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Accurate quantification of thermophilic spores in dairy powders

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ABSTRACT

Internationally, there are no official guidelines for the quantification of thermophilic spores in dairy products, which leads to variations in applied methodology. In this study, we assess the heat sensitivity of thermophilic spores, vegetative cells grown under laboratory conditions and spores in German dairy powders to determine appropriate heating conditions for accurate quantification of total thermophilic spores. The heat inactivation effect (80–95 °C) is limited for spores of *Anoxybacillus flavithermus* and *Geobacillus stearothermophilus* grown under laboratory conditions. However, for spores originating from whey, whey powder and skimmed milk powder (mostly identified as *A. flavithermus*), a different trend was observed; spore counts continuously reduced when heating time and temperature increased (80–98 °C, 10–30 min). The results indicate that data obtained using laboratory cultures cannot be extrapolated to commercial powders, and in this case, applying temperatures above 80 °C leads to an underestimation of spore counts in dairy powders.

1. Introduction

Powdered dairy products have high economic value and are well suited for global trading. They do not require strict storage conditions and have a long shelf life, as they are less prone to microbial growth due to low water activity ($a_w < 0.26$) (Hill & Smythe, 2012). Because vegetative cells are not able to survive the powder production process, the microbial load of milk powder is mostly due to heat stable endospores, as long as recontamination events are avoided (Reich et al., 2017).

Many previous studies have reported that endospores in dairy powders belong to the thermophilic bacilli group, and the microbiota is often dominated by the two non-pathogenic species *Anoxybacillus flavithermus* and *Geobacillus stearothermophilus* (Burgess, Lindsay, & Flint, 2010; Nazina et al., 2001; Pikuta et al., 2000; Ronimus et al., 2003; Sadiq, Flint, & He, 2018; Yuan et al., 2012). Both species are obligately thermophilic and believed to result from growth over the production process. Additionally, endospores of mesophilic bacilli are frequently isolated. *Bacillus licheniformis* is a mesophilic bacterium, with an extended temperature growth range up to 60 °C, and the third major spore-forming species found in powders worldwide (Burgess et al., 2010; Kent, Chauhan, Boor, Wiedmann, & Martin, 2016; Ronimus et al., 2003; Ruckert, Ronimus, & Morgan, 2004; Yuan et al., 2012).

The spore load of dairy powders has been reported to be highly variable between different batches of the same product, as well as between different product types, and covers between <10 cfu·g^{-1} and $> 10^5$ cfu·g^{-1} (Hill & Smythe, 2012; Kent et al., 2016). Besides the absence of pathogenic bacteria (e.g. *Staphylococcus aureus*), thermophilic spore populations have emerged as one of the most important parameters for production hygiene, and, as a result, stringent criteria have been set to limit spore content. In particular, specifications for acceptable thermophilic spore counts have been lowered, which poses a major challenge for manufacturers to fulfil. The US Dairy Export Council must adhere to a limit of 500 cfu·g^{-1} thermophilic spores (Watterson, Kent, Boor, Wiedmann, & Martin, 2014). However, in Ireland or China, the thermophilic count is not specified in detail, but rather the aerobic plate count is limited to $<10^4$ cfu·g^{-1} in Ireland (FSAI, 2014) and $< 10^3$ cfu·g^{-1} in China (Yuan et al., 2012). Global trading requires harmonised methods for quality control to ensure comparability of results between different countries, manufacturers and customers. Yet, unlike the

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quantification of mesophilic and highly heat-resistant spores, there is no standardised methodology for the enumeration of thermophilic endospores, which leads to a large variation in methods applied (Burgess et al., 2010; Coorevits et al., 2008; McGuiggan, McCleery, Hannan, & Gilmour, 2002; Miller et al., 2015; Murphy, Lynch, & Kelly, 1999; Ruckert et al., 2004; Rueckert, Ronimus, & Morgan, 2005; Yuan et al., 2012).

Culture-based quantification of bacterial spores includes a heating step to inactivate vegetative cells. According to Reich et al. (2017), the inactivation temperature of vegetative thermophilic spore formers exceeds common pasteurisation temperatures, but lies well below 80–85 °C. The standard methodology for mesophilic spores of aerobic bacilli includes a heat treatment at 80 °C for 10 min, (VDLUFA M 7.17.2; Frank & Yousef, 2004), whereas highly heat-resistant spores are enumerated after heating for 30 min at 106 °C (ISO/TS 27265:2009). In China, regulations demand a heat treatment at 100 °C for 30 min, including the equilibration of the sample (PSPRC, 2007), based on NEN 6809:1999. These parameters, however, have been shown to influence spore level detection as well as species recovery after heating (Kent et al., 2016). By increasing temperature, the counts of both mesophilic and thermophilic spores decreased, although the effect was much more pronounced for mesophilic spores.

This study analyses the impact of different temperature–time combinations on the thermophilic spore count in dairy powders of German origin, focusing on obligate thermophilic bacilli. Different temperature–time combinations were analysed for their effect on vegetative cells and spores of thermophilic spore formers grown in laboratory conditions. In addition, processed samples of skimmed milk powder (SMP), whey powder (WP) and whey from German manufacturers were analysed for their microbial composition and heat sensitivity of thermophilic spores. This data ultimately contributes to the effort of finding the optimal temperature–time combination for reliably inactivating vegetative cells while leaving spores unaffected to prevent over- or underestimation of thermophilic spore counts in dairy powders.

