Microbiota Dynamics and Diversity at Different Stages of Industrial Processing of Cocoa Beans into Cocoa Powder

Lídia J. R. Lima, Vera van der Velpen, Judith Wolkers-Rooijackers, Henri J. Kamphuis, Marcel H. Zwietering and M. J. Rob Nout

***Appl. Environ. Microbiol.* 2012, 78(8):2904. DOI: 10.1128/AEM.07691-11.**
Published Ahead of Print 10 February 2012.

AEM
Journals.ASM.org

Microbiota Dynamics and Diversity at Different Stages of Industrial Processing of Cocoa Beans into Cocoa Powder

Lídia J. R. Lima,[a] Vera van der Velpen,[a]* Judith Wolkers-Rooijackers,[a] Henri J. Kamphuis,[b] Marcel H. Zwietering,[a] and M. J. Rob Nout[a]

Laboratory of Food Microbiology, Wageningen University, Wageningen, The Netherlands,[a] and Cargill Cocoa and Chocolate, Wormer, The Netherlands[b]

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 with clone library construction, to analyze the samples. Aerobic thermoresistant spores (ThrS) (100°C; 10 min) were also isolated and characterized (identity, genetic diversity, and spore heat resistance), in view of their relevance to the quality of downstream heat-treated cocoa-flavored drinks. In the nibs (broken, shelled cocoa beans), average levels of total aerobic microorganisms (TAM) (4.4 to 5.6 log CFU/g) and aerobic total spores (TS) (80°C; 10 min; 4.3 to 5.5 log CFU/g) were significantly reduced ($P < 0.05$) as a result of alkalizing, while fungi (4.2 to 4.4 log CFU/g) and *Enterobacteriaceae* (1.7 to 2.8 log CFU/g) were inactivated to levels below the detection limit, remaining undetectable throughout processing. Roasting further decreased the levels of TAM and TS, but they increased slightly during subsequent processing. Molecular characterization of bacterial communities based on enriched cocoa samples revealed a predominance of members of the *Bacillaceae*, *Pseudomonadaceae*, and *Enterococcaceae*. Eleven species of ThrS were found, but *Bacillus licheniformis* and the *Bacillus subtilis* complex were prominent and revealed great genetic heterogeneity. We concluded that the microbiota of cocoa powder resulted from microorganisms that could have been initially present in the nibs, as well as microorganisms that originated during processing. *B. subtilis* complex members, particularly *B. subtilis* subsp. *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 ultrahigh-temperature-treated chocolate drinks.

Cocoa beans, the fruit seeds from the tropical tree *Theobroma cacao* L., are highly prized, as the solids and 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 semimanufactured or finished products are obtained for commercialization (16).

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 flavor (18). Different studies have shown that during the process, yeasts, lactic acid bacteria, and 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, postfermentation microbial ecology studies have been focused on the prevalence of mycotoxins in cocoa beans and derived products (7, 30), as well as survival of *Salmonella* during manufacture and storage of cocoa products (17, 25). 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 in 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 provide the opportunity to identify microorganisms with novel physiological traits (20, 35).

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 Fig. 1. The general principle of production (14, 16) consists of blending of cocoa beans from different origins, cleaning off impurities, and applying surface heat treatments to facilitate shell removal. Subsequently, the beans are broken and separated from the shell (nibs) and then undergo alkalizing by immersion in an alkaline solution (e.g., potassium carbonate) at high temperatures (up to 100°C). After alkalizing, the nibs are predried and roasted (the 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.

Preroasting bean treatments (Fig. 1, operations 4 and 5) and roasting (Fig. 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 flavor, destruction of *Salmonella* takes place (14). After roasting, only spore formers of the genus *Bacillus* and their relatives may survive (3). For this reason, the quantification of total aerobic microorganisms (TAM) is considered to be a suitable indicator of both roasting and global process conformity (14). However, in the cases of specific applications, such as manufacturing of ultrahigh-temperature (UHT)-treated cocoa-flavored 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 to the stability of the final drinks (19, 38). In the context of a previous work, where the microbiological composi-

Received 25 November 2011 **Accepted** 27 January 2012
Published ahead of print 10 February 2012
Address correspondence to Marcel H. Zwietering, marcel.zwietering@wur.nl.
* Present address: Division of Human Nutrition, Wageningen University, Wageningen, The Netherlands.
Supplemental material for this article may be found at http://aem.asm.org.

doi:10.1128/AEM.07691-11

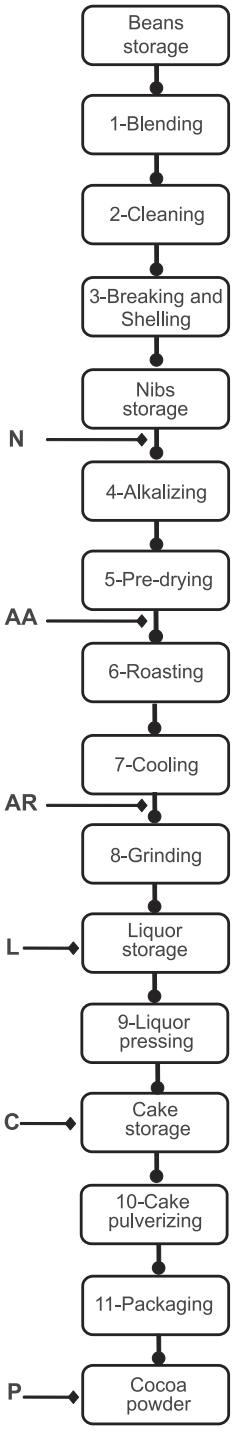

FIG 1 Diagram of operations involved in cocoa powder production in the studied factory. The sampled stages were Nibs (N), After Alkalizing and Predrying (AA), After Roasting (AR), Liquor Storage (L), Cake Storage (C), and Powder (P).

tion of commercial cocoa powder was investigated, we defined aerobic thermoresistant spores (ThrS) as spores able to survive a heat treatment of at least 100°C for 10 min, as opposed to the standard treatment of 80°C for 10 min for quantification of total spores (TS) (19). This more stringent treatment proved to be important, as appreciable differences were found 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 outset 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, to determine their identities, and to 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 molds and *Enterobacteriaceae* (10). To characterize the microbial diversity, PCR-denaturing gradient gel electrophoresis (DGGE), coupled with 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 reveal genetic heterogeneity. For selected strains, spores were produced under standardized conditions, and their wet-heat resistances were compared.

