (Table 3: 7-10 E), since strong DGGE bands were obtained until the end of the fermentation, whereas according to the culturing techniques, the occurrence of this species would have been restricted to the first 24 h.

However, the DGGE method may be restricted by inhibitory compounds that interfere with the PCR reaction or difficulties associated with the bias introduced by the preferential amplification of the 16S rRNA (ribosomal ribonucleic acid) gene of certain groups or species (Camu et al., 2007; Prakitchaiwattana et al., 2004). This constitutes a reason for the importance

of combining both culture-dependent and culture-independent techniques when studying such complex microbial fermentations.

Mechanisms of cocoa flavour precursors formation

The impact of the diffusion of metabolites into the beans can be classified into four main categories: acidification, subcellular decompartimentation, enzymatic degradation and components leaching. These physicochemical processes will be discussed in this section.

Citric acid is the main acid present in raw, unfermented cocoa cotyledons. Weissberger et al. (1971) found 9.8 $mg.g^{-1}$ of CA in experiments with beans from Trinidad. In Indonesia fermentations (Table 10: 4-5A) levels of 9.0 and 7.4 $mg.g^{-1}$ were quantified, while in Ghana (Table 10: 11A) these were in the order of 6 $mg.g^{-1}$. Succinic acid constitutes another acid that might be present at significant levels. In Ghana (Table 10: 11A) a level of 2.3 $mg.g^{-1}$ was reported, at the same time that the concentrations of LA and AA were null. In the course of the fermentation, while the concentration of CA decreased to 4, 3.5 and 2 $mg.g^{-1}$, respectively in Indonesia (4A), Indonesia (5A) and Ghana (11A), the concentration of all the other acids increased (Table 10). With regard to the final pH of the beans, Ardhana and Fleet (2003) reported a decrease from 6.3 to 5.1 and from 6.5 to 5.0, respectively in Indonesia (4A) and (5A) (Table 10).

The analysis of these metabolites in the pulp and beans reveals that LA diffusion occurs to a lesser extent in comparison to the other metabolites. This can be concluded from the lower amounts observed in the beans than in the pulp, while for the other metabolites the ratio pulp/beans was approximately equal or lower than one (Ardhana and Fleet, 2003; Camu et al., 2007; Camu et al., 2008a). Underlying reasons for this outcome have not been investigated.

The consequences of acidification and the accumulation of ethanol, as well as exposure to an atmosphere low in oxygen and high temperature have been extensively studied by Biehl and co-workers, in the sequence of leading investigations by researchers like Forsyth and Quesnel (Forsyth, 1952; Forsyth and Quesnel, 1957; Forsyth and Quesnel, 1963; Quesnel, 1965).

In vitro studies of fermentation-like conditions on the subcellular structure of raw, unfermented cocoa bean cotyledons revealed that an anaerobic atmosphere and incubation in the presence of 7.35 $mg.g^{-1}$ CA, pH 5.5 at 40°C, were able to induce seed germination (Biehl et al., 1982b).

However, increasing concentrations of acetic acid, ethanol and rise in temperature, not only prevented seed germination, but also induced seed death, evidenced by the loss in the germination ability and diffusion of the purple pigments throughout the parenchyma cells (Quesnel, 1965). In particular, experiments with incubations of cocoa beans in acetic acid solution with concentrations found during cocoa beans fermentation, increased temperatures and varying atmosphere conditions (air *vs*. nitrogen), were able to destroy the membranes of the hydrophilic compartments and induce fusion of lipid vacuoles. Such events, resulted in subcellular reorganizations: a continuous lipid phase was formed, which displaced water and

Table 10. Cotyledons conditions during cocoa bean fermentation

	A	J	K	L
	Country/Ref.	**pH**	**Sugars (mg g^{-1})**	**Cotyledons metabolites (mg g^{-1})**
4	Indonesia- Estate A (Ardhana and Fleet, 2003)	**pH_{0h}**: 6.3; **pH_{120h}:** 5.1	**F_{0h}**: 1.0; **F_{120h}**: 0.4 **G_{0h}**: 0.7; **G_{120h}**: 0.1 **S_{0h}**: 19; **S_{120h}**: 0	**CA_i**: 9; **CA_{120h}**: 4 **E_i**: 0.2; **E_{120h}**: 0.4 **LA_i**: 0.1; **LA_{120h}**: 2.0 **AA_i**: 1.0; **AA_{120h}**: 25
5	Indonesia- Estate B (Ardhana and Fleet, 2003)	**pH_{0h}**: 6.5; **pH_{72h}:** 5.0	**F_{0h}**: 0.8; **F_{72h}**: 0.3 **G_{0h}**: 0.6; **G_{72h}**: 0.1 **S_{0h}**: 18; **S_{72h}**: 0	**CA_i**: 7.4; **CA_{72h}**: 3.5 **E_i**: 0.2; **E_{24h}**: 52; **E_{72h}**: 1.6 **LA_i**: 0.1; **LA_{72h}**: 1.8 **AA_i**: 0.7; **AA_{72h}**: 15
11	Ghana (Camu et al., 2007)		**F_{0h}**: 0.7; **F_f**: 4.7 **G_{0h}**: 0.7; **G_f**: 5 **S_{0h}**: 12; **S_f**: 0	**CA_i**: 6; C_f: 2.0 **SA_i**: 2.3; **SA_f**: 5.0 **E_i**: 0; **E_f**: 7 **LA_i**: 0; **LA_f:** 2.8 **AA_i**: 0; **AA_f**: 6.4
12	Ghana- Heap 12 (Camu et al., 2008b)			**E_i**: 0; **E_f**: 1.8 **AA_i**: 0; **A_f**: 5.6
13	Ghana- Heap 13 (Camu et al., 2008b)			**E_i**: 0; **E_f**: 4.1 **AA_i**: 0; **A_f**: 7.0

Abbreviations: F- Fructose, G- Glucose, S- Sucrose, CA- Citric acid, E-Ethanol, LA- Lactic acid, AA- Acetic acid, M- Mannitol
Índices: i- Initial, f- Final.
Note: This table is the result of data conversion from graphs in the original articles, exception for the work by Ardhana and Fleet (2003).

organelles to the edges of the cell, and the components diffused out of their storage cells and spread across the parenchyma (Biehl et al., 1977; Biehl et al., 1982a). Parameters modulating this effect appeared to be the amount of undissociated AA (higher levels are accumulated as result of decreased pH in the seed) and the temperature. Quesnel (1965) demonstrated that, indeed, AA was the most effective agent inducing the seed death, while the temperature was shown to become the dominant factor when levels as high as 53°C were reached in the cocoa beans mass (which happened during very few fermentations as can be observed from Table 4. The role of LA in the seed death it is still unclear, but considering its low diffusion, it suggests that it will not have a preponderant effect (Biehl et al., 1985; Quesnel, 1965).

As consequence of the loss of membrane integrity, enzymes and their substrates, initially separated in individual compartments, converge, leading to a cascade of chemical reactions. It is in this apparent chaotic situation that resides one of the most valuable interests of cocoa bean fermentation because this leads to the formation of the cocoa flavour precursors (Figure 2). These precursors were shown to be reducing sugars, amino acids and peptides that during roasting react together, mainly through Maillard and Strecker degradation reactions, generating compounds like pyrazines and Strecker aldehydes. These last compounds contribute to the typical cocoa flavour (Reineccius et al., 1972b; Rohan, 1963; Rohan, 1964; Rohan and Stewart, 1966; Rohan, 1967). In this context, we define 'Cocoa flavour' as the totality of complex

olfactory, gustative or trigeminal sensations detected while roasting cotyledons of commercial cocoa (definition adapted from ISO 5492:1992 (ISO, 1992) by Almeida (1999)). 'Chocolate flavour' should be applied to the flavour resulting from the subsequent manufacturing operations and addition of other ingredients.

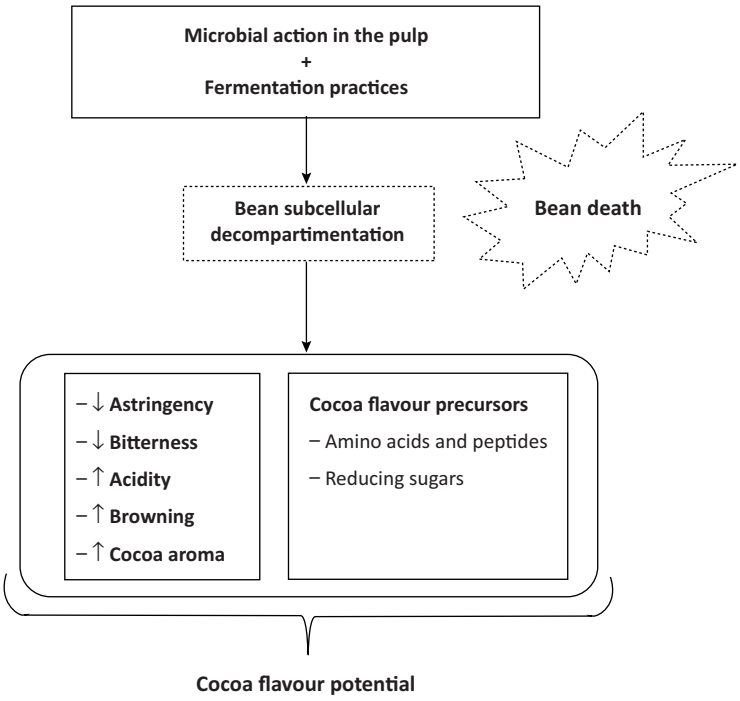

Figure 2. Changes in cocoa beans during fermentation related to the cocoa flavour.

The composition of the enzymatic system of cocoa seeds is still not fully understood. In the literature contradicting information about the presence of enzymes like polygalacturonase, cellulase or lipase is found (Lopez et al., 1978; Lopez and Dimick, 1991). Difficulties associated with sample preparation caused by polyphenol-rich extracts have been pointed out as one of the main reasons hampering the studies of enzyme activity in cocoa (Hansen et al., 1998; Lopez and Dimick, 1991). Nonetheless, there is a consensus on key enzymes responsible for the formation of cocoa flavour precursors, as well as some fermentative flavour compounds. These enzymes are invertase (EC 3.2.1.26), aspartic endoprotease (EC 3.4.23), carboxypeptidase (EC 3.4.17), polyphenol oxidase (EC 1.14.18.1) and glycosidases (Amin et al., 1998; Forsyth and Quesnel, 1957; Hansen et al., 1998; Lopez and Dimick, 1991; Sakharov and Ardila, 1999; Voigt et al., 1994a; Voigt et al., 1994c).

It was shown that during the anaerobic phase of the fermentation, corresponding to the initial stages where the oxygen tension is low, due to the tight packed structure of the beans, microbial oxygen utilization and carbon dioxide production, the enzymatic reactions are mainly of hydrolytic nature. Polyphenol oxidase becomes active during the later stages, when the pulp degradation creates conditions for the oxygen penetration in the mass. The terms 'anaerobic hydrolytic phase' and 'oxidative condensation phase' were employed by Forsyth and Quesnel (1963) to differentiate the period during which the enzymatic activities take place. However, in practice a considerable overlap between the anaerobic and the oxidative phases occurs, due to the heterogeneity of the cocoa bean mass.

Invertase activity is involved in the conversion of seed sucrose into fructose and glucose (although AA penetration in the cotyledons can also contribute to this conversion). Its location in the seed appears to be the shell (Lopez et al., 1978) and the cotyledons. It is active at pH levels between 4 and 5.5, with an optimum of 4.5 (Hansen et al., 1998). Cotyledon invertase appears to be very sensitive to the conditions during cocoa bean fermentation as only trace activity was detected after 2 days of fermentation (Hansen et al., 1998). During the fermentation, the sucrose present in the cotyledons is substantially degraded, at the same time that the levels of fructose and glucose increase, as result of sucrose hydrolysis (Table 10: 4-5K, 11K). In Ghana (Table 10: 11K) the authors registered an increase in fructose and glucose levels from 0.7 to 5 $mg.g^{-1}$. However, in both Indonesia fermentations (Table 10: 4-5K), instead of an increase, a decrease in the glucose and fructose levels was reported at the end of the fermentation. One could speculate on the possibility of degradation reactions of these sugars during the process or their exudation from inside the cotyledons, but surveillances on the quality of fermented commercial cocoa beans have been unanimous describing appreciable levels of reducing sugars. For example, reducing sugar levels of 2.2 $mg.g^{-1}$ and 6 $mg.g^{-1}$ were described by Hashim et al. (1999) and Reineccius et al. (1972b).

The major classes of seed proteins in unfermented cocoa beans are vacuolar storage proteins albumin and globulin, which constitute, respectively, 52 and 43% of the total seed protein (Biehl et al., 1982c; Voigt et al., 1993). The major albumin is a polypeptide with an apparent molecular weight of 19 kDa and the globulin fraction is constituted of polypeptides with an apparent molecular weight of 47, 31 and 14.5 kDa. In the globulin fraction, vicilin-type globulins are the only type found (Voigt et al., 1993). Voigt et al. (1993) observed that as result of the fermentation, the albumin fraction suffered a reduction of around 27%, whereas the decrease in globulin fraction was around 90%. *In vitro* proteolysis studies by Voigt et al. (1994a) showed that hydrophilic peptides and hydrophobic free amino acids were correlated with the formation of cocoa specific aroma. They found the enzymes aspartic endoprotease and the carboxypeptidase to be implicated in the generation of those products (Voigt et al., 1994a). In the sequence of this work, Voigt et al. (1994b) demonstrated in a very elegant way that the proteinaceous fraction of the flavour precursors was specifically derived from degradation of vicilin-like globulin proteins by the aforementioned enzymes (Voigt et al., 1994b). These findings could explain the previous observations by Biehl et al. (1985), who

showed that high concentration of proteolytic products was not necessarily correlated with high flavour potential. Interestingly, *in vitro* studies comparing the products of hydrolysis of globular storage proteins of different crops, showed that cocoa-specific flavour failed to be produced from sources other than cocoa and that this was linked with the particular chemical structure of the vicilin-like globulin (Voigt et al., 1994d).

The enzymes aspartic endoprotease and carboxypeptidase have different pH optima: this is 3 for the first, with an activity range between pH 3 to 7, and pH 6 for the later (Hansen et al., 1998). Both proteases displayed considerable stability during the fermentation and drying (Hansen et al., 2000). The potential of prolonged activity might partly explain why reducing sugars are found to be the limiting factor of flavour formation during roasting (Lerceteau et al., 1999; Reineccius et al., 1972b).

In what concerns variations of protein levels during the fermentation, de Brito et al. (2000) observed a decrease in protein levels in cotyledons from 220 $mg.g^{-1}$ to 18 $mg.g^{-1}$ after fermentation for 144 h and sun drying for 72 h, while the free amino acid content increased from 25.7 $mg.g^{-1}$ to 32.6 $mg.g^{-1}$. Hashim et al. (1998) reported an increase in free amino acids from 5.16 $mg.g^{-1}$ to 12.7 $mg.g^{-1}$ in fermentation experiments carried out in a rotary drum reactor, where the fraction of hydrophobic amino acids raised from 1.6 to 5.9 $mg.g^{-1}$. Rohsius et al. (2006) pointed out that the content of free amino acids in unfermented and well fermented cocoa beans was in the order of 2 $mg.g^{-1}$ to 4 $mg.g^{-1}$ in comparison to 8 $mg.g^{-1}$ to 25 $mg.g^{-1}$, respectively.

In the polyphenol group, the purple anthocyanidin fraction, constituted mainly by cyanidin-3-α-L-arabinoside- and cyanidin-3-β-D-galactoside, as well as the catechin and the proanthocyanidin fractions suffer modifications during the fermentation, due to enzymatic and non-enzymatic processes (Forsyth and Quesnel, 1957; Wollgast and Anklam, 2000). The glycosidase enzyme, present in cocoa cotyledons, hydrolyses the anthocyanidins to cyanidins and to the respective sugar moieties, galactose and arabinose (Forsyth and Quesnel, 1957). This results in the bleaching of the purple colour and release of reducing sugars that can participate as flavour precursors. By the fourth to fifth day of fermentation, the pigments are usually found to be almost totally hydrolysed (Ferrão, 1963). Hansen et al. (1998) found that glycosidases had an optimum pH of activity between 4 and 4.5 and were stable throughout the fermentation as well as to sun and artificial drying. The resulting cyanidins, besides being lost in the exudates, can be also decomposed, originating colourless compounds, or can undergo enzymatic oxidation in the oxidative phase (Forsyth and Quesnel, 1963).

By the time the oxygen starts to increase in the cotyledons, there are conditions for the activity of oxidase enzymes (mainly from polyphenol oxidase complex) and non-enzymatic oxidative reactions. The (-)-epicatechin is the major substrate for polyphenol oxidase, resulting in the production of quinones, very reactive compounds, which in turn can polymerize with other polyphenols or form complexes with amino acids and proteins. These processes have two important consequences: on the one hand, a brown colour is acquired by the beans and, on the other hand, high molecular weight insoluble compounds are formed, reducing the

astringency and bitterness of the beans (Ferrão, 2002; Forsyth and Quesnel, 1963; Hansen et al., 1998). The loss of polyphenols in the exudates of the fermentation also contributes to a reduction of astringency and bitterness (Kim and Keeney, 1984). The participation of proteins in these reactions has a positive effect on the flavour. Thompson et al. (2007) cite works were it was observed that the lower availability of peptides and proteins during roasting was associated with the reduction of off-flavours.

The process of insolubilisation of the polyphenols continues during sun drying, yielding cocoa beans that have a "milder taste", when compared with beans that are dried by artificial means (Almeida, 1999; Ferrão, 2002).

The polyphenol oxidase has an optimum pH of activity between 5.5 and 7 and is gradually inactivated as a result of the fermentation and drying.

In fermentations in Brazil, where cocoa beans had been fermented for 144 h and sun dryed for 72 h, the total polyphenol levels decreased from 231 $mg.g^{-1}$ to 157 $mg.g^{-1}$ (de Brito et al., 2000). On the other hand, in Ghana fermentation (11A) (Table 4) a reduction from 126 $mg.g^{-1}$ to 100 $mg.g^{-1}$ was observed after 144 h fermentation, being amplified during sun drying (10-14 days) to final levels of 22 $mg.g^{-1}$, which corresponded to a loss of 82% of polyphenols (Camu et al., 2008a).

With respect to the specific groups of polyphenols, Camu et al. (2008a), in studies of heap fermentations in Ghana, measured a decrease in the (-)-epicatechin content from 9-12 $mg.g^{-1}$ to 3.5-1.5 $mg.g^{-1}$ at the end of the fermentation. In the same fermentations, the (+)-catechin decreased from 2.5-3.5 $mg.g^{-1}$ to levels of 1.0-2.5 $mg.g^{-1}$. After sun drying the level of (-)-epicatechin and (+)-catechin was further reduced by 50 and 60%, respectively. Almeida and co-workers (Almeida and Leitão, 1995; Almeida, 1999) in fermentation studies conducted in São Tomé and Príncipe, where samples of cocoa beans had been dried either by sun or artificial drying, described a decrease in the (-)-epicatechin and procyanidins levels from 46 to 0.95 $mg.g^{-1}$ and from 81 to 12 $mg.g^{-1}$, respectively.

The fate of methylxanthines in the pigmented cells is reported as being reduced as result of loss in the exudates. Brunetto et al. (2007) registered a decrease in the level of theobromine from 9.2 to 6.4 $mg.g^{-1}$ in fermented dried samples, while the decrease in caffeine had been from 1.5 to 0.83 $mg.g^{-1}$ (a 45% drop). Although, Camu and co-workers detected lower levels of theobromine, they found the same decreasing trend in fermented dried cocoa beans samples in Ghana fermentation (11A) (Table 4). On the contrary, caffeine levels were reported to remain approximately constant (Camu et al., 2008a; Camu et al., 2008b).

During the fermentation, the starch remains insoluble in the cotyledons. This is the reason why starch has been poorly degraded by amylase (de Brito et al., 2000; Holden, 1959; Roelofsen, 1958).

Quality along the chain and research needs

Farming system

Worldwide, cocoa production faces many constraints, the most important of which are pests and diseases that cause an annual loss of production of 20 to 30% (Fowler, 1999; Gotsch, 1997). Witches broom, black pod, watery pod rot and swollen shoot are some examples of these diseases, but also insects, squirrels, rats and monkeys can consume significant quantities or ripe pods. Control of pests and diseases is achieved by sanitation and application of pesticides, combined with the use of resistant planting material and good growing practices (Fowler, 1999).

While in some areas cocoa growing is not viable at all, in others, due to more dry climatic conditions, it is possible to conduct an organic farming production. Organic cocoa, whose market share is estimated to be less than 0.5% of the total production, has the advantage of attracting premium prices from consumers and rendering increased revenue to farmers (ICCO, 2007a). Madagascar, Tanzania and Costa Rica are examples of cocoa suppliers for the organic market (ICCO, 2007a). Though the exact production methods may vary, general principles of organic production include the exclusion of most synthetic biocides and fertilisers, the management of soils through addition of organic materials, adequate growing practices (for example, regulation of tree density and pruning) and use of crop rotation (Prasad, 2005).

Several environmental benefits have been attributed to organic farming and were confirmed on the basis of available European literature in the area of soils, the farm ecosystem, ground and surface water protection and farm inputs and outputs (Padel, 2001). Moreover, it is an agricultural system that ensures the supply of food for future generations (Cacek and Langner, 1986). For example, Gosling et al. (2006) reported that low input systems such as organic farming are generally more favourable to arbuscular mycorrhizal fungi development, which in turn have the potential to substitute for fertilisers and biocides which are not permitted in organic systems. This means that the impact of organic farming on the macro and microbiota may be very different compared with the conventional farming system. In particular, it can have important implications for the profile and dynamics of cocoa beans fermentation. Nothing is known about this yet. Investigation in this type of systems could make available reliable data on the magnitude of the benefits of organic agriculture and help to improve the performance of organic methods.

The harvest

The harvest of cocoa pods is an operation that influences the quality and the yield of commercial cocoa. Linked to this operation is the assessment of pod ripeness, which is done using subjective factors, such as through colour observation or shine loss (Ferrão, 2002). Unripe pods are difficult to depulp, contain less sugars, fat, smaller nibs and compounds that contribute to the potential of flavour. In addition to these technological drawbacks, it has been noted that fermenting pods containing low pulp sugar content do not allow sufficient rise of

the temperature in the cocoa mass, lead to slower and less vigorous fermentations, and do not produce the desired cocoa flavour (Dimick and Hoskin, 1999; Schwan, 1998). With respect to the impairment in flavour formation, the impact of unripe pods appears to be weakly linked to the enzymatic system of the beans, since they are found to be sufficiently developed in immature beans (Hansen et al., 2000). On the contrary, overripe pods are more vulnerable to decay, there is the risk of bean germination and the pulp tends to become very liquid (Almeida, 1999; Ferrão, 2002).

Given the fructification cycle of cocoa, the gathering of perfectly ripe fruits would imply very frequent harvests, which is economically infeasible. In practice, the harvest is performed during a more or less long time-frame, depending as well on the availability of personnel. Therefore, pods with different degree of ripeness are often included in the fermentation (Ferrão, 2002; Lass, 1999). Due to this inevitable heterogeneity of fermenting batches, future studies should estimate the approximate proportion of unripe/ripe pods that can be included without compromising the quality of the final commercial cocoa beans.

Microbial interactions

In cocoa bean fermentation, the information on the type of microbial interactions that take place and their role shaping the course of the microbial dynamics and quality of the commercial cocoa beans is still insufficient. Several are the publications on fermented foods and beverages that have described an enhancement of LAB growth as result of carbon dioxide production by yeasts, and supply of simple sugars or growth factors like vitamins. It is possible that the co-occurrence of yeasts and LAB, as observed in most fermentations in Table 3 might be a reflection of mutualistic and synergistic interactions. It is questionable whether this is the only type of interaction that occurs. In the literature the ability of yeasts to inhibit mould growth in natural ecosystems and under laboratory conditions, through nutrient competition, production of toxic compounds (organic acids or ethyl acetate), cell wall-lytic enzymes and killer toxins is well documented. For instance, in maize dough fermentations *Saccharomyces cerevisiae* and *Issatchenkia occidentalis* inhibited the growth of mycotoxin producting moulds *Penicillium citrinum*, *Aspergillus flavus* and *A. parasiticus* (Jespersen et al., 1994). *S. cerevisiae* is a typical yeast involved in most cocoa beans fermentations, while *C. krusei* was found in fermentations in the Dominican Republic and Ghana (Table 6). This prompts the question whether the absence or the decline of mould populations in the fermentations summarised in Table 3 could have been influenced by presence of these yeasts in particular, or by other yeast species.

Many recent articles reported the absence or do not make reference to the occurrence of *Bacillus* spp. (Table 3). Interestingly, in these fermentations AAB, but especially LAB persisted throughout the fermentation and reached high levels, with LAB constituting the predominant group towards the later stages of the fermentation. On the contrary, in the majority of the fermentations where *Bacillus* spp. were present, these fermentations were characterised by a comparatively less pronounced development of both LAB and AAB. While the technological

practice of aeration appears not to prevent the presence of *Bacillus* spp. during cocoa beans fermentation (Ardhana and Fleet, 2003; Nielsen et al., 2007b), metabolites produced by LAB and AAB, namely LA and AA, could constitute a hurdle to the growth of *Bacillus* spp. Moreover, it is known that many strains of LAB are able to produce bacteriocins, which can have a narrow spectrum, impairing the activity of closely related species, or a wide spectrum, inhibiting the growth of a diverse group of Gram-positive microorganisms (Klaenhammer, 1988). Whether the LAB strains occurring during those fermentations had the ability to produce bacteriocins, active at the pH values found during the fermentation, is not known. This aspect raises the question of the significance of the absence of *Bacillus* spp. during cocoa beans fermentations, especially considering that in the tray fermentation (Table 3: 10G), claimed to produce commercial cocoa of better quality (Wood, 1975), *Bacillus* ssp. did not occur.

Camu et al. (2008a) found significant differences in the quality of commercial cocoa beans derived from fermentations performed with the same plant cultivar, during the same season, and with the same post-harvest practices (pod storage, fermentation method, type and duration of drying). The stronger cocoa flavour was obtained from a heap (heap 7) characterised by the lowest levels of ethanol and LA in the pulp (3.53 $mg.g^{-1}$ for both metabolites) and an AA concentration that was among the lowest (4.83 $mg.g^{-1}$). When the microbial levels of the corresponding fermentation are compared with the levels of another heap (heap 5), which contained the highest levels of pulp ethanol and LA, surprisingly, there was no connection with microbial levels of yeasts or LAB, as they were the same in both heaps. On the contrary, in heap 7 AAB persisted throughout the fermentation and attained the highest levels at the end, while in heap 5 they corresponded to 10% of the level registered in heap 7. The high ethanol levels in the final cocoa beans from heap 5 (8.15 $mg.g^{-1}$ *vs.* 3.53 $mg.g^{-1}$ in heap 7) are an indication of a less active metabolic activity of AAB. A hypothesis that can be raised is that the presence of high LA levels in heap 5 impaired the metabolic activity of AAB population, which did not occur in heap 7. Considering that LAB developed to the same levels in both heaps and that LA is a non-volatile acid, one might hypothesise about the presence of a possible microbial group that could have assimilated LA as a source of carbon. This could have been the case of species of *Bacillus*. The influence of homofermentative LAB can be ruled out as the authors showed in a previous publication the occurrence of solely heterofermentative species in those fermentations. With respect to the AAB group, the same species were found in these heaps.

Yeasts, LAB, AAB and, in some fermentations, *Bacillus* spp., constitute the most prominent microbial group, but other bacterial species (e.g. *Zymomonas mobilis*) and moulds were also reported in a few fermentations, during a certain period of time (Table 3). It is possible that many other unidentified species might be present (Camu et al., 2008b). The extent to which metabolites or enzymes produced by these minority groups impact the development of the major microbial groups remains unclear.

Metagenomics is an advanced and sophisticated stage of culture-independent genomic tools (Sleator et al., 2008; Venter et al., 2004). It involves direct isolation of DNA from a certain ecological niche, followed by cloning of the complete genomes and analysis of the

DNA library for sequences and functions of interest (Sleator et al., 2008). This approach has the advantage of overcoming the biases of preferential amplification of certain sequences in detriment of others as has been reported in PCR-DGGE. Once the data is collected it can be used in combination with the information derived from the culture-dependent technique to answer concrete questions related with the occurrence of functional genes, viz. the presence of bacteriocins or production of cell wall lytic enzymes; or to have access to the microbiome profile of a fermentation (Gill et al., 2006). The application of metagenomics, combined with high-throughput sequencing techniques, may open new insights and research fields in the domain of cocoa bean fermentation, with repercussions for the quality of the final product.

The acidification during the fermentation

Commercial cocoa beans from some countries tend to have more acidic characteristics than others. Cocoa beans from Malaysia, Brazil, São Tomé and Príncipe and some crop seasons from Indonesia are examples of this situation (Ferrão, 2002; Fowler, 1999; Jinap and Dimick, 1990). This has an adverse effect on the development of their international market, since cocoa products made with acidic cocoa beas are weaker in its specific flavour (Duncan et al., 1989) .

It is not yet totally understood what the main causes of the excessive acidification of cocoa beans are and the type of microbial succession and species associated with it.

High acidity content of commercial cocoa beans has traditionally been associated with the content of acetic acid, due to the negative impact of excessive acidification by acetic acid of cocoa beans during the fermentation on proteolysis reactions and development of cocoa flavour potential (Biehl et al., 1985). Later, Jinap and Dimick (1990) demonstrated a strong correlation between the concentration of acetic acid, the pH and titratable acidity of the fermented dried beans. However, studies on the acidity characteristics of fermented and roasted beans showed that while acetic acid contributes to the pH and titratable acidity, this correlation was weaker with respect to the perceived acid flavour. Instead, the levels of lactic acid were found to be the determinant factor for the acid flavour of cocoa and chocolate (Holm and Aston, 1993). In this respect, attention should be given to the population of specifically lactic acid bacteria.

Genetic differences in the planting material cultivated in Malaysia and West Africa were pointed out as the factor responsible for the high acidity of the commercial cocoa beans. The upper Amazon hybrids from Malaysia are characterised by a larger amount of pulp than the Amelonado type cultivated in West Africa (Carr, 1982). In Malaysia, when attempts were made to reduce the pulp content of cocoa beans prior to the fermentation a reduction in the acidity of cocoa beans and an increase in cocoa flavour were obtained. Experimental approaches consisted of storing the pods for 9 to 12 days or sun drying the surface of the mucilaginous beans by spreading them in layers, prior to fermentations in 0.32 to 0.42 m depth boxes (Biehl et al., 1990; Duncan et al., 1989; Meyer et al., 1989). The quality improvement was explained by the increase in the ratio surface/volume, which promoted yeasts respiratory metabolism in detriment of the fermentative. In addition, the fact that during pulp pre-conditioning there

was a decrease in the total amount of sugars, would have contributed to a reduced Crabtree effect during the process of fermentation (Biehl et al., 1989). As consequence, lower levels of ethanol, AA and LA were recorded in the pulp, there was a faster increase of the temperature of the fermenting mass and a decrease in beans pH to levels not below 5 (Biehl et al., 1989; Biehl et al., 1990; Meyer et al., 1989). Similarly, in Brazil, it was reported that removal of 20% of the cocoa pulp by mechanical means resulted in faster microbial progression and increased temperature and pH value of the cotyledon (Schwan and Lopez, 1987). Notwithstanding the possible genotype contribution to differences in the acidic characteristics of commercial cocoa beans from Ghana and Malaysia, it is interesting to observe that post-harvest processing in Ghana also includes pod storage, that can take from 2 to 3 days (Camu et al., 2007) to as long as 18 days (Jespersen et al., 2005).

An important question is how microbial successions of fermentations with or without pulp reduction differ. For example, it would be important to extend the analysis of the microbiota composition to other production countries, in order to verify whether the trend of occurrence of fermentative over respiratory yeasts correlates with cocoa beans with higher levels of AA and impaired quality.

Turning of cocoa beans mass is an important operation influencing the activity of the acidifying microbiota and supporting the enzymatic and non-enzymatic oxidation reactions in the beans. Studies conducted in Malaysia and Ghana revealed that turning of the bean mass after 48 h and 72 h, in box and heap fermentations, respectively, improved the quality of the resulting chocolate (Baker et al., 1994; Duncan et al., 1989). On the contrary, in recent studies in Ghana on the influence of heap turning for cocoa flavour attributes (Table 4: 12-13M), higher cocoa sensory scores were obtained from beans derived from the non-turned heap. Although non-turned heaps were characterised by pulp and beans AA levels approximately the same as in turned heaps, in turned heaps there was an accelerated ethanol decrease in the pulp that reached levels approximately close to zero. Besides, in the non-turned heaps, the levels of ethanol inside the beans were 5 times higher. Differences were found in LAB and AAB species in the two heaps (Table 7 and 8). To which extent the different species contributed to the flavour attributes of the resulting cocoa and what the contribution of yeasts species, was not mentioned.

The volatile fraction of the flavour

The sensory attributes of cocoa flavour are a combination of both a volatile and a non-volatile fraction. The non-volatile fraction of the flavour of fermented and roasted cocoa beans is conferred by the levels of polyphenols, methylxanthines and organic acids (Almeida, 1999; Stark et al., 2006). The volatile fraction of the flavour is by far more rich and complex, with more than 500 volatile compounds having been detected to date (Nijssen et al., 1996). This fraction is a combination of compounds naturally present in the fresh seed and compounds formed and lost during the fermentation, drying steps and roasting steps (Table 11).

Table 11. Volatile constituents of raw, fermented and dried and roasted cocoa beans[a,b,c]

	Raw beans	Fermented and dried beans	Roasted beans
Hydrocarbons			
C4-alkylbenzene		x [a]	
Cyclohexane			x [a]
Methylcyclohexane			x [a]
Styrene	x [a]		tr[a]
Toluene		x [a]	x [a]
Trimethylbenzene		x [a,c]	
Alcohols			
1-Pentanol		x [a]	x [a]
1-Phenylethanol	x [b]	x [b]	x [b]
2-Methylpropanol		x [a]	
2-Phenylethanol	x [a, b]	x [a, b]	x [a, b]
3-Methyl-1-butanol	x [b]	x [a, b]	x [a, b]
3-Methyl-2-butanol		x [b]	x [b]
Benzylic alcohol	x [b]	x [b]	x [b]
Furfurilic Alcohol	x [b]	x [b]	x [b]
2-Heptanol		x [a]	
3-Hexanol			x [a]
Linalol		x [b]	x [b]
Aldehydes			
3- Methylbutanal	x [b]	x [b]	x [b]
5-Methyl-2-furfural			x [a]
5-Methyl-2-phenyl-2-hexanal			x [a]
Benzaldehyde	x [a, b]	x [a, b]	x [a, b]
Hexanal	x [b]	x [b]	x [b]
Pentanal			x [a]
Phenylacetaldehyde	x [a, b]	x [a,b]	x [a,b]
Ketones			
Acetophenone	x [a]	x [a]	x [a]
2-Hexadecanone	x [a]	x [a]	
Acids			
Hexadecanoic acid			x [a]
Tetradecanoic acid			x [a]

Table 11. Volatile constituents of raw, fermented and dried and roasted cocoa beans[a,b,c] *(continued)*

	Raw beans	Fermented and dried beans	Roasted beans
Esters			
2-Phenylethyl acetate	x [b]	x [a,b]	x [a,b]
3-Methyl-2-butanol acetate		x [b]	x [b]
3-Methylbutyl acetate		x [a]	
Benzyl isothiocyanate	x [a]	x [a]	x [a]
Benzyl thiocyanate	tr[a]		
Ethyl acetate	x [b]	x [b]	x [a,b]
Ethyl cinnamate		x [a]	
Ethyl dodecanoate	x [a]		
Ethyl dodecanoate		x [a]	
Ethyl hexadecanoate	x [b]	x [a, b]	x [a, b]
Ethyl octadecanoate		x [a]	x [a]
Ethyl tetradecanoate		x [a]	
Methyl octadecanoate		tr[a]	x [a]
Methyl phenylacetate	x [a]		
Bases			
Pyridine			x [a]
Acetylpyrrole	x [b]	x [b]	x [b]
Methylpyrrole			x [a]
N-Ethylpyrrole			x [a]
2,3-Diethylpyrazine			x [b]
2,3-Dimethylpyrazine			x [b]
2,5-Diethyl-3-methylpyrazine			x [a]
2,5-Dimethyl-3-ethylpyrazine			x [a,b]
2,6-Diethyl-3-methylpyrazine			x [a]
2,6-Dimethylpyrazine			x [b]
2-Butyl-3,5-dimethylpyrazine			x [a]
2-Butyl-3-methylpyrazine			x [a]
2-Ethyl-3,5-dimethylpyrazine			x [b]
2-Ethyl-5-methylpyrazine			x [b]
2-Ethyl-6-pyrazine			x [b]
5-Methyl-2-furfural			x [a]
C5-alkylpyrazine			x [a]
Ethylpyrazine			x [b]
Methylpyrazine			x [a,b]

Table 11. Volatile constituents of raw, fermented and dried and roasted cocoa beans[a,b,c] *(continued)*

	Raw beans	Fermented and dried beans	Roasted beans
Tetramethylpyrazine		x [a,b,c]	x [a,b]
Trimethylpyrazine		x [a,b]	x [a,b]
S-compounds			
Dimethyl disulphide		tr[a]	x [a]
Nitriles and amides			
Dimethylformamide	x [a]	x [a]	x [a]
Phenylacetonitrile	tr[a]	x [a]	x [a]
Furans, furanones, Pyran, pyrones			
2-Ethylfuran			tr[a]
Methylfuran			x [a]

Tr- Trace

[a] (Gill et al., 1984)- raw cocoa beans were derived from ripe fruits; no information is presented on the cultivar, fermentation or roasting method.

[b] (Almeida, 1999)- raw cocoa beans from Forastero cultivar (São Tomé amelonado); fermentation in boxes for 136 h with turning after 48 h and 96 h, followed by sun drying; roasting at 156°C for 27 min.

[c] (Hashim et al., 1997b)- raw cocoa beans, derived from ripe fruit, from Forastero cultivar; fermentation in rotary drum reactor for 144 h.

Pyrazines are the main chemical group in the volatile fraction of the cocoa flavour, accounting for about 17% of total amount of volatile compounds. Esters, acids and hydrocarbons correspond to 12, 11 and 9%, respectively (Nijssen et al., 1996). Investigations aimed at characterizing the flavour potential of commercial cocoa beans have been concentrated on the content of free amino acids, oligopeptides and reducing sugars as precursors for the typical cocoa aroma formation (Biehl et al., 1989; Dimick and Hoskin, 1999; Hashim et al., 1999; Mabrouk, 1979; Reineccius et al., 1972b; Rohsius et al., 2006). However, additional volatile flavour characteristics such as 'fruity' and 'flowery' that are caused by the presence of esters and aldehydes formed via microbial synthesis, are also of high importance in the flavour. Nevertheless, the impact of the fermentation practices and the influence of specific species on the cocoa flavour development have been overlooked and this should be a point of attention.

Another noteworthy aspect is the origin of the pyrazine compounds in commercial cocoa beans. Dimethylpyrazines and tetramethylpyrazines are the main pyrazines quantified in commercial cocoa beans (Almeida, 1999; Hashim et al., 1997b). The temperatures reached during the fermentation and drying, although moderate in comparison to the temperatures during roasting, can contribute, to the final levels of pyrazines in commercial cocoa beans (Gill et al., 1984; Hashim et al., 1999; Hashim et al., 1997b). Pyrazines, can also be formed via microbial synthesis, notably by *Bacillus* spp. The first evidence of microbial synthesis was provided by Kosuge and Kamiya (1962), who showed that tetramethylpyrazine could

be produced by *B. subtillis*. A first proof of the implication of *Bacillus* spp. in the formation of pyrazines in cocoa was reported by Zak et al. (1972), who found a similar trend between tetramethylpyrazine formation and the proliferation of *B. subtilis* in the cocoa beans mass. Similarly, Romanczyk et al., (1995) proved the ability of strains of *B. cereus* isolated from cocoa fermentation of producing 2-acetyl-1-pyrroline and some alkylpyrazines. Hashim et al. (1997b) studied optimum conditions for the maximum formation of total pyrazines, trimethyl and tetramethylpyrazines in cocoa seeds fermenting for a period of six days in a rotary drum. They verified a considerable increase in the levels of tetramethyl and trimethhylpirazines after the third day or fermentation and a favourable influence of aeration. The influence of an increased aeration time in the increase of total pyrazines and trimethylpirazines could be related with the promotion of *Bacillus* spp. growth. Altogether, these data indicate the importance that *Bacillus* spp. might have during commercial cocoa bean fermentations, since pyrazines are a valuable attribute in the cocoa flavour (Table 11). However, the fact that these microbial species have also been implicated in the production of off-flavours, namely 2,3-butanediol, indicates the need of studying the properties of *Bacillus* and practices that could promote the formation of the desirable pyrazines, in detriment of off-flavours. With regards to the formation of butanediol, attention should be also given to members of *Leuconostoc* genus*, Lactococcus lactis* species and *Saccharomyces cerevisiae* (identified for instance during fermentations in Trinidad and Brazil, Table 7: 1-3), since their ability to ferment citrate into butanediol has been reported in dairy and wine fermentations (Herold et al., 1995; Hugenholtz, 1993).

The cocoa butter properties

Cocoa butter, a yellow fat extracted from fermented cocoa beans, is one of the most important ingredients of chocolate and it largely determines its physical properties. The most remarkable physical characteristic is the narrow melting range, between 32°C and 35°C. Its quick meltdown in the mouth produces a cool sensation, which is responsible for the pleasurable release of flavour (Hanneman, 2000).

Triacylglycerols (TAGs) are the main components of cocoa butter, representing around 97% of the total composition. The remaining fraction includes free fatty acids, mono- and diacylglycerols, phospholipids, glycolipids and unsaponifiable matter (Pontillon, 1998). In terms of its fatty acid composition, cocoa butter is a relatively simple fat. Three fatty acids, palmitic, stearic and oleic, generally account for over 95% of the fatty acids in cocoa butter. Of the remaining acids, linoleic acid is present at the highest level (Hernandez et al., 1991).

The chemical composition of cocoa butter, varies slightly according to type of cocoa tree, age of the plant, the country of origin and the season of harvesting, but also on processing factors like duration of fermentation. Those factors determine the exact composition of cocoa butter resulting in specific melting point and solidification (crystallization) behaviour (Meursing and Zijderveld, 1999; Talbot, 1999). Concerning the hardness, besides the above stated factors, the average daily temperature during the last few months of pod development also affects the characteristics of cocoa butter. Lower temperatures, give butters that are softer

or have a lower melting point (Fowler, 1999). Generally, cocoa butters made from Indonesian and Malaysian beans are harder than West African butters, which in turn are harder than Brazilian butters. These differences between cocoa butters can be found, especially in the ratio of TAGs of the type SOS/SOO (Table 12) (with S meaning 'saturated fatty acid' and O meaning 'oleic acid'). In very broad terms, SOS is a group of TAGs that are solid at room temperature, whereas SOO is a group of TAGs that are more liquid at room temperatures. Thus, Brazilian cocoa butter with a high level of SOO is less solid than Ghanaian cocoa butter, which in turn is less solid than Malaysian cocoa butter (Table 12) (Talbot, 1999).

Table 12. Triacylglycerol composition (%) of cocoa butters

Triacylglycerol	**Brazil**		**Ghana**		**Malaysia**
SSS	1.0	1.4	2	2.3	ns
SOS	63.7	76.8	78	84.0	85.3
SSO	0.5	0.4	ns	0.5	ns
SLiS	8.9	6.9	ns	6.8	3.9*
SOO	17.9	8.4	ns	5.1	3.1
OOO	8.0	6.1	ns	1.3	0.7
References	(Talbot, 1999)	(Talbot, 1999)	(Torbica et al., 2006)	(Talbot, 1999)	(Segall et al., 2005)

S= Saturated fatty acid (mainly palmitic and stearic acids): O= oleic acid: Li= linoleic acid
ns- not specified; *- does not include the fraction of triacylglyceride formed by the fatty acids palmitic-linoleic-stearic acids.

Not much is known about the impact of the fermentation on cocoa butter properties. In fact, it is not clear whether the fat suffers any quantitative and qualitative change in the storage cells. Making reference to practical field work developed in São Tomé and Príncipe in the 70's, Ferrão (2002) reported that quantitative modifications in the fat content due to fermentation and drying were found to be almost non-existent. The basis for that conclusion was the fact that fat is water insoluble and therefore it would not be lost in the fermentation sweatings. However, the same author states recent studies that suggest a modification in the relative proportion of quantitatively dominant fatty acids. Possible mechanisms involved in those modifications and the impact on the quality of the final product have not been yet fully explained. On the other hand, previous studies have confirmed that prolonged fermentation periods are associated with the increased formation of free fatty acids (FFA) (Lopez and Quesnel, 1973). These when present at low concentrations are regarded as contributing to the normal flavour of cocoa, but at higher concentrations contribute for lower fat melting point and flavour deterioration. Given the fact that the risk of oxidation reactions in cocoa butter is negligible, due to the low content of unsaturated fatty acids and the high content of natural antioxidants, the implication of (microbial) enzymes seems plausible (Lopez and Quesnel, 1973). In fact, certain filamentous

fungi and *Bacillus* spp. are able to produce lipases and therefore it is conceivable they could hydrolyse TAGs in the fat (Ardhana and Fleet, 2003; Lehrian and Patterson, 1983; Lopez and Quesnel, 1973).

On account of the higher price of cocoa butter compared with other technological products such as cocoa powder, seeds with high fat content, even though not processed at optimum conditions, will always find interested buyers. If there are changes in the properties of cocoa butter we may come across the conclusion that the fat characteristics on a given day of fermentation might have a more desirable technological quality for the production of a particular product. Therefore, there is the need to investigate and elucidate the effect of fermentation on the TAG composition, melting and solidification behaviour of cocoa butter in different stages of the fermentation process, giving special attention to the correlation of parameters with the occurring fermentative microbiota.

Quality requirements of commercial cocoa beans

Cocoa beans processors and chocolate manufacturers look for cocoa beans with a consistent ability to develop a strong cocoa flavour upon processing. In addition to the flavour potential attributes, the material yield and soundness constitute key criteria for the stage of commercialisation. The quality aspects of cocoa beans are frequently grouped into three main areas:

– **Economic** - related to the content of useful material and determine the price manufacturers are willing to pay in comparison to other cocoas. They include parameters like bean size, fat content or presence of germinated and infested beans.
– **Quality** - related to the flavour of the cocoa beans and include factors such as absence of off-flavours, presence of desirable ancillary flavours (for example floral, spicy and fruity) and some physical properties, such as cocoa butter hardness, melting and solidification behaviour. Based on these determinations it is decided whether cocoa will be included in blends or recipes for chocolate.
– **Wholesomeness** - related to food safety (mycotoxins, pesticides, heavy metals or foreign materials). The limits are regulated by the national food legislation in the country where the factory is located or through regulatory bodies like the European Union (EU) and the Food and Drug administration (FDA) in the USA.

Quality defects that can be found in cocoa beans include mouldy, smoky, acid, bitter, astringent, slaty, dull, putrid, lack of cocoa flavour, insect damaged, germinated and contaminated beans. The characters “bitter” and “astringent” are related with underfermentation problems; the term “slaty” beans refers to beans that were dried without undergoing fermentation; the terms “dull”, “putrid” and “lack of cocoa flavour” are associated with overfermentation practices; “contaminated” is used for beans that have absorbed off-flavours from other products, like rubber or oil based fuels (BCCCA, 1996; Fowler, 1999); and other not yet well known causes, as discussed previously.

Cocoa trading still lacks a single internationally accepted framework for grading of commercial cocoa beans. However, in cocoa physical markets, standard criteria established by the Federation of Cocoa Commerce Ltd (FCC) and the Cocoa Merchants' Association of America, Inc. (CMAA) are followed. For instance, the FCC makes a distinction between batches comprising less than 5% of mouldy and less of 5% slaty cocoa beans (well fermented cocoa beans) and batches with less than 10% mouldy and less than 10% slaty cocoa beans, where in both cases the content of foreign material should be below 1.5%. The classification of the cocoa beans according to this system is made by the use of the "cut test". This test involves cutting lengthwise 300 beans taken randomly from a sample, followed by the record of any defects and cotyledons colour (Almeida, 1999). During cocoa beans fermentation, this test is especially useful since it allows the expeditious monitoring of the status of the fermentation and the determination of its end point, as unfermented beans will show a purple colour and fully fermented beans a brown colour. However, a brown colouration is not always linked to well fermented cocoa beans, but instead may be associated with the occurrence of putrefaction reactions (Almeida and Leitão, 1995; Wood, 1975). In this context, the combination of the cut-test with analytical methods, facilitates a better characterisation of commercial cocoa bean batches, and may provide a way for a more fair remuneration among farmers.

We propose that other fermentation indexes to provide useful information on the flavour potential of the commercial cocoa beans could include the quantification of the following compounds:

- Volatiles produced during fermentation: specifically trimethyl- and tetramethylpyrazines (Table 11). Besides, certain compounds present in the raw seeds increase as result of the fermentation and these could also be used for this purpose. These include phenylacetaldehyde, benzaldehyde and 2-phenylethyl acetate (Almeida, 1999; Gill et al., 1984; Hashim et al., 1997b; Reineccius et al., 1972b);
- The ratio of reducing/total sugars (Rohan, 1967);
- The ratio between polyphenol fractions (Gourieva and Tserevitinov, 1979; Pettipher, 1986b);
- The ratio soluble nitrogen/ total nitrogen (Rohan and Stewart, 1967a);

In addition, based on the work by Kirchhoff et al. (1989), the ratio of hydrophobic/total free amino acids could also provide useful information on the flavour potential of the commercial cocoa beans.

Conclusions

The primary factor influencing the quality attributes of cocoa beans, the principal raw material for chocolate production, is the cocoa tree cultivar and genotype. The harvest and post-harvest processing determine the final quality of the commercial cocoa beans. This means that even

if the finest cultivars are selected, when the subsequent processing is not properly controlled and good agricultural and manufacturing practices employed the final quality will be impaired. In this sequence of processing, the fermentation is the most important step, since it is during the fermentation that biochemical reactions inside the cocoa beans take place, leading to the formation of the cocoa flavour precursors and fermentative flavour. The cocoa flavour is later fully developed during roasting.

During cocoa bean fermentation, different microbial groups are present. However, the comparison that we performed among different fermentations, showed that, on the contrary to what was believed so far, a well defined succession of the microbial groups does not always take place; and even in the same country and region the type of microbial species active throughout the process are not necessarily the same. While the presence of yeasts, LAB and AAB during fermentation gathers consensus in terms of their positive functional contribution for the final quality of commercial cocoa beans, the role of *Bacillus* spp. is still not very well understood and constitutes a subject of controversy. This has major implications, considering that in many fermentations where *Bacillus* spp. are reported they appear towards the later stages of the fermentation. It is pertinent to question whether fermentations should be halted at an earlier stage, in order to avoid their proliferation; and how the technological procedures should be adjusted to ensure that the desirable biochemical reactions take place inside the bean.

On the other hand, the role of microbial interactions during cocoa bean fermentation has been overlooked and, in many aspects, cocoa microbiologists rely on information derived from studies of other type of ecosystems. Cocoa bean fermentation is a domain that would benefit from the new insights that metagenomics bring, since it would allow the elucidation of, not only, specific genes that are being expressed in the time course of the fermentation, but also of the presence of microorganisms not detected by culturing techniques or culture-independent techniques like PCR-DGGE. Such microorganisms could have a modulating effect on the dynamics of the major microbial groups and ultimately affect the final quality of the fermented beans. The study of the functions of the microorganisms by use of metagenomics approaches could help in the future to devise strategies for the improvement of the fermentations by promoting specific microbial physiological traits.

In this review we also highlighted the need of better understanding aspects related to the microbial contribution to the acidic character of commercial cocoa beans, the volatile fraction of the flavour and the properties of the cocoa butter, together with the quantification of external factors to the fermentative microbiota, namely the degree of ripening of the pods and the type of farming system.

Since the culture-dependent techniques are still a very important component in the study of cocoa beans fermentation, a platform for the standardization of the microbiological methods should be established. This should include guidelines for the number and location of samples to be taken for the final composite sample, the media to be used for each microbiological group and the respective incubation conditions. This would have the advantage of permitting

reliable comparisons between countries and gaining of more knowledge leading to commercial cocoa beans of consistent better quality. Next, this platform should be extended to the use of culture-independent techniques, covering from the DNA extraction method to the conditions used to perform culture-independent analysis and amplicons identification.

Opportunities exist to elevate cocoa bean fermentation to levels similar to those of wine or beer fermentation (Schwan and Wheals, 2004). In fact, it was shown that controlled fermentations by use of starter cultures can produce commercial cocoa of good quality (Schwan, 1998). However, the fact that the practice of cocoa bean fermentation in the traditional way has allowed the production of cocoa beans of acceptable quality and price, might contribute to resistances or delays in the investment in more modern farm infrastructures. Moreover, commercial cocoa beans belong to a category of raw commodities, since high value is added in the different industrial products from them derived (chocolate, cocoa powder and cocoa butter). This contributes to the concentration of resources in the further stages of the chain.

Nevertheless, cocoa fermentation science is an exciting area, where fundamental research can be easily translated into knowledge with important practical applications. As consequence, it can be anticipated that findings leading to the improvement of the quality of commercial cocoa beans will successfully gradually transform this field.

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Chapter 3

Quality and Safety of commercial cocoa beans

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Cocoa beans supplied to the markets are highly heterogeneous. This results from differences in the planting material, cultivation conditions, post-harvest processing, final product sorting in the countries of origin as well as conditions during storage

The potential of cocoa beans to deliver the desirable cocoa flavour upon roasting is the most important quality attribute cocoa processors and chocolate manufacturers seek in commercial cocoa beans. However, the exact criteria for grading commercial cocoa bean batches depend on the stage in the chain. In the countries of origin, cocoa trade associations define standards dealing mainly with the yield of usable material, whereas at export terminals and laboratories in the importing countries, cocoa processors and chocolate manufacturers define more elaborate benchmarks related to the flavour, economic and safety factors as well as cocoa butter characteristics. In this chapter we describe the different type of standards and criteria which are used to evaluate the quality and safety of commercial cocoa beans. We also address the different categories of commercial cocoa beans, which include recent certifications such as 'UTZ CERTIFIED'.

We present in depth discussion about methods and approaches for quality and safety assessment. The importance of extending existing studies to define biomarkers distinguishing 'fine' from 'bulk' cocoa beans is also highlighted.

Although the quality and safety of commercial cocoa beans is still nowadays severely thwarted by pests and diseases, it is expected that the increasing affordability of advanced high-throughput sequencing approaches will pave the way to design effective cocoa protective strategies and to promote specific quality traits.

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Introduction

Cocoa epitomises one of the commodities with a relatively complex supply chain. This is due to the need for post-harvest processing in the country of origin prior to industrial transformation into a variety of commercial products. The post-harvest processing, consisting of cocoa bean fermentation and drying has a pivotal role in its value chain, as it determines the range of subsequent cocoa bean applications. Cocoa beans which have not undergone fermentation or have been poorly fermented are unsuitable for the manufacture of cocoa powder and chocolate, and can only be used for fat extraction. Thus, the progression of 'commercial cocoa beans' (i.e. beans which have undergone some kind of post-harvest processing prior to trading) in the chain, depends on their score on selected quality traits, ultimately determining the remuneration paid to farmers. In addition to quality parameters, the industrial requirement of assuring consumer health, adds a dimension of safety to the global evaluation of commercial cocoa beans.

To understand the factors that affect the quality and safety of commercial cocoa beans, it is important to critically analyse the chain of cocoa bean production. Six main features may be identified:

1. There are more than 40 producing countries in the world (ADM Cocoa, 2009; ICCO, 2011b) and within each country, and sometimes even regions within the same country, the planting material, the cultivation conditions and the exact post-harvest processing method vary, which results in commercial cocoa beans of distinct characteristics (yield of edible material, degree of bean fermentation, potential to produce chocolate of strong flavour, etc.) (Aculey et al., 2010; Baker et al., 1994; Clapperton et al., 1994; Davies et al., 1991; Motamayor et al., 2008; Nielsen et al., 2007b). The fact that about 90 to 95% of all cocoa in the world is produced by small-holder farmers, contributes to the high variability of cocoa characteristics in the market (ICCO, 2008).
2. It is estimated that between 20 to 40% of the global primary production of cocoa is annually lost, due to fungal diseases and insect attacks (Fowler, 2009; ICCO, 2011a). Black pod disease (caused by fungi-like *Phytophthora* spp.), witches' broom (caused by the fungus *Moniliophtora perniciosa*), mirids (insects from the family *Miridae*) and the cocoa pod borer moth (insect belonging to the species *Conopomorpha cramerella*), constitute the most widespread and devastating pests (pests are here defined as any organism that harms crops) (Bateman, 2009; Fowler, 2009). Control of these pests requires strategies employing various types of pesticides in combination with adequate phytosanitary practices;
3. The fermentation and post-fermentation handling of cocoa beans is still nowadays performed under very rudimentary conditions, relying on environmental contamination and open-air systems, where microbiological control is limited or non-existent. This inevitably results in batches containing, not only, high microbial levels, but, also, considerable proportion of adulterants and foreign material (Burndred, 2009). Moreover,

the absence of rigorously implemented processing protocols and absence of the use of defined microbial starter cultures, undermine the attainment of batches with homogenous quality;

4. Cocoa bean plays a major role in the economy of many countries, notably in West African countries (Akinnifesi et al., 2006; Folayan, 2010; Ntiamoah and Afrane, 2008), and for many farmers it is their only source of revenue. This means that, in order to increase throughput, some agronomic and processing practices may be neglected, impairing the quality of the final commercial product;
5. For some applications of cocoa beans, namely for cocoa butter extraction, bean fermentation is not always a requirement. Consequently, unless pre-agreements on separate circuits have been made, unfermented beans will contribute to increase the fraction of beans with flavour defects in the market;
6. Although dried cocoa beans constitute a reasonably stable product, precautions are needed to avoid infestation by insect pests and moulds during storage and transportation (Bateman, 2009; Dand, 1993). Although residue-free methods based on temperature and atmosphere control have proven to be technologically effective (Fowler, 2009), they are not cost-effective, and chemical strategies are still the basis of control.

All these factors determine the characteristics of commercial batches of cocoa beans and have influenced the establishment of a framework to evaluate their quality and safety, providing guidelines along which cocoa beans are traded.

In this Chapter, the quality and safety requirements of commercial cocoa beans are addressed, followed by a discussion of methods that are presently used to determine bean quality and safety. Attention is given to regulatory bodies and legislative aspects. Finally, we critically discuss and analyse future prospects in this field.

Quality and safety requirements for commercial cocoa beans

Standards for commercial cocoa beans

'Quality' is frequently defined as "the total of features and characteristics of a product or service that bears on its ability to satisfy given needs" (ASQC, 2010) or simply "fitness for use" (Luning et al., 2006). 'Safety' refers to the "need of absence of hazards with an acceptable risk" (Luning et al., 2006), with hazards comprising biological, chemical and physical agents or conditions, which can affect consumer health (ISO, 2005). Safety can be understood as an intrinsic attribute of quality. However, for clarity of explanation, we will keep these two concepts separated in this chapter.

Generally speaking, good quality commercial cocoa beans should be a synonym of excellent flavour potential (i.e. adequate proportion of precursor compounds of the cocoa flavour and balanced composition of metabolites of the 'fermentation flavour' (Lima et al., 2011a),

excellent colour (fully brown), high yield of usable material, and absence of contaminants. However, the exact criteria on which the quality of cocoa beans are assessed depend on the stage in the chain. Cocoa trade associations, namely the Cocoa Merchants' Association of America (CMAA) and the Federation of Cocoa Commerce in Europe (FCC), have developed 'standard trade contracts', which are used to trade cocoa beans in the countries of origin, and in the so-called 'actual markets'(Fowler, 2009; ICCO, 2011c). These standards are based on easily measurable and observable characteristics that require only simple equipment. In the United States of America (USA), the Food and Drugs Administration (FDA) and the Intercontinental Exchange (ICE) also set standards. Producing countries frequently use FDA/ICE standards as guides for the dissemination of quality parameters among farmers and cooperatives (Fowler, 2009). Further in the chain, cocoa bean processors and chocolate manufacturers, who seek to provide the market with products of consistent quality and characteristics (e.g. strong cocoa flavour), define more elaborate benchmarks (Dand, 1993; Fowler, 2009).

While some cocoa bean characteristics can be objectively detected or quantified, others, such as chocolate flavour, are highly subjective. As a consequence, the quality definition of cocoa beans is strongly dependent on the identification of negative aspects. Different types of defects can be found in cocoa bean batches (ADM Cocoa, 2009; BCCCA, 1996; Dand, 1993; Fowler, 2009). These are:

– 'Smoky'- beans contaminated by smoke flavour during artificial drying. This is considered the worst off-flavour, since the incorporation of minute quantities of cocoa beans contaminated with smoke is sufficient to result in chocolate with flavour defects;
– 'Mouldy'- beans infected by moulds. This only refers to contamination of the inside of the beans and not on the outside. The access to the inside of the bean can take place as result of shell fracture, due to damaged induced by insect attack. The use of as little as 3% of mouldy beans for chocolate making has been found to cause an unpleasant taste in chocolate. In addition to taste, mycotoxins could be formed, if moulds grow inside the beans;
– 'Under-fermented'- beans with a grey or violet coloration, indicating, respectively, beans which have not been fermented or beans of which the fermentation has been halted prematurely. They are commonly designated as 'slaty' beans. Under-fermentation results in cocoa beans which are excessively bitter, astringent and lack cocoa/ chocolate flavour;
– 'Over-fermented'- beans with a 'putrid' or 'dull' smell, resulting in chocolate with an unpleasant taste;
– 'Insect-damaged'- insect-penetrated and attacked beans, rendering cocoa beans unsuitable for manufacturing;
– 'Acidic'- beans with an excessively high content of organic acids, which might have resulted from fast, artificial drying or other not well known causes linked to the fermentation, discussed by Lima et al. (2011a). It is an undesirable characteristic, since even with neutralization of the acids during alkalising process, the chocolate flavour may be negatively affected (Dand, 1993);

– 'Germinated beans'- beans of which the shell has been pierced by the growth of the first root, leaving an opening favourable to attack by insects and moulds;
– 'Flat beans'- beans which contain no cotyledons or are incompletely developed.

Table 1 presents an overview of the contract standards that are used for grading the quality of commercial cocoa beans in the countries of origin.

Table 1. Comparison of cocoa bean contract standards (Source: Fowler, 2009)

	Description (Example of growth/ grade)	Bean count	Faults			Moisture (%)	Foreign material
			Mouldy (%)	Slaty (%)	Infested (%)		
FAO model of ordinance[a]	Grade 1	NS[b] (uniform in size)	3	3	3[c]	7.5	'Virtually free'
FCC[d]	Good fermented (main crop)	100/ 100g	5[e]	5	_[e]	NS	<1.5%[f]
ICE/CMAA[g]	Ghana (main crop)	1000/ kg	4[h]	10	4[h]	NS	NS

[a]FAO specifies that cocoa must be fermented, free of foreign odours and must not be adulterated. [b]NS- not specified
[c]Includes germinated and flat beans as well as insect damaged.
[d]FCC specifies that the beans should be uniform in size, homogeneous and fit for the production of foodstuff. The beans must be virtually free from contamination, which includes smoky, hammy or other off-flavour, taste or smell.
[e]Maximum 5% defectives (infested are included under mouldy).
[f]<1.5% waste passing through a 5 mm sieve. Additionally flat beans, bean clusters, broken beans and foreign material must not be excessive.
[g]ICE/CMAA specify that hammy (overfermented) or smoky cocoa are not deliverable.
[h]Maximum amount of mouldy and infested beans is 6% (FAO Administration Defect Action Levels).

Table 2 presents the quality and safety requirements for cocoa beans used by processors and chocolate manufacturers. In Table 1, the criteria basically deal with economic aspects, such as aspects related to the yield of usable material, while in Table 2, factors pertaining to physical properties, flavour and safety of cocoa beans are also included. In Table 2, economic and qualitative aspects are considered together as part of the quality components of commercial cocoa beans.

The quality and safety characteristics in Tables 1-2 vary within regions and country of origin. This variability and diversity determines the demand for certain types of cocoa in detriment of others, as well as the definition of their price in 'future markets' (exchanging platforms where agreements are made with respect to price and delivery) (ICCO, 2011c).

In the following sections, after dissecting the type of categories of commercial cocoa beans, we will present quality characteristics of cocoa butter (Table 2-B) and discuss aspects dealing with safety of cocoa beans, specifically contamination by mycotoxins, pesticides, heavy metals and hydrocarbon compounds (Table 2-C) as well as, occurrence of bacterial pathogens.

