Food Microbiology 83 (2019) 150–158

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Resistance of thermophilic spore formers isolated from milk and whey products towards cleaning-in-place conditions: Influence of pH, temperature and milk residues

Carolin Wedel[a,*], Mareike Wenning[b], Anna Dettling[b], Siegfried Scherer[b], Jörg Hinrichs[a]

[a] University of Hohenheim, Institute of Food Science and Biotechnology, Soft Matter Science and Dairy Technology, Stuttgart, Germany
[b] Technical University Munich, ZIEL-Institute for Food & Health, Chair of Microbial Ecology, Freising, Germany

ARTICLE INFO

Keywords:
Cleaning-in-place
Thermophilic spore formers
Milk powder processing
Anoxybacillus flavithermus

ABSTRACT

The occurrence of thermophilic spore formers in dairy powders is a major concern for producers worldwide. This study aims to investigate the resistance of thermophilic endospores towards cleaning solutions typically used for cleaning-in-place in dairy manufacturing plants. From eleven tested strains, all were able to survive an alkaline treatment (NaOH) at 65 °C for 10 min (0.5%), whereas at concentrations of 2% eight strains withstood the treatment. Acid solutions were more sporicidal. At 0.5% of HNO_3, only three strains survived the treatment. Milk impurities reduced the inactivation effect of the NaOH solutions towards thermophilic spore formers. For two selected strains, a detailed kinetic inactivation in NaOH and HNO_3 solutions at different temperatures was performed and non-log-linear inactivation curves were observed. This study highlights the risk of reusing cleaning solutions in dairies.

1. Introduction

In the last years, research was conducted to isolate primary microorganisms of interest that contaminate milk powder products, such as whey powder or skim milk powder. Those products are easy to ship and are exported to various countries all over the world. As the main organisms, the thermophilic spore forming species *Geobacillus stearothermophilus* and *Anoxybacillus flavithermus* were found (Sadiq et al., 2016; Watterson et al., 2014). Although these species, and thermophilic spore formers in general, are not pathogenic, high spore counts in powdered milk products are regarded as a problem for further processing. Customers assume powders with high spore counts to be an indicator for poor manufacturing practice (Burgess et al., 2010; Hill and Smythe, 2012). It is already known that thermophilic spore formers are widespread in the process environment of dairy powder plants and can survive, grow and sporulate during processing (Burgess et al., 2010; Reich et al., 2017; Sadiq et al., 2016; Scott et al., 2007). Crucial steps are those process conditions, in which critical temperatures of 40–70 °C such as separation, pasteurization and evaporation, are achieved (Reich et al., 2017; Scott et al., 2007). Due to their potential to form biofilms on stainless steel surfaces in their spore and vegetative form, this microorganisms group is able to contaminate a production site (Burgess et al., 2009; Zhao et al., 2013).

In milk processing companies, as cleaning procedure the so-called CIP (cleaning-in-place) is used. The cleaning involves the rinsing of the whole plant with water, a cleaning with alkaline, rinsing with water and subsequent acid solutions without demounting the plant. The solutions circulate through the different parts such as plate-heat-exchangers, separators, evaporators and the pipes. As cleaning solutions, NaOH and HNO_3 are the most commonly used chemicals. Concentrations range from 0.5 to 2% of the respective acid or base. While using CIP after the production, the reuse of the cleaning solutions is common (Kessler, 2002; Merin et al., 2002; Tamime, 2009). A lot of research has focused on the isolation of thermophilic and mesophilic microorganisms and their heat resistance (André et al., 2013; Stoeckel et al., 2016; Witthuhn et al., 2011; Yuan et al., 2012). In contrast, very little information is available about the resistance of thermophilic spores towards typical cleaning conditions. If spores are able to withstand cleaning trials, intensified through the reuse of cleaning solutions, a contamination of the next production run is the consequence.

To gain more information about the resistance of thermophilic spore formers towards cleaning procedures of dairies, the aim of this study was to investigate the sensitivity of *A. flavithermus* and *G. stearothermophilus* strains isolated from dairy powder environments towards cleaning solutions, *i.e.* NaOH and HNO_3 in concentrations of 0.5–2%. Furthermore, it should be investigated whether milk residues in the

* Corresponding author.
E-mail address: carolin.wedel@uni-hohenheim.de (C. Wedel).

https://doi.org/10.1016/j.fm.2019.05.002
Received 14 November 2018; Received in revised form 29 April 2019; Accepted 4 May 2019
Available online 10 May 2019

cleaning solutions, which are common in dairy industries, have an impact on the sporicidal effect of the solutions. Last, for strains being most resistant in the screening experiments, detailed inactivation kinetics of different heating times and temperatures should be determined and the kinetic parameters for three different concentration levels should be calculated.

2. Material & methods

2.1. Thermophilic microorganisms and preparation of spore suspensions

All thermophilic spore-forming strains were stored at −20 °C in 30% glycerin solution (Merck, Germany). The used microorganisms were sequenced via their 16S rDNA prior to this study. The strains were isolated form dairy products, such as milk and whey powder and intermediate products. The details to the isolated strains are given in Reich et al. (2017). The used strains all belong to the two species *Geobacillus stearothermophilus* and *Anoxybacillus flavithermus*.

For the preparation of the spore suspensions an adapted method from Witthuhn et al. (2011) was used, which was described in details elsewhere (Wedel et al., 2018). Briefly, the spores were streaked on TSA (Roth, Germany) and incubated in two subsequent liquid cultures to get a dense growth. The second liquid culture was plated on TSA plates (140 mm diameter), to which 0.1% (v/v) of sterile 0.1 M $MnCl_2$, 1 mM $FeSO_4 \cdot 7\ H_2O$ and 1 M $Ca(NO_3)_2 \cdot 4\ H_2O$ (Roth) was added. After a maximum of eight days, the spores and cells were harvested by pouring sterile phosphate buffer (2 mM KH_2PO_4 and 8 mM K_2HPO_4, Roth) on the plates. The spore pellet was pasteurized (80 °C, 10 min) and washed with the phosphate buffer at least 4 times by centrifugation (2,218 *g*, 7 min). The pellet was stored up to three days in ethanol (35% v/v). The final suspension was suspended in phosphate buffer and stored at 2 °C prior to use.