MATERIALS AND METHODS

Cocoa bean processing and sampling. A cocoa powder production line in the Netherlands was sampled during 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 Fig. 1 (when referring to the processing stages, the first letters are capitalized). The final cocoa powder fat content was between 10 and 12% (wt/wt). During the first three stages, we used a tracking system, which allowed us to collect samples in the same position on the conveyor. After grinding (Fig. 1, operation 8), samples corresponding to the same batch production were taken at Liquor, Cake, and Powder stages, based on the residence time data history. At each sampling site, approximately 200 g of sample was 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 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 (10). This allowed collection of 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 previously (19). Samples within a given batch were processed simultaneously.

Mesophilic (30°C for 3 days) and thermophilic (55°C for 2 days) 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 (19). 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 molds 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 ISO standard method 21528-1:2004 (15), using Violet Red Bile Glucose Agar (VRBGA) (Oxoid), an inhibitory medium for Gram-positive bacteria. 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 TS (80°C; 10 min) and aerobic ThrS (100°C; 10 min) were determined as described previously (19). 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 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 less than 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 they were stored as was previously performed (19). The strains were coded to reflect the isolation temperature, stage, and batch production (e.g., 30N1-1 corresponds to a strain that was isolated from a plate incubated at 30°C from nibs, batch I, and constitutes isolate number 1).

Molecular characterization of thermoresistant spore formers. 16S rRNA gene sequences were obtained with 8F and 1522R universal primers (11) as described previously (19) and compared to similar sequences of type and cultured strains in the GenBank database at the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/), using the BLAST method (1).

The genetic diversity of the strains was analyzed using the amplified fragment length polymorphism (AFLP) method (36), with the AFLP Analysis System Kit for Microorganisms (Invitrogen). This technique was performed as described previously (19), except that the primer combinations EcoRI-A/MseI-G and EcoRI-C/MseI-G were used, resulting in a high number of bands (30 to 75) for the large majority of the species. The reproducibility of this AFLP experimental setup had been successfully confirmed previously (19). Digital AFLP gel images were scored for dominant markers using AFLPQuantar (v1.05; KeyGene), and a phylogenetic tree was inferred using the method of Nei and Li and neighbor-joining methods implemented in Treecon v1.3b (34).

A selection of strains from the *Bacillus subtilis* complex (29) and a strain of *Bacillus licheniformis* were further identified to the species or subspecies level by partial sequencing of the gyrase A gene (*gyrA*). This was performed by the Bacteria Collection Laboratorium Voor Microbiologie of Ghent University (BCCM/LMG), using the primers and PCR conditions described by Chun and Bae (6).

Preparation of thermoresistant spore crops and wet-heat inactivation assay. Twenty-two strains isolated at the After Alkalizing, After Roasting, and Powder stages were selected to assess the wet-heat resistance capacities of their spore crops. The spore crops were produced on NA medium supplemented with different minerals (5). 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 greater than 95% free phase-bright spores for all strains. The spores were harvested, washed, and stored at 4°C in the dark for 1 month prior to the heat inactivation assay (19), during which time all spores remained phase bright, as monitored by phase-contrast microscopy. The concentration of working spore crops was adjusted to 7 to 8 log CFU/ml, based on the results of plate counts in nutrient broth (NB) (2.6 g/liter) solidified with agar bacteriological (AB) (Oxoid; 15 g/liter), after incubation of the plates for 3 days at 37°C and 2 days at 55°C. The heat resistance comparison was performed by subtracting the number of viable spores after heating at 110°C for 5 min from the number before heating, as was done previously (19).

Microbial genomic DNA extraction from cocoa samples. Five grams of nibs, liquor, cake, and cocoa powder and 10 g of nibs from After Alkalizing and After Roasting were aseptically weighed in a flow cabinet into a stomacher bag with a 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 aluminum 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 30°C and 55°C. 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 × *g* (Eppendorf 5804 RF; Rf-34-6-38). Subsequently, the supernatants (the phase of interest) were pipetted into clean Falcon tubes. The remaining pellets were washed (200 × *g*) with 10 ml of phosphate-buffered saline (PBS) buffer ($Na_2HPO_4 \cdot 2H_2O$, 57.7 mM, and $NaH_2PO_4 \cdot H_2O$, 42.3 mM, pH 7), and the supernatants were also added to the clean Falcon tube. The new tubes were then centrifuged at 9,000 × *g* for 5 min to collect the cell biomass. After centrifugation, the cell biomass was washed twice (9,000 × *g*) with 10 ml of PBS buffer. In the cases of liquor, cake, and powder samples, the cell biomass was diluted 1:5 (2 ml of sample/8 ml of PBS buffer) before being washed twice. 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 of Wang et al. (37) 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 albumin (Fermentas) in the PCR mixture, 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 to 5 tubes of lysing matrix. The bead beater FastPrep instrument (MP Biomedicals) was used during the extraction. Purified DNA was eluted (30 to 50 μl), pooled, and, when necessary, diluted in sterilized Milli-Q water (30 to 50 ng/μl). The DNA extracts were stored at −20°C.