2. Materials and methods

2.1. Processed dairy samples

Processed dairy samples (whey, WP and SMP) were obtained from 13 different companies. Altogether, the study included 17 samples of whey, 20 samples of WP and 23 different SMPs. Whey samples were used directly for thermal treatments, whereas the powdered dairy products had to be reconstituted. Therefore, the respective powders were dissolved homogeneously 1:10 in ¼ Ringer's solution (Merck).

2.2. Bacterial strain selection

All strains used in this study were originally isolated from dairy products like SMP or raw milk (see Table 1). Their identities were confirmed by 16S rDNA sequencing. Microorganisms were cryoconserved, stored at −80 °C and plated on tryptic-soy-agar plates (TSA, Roth) prior to use.

2.3. Preparation of spore and cell suspensions for heat experiments

2.3.1. Spore suspensions

Spore suspensions were prepared for three thermophilic strains: *A. flavithermus* G8748 and *G. stearothermophilus* EG1950 and EG1938. First, a culture in 7 mL tryptic soy broth (TSB, Merck) was inoculated and incubated at 55 °C for 24 h ± 30 min. Second, 200 µL culture was plated on sporulation media [TSA (Oxoid) where 0.1% (v/v) 1 mM $FeSO_4 \cdot 7H_2O$, 1 M $Ca(NO_3)_2 \cdot 4H_2O$ and 0.1 M $MnCl_2 \cdot 4H_2O$ (Roth) were added, in accordance with Wedel et al., 2018], and incubated at 55 °C for 5–13 days.

Table 1
Thermophilic bacilli used in this study and their respective isolation sources.[a]

Species	Strain	Food source
Anoxybacillus flavithermus	G8748[2]	Milk powder
	EG1851	Skim milk powder
	EG3109	Skim milk powder
	F48[1]	Skim milk powder
	G10613	Skim milk powder
Geobacillus stearothermophilus	EG1938	Skim milk powder
	EG1950	Skim milk powder
	EG1951	Skim milk powder
	EG1956[2]	Skim milk powder
	EG3113	Skim milk powder
	G6286	Raw milk
	G8742[2]	Milk powder

[a] References indicated by superscripts 1 and 2 are Lucking et al. (2013) and Reich et al. (2017), respectively.

Using microscopy, the degree of sporulation was evaluated. When the spore level was higher than the level of vegetative cells, at high spore levels, the culture was harvested. 5 mL phosphate buffer (2 mM KH_2PO_4, 8 mM K_2HPO_4, 4 °C) were used twice to carefully wash spores from the surface of the plate. To prevent aggregation of spores, the harvested spore suspension was stirred for 1–2 h in an ice bath. The next day, the pellet of the suspension was washed three times in 20 mL cold phosphate buffer. In between, the suspension was centrifuged at 4000 rpm and 4 °C for 7 min. Following the last washing step, the pellet was resuspended in 10 mL phosphate buffer and 10 mL 70% ethanol to inactivate remaining vegetative cells. After cool storage for 2–3 days, this washing step was repeated three times using cold phosphate buffer to remove the ethanol. The final spore suspension was stored at 4 °C. For thermal inactivation experiments, spore suspensions were diluted in TSB or milk (UHT-milk, 1.5% fat). The heat sensitivity of all strains was analysed in three replicates.

2.3.2. Vegetative cells

The heat sensitivity of vegetative cells of thermophilic bacilli was evaluated in TSB (Roth) and milk (UHT-milk, 1.5% fat) using suspensions from freshly grown day cultures. For the selected strains (*A. flavithermus* EG1851, F48, EG3109, G8748 and *G. stearothermophilus* EG1951, EG3113, G8742), a fresh dilution streak on TSA was used for the inoculation of an overnight (O/N) culture in 10 mL TSB. On the following day, 50 mL of TSB or milk were inoculated 1:500 using the O/N culture. After 6 h of incubation at 55 °C with shaking at 110 rpm, the culture was harvested to determine heat sensitivity. A centrifugation step (2000 × *g*, 15 min, room temperature = RT) was included to increase the initial cell count of the culture. The cell pellet was resuspended in 5 mL phosphate buffer or milk. For the thermal treatment, the resuspended cell culture was diluted 1:10 in phosphate buffer or milk, and the cell count before and after a distinct heat treatment was measured by plating on TSA. Each strain was analysed in at least three independent biological replicates.

2.4. Thermal treatment

The thermal treatment of spore suspensions, vegetative cells and processed samples was conducted in a temperature-adjusted water bath where the temperature was controlled continuously. Each treatment was conducted in a volume of 10 mL. A pilot tube was used for monitoring the temperature of the samples during each heating experiment and determining the start of the holding

time (time for equilibration < 4 min). Chinese regulations require the application of a heat treatment at 100 °C for 30 min (PSPRC, 2007). As 100 °C in an open water bath is achievable only at sea level, this study was conducted at the uppermost temperature of 98 °C. In this case, holding time began directly after placing the samples into the water bath; in contrast, for 80, 90 and 95 °C, the holding time began after equilibration of the samples to their respective temperatures.

Spore suspensions were analysed at 80, 90 and 95 °C for 0, 15 and 30 min. A heat treatment for 0 min represents the effect of equilibrating the sample without any holding time, and the test tubes were cooled directly after the respective temperature was reached. For dairy samples, a trial at 98 °C for 30 min was additionally analysed. Vegetative cells were analysed at 80 °C and a heating time of 0 and 30 min.

Processed samples were analysed once, whereas spore suspensions and vegetative cells were analysed in at least three independent replicates. Thermophilic cell counts were measured by plating on TSA and incubating the plates for 48 h at 55 °C.