2.2. Screening the sensitivity of thermophilic spores towards NaOH and HNO_3

2.2.1. Inactivation experiments in stainless steel tubes

With all thermophilic test strains, a screening of their sensitivity towards acid and basic conditions was conducted. As screening temperature a condition typical for cleaning in dairy production facilities was used (for both alkaline and acid cleaning 65 °C for 10 min). Three stock solutions for NaOH (Roth) and HNO_3 (Roth) were prepared: 0.55, 1.11 and 2.22% (w/v). Spores were diluted 1:10 in the cleaning solution to reach the concentration of 0.5, 1 and 2%. The pH-values of the solutions were controlled at room temperature (WTW, Weilheim, Germany) by adding phosphate buffer instead of spore suspension. Before the experiments started, the spore suspension was suspended in the respective cleaning solution and the initial spore count (N_0) at room temperature was measured. Directly afterwards, the inactivation experiments were carried out. Therefore, the mixture of spores and cleaning solutions was filled in stainless steel tubes (described in detail in Witthuhn et al. (2011)) and held in a temperature-controlled water bath (HAAKE S14P, Thermo Fisher Scientific) at 65 °C for 10 min. The time was started after the heating-up phase of 30 s. After heat treatment, the samples were cooled on ice, immediately serially diluted and plated on TSA plates. For samples that were near the limit of detection, the experiments were repeated and the solution was neutralized first and plated directly on TSA plates with a diameter of 140 mm.

2.2.2. Influence of milk residues on the inactivation of thermophilic spores

One aim of the study was, to find out if milk residues influence the effectiveness of the cleaning solutions towards the inactivation of thermophilic spores. Therefore, a part of the same spores as used for the inactivation experiments were centrifuged (2218 *g*, 7 min) and suspended in ultra-high-temperature (UHT)-treated milk from a local distributor (0.3% fat). The initial spore counts were measured again in the milk-spore-cleaning-suspension at room temperature and after the treatment. To see an effect of the milk residues only 2% of NaOH and 0.5% of HNO_3 were used, because the two concentrations showed a suitable inactivation effect. The procedure that followed afterwards was the same as described under 2.2.1.

2.2.3. Determination of spore count

For the determination of the thermophilic spore count, a serial dilution in a quarter Ringer's solution (Merck KGaA) was prepared and spread plated on TSA plates. The plates were incubated at 55 °C for

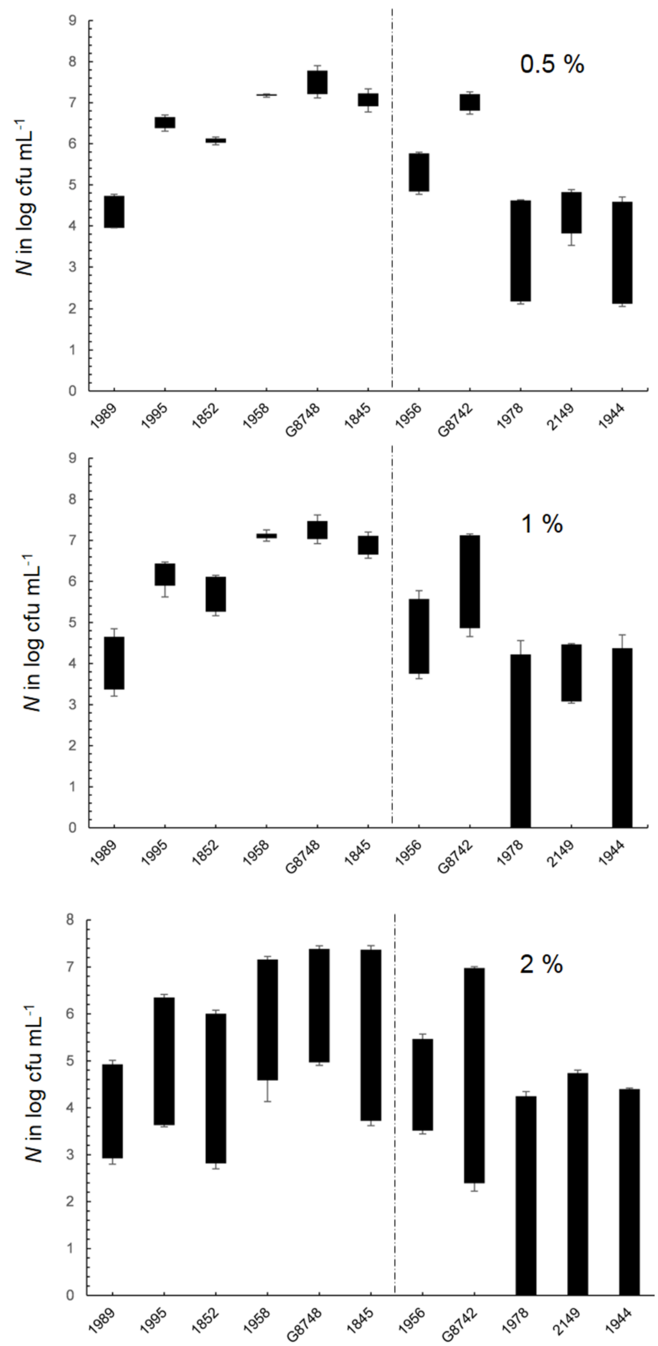


Fig. 1. Screening the resistance of thermophilic spore formers regarding alkaline solution (NaOH) for 10 min at 65 °C in the concentration of 0.5%, 1% and 2%. The top of the bar shows the initial spore concentration (± SD) the end of the bar shows the spore concentration after the treatment (± SD). Dashed lines show the border between *A. flavithermus* (left) and *G. stearothermophilus* (right) strains.

Table 1
Screening the resistance of thermophilic spore formers regarding acid solutions (HNO_3) for 10 min at 65 °C. The initial spore concentration in the cleaning solution (± SD) and the spore concentration after the treatment is shown.

	Con-centration HNO_3	0.5%		1.0%		2.0%	
	Strain number	Log N_0 before treatment	Log N_t after treatment	Log N_0 before treatment	Log N_t after treatment	Log N_0 before treatment	Log N_t after treatment
A. flavithermus	1989	5.9 ± 0.3	< 1	5.7 ± 0.1	< 1	5.8 ± 0.1	< 1
	1995	6.7 ± 0.7	< 1	6.3 ± 0.7	< 1	7.0 ± 0.1	< 1
	1852	6.0 ± 0.3	< 1	5.4 ± 0.4	< 1	5.7 ± 0.4	< 1
	1958	7.1 ± 0.1	< 1	6.8 ± 0.1	< 1	6.6 ± 0.1	< 1
	G8748	7.4 ± 0.4	3.0 ± 0.1	7.2 ± 0.1	< 1	6.9 ± 0.3	< 1
	1845	7.5 ± 0.1	1.9 ± 0.3	7.3 ± 0.1	< 1	6.8 ± 0.1	< 1
G. stearothermophilus	1956	5.6 ± 0.1	< 1	4.4 ± 0.3	< 1	5.2 ± 0.2	< 1
	G8742	7.0 ± 0.1	1.6 ± 0.3	6.8 ± 0.1	1.5 ± 0.2	6.9 ± 0.2	< 1
	1978	3.0 ± 0.7	< 1	< 1	–	< 1	–
	1944	4.2 ± 0.1	< 1	4.1 ± 0.1	< 1	3.7 ± 0.2	< 1
	2149	< 1	–	< 1	–	< 1	–

24–72 h. After the incubation, colonies were counted and the concentrations were calculated by the weighted arithmetic mean.

