Research Paper

Enumeration and Identification of Bacterial Spores in Cocoa Powders

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ABSTRACT

The presence of bacterial spores in cocoa powders is inevitable due to the cocoa bean fermentation process, during which members of the genera *Bacillus* and *Geobacillus* are typically present. Spores are a concern in heat-treated foods when they survive heat treatments and the finished product supports germination, growth, and potentially toxin production. In this study, available methods for the enumeration of total mesophilic and thermophilic spores (TMS and TTS, respectively) were evaluated, leading to the recommendation of one global method specifically for cocoa powders. The proposed method was validated during a ring test on seven selected cocoa powders and applied during routine analyses on commercial powders. The method includes dilution of cocoa powder using buffered peptone water, heating at 80°C for 10 min for TMS and TTS counts, and heating at 100°C for 30 min for a heat-resistant (HR) spore count. Tryptic soy agar is used as a recovery medium with a maximal concentration of cocoa powder of 2.5 mg/mL (to prevent growth inhibition) and a nonnutrient agar overlay to prevent swarming of bacteria. Plates are incubated for at least 72 h at 30°C for recovery of mesophilic bacteria and 55°C for thermophilic bacteria. Suitable alternatives to specific method parameters are provided. Median values of total spore concentrations are low (<400 CFU/g for TMS and <75 CFU/g for TTS), and concentrations of HR spores are very low (<5 CFU/g). Importantly, the relation between concentrations of HR spores in cocoa powder and incidence of spoilage of heat-treated beverages containing cocoa is currently unclear. In the powders included in this study, *Bacillus subtilis* and *Bacillus licheniformis* were the predominant spore-forming species identified (49 and 39%, respectively). Both species are known for high variability in spore heat resistance. The development of reliable and sensitive molecular methods is therefore required to assess the risk of spoilage caused by spores present in cocoa powders.

HIGHLIGHTS

- One recommended method for the enumeration of spores in cocoa is presented.
- The potential antimicrobial effect of cocoa can cause underestimations of counts.
- Concentrations of HR spores in cocoa are usually below the LOD (<100 CFU/g).
- The main bacterial species found in cocoa powders belong to the *B. subtilis* complex.

Cocoa powder is used globally as an ingredient in various processed foods and beverages. The cocoa powder is obtained by grinding the cocoa cake that has been separated from the cocoa butter in cocoa liquor, which has in turn been derived from cocoa beans. Cocoa powder is (in most cases) treated with an alkali substance for flavor and color development. Cocoa beans are fermented, dried, and roasted prior to grinding, to achieve optimal features of cocoa with respect to flavor and taste *(18)*. The fermentation process generally takes place at the location of cocoa bean harvest and involves a heap or box fermentation with microorganisms that are naturally present, such as yeasts, lactic acid bacteria, acetic acid bacteria, and toward the end of the fermentation, *Bacillus* species *(11)*. The latter group is able to produce heat-resistant spores, some of which may be able to survive after harvest and industrial processing *(19)*. This may pose issues for the stability of processed foods.

Spore-forming bacteria can be grouped according to the heat resistance of their spores and the range of growth

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temperatures of the organism. Generally, total spore concentrations are established by applying a heat treatment of 10 min at 80°C and plating on a suitable recovery medium, followed by incubation at 30 to 37°C for total mesophilic spores (TMS) or at 55°C for (facultative) total thermophilic spores (TTS). Concentrations of spores with a high heat resistance are determined by applying a heat treatment of 30 min for 100°C, followed by incubation at 30 to 37°C for heat-resistant (HR) mesophilic spore (HRMS) formers and at 55°C for HR thermophilic spore (HRTS) formers. Generally, the total concentrations of spores in cocoa powders range from below detection level to a maximum of 3.7 log CFU/g for TMS and a maximum of 3.1 log CFU/g for TTS *(19)*. The specification used in the cocoa industry to assess the overall microbial quality of powders is a total microbial count of 5,000 CFU/g, and no spore-specific specifications are currently in place. The concentrations of HR spores in cocoa powders are often very low, <10 CFU/g, which is below the detection limit of classical plating techniques. A previous study by Lima and coworkers *(19)* showed the presence of HRMS in 9 of 25 cocoa samples (ranging from 1.0 to 1.8 log CFU/g) and the presence of HRTS in 3 of 25 cocoa samples (ranging from <1 to 1.4 log CFU/g), as determined by a most-probable-number approach.

Routine microbial quality assessment of cocoa powders is generally performed using classical plating methods. An inventory of methods (as performed by the authors of this article) showed that over 35 different methods are applied within the market to enumerate total and HR spores in cocoa powders, including published standard methods for the microbiological examination of foods in general *(15)* and cocoa specifically *(10, 12)*. The methods mainly differ in the volumes of diluent used to dilute a sample, the heating parameters applied, the plating media used, and the incubation parameters applied for enumeration of meso-philic spore-forming species (e.g., ranging from 30 to 37°C and 48 to 72 h). The application of different methods influences the analytical outcome of spore counts in food ingredients and complicates interpretations of results in relation to risk assessments for the quality and stability of finished products *(8, 17)*. In addition, specifications for maximum spore concentrations in cocoa powder for quality control purposes are determined by customer demands and often vary among companies. Importantly, the low concentrations of HR spores that are generally detected in cocoa powder do not correlate with the occurrence of spoilage of ultrahigh-temperature (UHT)–treated liquid products.