2.5. Biodiversity of thermophilic spore formers

Reconstituted WP and SMP, as well as whey samples, were heated at 80 °C and kept for 10 min. Afterwards, the samples were cooled to RT, plated on TSA and incubated at 55 °C for 48 h. Subsequently, 25 pure colonies per sample were isolated randomly to allow for quantitative analysis of species composition. Pure colonies were identified using FTIR spectroscopy and selected isolates additionally by partial 16S rDNA sequencing. FTIR spectroscopy was conducted as described previously and after incubation of isolates at 55 °C for 24 h ± 0.5 h on TSA (Doll, Scherer, & Wenning, 2017; Oberreuter, Seiler, & Scherer, 2002; Wenning et al., 2014). Partial 16S rDNA sequencing was executed using primers 27f and 1492r, as described by Doll et al. (2017), and the EzTaxon server was used for identification (Yoon et al., 2017). For five samples of SMP, the biodiversity could not be determined because the number of pure isolates was not appropriate (less than 10 colonies). If spore counts were low, all colonies present were chosen (14 colonies at least).

2.6. Heat sensitivity of spores during growth at 55 °C

Two strains, *A. flavithermus* G10613 and *G. stearothermophilus* EG1956, were chosen to test the sensitivity of spores in regards to heat during growth and spore formation in liquid culture. A fresh dilution streak on TSA was used for the inoculation of an O/N culture (55 °C, 150 rpm, 10 mL 1.5% fat UHT-milk). On the next day, UHT-milk (1.5% fat) was inoculated 1:1000 using the O/N culture and incubated at 55 °C, shaking at 150 rpm. The culture was sampled directly after inoculation (= 0 h) to determine the inoculation level. Here the total thermophilic cell count and spore count after heat treatment at 80 °C and 10 min were measured by plating on TSA.

Further samples were taken every hour between 1 and 8 h for *A. flavithermus* G10613 and between 3 and 8 h for *G. stearothermophilus* EG1956. The last sampling was after 24 h of cultivation. For all samples, the total thermophilic cell count and spore counts after heating at 80, 90 and 95 °C for 10 min were determined by plating on TSA. All plates were incubated at 55 °C for 48 h. Thermal treatments were carried out in a thermal shaker while shaking at 600 rpm (T_{set} ± 1 °C). The temperature was controlled continuously and the heating time began when T_{set}−1 °C was reached. After the treatment, the samples were cooled directly to RT using tap water.

For *A. flavithermus* and *G. stearothermophilus*, seven and eight cultures were respectively grown and analysed independently.

3. Results and discussion

3.1. Heat resistance of thermophilic spores

Spore suspensions of three thermophilic test strains were prepared and tested for survival in milk and TSB after heating at temperatures between 80 and 95 °C. Initial spore counts prior to the heat treatment were between log 3.9 cfu mL^{-1} and log 5.7 cfu mL^{-1}. As expected, spores were largely unaffected by the heat treatment (Table 2). The maximum reduction for *A. flavithermus* G8748 as well as *G. stearothermophilus* EG1950 was 0.1 log in TSB and milk. The highest reduction was measured for *G. stearothermophilus* EG1938 at 90 °C for 30 min in TSB (log reduction = log 0.17 ± log 0.29). However, the decrease at 95 °C was less pronounced and an inactivation of EG1938 in milk could not be observed (Table 2). It is therefore assumed, that the differences observed may originate from methodological variance. Overall, thermophilic spores of *A. flavithermus* and *G. stearothermophilus* formed at laboratory conditions exhibit a high resistance to temperatures between 80 and 95 °C, as applied in this study.

The results are in line with previous studies that have investigated the heat sensitivity of thermophilic spores with a more intense heat treatment at 100 °C for 30 min (Sadiq et al., 2016; Wells-Bennik et al., 2018). Sadiq et al. (2016) focused on the heat resistance of thermophilic isolates from Chinese milk powders (incl. *G. stearothermophilus*) and observed 100% survival of spores after heat treatment of 100 °C for 30 min for all test strains. Similarly, the inactivation effect among 18 strains of *G. stearothermophilus* (of various origin) in the study of Wells-Bennik et al. (2018) was smaller than 0.2 log.

Only heat treatments of higher intensity at > 100 °C lead to a heat inactivation effect (Wedel et al., 2018; Yuan et al., 2012). $D_{121°C}$-values among the species *G. stearothermophilus* vary between different strains and are in the range of less than one to several minutes (e.g. Dogan, Weidendorfer, Müller-Merbach, Lembke, & Hinrichs, 2009; Guizelini, Vandenberghe, Sella, & Soccol, 2012; Rigaux, Denis, Albert, & Carlin, 2013; Wells-Bennik et al., 2018).

3.2. Heat sensitivity of thermophilic vegetative cells

Four strains of *A. flavithermus* and three strains of *G. stearothermophilus* were chosen to analyse the thermal inactivation behaviour of freshly grown vegetative cells at 80 °C. During a screening of thermophilic growth in milk and TSB (data not shown), some strains grew in TSB but not in milk. For other strains it was vice versa, and only one strain of the test set (EG3113) demonstrated sufficient growth in both TSB and milk (cell count higher than log 4 cfu mL^{-1} within 6 h).