2.3. Kinetic evaluation of inactivation data

A detailed kinetic of the inactivation in acid and alkaline cleaning solutions for two strains was obtained. In alkaline solution the strain 1956, in acid solution the strain G8742 was chosen (both belonging to the species *G. stearothermophilus)*, because the strains showed a high resistance in the respective cleaning solution. Temperatures from 50 to 70 °C (and 25 °C additionally for G8742) were chosen and per temperature, at least six time points were measured. All three concentrations (0,5, 1 and 2%) were used for the kinetic evaluation. The experimental procedure was the same as described under 2.2.

2.3.1. Modelling the data

To model the inactivation data, the Arrhenius-based-model according to equation (1) was used:

$$\frac{N_t}{N_0} = [1 + (n-1) \cdot N_0^{n-1} \cdot k_{ref} \cdot exp(-\frac{E_a}{R}) \cdot (\frac{1}{T} - \frac{1}{T_{ref}}) \cdot t]^{(\frac{1}{(1-n)})} \quad (1)$$

where N_t is the spore count (cfu·mL^{-1}) after the treatment at time t (s), N_0 the start spore count (cfu·mL^{-1}), n the order of reaction (no dimension), k_{ref} the death rate at the reference temperature T_{ref} (K), E_a the activation energy (J·mol^{-1}·K^{-1}), R the universal gas constant (8.314 J·mol^{-1}·K^{-1}) and T the absolute temperature (K). The Arrhenius-based-model with an order of reaction (n) unequal to 1 was chosen, because of the non-linearity of the inactivation curves.

The best solution for each concentration with the given data was solved with the program SigmaPlot™ (2013, Systat Software, USA).

Since the inactivation curves for the alkaline and acid treatments were not linear, the D_ϑ-values were calculated as the initial D_ϑ-values from the first linear part of the curve. At least three data points were taken for the linear regression.

The temperature dependence of the D-value is given by the z-value, where an increase of the z-value reduces the D -value to 1/10th. The z-value is given with equation (2) after Kessler (2002):

$$z = \frac{2.303 \cdot R \cdot T_{ref}^2}{E_a} \quad (2)$$

where T_{ref} is the reference temperature in K, R the universal gas constant, and E_a the activation energy.

Additional to the Arrhenius-based model, a modified Weibull-model proposed by Mafart et al. (2002) was used, to describe the experimental data. Its equation is given with

$$log\frac{N_t}{N_0} = -(\frac{t}{\delta})^p \quad (3)$$

where δ is the scale factor, p the shape factor and t the heating time. The shape factor models the curve depending on its value (< 1 concave upward, > 1 concave downward, equal to 1 linear inactivation). The δ value represents the time of the first decimal reduction.

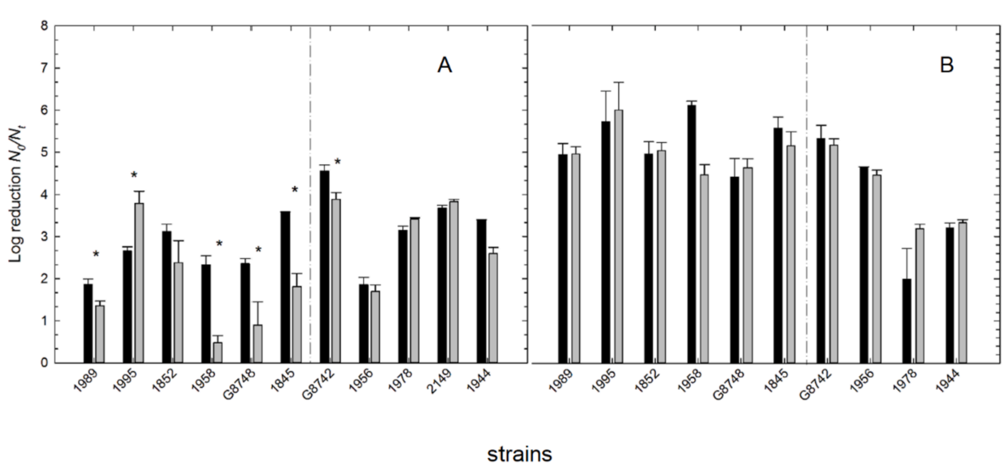


Fig. 2. Log reduction of thermophilic spores in A: 2.0% NaOH solution and B: 0.5% HNO_3 at 65 °C for 10 min without and with milk impurities. Significant differences are symbolized with an asterisk (p > 0.05).

For modelling the experimental values with the Weibull-model a free tool (GiNAFiT) described in Geeraerd et al. (2005) was used. The resulting parameters were given with standard error and an adjusted R-square value (regression coefficient).

2.4. Statistical analysis

All experiments were carried out at least in triplicate. To test a statistic significant difference a t-test was performed between the log reductions with milk residues and without (SigmaPlot, P-value < 0.05).

3. Results & discussion

3.1. Influence of pH and agent concentration on thermophilic spore formers survival

The results for the screening experiments of the resistance of thermophilic spore formers towards alkaline solutions are shown in Fig. 1. The three Figures show the reduction of thermophilic spores in NaOH solution with increasing concentration (0.5–2%) over a constant treatment at 65 °C for 10 min. All tested strains survived the treatment using the lowest concentration of 0.5% with the highest log-reduction being 2.4 and 2.3 for the strains 1978 and 1944, both belonging to the species *G. stearothermophilus*. Five from eleven tested strains were not reduced at all or below 0.5 log-levels in 0.5% NaOH, namely 1958, 1995, 1852, G8748 and 1845. All of these strains were assigned to the species *A. flavithermus*. By increasing the NaOH concentration from 0.5 to 1 and 2%, the lethality of the solutions towards the spores increased as well. The two sensitive strains 1978 and 1944 were not able to withstand the treatment in 1 and 2% NaOH solutions. In 1% NaOH solution, all of *A. flavithermus* strains were reduced 1-log or less. Only at a concentration of 2% of the alkaline solution all strains showed a reduction that ranged from 1.8 (1956) to a total inactivation of the spores (1978, 1944 and 2149).