Table 2. Commercial cocoa bean quality and safety requirements from the point of view of cocoa bean processors and chocolate manufacturers, and corresponding monitoring methods (Adapted from Fowler, 2009)

	Specifications or limits	Comments	Monitoring method	References
A- Economic aspects				
Moisture	– < 7% or 8%	– Mould growth prevention and prevention of edible material yield reduction	– ICA analytical method 43/1993 and ICA 1/1952	(ICA 1, 1952; ICA 43, 1993)
Bean size and size distribution	– Typically 100 beans/100g or 110 beans / 110 g. – Fraction of beans retained on certain sized sieves	– Influence on the yield of useful material and the unit operations in the factory, such as bean breaking and uniformity of whole bean roasting	– ICA analytical method 47/2001	(ICA 47, 2001)
Shell	– Typically 12-16% (w/w)	– Influence on the yield	– AOAC 968.10 and 970.23	(AOAC, 1974a; AOAC, 1974b)
Fat	– Typically 50-57% in dry nib	– Influence on the cocoa butter yield	– ICA analytical method 14/1972 and 37/ 1990	(ICA 14, 1972; ICA 37, 1990)
Foreign materials	– Absent or <1 to 5% (FCC)	– Influence on the purity and yield of edible material	– Cut-test	(ISO, 1977)
Flat, germinated and infested beans	– < 3% (FAO standard) and see Table 1	– Influence on the yield of edible material, purity and wholesomeness	– Cut-test	(ISO, 1977)
B- Qualitative aspects				
Unfermented (slaty) beans	– See Table 1	– Excessive slaty beans give an astringent taste and grayish colour to the chocolate	– Cut-test	(ISO, 1977)
Flavour and desirable ancillary flavours	– Various; often not specified	– Influence on the specific flavour characteristics of the final chocolate	– Sensorial analysis of chocolate	(ICA 12, 1971)
Off-flavours (e.g. smoky, hammy)	– Absent	– Influence on the specific flavour characteristics of the final chocolate	– ICA analytical method 44/1996	(ICA 44, 1996)

Table 2. Commercial cocoa bean quality and safety requirements from the point of view of cocoa bean processors and chocolate manufacturers, and corresponding monitoring methods (Adapted from Fowler, 2009) *(continued)*

	Specifications or limits	Comments	Monitoring method	References
Cocoa butter hardness	– Various	– Influence on the eating quality of chocolate (snap and melting properties)	– ICA analytical method 4/1962 and ICA analytical method 31/1988	(ICA 4, 1962; ICA 31, 1988)
Free fatty acids in cocoa butter	– Max. 1.75% (EU and Codex Standards)	– Increased levels contribute to a decrease in hardness of cocoa butter (Pontillon, 1998)	– ICA analytical method 42/1993	(ICA 42, 1993)
Unsaponifiable matter in cocoa butter	– Max. 0.35% (w/w) for pressed butter and max. 0.5% (w/w) for butter obtained by other methods (Codex Standard 86, 1981; European Council, 2000)	– Allows assessing the purity of cocoa butter	– ICA analytical method 23/1988	(ICA 23, 1988)
C- Safety aspects				
Mouldy beans	– 3-5% (see Table 1)	– Influence on the specific flavour characteristics of the final chocolate – Potential for mycotoxins and high level of free fatty acids	– Cut-test	(ISO, 1977)
Mycotoxins	– There are no specifications for cocoa beans – (Commission Regulation, 2006b; Commission Regulation, 2010a)	– Mycotoxins should be monitored due to the occurrence of different mycotoxin-producing fungi in the cocoa chain	– AOAC Official Method 2005.08	(AOAC, 2005; Commission Regulation, 2006a; Commission Regulation, 2010a)
Infested or insect damaged beans	– 3-5% (see Table 1)	– Influence on the wholesomeness	– Cut-test	(ISO, 1977)

Table 2. Commercial cocoa bean quality and safety requirements from the point of view of cocoa bean processors and chocolate manufacturers, and corresponding monitoring methods (Adapted from Fowler, 2009) *(continued)*

	Specifications or limits	Comments	Monitoring method	References
Pesticides	– Absent or below maximum residue limits (MRLs) defined by Commission regulations (Commission Regulation, 2005; Commission Regulation, 2008)	– Influence on consumer safety	– Residues analytical methods No. SANCO/ 10784/ 2009 from the European Commission (EC)	(European Commission, 2009)
Heavy-metals	– There are no specifications for cocoa beans (Commission Regulation, 2006a)	– May require regular monitoring due to existence of maximum levels for commercial cocoa butter and chocolate	– We provide the examples of lead- AOAC 934.07 and 972.25; and cadmium- AOAC 945.58 and 973.34	(AOAC, 1974c; AOAC, 1976; AOAC, 1993a; AOAC, 1993b)
Hydrocarbons	– There are no specifications for cocoa butter (Commission Regulation, 2006a)	– Can result from cocoa coming into contact with smoke during artificial drying or mineral oils in jute sacs used for their transport	– ISO 15302:1998, ISO/AWI 22959 and ISO/AWI 24054	(ISO, 1998; ISO, 2004b; ISO, 2004c)

Categories of commercial cocoa beans

Commercial cocoa beans are generally classified into 'bulk' cocoa and 'speciality' cocoa (ADM Cocoa, 2009; Dand, 1993; Fowler, 2009). Bulk cocoa beans consist of beans which generally derive from the widely cultivated *Forastero* variety of bean. They represent over 95% of the total cocoa traded in the world, being used for the manufacture of milk and dark chocolate, cocoa powder and extraction of cocoa butter (Fowler, 2009; ICCO, 2011d). Ivory Coast, Ghana, Indonesia, Nigeria, Cameroon, Brazil and Ecuador are some examples of suppliers of bulk cocoa beans. In this group, commercial cocoa beans from Ghana embody the regular supply of adequately fermented and dried cocoa beans, setting a reference against which all other cocoa beans are evaluated (Fowler, 2009). Commercial cocoa beans from the other countries have often been criticised for inconsistent quality, under-fermentation problems (specifically Ivory Coast, Nigeria, Ecuador and Indonesia) or off-flavour defects (e.g. smoky in Cameroon and Brazil and acidic in Brazil) (ADM Cocoa, 2009; Fowler, 2009).

Speciality cocoa is a broad category which comprises commercial cocoa beans with a distinguishing feature. Such features consist of: 'flavour' or 'fine', which are specific for a certain origin and are characterised by exquisite flavour traits; 'environmentally friendly', such as organic and rainforest protection-certified; cocoa produced under a system of pre-defined operations, as in UTZ certified; and cocoa marketed under a commitment to improve growers and farmers' livelihood, e.g. 'fair-trade' (Fowler, 2009).

Fine commercial cocoa beans have special ancillary flavour notes and/or a special colour, and are especially used to manufacture dark chocolate. This category of beans, which presently represents less than 5% of the total cocoa bean supply, originates from *Criollo, Trinitario* and a *Forastero* cross called *Nacional*. *Nacional* sub-varieties, also known as *Arriba*, are found in Ecuador. Ecuador is the world's largest supplier of fine cocoa, followed by Papua New Guinea, Venezuela, Madagascar, Indonesia, Trinidad and Tobago, São Tomé and Principe, Grenada and Jamaica (Fowler, 2009; ICCO, 2011b). In this group of nine countries, only five (Venezuela, Madagascar, Trinidad and Tobago, Grenada and Jamaica) grade the total output of cocoa beans exported as fine. For the remaining countries, this percentage varies between 1 to 75% (Fowler, 2009). Fine cocoa beans are known for special character descriptors, such as, 'fruity' (e.g. Jamaica and São Tomé and Príncipe), 'floral' (e.g. Ecuador and Papua New Guinea) and 'raisin' (e.g. Jamaica and Trinidad and Tobago). Besides commanding a higher price in the market, fine commercial cocoa beans, have their own supply circuits and a tighter quality control than bulk cocoa beans (Fowler, 2009).

The specific flavour requirements for both bulk and fine cocoa beans vary according to manufacturer's own house recipes for cocoa liquor and chocolate (Table 2-B).

In some regions, due to relatively dry climatic conditions, it is possible to conduct production by organic farming. Organic farming regions can be presently found in 18 countries, specifically, Madagascar, Tanzania, Uganda, Belize, Bolivia, Brazil, Costa Rica, Dominican Republic, El Salvador, Mexico, Nicaragua, Panama, Peru, Venezuela, Fiji, India, Sri Lanka and Vanuatu (ICCO, 2011e). Currently, the organic cocoa market share is estimated to be less than 0.5% of the total

production. Similarly to fine cocoa, organic cocoa has the advantage of attracting premium prices from consumers, rendering increased revenue to farmers (ICCO, 2011e).

UTZ CERTIFIED is a "worldwide certification program officially launched in 2002 that sets standards for responsible agricultural production and sourcing" (UTZ, 2011). The designation UTZ comes from the Mayan word 'Utz', which means "good" (UTZ, 2011). In 2007, a joint initiative of different cocoa bean processors and chocolate manufacturing companies together with the UTZ CERTIFIED organisation launched a framework for cocoa. The aim of the initiative was to increase transparency in the operations dealing with the primary production and post-harvest processing of cocoa beans, as well as to ensure better prices for farmers. Within this program, farmers are obliged to grow cocoa trees and process cocoa beans according to an internationally accepted 'Code of Conduct' (UTZ, 2009). The UTZ certification has been applied in the Ivory Coast, the Dominican Republic, Ghana, Nigeria, Peru, Tanzania and Vietnam, and extensions within these and other cocoa producing countries are foreseen (UTZ, 2011).

Rainforest alliance certification aims to promote the access of farmers to resources, the development of their livelihood and general regional economic growth, in equilibrium with the sustainable preservation of the ecosystems (Rainforest Alliance, 2011). In this certification, farmers should follow social and environmental practices defined in the standards established by the Sustainable Agriculture Network (SAN, 2011).

Fair trade is a social movement which seeks to promote and improve trading conditions for farmers, by creating credit and market access to farmers and shortening the path from primary production to consumer. Ultimately, the aim of this movement is to increase farmers' income and livelihood (Fair Trade, 2011).

Cocoa butter characteristics

Cocoa butter, a yellow fat extracted from cocoa beans, is a high priced component of cocoa bean and one of the most expensive commodity-based vegetable fats (ADM Cocoa, 2009). Cocoa butter is the ingredient in chocolate which determines its physical characteristics, such as the hardness, peculiar melting behaviour at human body temperature, plasticity, viscosity, soft texture and gloss (Hanneman, 2000; Liendo et al., 1997).

Cocoa butter is defined in the European directive 2000/36/EC (European Council, 2000) as the "fat obtained from cocoa beans or cocoa beans parts, with a free fatty acid content (expressed as oleic acid) not higher than 1.75% (w/w), and an unsaponifiable matter not higher than 0.5% (w/w), in the case of expelled and refined butter, and 0.35% (w/w) in the case of pressed butter". The definition in the Codex Standards (Codex Standard 86, 1981) is essentially the same as the European Directive.

Cocoa butter characteristics are largely determined by its triacylglycerols (TAGs), which represent about 97% of the total composition. The remaining fraction includes free fatty acids (FFAs), mono- and diacylglycerols, phospholipids, glycolipids and unsaponifiable matter (Pontillon, 1998). With respect to the content of fatty acids, three fatty acids generally account for over 95% of the total composition in cocoa butter: these are palmitic (C16:0), stearic

(C18:0) and oleic (18:1 cis-9) acids. Of the remaining acids, linoleic acid (C18:2(n-6)) makes up the highest fraction (Talbot, 2009a). However, the exact composition of cocoa butter is a function of the type of cocoa tree, age of the plant, country of origin and season of pod harvesting (Clapperton et al., 1994; Lehrian et al., 1980; Talbot, 2009a). Furthermore, factors such as type and duration of the fermentation could also play a role on its composition and physical characteristics, but very little information is available about this (Lima et al., 2011a).

In contrast with other fats, cocoa butter is predominantly composed (up to higher than 80%) of symmetrical monounsaturated TAG molecules, that is, molecules which bear oleic acid in the 2-position of glycerol, while the saturated fatty acid palmitic and/or stearic acid occupy the 3-positions. These TAGs are often referred to as SOS, where 'S' means saturated fatty acid and 'O' oleic acid (Talbot, 2009b). The remaining TAGs are of the following types: trisaturated (SSS), monounsaturated (SSU and SUS), diunsaturated (SUU and USU) and triunsaturated (UUU) (ADM Cocoa, 2009).

The content of SOS molecules in cocoa butter is responsible for its hardness, high stability and narrow melting range (ADM Cocoa, 2009; Talbot, 2009b). The presence of the natural antioxidant, tocopherol, also confers high stability to cocoa butter (Talbot, 2009b). The ratio between the TAG types SOS and SOO has been shown to vary across cocoa butter samples from different origins, resulting in cocoa butters with different hardness. For instance, Brazilian cocoa butters have a higher level of SOO than the Ghanaian, Malaysian and Indonesian butters and, therefore, are less solid at room temperature. On the other hand, Malaysian and Indonesian cocoa butters are harder than the Ghanaian butters. In addition to the origin, Lehrian and Keeney (Lehrian et al., 1980) demonstrated experimentally for pods from Brazilian plantations that a lower average daily temperature during the lasts months of pod development contribute to softer cocoa butters (Lehrian et al., 1980).

The aforementioned factors are taken into account for the purpose of chocolate making, where an ideal melting point and solidification (crystallization) behaviour of chocolate is sought (Talbot, 2009a).

In Table 2, criteria for cocoa butter quality are summarised. These contemplate aspects related to the amount of fat (Table 2-A), hardness, content of FFAs and unsaponifiable matter (Table 2-B). Cocoa industrials seek beans with a high fraction of fat and a low level of acidity and unsaponifiable matter (fraction of material in the fat which is not saponified by an alkali or that is not volatile (Dand, 1993)). The fraction of fat varies according to the type of cocoa population and genotype (Khan et al., 2008; Pires et al., 1998). With respect to FFAs, when present at low concentrations, these are regarded as contributing to the normal flavour of cocoa. However, at higher concentrations, FFAs contribute to softening of cocoa butter and to flavour deterioration, and, for this reason, a benchmark of maximum 1.75% FFAs was set to control the quality of cocoa butter (Table 2-B).

Safety of commercial cocoa beans

Mycotoxins

Mycotoxins are secondary metabolites of fungi that can cause acute or chronic disease in vertebrate animals upon ingestion of contaminated food or feed (Frisvad et al., 2007; Magan and Aldred, 2007). This definition does not include fungal toxins active against other living forms, such as bacteria and non-vertebrate animals, and toxins produced by members of *Basidiomycota* phylum (i.e. mushrooms and related fungi) (Frisvad et al., 2007).

Several mycotoxins have been described to date, but two of them are of special concern, due to their broad span of occurrence and high toxicity. These are Aflatoxins (AFs) and Ochratoxin A (OTA). AFs are notable as being potently carcinogenic, while OTA is notable as a nephrotoxic agent (Frisvad et al., 2007; Pfohl-Leszkowicz and Manderville, 2007). AFs are produced by certain species of the genus *Aspergillus* (e.g. *A*. *flavus* and *A. parasiticus*) and *Emericella* (e.g. *E*. *olivicola*). OTA is also produced by species within the *Aspergillus* genus (e.g. *A*. *carbonarius*), in addition to species of the genus *Penicillium* (e. g. *P*. *verrucosum*) and *Petromyces* (*P*. *alliaceus*) (Frisvad et al., 2007).

Not all strains within a given fungal species appear to have the ability to produce mycotoxins. Furthermore, production of mycotoxins depends on environmental factors, such as temperature, water activity, pH and type of growth medium (Amézqueta et al., 2008; Esteban et al., 2006; Frisvad et al., 2007; Mounjouenpou et al., 2008). For instance, higher production of OTA was observed for a strain of *A. carbonarius* when grown at 20°C than at 30°C (Esteban et al., 2004) and when the strain grew at a water activity of 0.90 than at 0.99 (Esteban et al., 2006). However, for another strain of *A. carbonarius* no toxin production was detected at water activities of 0.90 or below (Esteban et al., 2006). As a consequence, there may be little correlation between the presence of moulds in cocoa beans and the occurrence of mycotoxins (Fowler, 2009).

The Commission Regulation (EC) 1881/2006 (Commission Regulation, 2006b) sets maximum levels for different mycotoxins in foodstuff, including AFs and OTA. In this regulation, cocoa and cocoa derived products are not considered to be an important source of AFs or OTA and, therefore, no maximum limits have been established (Commission Regulation, 2010b) (Table 2-C). This regulation superseded an initial proposal to establish a maximum level of 2 µg/kg for OTA for commercial cocoa beans and cocoa-derived products (European Commission, 2003). Nevertheless, different research groups have been investigating the incidence of mycotoxins, especially OTA, in commercial cocoa beans and final commercial cocoa products as well as the ability of production of mycotoxins among fungal species isolated from these products.

Strains of *A. flavus, A. parasiticus* and *A. nomius* isolated from cocoa beans have been shown to be active producers of AFs, while strains from A. *carbonarius*, *A. niger* aggregate, *A. ochraceus*, *A. melteus* and *A. westerdijkiae* were implicated in the production of OTA (Amézqueta et al., 2008; Copetti et al., 2011a; Copetti et al., 2011b; Mounjouenpou et al., 2008; Sánchez-Hervás et al., 2008; Scott, 1969).

In a study by Bonvehi (2004), 76% of the analysed commercial cocoa beans originating from West Africa and Cameroon were positive for the presence of OTA, with levels varying from 0.1 to 3.5 µg/kg. Copetti et al. (2010) found 51% of sun-dried Brazilian cocoa beans to be positive for OTA, but only 1.2% had levels higher than 2 µg/kg (maximum level was 5.54 µg/kg). Similarly, de Magalhães et al. (2011) reported an incidence of 55% of OTA contamination in dried (bulk) Brazilian cocoa beans, but no samples had levels higher than 2 µg/kg. Curiously, in the latter study, OTA contamination of speciality cocoa (fine and organic) was much higher, reaching 100% incidence for fine cocoa from the early crop season. However, also in this case, the fraction of samples with levels higher than 2 µg/kg was low (11.1%, with an average level of 5.43 µg/kg).

In vitro studies on AF production have shown a high susceptibility of *A. parasiticus* to increasing levels of caffeine in cocoa beans (Lenovich and Hurst, 1979). This could be partially responsible for the low incidence of mycotoxins in cocoa.

Despite the fact that shelling of cocoa beans may give a reduction of OTA by levels between 50 and 95% (Amézqueta et al., 2005; Manda et al., 2009), OTA has been detected in a number of samples of cocoa mass (50%), cocoa cake (92.5%) and cocoa powder (93%), at levels ranging from 0.1 to 9 µg/kg (Bonvehí, 2004). Such findings emphasize the need for more stringent control of conditions for storing cocoa-derived products.

Bacterial pathogens

Salmonella is the most important bacterial pathogen of concern for cocoa processors and chocolate manufacturers, due to the implication of low levels of *Salmonella* in outbreaks of salmonellosis linked to consumption of chocolate (Hockin et al., 1989; Werber et al., 2005). The source of contamination of chocolate in the outbreaks reported by Hockin et al. (1989) and Werber et al. (2005) was unknown. In fact, difficulties in identifying the source of contamination by *Salmonella* has been stated as a major hindrance in the development of measures aimed at controlling *Salmonella* in commercial cocoa products (Burndred, 2009).

The concern about the occurrence of *Salmonella* in commercial cocoa products is reflected in the microbiological quality guidelines defined by industrial processors for cocoa liquor and cocoa powder. These guidelines specify the absence of *Salmonella* in 30 samples of 25 g, that is, in 750 g (Kamphuis, 2009).

Because of the nature of the post-harvest processing and conditions in the supply chain, *Salmonella* can be expected in commercial cocoa beans, although a recent study in Brazil revealed a very low incidence of *Salmonella* at different stages of the post-harvest (only one sample was positive in a total of 119) (da Silva do Nascimento et al., 2010). The presence of *Salmonella* in commercial cocoa beans, nevertheless, is not a reason for rejection of a commercial batch (Fowler, 2009).

Salmonella is unlikely to survive cocoa bean roasting; yet, it has been detected in a recent microbiological survey of commercial cocoa powder samples (Lima et al., 2011b). Such

findings suggest contamination by the microorganism after roasting and, therefore, the need for stringent control measures to prevent cross contamination of the heat-processed product.

Pesticides

Procedures for cocoa bean cultivation, which are based on good agricultural practices (GAP), are considered to be the essential first step in the control of pests. These include adequate canopy management, weeding, regular and complete harvesting, and sanitary pruning and disposal (ICCO, 2011a). However, these are not enough. The use of pesticides during cultivation of cocoa is necessary to allay or block the access of pests, which destroy or lead to loss of quality of the pod and render commercial cocoa beans unsuitable for manufacturing. Such pests comprise both fungi and insects.

Fungicides are the most common type of pesticides used in the primary production of cocoa and, within the fungicide group, copper and phenylamide-based compounds (e.g. metalaxyl) are the most widely used (Bateman, 2009). Copper-based fungicides and metalaxyl are applied, for instance, in the control of black pod disease. Copper-based fungicides are especially popular, but, as contact fungicides, they have shorter protective life duration than systemic fungicides (such as phenylamide compounds). However, the high costs of systemic fungicides, make their use economically unsustainable for farmers (ICCO, 2011a).

Copper-based fungicides are permitted in both conventional and organic farming systems (ICCO, 2011a). However, in organic farming their use has been restricted to a maximum application of 6 kg Cu/ha per year, as proposed in the standards by the International Federation of Organic Agriculture Movement (IFOAM) (IFOAM, 2010).

Insects are also recognised as a serious threat to cocoa cultivation worldwide. Their control requires the use of different type of insecticides, which are characterised by different mode of action and spectra (Bateman, 2009; ICCO, 2011a). For the control of mirids and the cocoa pod borer moth, the most widespread insect pests, broad spectrum organochlorine insecticides (e.g. lindane and endosulfan) were popular. Currently, these were substituted by less toxic and more environmentally friendly insecticides, such as organophosphorus compounds and neonicotinoids (mirids), and chlorpyrifos (cocoa pod borer moth). Chemicals from the pyrethroids class are used against both pests (Bateman, 2009).

Cocoa farmers must follow pesticide residue management strategies, such as the number of pesticides which are used, the correct method of spray application and the minimum number of days separating the last application of pesticides and the harvest (pre-harvest interval (PHI)) (Bateman, 2009). Because of the importance of the fermentation for cocoa bean quality, investigations on the impact of pesticides residues on the ecology of cocoa bean fermentation would seem pertinent.

During storage, cocoa beans are again susceptible to attack by moulds and insects. The control of moulds during this stage, and the subsequent stage of transport, is achieved by ensuring a low moisture level (this should be between 7 to 8%). No specific chemical agents are used to prevent their growth (Fowler, 2009). With respect to insects, the most effective

method of control is the use of phosphine (or phosphane), which is a toxic gas generated from sachets containing metal phosphides (Bateman, 2009). When correctly used, phosphides are considered to be safe and less likely to result in residue problems than, for instance, methyl bromide, which has now been banned in the European Union (EU) and restricted in the USA (Bateman, 2009). However, reports of increased insect resistance to phosphine over continued years of use, have resulted in the need to use increased concentrations to achieve the same levels of protection (Reddy et al., 2007). This underlines the need to foster the development of chemical-free strategies, which can offer long-term solutions.

Pesticide residues are highly regulated in Europe and in the USA, where specific information on maximum residue levels (MRLs) is provided according to the type of residue and product or group products (Table 2-C). In the EU, the pesticides database presents MRLs for cocoa, under the category of "tea, coffee, herbal infusions and cocoa" (European Union, 2010). For example, the MRLs for fungicides metalaxyl and metalaxyl-M is 0.1 mg/kg, while for copper-compounds this limit is 50 mg/kg. Regarding insecticides, MRLs for malathion (organophosphorus compound) and deltamethrin (pyrethroid compound) are, respectively, 0.02 and 0.05 mg/kg. A similar database hosted by the Codex Alimentarius can be found at http://www.codexalimentarius.net/mrls/pestdes/jsp/pest_q-e.jsp.

At the moment, the status of phosphine under the directive 91/414/EEC for placing plant protection products on the market (European Council, 1991), awaits conclusion (European Commission, 2008).

Heavy metals and hydrocarbon compounds

In addition to mycotoxins and pesticide residues, heavy metals and hydrocarbons are a matter of concern in the cocoa chain (Table 2-C). The occurrence of heavy metals in cocoa is considered rare but may occur as result of environmental contamination from pesticides (copper), gasoline (e.g. arsenic, lead), or cocoa trees grown in volcanic soil (cadmium) (Fowler, 2009). Hydrocarbons, namely polycyclic aromatic hydrocarbons (PAH), may contaminate cocoa beans due to the diffusion of mineral oils from jute bags, or direct contact with smoke during artificial drying with poorly maintained driers (Bateman, 2009; Fowler, 2009). Both in Europe and in the USA, no specifications for maximum limits for these contaminants have been set (Table 2-C) (Commission Regulation, 2006b; Fowler, 2009). Transport of commercial cocoa beans in jute bags is becoming less frequent, but in many parts of the world, cocoa is still dried by use of artificial sources of heat. This may indicate the need of assessing hydrocarbons levels for cocoa beans of certain origins.

No safety criteria have been defined for commercial cocoa bean's butter (Table 2-C). However, maximum levels for heavy metals, specifically arsenic (0.5 mg/kg), copper (0.4 mg/kg), lead (0.5 mg/kg) and iron (2 mg/kg) are defined in the Codex standard for cocoa butter to be used in the manufacture of chocolate (Codex Standard 86, 1981; Codex Standard 87, 1981). This implies the need for regular monitoring of these elements in cocoa beans.

Methods for quality and safety monitoring of commercial cocoa beans

Introduction

The examination and quality control of commercial cocoa beans is based on official analytical methods that have been long-time established. They are methods which were developed by the International Office of Cocoa, Chocolate and Confectionary (IOCCC), now known as the International Confectionary Association (ICA). In addition, methodology by the International Organization for Standardization (ISO), The International Union of Pure and Applied Chemistry (IUPAC) and the Association of Official Analytical Chemists (AOAC) serve as references (ADM Cocoa, 2009) (see Table 2). In practice, many laboratories have adjusted the published methods or developed their own methods, in view of the need for simplification or taking advantage of scientific advances (ADM Cocoa, 2009; Cargill Cocoa, 2011b).

The quality control of commercial cocoa beans takes place at several points along the chain: at the buying stations, where commercial cocoa beans from small farmers are acquired; at the export terminals, which are the gathering points for commercial cocoa beans from different buying stations, cooperatives and intermediate traders; and at the quality control laboratories in the importing countries (ADM Cocoa, 2009).

In the countries of origin, cocoa beans are essentially examined by means of visual inspection, making use of the so called 'cut-test', although the determination of moisture, pH, fat content and FFAs may also be performed prior to exportation, depending on the existence of company quality control laboratories (Cargill Cocoa, 2011a).

Different publications emphasise the need for implementing effective sampling methods prior to undertaking analyses. These will not be dealt with here, but we direct the reader to publications by the International Confectionary Association (ICA 45, 1996), Dand (1993), Cargill Cocoa (2011a), ICMSF (ICCO, 2011c) and ADM Cocoa (2009) for more information.

In Table 2, reference to methods used to evaluate the quality and safety of cocoa beans is made. In the sections below, we will give particular attention to methods used to assess the yield of useful materials, degree of fermentation, markers to differentiate between bulk and fine cocoa, cocoa butter quality and safety.

Cut-test

The cut-test is one of the most common methods to evaluate the sanitary and fermentation quality of commercial cocoa beans. It involves cutting lengthwise 300 beans taken randomly from a sample, followed by inspection and recording of defects (mouldy, insect and germination-damaged and flat) and the cotyledon colour (grey, violet and brown) (Dand, 1993). The cut-test has the advantage of not requiring specialised equipment (simply needing a knife or a Magra cutter) or advanced training. In addition to its usefulness in quality monitoring of commercial beans, the cut-test also allows an expeditious monitoring of the status of the fermentation, as in the course of the process, the colour of the cocoa bean changes from violet to brown. However, whilst the identification of visual defects can be unequivocally made, this is not

always the case with respect to assessing the degree of fermentation. A brown colour may be subjective, caused by putrefaction and difficult to score when analysing samples from different origins (Almeida and Leitão, 1995; Wood, 1975). Due to these difficulties, the cut-test needs to be combined with other analytical methods, which facilitate an objective characterisation of the degree of fermentation of commercial cocoa bean batches.

Advanced methods for monitoring the fermentation quality

The subjectivity and difficulty of standardisation of the cut-test to assess the degree of fermentation has been broadly acknowledged (Dand, 1993; Fowler, 2009). While the cut-test is important for its great convenience in cocoa producing countries, in the importing countries there is need for a more objective and reliable method, with well established databases, to correlate specific colour and flavour traits with other relevant quality parameters.

Alternative methods to the cut-test to assess the degree of fermentation of dried cocoa beans have been used in many laboratories. These are the determination of the colorimetric fraction OD_{460nm}/ OD_{525nm} (Gourieva and Tserevitinov, 1979) and colour measurement by means of a colour analyser (Aculey et al., 2010). Near Infrared spectroscopy (NIRS), fluorescence spectroscopy and Nuclear Magnetic Resonance (NMR) are highly sensitive, specific and relatively fast techniques that allow the simultaneous detection of different compounds in food systems (Caligiani et al., 2010; Christensen et al., 2006; Kaffka et al., 1982). NIRS and ^{1}H NMR have been used to quantify fat, proteins, carbohydrates, polyphenols and moisture in cocoa beans, cocoa liquor and powder (Caligiani et al., 2010; Kaffka et al., 1982; Permanyer and Perez, 1989; Ramirez-Sanchez et al., 2010; Whitacre et al., 2003). In particular, NIRS is routinely used for the quantification of moisture and fat in cocoa liquor and cocoa powder (Cargill Cocoa, 2011b). These methods are of considerable interest for cocoa industrials, since traditional analytical methods for the quantification of quality parameters, such as moisture and fat (Table 2-A) are slow, require special chemicals and do not produce immediately available results (Kaffka et al., 1982).

NIR, fluorescence spectroscopy and ^{1}H NMR were successfully applied to group cocoa bean samples with distinct degrees of fermentation, providing at the same time a satisfactory segregation of samples according to the origin and population type (Aculey et al., 2010; Caligiani et al., 2010). Davies et al. (1991) described the possibility of using NIRS to predict the potential quality of chocolate from the assessment of quality traits in the corresponding commercial cocoa bean batches.

As a result of the fermentation, a striking array of physico-chemical modifications occur in cocoa beans. These include hydrolysis of proteins, sugars and anthocyanidins, and oxidation and condensation reactions of polyphenols. In addition, different organic acids and other microbial metabolites, many of volatile nature, accumulate in the beans. Hence, the quantification of these compounds by using chromatographic techniques, such as high performance liquid chromatography (HPLC), (high-resolution) gas chromatography coupled to mass spectrometry (GC-MS) or olfactometry, can provide an objective means to ascertain the

extent to which the fermentation process has been properly conducted (Aculey et al., 2010; Almeida, 1999; Frauendorfer and Schieberle, 2008; Gill et al., 1984).

The potential of cocoa beans to deliver the desirable chocolate flavour is one of the most important quality parameters chocolate manufacturers seek in commercial cocoa beans. Sensory analysis, based on cocoa liquor or chocolate production on a pilot scale, is a frequently used method to classify cocoa beans and predict the flavour attributes of the final chocolate. This is done by a trained panel, using standardised methods and reference samples. During flavour evaluation of cocoa liquors, this may be mixed with an equal amount of granulated or powdered sugar and some water, after which the suspension, kept in a liquid form, is tasted and scored (ADM Cocoa, 2009).

Quantification of the following compounds in commercial cocoa beans could also offer useful information with respect to flavour potential:

a) Volatiles produced during fermentation: specifically trimethyl- and tetramethylpyrazines. Moreover, certain compounds present in the raw seeds increase as result of the fermentation and these could also be used for this purpose. These include phenyacetaldehyde, benzaldehyde and the 2-phenylethyl acetate (Almeida, 1999; Gill et al., 1984; Hashim et al., 1997a; Reineccius et al., 1972a);
b) The ratio epicatechins/ cathechins (Payne et al., 2010) and the content of anthocyanins (Pettipher, 1986a);
c) The ratio of reducing / total sugars (Rohan and Stewart, 1967c);
d) The ratio soluble nitrogen/ total nitrogen (Rohan and Stewart, 1967b);
e) The ratio of hydrophobic/ total free amino acids (Kirchhoff et al., 1989).

The aforementioned compounds are suitable ‘markers’ of the degree of fermentation and, when sufficient information is collected with respect to natural variability and country of origin, databases can be constructed to help making cost-effective predictions on the flavour potential, without the need to quantify all the different classes of compounds.

The increase in acidity of cocoa beans, as result of the microbial action on the pulp during the fermentation, is a key determinant of the quality of commercial cocoa beans (Biehl et al., 1985). The extent of acidification of cocoa bean cotyledons determines a fine equilibrium for the optimal activity of invertase, aspartic endoprotease and carboxipeptidase enzymes and, consequently, the formation of the precursors compounds of the cocoa flavour (Hansen et al., 1998; Voigt et al., 1994a).

Commercial cocoa beans from some countries tend to have more acidic characteristics than others. This is the case of cocoa bean batches from Malaysia, Brazil, São Tomé and Principe and in Indonesia. Conversely, cocoa bean batches from West Africa and the Dominican Republic, for instance, are considered to have a balanced level of acidity (Fowler, 2009; Jinap and Dimick, 1990). A high acidity content in cocoa beans has been negatively criticised for resulting in chocolate of weaker flavour than cocoa beans of intermediate or low acidity (Duncan et al., 1989).

The acidity in cocoa beans is determined by the titratable acidity (expressed in meq NaOH/g of sample) and the pH of cocoa beans filtrate. The titratable acidity of cocoa beans was shown to be highly correlated (*r*= 0.91) with the total volatile acid content - predominated by acetic acid – of cocoa beans, as well as with the pH (*r*=- 0.91) (Jinap and Dimick, 1990). This correlation was slightly lower with respect to content of lactic (*r*= 0.85) and citric acids (*r*= 0.61) (both the non-volatile acids). However, studies on acidity characteristics of fermented and roasted cocoa beans by Holm and Aston (1993) revealed that while acetic acid contributes to the pH and titratable acidity, the correlation was weaker regarding the perceived acid flavour. Instead, the levels of lactic acid were found to be the determinant factor for acidic cocoa and chocolate flavour.

Progress has been made with respect to the development of approaches for control of acidity during cocoa bean fermentation. In Malaysia, approaches consisting of pod storage for 9 to 12 days or sun-drying of fresh beans prior to fermentation led to a reduction in acidity and to an increase in cocoa flavour (Biehl et al., 1990; Duncan et al., 1989). Similarly, in Brazil, mechanical removal of about 20% of cocoa bean pulp yielded cocoa beans of higher pH value (Schwan and Lopez, 1987). Another factor which was identified as contributing to cocoa beans' high acidity is fast artificial drying. The reason is the formation of a hard crust in the bean, which hampers the volatilisation of acetic acid (Ziegleder, 2009). Altogether, these data underline the difficulty in establishing an uncontroversial root of the problem of high acidity in cocoa bean, especially since detailed comparative data on the evolution of microbial species in trials where pulp reduction methods have been employed were not generated. Linking of specific microbial fermentation profiles with the characteristics of roasted commercial cocoa beans could shed more light on the best procedure to fully control and standardise the acidity content in final products.

Biomarkers of 'fine' cocoa

In addition to the degree of fermentation and indices of flavour potential, a much sought index of quality is the one providing an objective differentiation between fine and bulk commercial cocoa beans. These characteristics are of considerable relevance for chocolate manufacturers. Different investigations have attempted to find biomarkers of fine cocoa, both focused on the planting material (genetic level) and on the characteristics of commercial cocoa beans (phenotypical level). For example, Lerceteau et al. (1997) used random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) of DNA extracts from leaves to investigate the genetic relationship among representatives of *Forastero*, *Trinitario* and *Criollo* populations. They concluded that although a "continuous genetic background" was found, some specific genetic traits differentiated *Nacional* sub-varieties from other *Forastero* populations. Motamayor et al. (2008) proposed a new classification for cocoa populations based on the analysis of microsatellite markers. This new classification encompasses 10 genetic groups, as opposed to the traditional genetic group formed by populations of *Forastero-Criollo-Trinitario*. In this investigation, representatives of *Criollo, Nacional and Amelonado* hybrids

were clearly segregated based on their distinct genetic profile, suggesting that microsatellites could be useful biomarkers relating the planting material and potential flavour characteristics of commercial cocoa beans.

Caligiani et al. (2010) used ^{1}H NMR to compare the composition of commercial cocoa beans originating from *Nacional*, *Criollo, Trinitario* and *Forastero* populations. *Arriba* (*Nacional*) beans were characterised by higher level of epicathechins, caffeic acid, sucrose and oligosaccharides and lower level of amino acids than other *Forastero* beans. On the other hand, *Criollo* beans had a low content of carbohydrates and high content of amino acids. Both the *Arriba* and *Criollo* beans had approximately the same total content of glucose plus fructose, as well as caffeine, this last compound being much higher than in the *Forastero* beans. The authors stressed the need of extending this type of analysis to a larger number of samples, in order to confirm the observed pattern to differentiate fine and bulk commercial cocoa beans.

Compared to bulk commercial cocoa beans, fine cocoas were found to have higher levels of linalool, a monoterpene contributing to a flowery and tea-like flavour in cocoa (Pino and Roncal, 1992; Ziegleder, 1990). Thus, the quantification of this volatile in samples of commercial cocoa beans, could also provide an objective categorisation of samples.

Nevertheless, it can be concluded that there is the need for more studies to establish objective criteria for fine cocoa bean classification.

Cocoa butter quality

Different parameters are defined to assess the quality of cocoa butter. These include the determination of the solid fat content (SFC) (cocoa butter hardness index), melting point (MP), iodine value (IV) (degree of unsaturation of a fat), FFAs content and peroxide value (PV) (degree of oxidative stability), among others (ADM Cocoa, 2009). However, only standards pertaining to cocoa butter hardness and content on FFAs are crucial during cocoa bean commercialisation, influencing the demand of cocoa beans of certain countries of origin (Table 2-B). The unsaponifiable matter or non-saponifiable fraction (NSF), being an index of non-volatile organic matter not natural to cocoa butter (e.g. contamination by mineral oil or shell fat), is more relevant at the stages of cocoa butter commercialisation for the purpose of chocolate manufacture.

The SFC is presently obtained by pulsed NMR, which gives the level of solid fat at a particular temperature. Differential Scanning Calorimetry (DSC) is an alternative method of SFC quantification, which has the advantage of determining the crystalline state of the fat (Löser, 2009).

The official determination of FFAs is based on an acid-base titration method (Table 2-B), but other methods have been reported for FFAs analysis in fat, namely chromatographic methods (GC, HPLC), Fourier transform infrared spectroscopy (FTIR), NIR and ^{1}H NMR (Procida and Ceccon, 2006; Satyarthi et al., 2009; Sherazi et al., 2007).

The origin of cocoa beans has not only an influence on the flavour of chocolate, but it also determines the characteristics of cocoa butter. Pyrolysis-mass spectrometry was successfully

applied to classify cocoa butters of African, Asian and South American origins and it also grouped the samples based on the type of technological treatments the cocoa butters had undergone, for instance, whether deodorisation had or not been applied (Radovic et al., 1998). Such analytical techniques for cocoa butter discrimination according to the country of origin are of great interest for cocoa manufacturers, due to the combination of speed and reliability.

Considerations on methods for safety monitoring

In Table 2-C, reference to methods for monitoring cocoa bean safety are summarized. The cut-test provides an expeditious way to assess the presence of moulds, infestation and damage by insects. With respect to the assessment of mycotoxins, pesticides, hydrocarbons and heavy metals, more elaborate analytical methods are required.

Liquid chromatography (LC or HPLC) coupled to tandem MS (or alternatively to fluorescent detection) is the most widely used method for mycotoxin analysis in food (AOAC, 2005; Commission Regulation, 2006a). Recently, Ultra-High performance liquid chromatography (UHPLC) has been developed to improve the sensitivity, resolution and speed of AFs and OTA quantification (Ibáñez-Vea et al., 2011). Other methodologies are based on NIRs, electrochemical immunosensor (ECIS) and enzyme linked immunosorbent assay (ELISA) (Aydin et al., 2007; Fernández-Ibañez et al., 2009).

The quantification of pesticides and hydrocarbons, such as PAH, is based on chromatographic techniques, namely GC-MS/LC-MS and GS-MS, respectively (European Commission, 2009; ISO, 2004b).

Regarding the determination of heavy-metals, a simple method based on the colorimetric determination of metal complexes with Dithizone (Dithizone method) (Hibbard, 1937) or based on Atomic Absorption Spectrometry is still recommended for the quantification of heavy metals in cocoa butter (Table 2-C). However, more sophisticated and sensitive methods are available for the simultaneous detection and quantification of different heavy-metals in food, such as, inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma atomic emission spectrometry (ICP-AES) (Baer et al., 2011).

While food safety management in cocoa bean processing plants and chocolate factories is based on rigorously implemented pre-requisite specifications and programs for Hazard Analysis and Critical Control Points (HACCP), this is not yet the case during post-harvest processing of cocoa beans (Burndred, 2009). UTZ certified (UTZ) is the first attempt to effectively standardise processing practices in the field and control the quality and safety of commercial cocoa beans.

No maximum levels for mycotoxins, heavy-metals and hydrocarbon compounds are set for commercial cocoa beans. The definition of maximum admissible limits in food depends on the availability of information derived from toxicological studies and technological innovations in analytical methods that provide gains of sensitivity and specificity. This emphasises the need for cocoa industrials to be updated on the release of new data with respect to these aspects, to continuously monitor the levels of mycotoxins, pesticides, heavy metals and hydrocarbons along the chain and to be alert on the emergence of new safety risks for the consumer.

Conclusions and future prospects

The quality of commercial cocoa beans results from the combination of many different factors which include the planting material and the agricultural and processing practices in the countries of origin. These are, in turn, affected by the agronomic microenvironment (e.g. climate, occurrence of pests), as well as the macroeconomic conjecture (e.g. the price of cocoa in future markets and availability of subsidies for farmers).

Table 3 presents our analysis of the Strengths, Weaknesses, Opportunities and Threats (SWOT) (Kotler and Armstrong, 2006) for the field pertaining to the quality and safety of commercial cocoa beans.

Although cocoa beans are spontaneously fermented, it is possible with a relatively simple technology to obtain a high quality material for the production of chocolate and cocoa powder (Table 3-S). Indeed, this is probably one of the reasons why the technological management of cocoa bean fermentations is not at the same level of that of beer or wine fermentations. At the same time, it can be expected that concerted efforts to increase the traceability and transparency of cocoa production and trading (UTZ, 2011) will allow a faster and more efficient communication and cooperation among stakeholders in the chain. This, in turn, is expected to have an enduring impact on the long-term commitment of farmers in the production of good quality cocoa beans (Table 3-S). This is definitely a strong point for the cocoa sector.

The fact that the chocolate taste is broadly appreciated (Table 3-O) constitutes a confidence for farmers who wish to invest in the long-term activity of cocoa cultivation and commercialisation. Furthermore, the elevation of cocoa to the status of a nutraceutical food and the increased consumer interest for premium, high-cocoa solid content chocolate, manufactured with beans from 'exotic' origins, creates an excellent opportunity for famers to invest in agricultural and processing practices aimed at quality upgrading. Capitalisation on quality might be of special importance for small countries that cannot compete in quantity with the main producing countries.

On the other hand, many weaknesses can be identified with respect to the quality and safety of commercial cocoa beans (Table 3-W). The primary weakness is the high susceptibility of cocoa to pests, which challenges the annual output of commercial cocoa beans. In addition, the fact that cocoa is mainly produced by small-holder farmers, often in remote regions, precludes the effective implementation of a widespread farm management system for the control of pests and protocols to guide the post-harvest processing of cocoa beans. At the same time, threats originating from the fact that in some regions cocoa production is highly dependent on the existence of external investments (ICCO, 2011f), may bring a fragile basis for the long-term (high-quality) production (Table 3-T).

Although more weaknesses than strengths were identified in this SWOT analysis, opportunities stemming from the increased affordability of high-throughput sequencing techniques and other molecular approaches (Table 3-O) pave the way to augment knowledge aimed at devising strategies to combat cocoa pests and promote specific quality traits (Argout et al., 2008; Argout et al., 2011).

Table 3. Strengths, Weaknesses, Opportunities and Threats (SWOT) analysis in the field pertaining to commercial cocoa bean quality and safety. Source: This work

S- Strengths	**W- Weaknesses**
– Compared with other crops cocoa is considered environmentally friendly, due to the relative low level of farming inputs and for supporting high level of biodiversity (Bateman, 2009; Fowler, 2009) – There is a great emphasis on the dissemination of knowledge on the sustainable use of pesticides in cocoa growing regions, integrated in a long-term strategy for pests and diseases management (Bateman, 2009) – Although cocoa beans are spontaneously fermented and characterised by low input technology, it is possible to produce a final product which allows manufacture of high quality chocolate – An increasing number of actions are being undertaken to promote the traceability of cocoa beans in the chain and improve the livelihood of farmers and their families (Fair Trade, 2011; UTZ, 2011) – The increasing trend towards the liberalisation of cocoa trade and industry in the countries of origin, contributes to the improvement of transparency in cocoa marketing and quality control over exported cocoa (ADM Cocoa, 2009) – Cocoa beans are a source of polyphenols with demonstrated beneficial health effects, including prebiotic action (Buijsse et al., 2006; Crozier et al., 2011; Tzounis et al., 2011)	– The high susceptibility of the cocoa to different pests, hampers the consistent (quantitative and qualitative) supply of cocoa – There are difficulties in breeding pest-resistant varieties adapted to different regions and countries, combining both vigour and high bean quality for chocolate manufacturing (Efombagn et al., 2011; McMahon et al., 2010; Micheli et al., 2010) – Although new cultivars resistant to major diseases have been identified during the last decades, studies have shown that the resistance may be overcome (Brown et al., 2005; de Albuquerque et al., 2010) – The cocoa sector faces many challenges derived from aging tree stocks, poor soil fertility management and widespread difficulty of farmers access to pest control strategies; but also improper use of chemicals and uncontrolled forest conversion (ADM Cocoa, 2009; Ayenor et al., 2004; McMahon et al., 2010) – In many countries a successful farm management system for the control of pests and implementation of good post-harvest processing practices for cocoa is absent – The supply of cocoa beans fluctuates from year to year, which has an influence on the price for farmers (ADM Cocoa, 2009) – Cocoa bean fermentation relies on the uncontrolled colonization of microorganisms from the environment, which creates difficulty in standardisation of quality – There is a great dependence of cocoa production from a small number of countries (mainly Ivory Coast followed by Ghana) (ICCO, 2011b)

O- Opportunities	T- Threats
– The chocolate taste is appreciated worldwide and there is opportunity to expand the consumption of chocolate to Asian and Arabic countries – There is an increased consumer awareness about the relationship between diet and disease and aging – There is an increasing demand for chocolate which fulfils social, ethical and environmental standards of sustainability (ICCO, 2010) – There is an increasing market for exquisite and differentiated products – New genomic tools, such as high-throughput sequencing and molecular biology approaches, like metagenomics, open avenues to improve the quality of the planting material (e.g. resistance to pests and flavour)) and the quality of commercial cocoa beans (Argout et al., 2011; Lerceteau et al., 1997)	– In some producing countries, cocoa is threatened by other crops which hold higher market prices or for which better subsidies are available (Fowler, 2009) – Political conflicts in the producing countries may jeopardise the supply of cocoa (Byrne, 2011) – In some areas, cocoa production or efforts to improve its quality control is highly dependent on the existence of external initiatives and subsidies (ICCO, 2011f) – Global economic turnover may preclude the existence of platforms for farmers' access to effective control strategies for pests, hindering their long-term commitment for the production of (high quality) cocoa

With respect to the characteristics of commercial cocoa beans, the post-harvest processing plays an essential central role on quality. Therefore, it can be anticipated that on-going research studying the influence of fermentation and drying on the quality traits of cocoa beans (e.g. Camu et al. (2008b) and Garcia-Armisen et al. (2010)) will transform the field of cocoa fermentation science and may gradually impact the quality of beans for trade in the market.

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Chapter 4

Microbiota of cocoa powder with particular reference to aerobic thermoresistant spore-formers

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The microbiological criteria of commercial cocoa powder are defined in guidelines instituted by the cocoa industry. Twenty-five commercial samples were collected with the aim of assessing the compliance with the microbiological quality guidelines and investigating the occurrence and properties of aerobic Thermoresistant Spores (ThrS). Seventeen samples complied with the guidelines, but one was positive for *Salmonella*, five for *Enterobacteriaceae* and two had mould levels just exceeding the maximum admissible level. The treatment of the cocoa powder suspensions from 100°C to 170°C for 10 min, revealed the presence of ThrS in 36% of the samples. In total 61 ThrS strains were isolated, of which the majority belonged to the *Bacillus subtilis* complex (65.6%).

Strains resporulation and spore crops inactivation at 110°C for 5 min showed a wide diversity of heat-resistance capacities. Amplified fragment length polymorphism analysis revealed not only a large intraspecies diversity, but also different clusters of heat-resistant spore-forming strains. The heat-resistance of spores of six *B. subtilis* complex strains was further examined by determination of their *D* and *z*-values.

We concluded that *B. subtilis* complex spores, in particular those from strain M112, were the most heat-resistant and these may survive subsequent preservation treatments, being potentially problematic in food products, such as chocolate milk.

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Introduction

The processing chain of cocoa beans (*Theobroma cacao* L. fruit seeds) for cocoa powder production starts in tropical countries, where farmers, after harvesting the cocoa pods, submit the beans to a process of natural fermentation, the "sine qua non" for the distinctive cocoa flavour development ensued by roasting (Lima et al., 2011a; Schwan and Wheals, 2004). Following the fermentation, the cocoa beans are dried and shipped to industrial plants, where intermediate or final products are manufactured. For the production of cocoa powder, cocoa beans from different origins are blended, roasted and processed into a mass, which is either subsequently partially defatted, to minimum levels of 20%, or between 10 to 20% (dry weight matter) and pulverised (Anon., 1981; Kamphuis, 2009). Prior to roasting, alkalisation of cocoa beans is generally applied to improve organoleptic and technological attributes of cocoa powder (Kamphuis, 2009).

Whereas diverse microorganisms are present at high numbers during cocoa beans fermentation, subsequent post-harvest and industrial processing operations allow only the survival of a microbiota dominated by the genus *Bacillus* and relatives (Barrile et al., 1971). The ability of members of this group to form endospores, in some cases of extreme heat-resistance, implies that they may survive industrial processes and pose spoilage and safety problems (Huemer et al., 1998; Oh and Cox, 2009; Oomes et al., 2007).

The general microbiological composition of commercial cocoa powder was investigated previously, revealing total aerobic microbial levels between 2 and 4.4 log CFU/g, with *B. licheniformis*, *B. cereus*, *B. megaterium* and *B. subtilis* constituting, respectively, 45, 20, 10 and 8% of the isolates (Gabis et al., 1970). In another study, in addition to total aerobic microorganisms, spores populations surviving heat treatments of 80°C and 100°C during 1 and 5 min were analysed, and this showed *B. subtilis* and *B. licheniformis* to represent 83% of the isolates (Mossel et al., 1974).

Although the aforementioned reports are of interest in comparative studies of cocoa microbiota, they offer less insight into the occurrence and properties of highly heat-resistant spores in cocoa powder. These are a problem for further preservation of cocoa products, namely in Ultra-High Temperature (UHT) treatment of chocolate-flavoured milk. To our knowledge, no published data are available about the occurrence, identity and thermal kinetic parameters of highly heat-resistant spores in cocoa powder. Furthermore, in view of the detection and control of these spores within the food industry, it is important to understand whether a highly heat-resistant spore phenotype is associated with a specific genotype, or if it can be found in isolates bearing considerable genotypic dissimilarity.

Presently, the only reference pertaining to the microbiological quality of commercial cocoa powder consists of guidelines defined by cocoa industrials (Dijk et al., 2007). These guidelines, which are the only reference the industry relies on, include specifications for total aerobic mesophiles (< 5000 CFU/g), moulds (< 50 CFU/g), yeasts (< 10 CFU/g), *Enterobacteriaceae* (absent in 1 g) and *Salmonella* (absent in 25 g).

This work reports the assessment of compliance of commercial cocoa powder samples with the microbiological quality guidelines and the investigation on the occurrence, levels and properties of aerobic Thermoresistant Spores in those samples. We defined 'Thermoresistant Spores', as spores able to survive a heat treatment of at least 100°C for 10 minutes. This enabled the isolation and identification of predominant Thermoresistant Spore-formers, characterisation of the diversity of heat-resistant properties of their spores produced under standardised conditions and appraisal of strains genotypic variability.

Materials and methods

Cocoa powder samples

Twenty-five cocoa powder samples (packages or tins of 125-500 g) were purchased in retail shops (21 different brands) or obtained from an industrial processor (4 samples). In total, 13 samples were from Europe, 3 from South America, 3 from Africa, and 6 from Asia (11 countries in total). They consisted of 100% cocoa powder, with fat contents between 10-12% and 20-22% (w/w) (in half of the cases, this information was not provided). One of these samples was sold in a solidified form, with an unspecified fat content ('cocoa-cookie'). No information was given on the packages with respect to the processing applied or the origin of the cocoa beans. All the samples were stored at room temperature and analysed before the expiration date.

Microbial groups enumeration and detection

The content of each cocoa powder package was aseptically transferred and mixed in a sterile polyethylene sampling bag. Ten grams of each sample were collected into a stomacher bag with filter and homogenised with 90 ml of Peptone Physiological Saline solution (PPS) (1 g Neutralized Bacteriological Peptone [NBP, Oxoid] and 8.5 g NaCl per liter) for 1 min, in a Stomacher lab-blender 400 (Seward Medical) by selecting the 'normal' speed setting. The preparation of the 'cocoa-cookie' was performed as described in Dijk et al. (2007) for cocoa mass. Subsequently, after serial tenfold dilutions in PPS, 1 ml of the appropriate dilution was pipetted into duplicate plates and the appropriate medium was distributed according to the pour-plating technique. An aliquot of the primary dilution was also used to measure the pH (WTW 525, electrode Sentix 4.1). Total Aerobic Microorganisms (TAM) were enumerated with Plate Count Agar (PCA, Oxoid), after incubation at 30°C (mesophiles) for 72 h and 55°C (thermophiles) for 48 h. Yeasts and moulds were enumerated in pour-plates of Oxytetracycline Glucose Yeast Extract Agar (OGYE, Oxoid) after incubation at 25°C for 5 days. *Enterobacteriaceae* detection was performed according to the method described in Dijk et al. (2007) with incubations at 37°C. Pre-enrichment of 1 g of cocoa powder was performed overnight (18h) in Buffered Peptone Water (BPW, Oxoid), followed by enrichment in *Enterobacteriaceae* Enrichment broth (EEB, Oxoid). Next, the incubated EEB was subcultured on a pre-poured plate of Violet Red Bile Glucose Agar (VRBGA, Oxoid) under microaerophilic conditions. Representative characteristic

colonies were confirmed by the oxidase and glucose fermentation tests. *Salmonella* was detected in 25 g of cocoa powder as described in Dijk et al. (2007), with the exception that the BPW was supplemented with Skim Milk Powder (SMP, Oxoid) 100 g/l. After overnight incubation for 18 h at 37°C, a secondary enrichment in Rappaport-Vassiliadis Soya broth (RVS, Oxoid) and Modified Semi-solid Rappaport Vassiliadis Soya medium (MSRV, Oxoid) was performed at 41.5°C for 24 h. Next, selective culturing was done at 37°C for 24 h on Brilliant Green Agar (BGA, Oxoid) and Xylose Lysine Desoxycholate Agar (XLD, Oxoid) from both previously incubated media. Presumptive *Salmonella* colonies were characterised by biochemical, serological and serotyping tests at the National Institute for Public Health and the Environment of The Netherlands (RIVM, Bilthoven). All media were prepared according to the manufacturers' instructions.

Total and thermoresistant spores enumeration and strains isolation

For aerobic Total Spores (TS) determination, 10 ml of the primary dilution were pasteurised at 80°C-10 min prior to pour-plating with PCA (TS were enumerated based on the same primary dilution as for general microbial groups in 2.2). Plates incubation conditions were as for described for TAM. The heat treatments were done in a screw-capped 120x15 mm stainless steel (type 304) tubes. The caps had a central hole of 8 mm, through which a removable silicone membrane of 3 mm thickness was visible. Heating was performed by immersing the tubes in a circulating bath filled with glycerol (Fluka 49780) at the desired temperature, followed by cooling with vigorous shaking in icy water at the end of the set time (at 80°C the come-up time was 120 s, while cooling to room temperature took 15 s [Squirell data logger, Eltek]).

For aerobic Thermoresistant Spores (ThrS) enumeration, fresh cocoa powder dilutions were made, after which heat treatments of 100°C-10 min, 100°C-30 min, 108°C-30 min, 110°C-10 min, 120°C-10 min, 150°C-10 min and 170°C-10 min were applied. This was followed by pour-plating with PCA and plates incubation at 30°C for 5 days and at 55°C for 3 days. In addition, for ThrS enumeration, 1 ml of heated cocoa powder suspension was serial-diluted in Tryptone Soya Broth (TSB, Oxoid) supplemented with SMP 100 g/l (Park et al., 1979), according to the three-tube Most Probable Number (MPN) technique (Blodgett, 2006). Tubes incubation was also at 30°C for 5 days and at 55°C for 3 days. Samples plating took place in a flow cabinet, with incorporation of negative control plates and tubes. After incubation, the result of each tube was confirmed by streaking 10 µL on a PCA plate. The MPN index and the confidence intervals were calculated using the Food and Drug Administration MPN Excel spreadsheet available online (Blodgett, 2006). Colonies were randomly selected from the lowest dilution of ThrS plates or plates from the MPN technique (when the level of ThrS counting plates was below the detection limit). They were purified by dilution streaks on Nutrient Agar (NA, Oxoid) and after growth, the macro and micromorphology were inspected for purity confirmation. Stock cultures were kept in Nutrient Broth (NB, Oxoid) with glycerol 25% (v/v) (Fluka, 49782) and stored at -80°C.

With exception of *Enterobacteriaceae*, *Salmonella* and ThrS, all the determinations were performed in duplicate independent experiments.

Thermoresistant spore-formers identification

Phenotypic characterisation

The isolates were first confirmed to the genus level by colony and cell morphology, catalase production and Gram reaction (Gregersen, 1978). Endospore formation was monitored by phase contrast microscopy, after inoculation on the sporulation medium supplemented with different minerals that is described by Cazemier et al. (2001).

Strains coding was according to their isolation temperature: M (Mesophilic) for strains isolated at 30°C and TT (Thermotolerant) for strains isolated at 55°C.

The ability of the strains to ferment and assimilate carbon sources was determined using API 50 CHB system (BioMérieux, France) according to the manufacturer's instructions. The results were analysed with the APIWEB tool v4.0 (https://apiweb.biomerieux.com/servlet). The type strains *Geobacillus stearothermophilus* DSM5934 and *Bacillus subtilis* DSM10 were included in the API test as comparison. They were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany).

Strains motility was determined by stab inoculation of tubes with motility medium (Casein Digest [CD, Difco] 20g/l, Meat Peptone [MP, Oxoid] 6.1 g/l and Agar Bacteriological [AB, Oxoid] 3.5 g/l), followed by incubation at 30ºC for 2 days. *Bacillus cereus* ATCC 14579 (Laboratory of Food Microbiology, Wageningen University) was used as a positive control.

Genotypic characterisation

DNA was isolated from cultures grown overnight using the Wizard genomic DNA purification kit (Promega Corporation), following the manufacturer's instructions. The DNA extracts were directly used to amplify the 16S rRNA gene using universal primers (Edwards et al., 1989). Amplifications were performed in the thermocycler GeneAmp PCR System 9700 (Applied Biosystems), with an annealing temperature of 56°C and using chemicals and Taq DNA polymerase from Fermentas. The PCR products were sequenced with the same set of primers by GATC Biotech (Germany). The resulting sequences were assembled in SeqMan (Lasergene v5.08, Dnastar Inc.). The contigs were compared to similar sequences (Altschul et al., 1990) in the GenBank database at the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/). The isolates were assumed to belong to a given species if the similarity between the query 16S rDNA sequence and the sequences in the databases was higher than 97% (Stackebrandt and Goebel, 1994). The nucleotide sequences have been deposited in GenBank under the accession numbers GQ340461-GQ340521. The tree builder tool provided by the Ribosomal Database Project (RDP) was then used to create a phylogenetic tree. The sequences of the type strains of *Bacillus subtilis* (DSM10) and *Clostridium sporogenes* (DSM795) were retrieved from the RDP and included in the alignment.

Strains genetic diversity was analysed using the Amplified Fragment Length Polymorphism (AFLP) method (Vos et al., 1995), with the AFLP Analysis System Kit for Microorganisms (Invitrogen). This technique was performed according to Myburg et al. (2001), with fragment separation and detection on a Li-COR automated sequencer. After preliminary screening of candidate primers and analysis of reproducibility (the same profile was sucessfully generated in duplicate independent experiments for 8 strains), two selective primer combinations, EcoRI-A/ MseI-C and EcoRI-C/MseI-G, were selected for DNA digestion (60-70 ng) and generation of strains fingerprints. Digital AFLP gel images were scored for the presence and absence of bands in the range 50-550 bp, using AFLPQuantar (v1.05, KeyGene). Both monomorphic and non-monomorphic bands were scored. Bands with the same mobility were scored as identical. The 2 binary data sets were subsequently combined and imported into Treecon v1.3b (van de Peer and De Wachter, 1994), where a phylognetic tree was inferred using Li and Nei (Nei and Li, 1979) and Neighbour joining methods (Saitou and Nei, 1987), implemented in Treecon.

Preparation spore crops of thermoresistant strains and heat inactivation assay

The induction of sporulation of the isolates was achieved as described above. For the preparation of overnight cultures, 25 ml of NB in an Erlenmeyer of 100 ml were inoculated with a loopful of cell culture from the glycerol stock and incubated at 200 rpm at 37°C, 45°C, 50°C or 55°C for 16 h in a water bath. One milliliter of the overnight culture was spread onto plates containing the sporulation media. These were incubated at the same temperature as growth occurred, until more than 95% of the spores had been released from the sporangium (2 to 4 days). Spores were harvested with 10 mL of Phosphate Buffer (PB) 10 mM pH 7.0 (tenfold dilution of 0.1 M K_2HPO_4/ KH_2PO_4 buffer). Tween 80 1 ml/l (Merck) was added to PB for isolates of *B. cereus* complex. The spores were purified by 4 successive centrifugational washings with PB at 4°C, during 5 days, at 1,157 g for 7 min and at 4,629 x *g* for 5 min, in an Eppendorf centrifuge (5804 RF, Rf-34-6-38) cooled at 4°C. For strains M27, TT45 and *B. subtilis* DSM10, gradient centrifugations in Nycodenz (Axi-Shield PoC) were used to separate the spores from the vegetative cells and debris. Briefly, 500 µl of concentrated spore suspension were resuspended in Nycodenz 20% (w/v) and gently pipetted on the top of a 1:1, 30: 60% gradient, in a 15 ml conical tube (Greiner bio-one). The tubes were centrifuged at 4,100 x *g* in a centrifuge Firlabo SW 12R, set at 20°C for 45 min. Following this step, the spores were washed six times with demineralised water and resuspended in PB. Working spore crops concentration were adjusted to levels between 7-8.5 log CFU/ml and these were stored at 4°C.

A comparative study of spores wet heat-resistance was performed at 110°C for 5 min, as described by Oomes et al. (2007), using the tubes and glycerol bath specified before in the 'Materials and Methods' section. Next, appropriate tenfold dilutions of the tubes were cultivated in duplicate pour-plates of NB 2.6 g/l solidified with AB 15 g/l. Plates incubation occurred at 37°C and 45°C for 5 days and 50°C for 3 days. The confirmation of the initial number of spores in the working spore suspension was achieved through injection of an unheated tube, followed by sampling as described above. For 6 strains, thermal inactivation parameters

were additionally determined. For this purpose, fresh spore crops were prepared and heated at different ranges: M65 (95-105°C); M95 (100-107.5°C); M35 (100-115°C); M22 (110-120°C); M1 (110-122.5°C); and M112 (117.5°C-130°C). Survivors determination was done in duplicate plates.

Thermoresistant spores survival data analysis

The survival curves were fitted with the log-linear (equation 1) and the Weibull models (equation 2) (Mafart et al., 2002), according to the equations below:

$$\log N(t) = \log N(0) - \frac{t}{D} \quad (1)$$

$$\log N(t) = \log N(0) - \left(\frac{t}{\delta}\right)^n \quad (2)$$

Where D is the decimal reduction time, δ is the first decimal reduction time and n is a shape parameter. Both models were fitted to the inactivation data in Microsoft Excel. For the Weibull model, parameters estimation was done with Excel Solver add-in and these were verified in TableCurve2D v.2.03 (Jandel Scientific), from which the confidence intervals for n were also obtained. The log-linear model was fitted to all the data points, but when log N(t) was higher than log N(0) (strain M112) an additional fitting without N(0) was also included. The number of data points in the survival curves was between 5 and 9 (except for M22 at 120°C and M112 at 130°C, where only 4 data points were available for the determinations, due to a fast inactivation). The goodness of fit of the models was determined by the mean square error of the model index (MSE_{model}) and analysis of the 95% confidence interval for the n-value (te Giffel and Zwietering, 1999). The temperature dependence of D and δ was expressed in the z-value concept. This value was calculated by the negative reciprocal of the slope of the plot of log D (or logδ) against the temperature. To obtain reliable estimates for the z-value, D or δ values 2.5 times higher than the measured experimental duration (12 min) were excluded.