PCR-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. (23) (Sigma). The PCR mixtures (50 μl) included 2 U of *Taq* DNA polymerase (Native; Fermentas), 5 μl of 10× *Taq* buffer, 1.5 mM $MgCl_2$ (Fermentas), 200 μM deoxynucleoside triphosphate mix (Fermentas), 0.4 μM each primer, 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 consisting of 30 s at 94°C, 20 s at 56°C, 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. (22) and further modified by Martín et al. (21). Five microliters of PCR product was loaded into the gel, with the exception of samples from After Alkalizing, After Roasting, and Liquor, where 20 μl was pipetted. A marker consisting of a mixture of amplicons obtained from diverse bacterial pure cultures was included to cover the region from low to high gradient concentrations and to facilitate gel normalization. The gels were silver stained according to the method of Sanguinetti et al. (31), and after overnight drying at 55°C, the gel images were digitized on a GS 800 calibrated densitometer (Bio-Rad) 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 Alkalizing, After Roasting, Cake, and Powder stages were amplified with 16S rRNA gene 8F and 1522F universal primers. PCR mixtures were set up as described above and amplified under the following conditions: 5 min at 95°C; 30 cycles, each consisting of 30 s at 95°C, 20 s at 56°C, and 1 min at 72°C; and a final extension of 30 min at 72°C. Purified DNA extracts

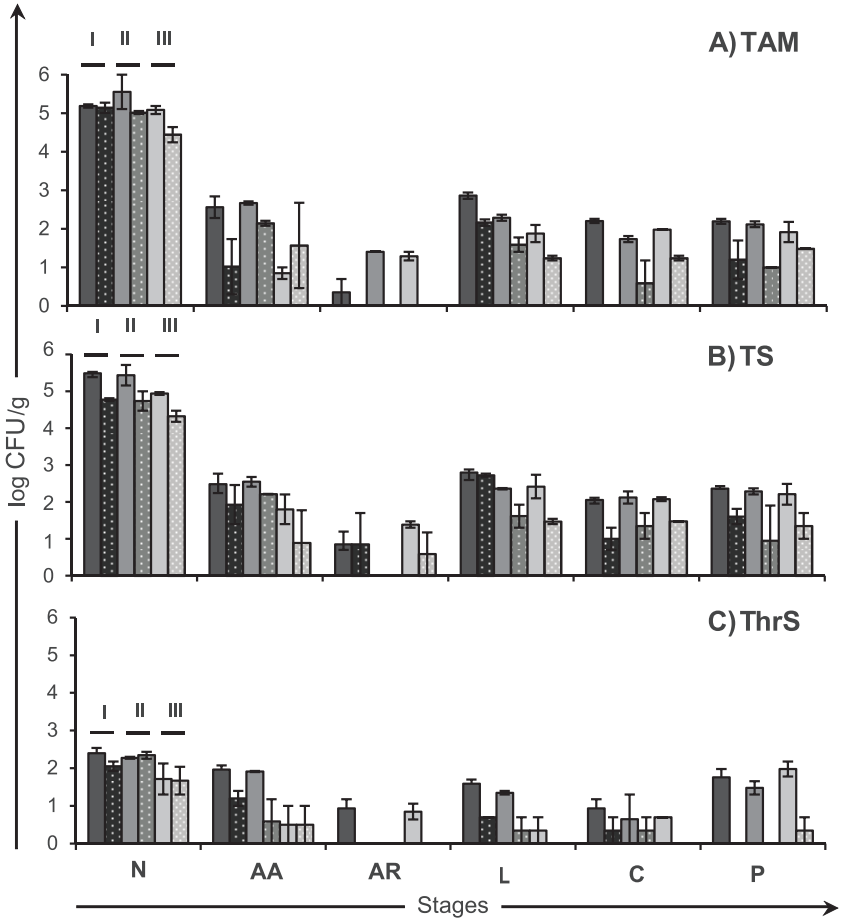

FIG 2 Microbial levels at different stages of cocoa bean processing into cocoa powder. See the legend to Fig. 1 for stage abbreviations. The bars correspond to the estimated average levels (log CFU/g) of TAM (A), aerobic TS (B), and aerobic ThrS (C) growing at 30°C (solid bars) and 55°C (stippled bars). Consecutive pairs of bars within each sampled stage correspond to the same batch production: batch I (black), batch II (dark gray), and batch III (light gray). The absence of bars indicates levels below the detection limit of the method (1 log CFU/ml) in two independent experiments. The error bars indicate standard errors of the mean.

(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 1,500 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′-AGAGTTTGATCMTGG CTCAG-3′). The sequences were trimmed to 850 bp and inspected for mistakes using Chromas v. 2.31 (Technelysium Pty Ltd.). The program Bellerophon (13) 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 analyzed 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 the Bionumerics software (22).

Statistical treatment of data. The plate count numbers of the two duplicate experiments were log transformed, and the average and standards error 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 analysis of variance (ANOVA) (PASW Statistica v.17.0).

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in GenBank under accession numbers 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 (Fig. 2A) and TS (Fig. 2B) at Nibs, with the levels varying between 4.3 and 5.6 log CFU/g (Fig. 2). Conversely, the estimated average levels of ThrS (Fig. 2C) 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*, they were 2.4 ± 0.11 log CFU/g, 2.8 ± 0.07 log CFU/g, and 1.7 ± 0.05 log CFU/g.

