Spores in dairy – new insights in detection, enumeration and risk assessment

ROBYN T EIJLANDER, 1 RINA VAN HEKEZEN, 1,† ANNIE BIENVENUE, 2 VICTORIA GIRARD, 3 ERIK HOORNSTRA, 4 NICHOLAS B JOHNSON, 5 ROLF MEYER, 5 ARJEN WAGENDORP, 1,‡ DONALD C WALKER 6 and MARJON H J WELLS-BENNIK 1*

1NIZO, Ede, The Netherlands, 2US Dairy Export Council, Arlington, VA, USA, 3R&D Microbiology, bioMérieux, La Balme-les-Grottes, France, 4Laboratory & Quality Services, FrieslandCampina, Leeuwarden, The Netherlands, 5Nestec Ltd., Nestlé Research & Development, Konolfingen 3510, Switzerland, and 6Abbott Laboratories, Columbus, OH, USA

The performance of the ISO method for the ‘Enumeration of the Specially Thermoresistant Spores of Thermophilic Bacteria in Dried Milk’ (ISO/TS27265; 2009) was compared with a more practical method. Both were tested for predictability of spoilage of UHT treated reconstituted milk. The data show that heating for 30 min at 100 °C has the same predictive value as heating for 30 min at 106 °C, provided that specifications are increased 1 log₁₀ and the use of TSA as a cultivation medium is recommended over PCMA. Predictability of spoilage using classical plating methods is furthermore discussed in relation to variation in spore heat resistance of spores commonly present in the dairy industry.

Keywords Milk powders, Spoilage bacteria, Heat treatment, Microbial survival, Quality assurance, Reconstituted milk.

INTRODUCTION

Bacterial contamination of foods may lead to reduced shelf life due to outgrowth of spoilage organisms and, in the case of pathogens, to foodborne illness upon consumption of contaminated products. To inactivate bacteria that may grow in finished products, many food products undergo a heat treatment. Pasteurisation treatments lead to inactivation of vegetative cells. However, bacterial spores will survive such treatments, after which they may germinate and grow in finished liquid products (reviewed in André et al. 2017). To ensure spore inactivation, much higher heat loads such as ultrahigh-temperature (UHT) treatments or retort sterilisation are required (reviewed in den Besten et al. 2018). But even after those heat treatment levels, some (very) heat-resistant spores may still be viable (Scheldeaman et al. 2006). Clearly, spores are a main concern for the food industry as they are the number one cause of spoilage of a wide range of processed foods due to their elevated heat resistance properties (reviewed in Setlow 2014).

Raw milk, in the bulk tank, can contain bacterial spores, including (highly) heat-resistant ones. These spores are ubiquitously present on the cow and in its environment and can be introduced into the milk at low concentrations (<10 to 7 × 10² cfu/mL; Coorevits et al. 2008) during milking. Furthermore, during the manufacturing of milk powder from raw milk, concentrations of spores may increase in the product due to concentration effects or due to attachment of spores and growth of vegetative cells of these bacterial species in processing equipment, followed by spore formation (Burgess et al. 2014; Jindal and Anand 2018). Spores of bacterial species that are often encountered in dairy products belong to a wide range of aerobic and anaerobic species with different optimal growth temperatures and growth requirements (Sadiq et al. 2016; Doll et al. 2017). Most mesophilic spore formers, including many Bacillus spp. and Brevibacillus spp., grow best at temperatures between 30 °C and 40 °C (and occasionally as high as 46 °C) (Willey et al. 2008). When spores of such organisms are

*Author for correspondence. E-mail: Robyn.Eijlander@nizo.com
†Present address: Fonterra Co-operative Group Limited, Amsterdam, The Netherlands.
‡Present address: smartfood R&D, Zetten, The Netherlands.

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viable and germinate, this may lead to spoilage when dairy products are stored at ambient temperate conditions throughout the distribution chain, especially during warm months or at geographical locations with relatively warm climates. Bacterial spore formers that are able to grow at high temperatures can be divided into facultative thermophiles and obligate thermophiles; obligate thermophilic species, such as *Anoxybacillus* spp. and *Geobacillus* spp., require minimum temperatures of around 45 °C for growth (with optimal temperatures between 55 and 65 °C), whereas facultative thermophiles (such as *B. subtilis*, *B. licheniformis* and *B. pumilus*) can grow at ambient conditions and at high temperatures (generally in a range from 10 to 60 °C; Burgess *et al.* 2014). Spoilage of low-acid dairy products caused by obligate thermophilic spore formers only occurs at elevated storage temperatures (above 45 °C, for instance in tropical climates) and is generally characterised by ‘flat-sour’ spoilage (Kalogridou-Vassiliadou 1992).

The optimal growth temperature of species often correlates with the thermal resistance of their spores (reviewed in den Besten *et al.* 2018), but not always (Sadiq *et al.* 2016). Thermophilic species, such as *Geobacillus* spp., are notorious for producing highly heat-resistant spores, which generally survive more severe heat treatments than spores of mesophilic species. Occasionally, spores of mesophiles or facultative thermophiles have higher heat resistances than spores of obligate thermophiles (den Besten *et al.* 2018).

Milk powders may contain much higher concentrations (exceeding levels of 10⁷ spores/g) of spores with high-level heat resistance than raw milk (Kent *et al.* 2016). Applying heat treatments that are required for the reduction of these spores will have significant impact on the quality of the finished product (e.g. taste, colour or nutritional value). Ingredients used to produce UHT-treated or sterilised foods generally have specifications for the concentration of heat-resistant spores surviving heat treatments of 30 min at 100 °C or at 106 °C, although it is not always clear which levels lead to problems in finished products.