Inactivation of *G. stearothermophilus* spores by alkaline solutions was previously investigated for one single strain (Pinho et al., 2015). At temperatures of 80, 100 and 110 °C in NaOH solutions ranging from 0.10 to 0.75 *M* (0.4–3%) log reduction values from 1 to 6 were observed between 4 and 24 min. Reasons for the inactivation mechanisms were discussed to be dissolving of proteins from the spore coat. The reduction was higher compared to our results but the temperatures applied were also higher (80–110 °C compared to 65 °C in our study). Another study was conducted to investigate the resistance of a single thermophilic spore former isolated from milk powder towards NaOH solutions (Knight and Weeks, 2008). The study showed, that for the tested strain the sporicidal effect increased when the concentration of the NaOH solutions was increased as well (from 0.5 to 2%). The conclusion of the authors was that cleaning solutions for reuse in dairy processing should be kept at elevated temperatures or higher concentrations should be used to avoid spore survival. Our results also suggest that at the highest concentration of 2% reuse of cleaning solutions might still lead to high survival rates of thermophilic spores. Some spores may carry over to a new production start and thereby contaminate the next run. Moreover, it was shown earlier, that caustic wash can indeed reduce the absolute value of thermophilic spores but the ability of the spores to attach on stainless steel surfaces is higher (Brent Seale et al., 2011). It was shown in their work that the spore coat from the investigated *G. stearothermophilus* strain was partially broken and on the same time, the hydrophobicity was increased. This enhances the attachment on stainless steel.

The difference between the two tested species, *G. stearothermophilus* and *A. flavithermus* towards their NaOH resistance was not reported until now. Our finding could be one reason why *A. flavithermus* strains are more often isolated in different milk products such as skim milk or whey powder and concentrate. *G. stearothermophilus* has a higher heat tolerance and higher growth optimum than *A.°flavithermus* (Burgess et al., 2014; Reich et al., 2017; Sadiq et al., 2016; Yuan et al., 2012) and would be therefore be expected as the main detected organisms but is less resistance towards NaOH.

Besides the resistance towards NaOH the acid sensitivity of the strain set of thermophilic spore formers was tested. The results for 0.5–2% treatment at 65 °C for 10 min are summarized in Table 1. Compared to the NaOH treatment, the acid solutions were significantly more lethal to the spores. At 0.5% HNO_3, only three of the eleven tested

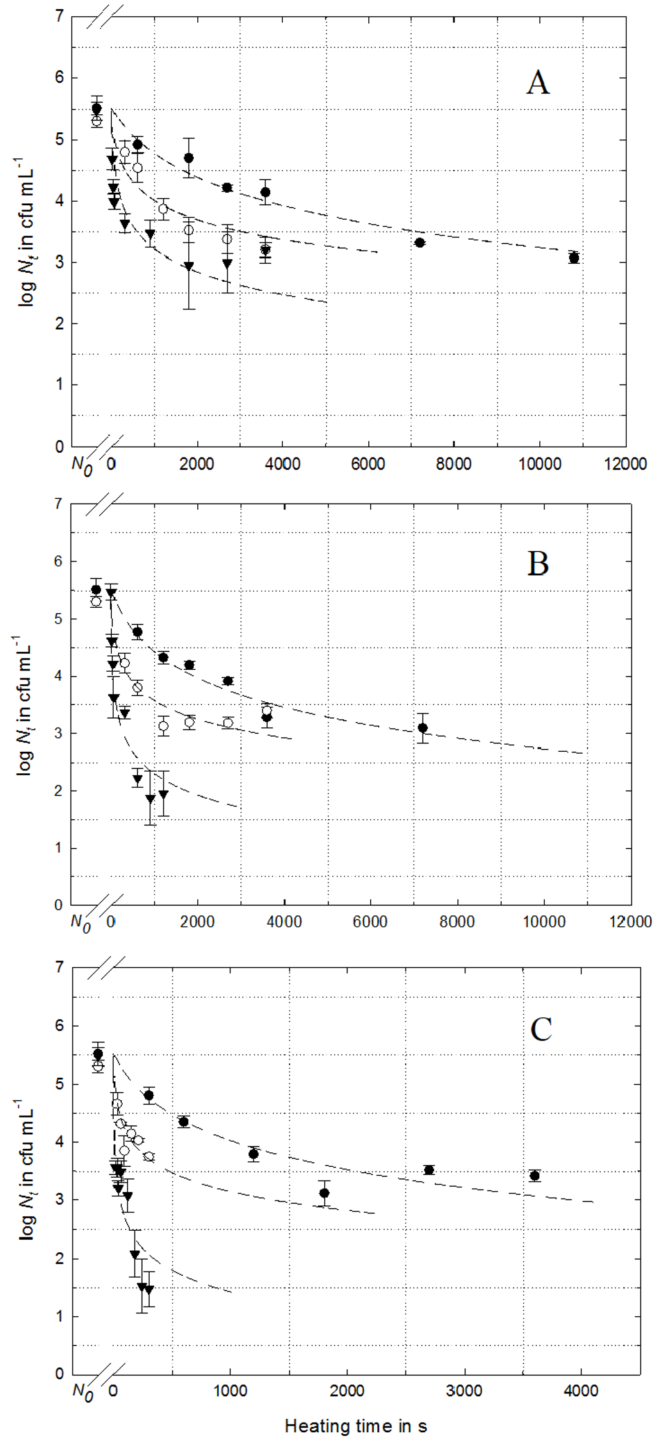


Fig. 3. Inactivation of *G. stearothermophilus* strain 1956 in NaOH • 0.5%, ○ 1% and ▼ 2% at 50 °C (A), 60 °C (B) and 70 °C (C). Dashed lines indicates the inactivation curves calculated with the Arrhenius equation using parameters from Table 2.

strains were able to survive the treatment. Two of the three strains survived with values < 2 log. Strain G8748 showed the highest resistance and a reduction of 4.4 log. The other two strains (1845 and G8742) were reduced by 5.6 and 5.4 log-levels. Only the strain G8742 was able to survive the treatment of a concentration at 1%. The two strains 1978 and 2149 were already inactivated before the initial spore count in HNO_3 at room temperature was measured. Therefore, no treatment with heat was performed. The other 8 strains were completely inactivated. At 2% HNO_3, all strains were inactivated and no surviving spores were observed.