There is a great need to reach agreement on the use of one standardized method for the enumeration of at least TMS and HRTS in cocoa powders. In addition, the agreed method should provide a clear overview of maximal concentrations and acceptable variations in spore counts as determined during routine microbiological testing of cocoa powders. In this study, various parameters of the desired method were tested and compared, including the influence of (i) different cocoa powder properties, (ii) liquids used for the dilution of cocoa powder, (iii) cocoa concentration, and (iv) incubation time and temperature combinations. A resulting "consortium method," with

TABLE 1. *List of cocoa powders used in this study*

Powder	pH[a]	% fat	Fineness (% <75 μm)[b]
NIZO2	8	23	99.7
NIZO7[c]	5.9	11	99.1
NIZO8	7.8	11	99.7
NIZO11	Unknown	11	Unknown
NIZO18	5.6	23	99.7
NIZO19	7	11	99.9
NIZO20	7.8	11	99.8
NIZO21	8.1	21	99.8

[a] The pH of the powders was determined by the provider of the cocoa powder by dispersion of cocoa powder in water and measuring the pH of the mixture.

[b] Fineness is the percentage of particles <75 μm, as determined by microsieving.

[c] This powder was not included in the ring test due to limited availability.

variations specifically for the enumeration of TMS, TTS, HRMS, or HRTS, was then validated by means of a ring test including eight separate laboratories, using seven selected cocoa powders with different physical properties (pH and fat content) and relatively high spore loads. Recovered bacterial sporeformers were identified to generate an overall assessment of the microbial spore-forming species that were present in all powders. In addition, the consortium method was applied by cocoa-producing companies that were involved in this study to assess concentrations of TMS and HRTS in cocoa powders.

The results are discussed in view of known heat resistance properties of spores of identified species and current hypotheses on the role of spores in cocoa in relation to spoilage incidents for UHT-treated beverages. Through this work, we offer a recommendation for the method (including specific variations) that should be applied for the enumeration of spores of aerobic bacteria in cocoa powders. The concentrations found when using this method are put in the context of the use of cocoa powder in heat-treated milk-based beverages.

MATERIALS AND METHODS

Cocoa powders. Cocoa powders were collected by the authors of this publication. Different powders, with a variety of levels of acidity and fat content, were specifically selected based on the fact that they contained elevated concentrations of spores. In total, eight powders were included in the method development and ring test, as listed in Table 1. The use of natural cocoa powders with spores was preferred over cocoa powders with spiked concentrations of laboratory-produced spores for the following three reasons.

(i) Producing spores in the laboratory and adding them to cocoa powders (e.g., after freeze drying) will influence the spore heat resistance in a nonnatural manner, causing differences in analytical outcome compared with the outcome with naturally present spores.

(ii) Mixing (freeze-dried) spores in a homogeneous manner in the cocoa powder is very challenging due to dust formation and the stickiness of cocoa powder. This could lead to great variations

in spore concentrations within cocoa batches and also between different samples of the same powder.

(iii) Adding spores to cocoa powders would be too different from the natural situation, where spores are formed during cocoa bean fermentation processes and are uniquely distributed within the cocoa powder during cocoa powder manufacturing (e.g., within cocoa butter particles), which cannot be reconstructed in the laboratory.

Dilution of cocoa. Per powder, 1.2-kg samples were mixed for 15 min using a standard drum hoop mixer at 2,800 to 250 rpm (Engelsmann Ludwigshafen, Germany) to obtain a homogenized mixture. Mixed powders were then aliquoted in 100-g samples in Gosselin high-density-polyethylene (HDPE) bottles (250 mL, 37-mm red cap with seal; Corning, Corning, NY) and stored at room temperature under dark conditions. Of the mixed aliquots, 10 g per sample was diluted by adding 90 mL of prewarmed (40°C *(12)*) buffered peptone water (BPW; per liter, 10 g of peptone, 5 g of NaCl, 9 g of $Na_2HPO_4 \cdot 12H_2O$, and 1.5 g of KH_2PO_4, pH 7.0 ± 0.2 *(12, 15)*), peptone physiological salt (PPS; per liter, 1 g of enzymatic digest of casein and 8.5 g of NaCl, pH 7.0 ± 0.2 *(15)*), or lactose broth (per liter, 5 g of peptone, 3 g of beef extract, and 5 g of lactose, pH 6.9 ± 0.2 *(26)*) in a sterile plastic (standard) stomaching bag (BA6041 Stomacher 400 classic standard bag, Seward, West Sussex, UK). Before taking the test portion, the laboratory sample should be shaken by hand or by mechanical means to ensure that the microorganisms are uniformly distributed (at least 30 s of vigorous mixing). The samples were homogenized in a stomacher (Stomacher 400 circulator, Seward) for 30 s at 230 rpm. After 30 s, the sample was inspected visually to see if any lumps were present. If so, the stomaching procedure was prolonged for another 30 s.

Heat treatments. Immediately after dilution (i.e., no waiting time), 10 mL of diluted sample was transferred to a sterile glass test tube (160 by 16 mm, thin walled, sterile, with cap) and heated directly to prevent germination of the spores. Samples were heated in a water bath for 10 min at 80 ± 1°C (total spores) or for 30 min at 100 ± 2°C (HR spores). (Note that when the method is applied at high altitude where water boils at [for instance] 95°C, a pressure pan or oil bath should be used to ensure a boiling temperature of 100 ± 2°C. Alternatively, the heating time can be prolonged to obtain the same F_0 value [number of minutes at 121°C] heat load. Assuming a z-value of 10°C, this means that the heating time should be extended to 2 h at 94 ± 1°C.) The level of the water was 2 to 3 cm higher than the suspension in the tube *(12)*. The time was started when the dummy (test tube in the water bath containing water with a thermometer) reached the desired temperature. After the indicated heating time, the tubes were taken out of the water bath and immediately (within 10 s) cooled under running tap water to <14°C.

