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

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ABSTRACT

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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1. 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., in press; 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 subsequently partially defatted to minimum levels of 20% or between 10 and 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 *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus megaterium* and *Bacillus 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

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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 min. 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.

2. Materials and methods

2.1. 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 in 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.

2.2. 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 in 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.

2.3. 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 screw-capped 120 × 15 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 serially 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.

2.4. Thermoresistant spore-formers identification

2.4.1. 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 *B. 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] 20 g/l, Meat Peptone [MP, Oxoid] 6.1 g/l and Agar Bacteriological [AB, Oxoid] 3.5 g/l), followed by incubation at 30 °C for 2 days. *B. cereus* ATCC 14579 (Laboratory of Food Microbiology, Wageningen University) was used as a positive control.

2.4.2. 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 *B. 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 successfully generated in duplicate independent experiments for 8 strains), two 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 phylogenetic tree was inferred using Nei and Li, and Neighbour joining methods.

2.5. Preparation of spore crops of thermoresistant strains and heat-inactivation assay

Isolates sporulation was achieved as described in section 2.4.1. 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 (2–4 days). Spores were harvested with 10 mL of Phosphate Buffer (PB) 10 mM pH 7.0 (tenfold dilution of 0.1 M K₂HPO₄/KH₂PO₄ 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 1157 g for 7 min and at 4629 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 4100 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 and 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 in the section 2.3. 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.

2.6. 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. 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 $\log D$ (or $\log \delta$) 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.

3. Results

3.1. Cocoa powder compliance with the microbiological quality guidelines

The microbiological composition of the analysed 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).

3.2. 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.

3.3. 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

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	Aerobic total mesophilic spores	Aerobic total thermophilic spores	Yeasts	Moulds	<i>Enterobacteriaceae</i> in 1g ^d	<i>Salmonella</i> in 25 g ^d
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 ^e	<1	0.4 (0.49)	<1	<1	<1	–	–
P12 /E/L	6.9	3.7 ^f (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^f (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	–	–
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

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.

^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)–10–12% (w/w); High (H)– 20–22% (w/w); not specified (ns) in the package.

^d +/– presence/absence.

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

^f 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.

Table 2Estimated 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
		<1 ^b	0.6	[−0.30; 1.41]	P15	M110
	100 °C-30 min	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
		<1	0.6	[−0.30; 1.41]	P4	M90
	108 °C-30 min	1.0	1.0	[0.34; 1.59]	P2	M88, M89
		1.0	0.6	[−0.30; 1.41]	P2	M94, M95
	110 °C-10 min	0.7	0.6	[−0.30; 1.41]	P2	M98
	120 °C-10 min	0.7	0.6	[−0.30; 1.41]	P2	M112
	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
		1.0	0.6	[−0.30; 1.41]	P12	TT96, TT97
	110 °C-10 min	1.0	0.6	[−0.30; 1.41]	P12	TT96, TT97

^a Samples with aerobic total 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 an 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.

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.

Fig. 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 *B. subtilis* complex, *B. licheniformis* and *B. cereus* complex, and group IV by the single species lineages of *Bacillus 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 *B. cereus* strains.

For 7 strains within the *B. subtilis* complex, 99% of similarity was found with *Bacillus 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, 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 *Bacillus 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).

The 16S rDNA sequence for strain TT50 resulted in 98% similarity with sequences mainly from *Geobacillus thermoleovorans*, *Geobacillus kaustophilus* and *Geobacillus caldopenax*. 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.

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 161 with the primer combination EcoRI-C/MseI-G. The number of fragments per strain varied between 50 and 83 for the first primer combination and from 37 to 65 for the second. The clustering of the AFLP profiles is depicted in Fig. 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).

3.4. Thermoresistant spores heat-resistance properties

Fig. 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.

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. Fig. 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

Fig. 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.

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 characterize 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 Fig. 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).

4. 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

Fig. 2. Thermoresistant isolates genetic relatedness on the basis of AFLP fingerprinting. The phylogenetic 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 Fig. 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.

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 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 and 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.

Members of *B. 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., in press).

The combination of the heat-resistance profile of the thermoresistant strains (Fig. 3), with the AFLP clustering pattern (Fig. 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*

Fig. 3. Thermal reduction (log cfu/ml) of thermoresistant spore crops at 110 °C for 5 min. 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 in bold.

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

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)	<i>D</i> -value (min)	<i>r</i> ²	MSE _{model}	<i>z</i> (°C) ^a	<i>r</i> ²	<i>D</i> _{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).

Fig. 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.

Fig. 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 ($\diamond$), M1 ($\blacktriangle$), M22 ($\ast$), M35 ($\bullet$), M65 ($\square$) and M95 ($+$).

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 crop. Strain M112 spores were the most heat-resistant under the studied conditions, with an estimated $D_{110^\circ\text{C}}$ 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 ($D_{120^\circ\text{C}}$ 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-temperature 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 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.

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Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.fm.2010.11.011.

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