Currently, different methods are used to enumerate heat-resistant spores in powders, generally consisting of a heat treatment followed by plating on cultivation media that allows for germination of spores and subsequent outgrowth. Importantly, results obtained with one method cannot be directly compared with results obtained using another method, especially if different heat treatments and media are used (Kent *et al.* 2016; Wells-Bennik *et al.* 2018). A method published by ISO (2009) for the enumeration of highly heat-resistant spores of thermophilic bacteria in dairy powders requires heating of reconstituted milk powder at 106 °C for 30 min, subsequent plating using plate count milk agar (PCMA, ‘BCP plate count skim milk agar with 0.2% mass fraction starch’) and incubation at 55 °C. However, for routine analyses the use of a heat treatment at 100 °C is preferred over a heat treatment at 106 °C. This is mainly due to limited facilities in factories that allow for heating at temperatures exceeding 100 °C. In addition, reproducibility of the heat treatments at temperatures above 100 °C may be a concern. In practice, different heat treatments and a wide range of cultivation media are used to assess the spore concentrations in powders, for which specifications have been drawn up to avoid risks of spoilage of finished products (Mchugh *et al.* 2017). If selected media do not sufficiently support germination and/or outgrowth of the spore species present, this may lead to severe underestimations of spore concentrations (Berendsen *et al.* 2015a; Wells-Bennik *et al.* 2018). Taken together, there is a strong need for global agreement on practical application of the best-performing enumeration method for heat-resistant spores (Kent *et al.* 2016), including for spores present in milk powders. This method should allow for reliable interpretation of spore concentrations in powders in relation to risk of spoilage of finished products.

In this study, the germination and outgrowth efficiency was investigated of spores of 38 strains that were isolated from dairy products, ingredients and dairy farm environments. Subsequently, milk powders from various geographical locations were collected to compare the performance of ISO/TS 27265:2009 with a practical method consisting of heating for 30 min at 100 °C and plating on TSA. Milk powders were furthermore reconstituted in water and subjected to UHT treatment followed by assessment of spoilage of the milk after storage at 37 °C and 55 °C, to link the analytical results to predictability of UHT product spoilage. The results of this study help to define a practical standard method for the enumeration of highly heat-resistant spores and provide increased insights in the interpretation of analytical results for spoilage risk assessment.

**MATERIAL AND METHODS**

**Bacterial strains used in this study**

Isolates from various relevant sources were collected by authors of this manuscript and are listed in Table 1. Isolates were cultured on plates containing tryptic soy agar (TSA) or brain–heart infusion (BHI) agar and were incubated at appropriate temperatures (Table 1). Single colonies were mixed with tryptic soy broth (TSB; Oxoid, Basingstoke, Hampshire, UK) and glycerol (final concentration 10%), frozen in liquid nitrogen and stored at –80 °C. Each isolate was identified using 16S rDNA sequencing (BaseClear, Leiden, The Netherlands), based on the V1.1-to-V3.2 region. Identification was performed using the RDP database (Cole *et al.* 2014). In addition, identification of all isolates was confirmed through matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) technology using VITEK® MS V3 (bioMérieux, Marcy l’Étoile, France).
Spore preparation
Cryostock cultures were spread on TSA plates (diameter 14.5 cm). Plates were incubated at the species-specific optimum temperature (37 °C, 45 °C, 55 °C) for ten days in a plastic bag to prevent evaporation. Spores were harvested as described previously (Berendsen et al. 2015b) and resuspended in 25 mL phosphate-buffered salt solution (PBS), after which spore formation was confirmed by phase contrast microscopy. Enumeration of spore suspensions was performed after pasteurisation at 80 °C for 10 min, followed by plating of serial dilutions on TSA (pour plates) and incubation at 37 °C, 45 °C, 55 °C or 60 °C, depending on the species (Table 1). Spore suspensions were stored in PBS at 4 °C.

Spore recovery media and incubation conditions
The recovery of spores derived from the isolates mentioned in Table 1 was determined on six different agar media listed in Table 2. All media were prepared by Tritium Microbiologie (Eindhoven, The Netherlands) based on the recipes provided in Table 2. For all media, agar was supplied by Lab M (Heywood, Lancashire, UK). To minimise the growth of spreading bacteria, a top layer of the same media was applied. Agar plates were incubated for 24 h for a

### Table 1 List of bacterial strains used in this study. Species were identified using 16S rDNA sequencing

| ID      | Species                                                                 | Source of isolation                        | Growth temp. (°C) |
|---------|-------------------------------------------------------------------------|--------------------------------------------|-------------------|
| NIZO1465 | Bacillus coagulans                                                      | Undisclosed                                | 37                |
| NIZO1961 | Bacillus sporothermodurans                                              | Undisclosed                                | 37                |
| NIZO2978 | Bacillus coagulans                                                      | Undisclosed                                | 37                |
| NIZO4225 | Geobacillus stearothermophilus                                          | Milk powder                               | 55                |
| NIZO4227 | Bacillus licheniformis                                                  | Milk powder                               | 37                |
| NIZO4229 | Bacillus subtilis/amyloidiiaceiens                                      | Undisclosed                                | 37                |
| NIZO4230 | Bacillus subtilis                                                       | Cocoa drink                               | 37                |
| NIZO4233 | Geobacillus debilis                                                     | Raw milk                                  | 60                |
| NIZO4234 | Bacillus thermoamylovorans                                             | Evaporated milk                           | 45                |
| NIZO4235 | Geobacillus stearothermophilus                                          | Medium-heat skim milk                     | 55                |
| NIZO4237 | Bacillus thermoamylovorans                                             | Evaporated milk                           | 45                |
| NIZO4240 | Anoxybacillus flavithermus                                             | Raw milk                                  | 55                |
| NIZO4241 | Geobacillus debilis                                                     | Evaporated milk                           | 60                |
| NIZO4244 | Geobacillus stearothermophilus                                          | Milk powder manufacturing plant           | 55                |
| NIZO4245 | Acetobacillus pallidus                                                  | Raw milk                                  | 55                |
| NIZO4249 | Aneurinbacillus thermoaeophilus                                         | Milk powder                               | 55                |
| NIZO4250 | Bacillus licheniformis                                                  | Milk powder                               | 37                |
| NIZO4251 | Geobacillus stearothermophilus                                          | Milk powder                               | 55                |
| NIZO4252 | Bacillus licheniformis                                                  | Milk powder                               | 37                |
| NIZO4253 | Bacillus licheniformis                                                  | Milk                                      | 37                |
| NIZO4255 | Anoxybacillus flavithermus                                             | Milk powder                               | 55                |
| NIZO4256 | Bacillus subtilis                                                       | Undisclosed                                | 37                |
| NIZO4259 | Bacillus subtilis                                                       | Milk                                      | 37                |
| NIZO4260 | Anoxybacillus flavithermus                                             | Milk powder manufacturing plant           | 55                |
| NIZO4263 | Geobacillus stearothermophilus                                          | Milk powder                               | 55                |
| NIZO4266 | Bacillus licheniformis                                                  | UHT milk                                  | 37                |
| NIZO4267 | Geobacillus caldoxylosilyticus                                          | Milk powder                               | 55                |
| NIZO4268 | Bacillus sonorenseis                                                    | Milk powder                               | 37                |
| NIZO4272 | Bacillus subtilis/moljaovensis/axarquensis/nalactiens                   | Milk powder                               | 37                |
| NIZO4274 | Bacillus licheniformis                                                  | Pea protein powder                        | 37                |
| NIZO4275 | Geobacillus caldoxylosilyticus                                          | Compost                                  | 55                |
| NIZO4276 | Bacillus subtilis                                                       | Condensed milk                            | 37                |
| NIZO4277 | Bacillus licheniformis                                                  | Milk powder                               | 37                |
| NIZO4279 | Bacillus licheniformis                                                  | Pea protein powder                        | 37                |
| NIZO4281 | Bacillus smithii                                                        | UHT cocoa drink with malt                | 55                |
| NIZO4284 | Acetobacillus pallidus                                                  | Compost                                  | 55                |
| NIZO4290 | Geobacillus thermodenitrificans                                         | Compost                                  | 55                |
| NIZO4294 | Bacillus thermoamylovorans                                             | Compost                                  | 37                |
maximum of 5 days (depending on the strain) at the optimal growth temperature per species or strain (see Table 1).