In TSB, the cells of the *G. stearothermophilus* strain G8742 were completely eliminated after equilibrating to 80 °C (0 min) (Fig. 1A). For the other four test strains (*A. flavithermus* EG1851 and F48, *G. stearothermophilus* EG1951 and EG3113) the cell count was decreased by at least 2.8 log. The inactivation effect did not increase with prolonged heating time (30 min), but ended in a plateau of equal cell counts and consequently resulted in a similar log reduction for these strains (Fig. 1A). In milk, the findings were comparable, as vegetative cells were either fully eliminated after equilibration to 80 °C (EG3109) or reduced by more than four log levels with a similar reduction for both heating times (Fig. 1B). Strain EG3113 displayed better growth in milk and reached an initial cell count that was 1 log level higher. As the spore levels were equal in both milk and TSB, we may attribute this effect to a larger fraction of vegetative cells that lead to the observed higher inactivation of 1 log level. As discussed above, thermophilic spores grown in lab culture can withstand temperatures <100 °C. The plateau

Table 2
Reduction of spores of *A. flavithermus* G8748 and *G. stearothermophilus* EG1950, EG1938 in milk and tryptic soy broth (TSB).[a]

Temp. (°C)	Heating time (min)	*A. flavithermus* G8748		*G. stearothermophilus* EG1950		*G. stearothermophilus* EG1938	
		TSB (log cfu·mL^{-1})	Milk (log cfu·mL^{-1})	TSB (log cfu·mL^{-1})	Milk (log cfu·mL^{-1})	TSB (log cfu·mL^{-1})	Milk (log cfu·mL^{-1})
80	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	15	0.01 ± 0.03	−0.05 ± 0.08	0.03 ± 0.01	0.07 ± 0.04	0.03 ± 0.11	−0.03 ± 0.03
	30	0.04 ± 0.01	0.03 ± 0.03	<0.01 ± 0.06	0.05 ± 0.03	0.06 ± 0.06	−0.01 ± 0.05
90	0	−0.01 ± 0.03	−0.03 ± 0.07	<0.01 ± 0.04	0.03 ± 0.04	0.03 ± 0.25	0.05 ± 0.24
	15	0.04 ± 0.02	0.03 ± 0.05	0.06 ± 0.02	0.07 ± 0.06	0.12 ± 0.27	<0.01 ± 0.24
	30	0.03 ± 0.02	0.10 ± 0.10	0.06 ± 0.01	0.05 ± 0.05	0.17 ± 0.29	−0.03 ± 0.21
95	0	−0.01 ± 0.02	0.01 ± 0.08	0.02 ± 0.05	0.04 ± 0.04	−0.10 ± 0.12	−0.08 ± 0.08
	15	<0.01 ± 0.01	0.05 ± 0.05	0.04 ± 0.01	0.11 ± 0.06	0.03 ± 0.15	−0.09 ± 0.04
	30	0.06 ± 0.03	0.11 ± 0.02	0.08 ± 0.03	0.05 ± 0.05	0.06 ± 0.14	−0.07 ± 0.02

[a] Thermal treatments were applied at 80, 90 and 95 °C. Cell counts were measured after heating times of 0, 15 and 30 min. Log reduction of cell counts after distinct heat treatment were calculated in relation to cell counts of the treatment at 80 °C and 0 min [$\log(N_{80°C,\ 0\ min}) - \log(N_t)$]. Each data point results from three replicates.

observed for all strains therefore likely represents spores that are not affected by the heat treatment. This spore count could be the result of sporulation during cultivation or residual spores of the inoculum.

In this study vegetative cells of seven test strains of *A. flavithermus* and *G. stearothermophilus* were highly sensitive to heat treatments at 80 °C. Thermal treatments of lower intensity (pasteurisation conditions at 63–73 °C) have previously been demonstrated to not be sufficiently effective for inactivating all vegetative cells, especially those of heat-stable strains (Reich et al., 2017). Thus, a treatment at temperatures below 80 °C may be insufficient, but 80 °C fully inactivates vegetative cells and only spores remain viable.

3.3. Analysis of processed dairy samples

Many different temperature–time combinations are used to determine thermophilic spore counts, and it is unclear to what extent they are comparable. Hence, we elucidated how heat treatments of various intensities affect spores occurring in dairy products. In addition, it was evaluated how heat resistance determined using laboratory spore suspensions compares to that of real samples. For this purpose, 17 samples of whey, 20 WP, as well as 23 SMP were analysed. Heating conditions were chosen between 80 and 98 °C and heating times varied between 10, 20, or 30 min to test heat inactivation effects in more detail than previously conducted for spore suspensions.

As samples were obtained from 13 different German companies and production sites throughout different seasons, the sample pool was highly diverse. Consequently, a large variance in microbial counts between samples of the same product type was observed. Total thermophilic cell counts determined without any heat treating ranged from log 1.4–4.1 cfu·mL^{-1} for whey, log 2.9–4.5 cfu·mL^{-1} for WP and log 2.6–5.4 cfu·mL^{-1} for SMP. Treating the samples at 80 °C for 10 min led to a minimal decrease in numbers, log 0.2 ± 0.2 cfu·mL^{-1} (SMP, WP), on average. No reduction was observed for whey samples.

To get an impression of microbial composition, the thermophilic microbiota of all samples were analysed after heating at 80 °C for 10 min. To allow for a quantitative evaluation, colonies were selected randomly and not by morphology. With six species in total, low biodiversity was detected. Independent of the product type (whey, WP, SMP), *A. flavithermus* was the dominant species, representing 85–93% of the isolates on average (Table 3). Occasionally, there were samples with lower fractions of *A. flavithermus*, but in each of the three products, there were many samples in which it was the only species detected (Table 3). *B. licheniformis*, a mesophilic spore-forming bacterium able to grow at the optimal thermophilic