After alkalizing and predrying of nibs, both mesophilic and thermophilic TAM and TS levels were in general significantly reduced ($P < 0.05$). In the case of ThrS, they were only significantly

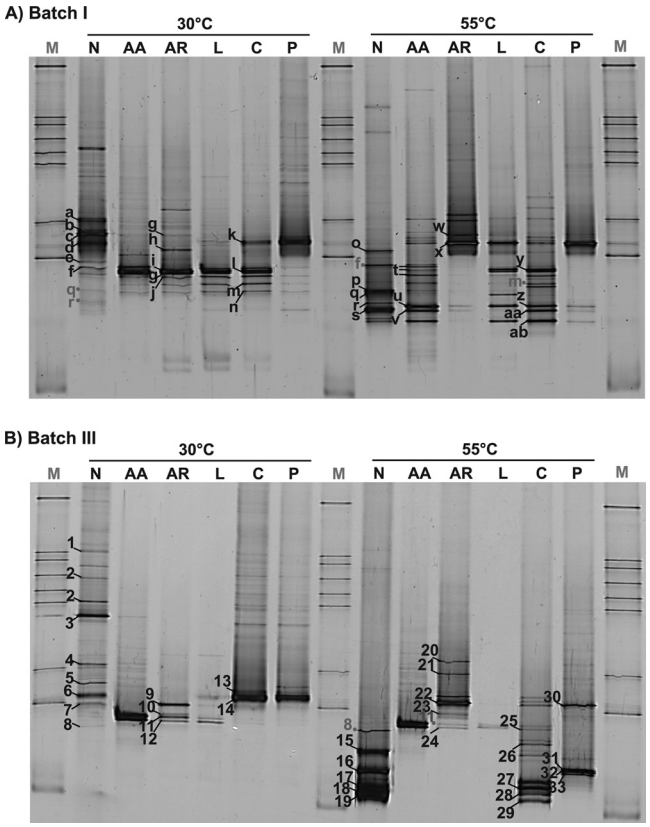

FIG 3 Changes in bacterial communities during cocoa bean processing into cocoa powder. The fingerprints were generated by PCR-DGGE of the V6-V8 region of the 16S rRNA gene for batch I (A) and batch III (B) (see the legend to Fig. 1 for stage abbreviations); M, marker. Each lane represents the fingerprints of amplified DNA extracts obtained after sample enrichment at 30°C or 55°C. The amplicons were resolved in a 30 to 60% denaturing gradient. Stages that were cloned and characterized in more detail are indicated with letters or numbers. The identities of the clone sequences are presented in Table 1. Letters or numbers in gray indicate band positions that were confirmed by running the PCR-DGGE amplicons of the cocoa DNA samples juxtaposed.

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 remained undetectable throughout processing.

Roasting of nibs further reduced the microbial levels. At this stage, conspicuous differences between TS and TAM were present, but they involved a very low number of colonies and/or high standard deviations, rendering the differences inaccurate. In contrast, samples from the Liquor stage 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.

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 setups, 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 from batch III. At Nibs, several bands were present, which could indicate great bacterial diversity. Clear differences were present in the profiles of nib samples enriched at 30°C and 55°C. Upon alkalizing 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 group quantification (Fig. 2), a higher number of bands was expected at Liquor than at After Roasting, but this was not the case, with the 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 the same migration pattern as another band at Nibs-30°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 bacterial primers, but only a relatively low number of distinct phylotypes were present (Table 1). These phylotypes belonged to the phyla *Firmicutes* and *Proteobacteria*, specifically, the classes *Bacilli* and *Gammaproteobacteria*. In the clone library of nibs enriched at 30°C (NibsI-30°C), sequence types of the *Enterococcus faecium* group, which includes the species *Enterococcus durans* and *E. faecium* (9), and the genera *Bacillus* and *Lysinibacillus/Rummeliibacillus* were detected, while in NibsIII-30°C, additional sequences affiliated with the genus *Citrobacter* were also annotated. At After Roasting-30°C from batches I and III, sequence types with high percentages of similarity to the *Pseudomonas putida* group, which includes *P. putida* and *Pseudomonas plecoglossicida* (2), the genera *Ornithinibacillus/Paucisalibacillus* and *Bacillus* 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 the *P. putida* group and members of the genus *Bacillus* were also detected in CakeI-30°C.

Clone libraries of samples enriched at 55°C revealed four phylotypes not detected in clone libraries enriched at 30°C. They were all members of the genus *Bacillus*, specifically *Bacillus coagulans* (NibsI-55°C), *Bacillus ginsengihumi* (NibsI-55°C), *Bacillus thermoamylovorans* (NibsIII-55°C, After AlkalizingI-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 those of the cocoa DNA samples concurrently in the same gel, we attempted to match the positions of the bands (Fig. 3). However, confirmation of the exact band identities would require sequencing of excised bands (22).

Diversity of aerobic thermoresistant spore formers and spore 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 *gyrA* gene-sequencing results. Table S1 in the supplemental material 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 the *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 were 98.5 to 99.7%, while with *B. subtilis* subsp. *spiz-*

TABLE 1 Phylogenetic affiliations of cloned 16S rRNA genes obtained from enriched cocoa DNA samples[a]