**Spore heat treatments**

Two individual samples of each spore suspension (2.5 mL, with concentrations of 7–8 log₁₀ spores/mL) were exposed to four different heat treatments, namely 30 min at 80 °C, 30 min at 100 °C, 30 min at 106 °C and 20 or 10 min at 115 °C. Heat treatments at 80 °C and 100 °C were performed in a water bath, whereas heat treatments at 106 °C (±0.5 °C) and 115 °C (±0.5 °C) were performed in a pressure pan (Vapour-Line Lite; VWR). Temperatures were registered using a dummy container with 2.5 mL water and a thermocouple. The selected heating time started once the desired temperature for each heat treatment was reached. Samples were cooled on ice immediately after heat treatment. Subsequently, samples were 10-fold serially diluted and plated on the agar plates.

**Milk powders**

Milk powders (listed in Table 3) were collected by the authors of this publication and were subjected to a prescreening, using heat treatments as described above, to determine spore levels. Powders were specifically produced (using prolonged run times under confidential conditions) and selected to contain high levels of spores and include maximum differences in types of powders with respect to origin (geography), heat classification (medium and high heat) and fat content (skimmed and whole milk). In total, 27 powders were categorized according to initial spore counts as determined in the prescreening; category A powders contained low concentrations of highly heat-resistant (HHR) spores (<10⁵ spores/g surviving 30 min 100 °C and <10⁴ spores/g surviving 30 min 106 °C and 10 min 115 °C), category B powders contained levels of HHR spores of >10⁵ spores/g upon heating for 30 min at 100 °C or >10³ spores/g surviving 10 min at 106 °C and category C powders contained high concentrations of HHR spores (>10⁷ spores/g upon heating for 10 min at 115 °C) (Table S2).

**Preparation, UHT treatment and spoilage assessment of reconstituted milk**

Reconstituted milk was prepared from the powders as follows. Samples (1.6 kg) from the milk powders described above were mixed in a blender bottle (4000 mL, Nalgene; Thermo Fisher, Waltham, MA, USA) for 30 min in a blender machine (Vatenmenger; J. Engelsmann akt.ges., Ludwigshafen am Rhein, Germany) to obtain an evenly distributed mixture of powder (preventing local high concentrations of spores). Powders were then dissolved through aseptic rehydration in reverse osmosis (RO) water (1:10) for 15 min at room temperature while stirring. Dissolved powders were subjected to UHT treatment using the Flow Low capacity Indirect Pasteurizer/Sterilizer (FLIP) device (NIZO, Ede, The Netherlands) at a flow rate of 5 L/h using a spiral length of 1000 cm for the holding section (with a holding time of 157.5 s). The sterility of the device was checked during and after cleaning with regular cultivation of flow-out samples on plates.

Four different reconstituted milk samples were heat-treated on one day with cleaning cycles between each batch. Each milk batch was subjected to two different UHT treatments that are commonly used in practice, based on the estimated HHR spore concentrations in the corresponding milk powders. Per milk batch, the highest heat treatment \( F_0 = 16.6 \) (129 °C) or \( F_0 = 8.4 \) (126 °C) was applied first, followed by the lower heat treatment \( F_0 = 8.4 \) (126 °C) or \( F_0 = 5.3 \) (124 °C). The \( F_0 \) value represents the time (in minutes) of a heat treatment at 121 °C. In other words, a heat treatment of 157.5 seconds at 129 °C is equivalent to a heat treatment of \( (F_0) \) = 16.6 min at 121 °C.