Results

Cocoa powder compliance with the microbiological quality guidelines

The microbiological composition of the tested commercial cocoa powder samples is presented in Table 1. Whereas yeasts were below the detection limit of 1 log CFU/g, other microbial groups were present at diverse levels. In total, eight out of the twenty-five samples did not comply with the microbiological quality guidelines: samples P10 and P20 had mould levels that just exceeded the maximum admissible levels; samples P1 to P4 and P8 were positive for *Enterobacteriaceae*; and sample P12 was positive for *Salmonella* (serotype nigeria).

Table 1. Estimated average levels (log CFU/g)[a] and occurrence of general microbiological groups in commercial cocoa powder samples

Sample code/ Origin[b]/ Fat[c]	pH	Total aerobic mesophiles	Total aerobic thermophiles	Total aerobic mesophilic spores	Total aerobic thermophilic spores	Yeasts	Moulds	Enterobacteriaceae in 1g[d]	Salmonella in 25 g[e]
P1/ E/ L	5.7	2.5 (0.01)	<1	2.0 (0.09)	<1	<1	<1	+	-
P2/ E/ L	6.9	2.0 (0.04)	<1	2.6 (0.36)	<1	<1	<1	+	-
P3/ E/ L	6.9	1.2 (0.21)	<1	2.1 (0.11)	<1	<1	<1	+	-
P4/ E/ ns	6.7	1.9 (0.16)	<1	1.8 (0.32)	<1	<1	<1	+	-
P5/ E/ ns	7.0	1.2 (0.09)	<1	0.4 (0.49)	<1	<1	<1	-	-
P6/ E/ ns	7.2	2.1 (0.14)	<1	2.2 (0.01)	<1	<1	<1	-	-
P7/ E/ ns	7.0	0.5 (0.71)	<1	0.9 (0.34)	<1	<1	<1	-	-
P8/ E/ ns	6.8	1.3 (0.00)	<1	1.0 (0.00)	<1	<1	<1	+	-
P9/ E/ ns	7.1	2.2 (0.00)	<1	2.2 (0.12)	0.4 (0.49)	<1	<1	-	-
P10/ E/ H	7.5	3.0 (0.15)	1.5 (0.28)	2.5 (0.06)	1.0 (0.43)	<1	**1.8** (0.59)	-	-
P11/ E/ ns	6.8	<1[f]	<1	0.4 (0.49)	<1	<1	<1	-	-
P12/ E/ L	6.9	3.7[g] (0.21)	3.2 (0.11)	3.7 (0.19)	3.1 (0.11)	<1	<1	-	+
P13/ E/ H	7.1	<1	<1	0.8 (1.13)	<1	<1	<1	-	-
P14/ SAM/ L	7.0	1.8 (0.12)	0.5 (0.71)	1.3 (0.38)	0.5 (0.71)	<1	<1	-	-
P15/ SAM/ L	6.9	1.8 (0.03)	0.6 (0.83)	2.0 (0.16)	0.7 (1.04)	<1	1.7[h] (0.37)	-	-
P16/ SAM/ ns	5.5	3.4 (0.03)	3.1 (0.21)	3.3 (0.06)	2.9 (0.34)	<1	<1	-	-
P17/ AF/ ns	6.8	1.2 (0.21)	<1	1.2 (0.28)	0.4 (0.49)	<1	<1	-	-
P18/ AF/ L	5.4	0.9 (0.34)	<1	1.0 (0.43)	0.4 (0.49)	<1	<1	-	-
P19/ AF/ L	5.2	1.6 (0.00)	1.1 (0.60)	1.4 (0.12)	0.6 (0.83)	<1	<1	-	-

Table 1. Estimated average levels (log CFU/g)[a] and occurrence of general microbiological groups in commercial cocoa powder samples *(continued)*

Sample code/ Origin[b]/ Fat[c]	pH	Total aerobic mesophiles	Total aerobic thermophiles	Total aerobic mesophilic spores	Total aerobic thermophilic spores	Yeasts	Moulds	Enterobacteriaceae in 1g[d]	Salmonella in 25 g[e]
P20/ AS/ ns	6.9	1.7 (0.17)	1.1 (0.12)	1.6 (0.19)	0.7 (0.21)	<1	**1.7** (0.25)	-	-
P21/ AS/ ns	6.9	0.5 (0.71)	<1	<1	0.7 (0.49)	<1	<1	-	-
P22/ AS/ ns	7.2	1.2 (0.71)	<1	0.4 (0.49)	<1	<1	<1	-	-
P23/ AS/ H	7.6	1.2 (0.09)	<1	<1	<1	<1	<1	-	-
P24/ AS/ H	7.3	2.6 (0.06)	1.4 (0.30)	2.6 (0.14)	2.4 (0.15)	<1	<1	-	-
P25/ AS/ ns	5.3	2.0 (0.03)	<1	1.9 (0.05)	0.7 (0.99)	<1	<1	-	-
Guidelines		**< 3.7**				**< 1**	**< 1.7**	**Absent in 1g**	**Absent in 25g**

[a] Mean values and standard errors of the means (between brackets) of two independent experiments are reported. For *Enterobacteriaceae* and *Salmonella* one single experiment was performed.

[b] E- Europe, SAM- South America, AF- Africa, AS- Asia.

[c] Fat content: Low (L)-10 to 12% (w/w); High (H)- 20 to 22% (w/w); not specified (ns) on the package.

[d, e] +/- presence/ absence.

[f] 1 log CFU/g correspond to the detection limits of total aerobic microorganisms and total aerobic spores. < 1 log CFU/g means that no colonies were found in any of the two independent experiments.

[g, h] For sample P12 the total aerobic mesophiles level without rounding is 3.65 log CFU/g and for sample P15 the moulds level is 1.66 log CFU/g. Therefore, both are considered (just) to comply with the guidelines.

Bold numbers and characters correspond to situations by which samples did not comply with the microbiological quality guidelines for cocoa powder. The microbiological quality criteria are indicated.

Occurrence and levels of total and thermoresistant spores

Apart from the Total Aerobic Microorganisms (TAM), also aerobic Total Spores (TS) were investigated. The level of mesophilic TS varied between 3.7 log CFU/g, in sample P12 and levels below the detection limit, in samples P21 and P23 (Table 1). On the other hand, thermophilic TS varied between 3.1 log CFU/g in sample P12 and levels below the detection limit for 12 out of the 25 samples.

Table 2 summarises the estimated average levels and the Most Probable Number (MPN) for mesophilic and thermophilic aerobic Thermoresistant spores (ThrS). Mesophilic ThrS were detected in nine out of the twenty-five samples, while thermophilic ThrS were detected in three. Plate count and MPN techniques showed a good correspondence, as the log numbers were within the 95% confidence interval of the MPN technique. For mesophilic ThrS, we found survivors until a heat treatment of 150°C-10 min (sample P2), whereas for thermophilic ThrS, survivors were found up to 110°C-10 min (sample P12). In total 61 thermoresistant spore-forming strains were isolated for further study, 48 of which isolated at 30°C and 13 at 55°C.

Thermoresistant spore-formers identification and molecular typing

All isolates were found to be Gram- and catalase- positive rods. Phase bright endospores were observed, with exception for strains M13 and TT50. For these strains, even with prolonged sporulation up to 15 days, increased $MnSO_4$ concentration from 3 to 6 g/l and incubation in the range 20°C-45°C for M13, and 45°C-70°C for TT50, the sporulation was not promoted, in the case of strain M13, or to a very insignificant level, in the case of strain TT50 (occasional spores were seen in several microscopic fields). For all other strains isolated at 55°C, higher spore yields were obtained at either 50°C or 45°C. For strains isolated at 30°C higher yield or faster spore production took place at 37°C. Figure 1 presents a phylogenetic tree based on the 16S rDNA sequence of the isolates (contigs varied between 1434 and 1490 bp). Four groups could be identified: group I, group II and group III, constituted, respectively, of members of *Bacillus subtilis complex*, *B. licheniformis* and *B. cereus* complex, and group IV by the single species lineages of *B. simplex* and *Geobacillus* spp. High percentages of similarities with known sequences in GenBank (98-100%) were found for the majority of the isolates (Supplementary data). In the case of members of *B. cereus* group these values were somewhat lower, but the blast search with sequences derived from the amplification with the forward primer resulted in 99% similarity to *Bacillus cereus* strains.

For 7 strains within the *B. subtilis* complex, 99% of similarity was found with *B. amyloliquefaciens* strains in the GenBank database. Considering that D-lactose fermentation is restricted among *B. subtilis* (Nakamura, 1987) and that *B. amyloliquefaciens* strains have a limited ability to use inulin (Logan and Berkeley, 1984), the isolates were presumptively identified as *B. amyloliquefaciens*. All the other strains were identified as members of *B. subtilis* complex (Roberts et al., 1994; Roberts et al., 1996). A high degree of 16S rDNA similarity exists among species of the *B. cereus* group as well (Lechner et al., 1998; Nakamura, 1998). However, none of the isolates had the typical *B. mycoides* colony morphology (Di Franco et al., 2002) and

all of them were motile, which allowed the exemption of species assignment as *B. mycoides* or *B. anthracis* (Sneath, 1986).

Table 2. Estimated average levels (log CFU/g) and Most Probable Number (MPN) of mesophilic and thermophilic thermoresistant spores in cocoa powder samples[a]

Incubation (°C)	Heat treatment	log CFU/g	log MPN/g	95% confidence limits	Sample code	Strain code of studied isolates
30	100°C-10 min	1.0	0.6	[-0.30; 1.41]	P20	M103, M104
		1.2	1.3	[0.85; 1.77]	P8	M21, M22
		1.2	1.3	[0.85; 1.77]	P15	M100, M101
		1.2	1.3	[0.85; 1.77]	P24	M107, M108, M109
		1.4	1.6	[0.99; 2.27]	P10	M31, M32, M34, M35
		1.5	1.6	[0.99; 2.27]	P9	M25, M27, M29, M30
		1.6	2.0	[1.35; 2.59]	P4	M13, M14, M16, M17
		1.7	2.0	[1.35; 2.59]	P12	M38, M40, M41, M42
		1.8	2.2	[1.65; 2.70]	P2	M1, M2, M4, M5, M7, M8
	100°C-30 min	<1[b]	0.6	[-0.30; 1.41]	P15	M110
		1.0	1.0	[0.34; 1.59]	P12	M70, M71
		1.3	1.0	[0.34; 1.59]	P4	M67, M68, M69
		1.4	1.3	[0.85; 1.77]	P2	M63, M64, M65, M66
	108°C-30 min	<1	0.6	[-0.30; 1.41]	P4	M90
		1.0	1.0	[0.34; 1.59]	P2	M88, M89
	110°C-10 min	1.0	0.6	[-0.30; 1.41]	P2	M94, M95
	120°C-10 min	0.7	0.6	[-0.30; 1.41]	P2	M98
	150°C-10 min	0.7	0.6	[-0.30; 1.41]	P2	M112
55	100°C-10 min	<1	0.6	[-0.30; 1.41]	P15	TT102
		<1	0.6	[-0.30; 1.41]	P20	TT106
		1.9	1.6	[0.99; 2.26]	P12	TT45, TT46, TT50, TT52, TT53
	100°C-30 min	1.9	1.6	[0.99; 2.26]	P12	TT77, TT80
	108°C-30 min	1.3	0.9	[0.26; 1.47]	P12	TT92, TT93
	110°C-10 min	1.0	0.6	[-0.30; 1.41]	P12	TT96, TT97

[a] Samples with total aerobic spores level below the detection limit, as ascertained in Table 1, were not included in the analysis (namely P23), while samples not shown in this table had all MPN tubes negative, corresponding to a MPN level below 0.5 log MPN/g and a higher confidence limit for the 95% confidence interval of 0.98 log MPN/g).
[b] 1 log CFU/g is the detection limit of the plate count based method.

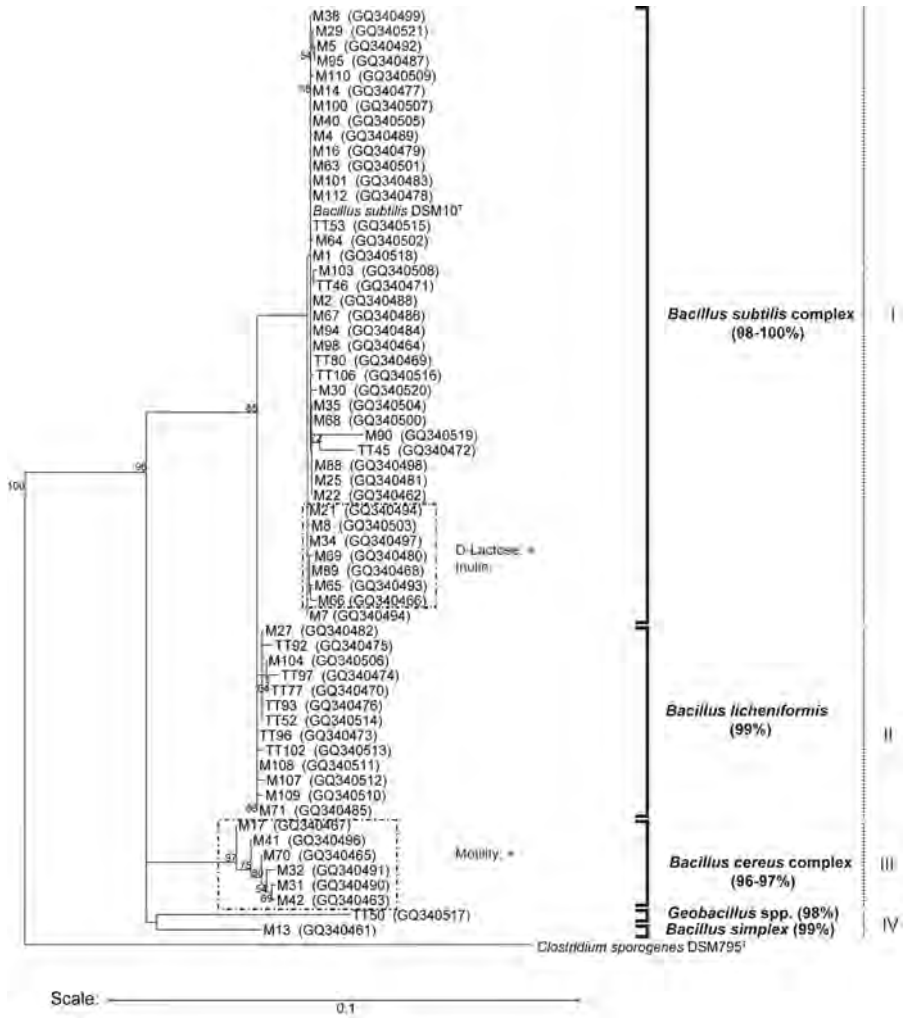

Figure 1. Phylogenetic tree of the thermoresistant spore-forming isolates based on nearly full 16S rRNA gene sequences. The phylogram was constructed with the tree builder tool in the Ribosomal Database project II (RDP). The bootstrap values are based on 100 bootstrap replications and are not shown on the nodes with lower than 50% bootstrap support. The scale bar (0.1) represents the number of nucleotide substitutions per sequence position. The sequence of *Clostridium sporogenes* DSM 795^T (type strain) was used as an outgroup. The GenBank accession numbers assigned to nucleotide sequences determined in this study, as well as the percentages of similarity with known sequences in GenBank are given between brackets. Lactose positive means that the isolates were able to ferment lactose, while inulin negative indicates the inability of its fermentation as ascertained by the API 50 CHB system. A positive motility indicates a diffuse growth spreading from the line of inoculation in the motility agar medium prepared as described in the materials and methods.

The 16S rDNA sequence for strain TT50 resulted in 98% similarity with sequences mainly from *Geobacillus thermoleovorans*, *G. kaustophilus* and *G. caldotenax.* Nonetheless, outcomes with equal similarity were also obtained for *G. stearothermophilus*, with which a distinct API 50 CHB profiles had been obtained (Supplementary data). Based on the gathered information, strain TT50 was solely classified as a member of the *Geobacillus* genus.

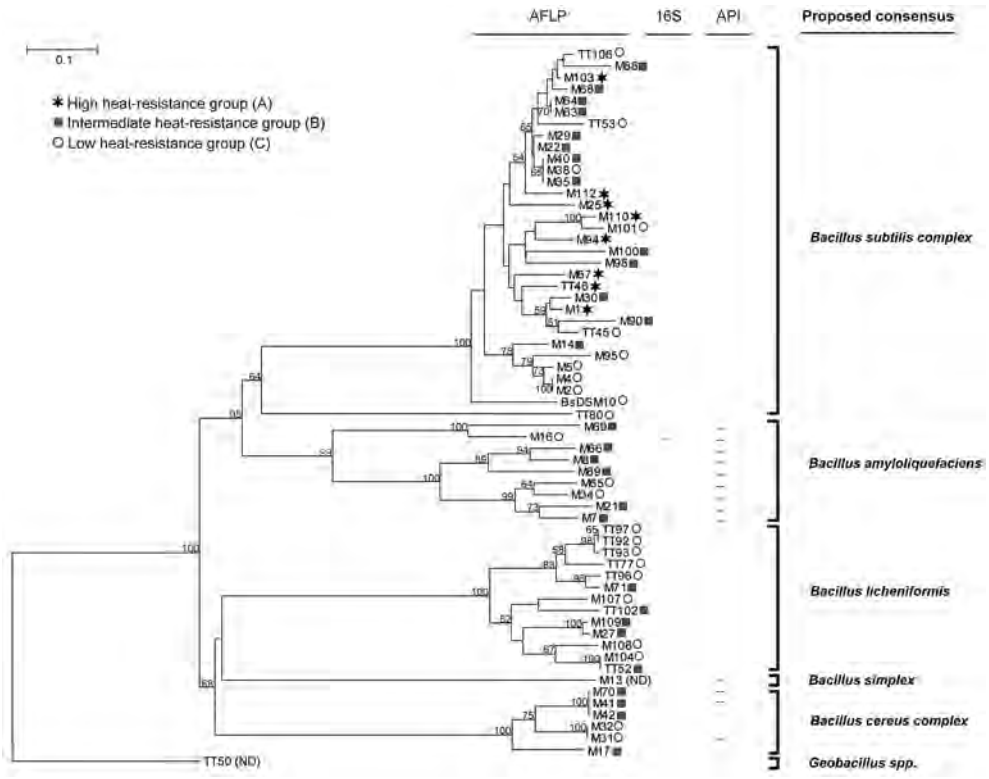

Figure 2. Thermoresistant isolates genetic relatedness on the basis of AFLP fingerprinting. The phylogenic tree was generated with the Treecon tool using Nei and Li's method, with 1000 bootstrap replications. Strain TT50 was used as an outgroup. Bootstrap values below 50% are not shown. Strains grouped by a vertical line are clonally related. Squares indicate the heat-resistance group according to Figure 3: High (*), intermediate (■) and low (○). The species assignment takes into account combined information from 16S rDNA sequence and phenotypical tests as described in materials and methods. (--) indicates the absence of correspondence between the technique and the AFLP clustering. ND, indicates that no spores could be produced. The abbreviations '16S' and 'API' show the correspondence of the 16S rDNA and API 50 CHB with the AFLP proposed consensus, respectively.

The AFLP analysis of the isolates yielded a total of 261 markers, of which 101 were obtained with the primer combination EcoRI-A/MseI-C and 160 with the primer combination EcoRI-C/MseI-G. The number of fragments per strain varied between 50 to 83 for the first primer combination and from 37 to 65 for the second. The clustering of the AFLP profiles is depicted in Figure 2. The tree displayed a similar topology with the one obtained based on the 16S rDNA sequences, with the difference that a subcluster of *B. amyloliquefaciens*, encompassing, as well, isolates M16 and M7 emerged. Twenty-eight polymorphic profiles were obtained within the *B. subtilis* complex (thirty-one strains), nine for *B. amyloliquefaciens* (nine strains), eleven for *B. licheniformis* (thirteen strains) and three for *B. cereus* group (six strains).

Thermoresistant spores heat-resistance properties

Figure 3 displays the comparative survival of the spore crops at 110°C for 5 min. The spores revealed substantially different heat-resistance: 24 spore crops out of 60, including the type strain of *B. subtilis*, were reduced to levels below the detection limit (group C); eight were highly heat-resistant, with a reduction lower than 1 log CFU/ml (group A); and for the remaining crops, reductions ranged between 1.3 and 5.0 log CFU/ml (group B). Strain M112 spores had an exceptionally high heat-resistance, showing rather an increase in spore counts as result of the heat treatment.

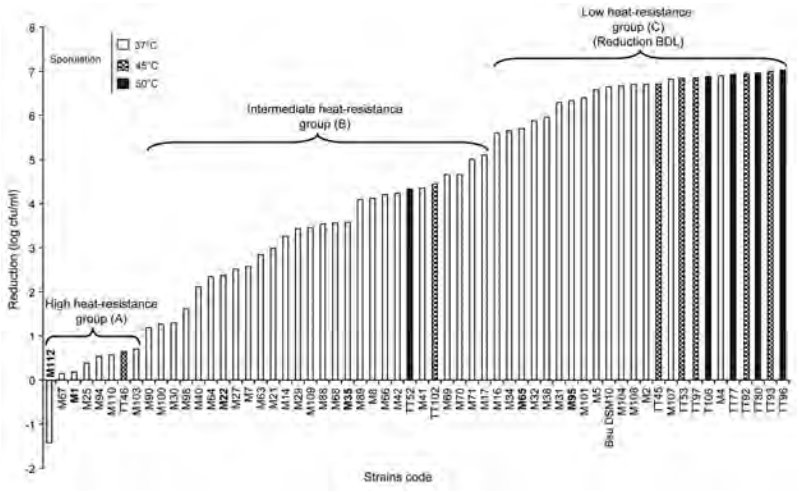

Figure 3. Thermal reduction (log CFU/ml) of thermoresistant spore crops at 110°C for 5 minutes. The spore batches were heated in phosphate buffer (PB) 10 mM, after sporulation at 37°C (☐), 45°C (▨) and 50°C (■). Reduction corresponds to the difference between the log number of unheated spores and the log number of survivors. BDL, corresponds to a reduction Below the Detection Limit of 1.7 log CFU/ml. Bsu DSM10 represents the type strain *Bacillus subtilis* DSM10. *D* and *z*-values were determined for the strains highlighted with an arrow.

For a selection of strains of the *B. subtilis* complex, representative of the 3 established heat-resistance groups, including one *B. amyloliquefaciens* strain, thermal kinetic parameters were determined. Figure 4 shows the survival curves for two of the most heat-resistant spore crops. In general, the survival plots yielded straight lines, except for strain M112 spores, which consistently displayed activation shoulders in the range 117.5-125°C.

Based on the criteria whether the confidence interval for the *n*-value of the Weibull model contained the value 1 (indicating that the Weibull model could be reduced to a log-linear model) and analysis of the MSE_{model} values (the lower, the better the fit of the applied model), the fitting performance of the log-linear and Weibull models was compared. The log-linear model was found to be adequate to fit the survival data in 18 out of 23 cases. For this reason, this model was chosen to globally characterise and compare the inactivation profile for all the spore crops (Table 3). Strain M112 spores were the most heat-resistant with an estimated D-value at 130°C of 0.18 min. Strains M65 and M95 spores were the most heat-sensitive and had comparable *D*-values in the range 100°C-105°C. The *z*-values varied between 6.4°C and 11.9°C, respectively, for M65 and M95 spore crops (Table 3 and Figure 5). For the three most heat-resistant spore crops, the estimated *z*-value varied between 6.5°C and 8.4°C. The regression coefficients for the *z*-value estimation were in general high (0.98-0.99), with exception for strains M65 and M112 spore crops, where these values were, respectively, 0.96 and 0.79 (Table 3).

Discussion

The trajectory of cocoa beans in the cocoa chain, determines not only the microbial load, but also the diversity and physiological properties of the microorganisms associated with cocoa beans derived-products, specifically cocoa powder.

It was found that commercial cocoa powder samples can have substantially different microbiological quality. For example, five of the analysed cocoa powder samples had extremely low levels of total aerobic mesophiles (lower than 1 log CFU/g or below the detection limit), while for one sample (P12) detectable levels up till 3.7 log CFU/g were registered. The latter, in conjunction with the detection of *Enterobacteriaceae*, moulds and *Salmonella* are indications of post-roasting contamination. In particular, the detection of *Salmonella* in one of the twenty-five samples is a matter of attention, due to the implication of low levels of *Salmonella* in outbreaks linked to chocolate (Werber et al., 2005).

For a few samples, namely P2, P3 and P15, the level of aerobic Total Spores (TS) was observed to be higher than that of Total Aerobic Microorganisms (TAM) (Table 1). This difference was particularly evident in sample P2, where, not only, a higher number of aerobic

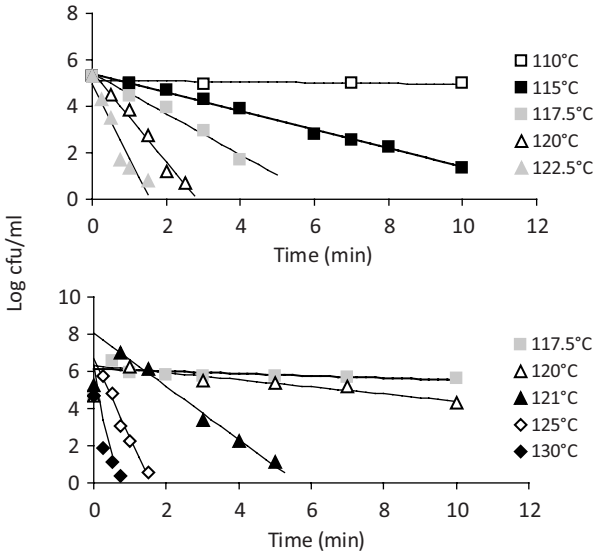

Figure 4. Survival curves of *Bacillus subtilis* complex spores crops of strains M1 (A) and M112 (B) in phosphate buffer 10 mM at 110°C (□),115°C (■), 117.5°C (■), 120°C (△), 121°C (▲), 122.5°C (▲), 125°C (◇) and 130°C (◆). The points correspond to average of duplicate plate count determinations.

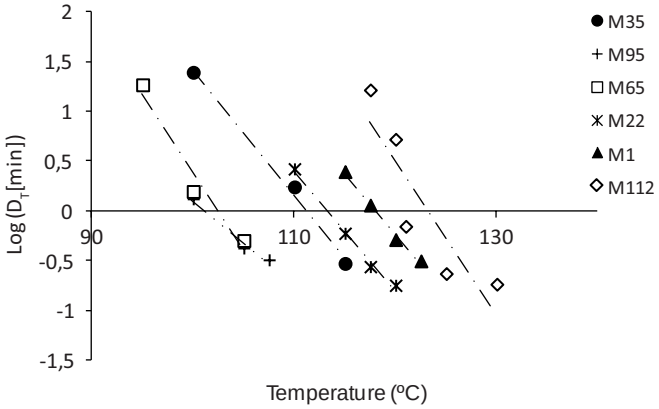

Figure 5. Least-square regression of log-transformed estimated *D*-values as function of the temperature for *Bacillus subtilis* complex spores in phosphate buffer 10 mM. *D*-values 2.5 times higher than the experimental duration were excluded. Spore crop of M112 (◇), M1 (▲), M22 (*), M35 (●), M65 (+) and M95 (+).

Thermoresistant spores (ThrS) were isolated, but also, the most heat-resistant spores were present (namely spores from strain M112) (Table 2). This difference could be explained by the phenomenon of spore activation, indicating the predominance of spore-formers in these cocoa powder samples. With respect to the occurrence of ThrS, these were detected in 36% of the samples, with levels which ranged from below 5% of TS (such as in the case of samples P12 and P24) to levels approximately the same as TS (for instance sample P8).

ThrS were isolated from 'Alkalised cocoa powders' (pH 7-8), but not from 'Natural cocoa powders' (pH 5-6) (Table 1-2). Whether this was a fortuitous outcome or influenced by the pH at which the cocoa powder suspension was heated, is something that would need to be investigated in a systematic way.

Table 3. *D*- and *z*-values calculated using the log-linear model for spores of *B. subtilis* complex strains in phosphate buffer 10 mM

Strain	Temperature (°C)	*D*-value (min)	r^2	MSE_{model}	*z* (°C)[a]	r^2	$D_{110°C}$ (min)
M65	95	18.4	0.849	0.00931	6.40	0.956	0.0669
	100	1.57	0.935	0.164			
	105	0.505	0.969	0.185			
M95	100	1.32	0.997	0.0216	11.9	0.978	0.183
	105	0.425	0.973	0.190			
	107.5	0.323	0.639	2.62			
M35	100	24.6	0.756	0.0148	7.94	0.994	1.44
	110	1.74	0.976	0.102			
	115	0.297	0.995	0.0251			
M22	110	2.64	0.996	0.0123	8.35	0.991	2.49
	115	0.596	0.983	0.0613			
	117.5	0.276	0.992	0.0509			
	120	0.179	0.970	0.177			
M1	110	57.3	0.263	0.0201	8.24	0.990	9.51
	115	2.48	0.994	0.0125			
	117.5	1.14	0.976	0.0263			
	120	0.516	0.982	0.0762			
	122.5	0.315	0.907	0.376			
M112[b]	117.5	16.4	0.491	0.0559	6.47	0.785	111
	120	5.23	0.948	0.0354			
	121	0.700	0.994	0.0578			
	125	0.235	0.986	0.0944			
	130	0.184	0.874	0.667			

[a] *D*-values 2.5 times higher than the experimental duration (12 min) were not included for the determination of the *z*-values.

[b] The kinetic parameters for the spore crop of M112 in the range 117.5-125°C are calculated without *N*(0).

Members of *Bacillus subtilis* complex were the predominant thermoresistant spore-forming species, with a fraction of 65.6% (40/61) of the total isolates, of which 14.8% (9/61) could be identified as *B. amyloliquefaciens*. *Geobacillus* spp. strain TT50 was the only true thermophilic strain in our collection. This is consistent with the reported Bacilli diversity during cocoa beans fermentation, where the participation of thermophilic species appears to be sporadic (Lima et al., 2011a).

The combination of the heat-resistance profile of the thermoresistant strains (Figure 3), with the AFLP clustering pattern (Figure 2) provided a range of thermal resistance and genotypic correspondences. Strains from *B. subtilis* complex formed the most heat-resistant spores and some of these isolates clustered in the same clade, for example, M67-M1. However, clades formed by strains producing spores with considerable different heat-resistance were also found, for instance, M30-TT45, M110-M101 and TT106-M68. Since the spores were produced under the same conditions, the nature of the underlying difference of spores heat-resistance should be bound to genetic factors that were not visible in the AFLP profiling. This genotypic diversity needs to be taken into account in strategies aimed at designing biomarkers for the detection and control of thermoresistant spore-formers within the food industry. Strikingly, also strains clonally related had spores with significantly different heat-resistance. This was the case of strains of *B. subtilis* complex M40/M38/M35 and strains M104/TT52 belonging to *B. licheniformis* species. It is conceivable that homoplasy and collision events in the AFLP profile might have hampered the discrimination of these strains (Meudt and Clarke, 2007), or that the strains constitute variants of the same strain (Avery, 2006). The approach to fully understand such phenomenon would require whole genome sequencing.

Some of the strains with a putative clonal origin, were isolated from samples of different countries or continents, such as *B. subtilis* complex strains M40-M35, and *B. licheniformis* strains M104-TT52. This could be an indication of a wide geographical distribution of the genotype in question, but the fact that cocoa powder is made up of blends of cocoa beans from different countries, together with the uncertainty of the clonal origin, imply that these observations must be interpreted carefully.

Another outcome from our results was the observation that after resporulation of the isolates, some spore crops displayed lower heat-resistance than expected, considering their isolation conditions (for instance, spores of *B. subtilis* complex strains M89 and M95 and *B. licheniformis* strains TT92, TT93, TT97 and TT96). This is not a new observation (Huemer et al., 1998). Different factors could explain this phenomenon, namely the fact that heat-resistance is not an absolute spore property, but dependent on the sporulation conditions and maturation circumstances after spores release from the mother cell (Bender and Marquis, 1985; Hornstra et al., 2009). Moreover, the occurrence of cell cultures prone to phenotypical heterogeneity, as mentioned before, could also influence the range of spores heat-resistance.

Six strains belonging to the *B. subtilis* complex, including one strain identified as *B. amyloliquefaciens*, were chosen to determine the thermal kinetic parameters *D* and *z*-values and illustrate the disparity in heat-resistance that can be found within this group (Table 3). The

comparison of the D_{ref} at 110°C shows the large difference in the heat-resistance of the strains spore crops. Strain M112 spores were the most heat-resistant under the studied conditions, with an estimated $D_{110°C}$ of 111 min. Its spore crop heat-resistance was 7 times higher than the one of another highly heat-resistant *B. subtilis* strain (A163), which had been isolated from a sterilised peanut chicken soup ($D_{120°C}$ of 0.7 min) (Kort et al., 2005; Oomes et al., 2007). Though, to precisely compare the heat-resistance of these spores, they would have to be produced under the same conditions.

The persistence of shoulders in the inactivation profile of strain M112 may be explained by spores activation. Pre-activation treatments to avoid shoulders were deliberately not applied, in order to characterise the spore crop resilience at UHT range, for which the estimated *D*-values can be useful in the processing of cocoa-derived products.

We concluded that spores from the *B. subtilis complex* have the potential of surviving subsequent preservation treatments, challenging downstream product quality stability, such as in chocolate milk.

It can be expected that the systematic study of highly heat-resistant spores in cocoa powder can contribute to better targeted processing and quality optimisation of cocoa-derived products.

Acknowledgements

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Supplemental data

Microbiota of cocoa powder with particular reference to aerobic thermoresistant spore-formers

Table S1. Phenotypical and genotypical identification of thermoresistant spore-forming isolates*

	Phenotype			Genotype			
Strain code	**Taxon**	**ID (%)**	**T index**	**Closest relative in GenBank**	**Similarity (%)**	**Closest accession no.**	**Complementary discussion/ information[a]**
M1	*Bacillus subtilis/amyloliquefaciens*	99.9	0.88	*Bacillus subtilis*	99	EF656456.1	
M2	*Bacillus subtilis/amyloliquefaciens*	96.1	1	*Bacillus subtilis*	99	EF656456.1	
M4	Bacillus subtilis/amyloliquefaciens	96.1	1	*Bacillus subtilis*	99	FJ263034.1	
M5	*Bacillus subtilis/amyloliquefaciens*	96.4	0.98	*Bacillus subtilis*	99	EF656456.1	
M7	*Bacillus subtilis/amyloliquefaciens*	99.5	0.89	Bacillus subtilis	99	EU257436.1	
M8	*Bacillus subtilis/amyloliquefaciens*	96.6	0.93	*Bacillus amyloliquefaciens*	99	FJ436406.1	D- Lactose: +; Inulin: -
M13	No identification			*Bacillus simplex*	99	FJ455076.1	
M14	*Bacillus subtilis/amyloliquefaciens*	96.1	1	*Bacillus subtilis*	99	AL009126.3	
M16	*Bacillus subtilis/amyloliquefaciens*	99.9	0.83	*Bacillus subtilis*	99	AY881638.1	
M17	*Bacillus cereus 1*	76.2	0.76	*Bacillus cereus*[b]	96	CP000227.1	
M21	*Bacillus subtilis/ amyloliquefaciens*	98.8	0.89	*Bacillus amyloliquefaciens*	99	AB301006.1	D- Lactose : +; Inulin: -
M22	*Bacillus subtilis/amyloliquefaciens*	59.9	0.82	*Bacillus subtilis*	99	EF656456.1	
M25	*Bacillus subtilis/amyloliquefaciens*	99.7	0.87	*Bacillus subtilis*	99	AY917141.1	
M27	*Bacillus licheniformis*	99.9	0.91	*Bacillus licheniformis*	99	CP000002.3	
M29	*Bacillus subtilis/amyloliquefaciens*	96.1	1	*Bacillus subtilis*	99	AL009126.3	
M30	*Bacillus subtilis/amyloliquefaciens*	84.9	0.55	*Bacillus subtilis*	99	AL009126.3	
M31	*Bacillus mycoides*	40	0.97	*Bacillus cereus*[b]	97	FJ435217.1	Motility: +
M32	*Bacillus cereus* 1	57.6	1	*Bacillus cereus*[b]	97	FJ435217.1	
M34	*Bacillus subtilis/amyloliquefaciens*	98.8	0.89	*Bacillus amyloliquefaciens*	99	AB301006.1	D- Lactose: +; Inulin: -

Table S1. Phenotypical and genotypical identification of thermoresistant spore-forming isolates* *(continued)*

	Phenotype			Genotype			
Strain code	**Taxon**	**ID (%)**	**T index**	**Closest relative in GenBank**	**Similarity (%)**	**Closest accession no.**	**Complementary discussion/ information[a]**
M35	*Bacillus subtilis/amyloliquefaciens*	95	0.92	*Bacillus subtilis*	99	AL009126.3	
M38	*Bacillus subtilis/amyloliquefaciens*	95	0.92	*Bacillus subtilis*	98	AB018484.1	
M40	*Bacillus subtilis/amyloliquefaciens*	97.5	0.82	*Bacillus subtilis*	100	AY881638.1	
M41	*Bacillus mycoides*	71.9	1	*Bacillus cereus*[b]	97	CP001177.1	Motility: +
M42	*Bacillus cereus 1*	95.8	0.87	*Bacillus cereus*[b]	96	FJ435217.1	
TT45	*Bacillus subtilis/amyloliquefaciens*	99.7	0.81	*Bacillus subtilis*	99	AY881638.1	
TT46	*Bacillus subtilis/amyloliquefaciens*	98.7	0.68	*Bacillus subtilis*	99	EF656456.1	
TT50	*Paenibacillus polymyxa*	84.7	0.71	*Geobacillus thermoleovorans* *Geobacillus kaustophilus* *Geobacillus caldotenax*	98 98 98	AY550103.1 BA000043.1 AY608937.1	
TT52	*Bacillus licheniformis*	99.9	0.69	*Bacillus licheniformis*	99	AJ582721.1	
TT53	*Bacillus subtilis/amyloliquefaciens*	96.1	1	*Bacillus subtilis*	99	EF656456.1	
M63	*Bacillus subtilis/amyloliquefaciens*	97.8	0.69	*Bacillus subtilis*	99	AL009126.3	
M64	*Bacillus subtilis/amyloliquefaciens*	97.8	0.69	*Bacillus subtilis*	99	AL009126.3	
M65	*Bacillus subtilis/amyloliquefaciens*	98.8	0.89	*Bacillus amyloliquefaciens*	99	AB301006.1	D- Lactose: +; Inulin: -
M66	*Bacillus subtilis/amyloliquefaciens*	70.4	0.79	*Bacillus amyloliquefaciens*	99	FJ436406.1	Lactose: +; Inulin: -
M67	*Bacillus subtilis/amyloliquefaciens*	99.9	0.69	*Bacillus subtilis*	99	EF656456.1	
M68	*Bacillus subtilis/amyloliquefaciens*	64.7	0.87	*Bacillus subtilis*	99	AL009126.3	
M69	*Bacillus subtilis/amyloliquefaciens*	88.2	0.8	*Bacillus amyloliquefaciens*	99	AB201122.1	D- Lactose: +; Inulin: -
M70	*Bacillus mycoides*	42.2	0.78	*Bacillus cereus*[b]	96	FJ393296.1	Motility: +
M71	*Bacillus licheniformis*	99.9	0.87	*Bacillus licheniformis*	99	EU344793.1	

Table S1. Phenotypical and genotypical identification of thermoresistant spore-forming isolates* *(continued)*

	Phenotype			Genotype			
Strain code	**Taxon**	**ID (%)**	**T index**	**Closest relative in GenBank**	**Similarity (%)**	**Closest accession no.**	**Complementary discussion/ information[a]**
TT77	*Bacillus licheniformis*	69.1	0.64	*Bacillus licheniformis*	99	AY071857.2	
TT80	*Bacillus subtilis/amyloliquefaciens*	98	0.86	*Bacillus subtilis*	99	AL009126.3	
M88	*Bacillus subtilis/amyloliquefaciens*	97.8	0.92	*Bacillus subtilis*	99	AY881638.1	
M89	*Bacillus subtilis/amyloliquefaciens*	94.6	0.87	*Bacillus amyloliquefaciens*	99	FJ436406.1	D- Lactose: +; Inulin:-
M90	*Bacillus subtilis/amyloliquefaciens*	80.4	0.74	*Bacillus subtilis*	98	FJ797594.1	
TT92	*Bacillus licheniformis*	99.9	0.91	*Bacillus licheniformis*	99	AY071857.2	
TT93	*Bacillus licheniformis*	80	0.49	*Bacillus licheniformis*	99	EU344793.1	
M94	*Bacillus subtilis/amyloliquefaciens*	53.3	0.91	*Bacillus subtilis*	99	AL009126.3	
M95	*Bacillus subtilis/amyloliquefaciens*	96.1	1	*Bacillus subtilis*	99	EF656456.1	
TT96	*Bacillus licheniformis*	99.9	0.95	*Bacillus licheniformis*	99	EU344793.1	
TT97	*Bacillus licheniformis*	99.8	0.87	*Bacillus licheniformis*	99	CP000002.3	
M98	*Bacillus subtilis/amyloliquefaciens*	99.6	0.8	*Bacillus subtilis*	99	AL009126.3	
M100	*Bacillus subtilis/amyloliquefaciens*	99.7	0.75	*Bacillus subtilis*	99	AL009126.3	
M101	*Bacillus subtilis/amyloliquefaciens*	95.4	0.98	*Bacillus subtilis*	99	AL009126.3	
TT102	*Bacillus licheniformis*	99.9	0.93	*Bacillus licheniformis*	99	EU344793.1	
M103	*Bacillus subtilis*	59.9	0.82	*Bacillus subtilis*	99	AY917141.1	
M104	Bacillus licheniformis	99.7	0.77	*Bacillus licheniformis*	99	EF105377.1	
TT106	*Bacillus subtilis/amyloliquefaciens*	99.8	0.87	*Bacillus subtilis*	99	AY881638.1	
M107	*Bacillus licheniformis*	99.8	0.87	*Bacillus licheniformis*	99	AY071857.2	

Table S1. Phenotypical and genotypical identification of thermoresistant spore-forming isolates* *(continued)*

	Phenotype			Genotype			
Strain code	**Taxon**	**ID (%)**	**T index**	**Closest relative in GenBank**	**Similarity (%)**	**Closest accession no.**	**Complementary discussion/ information[a]**
M108	*Bacillus licheniformis*	99.9	0.9	*Bacillus licheniformis*	99	EU344793.1	
M109	*Bacillus licheniformis*	99.9	0.85	Bacillus licheniformis	99	EU344793.1	
M110	*Bacillus subtilis/amyloliquefaciens*	99.3	0.91	*Bacillus subtilis*	99	AL009126.3	
M112	*Bacillus subtilis/amyloliquefaciens*	98.9	0.63	*Bacillus subtilis*	99	AY881638.1	
DSM10	*Bacillus subtilis/amyloliquefaciens*	99.8	0.86	NP			
DSM5934	*Geobacillus stearothermophilus*	99.9	0.95	NP			

* The phenotype is based on the results of API 50 CHB test. The genotype corresponds to nearly full 16S rRNA gene sequences that were compared with similar sequences in the RDP and GenBank databases.

[a] The lactose and inulin results were retrieved from API. They were highlighted to support the classification of the strains as *Bacillus amyloliquefaciens*. For *B. subtilis* strains acid production from lactose was negative but inulin was positive.

[b] For *Bacillus cereus* complex strains, the blast search with the sequence derived from the forward primer resulted in 99% similarity to *Bacillus cereus*.

Underlined fonts refer to non matching outcomes between the API tests and 16S rRNA gene sequences classification.

NP- Not Performed.

Chapter 5

Microbiota dynamics and diversity at different stages of cocoa bean industrial processing to cocoa powder

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We sampled a cocoa powder production line to investigate the impact of processing on the microbial community size and diversity at different stages. Classical microbiological methods were combined with 16S rRNA gene PCR-Denaturing Gradient Gel Electrophoresis, coupled to clone library construction, to analyze the samples. Aerobic Thermoresistant Spores (ThrS; 100°C-10 min) were also isolated and characterised (identity, genetic diversity and spore heat-resistance), in view of their relevance for the quality of downstream heat-treated cocoa-flavoured drinks.

In the nibs (broken, shelled cocoa beans), average levels of Total Aerobic Microorganisms (TAM) (4.4-5.6 log CFU/g) and aerobic Total Spores (TS; 80°C-10 min) (4.3-5.5 log CFU/g) were significantly reduced ($P< 0.05$) as result of alkalising; while fungi (4.2-4.4 log CFU/g) and *Enterobacteriaceae* (1.7-2.8 log CFU/g) were inactivated to levels below the detection limit, remaining undetectable throughout processing. Roasting decreased further the levels of TAM and TS, but these increased slightly during subsequent processing.

Molecular characterization of bacterial communities based on enriched cocoa samples revealed a predominance of *Bacillaceae*, *Pseudomonadaceae and Enterococcaceae* members. Eleven species of ThrS were found, but *Bacillus licheniformis* and the *B. subtilis* complex were prominent and revealed large genetic heterogeneity.

We concluded that the microbiota of cocoa powder resulted from microorganisms which could have been initially present in the nibs, as well as microorganisms which originated during processing. *B. subtilis* complex members, particularly of subspecies *subtilis*, formed the most heat-resistant spores. Their occurrence in cocoa powder needs to be considered to ensure the stability of derived products, such as Ultra-High Temperature treated chocolate drinks.

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Introduction

Cocoa beans, the fruit seeds from the tropics' endemic tree *Theobroma cacao* L., are highly prized, as the solids and the fat provide the basis for cocoa powder and chocolate production. Following bean fermentation and drying in the countries of origin, cocoa beans are transported to industrial plants where semi-manufactured or finished products are obtained for commercialization (Kamphuis, 2009).

The microbiota evolving during cocoa bean fermentation has been studied extensively, owing to its importance in the formation of the precursor compounds of the cocoa flavour (Lima et al., 2011a). Different studies have shown that during the process yeasts, lactic acid bacteria, acetic acid bacteria, as well as members of the genus *Bacillus* are typically present.

Given the field conditions under which cocoa bean fermentations are conducted, microbial ecology studies have been focused on the prevalence of mycotoxins in cocoa bean and derived products (Copetti et al., 2011a; Sánchez-Hervás et al., 2008), as well as survival of *Salmonella* during manufacture and storage of cocoa products (Krapf and Gantenbein-Demarchi, 2010; Park et al., 1979). Far less attention has been paid to the impact of cocoa bean industrial processing on the microbial community profile at the different stages of production and finished products. Besides being relevant from a quality and safety point of view, systematic microbiological surveys of 'industrial ecosystems' have fundamental interest, as they may grant the opportunity to identify microorganisms with novel physiological traits (Manaia and Moore, 2002; van Zuijlen, 2011).

Thus, the first aim of this work was to unravel microbial changes during cocoa bean industrial processing, focusing on population levels, structure and diversity. As a case study, we selected a cocoa powder production line in the Netherlands operating under the scheme outlined in Figure 1. The general principle of production (ICMSF, 2002; Kamphuis, 2009) consists of blending of cocoa beans from different origins, cleaning from impurities and applying surface heat treatments to facilitate shell removal. Subsequently, the beans are broken, separated from the shell (nibs) and then undergo alkalising, by immersion in an alkaline solution (e.g. potassium carbonate) at high temperatures (up till 100°C). After alkalising, the nibs are pre-dried and roasted (nib final temperature is typically between 105°C and 140°C). The roasted nibs are then finely ground into a mass (liquor), which is subsequently partially defatted. The resulting cake is finally pulverized and packed as commercial cocoa powder.

Pre-roasting bean treatments (Figure 1, operations 4-5) and roasting (Figure 1, operation 6) are critical control points in the process, and these processes are validated to ensure that, in addition to the primary development of the typical cocoa flavour, destruction of *Salmonella* takes place (ICMSF, 2002).

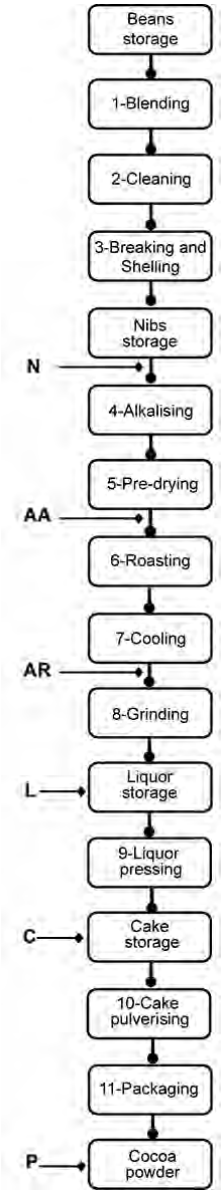

Figure 1. Diagram of operations involved in cocoa powder production in the studied factory. The sampled stages are Nibs (N), After Alkalising and pre-drying (AA), After Roasting (AR), Liquor storage (L), Cake storage (C) and Powder (P).

Upon roasting, only spore-formers of the genus *Bacillus* and relatives may survive (Barrile et al., 1971). For this reason, the quantification of Total Aerobic Microorganisms (TAM) is considered to be a suitable indicator of both roasting and global process conformity (ICMSF, 2002). However, in the case of specific applications, such as manufacturing of Ultra-High Temperature (UHT) treated cocoa-flavoured drinks, it is also of particular interest to understand the fate of highly heat-resistant spores during processing, since such spores may survive UHT treatments and pose a risk for the stability of final drinks (Lima et al., 2011b; Witthuhn et al., 2011). In the context of a previous work, where the microbiological composition of commercial cocoa powder had been investigated, we defined aerobic 'Thermoresistant Spores' (ThrS) as spores able to survive a heat treatment of at least 100°C for 10 minutes, as opposed to the standard treatment of 80°C for 10 minutes for Total spores (TS) quantification (Lima et al., 2011b). This more stringent treatment proved to be important, as appreciable differences were found when compared to the standard.

Presently, it is still not very well understood where ThrS present in cocoa powder originate from, that is, whether they are already present in cocoa beans at the onset of processing, or whether they are introduced at later stages. In order to design adequate intervention measures for ThrS control in cocoa powder, it is necessary to track them during processing, determine their identity and ascertain the persistence of specific genotypes. Therefore, the second aim of this work was to investigate the occurrence, levels and genotypic characteristics of ThrS.

Classical microbiological methods were used to quantify the levels of TAM, TS and ThrS during processing. Attention was also given to the occurrence of specific microbial groups that are monitored in commercial cocoa powder, namely yeasts and moulds, and *Enterobacteriaceae* (Dijk et al., 2007). To characterise the microbial diversity, PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE), coupled to clone library construction, was applied to analyze the samples. Isolated thermoresistant spore-forming strains were subjected to molecular methods for species identification and typed to unveil genetic heterogeneity. Of selected strains, spores were produced under standardized conditions and their wet heat-resistance was compared.

Materials and methods

Cocoa bean processing and sampling

A cocoa powder production line in the Netherlands was sampled at three different weeks between October and November 2008 (3 independent batch productions). The samples were collected at six stages, either at conveyors or at bulk storage stages as shown in Figure 1 (hereafter, when referring to the processing stages the first letters are capitalized). The final cocoa powder fat content was between 10-12% (w/w). During the first three stages we used a tracking system, which allowed us to collect samples in the same position in the conveyor. After grinding (Figure 1, operation 8), samples corresponding to the same batch production were taken at Liquor, Cake and Powder, based on residence time data history. At each sampling site, approximately 200 g of sample were aseptically collected in triplicate into sterile polystyrene jars and transported to the laboratory at room temperature. All cocoa samples were subsequently stored at room temperature, during which time no microbial growth took place (data not shown).

Enumeration and detection of microbial groups

The microbial enumerations were performed within 1 week after the samples from the same batch had been obtained. The samples were aseptically pooled together and mixed in a sterile polyethylene lockable sampling bag. In the case of cocoa liquor (solid at room temperature), the samples were transferred to polyethylene bags and tempered at 40°C in a water bath for 30 min prior to careful pooling (Dijk et al., 2007). This allowed collecting the necessary aliquot for analysis. When a second liquor aliquot was needed (all microbial enumerations were performed in duplicate) the liquor in the bag was first carefully crushed on a hard surface and the aliquot was taken subsequently. Next, the aliquot was homogenized with the diluent tempered to 40°C, after being allowed to melt for 30 min (17). All samples were diluted, plated and enumerated as described earlier (Lima et al., 2011b). Samples within a given batch were processed simultaneously.

Mesophilic (30°C for 3 days) and thermophilic (55ºC for two days) Total Aerobic Microorganisms (TAM) were enumerated by culturing 1 ml of the appropriate decimal dilution in duplicate pour-plates of Plate Count Agar (PCA, Oxoid), as performed previously (Lima et al., 2011b). An exception was the addition of a top layer of 1.5% Technical Agar (TA, Oxoid) for plates incubated at 55°C, in order to restrict colony spreading. Yeasts and moulds were enumerated in duplicate pour-plates of Dichloran-Glycerol Agar Base medium (DG18, Oxoid), supplemented with glycerol (49782, Sigma) and chloramphenicol (Oxoid) as indicated by the manufacturer, after incubation at 25°C for 5 days. *Enterobacteriaceae* were enumerated at 37°C, according to the ISO standard method 21528-1:2004 (ISO, 2004a), using Violet Red Bile Glucose Agar (VRBGA, Oxoid), an inhibitory medium for Gram-positives. To confirm the presence of *Enterobacteriaceae*, the oxidase and glucose fermentation tests were carried out on presumptive colonies, as recommended by the ISO method.

Aerobic Total Spores (TS; 80°C-10 min) and aerobic Thermoresistant spores (ThrS; 100°C-10 min) were determined as described before (Lima et al., 2011b). TS were determined based on the same primary dilution as for general microbial groups, with application of the heat treatment within 3 min after sample homogenization. In the case of ThrS, fresh primary dilutions per sample were prepared. TS plates were incubated as performed for TAM, while ThrS plates were incubated at 30°C for 5 days and 55°C for 3 days.

ThrS plates from the first duplicate experiment, corresponding to a number of colonies below or equal to 50, were selected to perform isolations. The square root of the total number of colonies was randomly picked up from both duplicate plates. The purity of the colonies was confirmed after dilution streaks on Nutrient Agar (NA, Oxoid) and these were stored as previously performed (Lima et al., 2011b). The strains were coded to reflect the isolation temperature, stage and batch production (e.g. 30N1-1 corresponds to a strain isolated from a plate incubated at 30°C, from Nibs, batch I and constitutes the isolate number one).

Molecular characterization of thermoresistant spore-formers

16S rRNA gene sequences were obtained with 8F and 1522R universal primers (Edwards et al., 1989) as described previously (Lima et al., 2011b) and these were compared to similar sequences of type and cultured strains in GenBank database at the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/), using the BLAST method (Altschul et al., 1990).

The genetic diversity of the strains was analyzed using the Amplified Fragment Length Polymorphism (AFLP) method (Vos et al., 1995), with the AFLP Analysis System Kit for Microorganisms (Invitrogen). This technique was performed as given earlier (Lima et al., 2011b), with exception that the primer combinations *Eco*RI-A/*Mse*I-G and *Eco*RI-C/*Mse*I-G were used, resulting in high number of bands for the large majority of the species (30-75). The reproducibility of this AFLP experimental set-up had been successfully confirmed before (Lima et al., 2011b). Digital AFLP gel images were scored for dominant markers using AFLPQuantar (v1.05, KeyGene) and a phylogenetic tree was inferred using Nei and Li, and Neighbor joining methods with Treecon v1.3b (van de Peer and De Wachter, 1994).

A selection of strains from the *Bacillus subtilis* complex (Rooney et al., 2009) and a strain of *B. licheniformis* were further identified to species or subspecies level by partial sequencing of the gyrase A gene (*gyrA*). This was performed by the Bacteria Collection Laboratorium Voor Microbiologie from Ghent University (BCCM/LMG), using the primers and PCR conditions described by Chun and Bae (2000).

Preparation of Thermoresistant spore crops and wet heat-inactivation assay

Twenty-two strains isolated at After Alkalising, After Roasting and Powder were selected to assess the wet heat-resistance capacity of their spore crops. The spore crops were produced on NA medium supplemented with different minerals (Cazemier et al., 2001). Mesophilic and thermotolerant strains were sporulated at 37°C for 72 h, whereas a thermophilic strain was

sporulated at 55°C for 48 h. This resulted in higher than 95% free phase bright spores for all strains. The spores were harvested, washed and stored at 4°C in the dark for one month prior to the heat-inactivation assay (Lima et al., 2011b), during which time all spores remained phase-bright, as monitored by phase-contrast microscopy. The concentration of working spore crops was adjusted to 7-8 log CFU/ml, based on the result of plate counts in NB 2.6 g/l solidified with Agar Bacteriological (AB, Oxoid) 15 g/l, after plates incubation for 3 days at 37°C and 2 days at 55°C. The comparison of heat-resistance was performed by subtracting the number of viable spores after heating at 110°C-5 min from the number before heating as performed previously (Lima et al., 2011b) .

Microbial genomic DNA extraction from the cocoa samples

Five grams of nibs, liquor, cake and cocoa powder, and 10 g of nibs from After Alkalising and After Roasting were aseptically weighed in a flow cabinet into a stomacher bag with filter (SM2, M-Tech Diagnostics). The samples were subsequently resuspended in 45 ml of NB by manually massaging the bags for 1 min and placed in an aluminium tin. Next, the bag was covered with a sterile polyethylene bag (SM1, M-Tech Diagnostics) and incubated overnight for 16 h under static conditions. Duplicates of each sample type were prepared for incubations at 30ºC and 55ºC. Negative controls of the medium were also included. Beakers with sterile water were placed next to the samples incubated at 55°C to reduce evaporation. Following overnight enrichment, the cocoa suspensions were manually homogenized in the bag, after which the content was aseptically transferred to a 50 mL falcon tube (Greiner Bio-One). Next, the tubes were centrifuged at 22°C for 5 min at 200 x *g* (Eppendorf 5804 RF, Rf-34-6-38). Subsequently, the supernatants (the phase of interest) were pipetted to clean falcon tubes. The remaining pellets were washed (200 g) with 10 ml of PBS buffer ($Na_2HPO_4.2H_2O$ 57.7 mM and $NaH_2PO_4.H_2O$ 42.3 mM, pH 7) and these supernatants were also added to the clean falcon tube. The new tubes were then centrifuged at 9,000 x *g* for 5 min to collect the cell biomass. After centrifugation, the cell biomass was washed twice (9,000 x *g*) with 10 ml of PBS buffer. In the case of liquor, cake and powder samples, the cell biomass was diluted 1:5 (2 ml of sample: 8 ml of PBS buffer) before twice washing. This was done to reduce the slurry-like viscosity of the samples. The resulting cell pellets were subsequently used for genomic DNA extraction.

The DNA extraction method by Wang et al. (2008) was tested. However, it did not result in suitable DNA extracts for consistent PCR amplification, even after repeated DNA purification steps (tris-phenol and ethanol precipitation), use of Bovine Serum Albumine (Fermentas) in the PCR mix, or use of Phusion® polymerase (FINNZYMES). The FastDNA® Spin Kit for soil (MP Biomedical) resulted in DNA freed from cocoa impurities and allowed consistent DNA amplification. The kit was used following the manufacturer's instructions, after subdividing each pellet resuspended in the provided buffers through 3-5 tubes of lysing matrix. The bead-beater FastPrep® Instrument (MP Biomedicals) was used during the extraction. Purified DNA was eluted (30-50 µl), pooled and, when necessary, diluted in sterilized milli-Q water (30-50 ng/µl). The DNA extracts were stored at -20°C.

PCR-Denaturing Gradient Gel Electrophoresis analysis (DGGE)

Microbial genomic DNA isolated from the enriched cocoa samples was used directly to amplify the V6-V8 region of the bacterial 16S rRNA gene, using the set of primers described by Nubel et al. (1996). PCR mixtures (50 µl) included 2 U of *Taq* DNA polymerase (Native, Fermentas), 5 µl of 10x *Taq* buffer, 1.5 mM $MgCl_2$ (Fermentas), 200 µM of deoxynucleoside triphosphate mix (Fermentas), 0.4 µM of each primers, 1 µl template DNA (10 to 50 ng/µl) and sterile Milli-Q water. The amplifications were performed in a GeneAmp PCR System 9700 (Applied Biosystems) with the following program: 5 min at 94°C; 35 cycles, each made-up of 30 s at 94°C, 20 s at 56ºC and 1 min at 72°C; and a final extension of 7 min at 72°C. DGGE was performed using the Dcode System apparatus (Bio-Rad), according to the method initially described by Muyzer et al. (1993) and as further modified by Martín et al. (2007). Five microliters of PCR product were loaded in the gel, with exception of samples from After Alkalising, After Roasting and Liquor, where 20 µl were pipetted. A marker made-up of a mixture of amplicons obtained from diverse bacterial pure-cultures, was included to cover the region from low to high gradient concentration and facilitate gel normalization. The gels were silver-stained according to Sanguinetti et al. (1994) and after overnight drying at 55°C, the gel images were digitized on a GS 800 calibrated Densitometer (BioRad) and normalized using Bionumerics software v.4.0 (Applied Maths) for data interpretation.

DNA clone library construction, sequencing and annotation of DGGE fingerprints

Microbial genomic DNA from Nibs, After Alkalising, After Roasting, Cake and Powder were amplified with 16S rRNA gene 8F and 1522F universal primers. PCR mixtures were set-up as described above and amplified using the following conditions: 5 min at 95°C; 30 cycles, each made-up of 30 s at 95°C, 20 s at 56°C, 1 min at 72°C and a final extension of 30 min at 72°C. Purified DNA extracts (QIAquick PCR Purification Kit, Qiagen) were stored overnight at 4°C and cloned in *Escherichia coli* JM109 High Efficiency Competent Cells (Promega) with the pGEM-T Easy Cloning Kit (Promega), following the manufacturer's instructions. A number of colonies covering at least five times the total number of bands at each stage were randomly picked up from the plates of each sample (recombinant colonies were distinguished by their white color). These plasmid-harboring clones were transferred with a sterile toothpick into 50 µl of Tris-EDTA buffer, lysed and amplified with T7 and Sp6 pGem-T-specific primers to confirm the appropriate size of the insert (approximately 1500 bp). In practice, for each DNA extract sample, 96 clones containing the plasmid with insert (a full 96 well microtiter-plate) were sent for sequencing at GATC Biotech (Germany), with the bacterial universal primer 16S-27f: 5'- AGAGTTTGATCMTGGCTCAG- 3'. The sequences were trimmed to 850 bp and inspected for mistakes using Chromas v. 2.31 (Technelysium Pty Ltd.). The program Bellerophon (Huber et al., 2004)& was used to discard chimeric sequences in the clone libraries. The tree builder tool provided by the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) was used to create a phylogenetic tree displaying the different sequence types. Representative clones from each phylogenetic group were compared to similar sequences in the GenBank database.

Clones bearing the sequences of interest were analysed by DGGE with the V6-V8 primers. Their DGGE bands were detected in the cocoa DNA extract sample fingerprints by comparison of the migration positions in Bionumerics software (Muyzer et al., 1993).

Data statistical treatment

The plate count numbers of the two duplicate experiments were log transformed and afterwards the average and standards errors of the mean were calculated. Significant statistical differences ($P< 0.05$) in the average levels of microorganisms were identified using a two-tailed independent Student's *t*-test or one-way ANOVA (PASW Statistica v.17.0).

Nucleotide sequences accession numbers

The sequences determined in this study have been deposited in the GenBank: JN366708 to JN366797 (ThrS isolates), JN366647 to JN366707 (clones) and JN366799 to JN366805 (*gyrA*).

Results

Changes in microbial group levels during processing

The survey of microbiological groups revealed high average levels of mesophilic and thermophilic TAM (Figure 2A) and TS (Figure 2B) at Nibs, with these levels varying between 4.3 and 5.6 log CFU/g (Figure 2). Conversely, the estimated average levels of ThrS (Figure 2-C) corresponded to less than 0.1% of mesophilic TS and less than 0.5% of thermophilic TS. With respect to fungi, average levels of 4.2± 0.04 log CFU/g, 4.4± 0.14 log CFU/g and 4.2± 0.17 log CFU/g were found, respectively for batches I, II and III; whereas for *Enterobacteriaceae*, these were 2.4± 0.11 log CFU/g, 2.8± 0.07 log CFU/g and 1.7± 0.05 log CFU/g.

After alkalising and pre-drying of nibs, both mesophilic and thermophilic TAM and TS levels were in general significantly reduced ($P< 0.05$). In the case of ThrS, these were only significantly reduced in batch I for thermophiles and batch II for mesophiles ($P< 0.05$). On the other hand, both fungi and *Enterobacteriaceae* groups were reduced to levels below the detection limit and these remained undetectable throughout processing.

Roasting of nibs further reduced the microbial levels. At this stage, conspicuous differences between TS and TAM were present but these involved a very low number of colonies and/or high standard deviations, rendering the differences inaccurate. In contrast, samples from Liquor exhibited slightly increased levels, but little variation was encountered among the last three stages within TAM, TS and ThrS; and the difference between mesophiles and thermophiles was more important at Powder for ThrS.