Batch	Stage[b]/band	% similarity/GenBank closest relative	GenBank accession no.
I	N/a	99/*Enterococcus durans* 99/*Enterococcus faecium*	JN366664
	N/b	98/*Lysinibacillus sphaericus*	JN366665
	N/c	100/*Bacillus circulans*	JN366666
	N/d	99/*E. durans* 99/*E. faecium*	JN366667
	N/e	96/*Rummeliibacillus stabekisii* 96/*Lysinibacillus fusiformis* 96/*L. sphaericus*	JN366668
	N/f	98/*Bacillus subtilis* 98/*Bacillus vallismortis* 98/*Bacillus amyloliquefaciens*	JN366669
	AR/g	99/*Pseudomonas plecoglossicida* 99/*Pseudomonas putida*	JN366651
	AR/h	99/*Ornithinibacillus* spp. 99/*Paucisalibacillus globulus*	JN366652
	AR/i	99/*Ornithinibacillus* spp. 99/*P. globulus*	JN366650
	AR/j	99/*Bacillus licheniformis*	JN366653
	C/k	99/*E. durans* 99/*E. faecium*	JN366656
	C/l	100/*P. plecoglossicida* 100/*P. putida*	JN366657
	C/m	99/*B. licheniformis*	JN366658
	C/n	99/*Bacillus clausii*	JN366659
	N/o	95/*B. licheniformis*	JN366670
	N/p	99/*B. licheniformis*	JN366671
	N/q	100/*B. licheniformis*	JN366672
	N/r	99/*Bacillus coagulans*	JN366673
	N/s	97/*Bacillus ginsengihumi*	JN366674
	AA/t	99/*B. subtilis*	JN366647
	AA/u	99/*Bacillus thermoamylovorans*	JN366648
	AA/v	99/*B. thermoamylovorans*	JN366649
	AR/w	99/*Bacillus* sp. 94/*B. thermoamylovorans*	JN366654
	AR/x	99/*Bacillus* sp. 94/*B. thermoamylovorans*	JN366655
	C/y	100/*P. plecoglossicida*	JN366661
	C/z	98/*B. thermoamylovorans*	JN366662
	C/aa	99/*B. thermoamylovorans*	JN366663
	C/ab	98/*B. thermoamylovorans*	JN366660
III	N/1	99/*B. circulans* 99/*Bacillus nealsonii* 99/*Bacillus benzoevorans*	JN366691
	N/2	99/*Citrobacter rodentium*	JN366692
	N/3	99/*C. rodentium* 99/*Citrobacter sedlakii*	JN366693
	N/4	99/*C. rodentium* 99/*C. sedlakii*	JN366694
	N/5	99/*L. fusiformis* 99/*L. sphaericus*	JN366695
	N/6	99/*E. durans* 99/*E. faecium*	JN366696
	N/7	98/*L. fusiformis* 98/*L. sphaericus*	JN366697
	N/8	99/*B. subtilis*	JN366698
	AR/9	99/*Ornithinibacillus* spp. 99/*P. globulus*	JN366675
	AR/10	100/*Bacillus* sp. 99/*Bacillus firmus*	JN366676
	AR/11	99/*Bacillus firmus*	JN366677
	AR/12	99/*P. plecoglossicida* 99/*P. putida*	JN366678
	C/13	99/*E. durans* 99/*E. faecium*	JN366684
	C/14	99/*E. durans* 99/*E. faecium*	JN366685
	N/15	98/*B. thermoamylovorans*	JN366699
	N/16	99/*B. licheniformis*	JN366700
	N/17	98/*B. coagulans*	JN366701
	N/18	96/*B. licheniformis* 96/*Bacillus oleronius*	JN366702
	N/19	98/*B. thermoamylovorans*	JN366703
	AR/20	99/*P. plecoglossicida* 99/*P. putida*	JN366679
	AR/21	99/*Bacillus* sp. 93/*B. thermoamylovorans*	JN366680
	AR/22	98/*Bacillus* sp. 94/*B. thermoamylovorans*	JN366681
	AR/23	99/*Bacillus* sp. 94/*B. thermoamylovorans*	JN366682
	AR/24	98/*P. plecoglossicida* 98/*P. putida*	JN366683
	C/25	100/*P. plecoglossicida* 100/*P. putida*	JN366689
	C/26	99/*B. clausii*	JN366690
	C/27	99/*B. thermoamylovorans*	JN366686
	C/28	99/*B. thermoamylovorans*	JN366687
	C/29	99/*B. thermoamylovorans*	JN366688
	P30	98/*Bacillus* sp. 94/*B. thermoamylovorans*	JN366706
	P/31	99/*B. licheniformis*	JN366705
	P/32	99/*B. licheniformis*	JN366707
	P/33	98/*B. coagulans*	JN366704

[a] The BLAST search at GenBank (National Center for Biotechnology Information) was based on sequences of 850 bp.
[b] The legend to Fig. 1 lists stage abbreviations.

izenii and *inaquosorum*, they were below 96%). Also, for a strain of *B. licheniformis* (30P3-1), the *gyrA* gene sequence was obtained. The similarity to the type strain of *B. licheniformis* was 100%, whereas with that of *B. sonorensis*, it was only 86.2%, confirming the suitability of 16S rRNA gene sequencing to distinguish isolates from these two taxa (24). Of the total of 10 species shown in Fig. 4, 9 were found at Nibs. Upon subsequent processing, mainly strains of the *B. subtilis* complex, together with *B. licheniformis*, prevailed (Table 2).

Cluster analysis of the AFLP banding patterns revealed great genetic heterogeneity among the strains. In total, 354 markers were scored in the gel shown in Fig. 4A and 269 in the gel shown in Fig. 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 for 5 min. Spores of the *B. subtilis* complex were the most heat resistant. 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 also found (strain 30P3-3). For *B. licheniformis*, only spores of strain 30AA2-1 showed relatively