After heating, the milk was cooled to room temperature in the FLIP and stored in sterile bottles in quadruplicate (in

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**Table 2** Enumeration media for spores

| Name                      | Acronym | Ingredients and suppliers                                                                 | Reference                  |
|---------------------------|---------|-------------------------------------------------------------------------------------------|----------------------------|
| Dextrose                  | DTA     | Glucose (BDH/VWR, Amsterdam, NL); tryptone (Difco, Lawrence, KS, USA); bromocresol purple (Merck, Schiphol-Rijk, NL) | Scheldeman et al. (2005), Witthuhn et al. (2011) |
| Brain–heart infusion agar | BHI     | BHI (Merck, Schiphol-Rijk, NL) + 1 mg/L vitamin B12 (Sigma-Aldrich, Steinheim, DL)         | ISO-IDF (2009)             |
| Plate count milk agar + starch | PCA + starch | PCA (Difco, Lawrence, KS, USA) + milk powder (Promex, Lochristi, BE) + 0.2% starch (Sigma-Aldrich, Steinheim, DL) | NEN 6809 (2014)           |
| Plate count agar + starch  | PCA + starch | PCA (Difco, Lawrence, KS, USA) + 0.2% starch (Sigma-Aldrich, Steinheim, DL)               | NEN 6809 (2014)           |
| Tryptic soy agar           | TSA     | TSA (Oxoid, Basingstoke, Hampshire, UK)                                                    | Ronimus et al. (1997)     |
| Tryptic soy agar + starch  | TSA + starch | TSA (Oxoid, Basingstoke, Hampshire, UK) + 1% starch (Sigma-Aldrich, Steinheim, DL)        | ISO-IDF (2009)             |
10-mL and 100-mL aliquots) at 37 °C and 55 °C (marked as 'day 0'). For four powders (NIZO1, NIZO5, NIZO23A and NIZO35), the UHT treatment and storage was repeated on a separate occasion to test reproducibility.

Spoilage of the heated samples was assessed by visual inspection of coagulation on various days during incubation, for a maximum of 13 days. Samples that did not show visible coagulation were furthermore tested by pH measurements and plating on TSA plates (incubation at 37 °C and 55 °C) on day 13.

Identification of species recovered from spoiled UHT treated reconstituted milk

A maximum of two bottles per spoiled product were used for the enumeration of spoilage bacteria. Serial dilutions were prepared and 0.1-mL aliquots spread on TSA plates. Plates were incubated at 55 °C for two days before enumeration. A maximum of ten colonies per spoiled product were selected from the plates. The isolates were prepared for identification by 16S rRNA sequencing (BaseClear) and MALDI-TOF analysis (bioMérieux, La Balme-les-Grottes, France) by resuspending a single colony in 0.2 mL peptone physiological salt (PPS) solution (Tritium PFZ P100.76.0200) in a 96-well plate. MALDI identification was performed using VITEK MS Plus system V3.0 (bioMérieux) according to the manufacturer’s instructions. Bacterial species cultivated on plates were deposited directly on the MS slide, covered by 0.01 mL of α-cyano-4-hydroxycinnamic acid matrix (CHCA; bioMérieux) and air-dried before analysis.

Recovery and identification of spores from milk powders

The concentrations and identities of spores in nine milk powders were determined. The selection of the milk powders was based on the outcome of the spore concentrations as established in the prescreening and the spoilage

Table 3

| Powder ID | Continent of origin | Type of product | Heat classification | Powder category |
|-----------|---------------------|-----------------|---------------------|----------------|
| NIZO1     | America             | Nonfat dry milk | Low                 | B              |
| NIZO2     | Europe              | 1% skim milk powder | Medium                  | C              |
| NIZO3     | Europe              | 1% skim milk powder | Medium                  | C              |
| NIZO5     | America             | Nonfat dry milk | High                | A              |
| NIZO8     | America             | Skim milk powder | Medium                | C              |
| NIZO10    | Europe              | 1% skim milk powder | Medium                  | C              |
| NIZO12    | America             | Nonfat dry milk | Low                  | B              |
| NIZO14    | America             | Skim milk powder | Medium                | A              |
| NIZO15    | America             | Skim milk powder | Medium                | A              |
| NIZO16    | Europe              | 1% skim milk powder | Medium                  | C              |
| NIZO17    | America             | Nonfat dry milk | Low                  | A              |
| NIZO19    | America             | Nonfat dry milk | Low                  | B              |
| NIZO20    | America             | Nonfat dry milk | Low                  | B              |
| NIZO21    | America             | Whole-milk powder | Medium                  | A              |
| NIZO22    | America             | Nonfat dry milk solids | Low                    | C              |
| NIZO23*   | Asia                | Full-cream milk powder | Medium                  | C              |
| NIZO23A*  | Asia                | Full-cream milk powder | Medium                  | C              |
| NIZO23B*  | Asia                | Full-cream milk powder | Medium                  | A              |
| NIZO24    | America             | Nonfat dry milk | Medium                | A              |
| NIZO25    | America             | Nonfat dry milk | Low                  | A              |
| NIZO28    | America             | Nonfat dried skim milk | Medium                  | C              |
| NIZO29    | America             | Nonfat dry milk | Low                  | B              |
| NIZO31    | America             | Skim milk powder | Medium                | A              |
| NIZO34    | America             | Skim milk powder | Medium                | A              |
| NIZO35    | Europe              | 1% skim milk powder | Medium                  | C              |
| NIZO40    | Europe              | Skim milk powder | ND                   | C              |
| NIZO41    | Australia           | Skim milk powder | ND                   | A              |

A: low concentrations of highly heat-resistant spores, spoilage not expected; B: medium levels of highly heat-resistant spores, spoilage possible; C: high levels of highly heat-resistant spores, spoilage expected; ND: not determined.

*NIZO23, NIZO23A and NIZO23B are three separate sample bags of the same powder batch that were collected at different time points during milk powder production.
assessment of reconstituted powders. The powders were rehydrated by adding 9 mL sterile water to 1 g of pre-mixed powder (from the original 1.6 kg powder sample described above). The dissolved powder was mixed for two min in a stomacher and left for 15 min at room temperature. Sample vials (10-mL Precision Thread Headspace Vial, round bottom (P/N:5134191); Grace Davison Discovery Trans Blue/White PTFE (P/N:5134188)) and heated in duplicate at 100 °C (steamer) or at 106 °C for 30 min (pressure cooker; heating-up time from 100 °C to 105.5 °C was <6 min). After heat treatment, each sample was cooled in a water bath at room temperature. Surviving spores in heat-treated reconstituted milk were then enumerated in duplicate by spread-plating serial dilutions on TSA or PCMA plates. Four separate plates per powder per heat treatment per recovery medium were incubated at 55 °C for a maximum incubation time of 72 h. Colonies were manually counted using a colony counter and average cfu/g calculated. A maximum of ten single colonies per powder per condition were used for colony identification by 16S rDNA sequencing (BaseClear) or MALDI-TOF analysis (bioMérieux, La Balme-les-Grottes, France; and De Gelderse Vallei, Ede, The Netherlands). When possible, colonies were picked based on different colony morphologies.