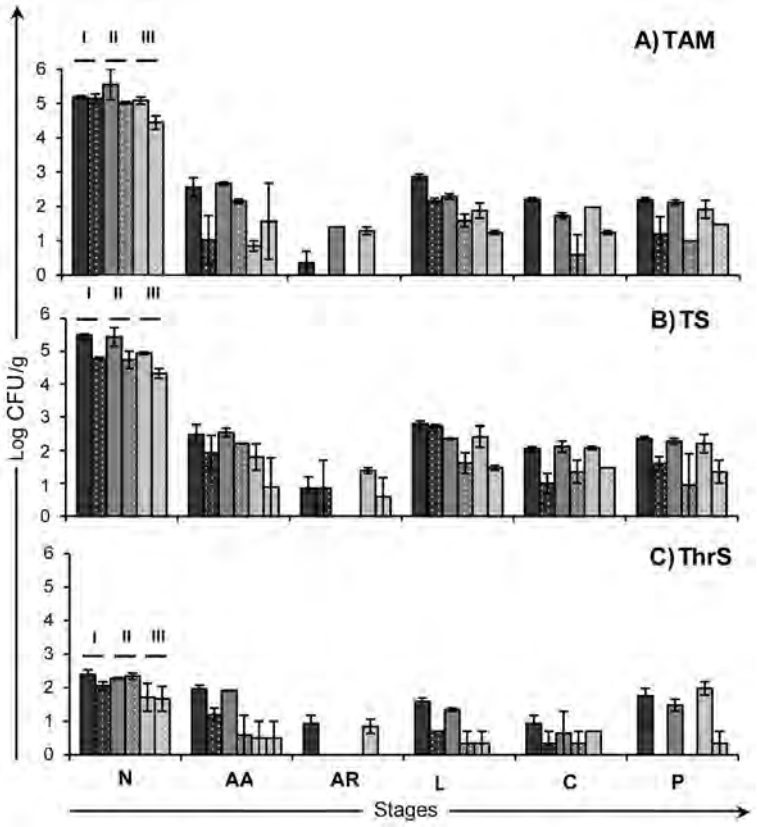

Figure 2. Microbial levels at different stages of cocoa bean processing to cocoa powder. See Figure 1 for stage abbreviations. The columns correspond to the estimated average levels (log CFU/g) of Total Aerobic Microorganisms (TAM) (A), aerobic Total Spores (TS) (B) and aerobic Thermoresistant spores (ThrS) (C) growing at 30°C (□) and 55°C (▨). Consecutive pairs of columns within each sampled stage correspond to the same batch production: Batch I (■), Batch II (■), Batch III (■). The absence of columns indicates levels below the detection limit of the method (1 log CFU/ml) in two independent experiments. The error bars are standard errors of the mean.

Changes in diversity of bacterial communities during processing assessed by PCR-DGGE of the 16S rRNA gene

Figure 3 shows the PCR-DGGE fingerprints for mesophilic and thermophilic bacterial communities for batches I and III. Due to low microbial levels after Nibs, sample enrichment was required prior to DNA extraction to obtain sufficient template for amplification. Efforts to optimize the DNA purification protocols in conjunction with improved PCR set-ups, including the use of more robust polymerases and polymerase protectants, failed to yield results.

The PCR-DGGE fingerprints from batch I showed a more complex profile than those of batch III. At Nibs, several bands were present, which could indicate large bacterial diversity. Clear differences were present in the profiles of nib samples enriched at 30°C and 55°C. Upon alkalising of nibs, a number of bands disappeared with mostly new bands emerging for both mesophilic and thermophilic populations. Notably, specific bands persisted across the different stages after Nibs in batches I and III. Intriguingly, considering the results of microbial groups quantification (Figure 2), a higher number of bands was expected at Liquor when compared to After Roasting, but this was not the case, with exception of fingerprints from LiquorI-55°C. Cake and Powder within batches I and III showed common bands, with the patterns having larger differences for incubations at 55°C. At these two stages, for enrichments at 30°C, the strong, upper band showed a same migration pattern as another band at Nibs30°C (specifically bands d and 6).

Clone library analysis

Enriched samples were successfully cloned and sequenced. Fourteen 16S rRNA gene clone libraries were constructed with Bacteria primers, but only a relatively few number of distinct phylotypes were present (Table 1). These phylotypes belonged to *Firmicutes* and *Proteobacteria* phyla, specifically *Bacilli* and *Gammaproteobacteria* classes. In the clone library of nibs enriched at 30°C (NibsI-30°C) sequence types of *Enterococcus faecium* group, which includes *E. durans* and *E. faecium* species (Devriese et al., 1993), *Bacillus* and *Lysinibacillus/ Rummeliibacillus* genus were detected, while at NibsIII-30°C, additional sequences affiliated with the *Citrobacter* genus were also annotated. At After Roasting-30°C from batches I and III, sequence types with high percentages of similarity to *Pseudomonas putida* group, which includes *P. putida* and *P. plecoglossicida* (Anzai et al., 2000), *Ornithinibacillus*/*Paucisalibacillus* and *Bacillus* genus were present. Samples of CakeI-30°C and CakeIII-30°C had sequence types of the *E. faecium* group in common, whereas sequence types of *P. putida* group and members from *Bacillus* genus were detected in CakeI-30°C additionally.

Clone libraries of samples enriched at 55°C revealed four phylotypes not detected in clone libraries enriched at 30°C. These were all members of the genus *Bacillus*, specifically *B. coagulans* (NibsI-55°C), *B. ginsengihumi* (NibsI-55°C), *B. thermoamylovorans* (NibsIII-55°C, After AlkalisingI-55°C, After Roasting-55°C and Cake-55°C) and a putatively new species of the genus *Bacillus* (After RoastingI-55°C, After RoastingIII-55°C and PowderIII-55°C).

By running the PCR-DGGE amplicons of clones with that of the cocoa DNA samples concomitantly in the same gel, we attempted to match the position of the bands (Figure 3). However, the confirmation of the exact band identities would require sequencing of excised bands (Muyzer et al., 1993).

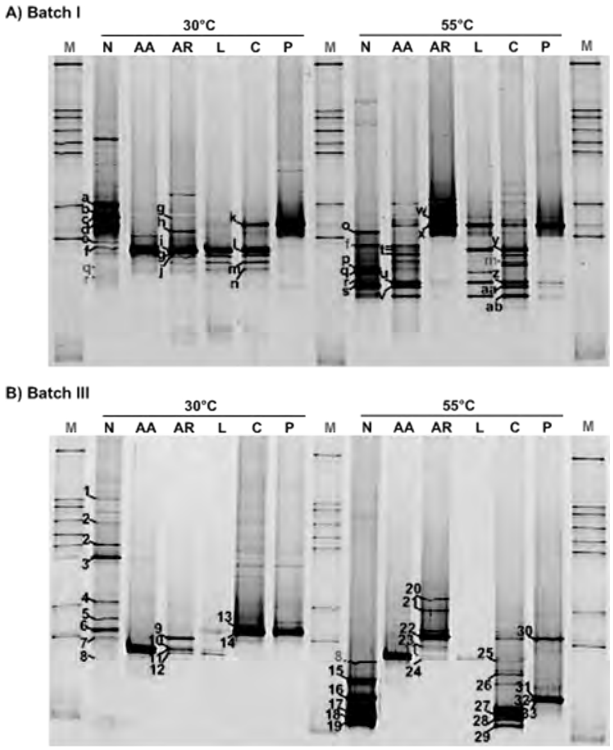

Figure 3. Changes in bacterial communities during cocoa bean processing to cocoa powder. The fingerprints were generated by PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) of the V6-V8 region of the 16S rRNA gene for Batch I (A) and Batch III (B). See Figure 1 for stage abbreviations; M- marker. Each lane represents fingerprints of amplified DNA extracts obtained after sample enrichment at 30°C or 55°C. The amplicons were resolved in a 30-60% denaturing gradient. Stages which were cloned and characterised in more detail are indicated with letters or numbers. The identity of the clone sequences is presented in Table 1. Arabics or numbers in grey indicate band positions that were confirmed by running the PCR-DGGE amplicons of the cocoa DNA samples juxtaposed.

Diversity of aerobic thermoresistant spore-formers and spores wet heat-resistance

Figure 4 presents the AFLP analysis of the isolated thermoresistant spore-forming strains and provides an overview of the 16S rRNA and *gyr*A gene sequencing results. Table S1 summarizes the details of 16S rRNA gene determination and isolate identification. The isolates were found to belong predominantly to the genus *Bacillus*, specifically *B. licheniformis* and *B. subtilis* complex. The *gyrA* gene sequences of three strains of the *B. subtilis* complex, namely strains 30AR1-1, 30AA2-6 and 30P3-3, were found to be closely related to the type strain of *B. subtilis* subsp. *subtilis* (sequence similarities of 98.5-99.7%, while with that of subspecies *spizizenii* and *inaquosorum* were below 96%). Also for a strain of *B. licheniformis* (30P3-1), the *gyrA*

Table 1. Phylogenetic affiliation of cloned 16S rRNA genes obtained from enriched cocoa DNA samples[a]

Stage[b]/Band	Batch I		Stage/Band	Batch III	
	% Similarity and Genbank closest relative	**GenBank given accession no.**		**% Similarity and Genbank closest relative**	**GenBank given accession no.**
N/a	99% *Enterococcus durans* 99% *Enterococcus faecium*	JN366664	N/1	99% *Bacillus circulans* 99% *Bacillus nealsonii* 99% *Bacillus benzoevorans*	JN366691
N/b	98% *Lysinibacillus sphaericus*	JN366665	N/2	99% *Citrobacter rodentium*	JN366692
N/c	100% *Bacillus circulans*	JN366666	N/3	99% *Citrobacter rodentium* 99% *Citrobacter sedlakii*	JN366693
N/d	99% *Enterococcus durans* 99% *Enterococcus faecium*	JN366667	N/4	99% *Citrobacter rodentium* 99% *Citrobacter sedlakii*	JN366694
N/e	96% *Rummeliibacillus stabekisii* 96% *Lysinibacillus fusiformis* 96% *Lysinibacillus sphaericus*	JN366668	N/5	99% *Lysinibacillus fusiformis* 99% *Lysinibacillus sphaericus*	JN366695
N/f	98% *Bacillus subtilis* 98% *Bacillus vallismortis* 98% *Bacillus amyloliquefaciens*	JN366669	N/6	99% *Enterococcus durans* 99% *Enterococcus faecium*	JN366696
AR/g	99% *Pseudomonas plecoglossicida* 99% *Pseudomonas putida*	JN366651	N/7	98% *Lysinibacillus fusiformis* 98% *Lysinibacillus sphaericus*	JN366697
AR/h	99% *Ornithinibacillus* spp. 99% *Paucisalibacillus globulus*	JN366652	N/8	99% *Bacillus subtilis*	JN366698
AR/i	99% *Ornithinibacillus* spp. 99% *Paucisalibacillus globulus*	JN366650	AR/9	99% *Ornithinibacillus* spp. 99% *Paucisalibacillus globulus*	JN366675
AR/j	99% *Bacillus licheniformis*	JN366653	AR/10	100% *Bacillus* sp. 99% *Bacillus firmus*	JN366676
C/k	99% *Enterococcus durans* 99% *Enterococcus faecium*	JN366656	AR/11	99% *Bacillus firmus*	JN366677

Table 1. Phylogenetic affiliation of cloned 16S rRNA genes obtained from enriched cocoa DNA samples[a] *(continued)*

Stage[b]/Band	Batch I		Stage/Band	Batch III	
	% Similarity and Genbank closest relative	**GenBank given accession no.**		**% Similarity and Genbank closest relative**	**GenBank given accession no.**
C/l	100% *Pseudomonas plecoglossicida* 100% *Pseudomonas putida*	JN366657	AR/12	99% *Pseudomonas plecoglossicida* 99% *Pseudomonas putida*	JN366678
C/m	99% *Bacillus licheniformis*	JN366658	C/13	99% *Enterococcus durans* 99% *Enterococcus faecium*	JN366684
C/n	99% *Bacillus clausii*	JN366659	C/14	99% *Enterococcus durans* 99% *Enterococcus faecium*	JN366685
N/o	95% *Bacillus licheniformis*	JN366670	N/15	98% *Bacillus thermoamylovorans*	JN366699
N/p	99% *Bacillus licheniformis*	JN366671	N/16	99% *Bacillus licheniformis*	JN366700
N/q	100% *Bacillus licheniformis*	JN366672	N/17	98% *Bacillus coagulans*	JN366701
N/r	99% *Bacillus coagulans*	JN366673	N/18	96% *Bacillus licheniformis* 96% *Bacillus oleronius*	JN366702
N/s	97% *Bacillus ginsengihumi*	JN366674	N/19	98% *Bacillus thermoamylovorans*	JN366703
AA/t	99% *Bacillus subtilis*	JN366647	AR/20	99% *Pseudomonas plecoglossicida* 99% *Pseudomonas putida*	JN366679
AA/u	99% *Bacillus thermoamylovorans*	JN366648	AR/21	99% *Bacillus* sp. 93% *Bacillus thermoamylovorans*	JN366680
AA/v	99% *Bacillus thermoamylovorans*	JN366649	AR/22	98% *Bacillus* sp. 94% *Bacillus thermoamylovorans*	JN366681
AR/w	99% *Bacillus* sp. 94% *Bacillus thermoamylovorans*	JN366654	AR/23	99% *Bacillus* sp. 94% *Bacillus thermoamylovorans*	JN366682
AR/x	99% *Bacillus* sp. 94% *Bacillus thermoamylovorans*	JN366655	AR/24	98% *Pseudomonas plecoglossicida* 98% *Pseudomonas putida*	JN366683

Table 1. Phylogenetic affiliation of cloned 16S rRNA genes obtained from enriched cocoa DNA samples[a] *(continued)*

Stage[b]/Band	Batch I		Stage/Band	Batch III	
	% Similarity and Genbank closest relative	**GenBank given accession no.**		**% Similarity and Genbank closest relative**	**GenBank given accession no.**
C/y	100% *Pseudomonas plecoglossicida*	JN366661	C/25	100% *Pseudomonas plecoglossicida* 100% *Pseudomonas putida*	JN366689
C/z	98% *Bacillus thermoamylovorans*	JN366662	C/26	99% *Bacillus clausii*	JN366690
C/aa	99% *Bacillus thermoamylovorans*	JN366663	C/27	99% *Bacillus thermoamylovorans*	JN366686
C/ab	98% *Bacillus thermoamylovorans*	JN366660	C/28	99% *Bacillus thermoamylovorans*	JN366687
			C/29	99% *Bacillus thermoamylovorans*	JN366688
			P30	98% *Bacillus* sp. 94% *Bacillus thermoamylovorans*	JN366706
			P/31	99% *Bacillus licheniformis*	JN366705
			P/32	99% *Bacillus licheniformis*	JN366707
			P/33	98% *Bacillus coagulans*	JN366704

[a]The BLAST search at GenBank (National Center for Biotechnology Information) is based on sequences of 850 bp. [b] See Figure 1 for stage abbreviations.

gene sequence was obtained. The similarity to the type strain of *B. licheniformis* was 100%, whereas with that of *B. sonorensis* was only 86.2%, confirming the suitability of 16S rRNA gene sequencing to distinguish isolates from these two taxa (Palmisano et al., 2001). Of the total of 10 species indicated in Figure 4, 9 were found at Nibs. Upon subsequent processing, mainly strains of *Bacillus subtilis* complex, together with *B. licheniformis*, prevailed (Table 2).

Cluster analysis of the AFLP banding patterns revealed large genetic heterogeneity among the strains. In total, 354 markers were scored in the gel of Figure 4A and 269 in the gel of Figure 4B. Only a limited number of strains appeared to be clonally related.

Figure 5 displays the comparative survival of spore crops of thermoresistant spore-forming strains at 110°C-5 min. Spores of *B. subtilis* complex formed the most heat-resistant spores. For six out of the nine spore crops no visible inactivation took place, but rather spore activation. However, a *B. subtilis* complex strain forming heat-sensitive spores was equally found (strain 30P3-3). For *B. licheniformis*, only spores of strain 30AA2-1 showed a relatively high heat-resistance when compared to the other *B. licheniformis* spore crops.

Discussion

This study describes the use of classical microbiological and PCR-based approaches to unravel the microbial community composition at different stages of cocoa bean processing to cocoa powder. Nibs was the stage with the highest levels and diversity, since other than cleaning and shelling no other major operations had been carried out. The analysis of the clone libraries from Nibs revealed about seven different phylotypes. As expected, based on the dynamics depicted in Figure 2, spore-formers were a predominant fraction of this diversity; but small differences between TAM and TS levels, in combination with the data in Table 1, indicates that not all aerobic microorganisms were spore-formers.

The assemblage at Nibs was less diverse than the one reported by Barrile et al. (1971), who identified about 20 species in cocoa beans, belonging to the *Bacilli*, *Gammaproteobacteria* and *Actinobacteria* classes. Species in common in the two studies included *B. licheniformis*, *B. coagulans* and *B. circulans*. Curiously, they did not find strains of the *B. subtilis* complex.

The analysis of the microbiota diversity along the different processing stages revealed some unexpected results: (i) phylotypes of *P. putida* group were retrieved from clone libraries at After Roasting and Cake, and corresponding band positions were found at Liquor (ii) the increase in TAM and TS levels at Liquor (Figure 2) was not always reflected in larger diversity; (iii) *E. faecium* group had the only phylotypes in the clone libraries at CakeIII-30°C and very few PCR-DGGE bands were present at this stage. However, the estimated average levels of TS and TAM at Cake and Powder were similar. Several factors could have contributed to these discrepancies. First, with respect to the persistence of phylotypes from *P. putida* and *Enterococcus faecium* groups, since these non-spore-forming species do not survive roasting, it is conceivable that they originated from conveyors linking the different stages and/or

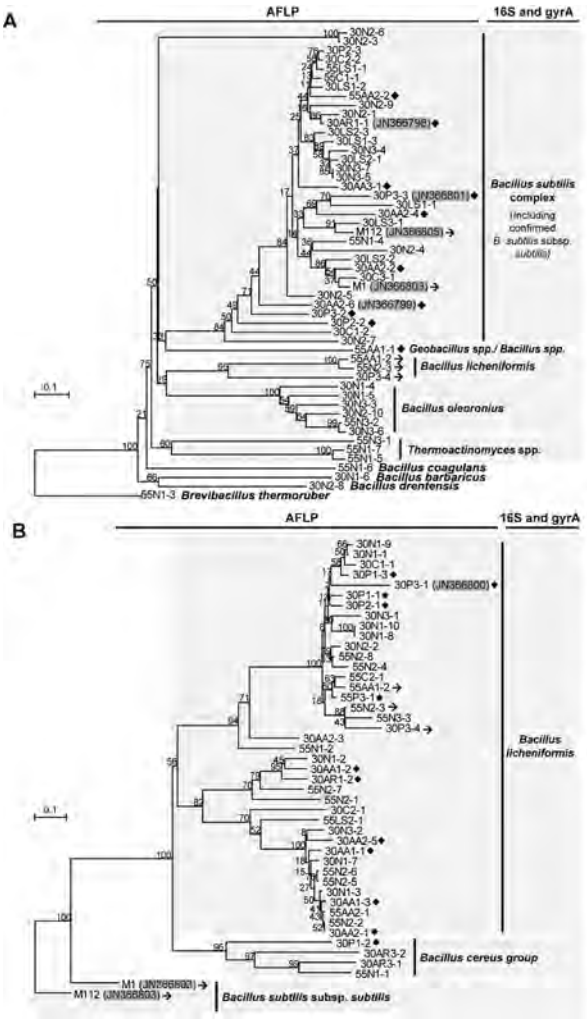

Figure 4. Amplified Fragment Length Polymorphism (AFLP) analysis of the thermoresistant spore-forming isolates. Bootstraps (%) are based on 1000 replications obtained with Treecon program. Phylogenetic tree displaying members of *Bacillus subtilis* complex (Rooney et al., 2009) and others species, with strain 55N1-3 as outgroup (A); and members of *B. licheniformis* and *B. cereus* group (Tourasse et al., 2011), with strain M112 as an outgroup (B). The scale bars corresponds to 0.1 nucleotide substitutions per site. Strains M112 and M1 had been characterised before (Lima et al., 2011b). The abbreviations '16S' and '*gyr*A' correspond to the 16S rRNA and gyrase A gene sequences, respectively. The Gyrase A gene accession numbers are given between brackets. All these strains were identified as *B. subtilis* subsp. *subtilis* or *B. licheniformis*. Strains with an arrow (→) were run in both gels of Figure A and B. Strains of which spore crops' heat-resistance was determined (Figure 5) are indicated by a diamond symbol (◆).

Table 2. Occurrence of thermoresistant spore-formers isolated at different stages during cocoa bean processing

Species (batch)	Thermoresistant spore-formers (No. /%)												Total
	N		AA		AR		L		C		P		
	30°C	55°C	30°C	55°C	30°C	55°C	30°C	55°C	30°C	55°C	30°C	55°C	
Bacillus barbaricus (I)	1/3.7	-	-	-	-	-	-	-	-	-	-	-	1
Bacillus cereus group (I, III)	-	1/5.6	-	-	2/50	-	-	-	-	-	1/10	-	4
Bacillus coagulans (I)	-	1/5.6	-	-	-	-	-	-	-	-	-	-	1
Bacillus drentensis (II)	1/3.7	-	-	-	-	-	-	-	-	-	-	-	1
Bacillus licheniformis (I, II, III)	10/37	10/56	6/60	2/50	1/25	-	-	1/50	2/40	1/50	5/50	1/100	39
Bacillus oleronius (I, II, III)	5/19	1/5.6	-	-	-	-	-	-	-	-	-	-	6
Bacillus subtilis complex (I, II, III)	10/37	1/5.6	4/40	1/25	1/25	-	7/100	1/50	3/60	1/50	4/40	-	33
Brevibacillus thermoruber (I)	-	1/5.6	-	-	-	-	-	-	-	-	-	-	1
Geobacillus spp./*Bacillus* spp. (I)	-	-	-	1/25	-	-	-	-	-	-	-	-	1
Thermoactinomyces spp. (I, III)	-	3/16.6	-	-	-	-	-	-	-	-	-	-	3
Total	27	18	10	4	4	0	7	2	5	2	10	1	90

See Figure 1 for stage abbreviations.

transport systems for intermediate storage of cocoa (see Figure 1). In particular, *Enterococcus* spp. are recognised for their ability to survive adverse conditions, such as drying (Devriese et al., 1993), which could have enabled them to persist in the product. Secondly, although a positive aspect of enrichment is that it detects only viable cells, it can introduce bias by favouring minor microbial groups (De Clerck et al., 2004a), namely the detected members from *P. putida* and *Enterococcus faecium* groups, and by failing to induce germination of very dormant spores (Ghosh and Setlow, 2009a). Thirdly, the presence of inhibitory compounds, such as polyphenols, could have hindered the growth of more sensitive strains (Park et al., 1979; Pearson and Marth, 1990). Finally, amplification artifacts (Prakitchaiwattana et al., 2004; Reysenbach et al., 1992) could have also led to changes of dominant species in the analyzed samples.

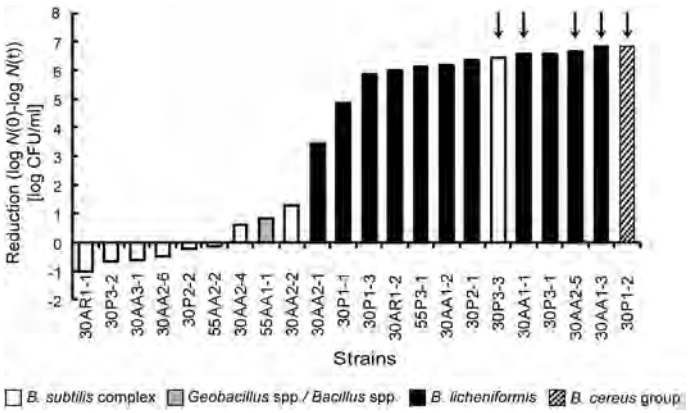

Figure 5. Thermal reduction (log CFU/ml) of Thermoresistant Spore crops at 110°C-5 min in phosphate buffer (PB) 10 mM, after sporulation at 55°C (strain 55AA1-1) or 37°C (all the other strains). Reductions resulting in counts below the detection limit of the method (1.7 log CFU/ml) are indicated with an arrow.

Several common phylotypes were identified at stages of both batches I and III. Standardization of blending and other operations to ensure a consistent brand-flavour, in conjunction with the factors discussed above, could possibly underlie the similarities in the microbiota in the two batches. Overall, strains of *B. licheniformis*, *B. thermoamylovorans*, a putative novel thermophilic species of the genus *Bacillus* and *P. putida* group members, were the most prominent phylotypes in the enriched aerobic fraction of cocoa during processing. Notably, we retrieved phylotypes of *Pseudomonas putida* group from samples enriched at 55°C. This is not the first report of thermotolerance in *Pseudomonas* spp. (Manaia and Moore, 2002), emphasizing the wide adaptive physiological capacity of members within this genus.

ThrS had already found in the nibs, but we could not trace strains isolated at Powder back to the ones at Nibs, due to the absence of clonally related strains at these stages. On the other hand, we cannot discard the possibility of persistence of strains in cocoa powder originating from nibs that could have only been detected by collecting a larger number of samples. Nonetheless, the large heterogeneity in AFLP profiles of *B. subtilis* and *B. licheniformis* suggests that new genotypes made their way into the process.

Strains of *B. subtilis* complex for which little or no apparent inactivation took place at 110°C-5 min, were present at various positions in the AFLP tree (Figure 4), indicating that the ability to form spores of high heat-resistance can be found in strains with substantial dissimilar genetic make-up. No clonally related strains were found with the heat-resistant *B. subtilis* spore-forming strains M112 and M1, which were isolated earlier from cocoa powder (Lima et al., 2011b).

Scheldeman et al. (2005) isolated around 20 different species surviving a heat treatment of 100°C-30 min from milking equipment swabs, of which *B. pallidus*, *Brevibacillus* spp. and *B. licheniformis* were the most widely represented species. Common species to this work were *B. licheniformis*, *B. oleronius* and *B. subtilis* complex. Strikingly, they isolated several strains of *B. amyloliquefaciens*. Our results were more in accordance with the study by van Zuijlen (2011), where strains of *B. subtilis* and *B. licheniformis* were the most frequently encountered species in soup ingredients and spoiled sterilized soups, after isolation treatments at 100°C-15 min.

In this study we concluded that the cocoa industry has to cope with microbiota comprising heat-resistant spore-formers, specifically from the *Bacillaceae* family, and non-sporeformers from *Pseudomonadaceae* and *Enterococcaceae* families. Based on enumeration of viable microorganisms, we observed that despite the fact that the levels in the first three stages were remarkably reduced, there could have been some survivors. Taken as a whole, the analysis of the results, based on quantification and molecular identification approaches, indicated that the microbiota of cocoa powder resulted from microorganisms which could have been initially present in the nibs, as well as microorganisms which originated during processing. However, the cocoa powder samples complied with the industrial guidelines, which specify maximum TAM levels of 3.7 log CFU/g (Dijk et al., 2007).

Future studies may provide additional information by using different enrichment media, including media compositions containing co-adjuvants to neutralise the potential inhibitory effect of polyphenols (Park et al., 1979); use of heat-activation steps prior to overnight enrichment to enhance the detection of spore-formers; and primers specific for members of the genus *Bacillus* and relatives.

Although we could not trace thermoresistant spore-forming strains of *B. subtilis* complex occurring in cocoa powder back to Nibs, we demonstrated the existence of a strain from After Roasting with the ability to resporulate into spores of high heat-resistance. Since the remaining processing stages do not include a killing step, these spores would persist in commercial cocoa powder. Therefore, their occurrence in cocoa powder needs to be taken into account to ensure the stability of downstream heat-preserved products, such as UHT chocolate drinks. Spoilage

incidents of UHT treated chocolate drinks have been linked to strains of *B. subtilis*, even though thermophilic spore-formers of the genus *Thermoanaerobacterium* and *Moorella* form the most heat-resistant spores reported thus far (Byrer et al., 2000). Yet, anaerobic spore-formers appear to be less important in the ecology of cocoa products (H. Kamphuis, pers. comm.).

The combined results of this and previous investigations (Lima et al., 2011b) allow us to conclude that owing to the high heat-resistance of *B. subtilis* complex members, in particular subspecies *subtilis*, these appear to be ultimate survivors of the cocoa powder production chain.

Acknowledgements

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Supplemental data

Microbiota dynamics and diversity at different stages of cocoa bean industrial processing to cocoa powder

Table S1 Thermoresistant spore-formers 16S rRNA gene FASTA sequence interpretation and proposed consensus

Batch	Stage[1] (Isolation temperature)	Strains code	Genbank closest accession no.	Closest relative in GenBank and respective Similarity %	GenBank given accession no.	Proposed Consensus[2]
I	N (30°C)	30N1-1	CP000002.3	98% *Bacillus licheniformis*	JN366790	*Bacillus licheniformis*
		30N1-2	AY017347.1	99% *Bacillus licheniformis*	JN366791	*Bacillus licheniformis*
		30N1-3	GU584875.1	99% *Bacillus licheniformis*	JN366792	*Bacillus licheniformis*
		30N1-4	EU430987.1	98% *Bacillus oleronius*	JN366793	*Bacillus oleronius*
		30N1-5	EU430987.1	99% *Bacillus oleronius*	JN366721	*Bacillus oleronius*
		30N1-6	GQ284513.1	98% *Bacillus barbaricus*	JN366722	*Bacillus barbaricus*
		30N1-7	GU250455.1	98% *Bacillus licheniformis*	JN366719	*Bacillus licheniformis*
		30N1-8	HQ003408.1	99% *Bacillus licheniformis*[3]	JN366713	*Bacillus licheniformis*
		30N1-9	AE017333.1	99% *Bacillus licheniformis*^{T}	JN366720	*Bacillus licheniformis*
		30N1-10	JF414759.1	99% *Bacillus licheniformis*[4]	JN366714	*Bacillus licheniformis*
	N (55°C)	55N1-1	CP001407.1	98% *Bacillus cereus*	JN366782	*Bacillus cereus group*
		55N1-2	AE017333.1	99% *Bacillus licheniformis*^{T}	JN366783	*Bacillus licheniformis*
		55N1-3	AB362290.1	99% *Brevibacillus thermoruber*	JN366784	*Brevibacillus thermoruber*
		55N1-4	EF656456.1	99% *Bacillus subtilis*	JN366715	*Bacillus subtilis* complex
		55N1-5	AY114167.1	100% *Thermoactinomyces vulgaris*	JN366723	*Thermoactinomyces vulgaris*
		55N1-6	AB362706.1	99% *Bacillus coagulans*	JN366734	*Bacillus coagulans*
		55N1-7	AF138739.1 AF138734.1	99% *Thermoactinomyces vulgaris* 99% *Thermoactinomyces intermedius*	JN366732	*Thermoactinomyces* spp.
	AA (30°C)	30AA1-1	GU584875.1	99% *Bacillus licheniformis*	JN366787	*Bacillus licheniformis*
		30AA1-2	AY017347.1	99% *Bacillus licheniformis*	JN366788	*Bacillus licheniformis*
		30AA1-3	GU584875.1	99% *Bacillus licheniformis*	JN366789	*Bacillus licheniformis*
	AA (55°C)	55AA1-1	BA000043.1	99% *Geobacillus kaustophilus*,	JN366780	*Geobacillus* spp./*Bacillus* spp.

Table S1 Thermoresistant spore-formers 16S rRNA gene FASTA sequence interpretation and proposed consensus *(continued)*

Batch	Stage[1] (Isolation temperature)	Strains code	Genbank closest accession no.	Closest relative in GenBank and respective Similarity %	GenBank given accession no.	Proposed Consensus[2]
			EU214615.1	99% *Geobacillus thermoparaffinivora*		
			FN428684.1	99% *Geobacillus thermoleovorans*		
			FN666246.1	99% *Geobacillus lituanicus*		
			AY608941.1	99% *Geobacillus stearothermophilus*		
			AY952967.1	99% *Bacillus caldotenax*		
			Z26924.1	99% *Bacillus caldolyticus*		
		55AA1-2	AE017333.1	99% *Bacillus licheniformis*^{T}	JN366781	*Bacillus licheniformis*
	AR (30°C)	30AR1-1	AL009126.3	99% *Bacillus subtilis*^{T}	JN366710	*Bacillus subtilis* subsp. *subtilis*
		30AR1-2	CP000002.3	99% *Bacillus licheniformis*^{T}	JN366755	*Bacillus licheniformis*
	A (55°C)	-----[5]	-----	-----		
	L (30°C)	30L1-1	AL009126.3	99% *Bacillus subtilis*^{T}	JN366711	*Bacillus subtilis* complex
		30L1-2	AL009126.3	99% *Bacillus subtilis*^{T}	JN366795	*Bacillus subtilis* complex
		30L1-3	AL009126.3	99% *Bacillus subtilis*^{T}	JN366796	*Bacillus subtilis* complex
	L (55°C)	55L1-1	AL009126.3	99% *Bacillus subtilis*^{T}	JN366794	*Bacillus subtilis* complex
	C (30°C)	30C1-1	AB553280.1	99% *Bacillus licheniformis*	JN366785	*Bacillus licheniformis*
		30C1-2	AL009126.3	99% *Bacillus subtilis*^{T}	JN366786	*Bacillus subtilis* complex
	C (55°C)	55C1-1	AL009126.3	99% *Bacillus subtilis*^{T}	JN366797	*Bacillus subtilis* complex
	P (30°C)	30P1-1	AE017333.1	99% *Bacillus licheniformis*^{T}	JN366733	*Bacillus licheniformis*
		30P1-2	FJ932761.1	99% *Bacillus thuringiensis*	JN366779	*Bacillus cereus group*
			JF705198.1	99% *Bacillus cereus*		
			HQ200405.1	99% *Bacillus anthracis*		
		30P1-3	CP000002.3	99% *Bacillus licheniformis*^{T}	JN366778	*Bacillus licheniformis*

Table S1 Thermoresistant spore-formers 16S rRNA gene FASTA sequence interpretation and proposed consensus *(continued)*

Batch	Stage[1] (Isolation temperature)	Strains code	Genbank closest accession no.	Closest relative in GenBank and respective Similarity %	GenBank given accession no.	Proposed Consensus[2]
	P (55°C)	-----[5]	-----	-----		
II	N (30°C)	30N2-1	AL009126.3	99% *Bacillus subtilis*^{T}	JN366742	*Bacillus subtilis* complex
		30N2-2	CP000002.3	99% *Bacillus licheniformis*^{T}	JN366743	*Bacillus licheniformis*
		30N2-3	CP000560.1	98% *Bacillus amyloliquefaciens*	JN366744	*Bacillus subtilis* complex
			GU045558.1	98% *Bacillus subtilis*		
		30N2-4	AL009126.3	100% *Bacillus subtilis*^{T}	JN366745	*Bacillus subtilis* complex
		30N2-5	AL009126.3	100% *Bacillus subtilis*^{T}	JN366746	*Bacillus subtilis* complex
		30N2-6	GU125640.1	99% *Bacillus amyloliquefaciens*	JN366747	*Bacillus subtilis* complex
		30N2-7	EF656456.1	99% *Bacillus subtilis*	JN366724	*Bacillus subtilis* complex
		30N2-8	AJ542505.1	98% *Bacillus drentensis*	JN366725	*Bacillus drentensis*
		30N2-9	JF414762.1	99% *Bacillus subtilis*	JN366773	*Bacillus subtilis* complex
		30N2-10	AY988598.1	99% *Bacillus oleronius*^{T}	JN366727	*Bacillus oleronius*
	N (55°C)	55N2-1	AY017347.1	99% *Bacillus licheniformis*	JN366740	*Bacillus licheniformis*
		55N2-2	GQ153850.1	99% *Bacillus licheniformis*	JN366741	*Bacillus licheniformis*
		55N2-3	GQ222400.1	99% *Bacillus licheniformis*	JN366736	*Bacillus licheniformis*
		55N2-4	GQ222400.1	99% *Bacillus licheniformis*	JN366737	*Bacillus licheniformis*
		55N2-5	GU250455.1	99% *Bacillus licheniformis*	JN366738	*Bacillus licheniformis*
		55N2-6	GU250455.1	99% *Bacillus licheniformis*	JN366739	*Bacillus licheniformis*
		55N2-7	CP000002.3	99% *Bacillus licheniformis*^{T}	JN366716	*Bacillus licheniformis*
		55N2-8	GQ222400.1	99% *Bacillus licheniformis*	JN366726	*Bacillus licheniformis*
	AA (30°C)	30AA2-1	CP000002.3	99% *Bacillus licheniformis*^{T}	JN366749	*Bacillus licheniformis*
		30AA2-2	AL009126.3	99% *Bacillus subtilis*^{T}	JN366750	*Bacillus subtilis* complex

Table S1 Thermoresistant spore-formers 16S rRNA gene FASTA sequence interpretation and proposed consensus *(continued)*

Batch	Stage[1] (Isolation temperature)	Strains code	Genbank closest accession no.	Closest relative in GenBank and respective Similarity %	GenBank given accession no.	Proposed Consensus[2]
		30AA2-3	CP000002.3	99% *Bacillus licheniformis*^{T}	JN366751	*Bacillus licheniformis*
		30AA2-4	AL009126.3	99% *Bacillus subtilis*^{T}	JN366752	*Bacillus subtilis* complex
		30AA2-5	CP000002.3	99% *Bacillus licheniformis*^{T}	JN366753	*Bacillus licheniformis*
		30AA2-6	AL009126.3	100% *Bacillus subtilis*^{T}	JN366754	*Bacillus subtilis* subsp. *subtilis*
	AA (55°C)	55AA2-1	FJ189777.1	99% *Bacillus licheniformis*	JN366748	Bacillus licheniformis
		55AA2-2	GQ199595.1	99% *Bacillus subtilis*	JN366718	*Bacillus subtilis* complex
	AR (30°C)	-----[5]	-----	-----		
	AR (55°C)	-----[5]	-----	-----		
	L (30°C)	30L2-1	AL009126.3	99% *Bacillus subtilis*^{T}	JN366756	*Bacillus subtilis* complex
		30L2-2	JF414762.1	99% *Bacillus subtilis*	JN366708	*Bacillus subtilis* complex
		30L2-3	AL009126.3	99% *Bacillus subtilis*^{T}	JN366758	*Bacillus subtilis* complex
	L (55°C)	55L2-1	AY017347.1	99% *Bacillus licheniformis*	JN366759	Bacillus licheniformis
	C (30°C)	30C2-1	CP000002.3	99% *Bacillus licheniformis*^{T}	JN366764	Bacillus licheniformis
		30C2-2	AL009126.3	99% *Bacillus subtilis*^{T}	JN366757	*Bacillus subtilis* complex
	C (55°C)	55C2-1	CP000002.3	99% *Bacillus licheniformis*^{T}	JN366762	Bacillus licheniformis
	P (30°C)	30P2-1	X68416.1	100% *Bacillus licheniformis*	JN366760	*Bacillus licheniformis*
		30P2-2	AL009126.3	99% *Bacillus subtilis*^{T}	JN366761	*Bacillus subtilis* complex
		30P2-3	AL009126.3	99% *Bacillus subtilis*^{T}	JN366763	*Bacillus subtilis* complex
	P (55°C)	-----[5]	-----	-----		

Table S1 Thermoresistant spore-formers 16S rRNA gene FASTA sequence interpretation and proposed consensus *(continued)*

Batch	Stage[1] (Isolation temperature)	Strains code	Genbank closest accession no.	Closest relative in GenBank and respective Similarity %	GenBank given accession no.	Proposed Consensus[2]
III	N (30°C)	30N3-1	GU584875.1	99% *Bacillus licheniformis*	JN366772	*Bacillus licheniformis*
		30N3-2	GQ153850.1	99% *Bacillus licheniformis*	JN366765	Bacillus licheniformis
		30N3-3	AY988598.1	99% *Bacillus oleronius*^{T}	JN366766	*Bacillus oleronius*
		30N3-4	AL009126.3	99% *Bacillus subtilis*^{T}	JN366728	*Bacillus subtilis* complex
		30N3-5	AL009126.3	99% *Bacillus subtilis*^{T}	JN366729	*Bacillus subtilis* complex
		30N3-6	AY988598.1	99% *Bacillus oleronius*^{T}	JN366730	*Bacillus oleronius*
		30N3-7	EF528288.1	100% *Bacillus subtilis*	JN366731	*Bacillus subtilis* complex
	N (55°)	55N3-1	AF138739.1 AF138732.1 AF138734.1	99% *Thermoactinomyces vulgaris*[6] 99%*Thermoactinomyces candidus* 99% *Thermoactinomyces intermedius*	JN366712	*Thermoactinomyces* spp.
		55N3-2	AY988598.1	99% *Bacillus oleronius*^{T}	JN366717	*Bacillus oleronius*
		55N3-3	GU584875.1	99% *Bacillus licheniformis*	JN366735	*Bacillus licheniformis*
	AA (30°C)	30AA3-1	AL009126.3	99% *Bacillus subtilis*^{T}	JN366767	*Bacillus subtilis* complex
	AA (55°C)	---	-----	The isolate lost viability		
	AR (30°C)	30AR3-1	FJ435217.1	96% *Bacillus cereus*[7]	JN366709	*Bacillus cereus* group
		30AR3-2	CP001407.1	99% *Bacillus cereus*	JN366768	*Bacillus cereus* group
			CP001903.1	99% *Bacillus thuringiensis*		
	AR (55°C)	-----[5]	-----	-----		
	L (30°C)	30L3-1	GQ199595.1	99% *Bacillus subtilis*	JN366769	*Bacillus subtilis* complex
	L (55°C)	-----[5]	-----	-----		
	C (30°C)	30C3-1	AL009126.3	99% *Bacillus subtilis*^{T}	JN366770	*Bacillus subtilis* complex
	C (55°C)	-----[5]	-----	-----		

Table S1 Thermoresistant spore-formers 16S rRNA gene FASTA sequence interpretation and proposed consensus *(continued)*

Batch	Stage[1] (Isolation temperature)	Strains code	Genbank closest accession no.	Closest relative in GenBank and respective Similarity %	GenBank given accession no.	Proposed Consensus[2]
	P (30°C)	30P3-1	CP000002.3	99% *Bacillus licheniformis*^{T}	JN366774	*Bacillus licheniformis*
		30P3-2	AL009126.3	99% *Bacillus subtilis*^{T}	JN366775	*Bacillus subtilis* complex
		30P3-3	AL009126.3	99% *Bacillus subtilis*^{T}	JN366776	*Bacillus subtilis* subsp. *subtilis*
		30P3-4	HM006898.1	98% *Bacillus licheniformis*	JN366771	*Bacillus licheniformis*
	P (55°C)	55P3-1	CP000002.3	99% *Bacillus licheniformis*^{T}	JN366777	*Bacillus licheniformis*

[1]Stage abbreviations correspond to Nibs (N), After Alkalising and pre-drying (AA), After Roasting (AR), Liquor storage (L), Cake storage (C) and Powder (P).
[2]Combined information from 16S rRNA and *gyr A* gene sequences with AFLP.
[3]The sequence identity is based on partial sequence derived from DNA amplification with the reverse primer. The sequence size is 1165 bp.
[4]The sequence identity is based on partial sequence derived from DNA amplification with the reverse primer. The sequence size is 1058 bp.
[5]For the indicated sample the level of Thermoresistant spores was below the detection limit of the method.
[6]The sequence identity is based on partial sequence derived from DNA amplification with the reverse primers. The sequence size is 810 bp.
[7]The sequence identity is based on partial sequence derived from DNA amplification with the forward primers. The sequence size is 864 bp.

Chapter 6

Compositional and ultrastructural factors that make *Bacillus subtilis* subsp. *subtilis* spores highly heat-resistant

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Spores of *Bacillus subtilis* subsp. *subtilis* span a large spectrum of wet heat-resistance. We selected five strains to investigate which physicochemical and ultrastructural factors set apart heat-resistant spores from heat-sensitive ones. Spore *D*-values were determined and correlated with levels of spore minerals, core water content, dipicolinic acid and the ratio between the core and sporoplast dimensions. In addition, the strains were tested for their growth performance at ranges from 37°C to 56°C.

The determined $D_{110°C}$ for spores of strains M112 and A163 were, respectively, 600- and 1000-fold higher than that of M5, which produced the least heat-resistant spores. Sporulation of strain M5 at 50°C compared to 37°C, led to nearly 18-fold increase in the $D_{110°C}$. In contrast, spores of strain M112 sporulated at 50°C did not show increased heat-resistance when compared to 37°C.

The average content of magnesium, manganese and calcium were the factors more strongly correlated with the spore heat-resistance ($r > 0.80$/ $\rho > 0.60$). This was followed by the core-sporoplast ratio ($r = -0.72$/ $\rho = -0.50$) and DPA ($r = 0.52$/ $\rho = 0.80$). However, of all factors, manganese appeared to be the best predictor of spore heat-resistance. This was corroborated by the observation that spores of M5 sporulated at 50°C had more than 2-fold higher content of manganese. Consequently, this mineral is proposed as a biomarker for *B. subtilis* subsp. *subtilis* spores wet heat-resistance.

Furthermore, high growth temperature capacity was shown not to be a requirement for the development of high heat-resistant spores.

Introduction

A classical problem in bacteriology concerns the understanding of the mechanisms which render endospores of the phylum *Firmicutes* resistant against wet heat. Such attention is in part driven by the fact that endospore wet heat-resistance (hereafter referred to as spore heat-resistance) constitutes a major hindrance in preservation processes within the food industry (van Zuijlen, 2011; Setlow and Johnson, 2007).

The acquisition and maintenance of spore wet heat-resistance are linked with relatively conserved structural features in *Bacillales* and *Clostridiales* (Atrih and Foster, 2001; Henriques and Moran, 2007; Paredes-Sabja et al., 2011). These features consist of a dehydrated spore core, the innermost spore compartment containing the DNA, ribosomes and enzymes as well as minerals, mainly calcium, complexed with pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]); a layer of modified peptidoglycan, constituent of the spore cortex, which surrounds the core and is responsible for maintaining its dehydrated state; and the coat, a structured protein layer surrounding the cortex, which protects the spore against chemicals and the cortex peptidoglycan from hydrolytic enzymes (Driks, 1999; Ghosh et al., 2008; Henriques and Moran, 2007; Popham et al., 1996; Setlow and Johnson, 2007). Binding of the spore DNA to small acid soluble proteins (SASPs) has also been shown to play an important role in spore stability and protection against damage, including wet heat (Fairhead et al., 1993; Setlow and Setlow, 1998).

Earlier studies comparing the physicochemical composition of spores across species showed that decreased core water content was one of the most important factors determining spore heat-resistance (Gerhardt and Maquis, 1989; Nakashio and Gerhardt, 1985; Paidhungat et al., 2000). Nonetheless, the water content alone could not explain the higher heat-resistance of spores of certain species, such as that of the thermophile *Geobacillus stearothermophilus*, when compared to that of mesophilic and thermotolerant species. The concomitant presence of certain minerals, specifically calcium and manganese were shown to play a crucial role in the high heat-resistance of *G. stearothermophilus* spores (Beaman and Gerhardt, 1986; Bender and Marquis, 1985). Another factor found to be correlated with spore heat-resistance was a reduction in the ratio between the core and the sporoplast (compartment made-up by the core and the cortex layer) dimensions (Beaman et al., 1982). More recently, intraspecies studies for *Clostridium perfringens* spores showed also a significant correlation between spore heat-resistance and reduced core-sporoplast ratio, low core water content and increased content of minerals, but principally iron (Orsburn et al., 2008).

Importantly, a central dogma related to spore heat-resistance is that thermophilism, that is, the ability of a microorganism to grow at high temperature would be the necessary condition for the production of spores of high heat-resistance (Beaman and Gerhardt, 1986; Warth, 1978). This view is supported by the observation that the most heat-resistant spore-formers reported thus far are thermophiles from the genus *Desulfotomaculum*, *Moorella* and *Thermoanaerobacterium* (Byrer et al., 2000; Goorissen, 2002; Stumbo, 1973). Within the

Bacillales order, *G. stearothermophilus* was regarded as the epitome of high heat-resistance, but more recently, the discovery of mesophilic and thermotolerant strains, specifically of *B. subtilis* and *B. sporothermodurans* species, of which spores had heat-resistance in the same order of magnitude or higher than the former, challenged this belief (André et al., 2012; Huemer et al., 1998; Lima et al., 2011b). *B. subtilis*, in particular, is not often considered to pose significant spoilage problems in high-temperature treated food products (Coleman et al., 2007; Setlow and Johnson, 2007). However, we and others have isolated *B. subtilis* subsp. *subtilis* strains from food ingredients or commercial products, forming spores with unusually high heat-resistance (Lima et al., 2011b; Lima et al., 2012; Oomes et al., 2007; van Zuijlen, 2011).

The serendipitous finding of such strains prompted us to investigate how spore compositional and ultrastructural factors known to contribute to spore wet heat-resistance compare among *B. subtilis* strains forming spores of substantially different heat-resistance. Only a few systematic studies for such spore properties have been carried out at intraspecies level. This information may be important for spore control within the food industry. Furthermore, although it is accepted that one or more proteins might be the target of spore killing (Coleman et al., 2007), we have no clue as to whether the targets are the same or not in different strains. The formulation of hypotheses in this field needs to be guided by the understanding on how compositional and ultrastructural factors vary within groups of heat-resistant and heat-sensitive spores.

Therefore, the aim of this investigation was to determine the relative contribution of physicochemical compositional factors, namely spore minerals, DPA and water content as well as structural factors, including the core-sporoplast ratio, for spore differential heat-resistance within a taxonomically well characterised group of *B. subtilis* strains. In addition, given the role of the sporulation temperature as a most important cue affecting the development of microorganisms (Farrell and Rose, 1967), we assessed the effect of spore formation at higher temperature on the spore heat-resistant phenotype and characteristics, for a strain both within the heat-sensitive and the heat-resistant group. Moreover, the relationship between the natural ability of a strain to grow at high temperature and its spore heat-resistant phenotype was examined.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Five *B. subtilis* subsp. *subtilis* strains identified to subspecies level on the basis of gyrase A gene (*gyrA*) sequencing were selected for this study. The *gyrA* gene sequencing was performed by the Bacteria Collection Laboratorium voor Microbiologie of Ghent University (BCCM/LMG), according to Chun and Bae (2000). The strains, the corresponding *gyrA* gene sequence accession number and origin were as follows: strains M5 (JN366806), M88 (JN366807), M1 (JN366803)

and M112 (JN366805) were isolated from cocoa powder (Lima et al., 2011b), while strain A163 (JN366802) was isolated from a sterilized soup (Oomes et al., 2007), and was kindly provided by André van Zuijlen (Unilever, Vlaardingen, The Netherlands). The strains were selected on the basis of differences in spore heat-resistance, as reported in the primary publications.

Stock cultures originating from a single colony were prepared in Nutrient Broth 13 g/l (NB, Oxoid) pH 7.0 and were kept frozen (-80°C) in the presence of 25% (v/v) glycerol (Fluka). The same batch of NB was used throughout this work.

Routine working-cultures were obtained by scraping the surface of a frozen vial, without thawing, with a 10 µl inoculation loop and by subsequently transferring the icy-cell suspension into 25 ml of NB in a 100 ml Erlenmeyer flask. The Erlenmeyers were then incubated for 16-18 h at 37°C in a shaking water bath (200 rpm; Julabo SW 24).

Amplified Fragment Length Polymorphism (AFLP) analysis

The strains were genotyped using the AFLP method (Vos et al., 1995), with the AFLP Analysis System Kit for Microorganisms (Invitrogen). This method was performed as reported previously (Lima et al., 2011b), with the exception that nine selective primer combinations were used to generate genomic fingerprints: i) *Eco*RI-A/*Mse*I-X and *Eco*RI-C/*Mse*I-V, where X corresponds to C and G, and V corresponds to A, C or G; ii) *Eco*RI-C/*Mse*I-CA and *Eco*RI-C/*Mse*I-CC; iii) *Eco*RI-A/*Mse*I-CA and *Eco*RI-A/*Mse*I-CC. The digital AFLP gel images were scored for dominant markers using AFLPQuantar (v1.05, KeyGene) and a phylogenetic tree was inferred using Nei and Li, and Neighbor joining methods implemented in Treecon v1.3b (van de Peer and De Wachter, 1994). *B. licheniformis* strain M109 (*gyrA* gene sequence JN366804) was included as an outgroup.

Growth assay

Twenty-five milliliters of NB were inoculated with 100 µl of an overnight grown working-culture and incubated for 16 h at 37°C. Next, the Absorbance at 600 nm (A_{600nm}) of these cultures was determined (spectrophotometer Novaspec II, Pharmacia Biotech) and adjusted to A_{600nm} of 1 with Peptone Physiological Saline solution (PPS) (1 g Neutralized Bacteriological Peptone [NBP, Oxoid] and 8.5 g NaCl per liter). Dilutions up to 10^{-5} were made in PPS for each cell suspension, using a 96 well microtiter-plate (Greiner Bio-One). Next, five microliters of each dilution were spotted (Neblett, 1976) onto four 12x12 cm plates (Nalgene Nunc International) containing freshly prepared Nutrient Agar (13 g/l NB solidified with 15 g/l Agar Bacteriological [AB, Oxoid]), and these were subsequently incubated at 37°C, 53°C, 55°C and 56°C for 72 h. To reduce the dehydration of the plates incubated in the range 53-56°C, these were wrapped with a polyethylene bag and beakers containing sterile water were placed in the incubators. Plate images were captured with a Casio Exilim-Z55 after 14 h of incubation, but the plates were immediately returned to the incubator to complete 72 h. After 72 h incubation, growth at the different dilutions was registered. A duplicate independent experiment was performed. The digital color images were imported into Adobe Photoshop CS5 Extended v12.0.3 (Adobe

Systems Incorporated), where minor adjustments in brightness/contrast were equally applied to all the images.

Preparation of spore crops

Overnight grown working-cultures at 37°C or 50°C (additionally for strains M5 and M112) were prepared in duplicate Erlenmeyer flasks. Per strain and sporulation condition, 40 petri dishes (9.4x1.5 cm, Greiner Bio-One) were inoculated by spread-plating 1 ml of the overnight culture on freshly prepared NA (13 g/l NB solidified with 15 g/l AB) pH 7.0 supplemented with minerals as described by Cazemier et al. (2001). The plates were then incubated at 37°C for 72 h or 50°C for 30 h (for strains M5 and M112 initially grown at 50°C), which corresponded to the release of nearly 99% free phase-bright spores, with exception of strain A163 for which a yield of about 90% was obtained, as monitored by phase contrast microscopy (Olympus BX40F4). All plates were turned up-side down after 8 h (plates incubated at 50°C) or 20 h (plates incubated at 37°C) incubation.

Following incubation the spores were harvested with 10 ml of Phosphate Buffer (PB) 10 mM pH 7.0 (tenfold dilution of 0.1 M K_2HPO_4/ KH_2PO_4 buffer) and collected into a 50 ml falcon tube (Greiner Bio-one). Spore crops of each strain were divided among 3-4 falcon tubes to facilitate the subsequent steps of purification. The spore crops were cleaned by daily rounds of centrifugational washings (Eppendorf 5804 RF, Rf-34-6-38), during a period of 3 weeks as described before (Lima et al., 2011b). Debris and vegetative cells were carefully removed from the apical region of the pellet with a sterile inoculation loop and the pellet was further purified by a modification of the swirling method (Long and Williams, 1958), which consisted of allowing a small volume (500 µl) of buffer to flow-off very slowly on top of the pellet and after swinging movements discharging of the supernatant. This led to highly purified spore crops (essentially 100% phase-bright spores) for all strains. The spores were inspected for the occurrence of clumping, but this was not observed and, consequently, there was no need to add surfactants to PB.

The spore crops of each strain were all prepared on the same day and handled in identical manner. In total, three independent spore batches per strain were prepared on different days and with freshly prepared medium and PB. The spore crops were stored in PB at 4°C in the dark and frequently monitored to ensure the use of stable crops of phase-bright spores.

The spore crops were coded to reflect the sporulation and recovery temperature after a heat treatment, e.g. 'M5(37)37' indicates that the sporulation took place at 37°C and that the spores were also recovered at 37°C.

Wet heat-resistance studies

The spore crops were assayed for their heat-resistance using the Kooiman method (Kooiman, 1973) as reported previously (Lima et al., 2011b). Working spore crops were prepared by appropriate dilution of the mother spore crop with PB (Lima et al., 2011b). Two heating regimes were applied: firstly, the spores were heated at 110°C for 1 min, 2.5 min and 5 min to establish

a basis of comparison for the heat-resistance of the spore crops (screening heat treatment); secondly, the spores were treated at different temperatures ranging from 100 °C to 122°C to generate survival curves. After the heat treatments, the number of survivors was quantified by enumeration in duplicate pour-plates of NB 2.6 g/l solidified with AB (15 g/l), upon incubation at 37°C for 5 days and 50°C for 3 days. Survival counts were made from plates with a number of colonies not higher than 300.

The heat-resistance was determined by subtracting the log number of viable spores after heating, from the log number before heating.

The data from the survival curves were fitted to adequate models to obtain thermal inactivation parameters as described below.

Fitting of survival models

The Geeraerd model (Geeraerd et al., 2006) was used to fit the heat-inactivation data of the spores. This model can account for the occurrence of inactivation shoulders and two subpopulations of spores with different heat-resistance:

$$\log N(t) = \log N(0) + \log\left[(1-f).e^{-k_{sen}\cdot t}\cdot \frac{e^{k_{sen}\cdot S_l}}{1+(e^{k_{sen}\cdot S_l}-1)\cdot e^{-k_{sen}\cdot t}} + f.e^{-k_{res}\cdot t}\cdot \frac{e^{k_{sen}\cdot S_l}}{1+(e^{k_{sen}\cdot S_l}-1).\, e^{-k_{sen}\cdot t}}\right]^{\frac{k_{res}}{k_{sen}}} \quad (1)$$

Where N is the number of surviving spores (CFU/ml) at time t and $N(0)$ is the initial number of spores. The parameters (1- f) and f represent the fraction of the heat-sensitive and the heat-resistant subpopulations, while k_{sen} and k_{res} (1/time unit) are the inactivation rates of the heat-sensitive and heat-resistant subpopulations, respectively. The parameter S_1 (time unit) represents the duration of the shoulder. The reciprocal of the inactivation rates was multiplied by Ln(10) to obtain the corresponding decimal reduction time (D-value), the time needed to reduce the population by 1 log unit (time unit).

The total number of datapoints in the heating interval was between 21 and 27. The model was fitted to all the inactivation datapoints in Microsoft Excel by using the solver add-in. Next the data fitting was verified with GInaFit (Geeraerd et al., 2006) and TableCurve2D v.2.03 (Jandel Scientific), where the confidence intervals for the model parameters were also obtained.

Model discrimination and goodness of fit

Stepwise exclusion of non-significant parameters was carried out. To assess whether model simplification was statistically accepted, the F-test was performed after exclusion of the parameters by comparing the f_{value} to the F_{table} as described by Zwietering et al. (1990).

In addition, different mathematical and statistical indices were determined to assess the extent to which the models described the data. These were the regression coefficient (r^2), the mean square error of the model (MSE_{model}) and the f_{ratio} (ratio between MSE_{model} and mean

square error of the data [MSE_{data}]), which was compared to the F_{table} at 95% confidence interval. When the f_{ratio} was smaller than the F_{table} the F-test was accepted, meaning that the model was adequate to describe the data (te Giffel and Zwietering, 1999).

Finally, the frequency of model acceptance was also considered for the purpose of model selection.

To enable a comparison of the determined *D*-values, the Bigelow model was used to compute normalised *D*-values at 110°C ($D_{110°C}$) (Bigelow, 1921):

$$\log D_{110^{\circ}C} = \log D_{ref} - \left(\frac{110 - T_{ref}}{z} \right) \quad (2)$$

Where D_{ref} is the decimal reduction time (min) at a reference temperature T_{ref}, $D_{110°C}$ is the decimal reduction time (min) at 110°C and the *z*-value is the increase in temperature (°C) leading to a reduction in *D* by a factor of 10. Published *z*-values for spores of strains M1, M112 and A163 were used for data conversion (Kort et al., 2005; Lima et al., 2011b). For the other strains an average of published *z*-values for *B. subtilis* strains was used (Condón et al., 1996; Conesa et al., 2003; Kort et al., 2005; Lima et al., 2011b; Nakayama et al., 1996; Palop et al., 1999b).

Spore minerals determination

The concentrations of elemental calcium, magnesium, potassium, iron and manganese were quantified by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES), according to the protocol by Orsburn et al. (2008). A volume of spore suspension corresponding to 4-7 mg dry weight (DW), as determined by air drying in duplicate in pre-weighed (Sartorius, BP 1105) aluminum foil cups at 100°C for 48 h, was used for quantification of minerals. The spore samples were analyzed by the certified laboratory Chemisch Biologisch Laboratorium Bodem from Wageningen University, along with an equivalent volume of 1.2 M HCL (37% HCL, Merck) for instrument calibration. All three independent spore crops were assayed simultaneously for all minerals. Triplicate readings per spore samples were collected.

Spore dipicolinic acid (DPA) determination

DPA was measured by a fluorescent assay as described by de Vries et al. (2004), with minor modifications. A volume of spores corresponding to 4-9 mg dry weight was resuspended in one milliliter sterile milli-Q water instead of Tris-HCl buffer for digestion at 121°C for 15 min. The supernatants (containing the DPA) were then collected into clean Eppendorf tubes (Greiner Bio-One) and 100 µl were assayed for DPA in the presence of terbium chloride ($TbCl_3$) 100 µM, which had been previously dissolved in milli-Q water. The measurements were performed in a 96 well UV-Star microplate (Greiner Bio-One), inserted in a Safire microplate reader (Tecan), in combination with XFLUOR4 software v4.40 (Tecan) and settings previously described (de Vries et al., 2004). A standard calibration curve of DPA (Sigma) in milli-Q water was obtained

for concentrations in the range 0-150 µM. Triplicates of points in the calibration curve and duplicates of each spore crop were obtained. The relationship between DPA concentrations and fluorescence (arbitrary units) was obtained by linear regression in Microsoft Excel, and the obtained equation (r^2> 0.99) was used to convert the fluorescence data into DPA concentrations for the individual spore samples.

Spore core water content determination

The core water content of decoated spores was determined by buoyant density sedimentation in gradients of Nycodenz (Axi-Shield Poc) according to Lindsay et al. (1985) and as described by Ghosh et al. (2009). Briefly, spore suspensions of the various strains with an A_{600nm} of 25 were pelleted in a benchtop centrifuge at 13,000 x *g* for 2 min. Next, the spores were decoated with a solution containing 0.5% (w/v) Sodium Dodecyl Sulphate (SDS; Sigma)-0.1 M Dithiothreitol (DTT; Sigma) in 0.1 M NaCl at pH 10, by incubation for 1 h at 65°C with continuous shaking (Eppendorf thermomixer, AG5355). The decoated spores were then washed five times by centrifugation in 0.1 M NaCl pH 10 and twice in milli-Q water, and subsequently equilibrated in 30% (w/v) Nycodenz (1.159 g/ mL). The viability of the SDS-DTT treated spores was confirmed by streak-plating on NA plates. The refractive index of Nycodenz solutions was determined in a precision refractometer (ATAGO 36642) at 19-20°C, and these measurements were subsequently converted to solution densities by use of a standard calibration curve (range 0-70% [w/v]) and the formula by Rickwood et al. (1982).

Nycodenz gradients in the range of 57-75% (w/v) were prepared in ultra-clear centrifuge tubes 14x89 mm (Beckman) with 1 mL layers of medium with 2% decreasing concentration. Four-hundred microliters of decoated and equilibrated spores corresponding to an A_{600nm} of 10 were layered over the Nycodenz gradients. The tubes were centrifuged in a Beckman XL-90 ultracentrifuge, provided with a swinging bucket rotor (SW41T) at 25,000 x *g* (14,000 rpm) for 45 min. The wet densities were determined by the banding positions of the spores at the interface immediately above and below a medium layer by comparison to a 12 ml calibrated ultra-clear tube. The determinations were performed simultaneously for all spore batches. The core water content of the spores was calculated according to the linear equation by Lindsay et al. (Lindsay et al., 1985).

Transmission Electron Microscopy (TEM) imaging and measurement of spore cross sections

Several TEM schemes previously published were tested with unsatisfactory results, due to difficulties in obtaining adequate embedding for samples of the most heat-resistant spores. Finally, by our modification of the protocol by Tabit and Buys (2010), we achieved the best results. Fresh spore crops had to be prepared for this assay, since during the time elapsed between completing the spore compositional analysis and finding an optimal TEM protocol (about eight months), spore aging occurred (evidenced by appearance of a large number of phase dark spores in the spore crops). The heat-resistance of these new spore crops was then

verified by heating at four distant time points retrieved from the individual spore survival curves (see section "wet heat-resistance studies"). There were no significant statistical differences in the *D*-values of the spores when compared to the previously determined ones, as assessed by a two-tailed independent Student's *t*-test ($P< 0.05$) (data not shown). In particular, for spores of strain M5(50)50 a biphasic inactivation curve continued to be observed.