The influence of acidic heating environments on the inactivation on *G. stearothermophilus* spores was investigated earlier (Iciek et al., 2008). A reduction of the pH-value from 6.0 to 4.0 reduced the heating time for a similar spore reduction by more than 1/3. Similar findings were obtained years ago, where the reduction of the pH-value in the food media reduced the $D_{115\ ^{\circ}C}$-values for *G. stearothermophilus* spores from 23 min (pH 7) to 4 min (pH 4.81) (Rodrigo et al., 1999). For the model organisms *B. subtilis* it was shown, that the destruction mechanisms of acid chemicals are due to DNA damage in the spore, whereas the reasons for the destructive effect of heat is also because of the inactivation of core enzymes and rupture of the spore's inner membrane (Setlow, 2006). A synergistic effect of the two spore destruction mechanics is suggested to explain the high lethal effect on thermophilic spores observed in this study.

To sum up the results, the alkaline treatment showed a very low inactivation effect compared to the treatment in acid. A survival of spores in alkaline cleaning solutions is possible, whereas acidified cleaning solutions are not likely to contain spores.

3.2. Influence of milk residues on the sensitivity of thermophilic strains towards NaOH (2%) and HNO_3 (0.5%)

In milk processing companies, cleaning solutions are reused several times, pH is controlled, and over time, chemicals are added to re-adjust pH. Therefore, milk residues such as proteins and contaminants can remain in the cleaning tank and recirculate in the next cleaning step. To simulate these conditions, we analyzed the effect of the cleaning solutions NaOH and HNO_3 on spore pellets in milk instead of phosphate buffer. The change in the sensitivity is shown for the alkaline treatment in Fig. 2. The log-reduction values were compared for the same spore suspensions suspended either in phosphate buffer or in milk with the same concentration of NaOH (2%). For six of eleven strains, the log-reduction in NaOH were statistically significant reduced ($p < 0.05$) by the presence of milk residues. For one strain, 1995, a higher inactivation rate with the milk residues was observed compared to no residuals. The differences for the other five strains ranged from 0.5 log (1989) to 1.8 log (1958) and the milk milieu led to a smaller reduction.

The same experiments were carried out for the acid treatment. Hereby, no significant differences in inactivation between milk residues and phosphate buffer were found (Fig. 2.). For strain 1958 however, in buffer a total inactivation was achieved while with milk some spores could be detected. In general, it would be possible that the impurities might affect the acid inactivation stronger as well, but since the acid treatment was more lethal to the spores, a higher start concentration would have been necessary.

Studies that investigate the effect of the protein content on the inactivation behavior in acid or alkaline solutions are lacking. For the inactivation through heat alone, an insignificant effect of the protein content up to 14.3% was shown recently for *B. cereus* (Stoeckel et al., 2014). We conclude that the impurities in the alkaline solutions lowered the potential to destruct the spore coat because the attacks from the alkaline solution are non-directional and are focused not only on the spores but on the protein pollution as well. The acid mechanism to kill the spores was not affected by the protein background in the media (Fig. 2).

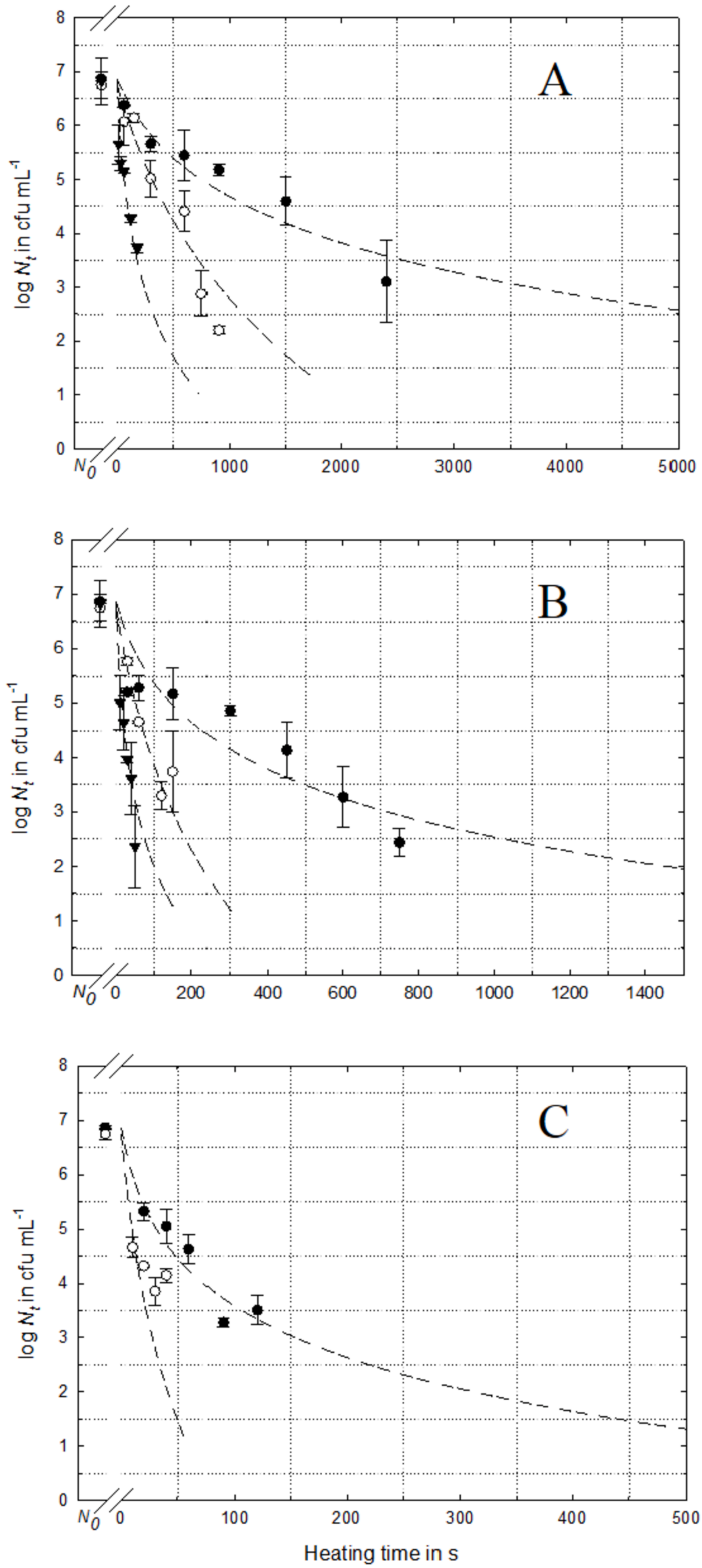
3.3. Determination of kinetic data for two selected strains