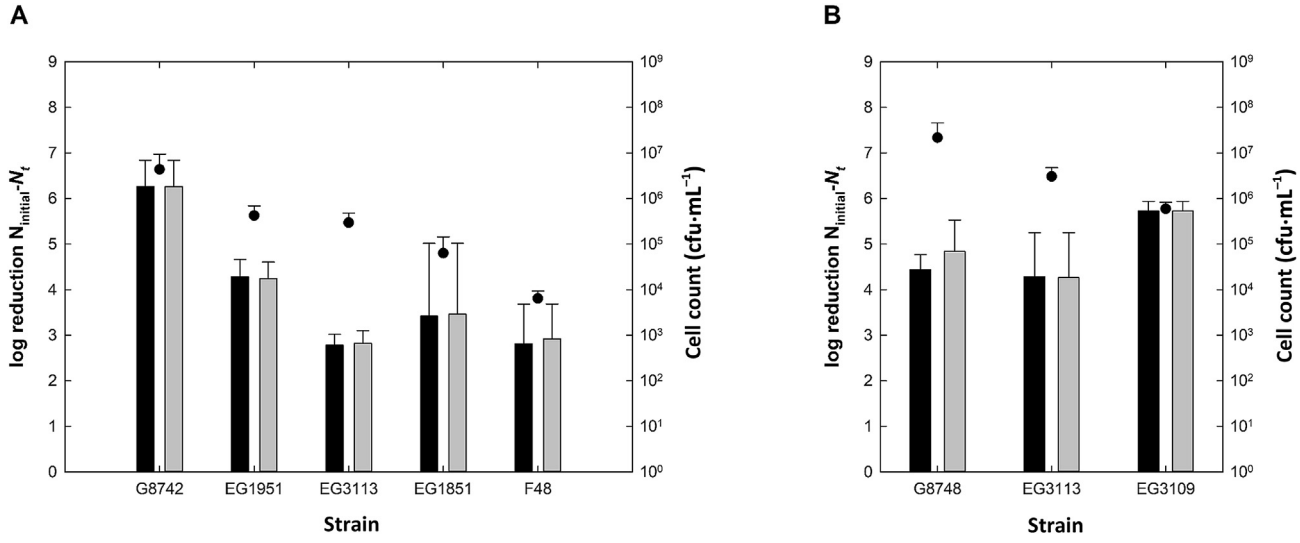

Fig. 1. Logarithmic reduction of cell counts for different strains of *A. flavithermus* (EG1851, F48, EG3109, G8748) and *G. stearothermophilus* (EG1951, EG3113, G8742). Cultures were grown for 6 h at 55 °C, 110 rpm, in tryptic soy broth (TSB, A) and milk (B). Cell counts were measured before the thermal treatment (initial cell count $N_{initial}$, ●) and after the treatment applied at 80 °C for 0 min (no holding time, ■) and 30 min (▒). The reduction [$\log(N_{initial}) - \log(N_t)$] was calculated. Each data point results from three independent biological replicates. Significant reduction for all strains ($p < 0.05$, paired t-test).

Table 3
Abundance of spore formers in processed dairy samples after heating at 80 °C for 10 min.[a]

Species	SMP (n = 18)			Whey (n = 17)			WP (n = 20)		
	average	range	X	average	range	X	average	range	X
Anoxybacillus flavithermus	85.4%	60–100%	18	92.7%	0–100%	16	92.2%	42–100%	20
Bacillus licheniformis	7.2%	0–30%	7	0.3%	0–5%	1	0.7%	0–5%	3
Geobacillus stearothermophilus	6.1%	0–35%	5	5.7%	0–89%	2	6.8%	0–58%	7
Aneurinibacillus thermoaerophilus	0.3%	0–5%	1	0.4%	0–7%	1			
Brevibacillus aydinogluensis	0.2%	0–4%	1				0.3%	0–5%	1
Paenibacillus cookii				0.3%	0–5%	1			
Not identified	0.8%	0–15%	1	0.6%	0–11%	1			

[a] Recovery and isolation at 55 °C. Abbreviations are: SMP = skimmed milk powder; WP = whey powder; n = number of samples; X = isolated from X samples per product type.

growth temperature of 55 °C, was the second highest representative among isolates from SMP, with fractions up to 30% in single samples and 7% on average (Table 3); for WP and whey samples, the abundance was even lower and <1% on average. Overall, *B. licheniformis* was isolated from only one fifth of all samples. The obligate thermophilic species *G. stearothermophilus* accounted for 6–7% of all isolates in samples of all product types and therefore represented the second highest fraction in whey and WP.

Other species like *Aneurinibacillus thermoaerophilus* and *Brevibacillus aydinogluensis* (both obligate thermophilic), as well as *Paenibacillus cookii* (mesophilic), were present in few samples in low abundance. Taken together, obligate thermophilic spores represented the major part of the microbiota of samples tested in this study; facultative thermophilic bacteria like *B. licheniformis* were of minor abundance. Prior analyses of milk powders have determined *A. flavithermus*, *B. licheniformis* and *G. stearothermophilus* as the most prominent representatives among the thermophilic microbiota at varying abundances, as summarised by Ruckert et al. (2004) and Pereira and Sant'Ana (2018) for different countries. Some studies primarily identified the facultative thermophilic *B. licheniformis* (e.g. Kent et al., 2016), whereas other studies are in accordance with our results and found a high prevalence of obligate thermophilic spores (e.g. Scott, Brooks, Rakonjac, Walker, & Flint, 2007). *B. licheniformis* is assumed to originate from raw milk, as its occurrence is ubiquitous in nature, whereas high counts of *Anoxybacillus* and *Geobacillus* are due to the production process itself (Miller et al., 2015; Scheldeman, Pil, Herman, De Vos, & Heyndrickx, 2005; te Giffel, Wagendorp, Herrewegh, & Driehuis, 2002).