Spore enumeration. The cooled sample was mixed using a vortex and, within 20 min after cooling, plated by transferring 1 mL to a sterile petri dish (90 mm) and adding 18 to 20 mL of liquid tryptic soy agar (TSA; per liter, 15 g of pancreatic digest of casein, 5 g of enzymatic digest of soya bean, 5 g of sodium chloride, and 15 g of agar *(8)* in duplicate [referred to as the -1 plate]). Alternatively, dextrose casein-peptone agar with the addition of bromocresol purple (BCP) was used (GCA; per liter, 10 g of peptone from casein, 5 g of D-(+)-glucose, 0.04 g of BCP, and 12 g of agar-agar). In addition, 1 mL of the sample was first diluted in 9 mL of sterile BPW and then plated similarly (referred to as the -2 plate). The samples were homogenized with the liquid

agar by a gentle circling motion to ensure homogeneous distribution of the cocoa, and plates were allowed to solidify at room temperature. Alternatively, two 0.5-mL samples or one 1-mL sample was transferred to two 90-mm or one 140-mm petri dish, respectively, and 18 to 20 mL of ±50 mL liquid TSA was added, respectively (-1 plate). Plates were allowed to solidify at room temperature. A nonnutrient (15% agar in sterile water *(14)*) overlay (10 mL for a 90-mm dish and 20 mL for a 140-mm dish) was added to the plate and allowed to solidify at room temperature. Duplicate plates were incubated aerobically at 30 ± 2°C (mesophiles) or 55 ± 2°C (thermophiles) for 72 h. Colonies were visualized using a light box and recorded as CFU. Importantly, and in contrast to what is regularly applied in practice, counts of <10 were not excluded in this study. When applicable, CFU per gram of cocoa powder was calculated according to a previously described method *(13)*. In brief,

$$N = \frac{\sum C}{V \times [n1 + (0.1 \times n2)] \times d}$$

where $\sum C$ is the sum of the colonies from all plates selected for the calculation from two successive dilutions, V is the volume of the inoculum placed on each dish (1 mL), $n1$ is the number of plates from the first dilution selected (2), $n2$ is the number of plates from the second dilution selected (2), and d is the dilution corresponding to the first dilution retained (10^{-1}).

Statistics. Data comparisons between multiple measurements of two test conditions were performed using an unpaired *t* test with a two-tailed *P* value in GraphPad Prism (version 5.01, GraphPad Software, Inc., San Diego, CA). A *P* value cutoff of 0.05 was applied for significance.

Bacterial identification using Vitek MS and MALDI Biotyper. Bacterial colonies were isolated from spore enumeration plates, transferred to fresh TSA plates, and incubated aerobically at 30 ± 2°C (mesophiles) or 55 ± 2°C (thermophiles). Clonality of isolated colonies was confirmed visually, after which they were stored in storage tubes (CryoBak, Copan Diagnostics, Murrieta, CA) for identification using mass spectrometry (MS; Vitek MS, bioMérieux Diagnostics, Marcy-l'Étoile, France) or matrix-assisted laser desorption ionization (MALDI) MS (MALDI Biotyper, Bruker Daltonics, Bruker Scientific Instruments, Billerica, MA) according to the manufacturer's instructions. For Vitek identification, each bacterial isolate was applied to two sample spots of a disposable barcode-labeled target slide (Vitek MS-DS, bioMérieux) by using a 1.0-μL loop, immediately overlaid with 1.0 μL of matrix solution (Vitek MS-CHCA, bioMérieux), and air dried. For instrument calibration, an *Escherichia coli* reference strain (ATCC 8739) cultured on TSA with 5% sheep blood was transferred to designated spots on the target slide using the procedure described above. After drying, the target slide was placed into the device and analyzed using the bioMérieux platform Myla. Samples were retested once upon a failed identification. Confirmation of identification on a subset of isolates, as well as a subset of samples that could not be identified by the Vitek MS, was performed by genetic identification by BNC Laboratories (Knoxville, TN) based on 16S rRNA gene sequencing.

For Biotyper identification, isolated colonies were smeared onto a position on a MALDI plate (Bruker Daltonics), overlaid with 1 μL of matrix (alpha-cyano-4-hydroxycinnamic acid, Bruker Daltonics), and left at room temperature until completely dry (maximum of 30 min). The manufacturer's bacterial test standard was used for instrument calibration. For each strain, two

TABLE 2. *Recommended method (consortium method) for the enumeration of aerobic bacterial spores specifically in cocoa powders*

Parameter	Description[a]
Test sample	10 g of cocoa powder, preferably from 1 kg of homogenized (mechanical mixing) sample
Diluent	BPW *(12, 15)*, 90 mL, prewarmed to 40°C
Dilution method	Add diluent to powder in stomaching bag, mix in stomacher for 30–60 s at 230 rpm
Sample	10 mL of hydrated sample in glass test tube *(12)*
Heat treatment	TS count, 10 min at 80 $\pm$ 1°C *(12, 15)*; HR spore count, 30 min at 100 $\pm$ 2°C in water bath; both counts, cool samples under running tap water *(12, 15)* or ice water to <14°C
Plating volume	1 mL on 18–20 mL of agar in 90-mm dish; for reliable 10^{-1} dilution, 0.5 mL each on 18–20 mL of agar in two 90-mm dishes or 1 mL on 43–45 mL of agar in 140-mm dish
Plating medium	TSA *(8)* in pour plates with nonnutrient agar overlay to prevent swarmers
Plating dilutions	TMS, 10-fold and 100-fold; TTS, HRMS, and HRTS, 10-fold
Incubation	Mesophiles, 30 $\pm$ 2°C for 72 h; thermophiles, 55 $\pm$ 2°C for 72 h *(12, 15)*

[a] Details of execution of the method are in "Materials and Methods." BPW, buffered peptone water; TS, total spores; HR, heat resistant; TSA, tryptic soy agar; TMS, total mesophilic spore count; HRMS, heat-resistant mesophilic spore count; TTS, total thermophilic spore count; HRTS, heat-resistant thermophilic spore count.

preparations of sample material were analyzed. Identification was based on the built-in library of the instrument. Scores were provided for each strain analyzed. Scores above 2.0 present a highly confident identification, while scores between 1.7 and 2.0 suggest a limited confidence in identification.