Two strains were selected (1956 and G8742 both belonging to the species *G. stearothermophilus*) for detailed investigations of kinetic inactivation data with different concentrations and temperatures over different holding times. The strains were chosen, because they showed the highest resistance to the respective acid or alkaline in the screening experiments (see Fig. 1 and Table 1). Moreover, a strain with a very high start concentration was needed to have enough data points to model correctly the inactivation curve. Both strains used, had a high spore concentration. The data log N_t over heating time and NaOH concentration is shown in Fig. 3. For all three temperatures (A: 50 °C, B: 60 °C, C: 70 °C) the trend observed was similar: With higher concentrations of NaOH solution a stronger inactivation effect was observed. All curves showed tailing and non-log-linear inactivation curves. This was considered for the calculation of the kinetic data (Table 2) and can be seen in the values for the order of reaction n. Indeed, for all three concentrations the order of reaction was > 1.5. The death rates (k_{ref}) were first decreased and increased strongly from 1 to 2% of NaOH concentration. The estimated parameters (Table 2) were used to plot survival curves with the Arrhenius equation for each cleaning condition (dashed lines Figs. 3 and 4). Since non-linear inactivation curves were observed, the $D_{60^{\circ}C}$-values were calculated from the first linear decrease in the curves from at least three points. At 0.5% the $D_{60^{\circ}C}$ -value was 29.7 min and was reduced by 2/3 if the concentration was doubled. From 1 to 2% NaOH concentration the $D_{60\ ^{\circ}C}$-value decreased again by more than 1/10th to 0.56 min. The connection of the D-value reduction over the concentration followed an exponential regression as it is shown in Fig. 5. The corresponding z-values for each concentration were calculated and decreased from 35.6 K to 13.7 at 2% NaOH concentration. By doubling the concentration (from 0.5 to 1% and 1–2%), the z-value decreased approx. to 10 K.

The results for the detailed kinetic data (strain G8742) in acid solution are shown in Fig. 4. Again, non-log-linear inactivation curves were observed for all tested temperatures (50, 60 and 70 °C). At 70 °C, the highest HNO_3 concentration resulted in a strong inactivation effect and therefore it was not possible to determine enough data points for this concentration/temperature-combination. Instead, to improve the accuracy of the model parameters, the inactivation at 25 °C for 2% was additionally obtained (data not shown). The data points at 25 °C were used to estimate the Arrhenius-parameters (n, E_a and k_{ref} at 2% HNO_3). At 50 °C an inactivation of 4-log values was reached between 40 and

Table 2
Kinetic parameters for the thermal inactivation in cleaning solutions with different concentration factors for the Arrhenius-based-model. With n order of reaction, E_a activation energy, k_{ref} the death rate at reference temperature and i the number of experiments for the data modelling. T_{ref} = 333.15 K.

Species and strains	Cleaning solution	$n \pm s$	$E_a \cdot 10^3 \pm s$ in J/mol	$k_{ref} \cdot 10^{-4} \pm s$ in s^{-1}	*D-value*$_{60^{\circ}C}$ in minutes	*z-value* in K	i
G. stearothermophilus 1956	0.5% NaOH	1.52 ± 0.08	60 ± 9	0.07 ± 0.05	29.7	35.6	20
	1% NaOH	1.91 ± 0.14	85 ± 15	0.006 ± 0.008	9.7	24.8	20
	2% NaOH	1.81 ± 0.11	154 ± 21	0.17 ± 0.16	0.56	13.7	25
G. stearothermophilus G8742	0.5% HNO_3	1.29 ± 0.07	147 ± 13	6.0 ± 5.0	3.7	14.5	22
	1% HNO_3	1.12 ± 0.05	161 ± 8	156 ± 90	0.58	13.1	18
	2% HNO_3	1.22 ± 0.06	130 ± 6	150 ± 118	0.21	16.3	18



(caption on next page)

Fig. 4. Inactivation of *G. stearothermophilus* G8742 in HNO_3 • 0.5%, ○ 1% and ▼ 2% at 50 °C (A), 60 °C (B) and 70 °C (C). Dashed lines indicates the inactivation curves calculated with the Arrhenius equation using parameters from Table 2.

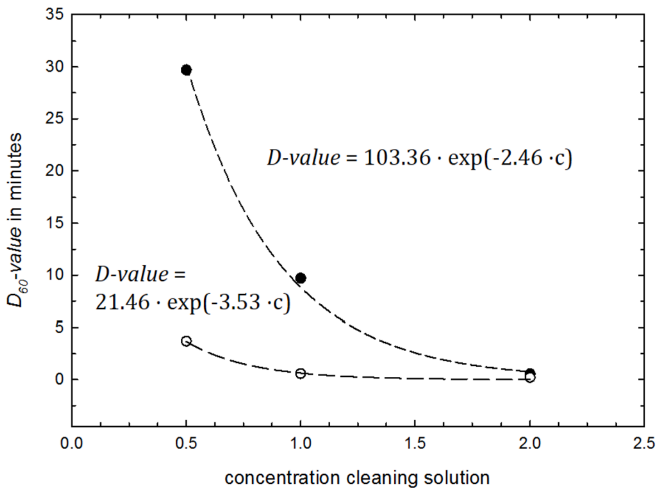


Fig. 5. Correlation of D_{60}-values of strains 1956 and G8742 in alkaline • and acid ○ solutions at different concentrations. The exponential regressions is shown.

4 min, depending on the concentration of the acid. At 70 °C between 1 and 2 min, the same inactivation of 4-log values was observed. Compared to the alkaline treatment, a high temperature dependency was observed. This is supported by the *z*-value (Table 2), ranging from 13.1 to 16.3 for the three concentrations. The order of reaction was stable as well for all concentrations. A logarithmic correlation of the concentration on the acid and alkaline solutions on the D_{60}-value was assumed (Fig. 5). With this correlation, it gets clear, that the concentration has a major impact on the efficiency of the spore destruction of the cleaning solutions. Table 3 shows the kinetic data for the Weibull-based model. The δ-value decreased strongly with the temperature for 0.5 and 1% NaOH and the acid inactivation. Thus, the dependency of the temperature can be seen. The shape-factor (*p*-value in Table 3) was < 1 for 17 of the 18 modelled curves. Therefore, a non-log-linear trend with concave curves are observed and are in accordance with the modelled parameters of the Arrhenius-based model. The regression coefficient showed for most of the curves a good fit (R^2 values > 0.9) but for six curves, a higher variability and thus higher standard errors were observed.