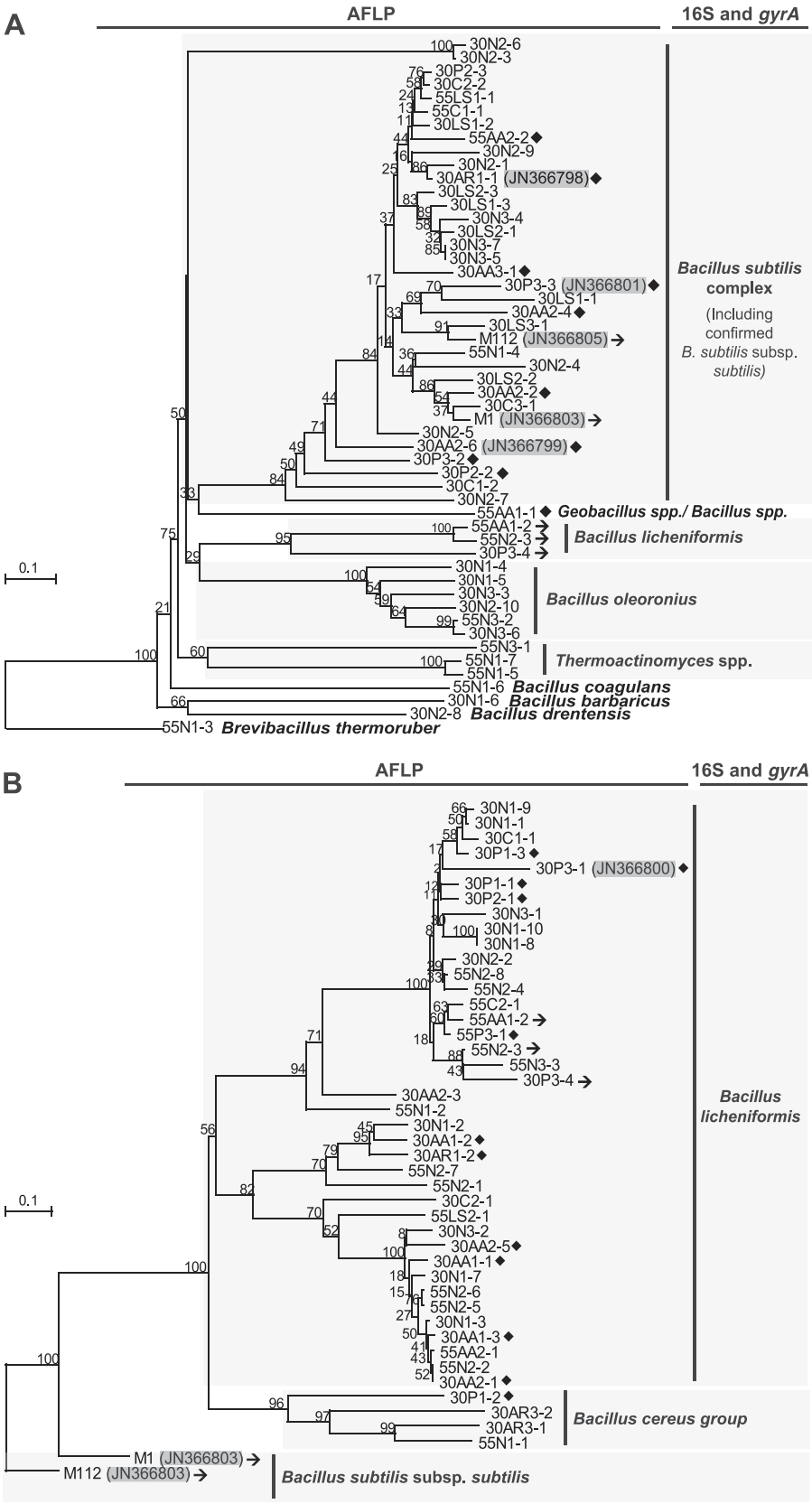

FIG 4 AFLP analysis of thermoresistant spore-forming isolates. Bootstraps (percent) are based on 1,000 replications obtained with the Treecon program. Shown are phylogenetic trees displaying members of the *B. subtilis* complex (29) and other species with strain 55N1-3 as an outgroup (A) and members of the *B. licheniformis* and *B. cereus* groups (33) with strain M112 as an outgroup (B). The scale bars correspond to 0.1 nucleotide substitution per site. Strains M112 and M1 had been characterized before (19). The abbreviations 16S and *gyrA* correspond to the 16S rRNA and gyrase A gene sequences, respectively. The gyrase A gene accession numbers are highlighted and given in parentheses. All the strains were identified as *B. subtilis* subsp. *subtilis* or *B. licheniformis*. Strains with arrows were run in both gels of Fig. 3A and B. Strains for which the spore crops' heat resistance was determined (Fig. 5) are indicated by diamonds.

TABLE 2 Occurrence of thermoresistant spore formers isolated at different stages during cocoa bean processing

Species (batch)	No./% of thermoresistant spore formers												
	N[a]		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	Total
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

[a] The legend to Fig. 1 lists stage abbreviations.

high heat resistance 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 compositions at different stages of cocoa bean processing into cocoa powder. Nibs was the stage with the highest levels and diversity, since other than cleaning and shelling, no 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 Fig. 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, indicate that not all aerobic microorganisms were spore formers. The assemblage at Nibs was less diverse than the one reported by Barrile et al. (3), who identified about 20 species in cocoa beans belonging to the classes *Bacilli*, *Gammaproteobacteria*, and *Actinobacteria*. Species common to the two studies included *B. licheniformis*, *B. coagulans*, and *Bacillus 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 the *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 (Fig. 2) was not always reflected in greater diversity; and (iii) the *E. faecium* group had the only phylotypes in the clone libraries at CakeIII-30°C, and very few PCR-DGGE bands were present at that 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 the *P. putida* and *E. 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 transport systems for intermediate storage of cocoa (see Fig. 1). In particular, *Enterococcus* spp. are recognized for their ability to survive adverse

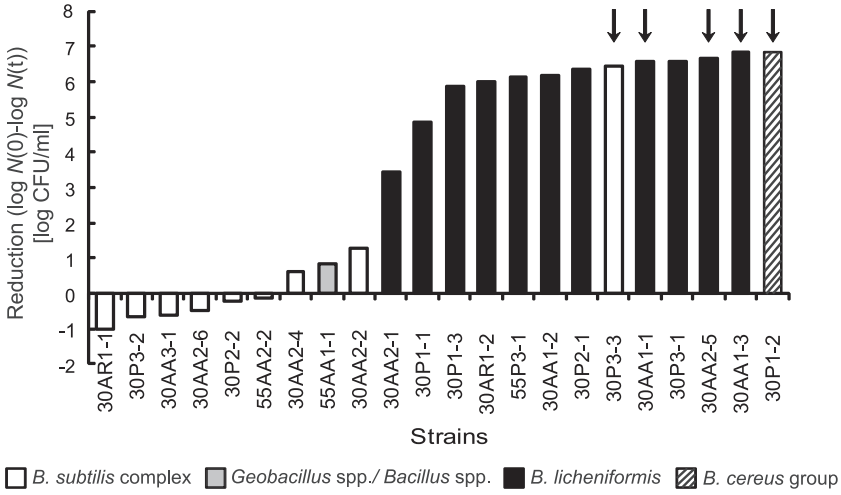

FIG 5 Thermal reduction (log CFU/ml) of thermoresistant spore crops at 110°C for 5 min in 10 mM phosphate buffer after sporulation at 55°C (strain 55AA1-1) or 37°C (all other strains). Reductions resulting in counts below the detection limit of the method (1.7 log CFU/ml) are indicated by arrows.