RESULTS

Consortium method for the enumeration of spores in cocoa powders. To reach agreement on a recommended method for the detection of total spores and HR spores of mesophilic and thermophilic bacteria in cocoa powders, specific parameters of such a method were first tested using a limited number of powders, namely, NIZO07, NIZO08, NIZO18, and NIZO21. In this test, cocoa powders with different properties (such as pH and fat content) were used, the influence of two frequently used diluents (PPS and BPW *(12, 15)*) was evaluated, and the most suitable incubation time was established. First, visible colonies on cocoa-containing plates were best observed after 72 h of incubation at either 30 or 55°C (data not shown). pH measurements of diluted cocoa powder prior to heating furthermore showed that BPW had a buffering effect specifically on nonalkalized powders (Supplemental Table S1), although no significant ($P < 0.05$) differences in spore counts were observed between samples that were diluted in PPS or BPW (Table S2). Importantly, for both sample types, a nonlinear relation between counts in the serial dilutions (10-fold and 100-fold) was observed for all powders (Table S2). This is assumed to result from compounds with inhibiting effects on microbial growth that are present in cocoa; the concentrations are lower in plates containing 10-fold-diluted cocoa (5 mg of cocoa per mL of agar medium) than in plates with 100-fold-diluted cocoa (0.5 mg of cocoa per mL of agar medium) *(6, 25)*. The inhibitory effect of cocoa was also observed when lactose broth was used as an alternative diluent (data not shown). In summary, the presence of ≥5 mg of cocoa per mL in agar plates likely results in underestimations of actual spore numbers that are present, especially when such numbers are low.

Interpretability of agar plates can be highly complicated when bacterial cells start to swarm across the agar layer.

Typically, bacteria with flagella (including many *Bacillus* species) have the ability to swarm, which is the coordinated group movement of bacterial cells through thin liquid films on surfaces *(21)*. To prevent such swarming, an agar overlay is commonly applied on top of the agar layer containing the sample. In this study, the appearance of swarmers after the application of a nutrient (TSA) and a nonnutrient (water) agar overlay was investigated. The number of swarmers detected after application of a nutrient overlay was significantly higher (11 of 96 plates) than after application of a nonnutrient overlay (1 of 96 plates) (data not shown). The recommended method (including specific method variations) for the detection of TMS, TTS, HRMS, and HRTS in cocoa powders is summarized in Table 2 and was evaluated in a coordinated ring test.

Ring test results. Evaluation of the recommended method for the enumeration of bacterial spores in cocoa powders involved eight different laboratories and included seven different cocoa powders (i.e., all of the cocoa powders listed in Table 1, with the exception of NIZO07). Variation in colonies on the plates with the lowest dilution (10-fold) was expected (due to the previously observed cocoa inhibitory effect), and low counts (<10 CFU/g) for several powders were expected. To represent the observed variation in analytical outcome between the different laboratories in the most intuitive manner, counts were reported as actual counts on plates and were not converted to CFU per gram.

An overview of the variation in results generated by the individual laboratories is shown in Figure 1, which is based on data on spore counts provided in Table S3. The results clearly show the highest degree of variation in counts for TMS when 1 mL of the 10-fold-diluted sample was plated using dishes with a diameter of 90 mm (Fig. 1, white boxes). Such variation in counts is much lower when determined using the 100-fold-diluted samples (Fig. 1, grey boxes). This is best demonstrated for cocoa powders with relatively high spore concentrations (NIZO08, NIZO20, and NIZO21). Additional data from one laboratory on spore counts determined using large plates (140 mm) show that, in