RESULTS

To determine the overall best-performing recovery medium for spores in dairy powders, the recovery of spores produced by 38 different bacterial strains of species that are often encountered in the dairy industry was investigated.

Performance of different plating media in the recovery of dairy-specific spore species

Spore recovery data for 38 strains, belonging to 16 bacterial species, are shown in Table S1 and are summarised in Table 4. Spores of eight strains did not survive a heat treatment of 30 min at 100 °C. Nine strains produced spores with very high heat resistance, surviving 20 min 115 °C, namely NIZO4245 and NIZO4284 (A. pallidus), NIZO4230 (B. subtilis), NIZO4234 (B. thermoamylovorans), NIZO1961 (B. sporothermodurans), NIZO4233 (G. debilis), NIZO4275 (G. caldoxylsilyticus), NIZO4235 and NIZO4263 (G. steatothermophilus).

Overall, TSA or TSA + starch showed the best performance with respect to recovery of spores, which is in agreement with

| Species                                    | Number of strains | Best recovery temp | Recovery on | Log10 reduction after |
|--------------------------------------------|-------------------|--------------------|-------------|-----------------------|
|                                            |                   |                    | BHI         | DT A PCA+ PMCA+ TSA  |
|                                            |                   |                    | 30 min 100 °C | 106 °C 115 °C |
| Aerobacillus pallidus                      | 2                 | 55 °C              | +++ ++ ++   | +++ +++ ++++++       |
| Aneurinibacillus thermoaueroophilus        | 1                 | 55 °C              | +++ +++ +++ | +++ +++ ++++++       |
| Anoxybacillus flavithermus                 | 3                 | 55 °C              | +++ +++ ++  | +++ +++ ++++++       |
| Bacillus caugulans                         | 2                 | 37/55 °C           | +++ +++ +++ | +++ +++ ++++++       |
| Bacillus licheniformis                     | 8                 | 37/55 °C           | ++ ++ ++   | +++ +++ ++++++       |
| Bacillus smithii                          | 1                 | 55 °C              | ++ ++ ++   | +++ +++ ++++++       |
| Bacillus sonorensis                        | 1                 | 37/55 °C           | +++ ++ ++  | +++ +++ ++++++       |
| Bacillus sporothermodurans                | 1                 | 37 °C              | +++ ++ ++  | +++ +++ ++++++       |
| Bacillus subtilis                         | 4                 | 37/55 °C*          | +++ +++ ++  | +++ +++ ++++++       |
| Bacillus subtilis/mojavensis/axaruquieni/malacitensis | 1     | 37 °C              | +++ +++ ++  | +++ +++ ++++++       |
| Bacillus subtilis/amyloliquefaciens        | 1                 | 37 °C              | +++ +++ ++  | +++ +++ ++++++       |
| Bacillus thermammuolovorans               | 3                 | 37/45/55 °C*       | +++ +++ +++ | +++ +++ ++++++       |
| Geobacillus caldoxylsilyticus              | 2                 | 55 °C              | ++ ++ ++   | +++ +++ ++++++       |
| Geobacillus debilis                        | 2                 | 60 °C              | +++ ++ ++  | +++ +++ ++++++       |
| Geobacillus steaothermodurificans         | 5                 | 55 °C              | +++ ++ ++  | +++ +++ ++++++       |
| Geobacillus thermodenitrificans            | 1                 | 55 °C              | +++ ++ ++  | +++ +++ ++++++       |

*Growth optimum depending on the strain.

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our previous study specifically on *G. stearothermophilus* spore recovery (Wells-Bennik et al. 2018). The addition of starch to TSA did not show a significant difference in recovery of spores of the bacterial species included in this study. Performance of PCMA + starch (ISO-IDF, 2009) was comparable with PCA + starch, while overall the recovery on BHI and DTA was not as good as on PCMA + starch (Table 4).

Sporules of the *Aer. pallidus* strain showed good recovery on most media, but not on DTA. This species produces spores with some of the highest heat resistances encountered and is able to grow at 37 °C and 55 °C. For *B. sporothermodurans*, spores were not recovered on PCA + starch and PCMA + starch, while on other media the recovery was good (Table 4). For *B. thermoanmylovorans*, the recovery was also much lower on PCA + starch and PCMA + starch than on other media when incubated at 37 °C. In addition, various strains producing heat-resistant spores showed ability to grow at 37 °C (*B. thermoanmylovorans*, *B. sporothermodurans*, *An. flavithermus*, *Aer. pallidus*), but not when cultured on PCMA + starch at 37 °C. The use TSA instead of PCMA or PCMA + starch is therefore recommended.

**Spoilage assessment of UHT treated reconstituted milk**

Milk powders (listed in Table 3) were used for the preparation of UHT treated reconstituted milk, which was stored at 37 °C and 55 °C to assess spoilage by visible coagulation, pH measurements and cfu analysis on TSA. Of the 27 products, ten showed spoilage upon storage at 55 °C. Most of these products spoiled within one week of storage, with only one exception (NIZO28). No spoilage was observed for UHT treated reconstituted milk samples that were stored at 37 °C. Further analyses of samples that did not show visible coagulation of milk proteins only revealed signs of spoilage in one case (NIZO10, underlined in Table 5). In this case, the pH measured was 5.57, which is an indication of spoilage. A summary of the spoilage results is shown in Table 5. All isolates from spoiled UHT treated reconstituted milk products were identified as *G. stearothermophilus* (Table S3).

**Comparison of TSA and PCMA as recovery media for spores present in milk powders**

Recovery of spores was investigated in more detail for a selection of powders (NIZO1, NIZO2, NIZO8, NIZO12, NIZO16, NIZO22, NIZO23A, NIZO28 and NIZO35). The selection was based on the spore load category (mainly group C powders) and the spoilage outcome of the reconstituted powders upon UHT treatment (Table 5). Average concentrations of surviving spores per g powder are presented in Table S4 and Figure 1. It must be noted that colonies on TSA plates were generally larger than on PCMA (after 48 h of incubation, data not shown), facilitating counting of colonies and minimising over- or underestimations due to inclusion or exclusion of pinpoint colonies.