The spore inactivation was evaluated by calculating the log reduction [$\log(N_{80°C,10\ min}) - \log(N_t)$] of each thermal parameter related to the treatment at 80 °C for 10 min. Heat treatments at different temperatures resulted in an increasing reduction of spore counts upon intensifying the treatment, which indicates at least an elevated heat sensitivity of spores in commercial powder samples from German manufacturers (Fig. 2). The spore counts in whey and WP were more affected compared to SMP samples, while WP samples exhibited the highest sensitivity and reduction. Except for the SMP treatment at 80 °C for 20 min, all other heat treatments led to a statistically significant ($p < 0.05$) reduction in spore counts. At 80 °C, thermophilic spore counts were reduced only slightly by 0.1–0.3 log (Supplementary material Fig. 1A and B). Higher temperatures of 90 and 95 °C further decreased the spore counts, and highest median reductions were observed at 98 °C [0.9 log (SMP) < 1.1 log (whey) < 1.3 log (WP)]. By increasing heating time (10 < 20 < 30 min) and temperature (80 < 90 < 95 < 98 °C), the reduction of spore counts continuously increased (Fig. 2; Supplementary material Fig. 1) and the level of significance for this

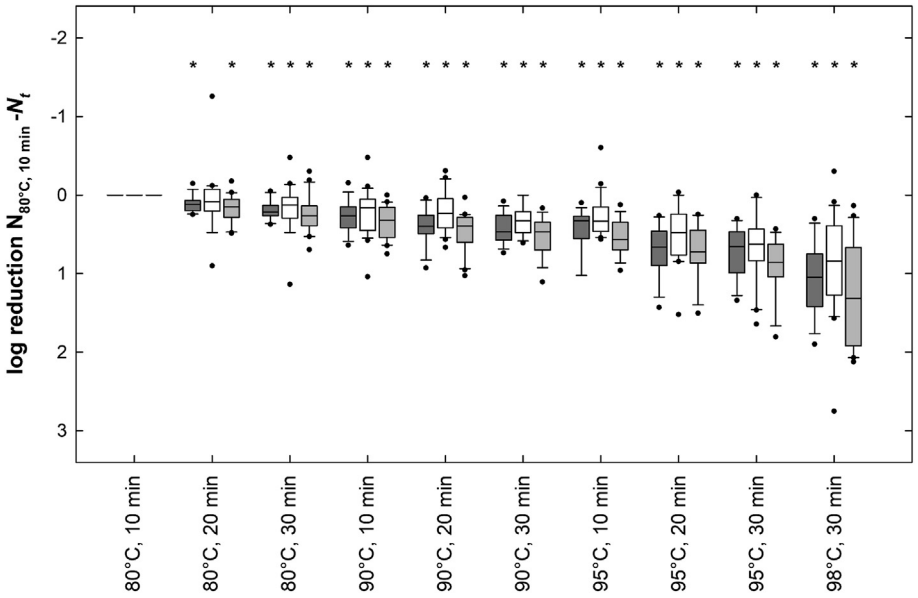

Fig. 2. Logarithmic reduction of thermophilic bacterial counts for processed dairy samples after heat treatments at 80, 90 and 95 °C for 10, 20 and 30 min excluding the time for equilibration and 98 °C for 30 min including equilibration of heating. Cell counts after heat treatments were related to the cell count at 80 °C, 10 min [$\log(N_{80°C,\ 10\ min}) - \log(N_t)$]. Analysis of whey (■; n = 17), whey powder (▩; n = 20), and skimmed milk powder (□; n = 23) is shown. A significant reduction ($p < 0.05$, paired t-test) compared with the 80 °C, 10 min treatment is marked with an asterisk (*).

effect increased by several orders of magnitude (e.g. $p < 1.9 \cdot 10^{-9}$ for WP at 95 °C for 10 min; Supplementary material Fig. 1D). There was a variance in reduction observed for SMP and WP, which was relatively low initially but increased for treatments at 95 °C (20 + 30 min) and largely increased at 98 °C (Fig. 2; Supplementary material Fig. 1C). Standard deviations for SMP and WP constantly ranged <0.3 log levels, but increased to approx. 0.6 log for the treatment at 98 °C (Supplementary material Fig. 1C). For whey, a continuous increase over intensified treatments was observed.

These findings do not correlate with those for spore suspensions and clearly indicate a difference in heat inactivation effects between thermophilic spores originating from dairy products in this study and laboratory spore suspensions. This discrepancy may be attributed to the presence of spores of mesophilic species, which have been shown to exhibit higher heat sensitivity than thermophilic spores (Andre, Zuber, & Remize, 2013; Kent et al., 2016). However, Kent et al. (2016) frequently isolated *B. licheniformis,* which is facultatively thermophilic, even after treatments at 106 °C. In addition, mesophilic spores were of low abundance (<10%) within the thermophilic microbiota of the present study. Thus, their selective inactivation cannot be causative for the decreasing spore counts observed. In addition, the reduction in SMP samples was lowest even though these samples contained the highest amounts of facultative thermophilic spores.

It is well known that the spore's characteristics, such as resistances or long-term survival, are influenced by many factors. Especially the environment (e.g. temperature, nutrients, osmolarity) of the sporulating mother cell influences the characteristics of the spores formed (Nguyen Thi Minh, Durand, Loison, Perrier-Cornet, & Gervais, 2011). Studies that have analysed the effect of temperature, pH, or nutrient availability on heat sensitivity of *G. stearothermophilus* strains found a strong dependency of heat resistance on optimal growth conditions. It was demonstrated that the heat resistance of *G. stearothermophilus* strains, as well as the spore yield, is highest at conditions providing the best growth (Durand, Planchon, Guinebretiere, Carlin, & Remize, 2015; Mtimet et al., 2015; Wells-Bennik et al., 2018). Such conditions are usually used for the preparation of laboratory spore suspensions, which hence result in spores of maximised heat resistance.