FIGURE 1. *Box plot representation of spore counts (CFU) on plates generated after execution of the consortium spore detection method by eight separate laboratories on seven different cocoa powders (also see Table S3). The counts on plates (y axis) are reported for two dilutions of hydrated cocoa (white, d1 [10-fold], and grey, d2 [100-fold]) after two different heat treatments (H, in °C) and two different incubation temperatures (I, in °C) (x axis). The median value is shown in each box as a horizontal bar, with data variation into the first and third quartiles extending to the outer edges of the boxes (25th to 75th percentile). The whiskers and outliers are represented according to the Tukey method.*
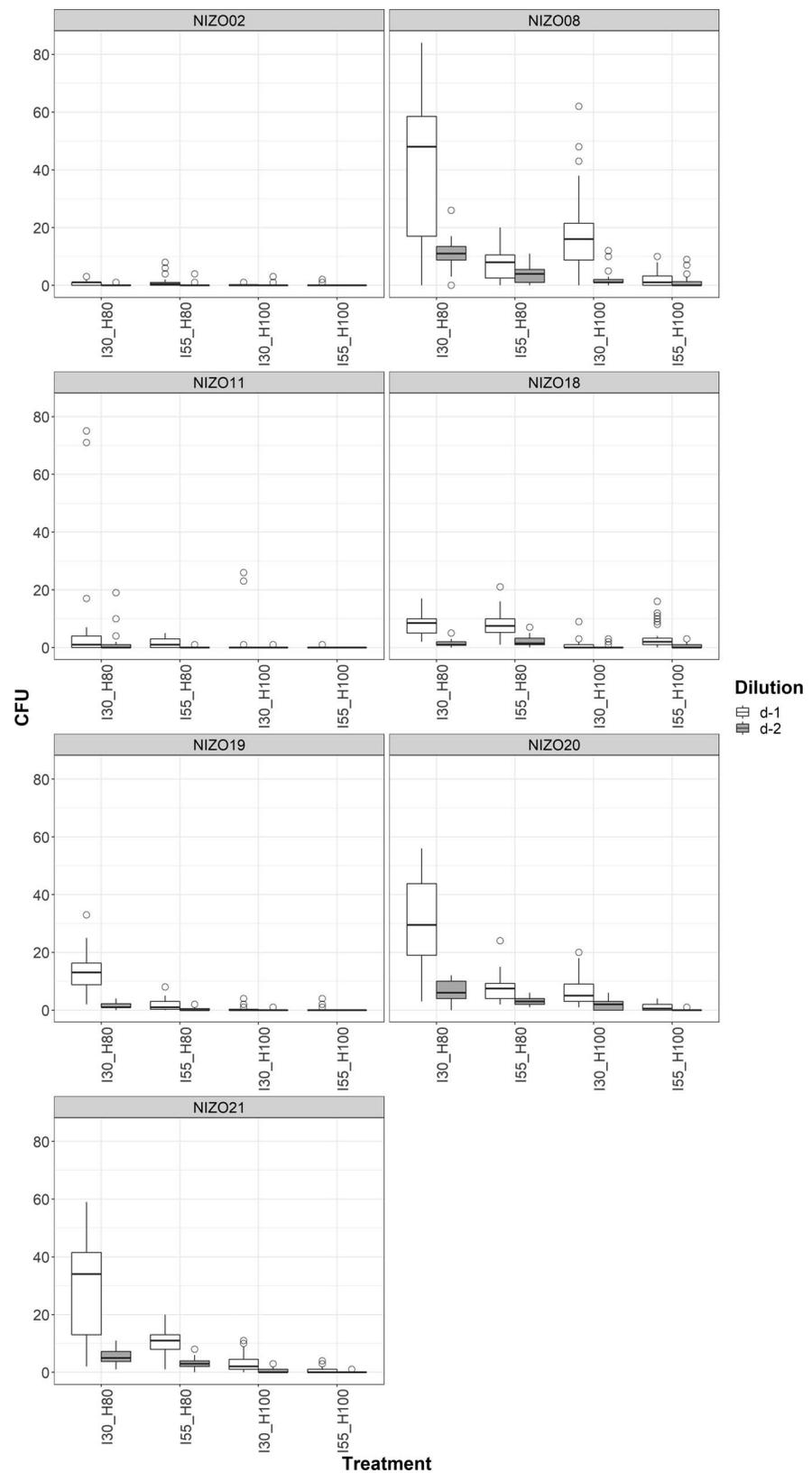
almost all cases, counts are increased compared with counts using regular dishes (90 mm), with differences exceeding 3-fold on three occasions (Table S4). This confirms our previous observation of a cocoa inhibitory effect on bacterial germination and/or outgrowth that is lifted when cocoa concentrations in plates are reduced to below 5 mg/

mL. The consortium method was thus adapted to always plate 1 mL of the 10-fold dilution on large dishes (140 mm) or two 0.5-mL amounts of the 10-fold dilution on regular-size dishes (90 mm). In practice, BCP was sometimes added to GCA plates to increase the reliability of colony counts (Table S5). BCP is a pH indicator that turns yellow when
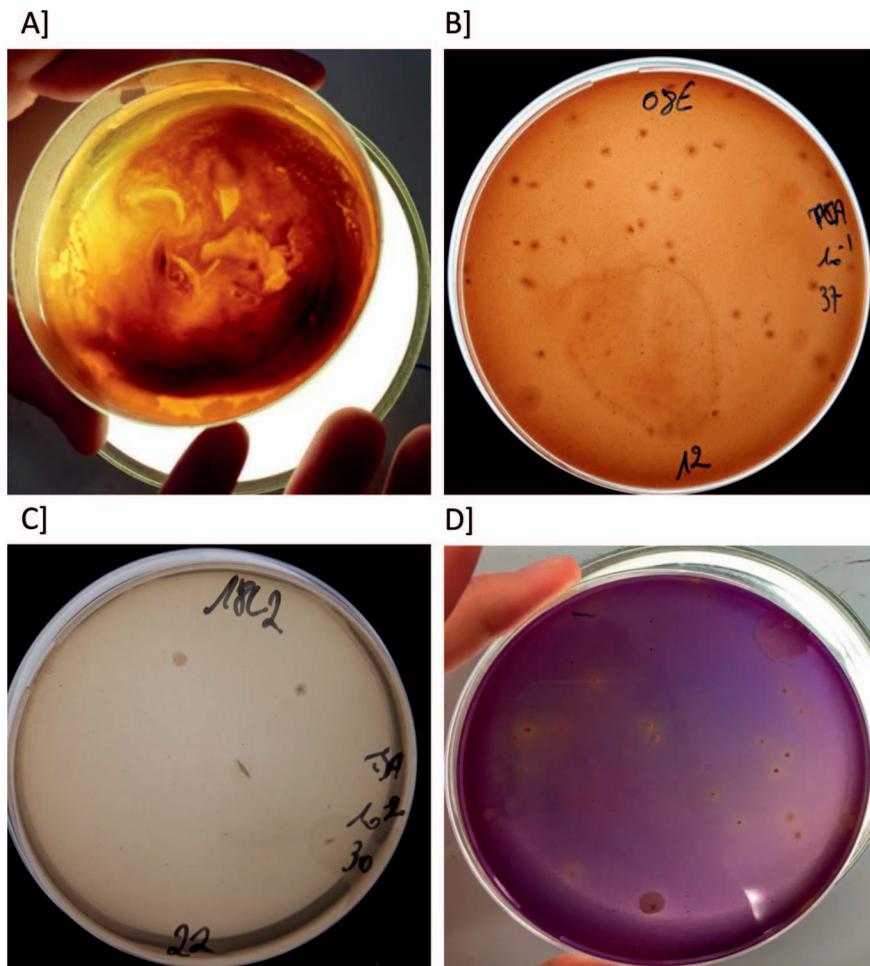
FIGURE 2. *Photos of plates taken after execution of the consortium method for detection of bacterial spores in cocoa. (A) Failure to generate pour plates in which the cocoa is well distributed will lead to dark or very dark patches of cocoa in the agar, complicating the distinction of bacterial colonies in the agar. (B) Example of a well-distributed 10-fold-dilution pour plate, for which a light box is required to clearly visualize bacterial colonies. (C) Example of a well-distributed 100-fold-dilution pour plate, in which the minimal concentration of cocoa enhances readability of colonies in the plate. (D) Using pour plates of GCA with added BCP increases visibility of colonies by a purple-to-yellow color transition.*