Pelleted spores were fixed in 4% (v/v) glutaraldehyde (EMS) buffered at pH 7.2 in 0.1 M PB for 18 h at 4°C. After three times washing with buffer, the samples were post-fixed for 64 h at 4°C with 1% (w/v) osmium tetraoxide (EMS) in 0.1 M PB pH 7.2. After five times washing with demi-water, the samples were dehydrated in a graded series of ethanol (10, 30, 50, 70, 90 and 100% ethanol), by pelleting the samples for 2 min at 10,000 x *g* and vortexing. Next, the samples were infiltrated with quetol resin (quetol-651 38.9 g, nadic methyl anhydride 44.6 g, dodenyl succinic anhydride 16.6 g, araldite RD2 2 ml and dimethylaminoethane 1 ml), by adding small amounts of resin during 6 h until the final concentration was raised to 65% (v/v). Subsequently, the samples were fully infiltrated with 100% quetol resin for three days, by performing three changes with 100% quetol. This was accomplished by centrifugation of the samples for 3 min at 14,000 x *g* and resuspension of the spores in 100% quetol. After infiltration, the spores were pipetted into BEEM capsules (EMS) and these were covered with fresh 100% quetol resin. The capsules were centrifuged for 3 min at 10,000 x *g* and polymerized in an embedding oven (Agar scientific, B702) for 48 h at 60°C. Ultra-thin sections (80 nm) of embedded sample were then made using a diamond knife (HA 3095) fitted in an ultra-microtome (Reichert Ultra Cut S). During sectioning, one micrometer distance was left between the sectioned areas. The sections were examined with a TEM JEM 1011Jeol and a number of micrographs per spore sample were obtained (between 151 and 183). Staining of the sections prior to observation with uranyl acetate and lead citrate was not required.

Images of transversally sectioned spores (circular or approximately circular), with well-defined core and pericortex membranes were selected to perform area measurements of the core and sporoplast. The core could be clearly distinguished from the cortex, as the latter appears as an unstained and featureless zone (Atrih and Foster, 1999). Area measurements were made in the imaging software iTEM (Olympus Soft Imaging Solutions, v5.0) by selecting the circular/ ellipse area function. The measurements were subsequently exported to Microsoft Excel, where cortex areas (difference between the sporoplast and core areas) and the ratio between the core and sporoplast area were computed.

Statistical analysis

Plate count survivor numbers were log transformed, averaged and the standard errors of the mean calculated in Microsoft Excel.

Technical replicates for each spore crop properties were averaged and subsequently entered in Excel to compute an overall average and the corresponding standard error of the mean.

Significant statistical differences ($P< 0.05$) in average levels of spore factors were identified by one-way ANOVA. Subsequently, the outcome of *post hoc* Bonferroni test was applied to resolve the ANOVA.

To determine the presence of differences in the *D*-values of the spores, the selected survival model was fitted to the data of each individual spore crop and a final average was subsequently calculated.

Core-sporoplast ratio values were imported into the statistical package PASW Statistica, where normality tests were performed (Kolmogorov-Smirnov and Shapiro-Wilk; $P< 0.05$) and frequency distributions were created. The number of bins in the histogram was calculated by taking the square-root of the total number of observations. In addition the Q-Q plots were inspected to help identify situations of serious departure from normality.

For correlation analyses both Pearson's (r) and the Spearman's rank (ρ) correlation coefficients were obtained. The Spearman's rank correlation coefficient compensates for the fact that a strong, yet nonlinear relationship between two variables might be present, which is not recognised by Pearson's. Furthermore, the Spearman's correlation coefficient is less sensitive to one or few outlying point pairs (Field, 2005). The correlation coefficients were classified as very high [0.90-1], high [0.70-0.89], moderate [0.40-0.69], low [0.20-0.39] and very low [0-0.19].

Multiple Linear Regression (MLR) was also used with the aim of expressing the relation between spore heat-resistance (dependent variable) with the different determined spore factors (predictors). The general mathematical function in MLR is a first-degree equation specified as:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_m X_m + \varepsilon \quad (4)$$

Where Y is the dependent variable (spore heat-resistance, expressed in $\log D_{110°C}$), Xm represents the m experimental factors tested (predictors), β_0 is the constant term, β_m represents the regression coefficients of the predictors, with each coefficient representing the "weight" (correlation) of the respective predictor; and ε is the predictive error, that is, the difference between the predicted and the observed values of Y. The results of the correlation analysis were used to enter the most important predictors in the model ($r> 0.6$). Hierarchical and backwards regression methods were used to determine the extent to which variation in critical spore properties could explain the spore heat-resistant phenotype ($P< 0.05$). Due to absence of biological replicates for the TEM data, the data of the single TEM experiment was used for input in the model as triplicate. Such procedure does not affect the assumptions of MLR (Field, 2005).

Different values or coefficients implemented in the utilised software were retrieved to assess the adequacy of the MLR model to describe the data. These were the regression coefficients r^2_{Adjusted}, the F change value and the Durbin-Watson (d) value. The F change test tells whether the change in the r^2 by adding one or more predictors to the model is significant

(the *F* change value is significant at *P*< 0.05) (Field, 2005). The Durbin-Watson (*d*) test informs whether adjacent residuals are correlated (test of autocorrelation). Values closer to 2 mean that the residuals are not correlated, while values less than 1 or greater than 3 mean that the assumption is not verified (Field, 2005).

All statistical analysis and tests were carried out using the PASW Statistics v17.0 (IBM-SPSS) software.

Results

B. subtilis strains characteristics and screening of spores heat-resistance

Cluster analysis of the AFLP banding patterns for the five strains revealed genetic heterogeneity without clonal relationships (Figure 1). In total, 808 markers were scored, of which 636 were exclusive to the *B. subtilis* strains. Interestingly, strains M5 and M88, which produced the least heat-resistant spores (Figure 2), clustered separately from the other strains. Reexamination of the tree topology by reshuffling the order of the strains or by obtaining tree representations with combinations of subsets of primer combinations, had no effect on the internal tree topology (data not shown), indicating that the reconstruction in Figure 1 is stable.

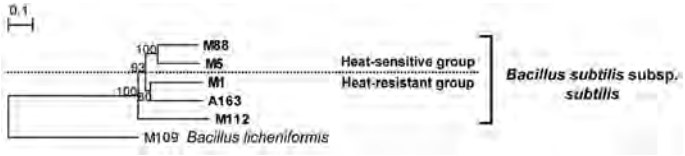


Figure 1. Amplified fragment length polymorphism (AFLP) cluster analysis of the *Bacillus subtilis* strains, based on nine selective primer combinations. *B. licheniformis* strain M109 was added as an outgroup. Bootstraps (percent) are based on 1000 replications obtained with Treecon program. The scale bar represents 1 nucleotide difference per 100 nucleotides.

Heat treatment at 110°C proved to be largely lethal for spores of strain M5, whereas for spores of strains M112 and A163 no visible spore inactivation, but rather activation, took place (Figure 2). Sporulation of strain M5 at higher temperature resulted in increased spore heat-resistance, with a reduction of only 3.0 log CFU/ml being quantified after 5 min (Figure 2; black bar). For spores of strain M112(50) spore activation was also observed, but it was less pronounced than for M112(37). In the case of strain M1, heating for 5 min did not further increase spore reduction, when compared to 2.5 min (Figure 2; light grey bar), which was the case for spores of strain M88. For this reason strain M1 was considered to belong to the group of heat-resistant spore-formers.

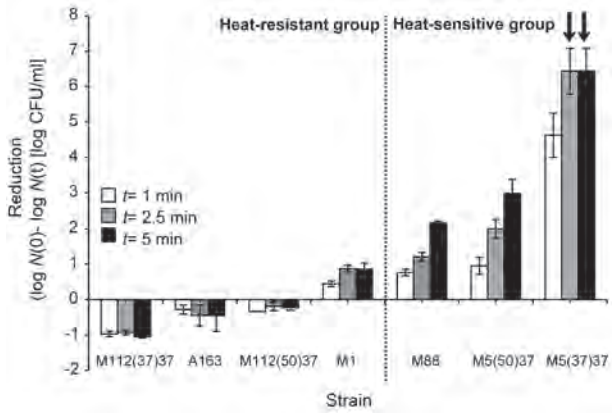


Figure 2. *Bacillus subtilis* spores thermal reduction (log CFU/ml) at 110°C for 1 min, 2.5 min and 5 min in phosphate buffer 10 mM. The strains were sporulated at either 37°C (all strains) or at 50°C (additionally for strains M5 and M112) and the heat-treated spores were recovered at 37°C. For spores of strains produced at two different temperatures, the sporulation and recovery temperatures are sequentially given. Reductions resulting in counts below the detection limit of the method (1.7 log CFU/ml) are indicated with an arrow.

The strains were tested for their growth performance at high temperature ranges. Strain M112 consistently showed poor growth at 55°C in two independent experiments. On the contrary, strains M5 and M88 grew at 55°C at all dilutions (Figure 3). At 56°C none of the strains showed growth.

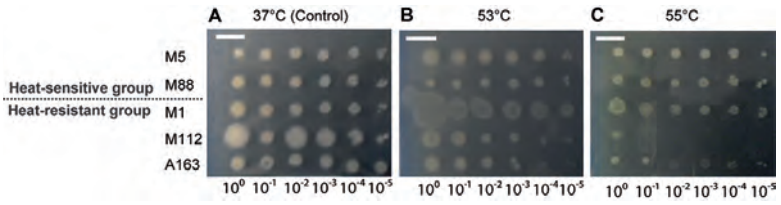


Figure 3. Effect of incubation temperature on the growth performance of stationary-phase *Bacillus subtilis* cells grown in nutrient broth. The dilution factors are indicated below the picture. The pictures were obtained after incubation for 14 h, but incubation for additional 58 h did not change this profile. For strain M112 only faint growth occurred at the dilution 10^{-1} at 55°C, while at the remaining dilutions no growth was shown. None of the strains showed growth at 56°C after 72 h. This experiment was performed in duplicate independent experiments and both duplicates showed the same result. One set is shown here. The scale bars correspond to 16 mm (**A**), 12 mm (**B**) and 15.7 mm (**C**).

Together these results support the hypothesis that the ability of a strain to grow well at high temperatures is not a requirement for production of spores of high heat-resistance.

Quantification of the heat-resistance of *B. subtilis* spores

Spore survival curves were obtained for the individually screened strains (Figure 2) to rank the heat-resistance of their spores. Figure 4 depicts spore survival curves at different heating temperatures. Visual inspection of the survival curves indicated strong deviations from linearity for spore crops of strain M5(50)50, where two spore subpopulations with different heat-resistance were systematically detected (Figure 4, c); and for spores of both strains M112 and A163, where activation shoulders were observed (Figure 4, h-i). Interestingly, shoulders did not appear when strain M112 was sporulated at 50°C.

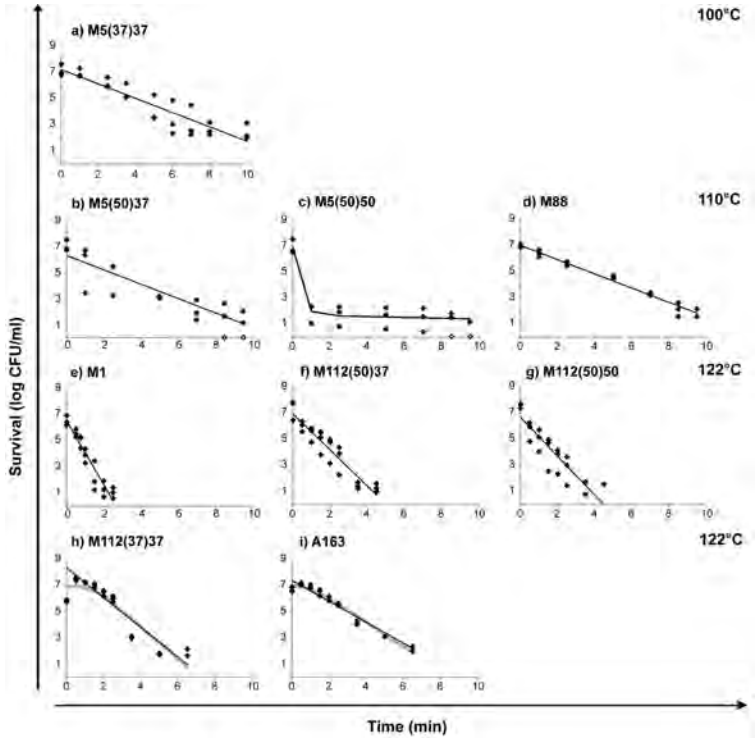


Figure 4. Survival curves of *Bacillus subtilis* spores in phosphate buffer 10 mM. The sporulation temperature is given between brackets followed by the recovery temperature. For the other strains both the sporulation and recovery took place at 37°C. The log-linear model (black line) was fitted to the data, which showed the best fitting performance in most cases. The log-linear model with shoulder is also included for spores of strains M112(37) and A163 (grey line). For spores of strain M5(50)50, the linear model is clearly not adequate and for this reason the data fitting is given with the Geeraerd log-linear model with tail.

The open diamonds indicate datapoints below the detection limit of the method of 1.7 log CFU/ml, and these were excluded from the process of data modeling.

Due to deviations from linearity, models other than the log-linear were assessed for their adequacy to describe the data. Table 1 summarises the results of the estimated parameters. The log-linear model was found to be adequate to fit the data in seven out of nine experimental conditions depicted in Figure 4, as demonstrated by the confidence intervals for the parameters estimates and the F-test. In the case of spores of strain M5(50)50, the Geeraerd log-linear model with tail provided the best fit. For spores of strains M112(37)37 and A163, the Geeraerd log-linear model with shoulder showed an improved fitting performance when compared to the log-linear model (Table 2). Still in the case of strain M112 the Geeraerd log-linear model with shoulder was not statistically accepted, since this model only accounts for flat activation shoulders. In addition, for strains M112(37)37 and A163, data fitting without log $N(0)$ was further performed. The log-linear model was accepted for the situation A163-$N(0)$, but not for M112(37)37-$N(0)$, due to structural deviation. Finally, for spores of M112(37)37, the Peleg model (Peleg, 2002) was tested since it accounts for concave activation shoulders. With the Peleg model, the mean square-error of the model (MSE) was slightly lower (MSE_{model}= 0.524), but it was not the preferred model due to larger number of parameters and because the parameters do not have biological significance.

Considering that the log-linear model was adequate to fit the survival data in a larger number of cases, this model was further selected to compare the D-values of the different spore crops. The comparison of the average $D_{122°C}$ among strains in the heat-resistant spore-forming group revealed statistical significantly differences [$F(4,10)= 43.44$, $P< 0.001$]. The heat-resistance of M1 spores was statistical significantly lower than that of all the other spores, whereas those of A163 where significantly higher ($P< 0.05$). On the other hand, the D-values of M112-$N(0)(37)(37)$ and M112(50)(37), or M112(50)(37) with M112(50)(50) did not show significantly differences ($P> 0.05$).

Sporulation of strain M5 at 50°C increased the heat-resistance of the spores to levels similar to those of M88. Indeed, no significant statistical differences were found in the average $D_{110°C}$ among spores of M5(50)37, M5(50)50 and M88 [$F(2,6)= 1.18$, $P= 0.34$].

A z-value of 7.99°C resulting from averaging z-values found in the literature for different spores of *B. subtilis* was used to derive the $\log D_{110°C}$ for spores of strain M5, M5(50) and M88. An estimated 600-fold and 1000-fold difference in the $D_{110°C}$ was obtained for spores of strain M112 and A163, respectively, when compared to spores of M5, indicating that strain A163 produced the most heat-resistant spores, whereas strain M5 produced the most heat-sensitive spores (see $D_{110°C}$ in Table 1).

Altogether, the large discrepancy in spore heat-resistance, from 0.1 min to 106 min for the $D_{110°C}$, suggests that important differences in the composition of the spores and possibly in their ultra-structure could be present.

Table 1. Model parameter estimates for the heat-inactivation of *Bacillus subtilis* spores in phosphate buffer 10 mM pH 7 and goodness of fit of the model

Spore crop	Model (no. parameters)	T (°C)	Model parameters[a]				Mathematical and statistical indices			$D_{110°C}$[c] (min)
			Log *N*(0) (log CFU/ml)	*D* (min)	D_{res} (min)	*Sl* (min)	r^2	MSE_{model}	f_{ratio}[b]	
M5(37)37[d]	Log-linear (2)	100	7.22 (0.27)	1.83 (0.16)	-	-	0.842	0.594	**0.563**	0.101
M88	Log-linear (2)	110	7.02 (0.11)	1.83 (0.07)	-	-	0.976	0.0918	**1.07**	1.83
M1	Log-linear (2)	122	6.50 (0.26)	0.411 (0.030)	-	-	0.905	0.450	**0.0672**	11.7
M112(37)37	Log-linear (2)	122	7.62 (0.32)	1.06 (0.12)	-	-	0.769	1.037	33.9	75.9
	Log-linear+ shoulder (3)	122	6.95 (0.31)	0.823 (0.149)	-	1.45 (0.49)	0.849	0.707	22.8	-
M112-*N*(0) (37)37	Log-linear (2)	122	8.36 (0.26)	0.882 (0.067)	-	-	0.903	0.499	14.5	62.9
A163	Log-linear (2)	122	7.40 (0.12)	1.25 (0.07)	-	-	0.903	0.148	**0.0193**	116
	Log-linear+ shoulder (3)	122	7.01 (0.12)	1.08 (0.05)	-	0.968 (0.234)	0.969	0.0079	**0.0103**	-
A163- *N*(0)	Log-linear (2)	122	7.70 (0.08)	1.14 (0.04)	-	-	0.981	0.0847	**0.468**	<u>106</u>
M5(50)37	Log-linear (2)	110	6.27 (0.37)	1.83 (0.22)	-	-	0.797	0.950	**0.657**	1.83
M5(50)50	Log-linear (2)	110	4.09 (0.67)	2.47 (0.84)	-	-	0.365	3.07	4.31	2.47
	Log-linear+ tail (4)	110	6.92 (0.39)	0.184 (0.046)	25.3 (12.2)	-	0.918	0.454	**0.637**	-
M112(50)37	Log-linear (2)	122	6.94 (0.25)	0.729 (0.055)	-	-	0.888	0.523	**0.794**	52.2
M112(50)50	Log-linear (2)	122	6.67 (0.34)	0.670 (0.077)	-	-	0.801	1.168	**0.000**	47.9

[a]The standard deviation of the mean of the parameters is given between brackets. The parameter *f* of the log-linear+tail model is close to zero and is not indicated in the table.
[b]Boldface indicate acceptance of given model with the F-test.
[c]The following *z*-values were used for data conversion: *z*= 7.99°C was used for data conversion for spores of M5 and M88 (averaged from literature data for *B. subtilis*; see materials and methods); *z*= 8.24°C for spores of M1 (Lima et al., 2011b); *z*= 6.47°C for spores of M112 (Lima et al., 2011b); *z*= 6.10°C (Kort et al., 2005) for spores of A163. Numbers which are underlined were used for subsequent correlation and multiple linear regression analyses.
[d]The sporulation and recovery temperatures are sequentially given.

Physicochemical composition of *B. subtilis* spores

Differences in elemental minerals were present across the strains (Table 2). Of the quantified minerals, calcium was the most abundant, while iron was the least. Magnesium was found to be the mineral element for which no significant statistical differences were present among the spores [F(6, 14)=0.17, *P*= 0.98]. The average levels for the remaining minerals varied between 12.3 to 24.2 µg/mg DW for calcium, 1.96-4.65 µg/mg DW for potassium, 0.097-0.186 µg/mg DW for iron and between 0.630-1.90 µg/mg DW for manganese. Spores of strains M5 and M112 behaved differently with respect to the pattern of accumulation of minerals when sporulated at higher temperature. Spores of M5(50) systematically revealed higher average levels of minerals than spores of M5(37). On the contrary, spores of M112(50) only revealed increased average level of iron and manganese. Overall, spores of M5(37) showed in general the lowest average mineral levels.

Spore DPA, density and core water contents are summarised in Table 3. The DPA content of the spores varied from 26.9-32.7 µg/ mg DW, with the lowest amount being detected for spores of M88 and the highest for spores of M112. Spore density values ranged from 1.318-1.335 g/ml, with corresponding water contents in the range 49.9-56.8 g/100g of core. No significant statistical differences were found in the average contents of core wet density ([F(6, 14)= 1.58, *P*= 0.224]), core water content ([F(6, 14)= 1.60, *P*= 0.22]) or DPA ([F(6,14)= 0.840, *P*=0.56]).

Although A163 spores were more heat-resistant than those of M112(37) and M112(50), the former spores did not have lower water content. This was also the case with respect to M88 spores when compared to those of M5. Still, A163 spores had lower water content than spores of strains M1, M88 and M5.

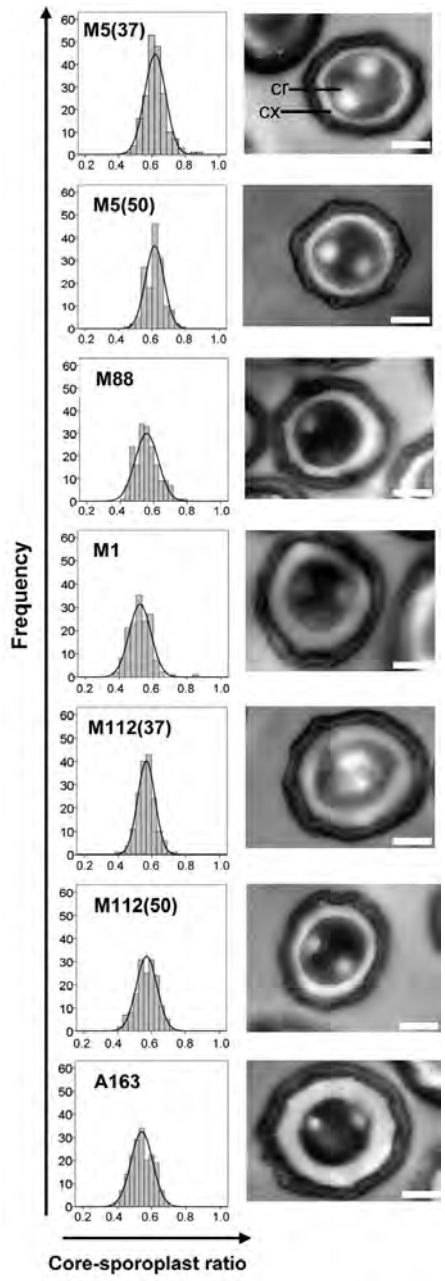
Table 2. *Bacillus subtilis* spores composition in mineral elements (µg/mg dry weight)

Strain	Calcium	Magnesium	Potassium	Iron	Manganese
M5(37)	12.3 (2.8)^{a}	0.946 (0.195)	1.96 (0.85)^{a}	0.104 (0.019)^{a}	0.630 (0.201)^{a}
M5(50)	16.6 (3.9)^{ab}	0.962 (0.226)	2.20 (0.50)^{abc}	0.166 (0.039)^{ab}	1.40 (0.28)^{ab}
M88	19.9 (1.4)^{ab}	1.03 (0.10)	4.65 (0.47)^{c}	0.127 (0.012)^{ab}	1.06 (0.09)^{ab}
M1	24.2 (1.7)^{b}	1.06 (0.08)	2.55 (0.28)^{bc}	0.157 (0.018)^{ab}	1.23 (0.15)^{ab}
M112(37)	23.1 (0.7)^{ab}	1.09 (0.09)	2.38 (0.15)^{bc}	0.186 (0.017)^{ab}	1.26 (0.11)^{ab}
M112(50)	20.6 (0.3)^{ab}	0.979 (0.025)	1.98 (0.09)^{b}	0.227 (0.015)^{b}	1.90 (0.08)^{b}
A163	20.8 (3.1)^{ab}	1.10 (0.22)	2.96 (0.47)^{bc}	0.097 (0.0125)^{a}	1.77 (0.16)^{b}

Standard errors of the mean of three independent spore crop preparations are given between brackets.
Mean values with no common letters differ significantly (*P*< 0.05) as determined by application of Bonferroni *post hoc* test.

Ultrastructure of *B. subtilis* spores

A successful protocol for fixation and embedding of all spore crops for visualisation by Transmission Electron Microscopy (TEM) was developed. This allowed the comparison of the relative dimensions of the core, cortex and sporoplast for all spore crops (Table 3). Statistical significantly differences were present in the average areas of the core [F(6, 1184)= 61,72, *P*= 0.000], cortex [F(6, 1184)= 104.24, *P*= 0.000] and core-sporoplast ratio [F(6, 1184)= 55,17, *P*= 0.000]. The core areas were significantly larger for spores of M112, and smaller for spores of A163 (*P*< 0.05). With respect to the cortex, spores of M1 were found to have the largest cortex area (*P*< 0.001), while both spores of M5 and M5(50) revealed the smallest (*P*< 0.05). Regarding the core-sporoplast ratio, the smallest values were verified for M5 and M5(50) (*P*< 0.001). On the other hand, no significant difference was found in this ratio among spores of M88 and spores of the most heat-resistant spores (*P*> 0.05), with exception for spores of M1.



We scrutinized further the diversity in the core-sporoplast ratio of each spore type. The representation depicted in Figure 5 shows no significant deviations from a normal distribution, as confirmed by normality tests and inspection of the Q-Q plots (data not shown). This representation emphasises the large heterogeneity in spore ratios within each spore crop, but also the overlap in ratios among the heat-resistant and the heat-sensitive spore groups is visibly present.

Figure 5. Frequency distributions of core-sporoplast area ratios (nm^2/nm^2) within a single spore crop of each *Bacillus subtilis* strain. A representative set of transmission electron microscopy images of a spore cross section is given. See Table 4 for details on the spore structure dimensions. The structural compartments in the spore are indicated: core (cr) and cortex (cx).

Table 3. *Bacillus subtilis* spores core density, core water content, DPA content and ultrastructural characteristics

Strain	Wet density (g/ml)	Water (g/100g core)	DPA (µg/mg Dry weight)	Core (nm^2)	Cortex (nm^2)	Core-sporoplast ratio (nm^2/ nm^2)
M5(37)	1.320 (0.005)	56.1 (1.8)	31.7 (7.5)	160,403(1,886) [c]	99,636 (1,637) [a]	0.617 (0.004) [d]
M5(50)	1.323 (0.003)	54.8 (1.2)	32.0 (0.5)	149,555 (2,519) [b]	92,947 (1,759) [a]	0.615 (0.004) [d]
M88	1.318 (0)[*]	56.8 (0)	26.9 (1.4)	140, 868 (2,138) [b]	113,299 (2,229) [b]	0.558 (0.005) [bc]
M1	1.323 (0.005)	55.8 (2.1)	31.8 (5.0)	170,012 (2,987) [c]	159,122 (3,186) [d]	0.522 (0.005) [a]
M112(37)	1.335 (0.002)	49.9 (0.7)	40.6 (4.5)	183,145 (3,023 [d]	139,359 (2,214) [c]	0.566 (0.004) [c]
M112(50)	1.325 (0.006)	54.1 (2.5)	32.1 (2.3)	144,252(1,749) [b]	108,784 (1,896) [ab]	0.572 (0.005) [c]
A163	1.321 (0.006)	55.4 (2.5)	32.7 (5.0)	130,549(1,822) [a]	112,110 (2,377) [b]	0.543 (0.005) [ab]

Standard errors of the mean of three independent spore crop preparations are given between brackets.

[*]A fourth significant figure (± $5x10^{-4}$) was added to calculate the ANOVA, due to null variance.

The number of transmission electron microscopy image sections analysed was as follows: M5= 197, M5(50)= 151, M88= 183, M1= 151, M112= 168, M112(50)=161 and A163=179.

Mean values with no common letters differ significantly ($P< 0.05$) as determined by application of Bonferroni *post hoc* test.

Interestingly, sporulation of the strains M5 and M112 at higher temperatures had a strong impact on the ultrastructure of the spore, by leading to reduced core and cortex areas in both spore crops of M5(50) and M112(50). However, the core-sporoplast ratio remained mostly unchanged, indicating that the modulatory effect of the temperature was exerted in the spore as a whole.

Dependence of spore heat-resistance on physicochemical composition and ultrastructural properties

Parametric and non-parametric correlational analyses between spore heat-resistance (sporulation at 37°C) and spore compositional and ultrastructural factors are summarised in Table 4.

The Pearson's correlation coefficient is given in the first line (r) and the Spearman's correlation coefficient (ρ) is given in the second line in grey italics (N=5), for spores of M5, M88, M1, M112 and A163 obtained at 37°C. The colour scheme is used in relation to the r coefficient: Black highlights r higher than 0.7; grey highlights r values between 0.4 and 0.7, while white highlights r below 0.4. *Correlation is significant with $P < 0.05$ or ** $P< 0.01$.

A positive and very high Pearson´s correlation coefficient was found between spore heat-resistance and the average content of magnesium, manganese and calcium ($r> 0.8$). For the core-sporoplast ratio, r was slightly lower ($r= 0.72$). In the case of calcium and the core-sporoplast ratio, the Spearman's correlation coefficient was lower than Pearson's indicating the occurrence of deviations from an unidirectional variation of the factors with $\log D_{110°C}$. The Pearson's correlation coefficient was found to be only moderate with core water content, DPA content and cortex area, while it was low with potassium, iron and core area. However, in the case of DPA, a very high Spearman's correlation coefficient was obtained with $\log D_{110°C}$. This indicates that DPA content, although not linearly correlated with the spore heat-resistance, is an important factor contributing for the spore heat-resistant phenotype. Other interesting observations are the existence of a very high and significant correlation between DPA and spore water content ($r= 0.95$ and $\rho= 0.90$) and core-sporoplast ratio with divalent cations, especially with calcium ($r= 0.89$ and $\rho= 0.70$).

Next, we evaluated the possibility of expressing spore heat-resistance as function of the determined factors. We used Multiple Linear Regression (MLR) for this purpose. Both the stepwise-backwards and hierarchical regression methods were tested to add possible predictors into the model and assess the significance of these predictors to describe together the spore heat-resistant phenotype. Table 5 summarises the results of the regression with the backwards regression method (the results with the hierarchical method were very similar).

We took advantage of the *a priori* knowledge on factors more strongly correlated with $\log D_{110°C}$ (both correlation with the average values and with individual independent replicates) to restrict the number of tested predictors to calcium, manganese, magnesium and core-sporoplast ratio, as recommended elsewhere (Field, 2005). Manganese and the core-sporoplast ratio were found to be the only factors which showed a significant correlation with spore

Table 4. Relationship between spore heat-resistance and spore properties in *Bacillus subtilis* strains

	Heat-resistance ($\log D_{110°C}$)	Calcium	Magnesium	Potassium	Iron	Manganese	Core water	DPA	Core area	Cortex area	Core-sporoplast ratio
Heat-resistance ($\log D_{110°C}$)	1										
Calcium	0.83 *0.60*	1									
Magnesium	0.99** *1.00***	0.99* *0.60*	1								
Potassium	0.04 *0.30*	0.21 *0.10*	0.15 *0.30*	1							
Iron	0.38 *0.00*	0.66 *0.60*	0.44 *0.00*	-0.14 *-0.20*	1						
Manganese	0.92* *1.00***	0.67 *0.60*	0.90* *1.00***	0.17 *0.30*	0.01 *0.00*	1					
Core water	-0.56 *-0.60*	-0.50 *-0.80*	-0.54 *-0.60*	0.43 *0.40*	-0.2 *-0.60*	-0.22 -0.60	1				
DPA	0.52 *0.80*	0.32 *0.60*	0.46 *0.80*	-0.63 *-0.30*	0.62 *0.30*	0.23 *0.80*	-0.95** *-0.90**	1			
Core area	0.00 *-0.10*	0.24 *0.50*	0.01 *-0.10*	-0.58 *-0.60*	0.83 *0.90**	-0.36 *-0.10*	-0.74 *-0.70*	0.67 *0.40*	1		
Cortex area	0.50 *0.30*	0.83* *0.90**	0.56 *0.30*	-0.17 *0.20*	 *0.80*	0.27 *0.30*	-0.53 *-0.60*	0.38 *0.30*	0.63 0.60	1	
Core-sporoplast ratio	-0.72 *-0.50*	-0.89 *-0.70*	 *-0.50*	-0.33 *-0.60*	-0.30 *0.00*	-0.74 *-0.50*	0.08 *0.20*	0.06 *-0.10*	0.11 *0.20*	0.70 *-0.60*	1

$\log D_{110°C}$ as indicated by the significance of the corresponding coefficients ($P< 0.05$). Although the average content of magnesium was shown to be highly correlated with spore $\log D_{110°C}$ (Table 4), due to high variation in the individual independent replicates, its effect became weaker. However, the Model 3 was the model which was statistically accepted, since it led to a significant F change value ($P< 0.05$) and the Durbin-Watson variable (d) was closer to 2. In model 3, manganese accounted for about 70% of spore heat-resistance, as indicated by the regression indice $r^2_{adjusted}$. A negative coefficient β_0, points towards a very low heat-resistance in the absence of this divalent cation, which is a plausible result. The linear equation pertaining to model 3, relating the heat-resistance of *B. subtilis* subsp. *subtilis* spores produced at 37°C with the content of manganese, can be written as follows:

$$\log D_{110°C} = 2.03 + 2.44\ Mn \quad (3)$$

With *Mn* being the concentration of manganese in µg/mg DW and $D_{110°C}$ having units in minutes.

Table 5. Multiple Linear Regression (MLR) models for describing of *Bacillus subtilis* spores heat-resistance

	Predictor[a]	Unstandardised coefficient	Coefficient significance	$r^2_{adjusted}$	Significance *F* change	Durbin-Watson (*d*)
Model 1	β_0	4.25 (5.26)	0.436	0.712	0.001	NA[b]
	β_1	0.012 (0.042)	0.782			
	β_2	-10.2 (7.86)	0.220			
	β_3	1.77 (0.56)	0.009			
Model 2	β_0	5.17 (3.98)	0.218	0.734	0.782	1.26
	β_2	-11.4 (6.29)	0.093			
	β_3	1.77 (0.54)	0.007			
Model 3	β_0	-2.03 (0.53)	0.002	0.687	0.000	1.52
	β_2	2.44 (0.43)	0.000			

The Backwards regression method was used to enter the predictors.

[a]Includes calcium (µg/mg dry weight) (β_1), manganese (µg/mg dry weight) (β_2) and the core-sporoplast ratio (β_3).
[b]NA-Not Available, i.e., no value resulted in the analysis.

Discussion

B. subtilis subsp. *subtilis* constitutes a subspecies characterised by large genetic diversity. This characteristic is manifested in the broad spectrum of heat-resistance of their derived spores (Lima et al., 2011b; Lima et al., 2012; Oomes et al., 2007). A comprehensive analysis of the relative contribution of physicochemical and ultrastructural factors modulating spore heat-resistance at intraspecies level, had been carried out for *C. perfringens* (Novak et al., 2003; Orsburn et al., 2008). In our work, we took advantage of the isolation of *B. subtilis* subsp. *subtilis* strains from processed foods, two of them forming spores of remarkably high heat-resistance to search for factors that could explain a high heat-resistant spore phenotype across strains of this species.

We found that the increased average content of manganese, calcium, and magnesium, and the decreased core-sporoplast ratio, but especially manganese, were the most important factors explaining high heat-resistance in *B. subtilis* subsp. *subtilis* spores. The observation that an increased content of manganese also corresponded with an increment in spore heat-

resistance for strain M5(50) when compared to M5(37), reinforces the essential role of this mineral for the differential heat-resistance in spores of this bacterial species. Conversely, while the spore core-sporoplast ratio appeared to be an important modulator of spore heat-resistance across the strains, it could not explain the increased heat-resistance within spores of the same strain when sporulated at higher temperature.

A remarkable outcome in this work was the relative magnitude of changes induced in spores of strains M5 and M112 upon sporulation at a higher temperature. While the heat-resistance of spores of strains M112(50)37 and M112(50)50 remained largely unchanged in comparison to M112(37)37, for spores of M5(50)37 an increase in 18-fold was found for $D_{110°C}$ when compared to that of spores of M5(37)37. The latter spores have approximately 5-fold lower $D_{100°C}$ than that of spores of the most studied strain of *B. subtilis*, strain 168 (Kort et al., 2005). Interestingly, this increase in the *D*-value was aligned with the observation that spores of M5(50) systematically displayed increased average accumulation of minerals and a slight lower average content of water, whereas this was not the case for spores of M112(50). The only feature found in common between spores of M5(50) and M112(50) was an increase in the average content of iron and manganese (more modest for spores of M112(50)), and a decrease in the average areas of the core and cortex (more modest for spores of M5(50)).

Manganese has been suggested to have an important role in the oxidative stress resistance, by protecting the cell against reactive oxygen species that can be generated by exposure at high temperatures (Aguirre and Culotta, 2012; Inaoka et al., 1999; Mols et al., 2009; Mols et al., 2011). Resistance against stresses may be conveyed to the cell by protection of the active site of enzymes, e.g. superoxide dismutase, and formation of non-proteinaceous manganese-based antioxidants (Aguirre and Culotta, 2012). Such role could be related to an increased accumulation of manganese in spores of M5(50) and M112(50) when produced at higher temperatures. A recent communication demonstrated that sporulation of a strain of *B. subtilis* in a medium with an increased concentration of manganese did not offer additional protection to the spore against inactivation by wet heat (Granger et al., 2011). This insensitivity of spore heat-resistance to increasing concentrations of manganese has two important consequences: (i) it is in line with experiments which showed that oxidative stress is not a major cause of spore inactivation (Setlow and Setlow, 1998); (ii) possibly indicates that the amount of manganese needed to fulfill a protective role in the spore and /or (outgrowing) cell is only required within a very narrow range.

The observation that strains within the same species respond differently to increases in sporulation temperature had been reported in other studies. For instance, while in *B. subtilis* ATCC 4673 sporulation at 50°C resulted in a 5-fold increase in the $D_{100°C}$ when compared to spores produced at 37°C, in *B. subtilis* A, growth at 49°C led to a decrease up to about 3-fold in $D_{100°C}$ when compared to sporulation at 37°C (Beaman and Gerhardt, 1986; Lindsay et al., 1990). Furthermore, contrary to the observation in this work, spores of *B. subtilis* A produced at 49°C, had lower manganese content, concomitantly with a reduction in the contents of calcium and magnesium. Nonetheless, an increase in spore heat-resistance with increased sporulation

temperature is the most reported trend (Beaman and Gerhardt, 1986; González et al., 1999; Melly et al., 2002; Palop et al., 1999a). Recent work (Baril et al., 2012b) clearly elucidates that spore heat-resistance does not increase indefinitely with the sporulation temperature, but shows a plateau after which the spore *D*-value decreases rapidly, independently of the heating temperature.

The heat-resistant behavior of spores of strain M5 towards increased sporulation temperature was in accordance with its growth performance at high temperature ranges. A parallel between the temperature for strain optimal growth and the temperature leading to the highest spore heat-resistance has been recently demonstrated (Baril et al., 2012a). This could indicate that the temperature resulting in spores of maximum heat-resistance could be substantially lower for spores of M112 when compared to M5.

The findings in this work showed some differences when compared to those by Orsburn et al. (2008) for spores of *C. perfringens*. By computing correlation coefficients between $\log D_{90°C}$ for *C. perfringens* strains in the study by Orsburn et al. (2008) with their determined factors, a high dependence of $\log D_{90°C}$ with the average content of iron (r= 0.80/ρ= 0.77) can be found, while with calcium and magnesium this dependence was only moderate ([r= 0.44/ρ= 0.47] and [r= 0.42/ρ= 0.45], respectively). On the other hand, the dependence of spore heat-resistance with the core-sporoplast ratio (r= -0.703/ρ= 0.236), the content of DPA (r= 0.419/ ρ= 0.450) and core water (r= -0.642/ρ= -0.544) were similar to this investigation. In an earlier study with spores of *C. perfringens*, Novak et al. (2003) reported a much lower correlation between spore heat-resistance and DPA (r= -0.138). In the two aforementioned studies, the content of manganese was not reported, thus it is not possible to hypothesize about a putative universal role of this mineral to predict spores differential heat-resistance at intraspecies level. Taken together, these findings suggest that the relative contribution of specific factors for the differential heat-resistance of spores might differ across species.

The range of minerals reported for *B. subtilis* varies extensively in the literature. For example, in the case of calcium, levels ranging between 12.2 to 44 µg/ mg DW have been reported (Atrih and Foster, 2001; Igura et al., 2003; Lindsay et al., 1990; Pendukar and Kulkarni, 1988), while, for manganese, levels as high as 6.03 µg/ mg DW (Atrih and Foster, 2001) to levels of 0.27 µg/ mg DW were published (Igura et al., 2003). However, the trend that spores show higher propensity to accumulate calcium, and propensity to accumulate manganese and iron in substantially lower concentration, is verified across works and appears to be valid for a large diversity of spore-forming species (Atrih and Foster, 2001; Bender and Marquis, 1985; Lechowich and Ordal, 1962; Orsburn et al., 2008). In addition to differences in methods, these variations might be explained by the fact that the exact mineral composition of spores is strongly determined by the medium composition and the sporulation temperature (Atrih and Foster, 2001; Ghosh et al., 2011; Granger et al., 2011; Lindsay et al., 1990).

Work with isogenic mutants of *B. subtilis* subsp. *subtilis* impaired in the synthesis of DPA, by a mutation in the *spoVF* operon (encoding DPA synthetase) and germination operons A, B and K (*Δger3*), demonstrated in a very elegant way, the important role of DPA-mineral complexes

in the displacement of water in the spore core and, consequently, its contribution for spore stability and resistance (Paidhungat et al., 2000). Such role was significantly corroborated in this work, where a very high and significant correlation between DPA and core water content was registered. This was also the case in the work by Orsburn et al. (2008), where Pearson's and Spearman's correlation coefficients of -0.74 and -0.64, between DPA and core water content can be computed, respectively. DPA levels between 3.8 to 15% of the spore DW have been reported for bacterial spores (Huang et al., 2007; Pendukar and Kulkarni, 1988). The levels of DPA in this work (2.7-4.06% DW) were somewhat lower when compared to this reported range. The corresponding molar ratio of DPA-calcium varied between 0.32 for spores of strain M1 to 0.62 for spores of M5(37). This is lower than the ratio of 1 mentioned for *B. subtilis* and other bacterial spores (Huang et al., 2007). However, other researchers have encountered deviations from this value not only in *B. subtilis*, but also in other species (Lindsay et al., 1990; Orsburn et al., 2008; Pendukar and Kulkarni, 1988). Differences in growth conditions and inherent genetic differences in *Bacillus* strains could contribute to such observed dissimilarities (Huang et al., 2007).

Spore core dehydration is mentioned as a most important factor determining spore heat-resistance (Beaman and Gerhardt, 1986; Popham et al., 1995). For this reason, it was expected that strain A163 would have a lower water content considering its high heat-resistance. However, this was not the case. This agrees with previous studies that concluded that the spore water content cannot be used as a sole predictor of spore heat-resistance (Kort et al., 2005; Orsburn et al., 2008). It is known that although water content is an important factor in spore heat-resistance, the total effect due to water is based on the net water activity in the spore core (Sunde et al., 2009). It is conceivable that other factors, such as the intrinsic thermostability of spore proteins, could add to the effect of partly dehydrated spore core, accounting for the higher heat-resistance of A163 spores (Warth, 1978).

Apart from the DPA, a role for the cortex peptidoglycan in the decrease of the core water content had been proposed. This was based on the hypothesis that reduction of the overall cortex matrix cross-linking would provide the cortex of higher volume and flexibility required for water loss during spore formation (Warth, 1985). However, this idea was later dismissed and a more passive role in maintenance of the dehydration state of the core was proposed (Popham et al., 1995; Popham et al., 1996). Indeed, variation in the extent of peptidoglycan cross-linking was observed within the group of heat-resistant and heat-sensitive spores of *C. perfringens* (Orsburn et al., 2008), pointing towards the possibility that the peptidoglycan structure, although being an important feature for spore stability, is of less importance for spore differential heat-resistance within species.

In this work, the inspection of the frequency distributions for the core-sporoplast ratio, as well as core and cortex area (data not shown), could not explain the biphasic inactivation pattern for spores of M5(50), since one single population emerged from the frequency distributions. This suggests that other factors underlie the occurrence of subpopulations of spores having different heat-resistance. Huang et al. (2007) demonstrated that in *B. subtilis*,

B. cereus and *B. megaterium*, spores vary with respect to their content of DPA-calcium and size. This is likely to impact the individual levels of water content and degree of heterogeneity in spore heat-resistance within a single spore crop (Huang et al., 2007). However, spores of M5(50) registered the lowest variance in DPA content and therefore, this factor alone could not explain the observed biphasic behaviour in spores of this strain either.

We attempted to probe the obtained MRL model, relating spore *D*-value with the content of manganese, to predict the heat-resistance of *B. subtilis* spores reported in the literature. Based on the data from Lindsay et al. (1990) a $D_{100°C}$ of approximately 5 min would be predicted. However, the experimentally determined $D_{100°C}$ was more than 10-fold higher. This shows that although the existence of such a model to predict spore heat-resistance would be desirable, its generalization can be difficult since sporulation, the technical assays and recovery conditions differ among studies.

For both *B. subtilis* (this work) and *C. perfringens* (Novak et al., 2003; Orsburn et al., 2008), although high correlations between spore heat-resistance and the above discussed factors were found, in no case did those values change consistently with the spore *D*-value, that is, they show deviations from a perfect correlation (r= 1, ρ= 1). Our data suggest that due to differences in the precise core composition of spores of strains displaying a similar heat-resistant phenotype, the specific target of spore killing, which is likely to be one or more proteins (Coleman et al., 2007; Setlow, 2006), varies among strains. This could be the case since the spore core composition, and in particular its dehydration state, affects protein immobilization and protein protection in the core (Sunde et al., 2009). Owing to the heterogeneity within a single spore crop (Huang et al., 2007), it is conceivable that such target(s) may also differ for individual spores.

In summary, this work sheds light onto factors setting heat-resistant spore-forming strains apart from heat-sensitive spore-forming ones in *B. subtilis* subps. *subtilis* strains. While a putative mechanism whereby manganese contributes to differential spore heat-resistant phenotype is not readily apparent from this work, this metal could serve as a biomarker to predict spore heat-resistance in spores belonging to this taxon.

A final conclusion is that the genetic make-up prevailed over abiotic (environmental) factors determining spore heat-resistance, as sporulation of strain M5 at 50°C did not lead to an increase of the spore crop *D*-value to levels registered for spores of strain M112 or A163. Understanding what common genomic denominator strains producing spores of high heat-resistance share, would be the next question to pursue.

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Chapter 7

Germination of highly heat-resistant *Bacillus subtilis* spores and relevance for cocoa-derived products

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The occurrence of highly heat-resistant *Bacillus subtilis* spores in food ingredients, such as cocoa powder, can challenge the stability of down-stream product applications, namely chocolate milk drinks. We assessed the germination response for spores of three highly heat-resistant and one heat-sensitive strain of *B. subtilis* subsp. *subtilis*, in L-alanine, a mixture of L-asparagine, glucose, fructose and potassium ions and nutrient broth at 22°C, 37°C and 45°C. Further information was obtained by characterising the germination in compounds and ingredients of chocolate milk drinks. The experiments were performed with absorbance- and culturing-based methods, as well as single-cell approaches, including flow-cytometry.

In the absence of heat shock activation, spores of all strains were mostly dormant to nutrient germinants. Upon sublethal heat shock activation, differences in temperature dependence and nutrient-induced germination were observed among the strains. Importantly, requirements in heat shock activation to induce germination appeared to be related to spore heat-resistance. At 22°C no appreciable germination took place for none of the spore crops.

We found that germination of spores of M112 and AR1 strains was triggered in milk and cocoa powder suspension, but to a very limited extent, even after heat shock activation treatment. Remarkably, cocoa powder supported a full cycle of spore germination-growth-sporulation.

Spores of M112 formed the most superdormant spores and it is shown that treatments in the Ultra-High Temperature range for milk are required to induce almost complete germination when the spores are recovered in the presence of a defined germinant or complex medium.

The applicability of the results for spore control in the food industry is discussed.

Introduction

Among endospore-forming *Firmicutes*, *Bacillus subtilis* is a frequently isolated species from a number of food production chains, such as of dairy products (Burgess et al., 2010; Scheldeman et al., 2005), indigenous fermented foods (Lima et al., 2011a; Parkouda et al., 2009), soups (Oomes et al., 2007), gelatin (De Clerck and De Vos, 2002) and cocoa powder (Lima et al., 2011b; Lima et al., 2012; Witthuhn et al., 2011). The ability of *Bacillus subtilis* to grow at a relative large range of temperatures, to metabolize various compounds, to adapt to different ecological niches and to produce spores that resist diverse physical and chemical insults (De Vos et al., 2009; Hecker and Volker, 2001; Setlow, 2006; Tam et al., 2006), is likely to contribute to its ubiquitous distribution in the environment.

We have recently shown that certain strains of *B. subtilis*, and in particular of *B. subtilis* subsp. *subtilis*, isolated from cocoa powder and intermediate products of cocoa manufacturing, are able to form spores of remarkably high heat-resistance (chapter 4 and 6). These spores have the potential of surviving industrial processes designed to kill the large majority of spores, thus posing a risk for the stability of downstream cocoa powder applications, such as Ultra-High Temperature (UHT) treated chocolate milk drinks (chapter 4). A peculiarity some spores of highly heat-resistant strains share, is a prominent activation shoulder in thermal destruction curves, which was systematically observed upon a heat treatment at 110°C for 5 min or higher (chapters 4-6). This behaviour constitutes an indication of extreme dormancy, since spores of these strains require substantial heat-activation to efficiently germinate and form colonies on plates.

Typically, the food industry deals with extremely dormant bacterial spores by employing what is knonw as “fail-safe” strategies (Ross, 1996). However, these strategies lead to over-processing, having as a consequence a detrimental effect on food quality and a negative impact on the costs and efficiency of production (Brul et al., 2006; van Zuijlen et al., 2010). A method which has been suggested for the control of extremely dormant spores, more precisely known as ‘superdormant spores‘ (Ghosh and Setlow, 2009a), in foods is the induction of their germination (series of events preceding the resumption of vegetative growth), followed by the application of a milder thermal regime (Gould et al., 1968). This constitutes an attractive strategy, since as a result of spore germination, the unique structure of the spore is lost, together with its specific properties of heat-resistance and dormancy (Moir et al., 2002; Setlow, 2003). However, the efficiency of coupling the germination to heat treatments has been thwarted by the observation that a number of individual spores do not germinate upon exposure to germinants, even after sublethal heat shock activation treatments (Broussolle et al., 2008; Ghosh et al., 2009; Gould et al., 1968; van der Voort et al., 2010).

Spore germination can be initiated by specific nutrient-germinants, such as amino acids, sugars and purine nucleosides, but also by non-nutrient compounds (e.g. calcium-dipicolinic acid) and physical treatments (e.g. high hydrostatic pressure) (Reineke et al., 2012; Setlow, 2003). *B. subtilis* has served as an important model species to better understand the process of germination, but these investigations have been mostly performed with laboratory strains

or a limited range of natural isolates which do not constitute a challenge for the food industry, due to low heat-resistance of their derived spores. It was demonstrated that heat-sensitive *B. subtilis* spores tend to germinate reasonably synchronously with nutrient-germinants, and that for some spores heat shock activation is not essential (Ghosh et al., 2009; Leuschner and Lillford, 1999; Moir and Smith, 1990; Paidhungat et al., 2000). On the contrary, we still lack data regarding the germination response of strains forming spores of high heat-resistance, in terms of behaviour to commonly used nutrient compounds known to induce germination in *B. subtilis* spores, influence of the incubation temperature and specific heat-activation requirements prior to germination. Highly heat-resistant spores which survive industrial processing can only proliferate and cause spoilage in foods if they germinate. Thus, generation of data about the germination behaviour of these spores is essential to allow the prediction of spoilage incidents and provide means for their control in foods.

In *B. subtilis*, reference nutrient compounds to study germination consist of L-alanine and a 4 compound mixture of L-asparagine, D-glucose, D-fructose and potassium ions (AGFK) (Wax and Freese, 1968). These nutrient-germinants are recognized by germinant receptors located in the spore inner membrane, termed GerA, GerB and GerK, with each receptor being formed by the subunits A, B and C. The receptor GerA responds to L-alanine, whereas the GerB and GerK are required together to respond to AGFK or to a mixture of L-alanine with GFK (Moir and Smith, 1990; Moir et al., 2002; Wax and Freese, 1968; Zhang et al., 2011).

In addition to studying spore germination in defined compounds, gathering of information about germination responses in (complex) food matrices is of considerable interest to realistically streamline spore germination predictions.

Therefore, the aim of this work was to study the germination response for a selection of *B. subtilis* strains isolated from cocoa products characterised by the formation of spores of high heat-resistance and activation shoulders upon heat-inactivation treatments. The germination behaviour of these strains was assessed in the presence of defined germinants, as well as in food matrices of relevance to the manufacturing of chocolate milk drinks. The effect of the incubation temperature on spore germination was also tested. Additional information was gathered by studying the effect of simulated UHT treatment on the germination and outgrowth capacity at single spore level by means of flow-cytometry, for one strain producing the most superdormant spores among all strains.

Materials and methods

Strains and culture conditions

Four strains of *Bacillus subtilis* subsp. *subtilis*, previously identified to species and subspecies level on the basis of 16S rRNA and gyrase A (*gyr A*) gene sequencing, were selected for this study (Lima et al., 2011b; Lima et al., 2012). Strains 30AA2-6 and 30AR1-1 were isolated from a cocoa powder processing line after the stage of cocoa nibs alkalising and roasting,

respectively, while strains 30P3-3 and M112 were isolated from commercial cocoa powder samples. Strains 30AA2-6, 30AR1-1 and M112 were chosen because spores of these strains had shown an activation shoulder upon heating at 110°C for 5 min, whereas spores of strain 30P3-3 were inactivated to levels below the detection limit (Lima et al., 2011b; Lima et al., 2012). For simplification, strains 30AA2-6, 30AR1-1, 30P3-3 are further referred to in this work as AA2, AR1 and P3-3.

Routine working-cultures of these strains were prepared by direct inoculation from a frozen vial, using a 10 µl inoculation loop, and by subsequently transferring the icy-cell suspension into 25 ml of Nutrient Broth 13 g/l (NB, Oxoid) pH 7.0 in a 100 ml Erlenmeyer flask. The Erlenmeyers were then incubated for 16-18 h at 37°C in a shaking water bath (200 rpm; Julabo SW 24).

Preparation of the spore crops

Spore production, purification and storage was performed as described in chapters 4 and 6. The sporulation medium consisted of Nutrient Agar (NA) (13 g/l NB solidified with 15 g/l Agar Bacteriological [AB, Oxoid]) pH 7.0, supplemented with minerals as described by Cazemier et al. (2001). The strains were sporulated for 72 h at 37°C, which corresponded to the release of 95-99% of free phase-bright spores. The spores were extensively purified by centrifugational washings in phosphate Buffer (PB) 10 mM pH 7.0 (tenfold dilution of 0.1 M K_2HPO_4/ KH_2PO_4 buffer), over a period of 4 weeks as described in detail in chapter 6. The spore crops of each strain were prepared on the same day and handled in identical manner. In total, three independent spore batches per strain were prepared (each set was prepared on different days and with freshly made sporulation medium and buffer).

Heat-inactivation assay

The wet heat-resistance of the prepared spore crops was evaluated at 110°C for 5 min by using the Kooiman method (Kooiman, 1973), as described before (Chapter 4). After the heat treatments, the number of survivors was quantified by enumeration in duplicate pour-plates of NB 2.6 g/l solidified with AB (15 g/l), after incubation at 37°C for 5 days. Survival counts were made from plates with a number of colonies between 10 and 300. The survival at 110°C-5 min was obtained by subtracting the number of viable spores after heating, from the number before heating.

Germinants

The germinants used in this study and their final concentration in the assay were as follows: L-alanine (Sigma) 10 mM, L-asparagine (Sigma) 3.3 mM, D-glucose 5.6 mM (AGFK assay; Merck) and 333 mM (milk component assay), D-fructose 5.6 mM (AGFK assay; Merck) and 205 mM (milk component assay), galactose 128 mM (Sigma), lactose 134 mM (milk component assay; Merck), KCl (Merck) 10 mM, kappa-carrageenan 0.02% w/v (Sigma) and NB 13 g/l (CM0001, Oxoid). The solutions were freshly prepared before each assay, by dissolving in PB 10 mM pH

7.0, followed by filter-sterilization with a 0.22 µM disposable syringe filter (Sartorius stedim biotech). All solutions were kept at room temperature prior to their use.

In addition, spores were assayed for their germination in Ultra-High Temperature (UHT) treated cow milk ('Friesche Vlag' 1.5% fat, 250 ml package, FrieslandCampina) and cocoa powder suspension 1.9% (w/v) (Van Houten, Cacao de Zaan). The cocoa powder was resuspended in the solution of kappa-carrageenan 0.02% (w/v) (El-Khair, 2009), which had been dissolved in PB.

Spore germination assay

Germination characterised by absorbance based-method

Non-heat shocked (NH) and heat shocked (HS) spores were tested for their germination capacity. Different heat-shock activation treatments were applied depending on the strain. This was 80°C for 10 min for strain P3-3, 100°C for 10 min for strains AR1 and AA2, and 100°C for 30 min for strain M112. The spores resuspended in PB were heat shocked in 1.5 ml eppendorf tubes (Greiner bio). After the heat shocks, the spores were cooled down in icy water and subsequently used in the assays. Preliminary assays showed that washing by centrifugation with buffer after the heat shock treatment had no effect on spore germination when compared to when these spores were not washed (data not shown). For this reason the step of washing was discontinued. Next, 20 µl of spores were resuspended in the germinant solution at an Absorbance of 600 nm (A_{600nm}) adjusted to 0.9-1 (spectrophotometer Novaspec II, Pharmacia Biotech) in a 96 well microtiter-plate (300 µl wells, Greiner Bio-One), placed on ice. The combined volume of spore suspension and germinant solution was 200 µl. Three replicate wells were used per independent spore crop and germinant type. Negative controls, consisting of HS and NHS spores resuspended in buffer were always included in the same microtiter-plate assay. Germination of HS and NH spores of the four strains, at the different nutrient-temperature combinations, were performed simultaneously for each individual spore crop.

In order to reduce evaporation losses and edge effects, the outermost rows and columns of each plate were filled with sterile milli-Q water and the edges of the plate with the cover were sealed with parafilm (Borucki et al., 2003). Spore germination was measured by monitoring the fall in the A_{600nm} (spectrophotometer VersaMax, Molecular Devices) which takes place during the transition from phase-bright to phase-dark spores. The assay was initiated after the spectrophotometer had been pre-warmed at the desired incubation temperatures of 22°C, 37°C and 45°C. The A_{600nm} was measured every 2 min for 18 h, after shaking for 30 s at time 0 h and 1 h and 18 h. The germination progress was monitored with the Softmax pro v.5.2 software. The germination response was described as the A_{600nm} drop (%) from an initial A_{600nm} of 0. At the end of the germination assay the plates were transiently placed at 4°C, and next, 2-3 wells per spore-germinant combination were visualised by phase-contrast microscopy (Olympus BX40F4). The different cell types (non-germinated or phase bright, germinated or

phase dark and vegetative cells) were counted in three to five microscopic fields and expressed as percentage.

Spores of all four strains were assessed for their germination response in the reference system for *B. subtilis*, specifically L-alanine and AGFK, as well as in NB. In addition, spores of strains AR1 and M112 were assayed for germination capacity in the presence of sugars found in milk. All three independent spore crops were assayed in different days and with freshly prepared germinant solutions.

Germination characterised by culturing based-methods

The germination of spores of strains AR1 and M112 was studied in the presence of UHT milk and cocoa powder suspension 1.9% (w/v). The milk and cocoa powder suspension (39.6 ml) were pipetted into 50 ml Falcon tubes (Greiner Bio-One) and subsequently pre-warmed at the incubation temperature of 37°C in a water-bath (Julabo SW 24). These media were next inoculated with HS spores to a final concentration between 10^4-10^5 CFU/ml and incubated in the water bath under static conditions for 48 h at 37°C. The tubes were sampled at eight time points, including at time 0 h (immediately after inoculation) for enumeration of Total Aerobic Microorganisms (TAM) and Total Spores (TS) (the same heat treatment as for heat shock activation, in order to kill vegetative cells).

At the set time, the Falcon tubes with inoculated milk or cocoa powder suspension were sampled by pipetting 1 ml into tubes with 9 ml of Peptone Physiological Saline Solution (PPS) (1 g Neutralized Bacteriological Peptone [NBP, Oxoid] and 8.5 g NaCl per liter) and serially diluted. Subsequently, one mililiter of the appropriate dilution was pour-plated in duplicate with NA and incubated at 37°C for TAM enumeration. In addition, for TS enumeration, 1.2 ml aliquot was taken and cultured as described for TAM, after heat shock activation treatment. Additionally for TS enumeration, culturing was performed in NA plates supplemented with 10 mM L-alanine, which were next incubated at 45°C. The plates were enumerated after incubation for 2 days and the results were expressed in log CFU/ml. A negative control of spores was performed by incubating HS and NHS spores in PB and sampling at time 0 h and 48 h. Negative controls of the milk and cocoa powder suspension were incubated concomitantly with the other inoculated tubes to assess for endogenous microbiota.

Two independent experiments were performed with two independently prepared spore crops, on different days and with freshly prepared media.

Simulated UHT treatment

Spores of strain M112 were subjected to a simulated UHT treatment at 135°C for 4 and 8 s to assess the effect of a more severe treatment on spore germination and outgrowth capacity. Two-hundred microliters of NH spores of strain M112 were injected with a syringe (Hamilton CR-700-200) into a stainless steel tube (described in chapter 4) containing 9.8 ml PB, which had been pre-warmed for 20 minutes at 135°C in a glycerol (Fluka 49780) bath (Julabo), using the materials and methods previously described (Lima et al., 2011b; Oomes et al., 2007) (final

spore concentration was 5.5-5.8 log CFU/ml, as determined by cuture-based methods). At the end of the set time the tubes were cooled down by vigorous shaking in icy water. After the UHT treatment, the content of the stainless steel tube was transferred into a 15 ml Falcon tube (Greiner Bio-one) and centrifuged (Eppendorf 5804 RF, Rf-34-6-38) for 8117 x *g* for 2 min to pellet the spores. The spores were subsequently resuspended in 10 ml L-alanine 10 mM or NB 13 g/l and incubated at 37°C in the water bath (Julabo SW24).

Flow-cytometry analysis of UHT-treated spores

After 2 h, 5 h and 24 h incubation, 1 ml of spore suspension was transferred to a 1.5 ml Eppendorf tube and subsequently centrifuged at 13,000 rpm for 1 min to pellet the spores in a benchtop centrifuge . The spores were washed once by centrifugation in filtered phosphate buffer saline (PBS) (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4), followed by resuspension in the PBS to give a final concentration of about 6 log CFU/ml. Spore germination and outgrowth was monitored by flow-cytometry, using the FACSCalibur flow-cytometer (Beckton Dickinson) and the fluorescent reporter dye Syto9 used at a final concentration of 1 µM (LIVE/DEAD BacLight bacterial viability kit, Invitrogen). Syto9 can penetrate across intact membranes and stain both living and dead cells (Haugland, 1999). Dormant spores stain poorly with Syto9, while the increasing spore permeability during germination leads to staining of the spore DNA and enhanced detected fluorescence. After staining the spore samples with Syto9 in the dark for 15 min at room temperature, the spores were pelleted at 13,000 rpm for 30 s and the supernatant was substituted by 1 ml of the PBS. The spores were resuspended to give a concentration of 10^6 CFU/ml. The samples were kept on ice prior to the analysis.

In the flow-cytometer apparatus, the operating software was the BD CellQuest Pro. (v4.0.2). The data rate was set at 600 events per second and the data acquisition was set to 20,000 events. The fluorescent emission from Syto9 was collected through a 530 nm band-pass filter using the FL1 detector, while the side scatter (SSC) threshold level was adjusted manually to 400 mV. A dot plot of FL1 versus SSC was chosen to monitor the data. The data was subsequently analysed with the Windows Multiple Document Interface software (WinMDI v. 2.9, USA; http//:facs.Scripps.edu/software.html).

Results

Germination in the presence of standard germinants

This work aimed at characterising the germination response for three natural isolates of *B. subtilis* subsp. *subtilis* with remarkably high heat-resistance (AA2, AR1, M112). To gain more insight into the extent to which the responses were specific to these highly heat-resistant spore-forming strains, a heat-sensitive strain (P3-3) was also selected.

We started by comparing the heat-resistance of the prepared spore crops by applying a heat treatment at 110℃ for 5 min, a temperature-time combination which was found previously to provide an adequate categorisation of the heat-resistance of spores isolated

from the cocoa powder production chain (chapter 4 and 5). No visible inactivation, but rather spore activation, was registered for spore crops of *B. subtilis* strains AA2, AR1 and M112, while spores of strain P3-3 were inactivated to levels below the detection limit of the method (Table 1). The results for the spore crops prepared in this work were in line with previously measured resistance for spores of these strains (Lima et al., 2011b; Lima et al., 2012).

Table 1. Spore thermal reduction (log CFU/ml) at 110°C for 5 min in phosphate buffer 10 mM and sublethal heat shock activation treatments used in the germination assays

Strain	Reduction (log CFU/ml)[a]	Heat shock (°C- min)
AA2	-0.50 (0.15)	100 -10
AR1	-1.0 (0.12)	100 -10
M112	-1.3 (0.07)	100 -30
P3-3	> 6.50 (0.18)	80 -10

[a] The reduction was obtained by subtracting the number of viable spores after heating from the number before heating. The detection limit of the method was 1.7 log CFU/ml. The standard error of the mean for the three independent spore crop preparations is given between brackets.

Next, spore germination was studied in the presence of compounds commonly tested in model strains of *B. subtilis* at 22℃, 37℃ and 45℃. These germinants consisted of L-alanine and a mixture of L-asparagine, glucose, fructose and KCl (AGFK). Figure 1 shows the germination data obtained by monitoring the drop in the initial Absorbance at 600 nm (A_{600nm}) over 14 h, upon application of sublethal heat shock activation. Table 2 presents the complete overview of the percentage of germinated spores for both heat shocked and non-heat shocked spores, as estimated by phase contrast microscopy.