**Identification of bacterial isolates from milk powders after heat treatment and plating**

An overview of the identified spore species in all powders is presented in Table 6. Species identified after a heat treatment of 30 min at 100 °C included spore formers of species of both facultative (*B. licheniformis*, *Brevibacillus* spp.) and obligate (*Geobacillus* spp., *An. flavithermus*, *Thermoactinomyces* spp.) thermophiles. Spoilage of the UHT treated reconstituted milk was only observed at 55 °C and not at 37 °C, which most likely indicates that spores of mesophilic bacteria (facultative thermophilic) did not survive the UHT treatments, or did not germinate and grow in reconstituted milk. For the milk powder analysis, the highest level of heat treatment tested, namely 30 min at 106 °C, resulted mainly in the cultivation of *G. stearothermophilus*, and in a few instances, *B. licheniformis* and *An. flavithermus* were also identified. *G. stearothermophilus* was identified as the only spoilage-causing organism for the recombined UHT-treated milk powders tested in this study.

**Linking analytical results to prediction of spoilage of UHT products**

In general, results on spoilage of UHT treated reconstituted milk, made from powders that were classified based on spore concentrations, were according to expectations (Tables 3 and 5), with a few unexpected results that are further discussed in the Discussion section. Ideally, spoilage limits in specifications are set considering the effective inactivation of spores during UHT processing, thereby preventing spoilage of the finished product. In relation to such predictability, our results indicate that if a spore specification of \(<10^2\) cfu/g is used when applying a heat treatment of 30 min at 106 °C, then the specifications should increase one log₁₀ to \(<10^3\) cfu/g when applying a heat treatment of 30 min at 100 °C (Figure 1), for an equivalent rate of spoilage. It must be noted, however, that specifications of concentrations of spores in powders after application of one specific detection method are not solely indicative for expected spoilage of product. An overview of the predictability of spoilage is shown in Table 7. This table shows a combination of spoilage results of UHT-treated products (Table 5) and outcomes of analytical test methods (Table S4) using the specification limits described above. From the ‘in-spec’ and ‘out-of-spec’ outcomes, a distinction was made between true positives (powder out-of-spec and spoilage observed), false positives (spoilage not observed but powder out-of-spec), true negatives (spoilage not observed and powder in-spec) and false negatives (spoilage observed but powder in-spec). These data clearly show that each method applied will generate false positives and false negatives, although the frequency may differ significantly. Generally, plating on TSA results in the lowest frequency of false-positive and false-negative analytical results, where false-negative results have the highest impact on the dairy business.

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Table 5 Spoilage results for four replicates of ultrahigh-temperature (UHT)-treated reconstituted milk assessed by visible coagulation

| Powder ID  | Category | UHT heat treatment | After storage at 37 °C | After storage at 55 °C |
|------------|----------|--------------------|------------------------|------------------------|
|            |          |                    | Replicate              | Replicate              |
|            |          |                    | 1     | 2     | 3     | 4     | 1     | 2     | 3     | 4     |
| NIZO1*, 3, 5*, 8, 14, 15, 17, 19, 20, 21, 24, 25, 29, 31, 34, 41 | F₀ = 5.3 | No     | No    | No    | No    | No    | No    | No    |
| NIZO2      | C        | F₀ = 5.3           | No     | No    | No    | No    | 4⁴    | 4     | 4     |
| NIZO10     | C        | F₀ = 8.4           | No     | No    | No    | No    | 6     | No    | No    |
| NIZO12     | B        | F₀ = 5.3           | No     | No    | No    | No    | No    | No    |
| NIZO16     | C        | F₀ = 8.4           | No     | No    | No    | No    | 3     | 3     | 3     |
| NIZO22     | C        | F₀ = 5.3           | No     | No    | No    | No    | 7     | 7     | 7     |
| NIZO23     | C        | F₀ = 8.4           | No     | No    | No    | No    | 7     | No    | No    |
| NIZO23A*   | C        | F₀ = 5.3           | No     | No    | No    | No    | 3⁴    | 5     | 5     |
| NIZO23B    | A        | F₀ = 5.3           | No     | No    | No    | No    | 4     | No    | No    |
| NIZO28     | C        | F₀ = 5.3           | No     | No    | No    | No    | No    | 12    | No    | No    |
| NIZO35*    | C        | F₀ = 8.4           | No     | No    | No    | No    | No    | No    |
| NIZO40     | C        | F₀ = 5.3           | No     | No    | No    | No    | 4⁴    | 4     | 4     |
|            |          | F₀ = 8.4           | No     | No    | No    | No    | 4     | No    | No    |

Spoiled samples are indicated by the number of days of incubation before spoilage was assessed. Nonspoiled samples by visible coagulation (‘no’), but with a pH value < 6.0, are underlined.

*For some powders, spoilage assessment through UHT treatment and incubation was repeated on a separate occasion for reproducibility.

#Spoilage also observed in the 10 mL samples.

Figure 1 Average cfu counts per gram milk powder after 30 min at 106 °C (a) or 30 min at 100 °C (b) heat treatment and plating on TSA (black bars) or PCMA (grey bars) media. The standard errors of the average values are indicated by error bars. [Correction added on 7 August 2019, after first online publication: Headings in Figure 1a,b are mixed up and is now corrected in this version.]
In conclusion, spore concentrations present in milk powder have a better predictive value for spoilage of UHT treated reconstituted milk product when established by plating on TSA compared with plating on PCMA. Heating for 30 min at 100 °C can be applied, provided that specifications are adjusted; for example, 10^3 cfu/g can be used when compared with 10^2 cfu/g after heating for 30 min at 106 °C. Heating for 30 min at 100 °C is preferred over 106 °C, because predictive results are comparable and heating at 106 °C is not practical.

**DISCUSSION**

Spore detection methods using classical plating techniques are practical and relatively cheap, but have several pitfalls that should be taken into consideration during data collection.