Detailed kinetic data for the inactivation of thermophilic spores in acidic and basic solutions are lacking. However, *z*-values for the treatment of thermophilic spores by heat alone are already known and range from 5.8 to 10.1 K (André et al., 2013). Thus, the temperature dependency for inactivation in acid or alkaline solutions compared to heat alone is low (in this study between 13.1 and 35.6 K).

Tailing of inactivation curves of bacterial endospores is a widely known phenomenon. There are different explanation discussed in literature, why this type of curve can be observed. As one reason for the tailing the clumping of spores to aggregates was discussed (Furukawa et al., 2005). A higher hydrophobicity due to the heat treatment was observed for the mesophilic spores in their study. An increase of the hydrophobicity is assumed for our data set as well, but more likely because of the acid or alkaline treatment and not because of the temperature increase which was very low in our study (from 50 to 70 °C). The tested temperatures were not in the dimension to affect already the thermophilic spores. Aggregates of the spores suspended in phosphate buffer were not observed under the microscope but may exist during alkaline treatment because of alterations in the spore coat such as the dissociation of protein bonds and the increase of the hydrophobicity. This already well-known phenomenon of clumping is schematically illustrated in Fig. 6 (A) as one explanation for the results. However, from our findings with milk contaminants the tailing might additionally as well occur because of pollution of dead spores. Moreover, the sporicidal potential of the OH^- ions decreased with the ongoing inactivation process (see Fig. 6 B). This effect should be investigated in more detail for future understanding of inactivation conditions with alkaline solutions. Another, well-discussed explanation for tailing are different resistances throughout the population (Cerf, 1977; Mamane-Gravetz and Linden, 2005). The variability in a population is known as 'bistability' (Veening et al., 2008) during the spore formation process and the described phenomena is added in Fig. 6 under C. All the three phenomena could occur as single events or in combination and therefore leading to the observed curves. Further experiments, e.g. higher pollution with dead spores or different conditions for spore production should be addressed for a better understanding and may help to improve CIP cleaning.

Table 3
Kinetic parameters for the thermal inactivation in cleaning solutions with different concentration factors for the Weibull model. With δ the scale factor or time for the first decimal reduction, *p* the shape factor and R^2 the regression coefficient.

Species and strains	Cleaning solution	Temperature in °C	δ ± *se* in s	*p* ± *se*	R^2
G. stearothermophilus 1956	0.5% NaOH	50	2431 ± 715	0.59 ± 0.09	0.97
		60	795 ± 401	0.43 ± 0.09	0.94
		70	464 ± 531	0.37 ± 0.17	0.81
	1% NaOH	50	662 ± 270	0.49 ± 0.10	0.95
		60	62 ± 124	0.20 ± 0.09	0.85
		70	62 ± 55	0.27 ± 0.11	0.85
	2% NaOH	50	9.1 ± 13	0.16 ± 0.03	0.93
		60	23 ± 22	0.33 ± 0.07	0.95
		70	26 ± 23	0.55 ± 0.17	0.85
G. stearothermophilus G8742	0.5% HNO_3	50	449 ± 167	0.73 ± 0.15	0.95
		60	107 ± 95	0.64 ± 0.26	0.85
		70	11 ± 9	0.53 ± 0.16	0.91
	1% HNO_3	50	263 ± 90	1.18 ± 0.31	0.95
		60	19 ± 18	0.60 ± 0.25	0.87
		70	1.5 ± 0.9	0.47 ± 0.09	0.97
	2% HNO_3	25	1718 ± 161	0.84 ± 0.05	0.99
		50	12 ± 7	0.40 ± 0.07	0.96
		60	6 ± 4	0.64 ± 0.16	0.94

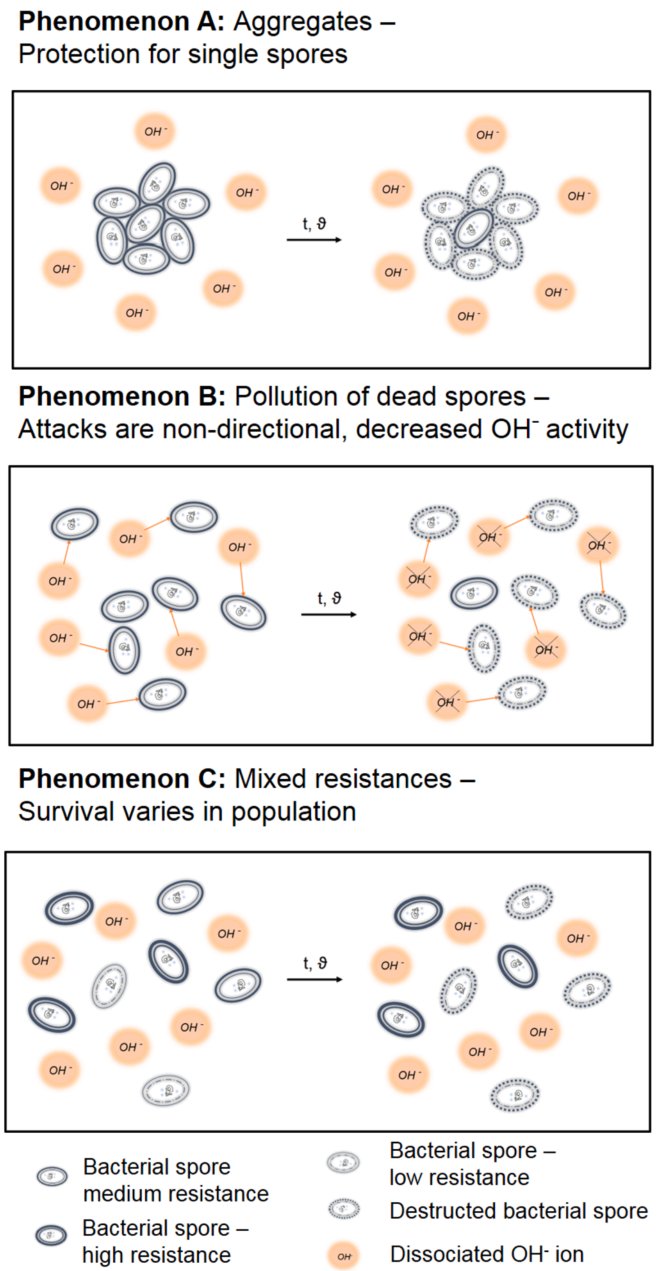


Fig. 6. Hypothesis for non-log-linear inactivation behavior in alkaline cleaning solution of thermophilic bacterial spores. Three explanations are schematically shown. A: The formation of spore aggregates due to increased hydrophobicity and thus a protection effect on single spores. B: The pollution of the solutions with dead spores lead to a decrease in the inactivation effect because the OH^- activity is lowered. C: Different resistances of single spores, which results in concave inactivation curves.