Spores of strain M112 were subjected to the longest heat shock activation, 30 min at 100℃, while spores of the remaining strains were activated at either 100℃ or at 80℃ for 10 min. Spores of all strains showed germination in the presence of L-alanine and AGFK (Figure 1 and Table 2), but the extent of germination was strain dependent and it was influenced by the incubation temperature. In heat shocked spores of strain AA2, incubation in the presence of L-alanine at 37℃ or AGFK at 45℃ were the conditions which led to the strongest drop in the A_{600nm}. The average drop was between 50% and 60% and it corresponded to the occurrence of 80% to 100% germinated spores (Table 2). In the case of strain AR1, the germination showed less dependence on the type of germinant and temperature when compared to strain AA2, although the germination rate appeared to be slightly higher at 45℃ than 37℃. In the case of strain AR1, the average drop in the A_{600nm} between 50% and 60% also corresponded with 80 to 100% germinated spores. Spores of strain M112 only showed an appreciable germination at 45℃. At 45℃, M112 spores responded with an equal magnitude to L-alanine and AGFK, giving a similar reduction in the A_{600nm} of little over 40%, which corresponded to 50-70% of germinated spores. Spores of the heat-sensitive spore-forming strain P3-3 showed a very

similar germination response of that observed for spores of the highly heat-resistant strain AA2.

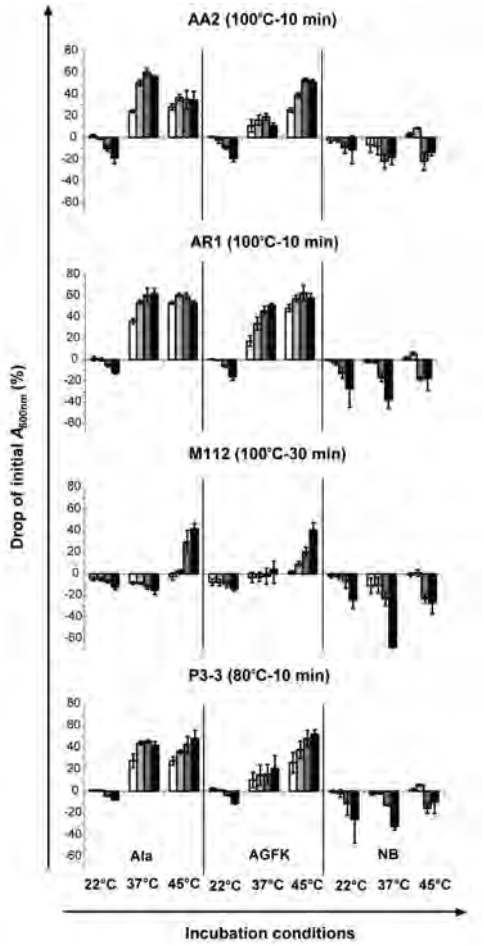

Figure 1. Drop of initial A_{600nm} (%) of heat shocked (HS) activated spores (the heat activation treatment is given between brackets) incubated in the presence of different nutrient-germinants and temperatures. The nutrient-germinants are L-alanine (Ala); a mixture of L-asparagine, D-glucose, D-fructose and potassium ions (AGFK); and nutrient broth (NB). The bars represent the different incubation time points: □ = 50 min incubation; ■ = 100 min incubation; ■ = 300 min incubation, ■ = 840 min incubation. The error bars correspond to the standard error of the mean for three independent spore crop preparations. See Table 2 for semi-quantitative information for phase-contrast monitoring of the 96 well microtiter plates and details of nutrient concentrations.

Table 2. Effect of germinants or germinant mixtures on *B. subtilis* spore population composition (%) in the germination assay in Figure 1, as assessed by phase-contrast microscopy[a]

Strain	T(°C)	PB		ALA		AGFK			NB			
		NH	HS	NH	HS	NH	HS		NH		HS	
		PD	PD	PD	PD	PD	PD	Veg	PD	Veg	PD	Veg
AA2	22	0	0	0	-	0	0	0	NA			
	37	0	0	0	+++	0	+	0	-	-	+	+
	45	0	0	+	++	+	+++	0	-	+	+	+
AR1	22	0	0	0	-	0	-	0	NA			
	37	0	0	-	+++	-	+++	0	+	-	+	+
	45	0	0	+	+++	+	+++	0	+	-	+	+
M112	22	0	0	0	0	0	-	0	NA			
	37	0	0	0	-	0	-	0	-	+	-	++
	45	0	0	0	++	-	++	0	-	+	+	++
P3-3	22	0	0	0	-	-	0	0	NA			
	37	0	0	-	++	+	++	0	+	-	+	+
	45	0	0	+	+++	++	+++	+	+	+	+	+

[a]The estimation of the population composition (%) of non-heat shocked (NH) and heat shocked (HS) activated spores (see Table 1) was performed by examination of 3-5 microscopic fields and counting of the different cell categories at the end of the germination assay. Two to three wells of the 96 well microtiter-plate for each independent spore crop (N=3 per strains) were sampled. The percentage of phase-dark (PD) and vegetative cells (Veg) is given for incubationss in L-alanine 10 mM (Ala); a mixture of L-asparagine 3.3 mM, D-glucose 5.6 mM, D-fructose 5.6 mM and potassium ions 10 mM (AGFK); nutrient broth (NB) 13 g/l; and phosphate buffer (PB) 10 mM pH 7.0 (negative control).
Germination/ vegetative cells: "0" = < 1%; "-" = 1~10%; "+" = 10~50%; "++" = 50~80%; "+++" = 80~100%. NA, means that no information is available.

In the absence of heat shock activation, the average drop in A_{600nm} was below 15% for all strains, independently of the nutrient-germinant and incubation temperature (data not shown). A notable exception was observed in the case of the combination AGFK-45°C for P3-3 spores, for which an average A_{600nm} drop of 25% was registered, corresponding 50-55% germinated spores.

The temperature of 22°C was not favourable for germination of none of the strains. The observed increase in the $A600_{nm}$ was explained by an experimental artifact.

In addition, nutrient broth was tested, due to its relevance as a general culturing media. The strains showed little reduction in the A_{600nm} (Figure 1). A maximum average drop in the A_{600nm} of 10% was observed for AA2, AR1 and P3-3 spores incubated at 45°C. A number of vegetative cells could be counted by phase-contrast microscopy, indicating that only a very small fraction of spores was able to germinate and resume vegetative growth.

The inspection of the composition of the germinated population (Table 2) showed a variable number of germinated spores and vegetative cells. In the absence of heat shock activation, the number of vegetative cells at 37°C varied between 5-10% (AA2, AR1 and P3-3)

and 20-30% (M112), whereas the number of germinated spores varied from below 5% (M112) up to 10-25% (AA2, AR1, P3-3). It was also observed that incubations at 45℃ contributed to a slight increase in vegetative cells when compared to 37℃. On the contrary, the increase in temperature had less effect on the number of germinated spores in this medium. After heat shock activation, the combined fractions of vegetative and germinated spores were higher than those of non-germinated spores for all strains and at the different incubation temperatures. The levels of vegetative cells varied from 30-50% (AA2, AR1, P3-3) to 50-60% (M112), while the levels of germinated spores ranged from 5-10% (M112) to 20-50% (AA2, AR1, P3-3).

Although outgrowth of cells of strain M112 at 37℃ led to a more prominent increase in the A_{600nm} than at 22℃ or 45℃ (Figure 1), this did not correspond to higher number of cells than that observed for incubations at 22℃ or 45℃. Instead the observed increase in the A_{600nm} was explained by cell elongation, as assessed by phase contrast microscopy.

Germination in the presence of chocolate milk drink related compounds and matrices

We subsequently focused on characterising the germination of spores of highly heat-resistant spore-forming strains in the presence of chocolate milk drinks related compounds and matrices. Since the germination behaviour of spores of strains AA2 and AR1 was similar, these experiments were focused on spores of strain AR1, which had shown slightly higher spore activation after applying the screening heat treatment at 110°C for 5 min. Spores of M112 were also selected since they showed distinct germination behaviour when compared to spores of AR1 and have high *D*-values (chapter 4 and 6).

Various sugars occurring in chocolate milk were tested. Raw milk contains about 4.6% (w/v) lactose (Walstra and Freese, 1984), and the concentrations of galactose, fructose and glucose were calculated based on the combined complete hydrolysis of both lactose (4.6% (w/v)) and sucrose (used in chocolate milk commercial formulations at levels of about 7.4% (w/v)). A standard temperature of 37°C was used, since it is also a general temperature for germination studies in *B. subtilis* and it was observed that at 22°C no appreciable germination had taken place. We had previously tested induction of germination by sucrose 7.4% (w/v) for a number of *B. subtilis* spores, including spores of strain M112, but no germination was observed at 37°C with or without heat shock activation after 10 h incubation (L. Lima and L. De Winter, unpublished results).

Figure 2 and Table 3 present the results of germination for AR1 and M112 in the presence of sugars. No germination took place in the presence of lactose after 14 h incubation, regardless of occurrence of heat shock activation treatment. With respect to the monosaccharides, important differences in the germination capacity for spores of AR1 and M112 were observed. M112 spores showed poor germination in the presence of galactose, fructose and glucose, even after heat shock activation, where germinated spores varied between 2 to 5%. Non-heat shocked spores of AR1 showed an average drop in the A_{600nm} below 5% for all monosaccharides. Conversely, heat shocked spores of AR1 exhibited an average drop in the A_{600nm} of about 5% in

the presence of galactose (around 7-15% germinated spores), of nearly 30% for fructose (45-50% germinated spores) and of about 45% for glucose (70-80% germinated spores) (Figure 2 and Table 3).

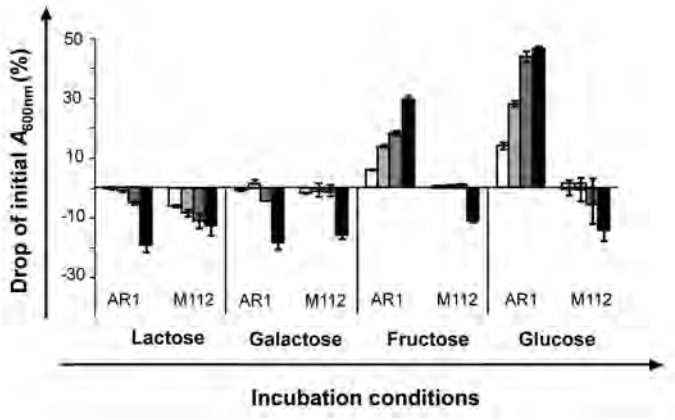

Figure 2. Drop of initial A_{600nm} (%) of heat shocked activated (HS) spores of strain AR1 and M112 incubated in the presence of different sugars at 37°C. The different bars represent the different incubation time points: □ = 50 min incubation; ■ = 100 min incubation; ■ = 300 min incubation, ■ = 840 min incubation. The error bars correspond to the standard error of the mean for three independent spore crop preparations. See Table 2 for semi-quantitative information for phase-contrast monitoring of the 96 well microtiter plates and details of sugar concentrations.

Next the germination capacity of spores of AR1 and M112 was assessed in the presence of UHT milk and cocoa powder suspension (1.9% w/v). Figure 3 illustrates the germination responses for heat shocked activated spores of AR1 and M112. In these assays, in addition to enumerating Total Aerobic Microorganisms (TAM), the level of Total Spores (TS) was quantified by heat shock activating the samples prior to plating.

Regarding the population of spores, while for AR1 a decrease of 1.2 log CFU/ ml in the level of TS occurred over the period of 48 h incubation, for M112 the average TS levels showed little change (grey line in Figure 4). This was also the case for TS which were recovered in plates supplemented with 10 mM L-alanine and incubated at 45°C (grey dots in Figure 3), the temperature which had been observed to result in significantly higher spore germination for M112. Concerning the population of TAM, an increase of 4 log CFU/ ml and 2.9 log CFU/ ml was registered for AR1 and M112, respectively, after 48 h (black line in Figure 4). Notably, the TAM curve of AR1 had a biphasic growth shape, suggesting outgrowth of a second group of germinated spores which was detected after 24 h.

Table 3. Effect of germinants or germinant mixtures on *B. subtilis* spore composition (%) in the germination assay in Figure 2, as assessed by phase-contrast microscopy[a]

	PB		Lactose			Galactose			Fructose			Glucose		
	NH	HS	NH	HS		NH	HS		NH	HS		NH	HS	
	PD	PD	PD	PD	Veg	PD	PD	Veg	PD	PD	Veg	PD	PD	Veg
AR1	0	0	0	0	0	0	+	0	-	+/++	0	-	++	0
M112	0	0	0	0	0	0	-	0	0	-	0	0	-	0

[a]The estimation of the population composition (%) of non-heat-shocked (NH) and heat-shocked (HS) activated spores (see Table 1) was performed by examination of 3-5 microscopic fields and counting of the different cell stages at the end of the germination assay. Two to three wells of the 96 well microtiter-plate for each independent spore crop (N=3 per strains) were sampled. The percentage of phase-dark (PD) and vegetative cells (Veg) is given for incubation in various sugars and potassium-phosphate buffer (PB) (negative control) at 37°C. The concentration of the germinants were as follows: lactose 134 mM; galactose 128 mM; D-fructose 205 mM; D-glucose 333 mM.
Germination/ Vegetative cells: "0" = < 1%; "-" = 1~10%; "+" = 10~50%; "++" = 50~80%; "+++" = 80~100%. NA, means that no information is available.

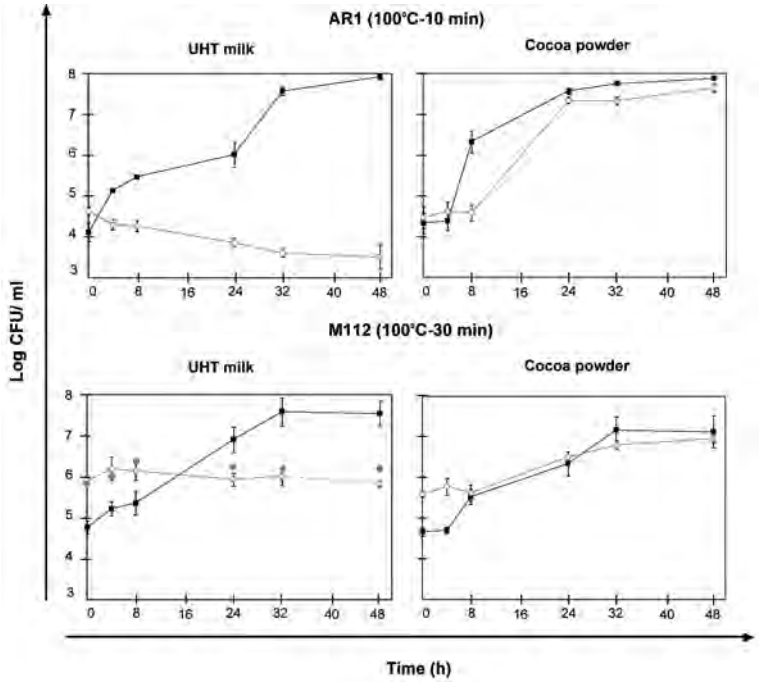

Figure 3. Germination and outgrowth behaviour of heat shocked spores of strains AR1 and M112 in the presence of UHT milk and 1.9% cocoa powder suspension at 37°C. Black lines correspond to Total Aerobic Microorganims, which were obtained by direct plating, while grey lines correspond to Total Spore counts (the samples were further heat shocked prior to plating). Grey dots show the recovery of heat shocked spores of M112 in nutrient agar plates supplemented with L-alanine which were incubated at 45°C. The error bars correspond to the standard error of the means for two independent spore preparations.

In the absence of heat shock activation, TS showed a decrease of 0.6 log CFU/ml for AR1, while for M112 a decrease of less than 0.4 log CFU/ml was quantified after 48 h incubation (data not shown). However, the TAM population reached similar final levels as observed in Figure 3, with the difference that a lag time with a duration of nearly 24 h for AR1 and 8 h for M112 was present.

Remarkably, in the presence of cocoa powder, after an initial lag phase of about 8 h, the TS population increased to similar levels of that of TAM for both AR1 and M112 (Figure 3). This means that cocoa powder was able to induce germination in spores of these two strains, and that the small fraction of spores which germinated and outgrew was able to complete a full cycle of sporulation.

The spores had been first tested for germination in the presence of pure carrageenan solution 0.02% by monitoring the drop in the A_{600nm} but, as expected, carrageenan did not induce a drop in the A_{600nm} or phase transition from phase bright to a phase dark spores (data not shown). This means that the observed effect of germination-resporulation was solely due to cocoa powder.

Throughout these experiments the endogenous TAM levels of milk and cocoa powder suspension were always below the detection limit and spores incubated in phosphate buffer (PB) remained phase bright, indicating no contribution of other factors for the observed responses in Figure 3.

Effect of simulated UHT treatments on the germination capacity

The previous experiments showed that strain M112 formed considerable more superdormant spores than strain AR1. Besides testing the effect of heat shock activation at 100°C, we assessed further the effect of a simulated Ultra-High temperature (UHT) treatment at 135°C for 8 s (without prior heat shock activation) on spore viability, as evidenced by subsequent ability to germinate and grow out. UHT treatments for milk are usually in the range of 135°C-150°C for 1 to 8 s, and these are meant to inactivate all microorganisms, including spores (Anon., 1992; Scheldeman et al., 2006).

In this set of experiments flow-cytometry was used to unveil details of population composition, while phase-contrast microscopy was performed in parallel to match the changes observed with flow-cytometer data with occurrence of different cell types. Figure 4 summarises some observations that were obtained in the presence of L-alanine 10 mM and nutrient broth. The transition from a dormant to a germinated spore increases the amount of dye which penetrates the spore and this is reflected in an increase in fluorescence (FL1-H). Morphological changes which accompany spore germination (increase in size) are shown in the forward scatter axis (FSC). Figure 4AI shows a population formed by mostly ungerminated spores. The signals of the dormant spores were not clustered into a single cloud of dots as it would be expected. In these experiments it was not possible to find out the reason for the displayed behaviour, but it is likely to be due to spore clumping, since it could result in spores of larger size and, therefore, of higher FSC values. Upon spore incubation in the presence of L-alanine 10 mM at

45°C for 14 h, there was a shift towards increased fluorescence, with approximately 70% of the spores being located in the upper region R2 (Figure 4AII). Spores in region R2 were germinated spores, as confirmed by phase-contrast microscopy. The estimations of germinated spores by phase-contrast microscopy were aligned with those performed with the WinMDI software for flow-cytometry.

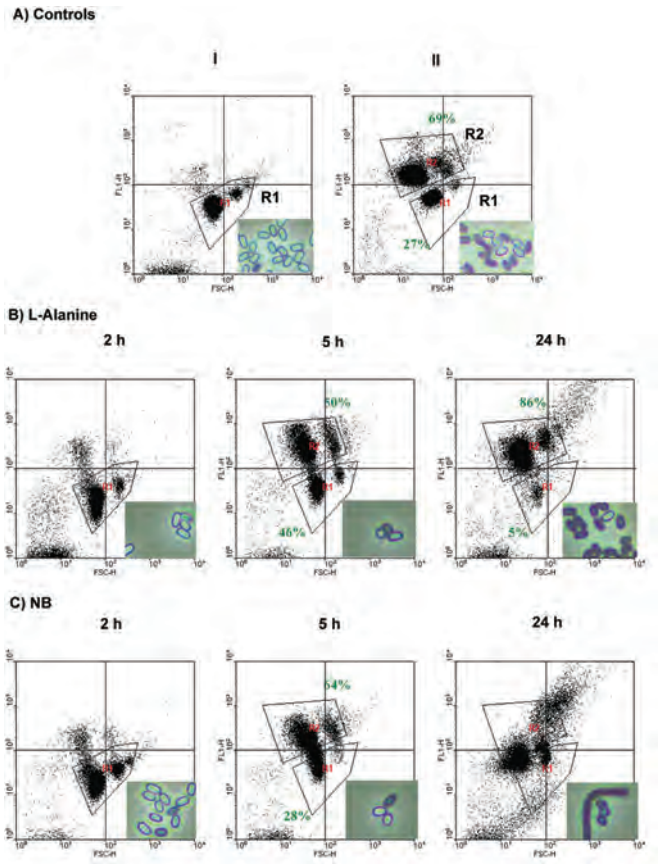

Figure 4. Representation of flow-cytometry dot-plots of green fluorescence (FL1-H) versus forward-scatter intensity (FSC-H) of Syto9 stained spores of *B. subtilis* strain M112. The insert corresponds to phase contrast microscopy observations. The fraction of the subpopulation which appeared in region 1 (R1) or region 2 (R2) was mentioned by a green number. Set A corresponds to control conditions: A.I- nearly 100% phase bright spores in phosphate buffer 10 mM; A.II- corresponds to 70% phase-dark and 30% phase bright M112 spores and it was obtained after incubating heat-shock activated spores in L-Alanine 10 mM for 14 h at 45°C. Set B and C correspond to the results of germination and outgrowth behaviour of spores upon simulated ultra-high temperature treatments at 135°C for 8 s, following incubation in L-alanine (B) or nutrient broth (C) at 37°C. Gates surrounding regions R1 and R2 were defined with WinMDI software.

Figure 4B and 4C depict the results of spore germination in L-alanine and nutrient broth at 37°C after subjecting the spores to the simulated UHT treatment. After 2 h incubation, the major spore fraction were ungerminated spores. After 5 h, the fraction of germinated spores was higher than that of ungerminated spores for both incubations in L-alanine and nutrient broth. After 24 h incubation, differences in the population composition for incubations in

L-alanine and nutrient broth were present. In the presence of L-alanine the fraction of germinated spores increased when compared to 5 h incubation, but about 5% of spores remained ungerminated. On the contrary, for spores incubated in nutrient broth, the number of ungerminated (unstained spores) located in the lower region of R1 was insignificant when compared to spores incubated in L-alanine. Instead, intermediately stained spores (partially phase-bright and partially phase-dark), constituted the predominant fraction of the population (located in between regions R1 and R2), indicating that a large number of spores had not been able to complete germination after 24 h. At the same time, actively growing cells were also observed (upper right quadrant).

Discussion

Bacterial spores are known to contaminate diverse ingredients used in the manufacturing of foods and, for this reason, understanding the conditions which lead to spore germination may provide means for their control (Carlin et al., 2000; Smelt et al., in press). The aim of this work was to characterise nutrient-germination responses in spores of three highly heat-resistant spore-forming strains of *B. subtilis* subsp. *subtilis*, strains AA2, AR1 and M112.The heat-sensitive spore-forming strain P3-3 was also included in the study to assess the extent to which the germination responses were exclusive to spores of heat-resistant spore-forming strain.

Currently, with exception of classical studies with spores of the thermophile *Geobacillus stearothermophilus* (e.g. Finley and Fields (1962) and Foerster (1983)), little knowledge is available with regard to germination responses for spores exhibiting very high heat-resistance. In this work it is shown that spore dormancy is independent of the spore heat-resistant phenotype. However, the degree of heat shock activation appeared to be related to spore heat-resistance, since spores of the heat-sensitive strain (P3-3) required a substantially lower temperature-time activation to yield similar germination responses than that observed for heat-resistant spores. Indeed, for spores of strains AA2 and AR1, little change in the A_{600nm} took place after applying a heat shock activation at 80°C for 10 min, when compared to the condition where the spores had not been heat-shocked. For this reason this treatment was increased to 100°C for 10 min. In the case of M112 spores, the heat shock activation treatment had to be further increased to 30 min, but this heat treatment still resulted in largely incomplete germination responses for the different nutrient-germinants. The requirements of heat shock activation treatment to stimulate germination in spores of the highly heat-resistant strains in

this work are similar to that reported for *G. stearothermophilus* (Feeherry et al., 1987; Finley and Fields, 1962; Foerster, 1983)

The germination temperature had a profound effect on the induction and rate of germination. For spores of AA2 and P3-3 specific nutrient-temperature combinations were more effective in the induction of germination, which was not the case for spores of AR1 and M112. Systematic studies on the effect of temperature on spore germination capacity were given for *B. megaterium.* In *B. megaterium* QMB1551, a maximal absorbance drop in the presence of glucose occurred between 30°C and 36°C after heat shock activation at 58°C for 5 min, while upon spore activation at 60°C for 5 min the range for spore germination was increased to 28-40°C (Levinson and Hyatt, 1970).

The nature of the applied heat shock activating treatments, and the rate and extent of germination in this work were considerably different from that of other *B. subtilis* strains. For instance, complete germination in non-heat shocked spores of *B. subtilis* CMCC 604, in the presence of L-alanine 10 mM, was reported to occur within 10-39 min at 37°C; and for this strain a heat-shock activation of 65°C for 10 min was reportedly sufficient to induce complete germination within 2.5 h for spores incubated at 20°C (Leuschner and Lillford, 1999). In another example, for spores of *B. subtilis* PS832 which had been activated at 70°C for 30 min, spore incubation in complex culturing medium (Luria Bertani) led to a drop in the A_{600nm} of 45% within less than 40 min at 37°C (Cortezzo et al., 2004).

Spores of strain M112 formed the most superdormant spores. Superdormant spores can be defined as spores which do not germinate under conditions which induce germination for the majority of the spore within a population (Ghosh and Setlow, 2009a). Even at 45°C incubation in L-alanine and AGFK, about 30 to 50% of spores did not germinate, indicating that there is heterogeneity in the optimal activation temperature for individual spores of this strain and germination capacity. We hypothesised that one possible reason for poor germination of spores of M112 in L-alanine could be due to the presence of alanine-racemase in the spore coats, an enzyme which by converting L-alanine into D-alanine leads to inhibition of spore germination by competitive binding. However, the addition of an inhibitor of alanine-racemase to the germination medium, D-cycloserine 2 mg/ ml (Gould, 1966; Stewart and Halvorson, 1953), did not change the germination behaviour or kinetics in L-alanine, independently of the germination temperature (data not shown)

In spores of *B. subtilis* PS533 it was demonstrated that superdormant spores had higher heat-resistance than fast germinating spores (Ghosh et al., 2009). In addition, those superdormant spores were found to have lower core water content (Ghosh et al., 2009), a factor which was shown in chapter 6 to be an important factor determining *B. subtilis* spores heat-resistance. Such spore properties, specifically high heat-resistance and low core water-content were described for spores of strain M112 in chapter 6.

Presently, there are three main theories which explain the occurrence of superdormant spores. One attributes spore superdormancy to the low water content in the spore core (Ghosh et al., 2009; Sunde et al., 2009), as mentioned above. However, it is not yet clear how

reduced core water content decreases spore germination (Ghosh et al., 2009). The second theory proposes that the extent of spore (super)dormancy is influenced by degradation of the pool of messenger RNAs (transcripts) during spore storage, such that unbalance between the existence of readily available transcripts and transcripts which require *de novo* synthesis hinder in some manner spore germination (Segev et al., 2012).The third theory postulates that the phenomenon of spore superdormacy occurs due to low level of germinant receptors in the spore inner membrane (Ghosh et al., 2012). The understanding of the mechanisms of spore superdormancy is complexed by the observation that this behaviour is modulated by the type and concentration of nutrient-germinants as well as the incubation temperature ((Ghosh and Setlow, 2009a; Ghosh and Setlow, 2009b; Yi et al., 2011); this work). Furthermore, superdormancy was shown to be an inducible property, since spores produced in rich medium had substantially higher germinant receptors than spores produce in poor medium (Ramirez-Peralta et al., 2012). However, it is possible that the impact of this last factor for spore superdormancy was minimised in this work, since all spores were produced under identical conditions.

It is known that germinant receptors are sensitive to the concentration of germinants (Ghosh and Setlow, 2009b; Gould et al., 1968; Yi et al., 2011). However, in this work it was not possible to draw conclusions about the effect of the concentration of sugars on germination, since in the AGFK assay the putative effect of glucose and fructose was masked by the presence of L-asparagine. L-asparagine was shown to be a determinant compound for the occurrence of a significant absorbance drop within 40 min incubation at 37°C for spores of *B. subtilis* 60127, in the presence of low concentrations of glucose and fructose (Wax and Freese, 1968). Therefore, L-asparagine could have also had a synergistic effect on germination induced by glucose and fructose in this work (Yi et al., 2011).

We showed that different chocolate milk related compounds were able to induce germination in spores of the highly heat-resistant strains of *B. subtilis*. Importantly, in addition, cocoa powder was able to induce a complete cycle of germination-growth-sporulation. This effect of cocoa powder had not been reported before in the literature.

Besides L-alanine and AGFK, other amino acids, simple sugars and purine nucleosides can trigger germination in *B. subtilis* (Setlow, 2003; Yi et al., 2011), but this is not the case for biopolymers, such as L-carragenanan. Wax and Freese (1968) reported initiation of germination of *B. subtilis* by more than 20 amino acids. Among these, L-alanine, L-asparagine, L-cysteine, L-glutamine, glycine, L-isoleucine, L-serine and L-valine could initiate germination alone. Although the concentrations of amino acids tested by Wax and Freese (1968) were higher than those usually present in cow milk (Guo et al., 2007), the synergistic effect of the different amino acids and minor quantities of glucose and galactose (Yi et al., 2011) present in cow milk, respectively, of 0.22 mM and 0.19-0.26 mM (Faulkner et al., 1981; Marschke and Kitchen, 1984) could possible trigger spore germination. Furthermore, cocoa powder is rich in protein (around 22%), starch (9.3%), sugars (0.6%), diverse macro and micronutrients and vitamins of the complex B (Cargill cocoa, H. Kamphuis personal communication). The exact

components triggering spore germination in cocoa powder remain an interesting aspect to study in more detail.

Sporulation is known to be induced by starvation from a carbon, nitrogen and/or phosphorous source (Piggot and Hilbert, 2004) and it requires or is further accelerated by the presence of minerals, such as manganese (Charney et al., 1951; Oh and Freese, 1976; Weinberg, 1964). The fact that cocoa powder is rich in diverse minerals, including manganese, could be involved in the occurrence of resporulation in the cocoa powder suspension.

Flow-cytometry is a high-throughput and highly sensitive technique which allows monitoring the degree of heterogeneity (composition) of cell populations. It offers a number of advantages over phase-contrast microscopy for population monitoring, such as speed and precision in population estimation. In this work, monitoring of germination by means of flow-cytometry showed differences during the recovery of UHT-treated spores (heat treatment at 135°C for 8s) in L-alanine and nutrient broth. L-alanine had higher stimulating effect over germination of UHT-treated spores than nutrient broth. The existence of a small fraction of spores (5%) which did not germinate after 24 h in L-alanine, and which was not present for spores recovered in nutrient broth, could correspond to heat-damaged spores (Lund and Peck, 1994). This is conceivable because it was shown that heat-damaged spores require rich media for damage-repair and to be able to complete germination and outgrowth (discussed by Coleman et al. (2007)).

In addition to performing UHT treatments at 135℃ for 8 s, we had also tested before the same temperature but for 4 s only. While the results of spore germination in L-alanine were essentially identical to those observed at 8 s, for incubations in nutrient broth, approximately 10% of spores remained unstained (ungerminated) (data not shown), meaning that in the absence of compounds that induce strong germination (such as high concentrations of L-alanine), the extent of heat-shock activation is the most critical factor to reduce the lag time prior to germination in complex medium for spores of M112.

Intriguingly, although spores of *B. subtilis* M112 are highly dormant, after prolonged storage at 4℃ (approximately 1 year), about 40 to 50% of the spores transit from phase bright into phase-dark, a phenomenon known as spore aging.

Taken as a whole, in chocolate milk drinks, milk and cocoa powder are both components which are able to induce spore germination in heat shock activated spores of highly heat-resistant strains. It was also observed that milk was able to trigger germination in a reduced number of non-heat shocked spores for both AR1 and M112 spores (data not shown). The findings in this work have also application for the formulation of beverages in the food industry. The new trend of substituting sucrose by glucose and fructose syrups (Hanover and White, 1993), increases the risk of spoilage, since both fructose and glucose are able to induce germination in surviving spores of *B. subtilis*. This study suggests that the use of sucrose and formulations with complex carbohydrates in combination with storage at low temperature, decreases the risk of spoilage. Time is also an important variable, since although time frames higher than 48 h where not tested in this work, the processes of spore aging, or whereby

the spore unexpectedly breaks its dormancy, implies that prolonged storage of commercial drinks should be avoided. The simulated UHT treatment used for spores of M112 indicated that the occurrence of spores in milk or cocoa powder with *D*-values similar to those of M112 (Chapter 4 and 6), might require industrial treatment at temperatures higher than 135°C to ensure product stability.

In conclusion, this work increases the knowledge about germination in highly heat-resistant spore-forming strains and the gathered information may provide a basis for their improved control within the food industry.

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Chapter 8

Genomic biomarkers of growth temperature preferences and spore heat-resistance: a meta-analysis with *Firmicutes*

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Growth temperature preference and spore wet heat-resistance are two phenotypes in endospore-forming *Firmicutes* that show divergent dependence on speciation. Despite extensive research about the mechanisms underlying spore wet heat-resistance, little is known about putative genomic determinants which make spores highly heat-resistant. A meta-analysis was performed to assess if growth temperature preference and spore heat-resistant phenotypes could be explained by genome base composition, gene content as well as amino acid composition of selected spore predicted proteins. The dataset comprising data from publicly available genomes, was augmented by genome sequencing of two *Bacillus subtilis* strains forming highly heat-resistant spores. In addition, the decimal reduction times (*D*-values) for spores of the sequenced strains of *Moorella thermoacetica* and *Thermoanaerobacterium thermosaccharolyticum* were determined. The strains were grouped according to growth temperature classes and spore heat-resistance groups to investigate the relation with the diverse genomic features.

A decreased genome size and an increased GC-content of the 16S rRNA gene were found to be the genomic features more strongly correlated with the optimal growth temperature (OGT), while contrary to previous studies, no correlation was found with the purine content of open reading frames. The GC-content of the 5S rRNA showed the highest correlation with the spore $\log D_{110^{\circ}C}$ and the strength of this correlation was similar to that observed with the OGT. Gene-trait matching approach allowed the identification of genes related to distinct growth temperature preferences and low heat-resistance spore production. In contrast, high heat-resistance spore formation could not be explained on the basis of shared gene content. For the studied protein set, large variability was present in the amino acid composition among strains within each phenotypic group, and, therefore, this feature showed limited resolution to yield genomic biomarkers.

Limited overlap was present in genomic features for the spore heat-resistant and growth temperature phenotypes. This comprehensive analysis at phylum level reconciles findings previously published as genomic determinants of thermophilism across Archaea and Eubacteria species. Our data points towards a most important role for previously identified characteristics of spore physicochemical composition and ultrastructural dimensions, as determinant factors underlying spore differential heat-resistance.

Introduction

The phylum *Firmicutes* harbours diverse and well-studied microorganisms. This phylum comprises microorganisms of the low GC-content genomic branch, which are presently divided into five classes, the *Bacilli*, *Clostridia*, *Erysipelotrichia*, *Thermolithobacteria* and *Negativicutes* (Ludwig et al., 2009; Marchandin et al., 2010). To date, more than 250 genera have been described (Euzéby, 2012).

The most remarkable properties of certain members within this phylum are their ability to form endospores resistant to a variety of environmental insults, such as wet heat, and their large spectrum of growth temperature ability. For instance, a decimal reduction time (*D*-value) at 121°C of more than three hours was reported for *Thermoanaerobacterium thermosaccharolyticum* spores, while spores of some pathogenic species are inactivated at boiling temperature (100°C, 1 atm) within a few minutes (Montville et al., 2005; Orsburn et al., 2008; Xezones et al., 1965). At the same time, whereas *Bacillus weihenstephanensi*s and *Clostridium putrefasciens* are able to grow at refrigeration temperatures (4-7°C) up till a maximum of 35°C to 43°C (Guinebretière et al., 2008; Lechner et al., 1998; Ross, 1965), *Desulfotomaculum kuznetsovii* requires growth temperature ranges between 50°C to 85°C (Nazina et al., 1987).

While maximal growth temperature is a property reasonably well conserved at the species level (Auger et al., 2008; Brown, 2000; Kort et al., 2008), this is not the case with respect to spore wet heat-resistance (hereafter referred to as heat-resistance). High heat-resistant spore phenotype appears to be restricted to a few strains and species within the phylum of *Firmicutes* (Byrer et al., 2000; Kort et al., 2005; Lima et al., 2011b).

The ability of a microorganism to produce spores of high heat-resistance has been traditionally linked to thermophilism, owing to the well documented spoilage by thermophiles in the canning industry, specifically from species of the genera *Geobacillus*, *Moorella*, *Desulfotomaculum* and *Thermoanaerobacterium* (Bender and Marquis, 1985; Stumbo, 1973). This is substantiated by the fact that the highest *D*-values registered thus far belong to thermophiles from the genera *Desulfotomaculum*, *Moorella* and *Thermoanaerobacterium* (Byrer et al., 2000; Goorissen, 2002; Xezones H et al., 1965). However, the recent discovery of thermotolerant strains of *B. sporothermodurans* and *B. subtilis* forming spores of comparable or higher heat-resistance than spores of thermophilic strains of *G. stearothermophilus* (André et al., 2012; Huemer et al., 1998; Lima et al., 2011b; van Zuijlen et al., 2010), challenged the paradigm of thermophilism as the "*sine qua non*" for high heat-resistance spore formation.

So far, knowledge regarding the mechanisms underlying spore differential heat-resistance has been mostly based on the characterisation of physicochemical and ultrastructural spore properties among strains forming spores of high and low heat-resistance (Beaman et al., 1982; Beaman and Gerhardt, 1986; Novak et al., 2003; Orsburn et al., 2008). These researches showed that spore heat-resistance was strongly correlated with a decreased water content in the spore core, an increased core content in certain minerals and a decreased ratio between

the dimensions of the core and of the sporoplast (the sporoplast is the compartment made-up by the core and the layer of peptidoglycan) (Beaman et al., 1982; Bender and Marquis, 1985; Nakashio and Gerhardt, 1985; Orsburn et al., 2008).

Putative genomic determinants of spore heat-resistance have only been investigated to a limited extent. Specifically, clustering approaches based on Amplified Fragment Length Polymorphism (AFLP) and ribotyping have been evaluated by our laboratory and others for their capacity to group strains of *B. subtilis* forming heat-resistant spores apart from strains forming heat-sensitive ones (Lima et al., 2011b; Oomes et al., 2007). However, these approaches did not provide consistent strain segregation according to their spore heat-resistant phenotype. The dearth of data about the genomic determinants of spore heat-resistance contrasts with the large body of information dealing with the genomic determinants of thermophilism in Prokaryotes [for reviews see e.g. Amelunxen and Murdock (1978), Das et al. (2006) and Sterner and Liebl (2001)].

The identification of genomic determinants or genomic biomarkers linked to a high heat-resistant spore phenotype is of utmost importance for a better understanding of factors influencing spore heat-resistance, for the elucidation on why some strains produce spores of high heat-resistance and others do not, and to allow the design of strategies to assist in the control of spores within the food and pharmaceutical industries, where these still challenge the implemented processes (Brul et al., 2011; De Clerck et al., 2004b).

Currently it is accepted that growth at high temperature is dictated by increased molecular stabilization at the DNA, RNA and protein level, these, in turn, being largely influenced by the base or amino acid composition in the primary structure (Jaenicke and Sterner, 2006; Vieille and Zeikus, 2001; Wada and Suyama, 1986). The mechanism by which sequence composition contributes to the thermostability of these biopolymers includes structure stabilisation by non-covalent forces, such as hydrogen bonds, van der Waals forces, hydrophobic interactions and ionic pairs (Šponer et al., 1996; Vieille and Zeikus, 2001), but many more mechanisms have been described, as discussed by Amelunxen and Murdock (1978) and Sterner and Liebl (2001).

Studies comparing DNA sequences of mesophilic with that of (hyper)thermophilic Eubacteria and Archaea species have identified a number of features correlated with thermophilism. These consist of an increased representation of purines (A and G bases) in protein coding sequences; an increased representation of GC-content in genes encoding structural and transfer ribosomes; and, depending on the taxonomic level, a relation with the overall GC-content of the genome and open reading frames (ORF) (Das et al., 2006; Galtier and Lobry, 1997; Lambros et al., 2003; Musto et al., 2004; Musto et al., 2006; Zeldovich et al., 2007).

In turn, analyses of amino acid compositions of predicted proteomes showed marked patterns towards an overrepresentation of charged, hydrophobic and aromatic amino acid residues (Cambillau and Claverie, 2000; Das et al., 2006; Haney et al., 1999; Zeldovich et al., 2007; Zhou et al., 2008). Specific amino acid substitutions have also been observed to take place in proteins from thermophilic microorganisms when compared to those of mesophilic

microorganisms. For example, Argos et al. (1979) a observed that glycine, serine, lysine and asparagine were substituted by alanine, threonine, arginine and glutamate in proteins from thermophiles when compared to that of mesophiles; while analyses of protein coding sequences by Zeldovich et al. (2007) revealed a high correlation between thermophilism and the occurrence of an amino acid set composed of isoleucine, valine, tyrosine, arginine, glutamate and leucine.

Since a number of genomes of endospore-forming *Firmicutes* have become publicly available during the past decade, this phylum offers an unique the opportunity to uncover, both genomic biomarkers of growth temperature preference and spore heat-resistance.
In this work we sought primarily to identify genomic biomarkers of spore heat-resistance in endospore-forming *Firmicutes*; but since many heat-resistant spore-formers are also thermophiles, the study was extended to reveal genomic determinants of thermophilism, assessing the extent to which an overlap in features for the two phenotypes exists. The main goal was to obtain features to allow an expedite phenotype prediction.

To address these questions, a comprehensive meta-analysis of genomic and phenotypic data was carried out (Figure 1), to explore three different hypotheses which could potentially explain those two phenotypes: (i) they result from features linked to the genome composition, such as base composition and size; (ii) they are related to the presence/absence of a specific gene or a set of genes; and (iii) they result from features linked to protein amino acid composition, specifically the frequency of specific amino acid residues.

Considering that high heat-resistance spore-formation can be found in strains with different growth temperature preferences (mesophiles, thermotolerants and thermophiles (Brown et al., 2012; Byrer et al., 2000; Derman et al., 2011; Lima et al., 2011b)), the assumption is that the genomic determinants of thermophilism and spore heat-resistance are not the same. On the other hand, in view of the observation that a plausible explanation of spore killing is due to the denaturation or inactivation of one or more critical spore protein (Coleman and Setlow, 2009; Coleman et al., 2007), it could be hypothesized that strains forming spores of high heat-resistance share adaptations towards higher protein thermostability, in line to what has been described as a survival strategy for thermophiles (Kumar et al., 2000; Sawle and Ghosh, 2011).

Since the data available for the spore heat-resistant phenotype are scarce, we also sequenced two of our strains of *B. subtilis* subsp. *subtilis* producing highly heat-resistant spores. We also determined the thermal kinetic parameters *D*- and *z*-values for the sequenced strains of *M. thermoacetica* ATCC 39073 and *T. thermosaccharolyticum* DSM 571. We bring to light genomic biomarkers linked to genome compositional features and gene content related to growth temperature preference, but no unequivocal biomarkers allowing the prediction of a high heat-resistant spore phenotype were identified.

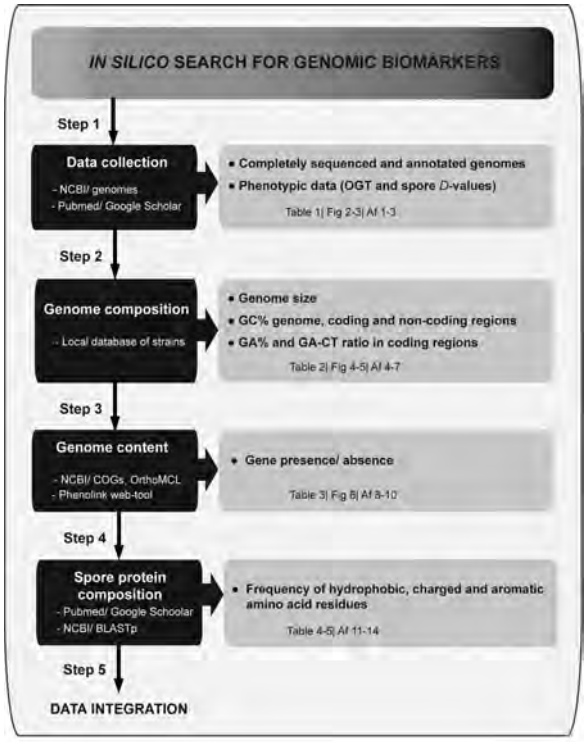


Figure 1. Bioinformatics workflow. Strategy followed in this work for an *in silico* search of genomic biomarkers of growth temperature preferences (OGT) and spore heat-resistant phenotype (*D*-values) in endospore-forming *Firmicutes*. 'Af' indicates additional files.

Methods

Data set and phenotype classification

A list of complete genomes of endospore-forming *Firmicutes* sequenced until 2010 was obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). In addition, since a few more genomes with published data for optimal growth temperature (OGT) and one with published data for spore decimal reduction time (*D*-value) became available in 2011, these strains were also included in the study (additional file 1).

Endospore formation was verified by consulting the primary publications or subsequent publications amending the species description. In addition, two strains of *Bacillus subtilis* subsp. *subtilis* isolated from cocoa powder and producing highly heat-resistance spores (Lima

et al., 2011b; Lima et al., 2012) were sequenced in the context of this work and included in the study.

The literature was scrutinized for published data on OGT- and spore *D*-values (by definition, the time needed at a given temperature to reduce a population by 90%). Care was taken to use OGT data, where a unique value was reported or OGT-values within a range not higher than ± 2°C (in this case the average value was taken). Regarding spore *D*-values, we collected data obtained in either buffer or water at neutral pH ranges (6.5-7.5). We discarded *D*-values that had been calculated for spores heated in fatty sauces or in the presence of high salt or sugar concentrations, since these factors change the original heat-resistance of the spore (Mazas et al., 1999; Rodrigo et al., 1999).The importance of collecting precise OGT-values for correlational studies has been admonished (Wang et al., 2006).

OGT-and *D*-values were not available for all strains (additional file 2). For this reason, with respect to the growth temperature phenotype, global species preferences were taken into account to classify the remaining strains. We constructed a decision tree based on information provided by Adams and Moss (2000), which was modified to account for 'thermotolerants' and 'hyperthermophiles', after careful study of the literature (Auger et al., 2008; Burgess et al., 2010; De Vos et al., 2009; Goorissen, 2002; Guinebretière et al., 2008; Lechner et al., 1998; Nazina et al., 2001) (Figure 2). Considering that spore *D*-values may vary considerably among strains and that very little is known with respect to the boundaries of spore heat-resistance at the species level, it was not possible to establish a similar system to classify the strains according to the spore heat-resistant phenotype.

To enable a comparison of the reported *D*-values for the different strains, the Bigelow model (Bigelow, 1921) (equation 1) was used to compute normalized *D*-values at 110°C ($D_{110°C}$):

$$\log D_{110^{\circ}C} = \log D_{ref} - \left(\frac{110 - T_{ref}}{z} \right) \quad (1)$$

Where D_{ref} is the decimal reduction time (min) at a reference temperature T_{ref}, $D_{110°C}$ is the decimal reduction time (min) at 110°C and the *z*-value is the increase in temperature (°C) leading to a 1 log *D* reduction. *z*-values were not available for all strains of which *D*-values were found. Hence, average values were calculated from data obtained from other strains within the same species or estimated by linear regression from the original publications.

To ensure a more balanced representation of both heat-sensitive and heat-resistant spore-formers in our database, the strains of *Moorella thermoacetica* ATCC 39073 and *Thermoanaerobacterium thermosaccharolyticum* DSM 571 were acquired and their thermal kinetic parameters were determined. The *M. thermoacetica* strain was kindly provided by Prof. Stephen W. Ragsdale (Department of Biological Chemistry, University of Michigan Medical School) (Pierce et al., 2008)t, while the *T. thermosaccharolyticum* strain was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany). The spore production, the heat-inactivation assay and the determination of the *D*- and *z*-values by linear regression were performed as described by André et al. (2012). The heat-inactivation assay

was carried out at temperature ranges of 125°C-135°C for the *M. thermoacetica* strain and of 107.5°C-117.5°C for the *T. thermosaccharolyticum* strain. The *D*-values were calculated from a total number of datapoints between 8-15.

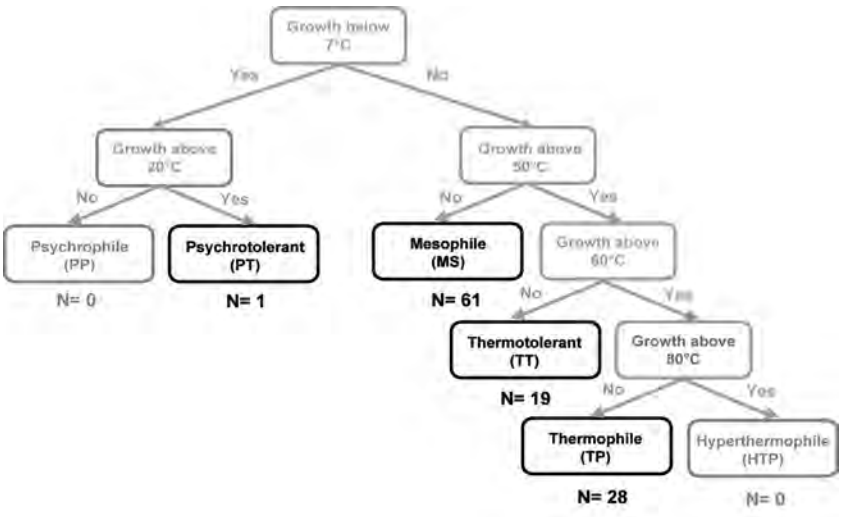


Figure 2. Decision tree for strain classification into a growth temperature class. The decision criteria are based on a survey of literature data and takes into account strain variation within species level.

The strains were classified into heat-resistant or heat-sensitive spore-formers, based on a cut-off value which was defined taking into account the *D*-values for *Clostridium botulinum* spores in buffer (pH 7). *C. botulinum* can be considered a spore-former producing spores of intermediate heat-resistance, although producing spores of substantially higher heat-resistance than that of other endospore-forming pathogenic species. An average $D_{121°C}$ of 0.21 min can be found by averaging the data for spores of 25 strains of *C. botulinum* in buffer (Brown et al., 2012). Assuming that the data originates from a normal distribution a $D'_{121°C}$, including the upper 95% confidence interval, of 0.32 min is obtained. Such $D'_{121°C}$ corresponds to a $D_{110°C}$ of 4.04 min, considering an average *z*-value of 9.99°C (Brown et al., 2012). Strains with a $D_{110°C} < 5$ min (0.7 log min) were classified as heat-sensitive.

Genomic data retrieval and base compositional analysis

Complete chromosomal and protein coding sequences were extracted from the GenBank ftp site at NCBI (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/). Structural RNAs (23S, 16S and 5S ribosomal RNAs) and transfer RNA (tRNA) sequences were obtained from the BacMAP site (http://wishart.biology.ualberta.ca/BacMap/) (Stothard et al., 2005). Subsequently, the GC-

and GA-contents of the genomic DNA, and the GC-contents of the structural and tRNAs were calculated. For the ribosomal RNAs, the multiple copies were concatenated prior computational quantification. All sequence manipulations were carried out using programs written in python or pearl.

Genome sequencing and annotation

Sequencing-grade genomic DNA was purified from an overnight culture of the *Bacillus subtilis* strains M112 and M1 (Lima et al., 2011b), using the Wizard genomic purification kit from Promega (A1125, Promega Corporation, United States of America), according to the manufacturer's instructions. The DNA was sent for sequencing at BaseClear (Leiden, The Netherlands) with Illumina Solexa technology. A 50-cycle paired-end read program was used with an Illumina genome analyzer II. The genome reads (total coverage of about 70x) were subjected to "pseudoassembly" using the genome of *B. subtilis* susbp. *subtilis* strain 168 as reference in a CLC Genomics Workbench v.4.0 (CLC GW). The assembled contigs were subsequently concatenated separated by repeated N characters (5 N's between the end of each contig), based on the *B. subtilis* 168 strain backbone. Next, genes were predicted in the Rapid Annotation using Subsystem Technology (RAST) server (http://rast.nmpdr.org/) (Aziz et al., 2008). The predicted proteins from RAST were subjected to alignment against an in-house built database for *Firmicutes* comprising the proteomic sequences described in the section above using BLASTP (Altschul et al., 1990), for functional annotation.

The tRNA were identified using the tRNAscan-SE v.1.23 (Lowe and Eddy, 1997), whereas the 16S ribosomal RNAs (rRNA) were obtained with the greengenes 16S rRNA gene database and workbench (http://greengenes.lbl.gov) (DeSantis et al., 2006). The 5S and 23S rRNA sequences were identified by a BLAST search against a local database made up of 5S and 23S rRNA sequences of the endospore-forming *Firmicutes* obtained from the GenBank ftp site. Gene functions were inferred on the basis of orthology in the conserved domains.

The draft genome scaffolds for *B. subtilis* subsp. *subtilis* M112 and M1 can be accessed from the authors via e-mail request.

Gene-trait matching

In order to screen for relationships between growth temperature preferences and spore heat-resistant phenotypes with the gene content in the genome, we performed Gene-Trait Matching (GTM) analysis, using the web-tool Phenolink (Bayjanov et al., 2012) (http://bamics2.cmbi.ru.nl/websoftware/phenolink). Phenolink is based on the random forest classifier (Breiman, 2001), which is a robust algorithm to identify features (in this case presence/absence of genes) important for a given phenotype. Phenolink filters the results of the random forest and, subsequently, allows the visualisation of gene relevance for a given phenotype by employing a colour code system.

An extensive comparative analysis for the overall gene content for the endospore-forming *Firmicutes* strains (additional file 1) was performed. First, the genes were assigned to Clusters

of Orthologous Groups (COG) (Tatusov et al., 1997) as described by Snel et al. (2002). Second, the remaining genes were clustered based on the Orthologs Markov Clusters (OMCLs) method (Li et al., 2003). By definition OMCLs are present in at least 2 strains and being more sparsely distributed across strains, they signify more specific gene functions. OMCLs might be related to orthologs (between 2 or more strains) or paralogs (duplication of genes within one strain). Overall, the analysis of gene content resulted in 3,180 COGs and 14,749 OMCLs. These data were used as genotypic input for all Phenolink analyses (.txt file).

Since there was only one psychrotolerant representative in the dataset, it was not possible to study this trait in detail and the strain was considered within the mesophiles. Three different types of metadata sets, comprising the strain code and corresponding phenotype (in the form of nominal categories) were prepared for analysis with Phenolink: (i) a metadata set of all endospore-forming *Firmicutes* (MDS-Firmicutes) divided into growth temperature classes as illustrated in Figure 2, specifically mesophiles, thermotolerants and thermophiles; (ii) a metadata set of strains with published OGT-values (MDS-OGT), which were divided into the mesophilic and thermophilic group members, with the mesophilic group comprising a psychrotolerant strain, mesophiles and thermotolerants; (iii) the metadata set of strains with published *D*-values (MDS-D), which were classifiedinto heat-sensitive and heat-resistant spore-formers, based on their values for the $\log D_{110°C}$ as described above. The datasets were uploaded at Phenolink as .txt files.

The data were analysed with default settings in Phenolink, which includes a maximum classification error of strains within a given class or group of 40% and exclusion of genes which show variance below 5%.

Since the phenotypes were unequally distributed among the classes or groups, a 'bagging' technique was selected in Phenolink to deal with class/group imbalance and prevent bias in feature selection and classification by the dominant phenotypes (i.e. phenotypes found more frequently among the strains). The bagging option 'multiple down-sizing' was chosen. In this technique the phenotype with fewest strains is set as reference and a random sample of strains in the dominant phenotype equal to two times the total number of strains in the smallest phenotype were then selected and tested against the strains in the smallest group, until all strains in the largest phenotypic group had been selected at least once.

The results of Phenolink classifications were assessed for their reliability in correctly classifying the strains to be part of their respective class/group (e.g., mesophile vs thermophile), by inspection of the table of classification accuracies (Out of Bag error-OOB error) for each class or group of strains, the list of misclassified strains and the table of positive contributions for each genes, as provided by the web-tool.

Analysis of gene interactions and context

To further analyse the top features resulting from Phenolink, the STRING tool (Szklarczyk et al., 2011) was used to obtain information about gene-gene interactions and context (e.g.

co-occurrence, neighboring relations). The database of Non-supervised Orthologous Groups (eggNOG) (Powell et al., 2012) was also consulted for annotation of relations in STRING.

Protein mining

Spore proteins derived from spore proteomic studies for strains of *Clostridium* spp., *B. subtilis* and *B. cereus* group were retrieved from the datasets provided by the original authors (Brul et al., 2011; DelVecchio et al., 2002; Kuwana et al., 2002; Lai et al., 2003; Lawley et al., 2009). We performed a selection of proteins which in principle would have homologs commonly present across different genera and involved in different cellular functions, e.g. protection responses, spore structure and germinationas well as metabolism (Lawley et al., 2009) (additional file 13).

Protein homolog sequences were downloaded from the GenBank ftp site at NCBI (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/). The identification of protein homologs for the different strains was performed with computational thresholds set at > 80% alignment coverage and 35% sequence identity over at least 75 amino acids for proteins longer than 100 amino acids, and over at least 40 amino acids for proteins shorter than 100 amino acids.

Protein information on (i) amino acid composition, (ii) aromaticity, (iii) charge and (iv) hydrophobicity was further analysed with custom made scripts. Since different hydrophobicity scales assign different scores for the hydrophobic character of each amino acid residue, we evaluated the results of two scales. We chose the scale by Kyte and Doolittle (KD) (1982), since this scale is the most widely used and it has been found to be among the best scales to predict the hydrophobic behaviour of amino acid residues (Palliser and Parry, 2001). Next to that, we used a scale by Wimley and White (Wimley and White interfacial hydrophobicity scale) (WW) (Wimley and White, 1996), which on the contrary to the Kyte and Doolittle scale, is an experimentally derived scale for all 20 amino acids, rather than theoretical. The WW scale is composed of experimentally determined values for the transfer of free energies of polypeptides from water to the bilayer interface, and therefore it is argued to have the advantage of including the hydrophobicity contributions of peptide bonds, in addition to that of peptide side chains, which other scales do not account for (Wimley and White, 1996). In hydrophobicity scales, the more positive the value, the more hydrophobic is the amino acid.

Statistical analyses

The identification of significant statistical differences in the average levels of genome and protein compositional features among strains in the different phenotypic classes or groups, were identified using the Kruskal-Wallis test, followed by Mann-Whitney tests with Bonferroni correction for the critical *P*-value; or using the Mann-Whitney test (significance level $P < 0.05$).

Correlation analyses between the OGT and $\log D_{110°C}$-values with genome and protein compositional features were obtained based on Pearson's (r) and the Spearman's rank (ρ) correlation coefficients. The Spearman's rank correlation coefficient (Spearman's correlation coefficient) compensates for the fact that a strong, yet nonlinear relationship between two variables might be present, which is not recognized by Pearson's. Furthermore, the Spearman's

correlation coefficient has the advantage of being less sensitive to one or few outlying points (Field, 2005). The correlation coefficients were classified as very high [0.90-1], high [0.70-0.89], moderate [0.40-0.69], low [0.20-0.39] and very low [0-0.19].

Multiple Linear Regression (MLR) was used with the aim of studying the relation between strain OGT and spore $\log D_{110℃}$ (dependent variables) with the different determined genomic compositional factors (predictors). The general mathematical function in MLR is a first-degree equation specified as given in the equation 2:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + ... + \beta_m X_m + \varepsilon \quad (2)$$

Where Y is the dependent variable, X_m represents the m experimental factors tested (predictors), β_0 is the constant term, β_m represents the regression coefficients of the predictors, with each coefficient representing the "weight" (correlation) of the respective predictor; and ε is the predictive error, that is, the difference between the predicted and the observed values of Y. The results of the correlation analysis were used to enter the most important predictors in the model (r> 0.6). Different values or coefficients implemented in the utilised software were retrieved to assess the adequacy of the MLR model to describe the data. These were the regression coefficients r^2_{Adjusted}, the F change value and the Durbin-Watson (d) value. The F change test tells whether the change in the r^2 by adding one or more predictors to the model is significant (the F change value is significant at P< 0.05) (Field, 2005). The Durbin-Watson (d) test informs whether adjacent residuals are correlated (test of autocorrelation). Values closer to 2 mean that the residuals are not correlated, while values less than 1 or greater than 3 mean that the assumption is not verified (Field, 2005).

All statistical analysis and tests were carried out using the PASW Statistics v17.0 (IBM-SPSS) software.

Results

Characteristics of the data set

To uncover genomic biomarkers of growth temperature preferences and spore heat-resistance, we started by performing an inventory of completely sequenced genomes of endospore-forming *Firmicutes* and corresponding phenotypic data (Figure 1- Step 1). This search yielded a selection of 107 genomes, of which 39 had published data for the optimal growth temperature (OGT) and 13 for spore heat-resistance (*D*-value) (additional files 1 and 2). In addition, two highly heat-resistant spore-forming strains of *Bacillus subtilis* subsp. *subtilis*, strains M112 and M1, belonging to our laboratory collection were sequenced (Table 1). Both the genome size and the genome GC-percentages for these strains were similar to that of the sequenced strain *B. subtilis* 168, which has a genome size of 4.2 Mb and a GC-content of 43.5% (additional file 1).

Table 1. Genome assembly statistics of *Bacillus subtilis* subsp. *subtilis* strains M112 and M1

Parameters	M112		M1	
	Count	Total bases	Count	Total bases
Reads	7,330,772	544,606,593	5,617,966	413,396,708
Matched	7,277,438	540,706,586	5,571,235	409,995,677
Not matched	53,334	3,900,007	46,731	3,401,031
Contigs	166	4,197,518	272	4,305,949
Paired reads	3,502,234	-	2,692,993	-
Genome GC (%)	-	42.93	-	43.03

The genomes of strains M112 and M1 were sequenced using Illumina technology. The genome reads were aligned to the genome of *Bacillus subtilis* subsp. *subtilis* strain 168 (NCBI reference number NC_000964.3).

The thermal inactivation parameters *D*- and *z*-values for spores of the thermophilic strains *Moorella thermoacetica* ATCC 39073 and *Thermoanaerobacterium thermosaccharolyticum* DSM 571 were also determined (Figure 3). Spores of the *M. thermoacetica* strain exhibited considerably higher heat-resistance than spores of *T. thermosaccharolyticum*. For instance, a *D*-value at 125°C of about 30 min was registered for the former, while for the latter, a *D*-value of 0.73 min was measured at 117.5°C (additional file 3). The estimated $D_{110°C}$ for *M. thermoacetica* was nearly 225-fold higher than that for *T. thermosaccharolyticum* (additional file 3). At the same time, the $D_{110ºC}$-value for *T. thermosaccharolyticum* spores were nearly 12 and 5-fold lower than that of spores formed by the thermotolerant strains M112 and M1, respectively. This supports the view that thermophilism is not a requirement for the development of spores with high heat-resistance.

Overall, based on the decision tree in Figure 2, the strains were classified into psychrotolerants (N=1), mesophiles (N=61), thermotolerants (N=19) and thermophiles (N=28). This dataset had a biased taxonomic distribution, as the genera *Bacillus* and *Clostridium* accounted for over half of the genomes (63%).

However, this bias was substantially reduced in the datasets for strains with published data for OGT (MDS-OGT) and spore *D*-value (MDS-D) (additional file 2), which included an acceptable level of diversity for subsequent computational analyses.

Genome base compositional comparison

Next, the variation of genome compositional features across the different growth temperature classes was assessed (Figure 1- Step 2). Mesophiles revealed significantly lower average values in the GC-content of the whole genome, ORF and rRNA and tRNA sequences than thermotolerants ($P< 0.025$) (Figure 4). On the contrary, no significant differences were found between the genome size of mesophiles and thermotolerants ($P> 0.025$). In turn, thermophiles had significantly lower genome size ($P< 0.025$) and significantly higher GC-content in DNA encoding ribosomal genes ($P< 0.025$) than that of thermotolerants.

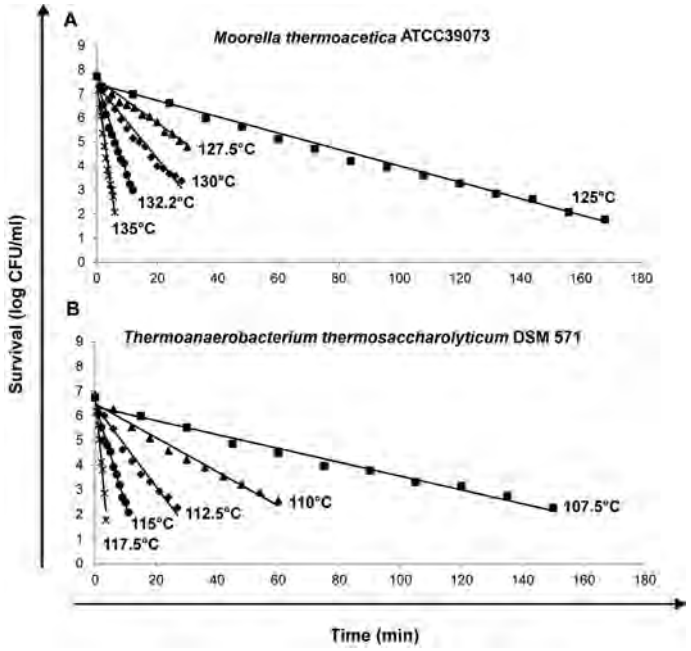


Figure 3. Thermal survival profiles of *Moorella thermoacetica* and *Thermoanaerobacterium thermosaccharolyticum*.