**Table 6** Spore-forming species isolated from dairy powders identified by 16S sequencing and/or MALDI-TOF. Powders indicated with a * did not show spoilage after a UHT treatment with a minimal \( F_0 \) value of 5.3

| Powder  | Medium | 30 min at 100 °C                      | 30 min at 106 °C                      |
|---------|--------|--------------------------------------|--------------------------------------|
| NIZO1*  | TSA    | Geobacillus stearothermophilus       | Bacillus licheniformis               |
|         | PCMA   | Geobacillus stearothermophilus       | Bacillus licheniformis               |
| NIZO2   | TSA    | Geobacillus stearothermophilus       | Geobacillus stearothermophilus       |
|         | PCMA   | Geobacillus stearothermophilus       | Geobacillus stearothermophilus       |
| NIZO8*  | TSA    | Geobacillus stearothermophilus       | Bacillus thermoalkalophilus          |
|         | PCMA   | Geobacillus stearothermophilus       | Bacillus licheniformis               |
| NIZO12  | TSA    | Geobacillus stearothermophilus       | Geobacillus stearothermophilus       |
|         | PCMA   | Geobacillus stearothermophilus       | Geobacillus thermodenitrificans      |
| NIZO16  | TSA    | Geobacillus stearothermophilus       | Bacillus licheniformis               |
| NIZO22  | TSA    | Geobacillus stearothermophilus       | Bacillus licheniformis               |
| NIZO23A | TSA    | Geobacillus stearothermophilus       | Anoxybacillus flavithermus           |
|         | PCMA   | Anoxybacillus flavithermus           | Anoxybacillus flavithermus           |
| NIZO28  | TSA    | Geobacillus stearothermophilus       | Bacillus licheniformis               |
|         | PCMA   | Bacillus licheniformis               | Bacillus licheniformis               |
| NIZO35* | TSA    | Geobacillus stearothermophilus       | Bacillus licheniformis               |
|         | PCMA   | Anoxybacillus flavithermus           | Bacillus licheniformis               |
interpretation. Firstly, presence of low concentrations of heat-resistant spores may not be detected due to the detection limit of the method (10 cfu/mL or g), even though such concentrations may still cause sterility issues in finished products. Secondly, suboptimal spore germination and outgrowth (due to sublethal damage caused by heat treatment or due to species and strain variation) can result in underestimations of spore numbers that are actually present (Berendsen et al. 2015a; Wells-Bennik et al. 2016). The current study demonstrates that spores of *B. sporothermodurans* were unable to germinate or grow on PCA and PCMA. Spores of *B. thermoamylovorans* and *B. sporothermodurans* species are notorious for their high heat resistance properties (Scheldeman et al. 2006) and may survive UHT sterilisation treatments. So far, *B. sporothermodurans* has only been detected in UHT milk, although the source of spores of this bacterium is unknown (Scheldeman et al. 2006; André et al. 2017). Considering our findings that *B. sporothermodurans* spores were not recovered on PCMA or PCA, presence of this organism in milk powders and spoiled products made thereof may have been missed when using PCA or PCMA for spore enumeration. A third pitfall is the incubation temperature that is used, as this determines whether thermophilic or mesophilic species are recovered. The incubation temperature will determine which species are able to grow either on plates during enumeration or in finished product throughout the distribution chain. Facultative thermophiles have the ability to grow below 40 °C (generally Bacillus species), while obligate thermophiles generally grow above 40 °C. However, this distinction is not so clear-cut in practice. Species that have traditionally been considered to be thermophilic species, such as *Aer. pallidus* (formerly *Geobacillus pallidus*), *An. flavithermus* and some strains of *G. steaothermophilus*, also showed an ability to grow at 37 °C (this study and Kakagianni et al. 2016). Finally, heat resistance of spores of specific bacterial species may be underestimated when this is based on a correlation with optimal growth temperatures. For instance, this study demonstrates that spores of several strains of *Bacillus* species that are generally recognised as mesophiles (*B. subtilis*, *B. thermoamylovorans*, *B. licheniformis* and *B. sporothermodurans*) were highly heat-resistant and survived heat treatments of 30 min at 106 °C or even 20 min at 115 °C.

The pitfalls of classical plating methods are clearly demonstrated by a few unexpected results in spoilage of UHT treated reconstituted milk products. For instance, reconstituted products using milk powders that were classified as group A (based on low levels of high-level heat-resistant spores) were not expected to spoil after UHT treatment with a minimum $F_0$ value of 5.3. However, in one case spoilage of the product was observed (NIZO23B; Table 5). In contrast, reconstituted milk made from two out of eleven powders from group C (NIZO3 and NIZO35) did not spoil after heat treatments with a minimum $F_0$ value of 5.3 (Table 5), even though spoilage was expected due to high levels of heat-resistant spores (Figure 1 and Table S2) and presence of significant concentrations of *G. steaothermophilus* spores (Table 6). Also, one powder (NIZO16) did not show a one log$_{10}$ reduction in spore counts after heating at 106 °C compared with 100 °C, which is indicative of the presence of primarily high-level heat-resistant spores. The source of these unexpected results could lie in important differences in heat resistance between spores of individual strains present in these powders, or differences in their ability to germinate and grow out in the reconstituted milk.

For pure spore isolates from dairy-relevant sources, a large variety in spore heat resistance between individual strains was observed. This variation was not limited to differences at the species level, but is clearly also apparent between different strains of the same species. For instance, of the six *B. subtilis* isolates studied (Table 1), one showed significant survival after a heat treatment of 20 min at

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**Table 7** Predictive value of four different spore detection and enumeration methods (heat treatment for 30 min at 100 °C or 106 °C and plating on TSA or PCMA medium followed by 2-day incubation at 55 °C) in relation to milk product spoilage after UHT at $F_0 = 5.3$ and $F_0 = 8.4$

| Method | $F_0 = 5.3$ | $F_0 = 8.4$ |
|--------|------------|------------|
| $30' 100 °C$ TSA | 33 | 20 |
| $30' 100 °C$ PCMA | 27 | 13 |
| $30' 106 °C$ TSA | 27 | 20 |
| $30' 106 °C$ PCMA | 27 | 13 |
| $F_0 = 5.3$ | True positives (%) | False positives (%) | True negatives (%) | False negatives (%) |
| $F_0 = 8.4$ | True positives (%) | False positives (%) | True negatives (%) | False negatives (%) |

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115 °C (NIZO4230, up to $9.7 \times 10^2$ cfu/mL), whereas the other five did not (Table S1).