Thermophilic spores in dairy products display considerable heterogeneity. First, dairy products contain spores from different species, and different strains of each species may be present as well. Each species and strain displays different heat resistance (Durand et al., 2015; Lucking, Stoeckel, Atamer, Hinrichs, & Ehling-Schulz, 2013; Sadiq et al., 2016). In addition, the formation of spores most probably occurs under varying conditions. A part of the spores originates from the raw product (e.g. raw milk) and may be environmental contamination. Another part of the spore microbiota originates from growth over the production process, which consists of different steps with varying processing conditions (e.g. temperature or osmotic pressure). Furthermore, spores of the same strain in a dairy product may be at different stages of the maturation process (physical state and composition), particularly if they are the result of production line growth.

The diversity of spore populations in dairy products (concerning species composition or process parameters that lead to different amounts of mature spores and individual heat resistance of occurring strains) explains the differences observed across all samples. Over the production process, the spores are likely formed continuously, which results in a spore distribution with varying degrees of spore maturation regardless of individual heat resistance. This finding appears to be congruent with the observed continuous reduction of spore counts during inactivation experiments even at temperatures below 100 °C (Fig. 2). Lower temperatures (80 °C) inactivate vegetative cells. Then, not fully maturated and more heat-sensitive prespores or forespores could be inactivated and only fully mature spores survive the highest temperatures and longest holding times.

3.4. Heat sensitivity of thermophilic spores is dependent on growth phase

To investigate if and to what degree the heat sensitivity of spores varies between different phases of bacterial growth, two thermophilic isolates were cultivated in UHT-milk, and the effect of heat treatments varying in intensity (80, 90, 95 °C) was monitored. Growth and sporulation behaviour differed between both strains analysed. *A. flavithermus* G10613 (Fig. 3A) exhibited a faster growth rate and much higher degree of sporulation compared to *G. stearothermophilus* EG1956 (Fig. 3B) when cultivated at 55 °C. Proliferation of vegetative cells began in the early growth phase, which is expressed by rising total cell counts, but nearly constant spore counts.

The low reduction of spore counts observed may be due to germination of spores from the inoculum during early growth. Spore counts began to rise in late exponential or early stationary phase (3–4 h) until the spores reached a sporulation level near 30% in late stationary phase. In the case of *G. stearothermophilus* EG1956 (Fig. 3B), spore and total cell counts increased in parallel at nearly the same rate and relative spore levels remained comparably low (<1%).

The heat sensitivity of the spores was indeed influenced by growth phase (Fig. 3). Mature spores from the inoculum displayed the highest heat resistance, and the heat sensitivity of spores increased until it reached its apex and declined again during the stationary phase. Focusing on the magnitude of the heat inactivation effect, *G. stearothermophilus* (Fig. 3B) was more heat sensitive than *A. flavithermus* (Fig. 3A) during growth. After 7 h (early stationary phase), the inactivation effect was maximum and the spore count was reduced by log 1.8 ± 0.8 after heating to 95 °C for 10 min (right axis). This reduction was significantly different from all other time points ($p < 0.05$). A less intense heat treatment at 90 °C produced a less pronounced inactivation effect (log reduction $N_{80\ °C,\ 10\ min} - N_t = 0.8 \pm 1.0$). Spores of *A. flavithermus* also demonstrated the highest heat sensitivity in the early stationary phase, between 4 and 6 h of cultivation, but were more heat resistant that those of *G. stearothermophilus* (log reduction $N_{80\ °C,\ 10\ min} - N_t = 0.8 \pm 0.2$ at 95 °C, 10 min after 5 h).

These results demonstrate the effect of the growth phase on the heat sensitivity of spores regardless of detailed growth characteristics, degree of sporulation, or absolute heat sensitivity. Particularly, spores that are newly formed in the phase of rising spore counts are more affected by heat treatments and inactivated to a higher degree. The heat inactivation effect diminishes as a constant spore level is reached. Thus, young spores are more sensitive to heat, suggesting that full maturation is necessary for maximal heat resistance. This finding corroborates the study of Sanchez-Salas, Setlow, Zhang, Li, and Setlow (2011) in which spores of *Bacillus subtilis* were found to acquire their high wet heat resistance late in sporulation. The authors observed a direct positive correlation between the last steps in the maturation of the forespore (DPA uptake, final decrease in spore's water content) and increasing heat resistance. This discovery could help to explain the difference in heat sensitivity between laboratory spores and spores in commercial powders in this study. Although these experiments have been conducted in liquid culture and spores developing in the production line mostly originate from growth in a biofilm or fouling layer, it is likely, that the effects observed also apply for spores in real samples. The developing spore needs to undergo the different steps of maturation regardless of whether it is sessile or planktonic.