Subsequently, the extent of dependence of OGT on genome base compositional features was ascertained. These correlations are depicted in Figure 5I. High and significant correlation with OGT was present for the genome size (r= -0.80, ρ= -0.80, P< 0.001) and for the GC-content of the 16S rRNA gene (r= 0.82; ρ= -0.81, P< 0.001) (Figure 5Ia and 5If). These high correlations are in agreement with the observation of significant statistical differences in the average values of the genome size and the 16S rRNA GC-content between mesophilic and thermophilic members in the MDS-OGT (P< 0.025) (additional file 4). The lowest Pearson's and Spearman´s correlation coefficients were found with the GC-content of tRNA gene and both with the GA-content and GA-CT ratio of predicted Open Reading Frames (ORF).

Considering that the genome size and the GC-content of the 16S rRNA gene showed Pearson's and Spearman´s correlation coefficients just below 0.70 (see the correlation matrix in additional file 6), we calculated the partial correlation between the OGT and the GC-content of the 16S rRNA gene, keeping the effect of the genome size constant. This reduced the Pearson's correlation coefficient slightly (r= -0.65, P< 0.01), but this coefficient remained both significant and relatively high.

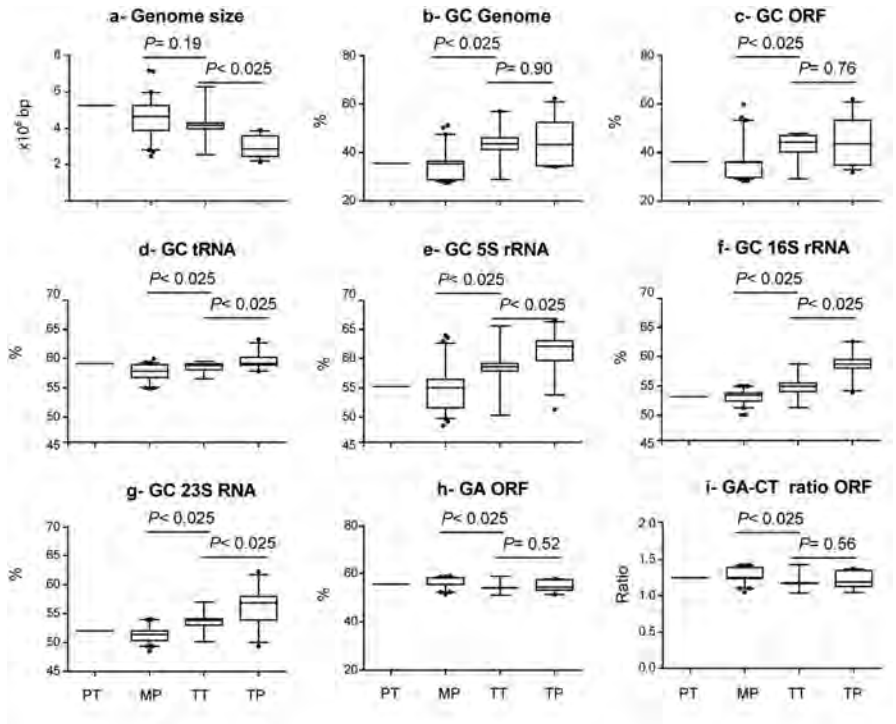


Figure 4. Variation of genome composition features across different growth temperature classes in endospore-forming *Firmicutes*. The classes correspond to psychrotolerants (PT, N=1), mesophiles (MP, N= 61), thermotolerants (TT, N= 19) and thermophiles (TP, N=28). Statistics were performed using the Kruskal-Wallis test, followed by Mann-Whitney tests with Bonferroni correction for the *P*-value.

Regarding spore heat-resistance, a high and significant correlation was only found between the log$D_{110°C}$-value and the GC-content of the 5S rRNA gene (r= 0.73; ρ= 0.69; P< 0.01). For the remaining genomic features, the correlation with the log$D_{110°C}$-values was only intermediate or low (the additional file 7 presents the complete correlation matrix for the different genomic compositional features). These findings were corroborated by ANOVA, since apart from the GC-content of 5S rRNA gene, where a borderline *P*-value was obtained (P= 0.05), no significant differences were found for the other genomic features between heat-resistant and heat-sensitive spore-formers (additional file 5).

Overall, although the Pearson's and Spearman's correlation coefficients were similar, there were a few divergences, such as in the case of the correlation between OGT with the GC-content of the 23S rRNA, where r= 0.31 and ρ= 0.60 (P> 0.01), for Pearson's and Spearman's, respectively. In this particular case, the discrepancy could be explained by one outlying point for *C. cellulovorans* 743B, of which 23S rRNA GC-content was 82.5%.

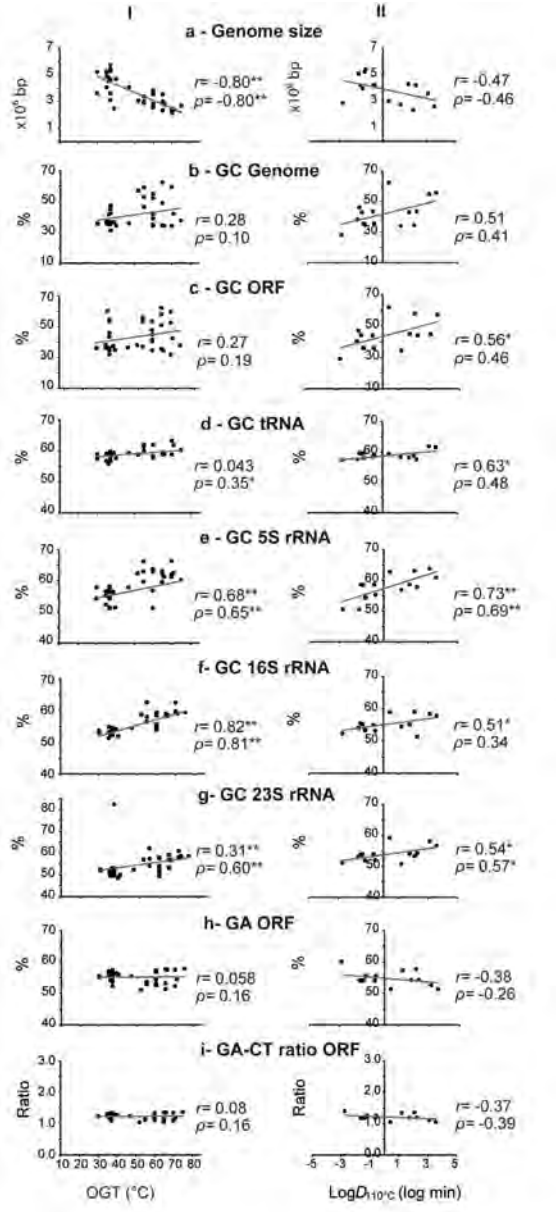


Figure 5. Correlation between genome compositional features with the phenotypes in study. Set I correspond to correlations with the Optimal Growth Temperature (OGT) of strains (N=39), while set II corresponds to correlations with the logarithm of the decimal reduction time of spores (log $D_{110°C}$) (N=15). Both Person's (*r*) and the Spearman's (ρ) correlation coefficients are given. *Correlation is significant with $P< 0.05$ or **$P< 0.01$.

In order to assess for the most important predictors for the OGT and spore log$D_{110°C}$, we performed Multiple Linear Regression (MLR) between the OGT and the spore log$D_{110°C}$ with the determined genomic features. Table 2 summarises the model which resulted in significant partial correlations. For the OGT, the genome size and the GC-content of the 16S rRNA gene were found to be the only factors which showed significant partial correlations with OGT, as assessed by the significance of the corresponding coefficients. These two predictors could explain roughly 78% of the variation of the OGT, as indicated by the adjusted regression coefficient ($r^2_{adjusted}$).

For spore heat-resistance, only the GC-content of the 5S rRNA gene appeared to contribute significantly by explaining about 50% of the variation in the log$D_{110°C}$. Although for the model-OGT and the model-log$D_{110°C}$ the Durbin-Watson statistic is accepted, neither model led to a significant *F* change, indicating that the description of the data by the model is not statistically significant. However, it can be argued that in the case of the model-OGT, the high regression coefficient makes it statistically more trustable than the model-log$D_{110°C}$, and, therefore, the role of the genome size and the GC-content of the 16S RNA gene as predictors of strains growth temperature preference.

Table 2. Multiple Linear Regression (MLR) models for the prediction of the OGT and spore log$D_{110°C}$-value in endospore-forming *Firmicute*s

	Predictor	Unstandardised coefficient[a]	Significance coefficient[b]	$r^2_{adjusted}$	Significance *F* change	Durbin-Watson statistic (*d*)
Model-OGT	Constant (β_0)	-68.4 (30.9)	0.03	0.78	0.10	1.66
	Genome size (bp) (β_1)	-5.8x10^{-6} (0.00)	0.00			
	GC 16S rRNA gene (%) (β_2)	2.5 (0.49)	0.00			
Model- log$D_{110°C}$	Constant (β_0)	-19.9 (5.2)	0.02	0.50	0.44	1.53
	GC 5S rRNA gene (%) (β_1)	0.35 (0.09)	0.02			

[a] Standard errors of the mean are given between brackets.
[b] Coefficients are significant at $P < 0.05$.

Gene-trait matching

To relate gene content with phenotype, we performed Gene-Trait Matching (GTM) using Phenolink (Figure 1- Step 3) (Bayjanov et al., 2012). The most relevant genes that were considered were those found to be important for the phenotype in study and sufficiently present among strains of that phenotype, while sufficiently absent in strains of the opposite phenotype (bright red against bright green features) (additional files 8-10). Table 3 summarises

those results of Phenolink classifications according to the growth temperature class and spore heat-resistance group.

Genes setting apart mesophiles and thermotolerants from thermophiles were present in the four main categories of Cluster of Orthologous Groups (COG), which include "information storage and processing", "cellular processes and signalling", "metabolism", and "poorly characterised" functions. Thermophiles could not be distinguished from mesophiles and thermotolerants on the basis of genes related to metabolism. At the same time, there were a few genes across all four categories which appeared to distinguish mesophiles from thermotolerants. However, a classification error for thermotolerants of 27% was obtained, while for mesophiles and thermophiles, this was 7.2% and 0%, respectively (data not shown). This indicates that the thermotolerant phenotype is more difficult to predict. The impact of the classification error was lower when Phenolink was run with mesophiles and thermotolerants together (mesophilic group in the MDS-OGT), decreasing the error to 6.8% (2.1% for strains in the thermophilic group) (data not shown).

In Table 3, Orthologous Groups (OGs) specifically associated with mesophiles determined from the PhenoLink analysis (Additional file 8) were COG1696 (no. 19), COG1882(30), COG1178(36), COG1528(37), COG1357(56) and OMCL_154(67). This group of genes included diverse transporters, a metabolic enzyme and genes of unknown function. For thermotolerants, these OGs comprised genes pertaining to transport (COG2851(31), COG1972 (33)), RNA metabolism (COG1510(1)), signalling processes (COG1119(39), OMCL_985(16)), compound synthesis (COG3424(41)), as well as poorly characterised functions (OMCL_39(70), OMCL_582(71), OMCL_615(72), OMCL_677(73) and COG1755(59)). Inspection of supporting evidence in STRING (Szklarczyk et al., 2011) of all OGs identified for thermotolerants (N=9), revealed that the COG1755(59), encoding for an unknown conserved protein in bacteria, is co-localised and co-occurs with COG3424(41), pertaining to a gene encoding for naringenin-chalcone synthase, an acyltransferase enzyme (data not shown).

Genes identified as important for thermophiles consisted of genes encoding proteins involved in DNA metabolism (COG1583(8) and COG1203(44)), an enzyme possibly involved in the synthesis of peptidoglycan (OMCL_856(23)) and genes encoding for unknown functions (COG3543(63) and COG4636(66)). Analysis of all genes relevant for thermophiles in STRING (N=7), showed co-localisation and co-occurrence relations between a predicted helicase (COG1203(44)) and the DNA repair enzyme (COG1583(8)) (data not shown).

In turn, we found 27 genes dominantly present among mesophiles and thermotolerants and poorly represented among strains of the thermophilic group. The most important genes (N=17) (see additional file 9 and as assessed by the table of positive contributions for the gene) included functions related to DNA metabolism, protein quality control, general metabolism, transport, cell wall, cell defense and poorly characterised functions. OGs found in common for mesophiles and thermotolerants, by running Phenolink with the MDS-Firmicutes and the MDS-OGT were COG1272(47), COG1346(18), COG1671(57) and COG2827(10). Profiling of all the identified genes for members of the mesophilic group revealed a number of interactions

among the different genes (Figure 6A). For instance the protein encoded by COG2378(2) could potentially be involved in the regulation of the activity of the β-lactamase encoded by COG2094(9). It also revealed that genes encoded by COG0652(28) and COG1253(16) involved in protein modification and quality control, respectively, are co-localized and their co-expression was observed experimentally. An uncharacterised protein encoded by COG1357(56) showed a co-localization and co-expression relation with two proteins with protein modification functions, which suggests a similar function.

With regard to the spore heat-resistant phenotype, genes prevalent among heat-sensitive (N=10) and a single gene prevalent among heat-resistant spore-formers were identified (Table 3 and additional file 10). For the former group, all strains were adequately classified (classification error of 0%), while for the latter, strain M112 was misclassified (1 out of 6, resulting in a classification error of 17%) (additional file 10). The genes which were important to set apart heat-sensitive strains from heat-resistant strains included OGs pertaining to DNA and RNA metabolism (OMCL_231(4) and COG2827(10)), membrane/ envelope biogenesis (COG0677(17) and OMCL_269(22)), an enzyme related to sulfurtransferases (COG2897) and proteins with less well characterised function. No interrelations were present among these genes found predominately among heat-sensitive spore-formers, as assessed with STRING.

The COG0863(7), encoding for a DNA modification methylase, was identified as an important gene common to heat-resistant spore-formers in the MDS-D. This gene was absent in strain M112 and all heat-sensitive spore-forming strains. However, by searching for this COG in the MDS-Firmicutes, it was observed that it is a relatively frequent protein among mesophiles (34%), thermotolerant (26%) and thermophiles (57%), some of them constituting strains which are not expected to produce spores of high heat-resistance, as obtained from semi-quantitative data published, for instance, for *B. cereus* 10975 (de Vries, 2006). This outcome indicates that the gene encoded by COG863 is not an important feature to account for the underlying mechanisms of spore heat-resistance.

In general, genes were not found to be related to more than one phenotype. The only exception was the COG2827(10), encoding for an endonuclease, possibly involved in RNA metabolism, which was found in common among mesophiles, thermotolerants and heat-sensitive spore-formers. This COG was absent in all heat-resistant spore-formers in the MDS-D.

It was further obtained the gene context for COGs of which function was unknown, using STRING. This analysis showed that for thermophiles, COG3543(63) and COG4636(66) were not related (data not shown). COG4636(66) is fused with a dioxygenase and co-localized with enzymes involved in the covalent modification of sugars (Figure 6B-i). In turn, COG3543(63) could have a function in stress responses in the cell, as it appeared co-localized with proteins involved with resistance to β-lactamase (NOG09683) and redox homeostasis (NOG39461) (Figure 6B-ii). Figure 6C illustrates the network of genes for COG2013(60) and COG3832(64) obtained for heat-sensitive spore-forming strains. A role in stress response mechanisms in the cell can be hypothesised for the protein encoded by COG2013(60) as it appeared to be fused

Table 3. Results of Phenolink classifications pertaining to growth temperature and spore heat-resistant phenotypes. The data is organized according to functional category groups and corresponding sub-categories (Tatusov et al., 2003)

No.	Orthologous group (OG)[a]		Description	Growth temperature class			Spore heat-resistance group		GEE[b]
				Mesophile	Thermo-tolerant	Thermo-phile	Heat-sensitive	Heat-resistant	
			INFORMATION STORAGE AND PROCESSING						
	K	**Transcription**							
1		COG1510	Predicted transcriptional regulators		+				NE
2		COG2378	Predicted transcriptional regulator	+	+				NE
3		COG3682	Predicted transcriptional regulator	+	+				NE
4		OMCL _231	MarR family transcriptional regulator; hypothetical protein				+		NE
	L	**Replication, recombination and repair**							
5		COG0270	Site-specific DNA methylase	+	+				E
6		COG0708	Exonuclease III	+					NE
7		COG0863	DNA modification methylase					+	NE
8		COG1583	Uncharacterised protein predicted to be involved in DNA repair (RAMP superfamily)			+			NE
9		COG2094	3-methyladenine DNA glycosylase	+	+				NE
10		COG2827	Predicted endonuclease containing a URI domain	+	+		+		NE
			CELLULAR PROCESSES AND SIGNALING						
	V	**Defense mechanisms**							
11		COG1680	Beta-lactamase class C and other penicillin binding proteins	+	+				NE
12		COG2720	Uncharacterised vancomycin resistant protein	+	+				NE

Table 3. Results of Phenolink classifications pertaining to growth temperature and spore heat-resistant phenotypes. The data is organized according to functional category groups and corresponding sub-categories (Tatusov et al., 2003) *(continued)*

No.	Orthologous group (OG) [a]		Description	Growth temperature class			Spore heat-resistance group		GEE [b]
				Mesophile	Thermo-tolerant	Thermo-phile	Heat-sensitive	Heat-resistant	
	T	**Signal transduction mechanisms**							
13		OMCL_83	ATP/GTP-binding protein; ATPase; hypothetical protein	+	+				NE
14		OMCL _224	ABC transporter ATP-binding protein; hypothetical protein				+		NE
15		OMCL _318	Factor required for KinB signal transduction and activation of the phosphorelay to sporulation		+				NE
16		OMCL _985	Ser/Thr phosphatase; Indirect negative regulator of sigma-B activity		+				NE
	M	**Cell wall/ membrane/ envelope biogenesis**							
17		COG0677	UDP-N-acetyl-D-mannosaminuronate dehydrogenase				+		NE
18		COG1346	Putative effector of murein hydrolase	+	+				NE
19		COG1696	Predicted membrane protein involved in D-alanine export	+					NE
20		COG2891	Cell shape-determining protein		+				NE
21		OMCL _35	Lipoprotein; sporulation lipoprotein YhcN/YlaJ, hypothetical protein			+			NE
22		OMCL _269	Spore coat protein; Spore coat protein CotD; hypothetical protein				+		NE
23		OMCL _856	Glycosyltransferase group I; hypothetical protein			+			NE

Table 3. Results of Phenolink classifications pertaining to growth temperature and spore heat-resistant phenotypes. The data is organized according to functional category groups and corresponding sub-categories (Tatusov et al., 2003) *(continued)*

No.	Orthologous group (OG) [a]		Description	Growth temperature class			Spore heat-resistance group		GEE [b]
				Mesophile	Thermo-tolerant	Thermo-phile	Heat-sensitive	Heat-resistant	
	N	**Cell motility**							
24		OMCL _398	Flagella biosynthesis protein FliZ		+				NE
	U	**Intracellular trafficking, secretion and vesicular transport**							
25		COG2805	Tfp pilus assembly protein, pilus retraction ATPase PilT			+			NA
	O	**Posttranslational modification, protein turnover, chaperones**							
26		COG0229	Conserved domain frequently associated with peptide methionine sulfoxide reductase	+	+				NE
27		COG0326	Molecular chaperone, HSP90 family	+	+				NE
28		COG0652	Peptidyl-prolyl cis-trans isomerase (rotamase)- cyclophilin family	+	+				NE
			METABOLISM						
	C	**Energy production and conversion**							
29		COG1227	Inorganic pyrophosphatase/ exopolyphosphatase	+	+				NA
30		COG1882	Pyruvate-formate lyase	+					NA
31		COG2851	H+/citrate symporter		+				NE
	E	**Amino acid transport and metabolism**							
32		COG1509	Lysine 2,3-aminomutase	+	+				NA
	F	**Nucleotide transport and metabolism**							
33		COG1972	Nucleoside permease		+				NA

Table 3. Results of Phenolink classifications pertaining to growth temperature and spore heat-resistant phenotypes. The data is organized according to functional category groups and corresponding sub-categories (Tatusov et al., 2003) *(continued)*

No.	Orthologous group (OG) [a]	Description	Growth temperature class			Spore heat-resistance group		GEE [b]
			Mesophile	Thermo-tolerant	Thermo-phile	Heat-sensitive	Heat-resistant	
	P	**Inorganic ion transport and metabolism**						
34	COG0288	Carbonic anhydrase	+	+				NE
35	COG0659	Sulfate permease and related transporters (MFS superfamily)	+	+				NE
36	COG1178	ABC-type Fe3+ transport system, permease component	+					NA
37	COG1528	Ferritin-like protein	+					NA
38	COG2897	Rhodanese-related sulfurtransferase				+		NA
	Q	**Secondary metabolites biosynthesis, transport and catabolism**						
39	COG1119	ABC-type molybdenum transport system, ATPase component/ photorepair protein PhrA		+				NA
40	COG2162	Arylamine N-acetyltransferase		+				NA
41	COG3424	Predicted naringenin-chalcone synthase		+				NA
42	OMCL _575	Thiol oxidation management factor, acetyltransferase; GCN5-related N-acetyltransferase; hypothetical protein		+				NE
		POORLY CHARACTERISED						
	R	**General function prediction only**						
43	COG0655	Multimeric flavodoxin WrbA	+	+				NA
44	COG1203	Predicted helicases			+			NA
45	COG1242	Predicted Fe-S oxidoreductase	+	+				NE
46	COG1253	Hemolysins and related proteins containing CBS domains	+	+				NE
47	COG1272	Predicted membrane protein, hemolysin III homolog	+	+				NE

Table 3. Results of Phenolink classifications pertaining to growth temperature and spore heat-resistant phenotypes. The data is organized according to functional category groups and corresponding sub-categories (Tatusov et al., 2003) *(continued)*

No.	Orthologous group (OG)[a]	Description	Growth temperature class			Spore heat-resistance group		GEE[b]
			Mesophile	Thermo-tolerant	Thermo-phile	Heat-sensitive	Heat-resistant	
48	COG1380	Putative effector of murein hydrolase LrgA	+	+				NA
49	COG2110	Predicted phosphatase homologous to the C-terminal domain of histone macroH2A1			+			NA
50	COG2910	Putative NADH-flavin reductase		+				NA
51	COG3180	Putative ammonia monooxygenase		+				NA
52	COG3173	Predicted aminoglycoside phosphotransferase				+		NE
53	COG3481	Predicted HD-superfamily hydrolase	+	+				NA
54	COG3973	Superfamily I DNA and RNA helicases	+	+				NA
55	COG4224	Uncharacterised protein conserved in bacteria	+	+				NA
S	**Function unknown**							
56	COG1357	Uncharacterised low-complexity proteins	+	+				NE
57	COG1671	Uncharacterised protein conserved in bacteria	+	+				NE
58	COG1714	Predicted membrane protein/domain		+				NE
59	COG1755	Uncharacterised protein conserved in bacteria		+				NE
60	COG2013	Uncharacterised conserved protein				+		NA
61	COG2357	Uncharacterised protein conserved in bacteria	+	+				NE
62	COG2996	Uncharacterised protein conserved in bacteria	+	+				NA
63	COG3543	Uncharacterised conserved protein			+			NA
64	COG3832	Uncharacterised conserved protein				+		NA

Table 3. Results of Phenolink classifications pertaining to growth temperature and spore heat-resistant phenotypes. The data is organized according to functional category groups and corresponding sub-categories (Tatusov et al., 2003) *(continued)*

No.	Orthologous group (OG)[a]	Description	Growth temperature class			Spore heat-resistance group		GEE[b]
			Mesophile	Thermo-tolerant	Thermo-phile	Heat-sensitive	Heat-resistant	
65	COG4198	Uncharacterised conserved protein	+					NA
66	COG4636	Uncharacterised protein conserved in cyanobacteria			+			NA
67	OMCL _154	Hypothetical protein	+					NA
68	OMCL _210	Hypothetical protein				+		NA
69	OMCL _310	Hypothetical protein; YetN		+				NE
70	OMCL _393	Hypothetical protein; YlqD protein		+				NE
71	OMCL _582	Hypothetical protein		+				NA
72	OMCL _615	Hypothetical protein		+				NA
73	OMCL _677	Hypothetical protein; YmaF protein		+				NE

The table summarises genes which are important and sufficiently present within a class or group, and sufficiently absent in the opposite class or group. In the case of the growth temperature phenotype the results of classifications with three classes (mesophiles, thermotolerants, thermophiles) or only two classes (mesophilic and thermophilic group) (see methods for details) are given. The additional files 9-11 provide the complete results for the individual classes/group. Phenolink was run with default parameters, which include feature selection based on 75% phenotype presence and 75% phenotype absence in an opposite class/group; correlation cut-offs of 0.95 (Spearman's correlation coefficient) and 0.98 (Pearson's correlation coefficient); a maximum classification error of 40%, exclusion of genes which show variance below 5%; and multiple-down-sizing to address class imbalance.

[a]Orthologous groups encompass both Clusters of Ortholog groups (COGs) and Orthologs Markov Clusters (OMCLs). COGs correspond to genes that have been annotated in a large number of sequenced genomes. OMCLs (Li et al., 2003) are composed of multiple genes in different group of strains, which have similar, yet, not necessarily, the same annotation.

[b]This column shows Gene Essentiality as compiled in the OGEE database by Chen et al. (2012): E- essential; NE- non-essential (tested in one or more endospore-forming Firmicutes, including *B. subtilis* strain 168 as described by Kobayashi et al. (2003); NA- not available (not tested).

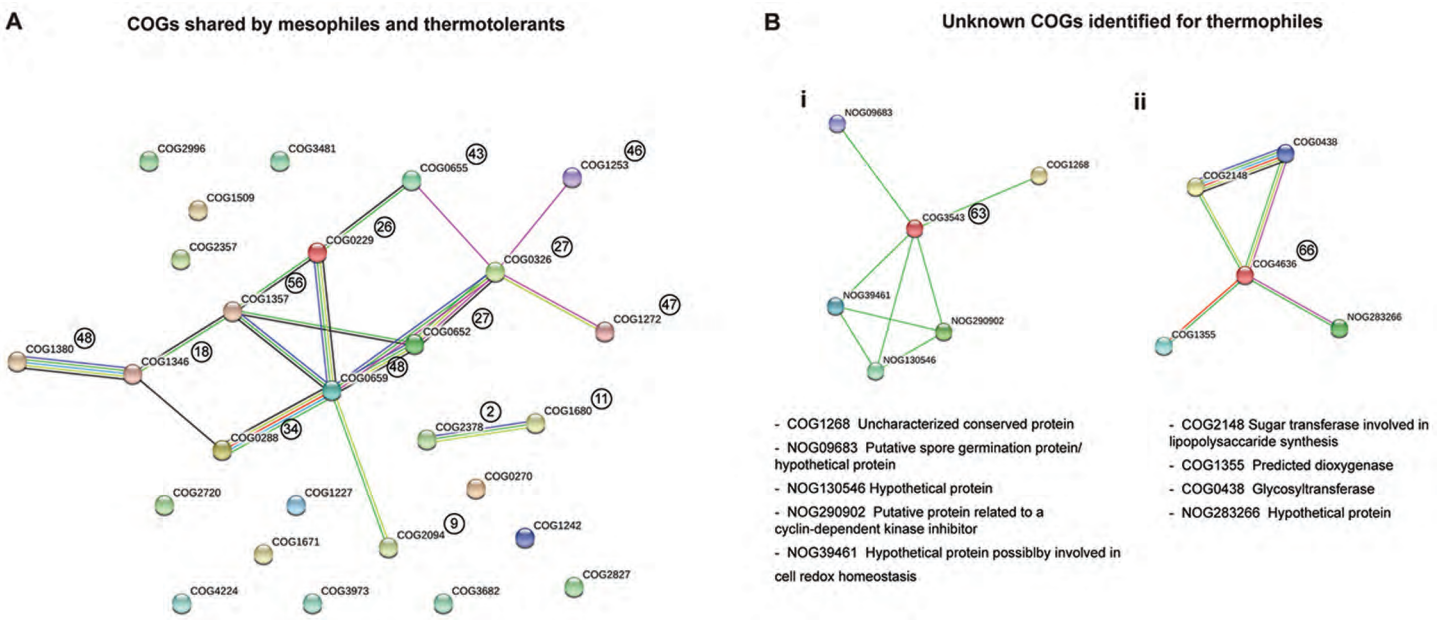
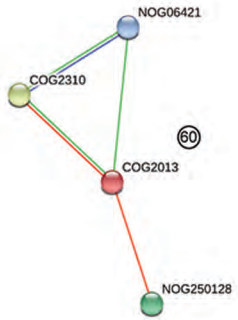


Figure 6. Profiling of gene context interactions of selected features obtained in Phenolink. The interaction networks as displayed in STRING (Szklarczyk et al., 2011) are given for all genes identified in common among mesophiles and thermotolerants (**A**). Additionally, the gene context for important COGs for the different phenotypes belonging to unknown functions is shown (**B-C**). Dark green lines indicate genes co-localized (neighborhood) in the genome (possibly operon strucures); red lines indicate genes fused into a single operon; dark blue lines indicate statisitically significant co-occurrence across mutliple genomes, black lines indicate genes showing co-expression in mycroarray experiments, pink lines indicate experiments, light blue lines indicates annotation in databases and light green lines indicate genes determined by text mining. NOG corresponds to the class of Non-supervised Orthologus groups (Powell et al., 2012). See Table 2 for the description of the numbered COGs. Other COGs were obtained as described at the Clusters fo Orthologous Groups database (Tatusov et al., 1997).

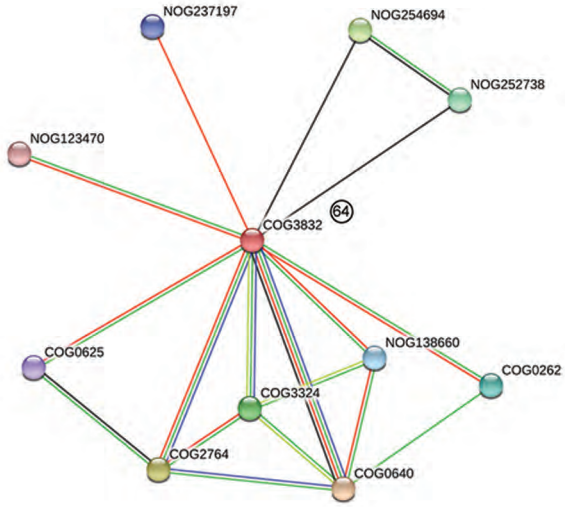
C **Unknown COG identified for heat-sensitive spore-formers**

i



- COG2310 Uncharacterized protein involved in stress response, homologs of TerZ and putativecAMP-binding protein CABP1
- NOG250128 Hypothetical protein

ii



- COG0640 Predicted transcriptional regulator
- COG2764 Uncharacterised protein conserved in bacteria
- NOG254694 Protein involved in RNA elongation
- COG3324 Predicted enzyme related to lactoylglutathione lyase
- NOG252738 Hypothetical protein
- COG0262 Dihydrofolate reductase
- NOG138660 Hypothetical protein
- NOG237197 Hypothetical protein
- COG0625 Gluthatione S-transferase
- NOG123470 Activator of HSP90 ATPase 1 family protein

to a protein involved in stress response and a transcriptional regulation (NOG250128) (Figure 6C-i). In the case of COG3832(64) (Figure 6C-ii) a complex relation with 10 different OGs was obtained. In particular, the observation of simultaneously "neighbourhood", "gene fusion", co-occurrence" and "co-expression" of COG3832(64) with the transcriptional regulator encoded by COG0640 could indicate that it is a protein under control of the regulator COG0640.

Taken together the results of GTM suggest that shared gene content could be an important factor shaping the growth temperature phenotype of microorganisms across different genera. It also revealed a number of genes which could potentially be either directly or indirectly involved with the inability of strains to form spores of high heat-resistance. On the contrary, the results indicate that high heat-resistant spore phenotype cannot be explained on the basis of shared gene content.

Amino acid composition comparison in spore proteins

We next analysed spore protein homologs for their content of hydrophobic, aromatic and charged amino acids (Figure 1-Step 4). Of an initial set of 121 spore proteins pertaining to 18 functions in the cell (additional file 11), 54 homologs were identified for all strains in the MDS-OGT and 70 for all strains in the MDS-D (additional file 12). The additional file 13 summarises the results of the comparison of these amino acid compositional features between strains in the mesophilic and in the thermophilic group, while the additional file 14 presents the comparison between heat-sensitive and heat-resistant spore-formers. Protein homologs were found for 13 cell functions in both MDS-OGT and MDS-D. Table 4 and Table 5 provide an overview of the most relevant results, specifically proteins for which intermediate or high

Pearson's and Spearman's correlation coefficients were supported by significant statistical differences ($P< 0.05$) in the mean values for the content of hydrophobic, aromatic or charged amino acids between strains belonging to the two phenotypic groups.

In the MDS-OGT, out of 54 protein homologs, 14 proteins based on the Kyte-Doolittle (KD) scale and 9 proteins based on the Wimley and White (WW) scale, showed significant differences with respect to their content of hydrophobic/hydrophilic amino acids, between members in the mesophilic group and the thermophilic group (Table 4). Members of the thermophilic group showed in most cases a significant overrepresentation of hydrophobic amino acids in calculations based on both scales, as assessed by the sign of the correlation coefficients (positive implies increased hydrophobicity with increasing OGT). This was the case for GyrA (DNA gyrase subunit A, no.17) protein which exhibited, based on the KD scale, a Pearson's and Spearman's correlation coefficient of 0.55 ($P < 0.05$). The GyrA(17) was the protein which based on KD scale showed the highest correlation between the OGT and the amino acid hydrophobic content, followed by the RpsI (30S ribosomal protein S9 protein, 32) ($r= 0.50$; $\rho= 0.53$, $P< 0.05$). Conversely, the protein Adk (Adenylate kinase, 20) had the highest negative correlation with OGT, indicating an increased content of hydrophilic amino acids across thermophilic members ($r= -0.51$; $\rho= -0.52$, $P< 0.05$) when compared to their mesophilic counterparts. Based on the WW scale, the protein ClpX (ATP-dependent Clp protease ATP-

binding subunit, 5) had the highest positive correlation coefficient (r= 0.49; ρ= 0.41, P< 0.05), while the protein GroEL (60 kDa chaperonin, 3) had the highest negative correlation coefficient (r= -0.53; ρ= -0.59, P< 0.05). Although the outcome of amino acid hydrophobicity content evaluation based on KD and WW scales showed a number of dissimilarities, there were also a few proteins which were simultaneously identified as having a significant different number of hydrophobic/hydrophilic amino acid residues by the two scales. This was the case for the GroEL(3),ValS (valyl-tRNA synthetase, 15) and DeoB (phosphopentomutase, 21).

Regarding the content of aromatic amino acids, 12 out of 54 homologs showed significant differences between mesophilic and thermophilic members, whereas in the case of the content of charged amino acids, 17 proteins were identified. The highest correlation between OGT and protein aromatic content was present for the DnaK (chaperone protein, 4) (r= 0.66; ρ= 0.63, P< 0.05), followed by the RplR (50S ribosomal protein L18 protein, 29) (r= 0.59; ρ= 0.60, P< 0.05). Regarding the content of charged amino acids, the protein RplR(29) showed the highest correlation with OGT (r= 0.63; ρ= 0.66, P< 0.05), after GroEL(3) (r= 0.69; ρ= 0.71, P< 0.05), with the charged amino acid content being the amino acid compositional feature which showed the highest significant correlation with OGT. GroEL(3) was a particular case since it showed simultaneously an increased content of hydrophilic amino acids based on the two scales, and an increased content of charged amino acids.

The comparison of amino acid features in protein homologs of heat-sensitive and heat-resistant spore-formers in the MDS-D (Table 5), revealed that although a larger number of protein homologs had been identified when compared to the MDS-OGT, the number of proteins showing significant differences in the amino acid composition in the two groups was substantially lower. Out of 70 protein homologs, 2 and 7 proteins showed significant differences in the hydrophobic content based on the KD and WW scale, respectively, while regarding the content of aromatic and charged residues this number was respectively, 7 and 9. Conversely, a slightly larger number of proteins showed a high correlation (r or ρ > 0.7) with increasing spore D-value, as highlighted in black boxes.

Based on the KD scale a significant dif ference between spore D-value and the hydrophobic amino acid content was identified for the FftsZ (cell division protein, 5) (r= 0.77; ρ= 0.61, P< 0.05), followed by the Spo0A protein (stage 0 sporulation protein, 7) (r= 0.55; ρ= 0.55, P< 0.05), while based on the WW scale high correlations were found for the GpmI (2,3-bisphosphoglycerate-independent phosphoglycerate mutase, 17) (r= 0.77; ρ= 0.76, P< 0.05), PykF (Pyruvate kinase, 18) (r= 0.75; ρ= 0.62, P< 0.05) and ValS (valyl-tRNA synthetase) (r= 0.71; ρ= 0.68, P< 0.05) proteins. Regarding the content of aromatic and charged amino acids, 7 and 9 proteins, respectively, showed significant difference between heat-resistant and heat-sensitive spore-formers. DacF (D-alanyl-D-alanine carboxypeptidase, 21) was the protein which showed the highest positive correlation with increased spore D-value (r= 0.69; ρ= 0.63, P< 0.05). The protein CyM (putative O-acetylserine sulfhydrylase, 14) (r= -0.83; ρ= -0.86, P< 0.05) and RpoC (DNA-directed RNA polymerase beta chain, 23) (r= -0.70; ρ=-0.64, P< 0.05) also exhibited high correlation coefficients with increased D-values, but in both cases the

Table 4. Comparison of amino acid compositional features between members in the mesophilic and thermophilic group (MDS-OGT)[a]

No.	Protein code	Hydrophobicity index[b]						Aromaticity index			Charge		
		KD			WW								
		P-value	*r*	*ρ*	*P*-value	*r*	*ρ*	*P*-value	*r*	*ρ*	*P*-value	r	ρ
	Conserved hypothetical												
1	CD079	0.07	-0.20	-0.24	**0.03**	-0.39	-0.34	0.66	0.03	0.01	**0.01**	0.45	0.44
	Cell processes												
2	ClpC	0.47	0.23	0.16	0.07	-0.36	-0.45	0.38	0.09	0.10	**0.00**	0.53*	0.58*
3	GroEL	0.02	-0.38	-0.43*	**0.00**	-0.53*	-0.59*	0.61	0.27	0.22	**0.00**	0.69**	**0.71****
4	DnaK	0.29	0.26	0.17	**0.00**	0.46*	0.46*	**0.00**	0.66	0.63	0.35	-0.19	-0.15
5	ClpX	0.74	0.04	-0.04	**0.01**	0.49*	0.41*	0.58	0.16	0.29	0.89	-0.04	0.06
6	ClpP1	**0.01**	0.43*	0.45*	0.32	0.23	0.15	**0.03**	0.42	0.43	**0.02**	0.38	0.38
	Transport, binding proteins												
7	SecA1	0.19	0.28	0.17	0.34	0.10	0.02	**0.01**	0.46*	0.43*	0.09	0.39	0.40*
	Adaptation												
8	MreB2	0.65	0.06	0.01	0.25	-0.22	-0.25	**0.03**	-0.28	-0.39	0.07	0.30	0.32
9	FftsZ	**0.02**	0.49*	0.43*	0.90	0.04	-0.02	0.18	0.15	0.23	0.21	0.31	0.26
	Spore structure, sporulation, germination												
10	Spo0A	**0.00**	0.49*	0.49*	0.44	0.14	0.00	0.54	0.15	-0.02	0.82	-0.07	0.00
11	Gpr	**0.01**	0.47*	0.46*	0.16	0.24	0.31	0.40	-0.22	-0.23	0.17	-0.19	-0.29
	Macromolecule degradation												
12	ComR	**0.03**	0.37	0.32	0.08	-0.19	-0.28	0.54	-0.15	-0.07	**0.00**	0.45*	0.48*

Table 4. Comparison of amino acid compositional features between members in the mesophilic and thermophilic group (MDS-OGT)[a] *(continued)*

No.	Protein code	Hydrophobicity index[b]						Aromaticity index			Charge		
		KD			WW								
		P-value	*r*	*ρ*	*P*-value	*r*	*ρ*	*P*-value	*r*	*ρ*	*P*-value	r	ρ
Macromolecule synthesis, modification													
14	AlaS	0.99	0.08	0.06	0.59	-0.11	-0.15	0.67	-0.10	-0.20	**0.01**	0.52*	0.54*
15	LeuS	0.27	-0.13	-0.22	0.39	-0.12	-0.20	**0.02**	0.48*	0.39	**0.01**	0.50*	0.48*
16	ValS	**0.02**	0.44*	0.43*	**0.00**	0.32	0.38	0.53	-0.18	-0.27	0.49	-0.08	-0.09
17	NusA	0.11	0.39	0.31	**0.01**	0.44*	0.43*	**0.02**	-0.41*	-0.51*	**0.01**	-0.47*	-0.44*
18	GyrA	**0.00**	0.55*	0.55*	0.76	0.06	0.01	0.48	0.01	-0.09	0.08	0.34	0.32
Amino acid biosynthesis													
19	CysK	0.64	0.00	-0.07	0.90	-0.02	-0.08	0.10	-0.13	-0.27	**0.02**	0.34	0.42*
20	CysM	0.78	0.05	-0.01	0.53	-0.08	-0.16	0.21	-0.11	-0.21	**0.01**	0.40*	0.47*
Central/intermediary metabolism													
21	Adk	**0.01**	-0.51*	-0.52*	0.06	-0.44*	-0.48*	0.95	0.09	-0.02	**0.04**	0.51	0.51
22	DeoB	**0.03**	0.41*	0.41*	**0.00**	0.44*	0.40*	0.23	0.23	0.18	0.05	-0.33	-0.32
23	IscS2	**0.01**	0.36	0.34	0.39	0.02	-0.06	0.10	-0.37	-0.44	0.79	0.13	0.16
24	GapdH	0.65	0.15	0.08	0.39	-0.14	-0.09	**0.02**	0.44*	0.39	**0.00**	0.47*	0.50*
Energy metabolism													
25	Eno	0.07	0.22	-0.09	0.13	0.97	-0.16	**0.03**	-0.36	-0.38	0.12	-0.05	-0.10
26	AtpD	0.38	0.21	0.17	0.26	0.46	0.12	0.07	0.24	0.32	**0.02**	0.36	0.35
Cell surface													
27	DacF	0.17	0.31	0.28	**0.03**	0.40*	0.42*	**0.01**	0.36	0.48*	1.00	-0.04	0.00

Table 4. Comparison of amino acid compositional features between members in the mesophilic and thermophilic group (MDS-OGT)[a] *(continued)*

No.	Protein code	Hydrophobicity index[b]						Aromaticity index			Charge		
		KD			WW								
		P-value	*r*	*ρ*	*P*-value	*r*	*ρ*	*P*-value	*r*	*ρ*	*P*-value	r	ρ
		Ribosome constituents											
28	RplC	0.42	0.14	0.26	0.56	0.15	0.11	0.08	0.30	0.29	**0.02**	0.44*	0.48*
29	RplR	**0.03**	-0.33	-0.38	0.09	0.16	0.09	**0.00**	0.59*	0.60*	**0.00**	0.63*	0.66*
30	RpsM	**0.04**	-0.30	-0.19	0.15	-0.28	-0.32	**0.02**	0.31	0.35	**0.01**	0.56*	0.52*
31	RpsD	**0.00**	-0.35	-0.34	**0.01**	-0.20	-0.38	0.60	0.13	0.03	0.02	0.38	0.43*
32	RpsI	**0.00**	0.50*	0.53*	0.92	0.09	-0.03	**0.01**	-0.41*	-0.41*	0.56	0.01	0.12

[a] Bold numbers for the P-value indicate that the difference in the mean values for strains in the mesophilic and in the thermophilic group are statistically significant ($P < 0.05$), as assessed by the Mann-Whitney test (2-tailed). the Pearson´s correlation coefficient (r) and the Spearman´s correlation coefficient (ρ) are given subsequently. The colour scheme highlights the strength of the correlation: black for correlations higher than 0.7, grey for correlation between 0.4 and 0.7 and white for correlation below 0.4. * Correlation is significant with $P < 0.05$ or ** $P < 0.01$.

[b] The hydrophobicity content of the protein is expressed in terms of the Kyte and Doolitle (KD) and Wimley and White (WW) hydrophobicity scales.

Only proteins with intermediate or high correlation values and which are supported by significant *P*-values are shown (see Additional file 14 for the complete list)

Table 5. Comparison of amino acid compositional features between heat-resistant and heat-sensitive spore-formers (MDS-D)[a]

No.	Protein code	Hydrophobicity[b]						Aromaticity			Charge		
		KD			WW								
		P-value	*r*	*ρ*	*P*-value	*r*	*ρ*	*P*-value	r	*ρ*	*P*-value	*r*	*ρ*
	Conserved hypothetical												
1	CD079	0.15	0.46	0.38	0.04	0.54	0.54	0.58	-0.32	-0.10	0.80	-0.17	-0.05
	Cell processes												
2	GroEL	0.23	-0.28	-0.19	0.18	-0.51	-0.44	0.31	-0.05	-0.04	0.03	0.54*	0.45*
3	DnaK	0.25	-0.04	-0.10	0.27	0.45	0.40	**0.01**	0.45*	0.36	0.79	-0.15	-0.15
4	ClpP1	0.50	0.37	0.16	0.53	-0.15	-0.07	0.11	0.49	0.48	**0.00**	0.62**	**0.71****
	Adaptation												
5	FftsZ	**0.01**	**0.77**	0.61	0.64	0.51	0.36	0.89	0.11	0.10	0.37	0.18	0.25
	Spore structure, sporulation, germination												
6	Obg	0.40	0.27	0.28	**0.03**	0.40*	0.38	0.09	-0.69	-0.60	0.08	-0.59	-0.44
7	Spo0A	**0.03**	0.55*	0.66*	0.76	0.30	0.11	0.95	0.17	0.20	0.39	-0.54	-0.41
8	Gpr	0.26	0.48	0.31	**0.03**	0.69*	0.50*	0.44	-0.12	-0.03	**0.02**	-0.67*	-0.51*
	Macromolecule synthesis, modification												
9	ValS	0.05	0.51	0.48	**0.02**	**0.71****	0.68**	0.75	-0.26	-0.17	0.84	-0.39	-0.32
10	LysS	0.05	0.63	0.47	0.15	0.65	0.39	**0.03**	-0.35	-0.40*	**0.01**	-0.66*	-0.46*
11	RpoB	0.66	-0.13	-0.04	**0.01**	-0.59*	-0.58*	0.67	0.06	-0.11	0.00	0.57*	0.55*
12	RpoC	0.27	-0.19	-0.05	0.22	-0.29	-0.14	**0.00**	-0.70	-0.64	0.06	0.41	0.29
	Amino acid biosynthesis												
13	CysK	0.88	0.51	0.30	0.12	0.55	0.44	0.14	-0.66	-0.54	0.59	-0.28	-0.32
14	CysM	0.15	0.62	0.61	0.50	0.35	0.34	0.00	-0.83**	-0.86**	0.86	-0.10	-0.13

Table 5. Comparison of amino acid compositional features between heat-resistant and heat-sensitive spore-formers (MDS-D)[a] *(continued)*

No.	Protein code	Hydrophobicity[b]						Aromaticity			Charge		
		KD			WW								
		P-value	*r*	*ρ*	*P*-value	*r*	*ρ*	*P*-value	r	*ρ*	*P*-value	*r*	*ρ*
	Central/intermediary metabolism												
15	GapdH	0.99	0.22	0.18	0.69	0.11	0.16	0.98	0.17	-0.02	**0.01**	0.60*	0.69*
	Energy metabolism												
16	Eno	0.34	0.23	0.20	0.38	0.33	0.17	**0.01**	-0.66*	-0.60*	**0.02**	-0.61*	-0.44*
17	GpmI	0.07	0.67	0.73	**0.02**	**0.77****	**0.76****	0.67	-0.02	0.09	0.18	-0.60	-0.57
18	PykF	0.44	0.36	0.19	**0.04**	**0.75***	0.62	0.37	0.14	0.41	0.58	0.06	-0.11
19	AtpD	0.07	0.46	0.39	0.07	0.61	0.58	0.50	-0.31	-0.26	0.30	-0.42	-0.42
	Fatty acid biosynthesis												
20	FabZ	0.21	0.37	0.33	0.08	0.44	0.43	0.78	-0.04	-0.11	0.05	-0.50	-0.47
	Cell surface												
21	DacF	0.34	0.59	0.32	0.06	0.65	0.62	**0.02**	0.69*	0.63*	0.34	-0.52	-0.45
	Ribosome constituents												
22	RplA	0.90	-0.17	-0.03	0.98	-0.22	-0.13	0.49	-0.02	0.13	0.01	0.64*	0.58*
23	RplC	0.98	0.05	-0.01	0.20	0.29	0.28	**0.02**	0.68*	0.49*	**0.03**	0.45*	0.34

[a] Bold numbers for the *P*-value indicate that the difference in the mean values for heat-sensitive and heat-resistant spore-formers are statistically significant ($P < 0.05$), as assessed by the Mann-Whitney test (2-tailed). the Pearson´s correlation coefficient (*r*) and the Spearman´s correlation coefficient (ρ) are given subsequently. The reader is referred to footnotes of Table 3 for further descriptions in this table.

Only proteins with intermediate or high correlation values and which are supported by significant *P*-values are shown (see Additional file 14 for the complete list).

correlations were negative, meaning that for those proteins, strains in the mesophilic group had a larger content of aromatic amino acids than strains in the thermophilic. The ClpP1 (ATP-dependent Clp protease proteolytic subunit, 4) (r= 0.62; ρ= 0.71, P< 0.05), followed by the GapDH (Glyceraldehyde-3-phosphate dehydrogenase, 15) (r= 0.60; ρ= 0.69, P< 0.05) proteins, showed the highest correlation with spore log$D_{110°C}$-value and and these correlations being positive indicate a trend towards increased content of charged amino acids in those proteins in heat-resistant spore-forming strains.

By inspecting the strains simultaneously in the MDS-OGT and MDS-D, it was attempted to understand whether protein features exhibiting a high correlation with log$D_{110°C}$ constituted eventual false-positives. To this end, the raw values of protein mean hydrophobicity and the amino acid percentages were compared with both the raw and average values from strains in the heat-resistant and heat-sensitive group. This further examination showed a number of mesophilic strains belonging to the MDS-OGT, which had amino acid profile contents similar to strains in the heat-resistant group, but those constituted strains we believe do not form spores of high heat-resistance. This included for instance, strains of *Desulfitobacterium hafniense*, *Clostridium ljungdahlii*, *C. phytofermentans* or *Oceanobacillus iheyensis*. This suggests that the highlighted protein compositional features in Table 5 are only valid for the dataset comprising the MDS-D and cannot be used as biomarkers of spore heat-resistance.

Taken together, protein compositional features studied for the protein dataset in this work did not provide means to distinguish strains belonging to different heat-resistant spore-forming groups and revealed relatively little resolution to distinguish strains belonging to the mesophilic and to the thermophilic group.

Discussion

The mechanisms underlying the capacity of a strain to thrive naturally at high temperatures and to produce spores of high heat-resistance have attracted considerable attention over the last half-century (Amelunxen and Murdock, 1978; Das et al., 2006; Gaughran, 1947; McDonald and Matney, 1963; Stewart and Halvorson, 1954; Xezones H et al., 1965). Both thermophilism and spore heat-resistance are highly complex phenotypes and, for this reason, understanding the underlying mechanisms requires approaches tackling these problems from distinct perspectives. This paper describes a meta-analysis of genomic and phenotypic data across endospore-forming *Firmicutes* aimed at identifying genomic biomarkers of growth temperature preference and spore heat-resistant phenotype.

As more bacterial genomes become publicly available, it is of interest to assess whether findings pertaining to the genomic determinants of thermophilism verified across studies comparing Archaea and Eubacteria species hold true at the level of a single phylum. At the same time, approaches focused on shared gene content are scarcer. On the other hand, although much research has been done regarding the physicochemical and ultrastructural

mechanisms involved in spore heat-resistance (the most recent review is given by Setlow and Johnson (2007)), less attention has been devoted to the understanding of regulation of this property at the genomic level or putative genomic factors involved in spore heat-resistant phenotype.

The working metadata set in this investigation was enlarged by genome sequencing of two of our *B. subtilis* subsp. *subtilis* strains M112 and M1, which produce spores of substantially higher heat-resistance than that of the type strain 168. In addition, the *D*- and *z*-values for *M. thermoacetica* and *T. thermosaccharolyticum* were determined. We found that while the range of *D*-values for *M. thermoacetica* was in agreement with previously published data (Byrer et al., 2000), this was not the case for spores of *T. thermosaccharolyticum*, as a 63-fold lower $D_{110°C}$ was estimated when compared to spores of another strain characterised by Xezones et al. (1965). Although these results do not contradict in general the observation that spores of these species are among the most heat-resistant spore-formers, it emphasises the importance about collecting precise phenotypic data, but especially *D*-values, for this type of *in silico* studies. This work reflects this effort in using an approach with precise phenotypic data for quantitative determinations and statistical assessment of data significance.

Genomic biomarkers of growth temperature preferences

The genome size and the GC-content of the 16S rRNA gene were the genomic features which had the highest correlation coefficient with the OGT and showed the best predictive capacity to explain that variable. Reduction in the genome size as result of increasing growth temperature has been suggested as a requirement to increase the efficiency and the speed in metabolic processes in the cell at high temperature. Genome size reduction has also been noted in bacteria thriving through parasitic lifestyles (Crisan, 1973; Das et al., 2006).

A number of studies have contradicted initial theories that claimed the existence of a direct correlation between the genome GC-content and the OGT (reviewed e.g. by Hurst and Merchant (2001)), stimulated by the findings that G and C base pairs contributed to increased structural stability, as a result of stacking interactions and hydrogen bonds (Šponer et al., 1996). Absence of such correlation was also confirmed in this work. It is meaningful to note that studies in the 60s had already shown that differences between bacterial OGT and the thermal stability of DNA were insignificant as the base compositions and the melting characteristics of the DNA of thermophiles were comparable to those of mesophiles (Campbell and Pace, 1968). However, Musto et al. (2006) by undertaking studies analysing the relationship between genomic GC-content and the OGT at the family level, found high correlations for a number of families, in which the *Bacillaceae* had the highest Pearson's correlation coefficient (r= 0.80; N=13). By calculating this coefficient for the *Bacillaceae* members in our MDS-OGT, we also found high and significant correlation coefficients (r= 0.76 and ρ= 0.68 (N=10)), meaning that factors other than the growth temperature contribute primarily to the genomic GC-content (Hildebrand et al., 2010; Wang et al., 2006; Wu et al., 2012). In particular, it is interesting to note that recent evidence has been gained, indicating that the occurrence of different

combinations of DNA polymerase III alpha subunit isoforms play a dominant in the GC-content variability across eubacterial genomes (Wu et al., 2012).

Absence of a correlation between OGT and GC-content of ORF was also described (Das et al., 2006; Lambros et al., 2003) and confirmed in this work, although the average GC-content of ORF for mesophiles in the MDS-Firmicutes appeared to be significantly higher than that of thermotolerants and thermophiles.

In turn, it has been demonstrated that OGT is correlated with the GC-content of the rRNA genes, in particular the GC-content of double-stranded stem and loop regions (Das et al., 2006; Galtier and Lobry, 1997; Wang and Hickey, 2002). The trend in our data for endospore-forming *Firmicutes* was indeed largely in accordance with this. Concomitantly, while an independence of the GC-content of rRNAs from the genomic GC-content has been postulated (Wang and Hickey, 2002), we found systematically significant Pearson's correlation coefficient above 0.59, for the relation between genomic GC-content and the GC-content of the 5S rRNA and 16S rRNA, for boh the MDS-OGT and MDS-D (Additional files 6 and 7).

Unlike what has been described, we demonstrated the absence of correlation between the OGT and the GA-content, as well as, the GA-CT ratio, although the ANOVA showed significant differences between mesophiles, on the one hand, and thermotolerants and thermophiles on the other (Das et al., 2006; Lambros et al., 2003; Zeldovich et al., 2007). Increased substitution of base C by A in species adapted to grow at higher temperatures could be due to the fact that heat induces deamination of base C at a higher rate than base A (Ehrlich et al., 1986). On the contrary, Lambros et al. (2003) also reported a high purine load for a number of mesophiles, indicating that more studies might be needed to establish a consistent trend for variation across different taxonomical levels.

The approach based on the search for phenotype shared gene content showed individual enrichment for COGs in mesophiles, thermotolerants and thermophiles, including functions related to DNA replication and repair, diverse cellular processes and signalling pathways in the cell, as well as several other functions for which little information is still available. DNA repair systems largely confined to thermophilic and hyperthermophilic Eubacteria and Archaea members have been identified (Makarova et al., 2002). Interestingly, the system discovered by Makarova et al. (2002) was composed of COGs which were also identified in this work as a signature for thermophilic *Firmicutes*. These were COG1583 (Table 3, no. 8), a predicted protein from the Repair Associated Mysterious Protein (RAMP) and COG1203(43), a predicted helicase. Remarkably, COG2827(10), encoding an endonuclease involved in excision repair with an UvrC-Intron-type (URI) domain (Aravind et al., 1999) was recovered as a signature for mesophiles and thermotolerants (it was present in a very limited number of thermophiles). This endonuclease has been postulated to belong to a repair system for DNA double-strand breaks homologous to the eukaryotic DNA end-binding Ku protein (Aravind et al., 1999). Our findings could corroborate the existence of DNA-repair specific systems in strains exhibiting differences in growth temperature preferences (Makarova et al., 2002; Makarova et al., 2003).

Although several COGs encoding enzymes involved in the energy metabolism and transport emerged for mesophiles and thermotolerants, this was not the case for thermophiles. Similarly, it could be expected that thermophiles would share specific systems involved in the regulation of protein quality control, e.g., chaperons, such as the heat-shock protein Hsp90 (COG0326(27)), which was recovered for members of the mesophilic groups. This could suggest diversity in the precise mechanisms of high temperature growth adaptation for thermophiles.

Thermotolerant strains have higher maximum growth temperature limits than their mesophilic counterparts and this was reflected in all studied genomic compositional features, with exception for the genome size. Moreover, such phenotypic difference was reflected in the recovery of several COGs specific to each growth temperature class. The most notable COGs distinguishing mesophiles from thermotolerants (shared by a large number of mesophiles, while sufficiently absent among both thermotolerants and thermophiles), fell within the categories related to iron transport and metabolism (Table 3-COG1178(36) and COG1528(37)). For thermotolerants, COGs underlying functions related to the modification of molecules by transfer of acetyl groups (COG2163(40), COG3424(41) and COG1755(59)) appeared to be the strongest features shared by thermotolerants strains, while sufficiently absent in the other two classes.

Through comparative analysis of mesophilic and thermophilic protein sequence homologs, we identified some proteins with increased in the average content of hydrophobic, aromatic and charged amino acids, or combinations of these features. In particular, the charge content of the GroEL protein, a molecular chaperone involved in protein folding (Zeilstra-Ryalls et al., 1991) could serve as a factor to predict the growth temperature class of endospore-forming *Firmicutes*, since it showed a high correlation of roughly 0.70 with the OGT (the average charge content in the thermophilic group was 28.4%, against 27.1% in the mesophilic one (data not shown)). Other than the GroEL protein, the correlation coefficients for the other proteins studied were always below 0.70. This precludes a role for the amino acid composition in the studied protein data-set, as a suitable biomarker for strain growth temperature preference. The absence of a larger number of proteins displaying significant differences in the composition of the predicted proteins, could be explained by the growing body of data showing that, rather than sequenced-based, protein structural factors might be more important to explain increased thermostability of proteins from thermophiles (Dill et al., 2011; Kumar et al., 2000; Sadeghi et al., 2006; Sawle and Ghosh, 2011; Szilágyi and Závodszky, 2000). In this regard, hydrogen bonds and salt bridges have been consistently shown to increase with the OGT of microorganisms in selected protein datasets (Kumar et al., 2000; Sadeghi et al., 2006; Szilágyi and Závodszky, 2000). In addition, proteins from both mesophiles and thermophiles exhibit high variability in their relative stabilities (Dill et al., 2011), and therefore, it is conceivable that a different protein datasets could yield slightly different results. Furthermore, as observed earlier differences between mesophiles and thermophiles may also be influenced by phylogeny, thus, protein melting temperature may instead be more appropriate to measure protein thermostability, and perform optimal growth temperature predictions (Kumar et al., 2000). Various external

factors, including divalent cations and co-factors in metabolic enzymes, may also contribute to protein stabilisation (Amelunxen and Murdock, 1978; Sterner and Liebl, 2001), influencing the fate of certain proteins in cells thriving at high temperatures.

Genomic biomarkers of spore heat-resistance phenotype

The analysis of spore *D*-values across species showed that formation of high heat-resistant spores is independent of the growth temperature preference of strains. Nevertheless, the most heat-resistant spore-formers registered thus far are thermophiles, specifically *D. kuznetsovii*, followed by *M. thermoacetica* and *T. thermosaccharolyticum* strains (Byrer et al., 2000; Xezones et al., 1965). As expected, genomic base compositional features which were found to have a high correlation with the OGT, showed lower correlation with spore log$D_{110°C}$, and vice-versa. A remarkable exception was seen for the GC-content of the 5S rRNA gene, of which correlation coefficient was similar for both strains in the MDS-OGT and MDS-D. The 5S rRNA is a small gene of about 120 nucleotides, of which secondary structure is highly conserved in both prokaryotes and eukaryotes (Szymanski et al., 2002). In prokaryotic ribosomes, the 5S rRNA binds the ribosomal proteins L5, L18 and L25 (Moore, 2001). Its putative contribution for spore heat-resistance, if any, is not clear. Although possessing a high correlation with the spore log$D_{110°C}$, as assessed by MLR, its predictive capacity can be debated and the selection of this gene as a biomarker of spore heat-resistance will require further studies.

On the contrary, the GTM approach did not yield any gene specifically linked to high heat-resistant spore phenotype. This is in agreement with earlier findings that strains clustering on the basis of shared dominant genomic fragments generated by AFLP do not necessarily result in strain segregation according to the heat-resistance of their derived spores (Lima et al., 2011b). On the other hand, assuming that phenotypes are found to be related by genes shared qualitatively (Snel et al., 1999), it could be expected that a number of gene categories would emerge as a common denominator for strains forming spores of high heat-resistance, in parallel to what was found for the group of strains forming spores of low heat-resistance.

Another remarkable finding in this study was the absence of a strong relationship between spore heat-resistance and protein compositional features. These findings contradicted our initial hypothesis that a possible factor underlying spore differential heat-resistance could be related to a higher thermostability of spore proteins. As discussed for the trends in the MDS-OGT, it is conceivable that this simply signifies that rather than the primary structure, the secondary structure should be inspected for the observation of trends related to thermostability of proteins in thermophiles and heat-resistant spore-formers. Consequently, the hypothesis that high heat-resistant spore phenotype is independent of an increased protein thermostability cannot be completely discarded at this point.

Spore inactivation has been demonstrated not to occur as a result of DNA damage, oxidative stress or the inability of the spores to synthesize heat-shock proteins upon subsequent germination and outgrowth in *B. subtilis* spores (Fairhead et al., 1993; Melly and Setlow, 2001; Setlow and Setlow, 1994; Setlow and Setlow, 1998). More recent research, provided solid

evidence that spore killing takes place as a result of the denaturation or inactivation of one or more critical spore enzymes, and possibly metabolic enzymes in spores of *B. subtilis*, *B. cereus* and *B. megaterium* (Coleman et al., 2007). Spore proteins are protected against damage in the spore core, primarily due to substantially reduced water content, when compared to the original content in the vegetative state (Setlow and Johnson, 2007).

Overall, the findings in this work could imply that the physicochemical composition and ultrastructural dimensions of the spore in its dormant state, might be the most important determinants of spore heat-resistance. If this is the case, one of the priorities in follow-up studies would be to determine spore physicochemical and ultrastructural factors known to influence spore heat-resistant phenotype for *D. kuznetsovii*, *M. thermoacetica* and *T. thermoanaerobacterium* strains and compare these factors with that of spores of other heat-resistant spore-forming strains. Simultaneously, considering that a number of proteins were found in common in strains forming spores of low heat-resistance, it is plausible to think that the absence of a critical target protein in spores of heat-resistant spore-formers could underlie their superior resistance.

Further research may provide more information about this question by (i) performing deletion mutants in the identified non-essential COGs for heat-sensitive spore-forming strains; and (ii) by undertaking GTM and protein compositional feature analysis for a collection of strains within the same species and which display differences in the spore heat-resistant phenotype. The reasoning for this is that by keeping variables, such as strain phylogenetic distance, and respiratory metabolism, constant, a larger number of shared genes that would have the potential of being related to the spore heat-resistant phenotype would emerge. Candidates for these study should include strains of *B. subtilis*, since it is a bacterium amenable to genetic manipulation (Dubnau, 1991) and has a large stock of mutant strains which can be quickly screened for spore heat-resistance (Flórez et al., 2009).

In conclusion, this study enhances our knowledge about factors related to spore heat-resistant phenotype and growth temperature preferences in endospore-forming *Firmicutes*, by providing a number of leads which have the potential of explaining those phenotypes. In particular, the determination of the 16S rRNA and genome GC-contents, implemented in the MRL model, might provide adequate approximations for the OGT in routine studies for sequenced endospore-forming *Firmicutes*.