the available glucose in the medium is consumed by the bacterial cells, leading to an acidification of the agar in the immediate vicinity of the colony. This addition can help in the distinction between bacterial colonies and cocoa particles on plates, especially when alkalized powders are used (i.e., dark-colored plates as a result of the presence of cocoa). One should, however, take into account that not all bacterial colonies on the plate cause such acidification and that all visible colonies should be counted, even when colonies are not surrounded by a yellow halo.

Figure 2 shows a selection of photos from plates that illustrate the increased complexity of distinguishing bacterial colonies when samples are not distributed in a homogeneous manner (Fig. 2A), which is improved when (i) cocoa is distributed homogeneously in the agar (Fig. 2B), (ii) the concentration of cocoa is decreased (Fig. 2C), or (iii) BCP is added to GCA plates (Fig. 2D).

Identification of isolates on plates. To obtain an overview of bacterial spore-forming species present in the cocoa powders included in this study, a total of 369 colonies obtained during the ring test were identified using MALDI-time of flight (TOF) MS analyses and, in some cases, genetic identification (Tables 3 and S6). These results clearly show a predominant presence of *Bacillus* spp. (94% of all colonies identified), the majority of which are *Bacillus subtilis* (*amyloliquefaciens/vallismortis*) and *Bacillus li-*

cheniformis (88% of all colonies identified). The identifications of species belonging to the *B. subtilis* complex were further distinguished by 16S rRNA sequencing. Several strains of these facultative thermophilic species were able to

TABLE 3. *Abundances of spore-forming species identified in the cocoa powders included in this study*

Taxon[a]	Value (%) for[b]:			
	TMS	TTS	HRMS	HRTS
B. subtilis/amyloliquefaciens/ vallismortis	32.2	0.0	16.0	1.2
B. licheniformis	17.8	13.3	5.0	2.4
B. horneckiae	0.3	0.0	0.0	0.0
B. circulans	0.9	0.0	0.0	0.0
B. clausii	1.8	0.0	0.0	0.0
B. smithii	0.0	0.3	0.0	0.0
B. thermoamylovorans	0.0	0.3	0.0	0.3
B. altitudinis/pumilus	2.7	0.0	0.0	0.0
G. thermodenitrificans	0.0	0.6	0.0	0.6
Aeribacillus pallidus	0.0	0.6	0.0	0.0
Thermoactinomyces spp.	0.0	3.8	0.0	0.0

[a] *B.*, *Bacillus*; *G.*, *Geobacillus*.
[b] Abundances are based on colony identifications provided in Table S6. TMS, total mesophilic spores; TTS, total thermophilic spores; HRMS, heat-resistant mesophilic spores; HRTS, heat-resistant thermophilic spores.

grow at both 30 and 55°C and produce spores with high heat resistance (surviving 30 min at 100°C). Other species that are known to be able to produce spores with high heat resistances were also detected, albeit with lower frequencies. This included *Bacillus thermoamylovorans* and *Geobacillus thermodenitrificans,* both of which were detected after incubation at 55°C.

In addition to sporeformers, a few nonsporeformers were identified on nine occasions (Table S6). Colonies from *Staphylococcus* spp. and *Micrococcus luteus* were found in plates from four different cocoa powders and were identified at two different geographical locations (Europe and the United States), using two different MALDI-TOF MS databases. Despite the identification of such species in multiple powders by different laboratories, the presence of nonsporeformers in the plates is best explained by the manual labor involved in producing cocoa powders or by postprocessing laboratory contamination (considering the heat treatment that the cocoa samples endured prior to plating). The frequency of such species identified was extremely low, and thus, no significant influence on total spore counts is expected. Also, there is no indication that these species survive UHT treatment of cocoa-containing products.

Spore concentrations regularly detected in cocoa powders. The consortium method for the detection of bacterial spores, especially HR spores, in cocoa powders was applied during routine microbiological testing of additional cocoa powders in two separate laboratories. Variations in concentrations of TMS, TTS, HRMS, and HRTS detected are shown in Figure 3. No significant differences ($P < 0.005$) in spore concentrations in the same powders were observed when GCA with BCP was used instead of TSA as a recovery medium (data not shown), indicating that this recovery medium serves as a suitable alternative to TSA. The results show that, even for cocoa powders specifically selected to contain spores that exceed regular levels, median values of spore concentrations are relatively low (<400 CFU/g for TMS and <75 CFU/g for TTS). The concentrations of spores with high heat resistance (surviving 30 min at 100°C) are very low (<5 CFU/g) and are thus below the detection limit of classical plating methods.