conditions, such as drying (9), which could have enabled them to persist in the product. Second, although a positive aspect of enrichment is that it detects only viable cells, it can introduce bias by favoring minor microbial groups (8), namely, the detected members of the *P. putida* and *E. faecium* groups, and by failing to induce germination of very dormant spores (12). Third, the presence of inhibitory compounds, such as polyphenols, could have hindered the growth of more sensitive strains (25, 26). Finally, amplification artifacts (27, 28) could also have led to changes in the dominant species in the analyzed samples.

Several common phylotypes were identified at stages of both batches I and III. Standardization of blending and other operations to ensure a consistent brand flavor, 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 the *P. putida* group from samples enriched at 55°C. This is not the first report of thermotolerance in *Pseudomonas* spp. (20), emphasizing the wide adaptive physiological capacity of members of the genus.

ThrS had already been 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 only have been detected by collecting a larger number of samples. Nonetheless, the great heterogeneity in AFLP profiles of *B. subtilis* and *B. licheniformis* suggests that new genotypes made their way into the process.

Strains of the *B. subtilis* complex for which little or no apparent inactivation took place at 110°C for 5 min were present at various positions in the AFLP tree (Fig. 4), indicating that the ability to form spores with high heat resistance can be found in strains with substantially dissimilar genetic makeups. 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 (19).

Scheldeman et al. (32) isolated around 20 different species that survived heat treatment of 100°C for 30 min from milking equipment swabs, of which *Bacillus pallidus*, *Brevibacillus* spp., and *B. licheniformis* were the most widely represented species. *B. licheniformis*, *Bacillus oleronius*, and the *B. subtilis* complex were encountered by Scheldeman et al. (32) and in the present study. Strikingly, they isolated several strains of *Bacillus amyloliquefaciens*. Our results were more in accordance with the study by van Zuijlen (35), where strains of *B. subtilis* and *B. licheniformis* were the species most frequently encountered in soup ingredients and spoiled sterilized soups after isolation treatments at 100°C for 15 min.

In this study, we concluded that the cocoa industry has to cope with microbiota comprising heat-resistant spore formers, specifically from the family *Bacillaceae*, and non-spore formers from the families *Pseudomonadaceae* and *Enterococcaceae*. 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 that could have been initially present in the nibs, as well as microorganisms that originated during processing. However, the cocoa powder samples complied with the industrial guidelines, which specify maximum TAM levels of 3.7 log CFU/g (10).

Future studies may provide additional information by using different enrichment media, including medium compositions containing coadjuvants to neutralize the potential inhibitory effect of polyphenols (25), 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 their relatives.

Although we could not trace thermoresistant spore-forming strains of the *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 with 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-treated chocolate drinks. Spoilage incidents with UHT-treated chocolate drinks have been linked to strains of *B. subtilis*, even though thermophilic spore formers of the genera *Thermoanaerobacterium* and *Moorella* form the most heat-resistant spores reported thus far (4). However, anaerobic spore formers appear to be less important in the ecology of cocoa products (H. Kamphuis, personal communication).

The combined results of this and previous investigations (19) allow us to conclude that, owing to the high heat resistance of *B. subtilis* complex members, in particular *B. subtilis* subsp. *subtilis*, they appear to be the ultimate survivors of the cocoa powder production chain.

ACKNOWLEDGMENTS

L.J.R.L. was supported by the Portuguese Foundation of Science and Technology (grant SFRH/BD721929/2005).

We are grateful to Hans Heilig for his help in setting up the DGGE technique at our laboratory and his expert advice during this study. We also appreciate the assistance of Gert Slot during sampling. We thank Fien Meijer-Dekens for running the AFLP gels and Tjakko Abee for critically reading the manuscript and providing insightful advice.

REFERENCES

1. **Altschul S, Gish W, Miller W, Myers E, Lipman D.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
2. **Anzai Y, Kim H, Park JY, Wakabayashi H, Oyaizu H.** 2000. Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. Int. J. Syst. Evol. Microbiol. **50**:1563–1589.
3. **Barrile JC, Ostovar K, Keeney PG.** 1971. Microflora of cocoa beans before and after roasting at 150°C. J. Milk Food Technol. **34**:369–371.
4. **Byrer DE, Rainey FA, Wiegel J.** 2000. Novel strains of *Moorella thermoacetica* form unusually heat-resistant spores. Arch. Microbiol. **174**: 334–339.
5. **Cazemier AE, Wagenaars SFM, Ter Steeg PF.** 2001. Effect of sporulation and recovery medium on the heat resistance and amount of injury of spores from spoilage bacilli. J. Appl. Microbiol. **90**:761–770.
6. **Chun J, Bae KS.** 2000. Phylogenetic analysis of *Bacillus subtilis* and related taxa based on partial *gyrA* gene sequences. Antonie Van Leeuwenhoek **78**:123–127.
7. **Copetti MV, Iamanaka BT, Frisvad JC, Pereira JL, Taniwaki MH.** 2011. Mycobiota of cocoa: from farm to chocolate. Food Microbiol. **28**:1499–1504.
8. **De Clerck E, De Vos J, De Vos P.** 2004. Molecular characterisation of bacterial contamination in semi-final gelatine extracts, using denaturing gradient gel electrophoresis. Syst. Appl. Microbiol. **27**:612–619.