Taken together, the results of this study indicate that significant differences exist between heat resistances of spores of at least *Bacillus* and *G. stearothermophilus* strains, which has implications for assessment of spoilage risks of products made from milk powders when this is based on spore counts determined by classical spore plating methods.

**Spore heat resistance of Geobacillus stearothermophilus**

Heat resistance of spores is influenced by various factors, including the genetic make-up of the strain (Berendsen et al. 2016a), the environmental conditions under which a spore is formed (Setlow 2014) and the matrix in which the spore resides (den Besten et al. 2018). In the literature, there are many contradictory results reported with respect to heat resistance of spores of individual dairy-related isolates, which is likely to occur when results based on different methods are compared (Kent et al. 2016; Sadiq et al. 2016). Such differences in findings may furthermore be attributed to unclarity in identification of species or may be caused by strain-to-strain variation.

In this study, *G. stearothermophilus* was identified as the main spoiler of UHT treated reconstituted milk products. The 95% confidence interval of the log₁₀ *D*-values (a measure of heat resistance) for spores of *G. stearothermophilus* is shown in Figure 2, based on a meta-analysis of previously reported *D*-values (André et al. 2013; Rigaux et al. 2013; Wells-Bennik et al. 2018). Based on these data, one log₁₀ reduction at 100 °C requires heating times of 1.45, 2.32 and 3.19 log₁₀ min (i.e. 28, 208 and 1535 min) for the least heat-resistant, the mean and the most heat-resistant spores of *G. stearothermophilus*, respectively (Figure 2). At 106 °C, heating times required for one log₁₀ reduction are

![Figure 2](image-url)
0.86, 1.73 and 2.60 log_{10} min (i.e. 7, 53 and 394 min) for the least heat-resistant, the mean and the most heat-resistant spores of *G. stearothermophilus*, respectively. Thus, when applying heat treatments of 30 min at 100 °C or 106 °C, spores of *G. stearothermophilus* will generally show <1 log_{10} unit inactivation. Figure 2 furthermore indicates the most heat-resistant spores of *G. stearothermophilus* are not or only slightly reduced upon a UHT treatment at \( F_0 = 5.3 \). When plotting spore \( D \)-values of various other spore formers encountered in dairy products (*B. sporothermodurans* (Huemer et al. 1998), *G. thermoglucosidans* (Zhao et al. 2013), *A. flavithermus* (Witthuhn et al. 2011) and *Bacillus* species (Berendsen et al. 2015b; 2016b)) against the 95% confidence interval of \( D \)-values for spores of *G. stearothermophilus*, it stands out that the heat resistance of spores of some strains *Bacillus* spp. is comparable with that of spores of *G. stearothermophilus*. Even though this was not observed for the milk powders used in this study, such spores may also survive UHT heat treatments and cause spoilage.

**Practical impact on spoilage potential**

The outcomes of this study are limited to the types of milk powders that were available for this study (Table 3), the bacterial spore species present in these powders (Table 6) and translation to predictability of spoilage of simple UHT milk products only (i.e. excluding complex products based on whey powders, additions of minerals and vitamins and/or low-acid (canned) milk products). In addition, this study does not take into account product spoilage caused by recontamination after UHT treatment (Doll et al. 2017). Based on the powders used and the spoilage seen for reconstituted milk made thereof, this study focusses on thermophilic spore formers, as the milk powders contained very low concentrations of mesophilic spore formers (Table S2) and no spoilage was observed after incubation at 37 °C (Table 5).

Together with *B. licheniformis*, *G. stearothermophilus* has previously been identified as the most predominant spore-forming species in milk powders (Kent et al. 2016; André et al. 2017), with the plate heat exchanger and the evaporator being the most common sources of contamination of these spores that have been identified during milk powder manufacturing (Scott et al. 2007). In practice, however, presence of viable *G. stearothermophilus* spores in UHT finished products will only have serious implications when such products are exposed to elevated temperatures (>37 °C) at some point within the supply or consumer chain.

In summary, there is no plating method for detection of spores in dried milk that could be used in combination with a specification that will result in 100% predictability of spoilage of UHT finished products, which is in agreement with findings previously published by Kent and co-workers (Kent et al. 2016). Nevertheless, similar predictability of spoilage was found after application of a heat treatment of 30 min at 100 °C as for a heat treatment of 30 min at 106 °C when specifications are increased by 1 log_{10} (e.g. from 10^2 cfu/g to 10^3 cfu/g). In addition, the use of TSA as a plating medium instead of PCMA is highly recommended for increased recovery of spores that can cause spoilage in UHT treated finished products. It should be noted that false positives and (more importantly) false negatives will always occur when using classical plating methods for spore detection, which is most likely due to significant strain-to-strain variation in spore heat resistance and/or germination efficiency. This stresses the importance of understanding spoilage potential of bacterial spores on a deeper level as well as the need for an ability to distinguish this immediately using a practical assay. A better understanding of molecular mechanisms underlying elevated heat resistance, for instance, can help in the development and/or improvement of practical molecular detection methods.

**ACKNOWLEDGEMENTS**

The authors would like to thank Kevin van Koerten for the preparation of Figure 2.

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SUPPORTING INFORMATION

The following supporting information is available for this article:

Table S1 Recovery of spore forming strains after heat treatments of 30 min at 80, 100, 106 °C or 20 min at 115 °C on 6 different media after incubation at 37 °C (red) or 55 °C (blue).

Table S2 Spore recovery data from milk powder pre-screen and categorization of powders.

Table S3 Identification of bacterial isolates from spoiled UHT products by 16S sequencing and MALDI-TOF.

Table S4 Spore recovery from milk powders.

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