DISCUSSION

Bacterial spores are naturally present in cocoa powders, either through cocoa bean fermentation processes or via postroasting contamination (20). In the cocoa powders used in this study, *B. subtilis* and *B. licheniformis* were the predominant spore-forming species identified (49 and 39%, respectively), which is in agreement with previous studies on spores in cocoa (19, 22). These species are notorious for showing great variations in spore properties (27), and some individual strains are known to produce spores with very high heat resistance (3, 4, 19). Some isolates of these species were shown to produce spores with decimal reduction times (D-values) at a given temperature similar to those of the thermophilic sporeformer *Geobacillus*
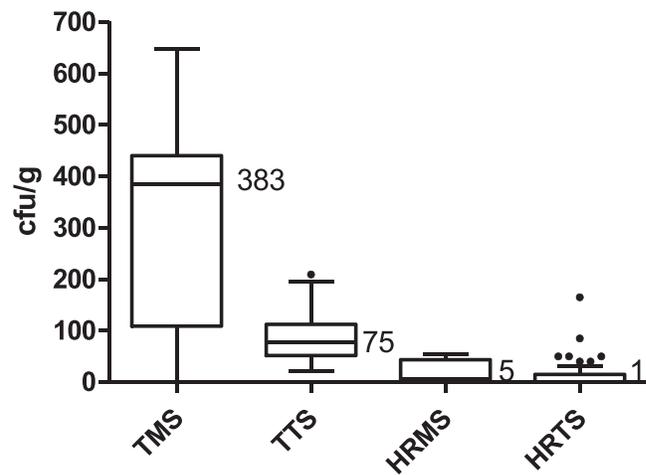

FIGURE 3. *Box plot representation of concentrations of total mesophilic spores (TMS; 41 measurements), total thermophilic spores (TTS; 20 measurements), heat-resistant (HR) mesophilic spores (HRMS; 20 measurements), and HR thermophilic spores (HRTS; 41 measurements) detected in selected cocoa powders. Spore concentrations (CFU/g) were determined through routine implementation of the consortium spore detection method (Table 2) on a specific selection of available cocoa powders. The median value is shown in each box as a horizontal bar and is furthermore specified in the graph. Data variations into the first and third quartiles are represented by the boxes (25th to 75th percentile). The whiskers and outliers are represented according to the Tukey method.*

stearothermophilus (7, 8), which is a notorious spoilage organism in both the dairy industry (23) and the canning industry (1). Spoilage by this microorganism mainly occurs at temperatures of >40°C, as most isolates of this species are thermophilic (28). This is not the case for *Bacillus* spp., which can also grow efficiently at ambient temperatures.

Reliable enumeration of spores, particularly HR spores, in cocoa powders is important for assessing the spore-specific microbial quality of these powders that are used to produce finished beverages. The efforts described in this study have resulted in a recommendation for one general method (with specific method parameters for the detection of TMS, TTS, HRMS, and HRTS) for this purpose (Table 2), with the following notes: (i) BPW is the recommended diluent used for the dilution of cocoa, but PPS may be used as a suitable alternative; (ii) TSA is the recommended recovery medium, as this medium was shown to support germination and growth of the largest variety of *Bacillus* and *Geobacillus* species (8); (iii) to increase the visibility of CFUs on cocoa-containing plates, GCA plus BCP may be used as an alternative plating medium, although this is not the preferred medium; (iv) the concentration of cocoa should be diluted to ≤2.5 mg/mL by plating two 0.5-mL amounts of the 10-fold dilution on 90-mm dishes or 1 mL on a 140-mm dish to prevent microbial growth inhibition on the plates (Fig. 1 and Table S4); and (v) a nonnutrient agar overlay should be added to the pour plates to prevent swarming of recovered bacteria and increase readability of the plates.

Variability in counts during the application of classical plating methods is common, due to technical variations (within and between laboratories) and biological variations

(within and between cocoa powder samples and bacterial species and strains). Specifically, for spore detection in cocoa powders, technical variation is increased when cocoa concentrations exceed 2.5 mg/mL (Fig. 1). To minimize technical variation in spore counts, the recommended method described in this manuscript includes dilution of cocoa concentrations and application of an agar overlay to prevent swarming of bacteria on plates (points (iv) and (v) mentioned above, respectively).

Importantly, the correlation between concentrations of spores, especially HR spores, in cocoa and the incidence of spoilage of UHT-treated beverages that contain cocoa as an ingredient is currently unclear. The concentrations of highly HR spores in cocoa powders are very low (<10 CFU/g). During execution of the ring test using selected powders (Table 1), 141 plate counts of a total of 1,723 (as performed by the different laboratories) were reported to be >10 CFU on plates for HRMS (counts of <10 are deemed unreliable during routine microbiological testing and are generally not included (13)). Such counts were only reported for four of seven powders (10-g samples), even though all cocoa powders included in the ring test were specifically selected to contain elevated levels of bacterial spores. For HRTS, only 6 of 1,692 reported counts were >10 CFU on plates, for only two of the seven powders that were selected (Table S3). During routine spore testing using other available cocoa powders, the median concentration of HRMS that was detected was 5 CFU/g, against 1 CFU/g for HRTS (Fig. 3). Importantly, such concentrations are calculated from counts that are <10 CFU on plates, which would normally not be reported due to unreliability and being below the detection limit of the plating assay. Notably, all cocoa powders included in this study were specifically selected based on detectable concentrations of total spores. Therefore, the actual concentrations of HRMS and HRTS are expected to be even lower in practice.