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Supplemental files

Additional files can be accessed at https://docs.google.com/open?id=0B9Gk5JHOac3_ZHJWUTNCN2FpUGs

Chapter 9

General discussion and future perspectives

General discussion

Introduction

Cocoa bean fermentation has been studied for more than a century for its importance in the formation of the essential precursor compounds of the cocoa flavour. These precursors react together during the industrial step of cocoa bean roasting and are responsible for the typical taste and flavour of chocolate. Although the quality of commercial cocoa beans depends on the combination of multiple factors, including the type of plant population, climate and soil conditions, cocoa bean fermentation plays the most important role.

Cocoa bean fermentation is attained by the action of different groups of microorganisms which develop spontaneously and in a largely uncontrolled manner on the cocoa bean pulp. At the start of this PhD project, the knowledge regarding the type of ecological succession and relevance of specific microbiological groups for cocoa bean quality was scattered and to a certain extent fragmentary. In the first part, the state of the art of cocoa fermentation literature was reviewed and the data regarding the microbial ecology of cocoa bean fermentations conducted across the world was critically evaluated. The primary objective was to interlink post-harvest processing practices and characteristics of the fermenting microbiota, with the quality of commercial cocoa beans obtained. This literature survey resulted in the identification of a number of research needs. These research needs are relevant within a framework that aims at providing a basis for consistent implementation of agricultural and operational measures to improve the quality of commercial cocoa beans.

Bacterial spores are resistant cell types, which may survive processing and preservation methods in the food industry. Indeed, these are still considered one of the most important challenges in food preservation (Smelt et al., 2008). Their presence in commercial products is a concern for food processors, since it may affect product shelf-life, quality and consumer health. The second part of this thesis focused on the microbiota composition and on the occurrence, diversity, and properties of heat-resistant spores in cocoa bean-derived products, such as cocoa powder. Cocoa powder finds application as an ingredient in heat-preserved drinks, namely Ultra-High Temperature (UHT) treated chocolate milk. Therefore, the presence of highly heat-resistant spores in cocoa powder, with the potential of surviving downstream processing treatments, challenges the stability of those final commercial products. In particular, *Bacillus subtilis* had been implicated in spoilage of chocolate milk drinks, but this association was not well documented. Following a “bottom-up” chain approach (from cocoa powder to cocoa beans), the occurrence of Thermoresistant Spores (ThrS; spores which survive at least a heat treatment of 100°C for 10 min) was assessed; and the wet heat-resistance (hereafter referred to as heat-resistance) of their derived spores was characterised *in situ* and *in vitro*. The level and diversity of ThrS were evaluated against the level and diversity of general microbiological groups, and compared with the level of Total Spores (TS; spores which survive a heat treatment of 80°C for 10 min). Following the isolation of ThrS, a selection of strains was characterised for key spore properties, specifically spore heat-resistance, physicochemical composition,

ultrastructure and germination. Motivated by these experiments, an approach was devised to search for genomic biomarkers of spore heat-resistance.

In the following sections the most important findings obtained in the course of this thesis project will be discussed. Finally, future perspectives in the field of cocoa fermentation science and spore research will be presented.

High quality commercial cocoa beans can be obtained by a reduced number of key players developing during the fermentation

In chapter 2, a comprehensive comparison of fermentations conducted across producing countries was performed. A core predominant microbiota comprising yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) was identified, independently of the farm and cocoa fermentation method. Species which appeared to be indigenous to cocoa bean fermentations were *Hanseniaspora guilliermondii* (anamorph *Kloeckera apis*), *Issatchenkia orientalis* (synonym *Pichia kudriavzevii*) and *Saccharomyces cerevisiae* in the yeasts group; *Lactobacillus fermentum* and *L. plantarum* in the LAB group; and *Acetobacter aceti* and *A. pasteurianus* within the AAB group. An important conclusion of the survey in chapter 2 was that although cocoa bean fermentation takes place in the form of a microbial succession, where microbial groups exhibit metabolic interdependence, a well-defined succession does not take place across countries. This is contrary to what had been initially described as a characteristic of cocoa bean fermentations (Schwan and Wheals, 2004). Therefore, it is probably more correct to refer to “microbial functionality”, or even “microbial activities” as has been recently proposed (Lefeber et al., 2012). Microbial activities during cocoa bean fermentation appear to be primarily determined by the characteristics of the cocoa bean pulp (acidity, the total amount of fermentable sugars), the fermentation method (e.g. heaps or boxes), the size of the fermenting mass, the temperature achieved in the fermenting mass and whether turning is or not performed. In chapter 2, the impact of these variables for differences in the temporal distribution of the identified core microbiota across fermentations was highlighted.

After the review in chapter 2 had been accepted for publication, several publications dealing with cocoa bean fermentations in Ghana, Ivory Coast, Brazil, Malaysia and Ecuador, became available (Daniel et al., 2009; Garcia-Armisen et al., 2010; Lefeber et al., 2011a; Lefeber et al., 2012; Papalexandratou et al., 2011a; Papalexandratou and De Vuyst, 2011; Papalexandratou et al., 2011b; Papalexandratou et al., 2011c). These investigations confirmed the aforementioned species of yeasts, LAB and AAB as dominant in the course of cocoa bean fermentations. Furthermore, other species of *Hanseniaspora* (specifically *H. opuntiae* (Daniel et al., 2009; Papalexandratou and De Vuyst, 2011)), *Leuconostoc pseudomesenteroides* (Papalexandratou et al., 2011a; Papalexandratou et al., 2011b), *Fructobacillus* spp. (Lefeber et al., 2011a; Papalexandratou et al., 2011b; Papalexandratou et al., 2011c), *A. ghanensis* and *A. senegalensis* appeared to be important for fermentations in Ghana (Camu et al., 2007; Camu et al., 2008b; Garcia-Armisen et al., 2010).

The recent studies also helped to further elucidate the role of other microbial species, of which beneficial contribution for cocoa bean fermentation was still controversial. These include members of endospore-forming species of the genus *Bacillus*. These members have been typically described in fermentations in Brazil and more recently in Ivory Coast (Ouattara et al., 2008; Ouattara et al., 2010; Papalexandratou et al., 2011a; Schwan et al., 1986). A role in the production of desirable pyrazines (tetrametylpyrazines) had been hypothesised (Zak et al., 1972) for *Bacillus* members, while an important function in pulp degradation was claimed, following demonstrations of the capacity of production of pectinolytic enzymes *in vitro* (Ouattara et al., 2008; Ouattara et al., 2010). On the other hand, Schwan (1998) making use of defined starter cultures applied to sterile cocoa beans, demonstrated that it was possible to produce high quality commercial cocoa beans in the absence of proliferation of *Bacillus* species. This outcome was corroborated recently (Lefeber et al., 2011b; Lefeber et al., 2012). This suggests that *Bacillus* species, moulds and some *Enterobacteriaceae* species which were also reported at early stages of fermentations in Ecuador and Brazil (Papalexandratou et al., 2011b; Papalexandratou et al., 2011c) are not essential to bring about the changes which make commercial cocoa beans a valuable commodity to produce chocolate. Furthermore, Lefeber et al. (2012) demonstrated that the sole activities of LAB and AAB were not sufficient to lead to commercial cocoa beans of consistent quality. Instead, the combined activity of yeasts, LAB and AAB, which is translated into the specific activities of ethanol production, citric acid conversion and production of lactic and acetic acid, is required. Altoghether, the outcome of fermentation studies making use of defined starter cultures (Lefeber et al., 2011b; Schwan, 1998) supports the idea that high quality commercial cocoa beans can be obtained not only by reduced number of microbial groups, but also by reduced number of strains.

Studies employing starter cultures have shown that these cultures can be important to increase quality consistence (Lefeber et al., 2012; Schwan, 1998), which is still lacking throughout many cocoa producing countries in the world (Fowler, 2009). Although the widespread use of starter cultures is challenging and not foreseeable in the nearby future (Schwan, 1998), starter cultures can be of interest for smaller batch productions aimed at selected market segments, where combinations of yeasts, LAB and AAB strains can be exploited to produce commercial cocoa beans with exquisite (volatile) flavour compounds. Indeed, besides pyrazines, other flavour attributes such as 'fruity' and 'floral', which have a microbial origin, are considered important attributes for 'speciality' commercial cocoa beans (Chapter 3) and opportunities exist to exploit cocoa bean fermentations to maximise these attributes.

Another recent publication was focused on the characterisation of organic farm fermentations in Brazil (Papalexandratou et al., 2011c). In chapter 2 it was discussed that an interesting topic for future research would be to compare the fermentation profile of an organic farming system with that of a conventional one. This could be of interest due to differences in the agricultural practices in the two systems. The research by Papalexandratou et al. (2011c) revealed an unexpectedly limited diversity of microbial groups (predominantly of *L. fermentum* and *A. pasteurianus*, within the LAB and AAB groups, respectively) in fermentations from an

organic farming system in Brazil, which was associated with the production of high quality cocoa and chocolate. The microbial community in that fermentation was less diverse than the one from a conventional farming system within the same production region (Papalexandratou et al., 2011a). The authors concluded that the operational practices which are based on the separation of healthy and infected fruits or beans, together with the method and duration of the fermentation, were determinant factors influencing the course of the fermentation process and, ultimately, cocoa bean quality (Papalexandratou et al., 2011a; Papalexandratou et al., 2011c).

High acidity is considered a defect in commercial cocoa beans (chapter 3; Fowler (2009)) and in chapter 2, the implications of this characteristic were addressed and several factors which should be investigated further, were discussed. The study by Papalexandratou et al. (2011a), comparing heap and box fermentations in Ivory Coast and Brazil, presented evidence that excessive acidification in commercial cocoa beans in Brazil was not dependent on the fermentation method (fermentation in heaps *versus* boxes). Instead, high acidic cocoa beans were correlated with the use of unripe and diseased pods, which led to the development of a large diversity of homo- and heterofermentative LAB and a two-fold increase of lactic acid, when compared to levels quantified in Ivorian fermentation. Furthermore, Papalexandratou et al. (2011a) registered a too early and dominant proliferation of AAB in box fermentations in Brazil, which explained the higher levels of acetic acid compared to those in cocoa beans in Ivory Coast. This signifies that the perceived acidic taste of commercial cocoa beans could be due to the combination of both lactic acid and acetic acid, resulting from an unbalanced (atypical) development of the acidifying microbiota.

An additional point discussed in chapter 2 concerned the importance of using advanced culture-independent approaches, such as metagenomics, in an attempt to reveal microbial species which could have not been detected by culturing based-methods or culture-independent approaches such as PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE). Notably, cloning and sequencing of the 16S rRNA gene of total genomic DNA extracts originating from fermentations in Ghana and in Brazil, which had been studied earlier by using culture-base methods and PCR-DGGE, only revealed additional species of *Gluconacetobacter*, thus indicating that the large majority of species had been already been identified in the studied fermentation sites (Camu et al., 2007; Garcia-Armisen et al., 2010). In a recent pioneering metagenomics study of cocoa bean fermentation in Brazil, the authors concluded that such approach revealed wider diversity of Ɣ-*Proteobacteria* and yeasts species than had been found for the same samples analysed by culturing and PCR-DGGE approaches (Illeghems et al., 2012).

Taken together it can be concluded that the long way cocoa fermentation science has gone is starting to pay-off, as initiatives aimed at supplying commercial cocoa beans of consistent quality to the markets are started to be established. At the same time diversification of the range of commercial cocoa bean products (‘speciality cocoa’), benefits the livelihood of the farmers and this has direct consequences for the long-term commitment to produce high quality cocoa (chapter 3).

B. subtilis subsp. *subtilis* are ultimate survivors of the cocoa powder production chain

Strains of *B. subtilis* complex and of *B. licheniformis* were the predominant thermoresistant species found in commercial cocoa powder samples (Chapter 4). However, strains of *B. subtilis* complex were notable for exhibiting the highest heat-resistance *in situ* and *in vitro*. In particular, spores of *B. subtilis* subsp. *subtilis* strain M112 showed the highest heat-resistance, with a *D*-value at 130°C ($D_{130°C}$) of 11 s being measured after resporulation under laboratory conditions. The extrapolation of the determined $D_{130°C}$ to 135°C, the typical minimum UHT temperature (Anon., 1992), results in $D_{135°C}$ of 1.8 s. Considering that UHT treatments for milk may last 1-8 s at 135°C (Anon., 1992; Scheldeman et al., 2006), survival of spores of M112 in milk could take place if milk would be treated during less than 1.8 s. In practice, survival of spores upon application of a given heat treatment would depend on the initial level and heat-resistance characteristics of the spores, as determined by previous history of the spore in the chain, namely the sporulation conditions in the original ecological niche.

The $D_{130°C}$ for spores of M112 was comparable to that for spores of the thermophile *Geobacillus stearothermophilus* C953, for which a $D_{130°C}$ of 9.6 s was reported (Huemer et al., 1998). Yet, both $D_{130°C}$ were lower than that for spores of *B. sporothermodurans* strain J16, for which a $D_{130°C}$ of 42.2 s was measured (Huemer et al., 1998). So far, strains of *B. sporothermodurans* are the ones which have been demonstrated to form the most heat-resistant spores within the genus *Bacillus*.

Altogether, the gathered data showed that cocoa powder can indeed be a vehicle of highly heat-resistant spores. However, it was also clear that high heat-resistance spore formation is not an ubiquitous property among *B. subtilis* complex strains.

Sampling of a cocoa powder production line at six different processing stages from cocoa nibs (shelled and broken cocoa beans) to cocoa powder (Chapter 5) revealed that spores of *B. licheniformis* and *B. subtilis*, were the dominant ThrS surviving an isolation heat treatment of 100°C for 10 min at all stages. In particular, the observation that spores of *B. licheniformis* and *B. subtilis* were predominant ThrS also in cocoa powder was in agreement with the outcome of the investigation in chapter 4. Moreover, it was demonstrated in chapter 5 that spores of *B. subtilis* complex, including two strains confirmed as *B. subtilis* subsp. *subtilis*, isolated at the stages of 'After Alkalising', 'After Roasting' and 'Cocoa Powder', were the ones of which spores showed the highest heat-resistance at 110°C for 5 min. Considering that subsequent steps following roasting do not include a heating step, ThrS present at the stages of 'After Roasting' may be present in cocoa powder. Therefore, the combined results of chapters 4 and 5 allowed the conclusion that owing to the high heat-resistance of *B. subtilis* complex members, in particular subspecies *subtilis*, these are ultimate survivors of the cocoa powder production chain. Consequently, this implies that the presence of *B. subtilis* complex strains in cocoa powder needs to be taken into account to ensure the stability of cocoa powder-derived products, such as UHT treated drinks.

In the present project it was shown that ThrS (100°C-10 min) were already present in the raw material, cocoa nibs, but these constituted less than 0.5% of the population of TS (Chapter 5). Reports concerning the occurrence of ThrS in spices and in other ingredients for the manufacturing of soups as well as in samples collected from a dairy environment (fodder, raw milk and milking equipment) have been published by Oomes et al. (2007), Scheldeman et al. (2005) and van Zuijlen (2011). These investigations showed that ThrS surviving heat treatments of 100°C for 15-30 min ranged from levels below the detection limit up till levels corresponding to 100% of that of TS (80°C 10-15 min). These results emphasise the large discrepancy of spore heat-resistance in nature, that ThrS are not restricted to a particular ecological niche and that these can be found in diverse food chains. Overall, this also implies that given the processing conditions in the cocoa bean production chain, it is very difficult to control the type of microbiota in cocoa beans and it can be expected that these will most likely be a source of ThrS. Still, it is of interest in future studies to search for possible ecological niches associated with spores of high heat-resistance, as this knowledge may have important implications for ThrS control in the cocoa chain.

An intriguing aspect of the overall thermoresistant microbiota of the cocoa powder production chain is the extremely low number of true thermophilic species, such as those from the genus *Geobacillus*. Of 151 thermoresistant strains isolated in the course of this work (Chapter 4 and 5), only 2 were potentially closely related to species of the genus *Geobacillus*, as assessed by sequencing of the 16S rRNA gene. Instead, thermotolerant strains of *B. licheniformis* and of *B. subtilis* complex were systematically isolated from plates incubated at 55°C. Although *Geobacillus* spp. are infrequently reported in cocoa bean fermentations (Chapter 2), these were found in fermentations in Trinidad (Ostovar and Keeney, 1973) and Brazil (Schwan et al., 1986; Schwan, 1998), and were reported in both raw and roasted cocoa beans in a study by Barrile et al. (1971). Thermophilic species of the genus *Geobacillus* form spores of high heat-resistance (Beaman and Gerhardt, 1986; Head et al., 2008; Huemer et al., 1998; Periago et al., 1998; Prevost et al., 2010; Sevenier et al., 2012), and for this reason, it could be expected that spores of this genus would be an important fraction of the thermophilic population of ThrS in cocoa samples, including cocoa powder. ThrS belonging to *Geobacillus* spp. surviving heat treatments of 100°C for 30 min, were isolated from fodder and green crop in the study by Scheldeman et al. (2005), and have been frequently associated with milk powder (Burgess et al., 2010), commercial batches of gelatin (De Clerck et al., 2004c) and sterilised canned vegetables (Sevenier et al., 2012). The poor representation of *Geobacillus* species in the cocoa chain among the population of ThrS remains an intriguing question which could be a subject of attention in the future by assessing the need of using isolation media supplemented with growth factors, such as vitamins (De Vos et al., 2009; Scheldeman et al., 2005) or higher incubation temperatures for effective strain isolation.

Natural isolates of *B. subtilis* subsp. *subtilis* are excellent models to unravel the mechanisms of spore differential heat-resistance

The study in chapter 6 aimed at understanding the reason why spores of different strains display dissimilar heat-resistance. This question was tackled by comparing physicochemical and ultrastructural spore properties in 5 strains of *B. subtilis* subsp. *subtilis*, well characterised at the molecular level, and of which spore $D_{110°C}$ spanned a range from 0.10 min up to 106 min. It was found that the average content of manganese, magnesium and calcium, followed by the ratio between the spore core and sporoplast dimensions were the factors most highly correlated with the spore heat-resistant phenotype. Manganese was proposed as a physiological biomarker for spore heat-resistance in *B. subtilis* subsp. *subtilis*. In practical terms, this means that the prediction of spore heat-resistance could be performed using an assay for determining manganese in spore lysates by inductively coupled plasma-atomic emission spectroscopy, which allows for higher throughput and is substantially faster than the use of plate-count based techniques for the comparison of spore D-values.

The importance of calcium, manganese and magnesium as well as reduced spore core-sporoplast ratio, had been found before to constitute critical factors underlying spore differential heat-resistance across strains of *G. stearothermophilus*, *B. cereus*, *B. subtilis* and *B. megaterium* (Beaman et al., 1982; Beaman and Gerhardt, 1986; Bender and Marquis, 1985). We showed that the importance of these factors to explain a high heat-resistant spore phenotype remains at intraspecies level.

Interestingly, although prokaryotes and also eukaryotes, accumulate iron in levels substantially higher than those of manganese (Eide et al., 2005; Outten and O' Halloran, 2001; Rosenfeld et al., 2010) in bacterial spores the manganese-iron ratio is inverted and manganese levels are substantially higher than those of iron (Chapter 6). The Mn-Fe ratio in this work varied between 18, for spores of A163 which formed the highest heat-resistant spores, to only 6 for spores of strain M5, of which spores were the most heat-sensitive (Chapter 6).

In spores of *B. subtilis* that accumulate high levels of dipicolinic acid, Mn-dipicolinic acid complexes were shown to provide protection against protein oxidative damage (Granger et al., 2011). However, oxidative damage is not a reason of spore killing by wet heat in *B. subtilis* spores (Setlow and Setlow, 1998). Unfortunately, the exact mechanism whereby manganese could contribute to higher heat-resistance in spores, besides a possible role in the decrease of spore core water by Mn-dipicolinic complexes (Setlow, 2006), could not be concluded from this study. The precise role of this mineral for spore differential heat-resistance remains to be established.

In chapter 7, the germination capacity of *B. subtilis* subsp. *subtilis* spores, with special reference to highly heat-resistant spores, was studied. It was shown that for spores of all tested strains, including those of a heat-sensitive spore-forming strain, sublethal heat-shock activation was a requirement to induce appreciable germination. Such requirement is an indication of spore dormancy. In addition, the observation that for spore crops of all strains, there were conditions that did not induce complete spore germination within the population,

meant that spore crops were also characterised by what is known as superdormant spores (Ghosh et al., 2009). As discussed in chapter 7, one of the factors found to be correlated with spore superdormancy was a reduced spore core water content, which was in turn related to higher spore heat-resistance and higher requirements for heat shock activation to induce spore germination (Ghosh et al., 2009). This could possibly explain the extreme superdormancy for spores of M112 when compared to that of the other tested strains, since the work in chapter 6 showed that spores of M112 had the lowest average core water content.

Other factors besides reduced core water content were found to be related to spore superdormancy. This includes a reduced number of germination receptors in the spore inner membrane (Ghosh et al., 2012; Yi et al., 2011) and processes related to the extent of mRNA degradation during spore storage, under a mechanism not yet fully understood (Segev et al., 2012). It is plausible that in situations where the core water content in highly heat-resistant spores is not sufficiently low, such as found for spores of *B. subtilis* strain A163 (chapter 6), the number of germinant receptors and/or the processes in the spore related with the degradation of the mRNA might play a preponderant role contributing to poor spore germination.

Although spore superdormancy in *B. subtilis* (strain PS533), *B. megaterium* (strain QM B1551) and *B. cereus* (strain T) was shown to be a characteristic occurring in spores exhibiting higher heat-resistance (Ghosh et al., 2009), a relationship between spore superdormancy and heat-resistance was absent across a collection of 22 natural isolates of *B. cereus* strains (de Vries, 2006). This implies that the exact factor(s) playing a role for spore superdormancy may vary not only at intraspecies level, but also at interspecies level. In sum, the problem of spore superdormancy appears to be complex and more systematic studies are required to understand the exact circumstances leading to the occurrence and breaking of spore superdormancy across strains.

The occurrence of superdormant spores has important implications for the control of spores in the food industry. On the one hand, it precludes the use of spore inactivation strategies based on induction of germination, followed by milder processing treatments, and, on the other hand, it signifies that food preservation regimes may have to be more severe to effectively inactivate such spores. The occurrence of superdormant spores, with substantially higher heat-resistance than the majority of the spore population has also implications for endospore-formers detection. Currently, TS are detected by heat-shock activation at 80°C for 10 min. Such treatments are adequate to detect spores of intermediate and low heat-resistance, but may fail to detect spores of substantially higher heat-resistance. This indicates that higher heat treatments are required in parallel to the standard treatment of 80°C-10 min to account for spores of all heat-resistance capacities. In chapter 7 it was found that in the case of highly heat-resistant and superdormant spores of strain M112, even heat treatments of 100°C for 30 min were not sufficient to induce complete germination. This shows that more work is needed to identify an optimal temperature interval leading to maximal spore activation in strains prone to form a large number of superdormant spores, while at the same time ensuring absence of spore inactivation.

The phenomenon of high heat-resistance spore formation has been mostly tackled from a physiological perspective, which pertains directly to the spore composition and structure. In chapter 8, a strategy was executed in order to identify putative genomic determinants of spore heat-resistance. This was undertaken across species of the *Firmicutes* phylum, by performing a meta-analysis of genomic and phenotypic data. It was assessed whether a high heat-resistant spore phenotype could be explained on the basis of genome compositional features, gene content or amino acid composition of spore predicted proteins. Since a number of endospore-forming *Firmicutes* are also thermophiles, the study was extended to assess for putative genomic determinants of thermophilism.

This approach did not yield any genomic biomarker of spore heat-resistance. On the contrary, features related to genome base composition, gene content and protein amino acid composition set apart strains with different growth temperature preferences. Furthermore, strains forming spores of low heat-resistance shared a number of genes which were mostly absent in strains forming spores of high heat-resistance. The first major outcome from this study was the confirmation of the independence of putative genomic determinants of thermophilism from that of spore high heat-resistance, as had already been noted in chapters 4 and 6. The second major conclusion was that considering that a common genomic denominator was not present across strains forming spores of high heat-resistance, this could mean that the spore physicochemical and structural properties are the primary determinants of spore heat-resistance. For this reason, follow-up studies focused on the characterisation of spore composition and ultrastructure of spores from strains of *Desulfotomaculum kuznetsovii*, *Moorella* thermoacetica and *Thermoanaerobacterium thermosaccharolyticum,* which are the most heat-resistant spore-formers described so far (chapter 8), could give an important contribution to solving the puzzle of spore high heat-resistance.

Future studies aiming at correlating spore heat-resistance with putative spore factors within phylum level can be sharpened by assays which do not depend on the recovery conditions of the spore, eliminating variables such as nutritional or oxygen requirements. An assay by Kort et al. (2005) showed that spore heat-resistance can be deducted from the minimum temperature at which maximal release of spore dipicolinic acid occurs. Such assay has the advantage of being faster than the classical determination of spore heat-resistance by culture-based methods.

As also discussed in chapter 8, another way of looking to the problem of spore differential heat-resistance is to search for putative genomic biomarkers among strains that are phylogenetically more related, such as strains belonging to the same species. Here, such an analysis was undertaken by performing Gene-Trait Matching (GTM) among the sequenced strain of *B. subtilis* subsp. *subtilis* strain 168 and our own sequenced strains of *B. subtilis* subsp. *Subtilis*, strains M112 and M1. Figure 1A presents a Venn diagram which provides an overview of the number of shared and unique genes for these 3 strains. The two natural isolates, strains M112 and M1 shared a larger number of genes than with the model laboratory strain 168. By zooming into the functional category of Orthologous Groups (OGs) shared by strain M112 and

M1 and absent in strains 168 (N= 261 genes) (Figure 1B and 1C), it is observed that the large majority of shared OGs between M112 and M1, fall into categories which have been poorly characterised ('general function prediction only' or 'function unknown'). The annotated COGs fell into a very restricted category and sub-categories, consisting mainly of transposases and genes involved in amino acid transport.

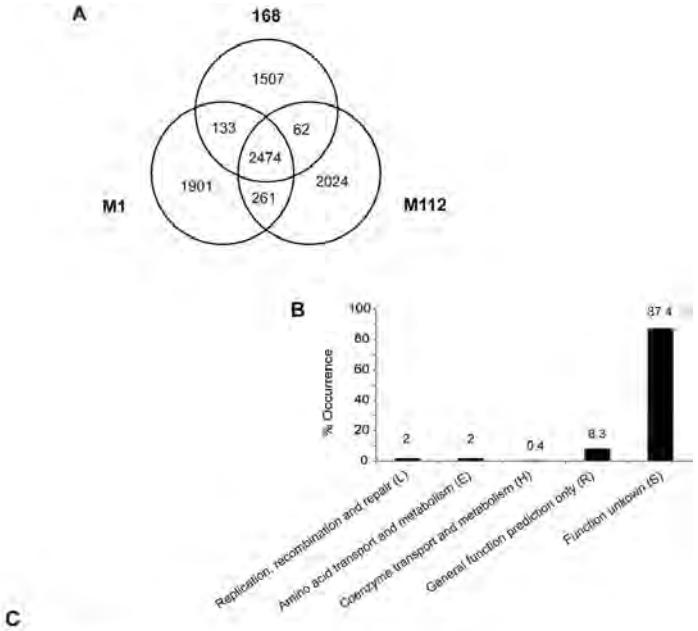
The genes in common for strains M112 and M1, are potential candidate biomarkers for high heat-resistant spore phenotype but such genes are not necessarily implicated in a high heat-resistant spore phenotype. The genes in common between strains M112 and M1 could be further reduced by sequencing other highly heat-resistant and heat-sensitive spore-forming strains.

Future perspectives

Significant advances have been achieved during the last decade in the area of cocoa fermentation science. A particularly remarkable milestone consists of the finding that high quality cocoa beans could be achieved with a reduced number of key species and strains (Lefeber et al., 2011b; Lefeber et al., 2012; Schwan, 1998). The use of starter cultures for cocoa bean fermentation constitutes a promising approach to ensure consistence in quality. It can be expected that the final choice of strains to be used in each country will benefit from screening of strain combinations to enhance certain quality traits, such as the presence of exquisite compounds in the volatile fraction of the cocoa flavour (chapters 2-3).

Currently, great opportunities exist to extend the use of metagenomic approaches to study complex fermentations, such as that of cocoa beans. With the prices of next generation sequencing techniques dropping every year, the availability of a large range of sequencing platforms and improved pipelines to handle the large datasets generated by metagenomics (Desai et al., 2012; Scholz et al., 2012), such approaches are becoming more and more affordable to a larger number of laboratories. New developments in the field of metagenomics include the access to the microbial community gene expression profile, by sequencing of environmental transcripts (metatranscriptomics), but also access to microbial community activities, by characterisation of whole protein profiles (Su et al., 2012). The combination of metagenomics, metatranscriptomics and metaproteomics, in conjunction with metabolomics, will lead to a comprehensive picture of cocoa bean fermentations, including a fine mapping and interlink between microbial diversity and group functionalities, as well as reveal interactions among different microbial groups.

Another interesting aspect about cocoa is its nutraceutical potential. Cocoa beans have the highest polyphenol (flavanol) content of all foods (Vinson et al., 1999), but as a result of the fermentation this content decreases significantly (chapter 2). Although good quality commercial cocoa beans cannot be produced by too short fermentation, a relatively shorter fermentation which could still allow for the sufficient development of cocoa flavour precursors



C

Orthologous group (OG)		Description
		Information storage and processing
L	**Replication, recombination and repair**	
	COG2801	Transposase and inactivated derivatives
	COG2963	Transposase and inactivated derivatives
	COG3666	Transposase and inactivated derivatives
	COG5655	Plasmid rolling circle replication initiator protein and truncated derivatives
	COG4644	Transposase and inactivated derivatives, TnpA family
		Metabolism
E	**Amino acid transport and metabolism**	
	COG0410	ABC-type branched-chain amino acid transport systems, ATPase component
	COG0559	Branched-chain amino acid ABC-type transport system, permease components
	COG0683	ABC-type branched-chain amino acid transport systems, periplasmic component
	COG0747	ABC-type dipeptide transport system, periplasmic component
	COG4177	ABC-type branched-chain amino acid transport system, permease component
H	**Coenzyme transport and metabolism**	
	COG2091	Phosphopantetheinyl transferase

Figure 1. *In silico* mining for genes in *B. subtilis* subsp. *subtilis* strains 168, M1 and M112. (**A**) Venn digram showing the number of shared and unique genes among the strains. (**B**). Representation of the distribution of orthologous groups (OGs) shared between strains M1 and M112 (N=261). (**C**) List of Cluster of Orthologous groups (COGs), shared between strains M1 and M112, within the sub-functional categories 'Replication, recombination and repair', 'Amino acid transport and metabolism' and 'Coenzyme transport and metabolism'.

and the desirable components of the volatile fraction of the flavour is of high interest for the development of chocolate with added nutraceutical properties. It is already possible to find chocolate in the market which has been fermented in a way which preserves a substantially higher content of total polyphenols. Commercial cocoa beans with these characteristics are still produced in a very small scale, but in the future such production process could be extended and optimised across producing country.

Considering the current applications of *Bacillus* species in a wide number of scientific domains it is clear that fundamental research into *Bacillus* taxonomy, spore formation, spore heat-resistance and spore germination are of practical utility. In particular, spore heat-resistance and germination phenotypes are key bacterial spore properties for the food industry. The ideal situation would be to explain differences in these two spore properties among strains on the basis of simple changes, for instance, changes in a given DNA sequence or due to the presence or absence of a specific gene. However, both spore heat-resistance and germination are complex cellular processes, since they depend on multiple factors.

Although the analysis of spore physicochemical composition and ultrastructure revealed manganese as a biomarker for *B. subtilis* subsp. *subtilis* spores heat-resistance, the scientific question regarding factors underlying bacterial spores differential heat-resistance should be

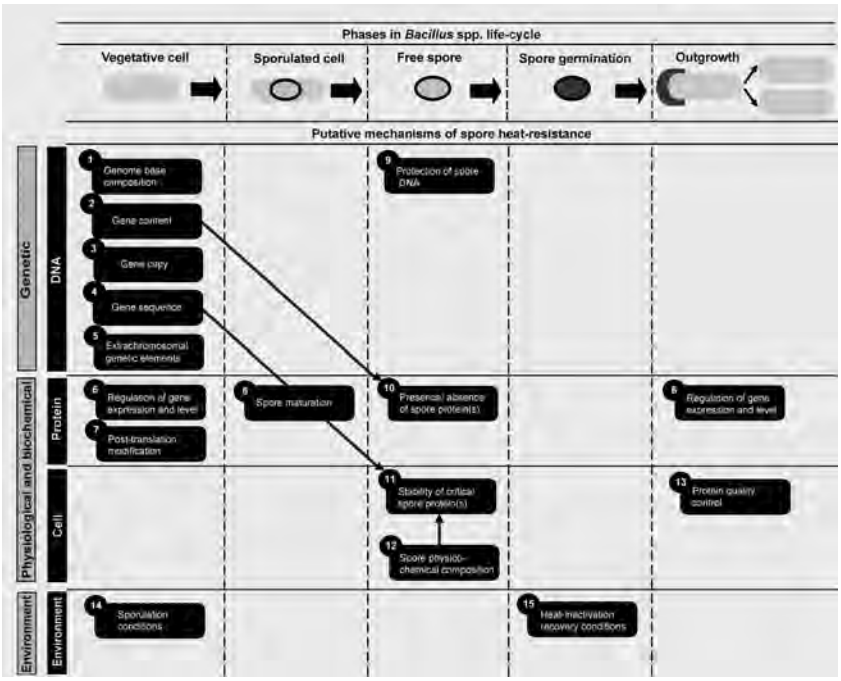


Figure 2. Schematic representation of a framework for putative factors governing *Bacillus* spores differential wet heat-resistance at different levels (DNA, protein, cell and environment). The arrows link related factors. See Table 2 for details about these factors. Source: This work.

Table 1. Discussion of the framework presented in Figure 2 for putative factors governing *Bacillus* spores differential wet heat-resistance

No.	Putative Factor	Description	Evidence so far of implication in spore differential heat-resistance
1	**Genome base composition**	– Consists of features such as whole genome size and genome base composition of whole genome, of open reading frames (ORFs) and ribosome RNA genes	– No This possibility has been appraised in the study in chapter 8
2	**Gene content**	– Pertains to the presence or absence of genes	– Yes Chapter 8
3	**Gene copy**	– Some genes are represented by multiple copies in the genome (e.g. ribosomal RNA genes) – It also includes DNA sequence repeats	– No
4	**Gene sequence**	– Specific sequence of a given gene – Could also include aspects related to the occurrence of Single Nucleotide Polymorphisms (SNPs)	– No
5	**Extrachromosomal elements**	– Includes plasmid-borne, transposon and phage related phenotypes	– No
6	**Regulation of gene expression and level**	– Respects processes whereby gene regulation takes place in the cell, including DNA methylation and processes involved in phenotypic variation	– No Transcriptomic analysis where gene expression of *B. subtilis* strain PS832 (heat-sensitive sporeformer) was compared with that of strain A163 (heat-resistant sporeformer), revealed a number of genes differentially expressed (Oomes and Brul, 2004). However direct connection with spore differential heat-resistance has not been established
7	**Post-translation modification**	– Includes processes such as protein phosphorylation, which has a central role for cell regulation and signalling, but many other processes were described (Witze et al., 2007). It also includes processes involved in protein folding into functional proteins (Rutherford, 2003). These processes impact protein activity	– No Entry in sporulation is determined by phosphorylation of Spo0A gene (Dubnau and Losick, 2006). Activation of Spo0A is not synchronous and temporal distribution of spore formation could have implications for subpopulation of spores with different heat-resistant properties

Table 1. Discussion of the framework presented in Figure 2 for putative factors governing *Bacillus* spores differential wet heat-resistance *(continued)*

No.	Putative Factor	Description	Evidence so far of implication in spore differential heat-resistance
8	**Proper spore maturation**	– Maturation of the forespore requires correct transport and deployment of proteins, DPA, minerals, etc. from the mother cell into the developing forespore (Meisner et al., 2008; Setlow and Johnson, 2007). In addition, this factor could include aspects related to the activity and affinity of transporters related for example with differential accumulation of mineral elements	– No
9	**Protection of DNA**	– It is well known that protection of spore DNA by SASPs is essential for spore survival against wet-heat and it is also known that spore killing by wet heat is not due to DNA damage (Fairhead et al., 1993; Setlow and Setlow, 1994). It remains to be established whether factors like extent of DNA compacting are involved in spore differential heat-resistance	– No
10	**Presence/absence of spore protein(s)**	– It has been proposed that the reason why spore killing takes place is due to the inactivation or denaturation of one or more spore protein (Coleman et al., 2007; Coleman et al., 2010). It can be hypothesised that spore heat-resistance could reside in the absence of (a) critical protein(s) – The comparative proteomics study by (Abhyankar et al., 2011) revealed a number of proteins found to be unique to spores of strain A163 (HHR) and which are absent in spores of strain 168 (HS)	– No This possibility has been discussed in chapter 8

Table 1. Discussion of the framework presented in Figure 2 for putative factors governing *Bacillus* spores differential wet heat-resistance *(continued)*

No.	Putative Factor	Description	Evidence so far of implication in spore differential heat-resistance
11	**Stability of critical spore protein(s)**	– Intrinsic protein stabilisation can be hypothesised by analogy to what happens with thermophiles and hyperthermophiles (Sadeghi et al., 2006; Vieille and Zeikus, 2001) – In addition, different extrinsic factors have been discovered as playing a role in protein stabilisation. Cations such as calcium and magnesium have been reported to increase the thermostability of enzymes (Barton, 2005). Sugars, salts and amino acids have also been implicated in protein protection against thermal denaturation (Sterner and Liebl, 2001) – The degree of protein hydration in the spore could also play a role in the spore different heat-resistant phenotype (Sunde et al., 2009)	– No This possibility has been discussed in chapter 8
12	**Spore physicochemical composition**	– This involves factors such as spore mineral content, DPA, water content and ratio of spore core-sporoplast. In chapter 6 we showed that in *B. subtilis* subsp. *subtilis*, manganese could be used as a physiological biomarkers for spore differential wet heat-resistance; while in *Clostridium perfringens* the content of iron and the ratio of spore core-sporoplast would appear to be the strongest candidates for biomarkers (Orsburn et al., 2008)	– Yes
13	**Protein quality control**	– This respects damage repair of spore proteins by heat shock proteins	– There is evidence that heat shock proteins do not play a role in spore heat-resistance (Melly and Setlow, 2001).
14	**Sporulation conditions**	– It respects all variables which affect spore growth and sporulation, such as T, pH and physiological state of the cell	– Yes Reviewed in chapter 1 and studied in chapter 6
15	**Heat-inactivation recovery conditions**	– It respects all variables playing a role in spore apparent heat-resitance	– Yes Reviewed in chapter 1 and studied in chapter 6

placed in a larger perspective. Figure 2 presents a proposal for a working framework summarising putative factors involved in spore differential heat-resistance. Table 2 presents the discussion of the points in Figure 2.

The framework in Figure 2 is divided into genetic factors, physiological/biochemical and environmental factors. In this thesis, a number of these factors were studied, namely factor 1, 2, 12, 14 and 15. Of the studied factors, numbers 2, 12, 14 and 15 showed clear contributions for spore differential heat-resistant phenotype.

A comprehensive understanding of all factors contributing to the spore heat-resistant phenotype is an important step to allow the construction of a mechanistic model. In turn, the mechanistic understanding of spore heat-resistance may lead to the identification of targets for spore inactivation. A mechanistic model would deal with questions of why manganese would be important for a high heat-resistant spore phenotype and the operational fashion it contributes to that phenotype. In this line of reasoning, additional questions to address would be to understand why certain spores accumulate more manganese than others. Such a question could be addressed by characterising the number and activity of (manganese) transporters during forespore maturation (Eisenstadt et al., 1973; Meisner et al., 2012). The framework in Figure 2 could be useful in future studies aimed at identifying further factors involved in the diversity of spore heat-resistance, at both phylum, species and intraspecies level.

Concurrent with the identification of factors underlying spore heat-resistance and subsequent construction of mechanistic models, it is of interest to discover the targets of spore killing. Elegant studies conducted with strains of *B. subtilis*, *B. megaterium* and *B. cereus*, provided evidence that spore killing appears to be linked to the denaturation/ inactivation of protein(s) with metabolic or macromolecular synthesis functions, and that cortex lytic enzymes are not primarily targeted (Coleman and Setlow, 2009; Coleman et al., 2007; Coleman et al., 2010; Setlow and Johnson, 2007). However, the identity of the target protein is not known yet. On the contrary, in *Clostridium botulinum* the observation that subletally damaged spores were recovered in medium supplemented with lyzosyme, points towards inactivation of the cortex lytic enzyme system in the spore (Lund and Peck, 1994; Stringer et al., 1999). In chapter 6, we speculated that due to differences in the exact composition of the spore core, it is even plausible that the exact target(s) of spore killing vary across strains. The identification of the reason behind spore killing is certainly a topic of considerable interest as it may hold means for optimised heat-preservation treatments in the food industry.

As reflected in factor 13, spore heat-resistance is not an absolute spore property, but it is highly dependent on the sporulation conditions. Several studies have demonstrated the importance of variables such as the availability of minerals in the sporulation medium and the sporulation temperature in the modulation of spore heat-resistance and impact for the measured *D*-value (see review in chapter 1). In this work it was observed that although strain M112 formed spores of very high heat-resistance, the measured heat-resistance after strain resporulation under laboratory conditions, was substantially lower when compared

to its original isolation treatment *in situ* (150°C for a total of 10 min, under nonisothermal conditions). The reason for the discrepancy between the measured heat-resistance *in situ* and the measured heat-resistance *in vitro* is not known. It is of interest to explore combination of factors leading to maximal spore heat-resistance and to discover the boundaries of spore heat-resistance (minimum and maximal) as influenced by combination of different levels of modulating factors.

Targetting spore germination as a method for spore control, either by promoting controlled germination or preventing germination, may become more and more important, due to increasing trends towards mild-preservation treatments in the food industry (Abee and Wouters, 1999; Carlin et al., 2000). Such mild-preservation treatments, may not inactivate spores, but only induce sublethal damage (Lund and Peck, 1994; Smelt et al., in press). For this reason, knowledge regarding germination and outgrowth of sublethally damaged spores as function of varying recovery conditions will be instrumental to effectively implement mild-preservation strategies in the food industry.

A common spore behaviour which has been described in studies of spore heat-resistance and germination is the existence of heterogeneity within a single spore population (e.g. Huang et al. (2007) and this work). Heterogeneity constitutes a challenge in predictive modelling, due to increased uncertainty in the estimation of parameters.

Population heterogeneity has been proposed to be a strategy whereby microorganisms increase the chance of survival by adapting to changing environments (de Jong et al., 2011; Dubnau and Losick, 2006). Whether, heterogeneity in heat-resistance and germination phenotype result from isogenic populations (the individuals share the same genomic information) or polygenic populations (the individuals only share parts of the genome) (de Jong et al., 2011) is not well understood yet. Importantly, the occurrence of spore heterogeneity could be related with the reason why it is observed that spores of *B. sporothermodurans* apparently lose their heat-resistance after multiple passages (Huemer et al., 1998). This could simply result from the selection of a cell within the population which forms spores of lower heat-resistance.

The study of spore heterogeneity has been shown to be facilitated by the use of gradient centrifugation (Ghosh, Zhang et al. 2009) and can be also performed by fluorescence-activated cell sorting (FACS) (Abee and Wouters, 1999; Tracy et al., 2010), which are both techniques which allow the separation of subpopulations with different characteristics. Recently, single-cell genomic techniques based on the amplification of DNA directly from individual cells have been developed (Kvist et al., 2007; Lasken, 2007; Raghunathan et al., 2005). These techniques allow sequencing of complete genomes from single cells and in this manner, allow mapping putative genomic differences in the observed individual phenotype. Raman microspectroscopy combined with optical tweezers is being used to assess the composition of individual cells and spores (Huang et al., 2004), and in particular this has proven to be useful explaining differences in heat-resistance properties among individual spores (Huang et al., 2007).

Concluding remarks

The results described in this thesis present an overview of the microbial functionality of cocoa bean fermentation and of the factors determining the quality of commercial cocoa beans. In addition, further progress has been made towards the understanding of the microbiota composition, but principally about the ecology, diversity and properties of heat heat-resistant bacterial spores in the cocoa chain. The combination of phenotypic, genotypic and genomic data allowed the characterisation of a number of thermoresistant isolates of which data are of importance for subsequent stages of downstream processing of cocoa powder derived-drinks. Furthermore, spore compositional and ultrastructural characteristics as well as genomic features were evaluated for their capacity to yield biomarkers of spore differential heat-resistance. This study also yielded a new framework that can be used as a basis for future investigations aimed at contributing to the understanding of the mechanisms of spore differential heat-resistance.

The gathered phenotypic data regarding both spore heat-resistance and germination are important inputs for predictive modelling approaches to contribute to the production of safe and high quality food.

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Summary

The cocoa bean (*Theobroma cacao* L.) production chain includes a number of operations which culminate with the manufacturing of the beloved chocolate as well as a range of other commercial products, including cocoa powder and cocoa butter. The operations in this chain are distinguished into the stages of cocoa post-harvest and of industrial processing. Cocoa bean fermentation constitutes an essential and critical step during the post-harvest processing for chocolate and cocoa powder manufacturing, since it leads to biochemical changes inside the beans that are vital to the development of the precursor compounds of the typical cocoa flavour. These transformations are induced by the combined action of different groups of microorganisms which develop on the cocoa bean pulp. Following the post-harvest stage, 'commercial cocoa beans' undergo a series of defined industrial operations, including roasting, which is the operation whereby the full cocoa flavour is formed. In addition to cocoa flavour formation, the industrial processing operations need to ensure the destruction of (pathogenic) microorganisms, in compliance with microbiological quality guidelines.

Cocoa bean fermentation is a low input process, which is still nowadays conducted in a spontaneous and uncontrolled way throughout the world. Nevertheless, during the last 60 years significant advances have been made regarding the understanding of the complex biochemical reactions which take place during cocoa bean fermentation and the way these reactions impact cocoa bean quality. In this project it was found of interest to critically evaluate the state of the art about cocoa fermentation literature, in order to interlink characteristics of fermentations practiced around the world with the quality of commercial cocoa beans obtained.

Owing to their high heat-resistance, certain bacterial spores may survive industrial processing and may be present in final commercial products. The second part of this project focused on acquiring knowledge about the occurrence, diversity and properties of such heat-resistant spores in cocoa bean-derived products, namely cocoa powder, due to the implication of this ingredient in spoilage incidents of Ultra-High Temperature (UHT) treated chocolate milk drinks.

In chapter 2, fermentations conducted in Trinidad and Tobago, Brazil, Indonesia, The Dominican Republican and Ghana were compared and the mechanisms whereby cocoa flavour precursors and other components of the flavour are formed were reviewed. The following factors of relevance for the quality of commercial cocoa beans were addressed: (i) the impact of the farming system, (ii) the impact of the ripening state of the pods, (iii) the role of microbial interactions on the fermentation profile, (iv) the problem of high cocoa bean acidification, (v) the importance of the volatile fraction of the flavour (vi) and the impact of fermentation on the cocoa butter properties. All these questions are related to the quality of cocoa beans at different levels (economic, qualitative and safety) and ultimately affect farmer's revenues, as discussed in chapter 3. A core microbiota comprising yeasts, lactic acid bacteria and acetic

acid bacteria common across fermentations in the aforementioned countries was identified. It was highlighted that contrary to what has been published before, a well-defined microbial succession does not take place across countries.

In the second part, commercial cocoa powder samples obtained from retail shops and from a cocoa industry as well as cocoa samples collected along a cocoa powder production line (chapter 4 and 5) were examined for their composition in general microbial groups, making use of culture-dependent and culture-independent techniques. In addition, the occurrence of Thermoresistant Spores (ThrS) that is spores with the ability to survive heat treatments of at least 100°C for 10 min was assessed. Surviving ThrS were identified and genotyped, and the heat-resistance of these spores was compared after resporulation under standardised conditions. Members of *B. licheniformis* and of *B. subtilis* complex were the predominant species in the ThrS library. However, members of *B. subtilis* complex, including species of *B. subtilis* subsp. *subtilis*, were notable for forming the most heat-resistant spores *in situ* and *in vitro*. Altogether, the data showed that cocoa powder can be a vehicle of highly heat-resistant spores and that strains of *B. subtilis* are potentially problematic for downstream preservation treatments of cocoa powder derived products, such as UHT treated chocolate milk drinks.

Both data in chapters 4 and 5 showed that high heat-resistance spore formation in *B. subtilis* was only found in a limited number of strains, which had distinct genotype. In chapter 6, we compared physicochemical and ultrastructural spore properties of 5 strains of *B. subtilis* subsp. *subtilis*, of which spore $D_{110°C}$ spanned a range from 0.10 min up to 106 min. We found the highest correlation between the log$D_{110°C}$ and the average content of magnesium, manganese and calcium, of which manganese constituted the best predictor of spore heat-resistance. This is the first time that such a dependence from manganese for *B. subtilis* subsp. *subtilis* species was demonstrated and it means that manganese could be a biomarker for B*. subtilis* subsp. *subtilis* spores wet heat-resistance.

In chapter 7, the germination capacity of three highly heat-resistant and one heat-sensitive strain of *B. subtilis* subsp. *subtilis* was assessed in the presence of reference compounds used to study germination in *B. subtilis* as well as compounds and ingredients related to chocolate milk. This study was performed with absorbance- and culturing-based as well as advanced single cell-based methods by means of flow-cytometry. It was shown that although sublethal heat shock activation was required for all strains to break spore dormancy and induce appreciable germination, the requirements of heat shock activation appeared to be related to the spore heat-resistant phenotype. Still, depending on the precise germinant and incubation temperature a variable fraction of superdormant spores was identified in spore crops of all strains. Strain specific responses to combinations of germinants and incubation temperature were observed and it was found that different compounds related to chocolate milk formulations are able to induce spore germination, including milk and cocoa powder. Flow-cytometry allowed a detailed quantification of the germinating spore population and may be of interest to study damage repair processes in spores which are subjected to sublethal heat preservation treatments and recovered in different media.

In addition to a physiological strategy, a genomic approach was executed with the aim of finding a common genomic denominator underlying a high heat-resistant spore phenotype within the phylum *Firmicutes* (Chapter 8). Publicly available genomes of *Firmicutes* and genomes of two *B. subtilis* subsp. *subtilis* strains isolated in this project and producing highly heat-resistant spores were mined for a number of genomic features, namely genome base composition, gene content and amino acid composition of spore predicted proteins. These genomic features were correlated with spore decimal reduction time (*D*-value) as well as optimal growth temperature (OGT), since a number of strains forming spores of high heat-resistance are also thermophiles. High heat-resistant spore phenotype could not be explained by any of the investigated features, while for growth temperature preference, high correlation was found between OGT and reduced genome size and increased GC-content of the 16S rRNA gene. Moreover, features related to gene content and protein amino acid composition also resulted in leads concerning growth temperature preferences. This study suggests the primary role of spore physicochemical composition and ultrastructural characteristics as determinant factors of spore differential wet heat-resistance.

In sum, the work compiled in this thesis presents suggestions to delve into the functionality of the microbiota involved in cocoa bean fermentation and to improve the quality of commercial cocoa beans. Furthermore, this study contributes towards the understanding of the microbiota composition, but principally about the ecology, diversity and properties of heat-resistant bacterial spores in the cocoa chain. Moreover, biomarkers for spore heat-resistant phenotype were obtained.

The gathered phenotypic data concerning spore heat-resistance and germination characteristics are useful input data for predictive modelling and to assist in the production of safe and high quality food.

Samenvatting

De productieketen van cacaobonen (*Theobroma cacao* L.) omvat diverse stadia die uiteindelijk resulteren in de zo geliefde chocolade en een aantal andere commerciële producten zoals cacaopoeder en cacaoboter. In deze keten worden onderscheiden (i) de verwerking van cacaovruchten na de oogst, gevolgd door (ii) een industrieel verwerkingsproces. Bij de verwerking van de vruchten speelt bonenfermentatie een belangrijke rol vanwege de biochemische veranderingen waarbij in de bonen precursors ontstaan van het typische cacao-aroma. Deze biochemische reacties worden uitgevoerd door microorganismen die zich in de vruchtpulp ontwikkelen. Tijdens het industriële proces worden zgn. "commerciële cacacaobonen" onderworpen aan diverse bewerkingen zoals roosteren waardoor het uiteindelijke cacao-aroma wordt gevormd. Tegelijkertijd worden tijdens dit proces ongewenste microorganismen gedood opdat het eindproduct aan microbiologische kwaliteitscriteria voldoet.

Cacaobonen worden nog steeds wereldwijd met eenvoudige middelen en op natuurlijke en ongecontroleerde wijze gefermenteerd. Er is echter tijdens de laatste 60 jaar veel inzicht verkregen in de complexe biochemische reacties tijdens de fermentatie en hun belang voor de cacaokwaliteit. In dit promotieonderzoek werd eerst de literatuur m.b.t. cacaofermentatie kritisch geanalyseerd om gangbare fermentatiepraktijken in verband te kunnen brengen met kwaliteitskenmerken van commerciële cacaobonen.

Door hun grote hitteresistentie kunnen bepaalde bacteriesporen het industriële proces overleven waardoor ze aanwezig kunnen zijn in commerciële cacaoproducten. In het promotieonderzoek werd aandacht besteed aan het vóórkomen, de verscheidenheid en de eigenschappen van zulke hitteresistente sporen in cacaoproducten zoals cacaopoeder, omdat deze bederf kunnen veroorzaken in UHT (ultrahoge temperatuur) verhitte chocolademelk.

In hoofdstuk 2 werden fermentatiepraktijken in Trinidad en Tobago, Brazilië, Indonesië, de Dominicaanse Republiek en Ghana vergeleken, en de vorming van cacao-aromabestanddelen en voorstadia beschouwd. De voor de kwaliteit van commerciële cacao relevante factoren zoals: (i) de agrarische omstandigheden, (ii) de rijpingsgraad van de cacaovrucht, (iii) microbiële interacties tijdens de fermentatie, (iv) ongewenste verzuring van cacaobonen, (v) de vluchtige aromabestanddelen, en (vi) de invloed van de fermentatie op de eigenschappen van cacaoboter, werden nader beschouwd. Zoals besproken in hoofdstuk 3 beïnvloeden bovengenoemde factoren de economische waarde, kwaliteit en veiligheid van cacaobonen, en daarmee het inkomen van de cacaoboer. Een algemeen voorkomende combinatie van gisten, melkzuurbacteriën en azijnzuurbacteriën werd in alle fermentaties in bovengenoemde landen geconstateerd. In tegenstelling tot eerdere publicaties bleek er geen sprake te zijn van een algemeen geldende opvolging (successie) van microorganismen.

In het tweede gedeelte werden commerciële cacaopoeders verkregen uit winkels en van een cacaoverwerkende fabriek, alsmede cacaomonsters van verschillende stadia tijdens het

industriële proces (hoofdstuk 4 en 5) onderzocht. Deze werden geanalyseerd m.b.v. kweek- en moleculaire methoden, op groepen microorganismen en op hitteresistente sporen (HS) d.w.z. die 10 minuten verhitting bij 100°C overleven. Deze werden geïdentificeerd en gegenotypeerd, en hun hitteresistentie werd vergeleken na hersporulatie, onder gestandaardiseerde omstandigheden. In de verzameling HS bleken *Bacillus licheniformis*, en stammen uit het *B. subtilis* complex te overwegen. Echter, *B. subtilis* complex stammen, waaronder *B. subtilis* ssp. *subtilis* soorten, vormden de meest hitteresistente sporen zowel *in situ* (cacao) als *in vivo* (laboratoriumkweek). Dit toonde aan dat cacaopoeder hitteresistente sporen kan bevatten, en dat *B. subtilis* stammen problemen zouden kunnen veroorzaken in de conservering, zoals bij UHT verhitting van chocolademelk.

In hoofdstukken 4 en 5 werd aangetoond dat hoge hitteresistentie in *B. subtilis* voorkomt bij slechts een beperkt aantal stammen van een bepaald genotype. In hoofdstuk 6 werden fysisch-chemische en ultrastructurele eigenschappen vergeleken van sporen van vijf *B. subtilis* ssp. *subtilis* stammen met een hitteresistentie variërend van 0,1 tot 106 minuten $D_{110°C}$ (Decimale Reductietijd bij 110°C). De beste correlatie werd gevonden tussen log $D_{110°C}$ en de concentratie van magnesium, mangaan en calcium in de sporen, waarbij mangaan de beste index leek te zijn voor sporenhitteresistentie. Dit is de eerste keer dat zulk een afhankelijkheid voor mangaan van *B. subtilis* ssp. *subtilis* stammen werd aangetoond, hetgeen betekent dat mangaan beschouwd zou kunnen worden als een biomarker voor *B. subtilis* ssp. *subtilis* hitteresistentie.

In hoofdstuk 7 werd het ontkiemingsvermogen van drie hitteresistente en één hittegevoelige stam van *B. subtilis* ssp. *subtilis* gemeten in aanwezigheid van gangbare referentie ontkiemingsstimulatoren van *B. subtilis,* alsook stoffen en ingrediënten die in de chocolademelkfabricage worden gebruikt. Deze studie werd verricht m.b.v. lichtabsorptie, kweekmethoden en waarneming van afzonderlijke sporen d.m.v. flow-cytometrie. Het bleek dat sub-lethale hitteschokactivering nodig was om de sporen te laten “ontwaken” en ontkieming te veroorzaken. De benodigde intensiteit van hitteschok verschilde per hitteresistent fenotype. Desondanks werd, afhankelijk van ontkiemingsomstandigheden, in alle stammen na ontkieming een aantal “superdomant” (levend maar niet te ontwaken) sporen aangetroffen. Stam-afhankelijke reacties op ontkiemingsmiddel en -temperatuur werden waargenomen; ingrediënten voor chocolademelk zoals melkpoeder en cacaopoeder veroorzaakten ontkieming van sporen. Met flow-cytometrie konden ontkiemende sporenpopulaties worden gekwantificeerd; dit zou van belang kunnen zijn bij het onderzoek van herstelprocessen in sporen die aan sub-lethale hitteconserveringsbehandelingen worden onderworpen, tijdens hun herstel in verschillende microbiologische media.

Naast de fysiologische aanpak werd ook genoomanalyse verricht om een gemeenschappelijke basis te vinden voor hoge hitteresistentie binnen het fylum *Firmicutes* (hoofdstuk 8). De basensamenstelling, genensamenstelling in het genoom, en de aminozuursamenstelling van voorspelde sporeneiwitten werden bepaald in (i) *Firmicutes* genomen beschikbaar in het publieke domein (www), en (ii) twee eigen gesequencede stammen van *B. subtilis* ssp. *subtilis*.

De genoomeigenschappen werden gecorreleerd met hitteresistentie (D-waarden) en Optimum Groei Temperatuur (OGT), aangezien een aantal stammen met hoog hitteresitente sporen tevens thermofiel zijn. Het fenotype “hoog hitteresistent” kon echter niet worden verklaard uit genoomgegevens, maar een sterke correlatie werd gevonden tussen OGT en kleinere genoomafmeting en hoger GC-gehalte van het 16S rRNA gen. Verder zijn er ook aanwijzingen voor een verband tussen OGT en de gensamenstelling, en eiwit-aminozuursamenstelling. Dit onderzoek duidt op een belangrijke rol van fysisch-chemische samenstelling en ultrastructuur van sporen in hun hitteresistentie.

Samenvattend levert dit proefschrift suggesties voor verder onderzoek naar de functionaliteit van de fermentatiemicroorganismen van cacaobonen, en ter verbetering van de kwaliteit van commerciële cacaobonen.

Verder is dit proefschrift een bijdrage aan de kennis van de microbiologische samenstelling, en vooral van de ecologie, verscheidenheid en eigenschappen van hitteresistente bacteriesporen in de productieketen van cacao. Bovendien werden biomarkers voor hitteresistentie verkregen. Verzamelde fenotypische gegevens over hitteresistentie en ontkiemingseigenschappen kunnen worden toegepast in voorspellende modellen en in de productie van veilig voedsel van hoge kwaliteit.

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List of publications

Cocoa fermentation science

Lima, L.J.R., Almeida, M.H., Nout, M.J.R. and Zwietering, M.H. (2011). *Theobroma cacao* L., "The food of the gods": Quality determinants of commercial cocoa beans with particular reference to the impact of the fermentation. *Crit. Rev. Food Sci.* 52: 731-761.

Lima, L. J. R. and Nout, M. J. R. (in press). Quality and safety of cocoa beans. In: Schwan, R. F. and Fleet, G. H. Ed. Cocoa and Coffee Fermentations Fermented Foods and Beverages Series. CRC Press/Taylor and Francis Group.

Almeida, M.H., Batalha, P., **Lima**, L.J.R., Leitão, A.E. and Espírito-Santo, S. Catongo cocoa beans from São Tomé and Príncipe: Physicochemical and sensorial evaluation (In revision for resubmission).

Microbiological quality of cocoa and characteristics of thermoresistant microbiota

Lima, L. J. R., Kamphuis, H. J., Nout, M. J. R. and Zwietering, M. H. (2011). Microbiota of cocoa powder with particular reference to aerobic thermoresistant spore-formers. Food Microbiol. 28:573-582.

Lima, L. J. R., V. van der Velpen, J. Wolkers-Rooijackers, H. J. Kamphuis, M. H. Zwietering and M. J. R. Nout. 2012. Microbiota dynamics and diversity at different stages of cocoa bean industrial processing to cocoa powder. Appl. Environ. Microbiol. 78:2904-2913.

Lima, L. J. R., Kizzie-Hayford, N., Smid, E. J., Nout, M. J. R. and Zwietering, M. H. Compositional and ultrastructural factors that make *Bacillus subtilis* subsp. *subtilis* spores highly heat-resistant (Submitted).

Lima, L. J. R., Gao, W., Zwietering, M. H. and Nout, M. J. R Germination of highly heat-resistant *Bacillus subtilis* spores and relevance for cocoa-derived products (In revision).

Lima, L. J. R., Schaap, P. J., Mobegi, F. M., André, S., Nout, M. J. R., Zwietering, M. H., van Hijum, S. A. F. T. and Smid, E. J. Genomic biomarkers of growth temperature preference and spore heat-resistance: a meta-analysis with *Firmicutes* (In revision).

Lima, L. J. R. and Nout, M. J. R. *Bacillus* spores heat-resistance mechanisms revisited (In preparation).

Others

Lima, L. J. R., Vida, J., Prista, C., Malfeito-Ferreira, M., Gonçalves, P., Peinado, J. M. and Loureiro-Dias, M. C. Effect of ethanolic stress on the fructophilic behaviour of *Zygosaccharomyces baillii* (In preparation).

About the author

Lidia Josélia Rebelo Lima was born in Lisbon, Portugal, on August 7th 1980. She graduated in 2004 as a food engineer by the Higher Institute of Agronomy, Technical University of Lisbon, Portugal (final mark of 16 over 20). Subsequently, in 2005, she obtained a diploma in industrial engineering by the Faculty of Sciences and Technology, New University of Lisbon (final mark 15 over 20), by completing the curricular part of the master. During her food engineering studies she became particularly interested in cell bioenergetics and went on to do her final year thesis in physiology of sugar transport in spoilage yeasts under Professor Maria Conceição Loureiro-Dias and Dr. Catarina Prista. Following her graduation in food engineering she worked as junior researcher in the same laboratory in a project aimed at developing molecular tools to characterise the fructophilic behaviour of *Zygosaccharomyces bailli*. While fully in love with yeast biology and biotechnology, she decided to pursue her dream of performing her PhD studies in the area of cocoa bean fermentation, processing and quality, a step she could accomplish by being awarded a grant from the Portuguese Foundation for Science and Technology. In February 2006, she started her PhD project at Wageningen University, Laboratory of Food Microbiology, under Dr. Rob Nout, Dr. Henry Kamphuis and Professor Marcel Zwietering. The outcome of her thesis project is presented in this booklet. She presently combines interests in the domain of food processing and preservation with the use of multidisciplinary strategies to unravel mechanisms of cell survival in food related environments. She holds a scientist position in meat preservation at Purac, where she is keenly engaged in developing innovative preservation strategies for different meat segments.

Overview of completed training activities

VLAG graduate School

Discipline specific activities

Courses

Molecular Food Microbiology, KVL, Denmark, 2006
Bioinformatics- A user's approach, VLAG, 2007
Genetics and Physiology of Food Associated Microorganisms, VLAG, 2007
Food Fermentation, VLAG, 2008
Management of Microbiological Hazards in Foods, VLAG, 2008

Conferences/Meetings

The 21st International ICFMH Symposium Food Microbiology, 2008
Congress Spore-Forming Bacteria in Food, 2009

General courses

Information Literacy, WGS, 2006
Academic Writing II, CENTA, 2006
Project and Time Management, WGS, 2007
Advanced Statistics, EPS/ PE&RC, 2007
Scientific Writing, CENTA, 2007
Bionumerics and GelCompar Workshop, Applied Math Belgium, 2008

Other activities

Preparation of the PhD Research Proposal, 2006
Organisation and participation in the PhD trip to Canada, 2008
Supervision of BSc. and MSc. Students, 2007-2011
Supervisor at Food Microbiology course, 2006
Lecturer and supervisor at the Food Fermentation course, 2007-2009
Meetings Laboratory of Food Microbiology, 2006-2010

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