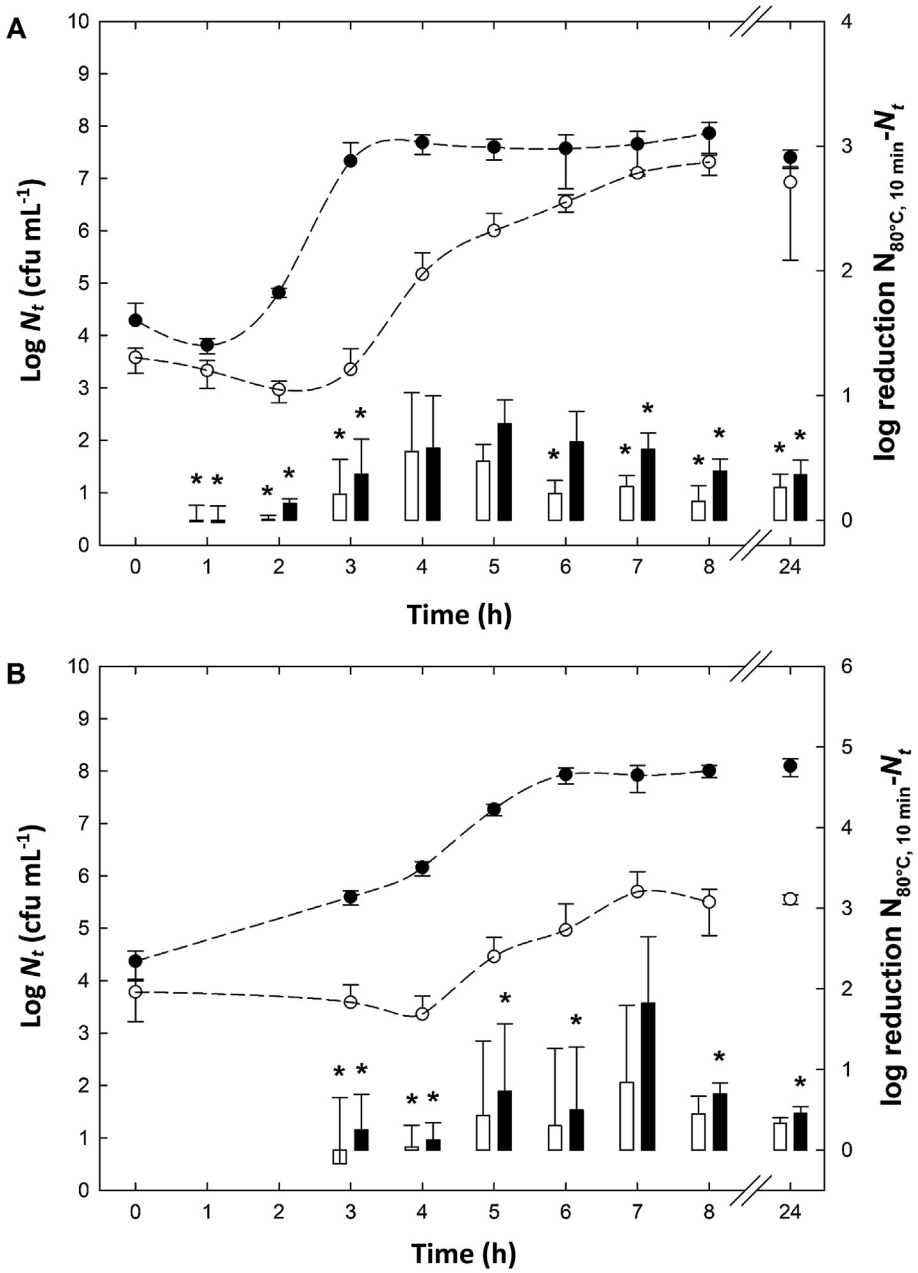

Fig. 3. Heat sensitivity of thermophilic spores dependent on the growth phase in milk. *A. flavithermus* G10613 (A, n = 7) and *G. stearothermophilus* EG1956 (B, n = 8) were cultivated for 24 h at 55 °C. Total cell counts (●) and spore counts (○; 80 °C, 10 min) were measured. Heat treatments at 90 °C (□) and 95 °C (■) for 10 min excluding the time for equilibration were applied to the samples, and the log reduction [$\log(N_{80°C, 10 min}) - \log(N_t)$] in relation to the spore count after the 80 °C, 10 min treatment was calculated. For data marked with (*), the reduction was significantly different ($p < 0.05$, paired t-test) from the reduction at 5 h (A) and 7 h (B).

The spore population in real samples is highly diverse and includes differently maturated spores, which leads to a certain variance in heat resistance. This variance results in a continuously increasing reduction of spore count as the temperature–time relation is increased, with relatively low standard deviations observed. However, the variance within one product type increases starting with the treatment at 95 °C for 20 min, and it is more pronounced for SMP and WP than for whey (Supplementary material Fig. 1C). This effect may be attributed to specific strain characteristics rather than to the maturation stage of the spores. Some samples exhibited very low spore reduction even at 98 °C for 30 min, whereas others' spores were diminished by almost 2 log levels. The critical conditions for selecting heat-resistant spores appear to be temperatures at or above 95 °C applied for 20 min or longer.

4. Conclusions

In the quantification of thermophilic spores, the heating step to inactivate all vegetative cells should ideally not affect the spores to prevent underestimation of spore counts. Temperatures above 90 °C, however, correspond to decreasing counts of thermophilic spores up to 1 log level or more in commercial powders used in this study (from German manufacturers), which cannot be attributed to a selective inactivation of mesophilic species. Instead, heat resistance of those spores may be lower compared with lab cultures because not all spores are fully maturated or have been formed under suboptimal conditions, which lead to less pronounced heat resistance. If the total thermophilic spore count is to be determined in commercial powders manufactured under similar conditions, temperatures <90 °C

therefore provide more realistic results than temperatures of 100 °C or higher. Based on the results obtained in the present study, we propose the application of 80 °C for 10 min to differentiate between spores and vegetative cells in similar powders, which is the same for mesophilic spores. In cases where highly heat-resistant spores are the object of investigation, temperatures of 100 °C or higher [e.g., according to ISO/TS 27265, IDF/RM 218:2009(E)] are suitable.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2019.07.003.

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