During the production of chocolate milk, cocoa powder is added at approximately 1% (w/v) to milk. The product is subsequently heated. Depending on the heat resistance of spores and the heat treatment applied, spores may survive the heating process and cause spoilage of finished product, even if only one spore per packaging unit survives. Based on the analyses of this study and previous studies (19, 22), the majority of spore-forming species encountered in cocoa belong to the *B. subtilis* group. For *B. subtilis,* two subgroups can be distinguished based on heat resistance of spores (4, 7), with one group producing spores that are relatively heat sensitive and another group producing spores that are relatively heat resistant (HRMS). Each subgroup shows different inactivation kinetics, for which the cause was defined to be the presence of a genetic element ($spoVA^{mob}$) present only in the subgroup of *B. subtilis* strains that produce spores with high heat resistance (2).

We would like to illustrate the relevance of the findings and conclusions of this study for industry by demonstrating a hypothetical worst-case scenario on risk of spoilage of UHT-treated chocolate milk: (i) a total of 1,000 packs (1-L volume) of chocolate milk is produced with 1% (w/v) cocoa powder; (ii) the cocoa powder contains a relatively high level of contamination of 10 CFU of HRMS per g (as

TABLE 4. *Expected spoilage rate in liquid product under optimal growth conditions*

| Spore contamination | Expected % of 1-L packs spoiled at[a]: | | | | | |
| | F_0 5 | | F_0 10 | | F_0 15 | |
	Mean	+95	Mean	+95	Mean	+95
B. subtilis HR group	0	100	0	92	0	9
G. stearothermophilus	82	100	1	100	0	14

[a] The expected spoilage rates are based on mean and upper 95% inactivation kinetics when applying different heat treatments (F_0, min at 121°C), considering a high-level contamination of 10 CFU of *Bacillus subtilis* spores per g (HR group (4)) or *Geobacillus stearothermophilus* in cocoa powder and assuming equal distribution of surviving spores in 1,000 1-L packs.

determined by the method described in Table 2), which leads to 100 HRMS/L in the product; (iii) all the spores belong to the HR subgroup of *B. subtilis* strains (4); (iv) the prepared product is heat treated using a relatively low F_0 value of 5 (equivalent to 5 min at 121°C); and (v) all surviving spores are distributed equally among all filled packs. Such low heat treatment would still be sufficient to inactivate all spores in case these spores express the mean heat resistance (*D*-value) and, thus, no spoilage will be observed (Table 4). However, if all spores in the powder were to show extremely high-level heat resistances (i.e., upper 95%), then all 1,000 packs are expected to spoil after an F_0 5 heat treatment. An arbitrary heat treatment of F_0 15 (equivalent to 15 min at 121°C) would still result in spoilage but would reduce the expected number of spoiled packs to 9% (Table 4). Notably, these predictions assume full exposure of the spores to wet heat treatment and the most favorable conditions for spore germination and (out)growth.

In cocoa, the species detected after incubation at 55°C (HRTS) are expected to be largely the same as the species detected at 30°C (due to facultative thermophilic growth properties of *Bacillus* species members). It must be noted, however, that other spore-forming species with different inactivation kinetics (such as *Geobacillus* spp. (28)) may be present on occasion (Table 3), the chance of which is increased when the chocolate milk is made using reconstituted milk powder (8). *G. stearothermophilus* is a notorious sporeformer associated with spoilage. As can be seen from the results in Table 4, the expected spoilage rate is expected to be slightly higher (14%) if HR-group *B. subtilis* is replaced by *G. stearothermophilus* in the upper 95% worst-case scenario described above. This confirms that specific strains of *B. subtilis* (containing one or more $spoVA^{mob}$ genetic elements) can be placed in a similar risk category of spoilage organisms as *G. stearothermophilus*.

Spoilage of cocoa-containing liquid products due to spores occurs occasionally and unexpectedly, which could be owing to a variety of reasons: the occasional presence of highly HR spores as demonstrated above, but also, through insufficient processing or postprocessing contamination. One other hypothesis is that insufficient hydration of the cocoa powder prior to processing of the product leads to the occurrence of "dry pockets" inside dispersed cocoa powder. In such dry pockets, spores may experience dry heat

conditions during UHT treatment. Spores are many times more resistant to dry heat than to wet heat (24). If spores are exposed locally to dry heat during heat treatments, this may result in survival that would not occur if the product was properly wetted. To our knowledge, this hypothesis has not yet been proven and would in the first place require a definition of "proper wetting" of cocoa powder. In addition, a suitable experimental setup should be developed to link total and highly HR spore concentrations in cocoa with measurements of cocoa powder wettability to spoilage rates in UHT-treated finished product containing cocoa, which will be the next direction of this research.

Finally, it needs to be noted that low concentrations (<100 CFU/g) of spores cannot be detected accurately and reliably using classical plating methods owing to the detection limit of such methods. In addition, these methods do not provide information on strain-specific variations, which is important for informed risk assessment (Table 4). Alternatively, more sensitive (e.g., molecular) detection methods for low or very low numbers of (notorious) HR spores could be developed for more accurate risk assessment (8), supported by predictive modelling techniques. However, such methods are relatively expensive to apply during routine microbiology testing and are currently mostly applied for the detection of (foodborne) pathogens for the assessment of food safety (5, 9, 16). Extensive research would be needed for the development and validation of both general and specific molecular detection assays relevant for cocoa powder.

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SUPPLEMENTAL MATERIAL

Supplemental material associated with this article can be found online at: https://doi.org/10.4315/JFP-20-071.s1; https://doi.org/10.4315/JFP-20-071.s2; https://doi.org/10.4315/JFP-20-071.s3; https://doi.org/10.4315/JFP-20-071.s4; https://doi.org/10.4315/JFP-20-071.s5; https://doi.org/10.4315/JFP-20-071.s6

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