9. **Devriese LA, Pot B, Collins MD.** 1993. Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. J. Appl. Bacteriol. **75**:399–408.
10. **Dijk R, et al.** 2007. Microbiologie van voedingsmiddelen: methoden, principes en criteria, 4th ed. Noordervliet Media B.V., Houten, The Netherlands.
11. **Edwards U, Rogall T, Blocker H, Emde M, Bottger EC.** 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. **17**: 7843–7853.
12. **Ghosh S, Setlow P.** 2009. Isolation and characterization of superdormant spores of *Bacillus* species. J. Bacteriol. **191**:1787–1797.
13. **Huber T, Faulkner G, Hugenholtz P.** 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics **20**:2317–2319.
14. **International Commission on Microbiological Specifications for Foods.** 2002. Microbiological testing in food safety management. Microorganims in food, vol 7. Kluwer Academic, New York, NY.
15. **ISO.** 2004. International standard ISO 21528-2:2004. Microbiology of food and animal feeding stuffs. Horizontal methods for the detection and enumeration of *Enterobacteriaceae*. Part 2: Colony-count method. International Organization for Standardization, Geneva, Switzerland
16. **Kamphuis H.** 2009. Production and quality standards of cocoa mass, cocoa butter and cocoa powder, p 121–141. *In* Beckett ST (ed), Industrial chocolate manufacture and use, 4th ed. Blackwell Publishing Ltd., Oxford, United Kingdom.
17. **Krapf T, Gantenbein-Demarchi C.** 2010. Thermal inactivation of *Salmonella* spp. during conching. Food Sci. Technol. **43**:720–723.
18. **Lima LJR, Almeida MH, Nout MJR, Zwietering MH.** 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.
19. **Lima LJR, Kamphuis HJ, Nout MJR, Zwietering MH.** 2011. Microbiota of cocoa powder with particular reference to aerobic thermoresistant spore-formers. Food Microbiol. **28**:573–582.
20. **Manaia CM, Moore ERB.** 2002. *Pseudomonas thermotolerans* sp. nov., a thermotolerant species of the genus *Pseudomonas* sensu stricto. Int. J. Syst. Evol. Microbiol. **52**:2203–2209.
21. **Martín R, Heilig GHJ, Zoetendal EG, Smidt H, Rodríguez JM.** 2007. Diversity of the *Lactobacillus* group in breast milk and vagina of healthy women and potential role in the colonization of the infant gut. J. Appl. Microbiol. **103**:2638–2644.
22. **Muyzer G, De Waal EC, Uitterlinden AG.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59**:695–700.
23. **Nubel U, et al.** 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. **178**:5636–5643.
24. **Palmisano MM, Nakamura LK, Duncan KE, Istock CA, Cohan FM.** 2001. *Bacillus sonorensis* sp. nov., a close relative of *Bacillus licheniformis*, isolated from soil in the Sonoran Desert, Arizona. Int. J. Syst. Bacteriol. **51**:1671–1679.
25. **Park CE, Stankiewicz ZK, Rayman MK, Hauschild AHW.** 1979. Inhibitory effect of cocoa powder on the growth of a variety of bacteria in different media. Can. J. Microbiol. **25**:233–235.
26. **Pearson LJ, Marth EH.** 1990. Inhibition of *Listeria monocytogenes* by cocoa in a broth medium and neutralization of this effect by casein. J. Food Prot. **53**:38–46.
27. **Prakitchaiwattana CJ, Fleet GH, Heard GM.** 2004. Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. FEMS Yeast Res. **4**:865–877.
28. **Reysenbach AL, Giver LJ, Wickham GS, Pace NR.** 1992. Differential amplification of rRNA genes by polymerase chain reaction. Appl. Environ. Microbiol. **58**:3417–3418.
29. **Rooney AP, Price NPJ, Ehrhardt C, Swezey JL, Bannan JD.** 2009. Phylogeny and molecular taxonomy of the *Bacillus subtilis* species complex and description of *Bacillus subtilis* subsp. *inaquosorum* subsp. nov. Int. J. Syst. Evol. Microbiol. **59**:2429–2436.
30. **Sánchez-Hervás M, Gil JV, Bisbal F, Ramón D, Martínez-Culebras PV.** 2008. Mycobiota and mycotoxin producing fungi from cocoa beans. Int. J. Food Microbiol. **125**:336–340.
31. **Sanguinetti CJ, Neto ED, Simpson AJG.** 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques **17**:914–921.
32. **Scheldeman P, Pil A, Herman L, De Vos P, Heyndrickx M.** 2005. Incidence and diversity of potentially highly heat-resistant spores isolated at dairy farms. Appl. Environ. Microbiol. **71**:1480–1494.
33. **Tourasse NJ, et al.** 2011. Extended and global phylogenetic view of the *Bacillus cereus* group population by combination of MLST, AFLP, and MLEE genotyping data. Food Microbiol. **28**:236–244.
34. **Van de Peer Y, De Wachter R.** 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comp. Appl. Biosci. **10**:569–570.
35. **van Zuijlen ACM.** 2011. Using ribotyping to trace foodborne aerobic sporeforming bacteria in the factory: a case study. *In* Brul S, Fratamico PM, McMeekin TA (ed), Tracing pathogens in the food chain. Woodhead Publishing Limited, Cambridge, United Kingdom.
36. **Vos P, et al.** 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. **23**:4407–4414.
37. **Wang HY, Zhang XJ, Zhao LP, Xu Y.** 2008. Analysis and comparison of the bacterial community in fermented grains during the fermentation for two different styles of Chinese liquor. J. Ind. Microbiol. Biotechnol. **35**: 603–609.
38. **Witthuhn M, Lücking G, Atamer Z, Ehling-Schulz M, Hinrichs J.** 2011. Thermal resistance of aerobic spore formers isolated from food products. Int. J. Dairy Technol. **64**:486–493.