4. Conclusion

This study provides important information about the resistance of thermophilic spore formers towards typical cleaning conditions used in milk processing companies. A screening of isolated strains belonging to the species *G. stearothermophilus* and *A. flavithermus* was conducted and it was demonstrated, that alkaline solutions used in common concentrations of 0.5–2% are not sufficient to eliminate all the tested thermophilic spores. Compared to that, acid solutions are more effective to destruct thermophilic spores. Reduced efficiency regarding spore inactivation was observed for cleaning solutions polluted with milk, which simulated the reuse-application during CIP-procedures. These milk residues, mainly because of retained protein in the alkaline solution, decrease the inactivation effect on thermophilic spores. One recommendation for milk powder manufacturers is to minimize the storage duration for alkaline solutions and to keep the cleaning solutions at high temperatures to reduce the recontamination potential for the next process run. More importantly, our data imply that a long aicd cleaning might serve as a disinfection of the plant and could be one solution for the problem of high-contaminated milk and whey powders.

Acknowledgments

The authors would like to thank Birgit Schilling, Kristiane E. Luma and Barbara Wack for helping with the experiments. This IGF Project of the FEI was supported via AiF within the program for promoting the Industrial Collective Research (IGF) of the German Ministry of Economic Affairs and Energy (BMWi), based on a resolution of the German Parliament Project AiF 19825 N.

References

André, S., Zuber, F., Remize, F., 2013. Thermophilic spore-forming bacteria isolated from spoiled canned food and their heat resistance. Results of a French ten-year survey. Int. J. Food Microbiol. 165 (2), 134–143.
Brent Seale, R., Flint, S.H., James Mcquillan, A., Bremer, P.J., 2011. Effect of NaOH (caustic wash) on the viability, surface characteristics and adhesion of spores of a Geobacillus sp. isolated from a milk powder production line. Lett. Appl. Microbiol. 52 (2), 104–108.
Burgess, S.A., Brooks, J.D., Rakonjac, J., Walker, K.M., Flint, S.H., 2009. The formation of spores in biofilms of Anoxybacillus flavithermus. J. Appl. Microbiol. 107 (3), 1012–1018.
Burgess, S.A., Flint, S.H., Lindsay, D., 2014. Characterization of thermophilic bacilli from a milk powder processing plant. J. Appl. Microbiol. 116 (2), 350–359.
Burgess, S.A., Lindsay, D., Flint, S.H., 2010. Thermophilic bacilli and their importance in dairy processing. Int. J. Food Microbiol. 144 (2), 215–225.
Cerf, O., 1977. A REVIEW tailing of survival curves of bacterial spores. J. Appl. Bacteriol. 42 (1), 1–19.
Furukawa, S., Narisawa, N., Watanabe, T., Kawarai, T., Myozen, K., Okazaki, S., Ogihara, H., Yamasaki, M., 2005. Formation of the spore clumps during heat treatment increases the heat resistance of bacterial spores. Int. J. Food Microbiol. 102 (1), 107–111.
Geeraerd, A.H., Valdramidis, V.P., Van Impe, J.F., 2005. GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. Int. J. Food Microbiol. 102 (1), 95–105.
Hill, B.M., Smythe, B.W., 2012. Endospores of thermophilic bacteria in ingredient milk powders and their significance to the manufacture of sterilized milk products: an industrial perspective. Food Rev. Int. 28 (3), 299–312.
Iciek, J., Błaszczyk, I., Papiewska, A., 2008. The effect of organic acid type on thermal inactivation of Geobacillus stearothermophilus spores. J. Food Eng. 87 (1), 16–20.
Kessler, H.G., 2002. Food and Bio Process Engineering - Dairy Technology. Verlag A. Kessler, Germany.
Knight, G.C., Weeks, M.G., 2008. Conditions for inactivation of thermophilic spores in NaOH solutions for reuse applications. Aust. J. Dairy Technol. 63 (3), 82–86.
Mafart, P., Couvert, O., Gaillard, S., Leguerinel, I., 2002. On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model. Int. J. Food Microbiol. 72 (1–2), 107–113.
Mamane-Gravetz, H., Linden, K.G., 2005. Relationship between physiochemical properties, aggregation and u.v. inactivation of isolated indigenous spores in water. J. Appl. Microbiol. 98 (2), 351–363.
Merin, U., Gésan-Guiziou, G., Boyaval, E., Daufin, G., 2002. Cleaning-in-place in the dairy industry: Criteria for reuse of caustic (NaOH) solutions. Lait 82 (3), 357–366.
Pinho, S.C., Nunes, O.C., Lobo-da-Cunha, A., Almeida, M.F., 2015. Inactivation of Geobacillus stearothermophilus spores by alkaline hydrolysis applied to medical waste treatment. J. Environ. Manag. 161, 51–56.
Reich, C., Wenning, M., Dettling, A., Luma, K.E., Scherer, S., Hinrichs, J., 2017. Thermal resistance of vegetative thermophilic spore forming bacilli in skim milk isolated from dairy environments. Food Control 82, 114–120.
Rodrigo, F., Rodrigo, C., Fernández, P.S., Rodrigo, M., Martínez, A., 1999. Effect of acidification and oil on the thermal resistance of Bacillus stearothermophilus spores heated in food substrate. Int. J. Food Microbiol. 52 (3), 197–201.
Sadiq, F.A., Li, Y., Liu, T., Flint, S., Zhang, G., Yuan, L., Pei, Z., He, G., 2016. The heat resistance and spoilage potential of aerobic mesophilic and thermophilic spore forming bacteria isolated from Chinese milk powders. Int. J. Food Microbiol. 238, 193–201.
Scott, S.A., Brooks, J.D., Rakonjac, J., Walker, K.M.R., Flint, S.H., 2007. The formation of thermophilic spores during the manufacture of whole milk powder. Int. J. Dairy Technol. 60 (2), 109–117.
Setlow, P., 2006. Spores of Bacillus subtilis: their resistance to and killing by radiation, heat and chemicals. J. Appl. Microbiol. 101 (3), 514–525.
Stoeckel, M., Atamer, Z., Hinrichs, J., 2014. Thermal inactivation of Bacillus cereus spores in micellar casein concentrates-effect of protein content and pH development. Dairy Sci. Technol. 94 (6), 539–548.

Stoeckel, M., Lücking, G., Ehling-Schulz, M., Atamer, Z., Hinrichs, J., 2016. Bacterial spores isolated from ingredients, intermediate and final products obtained from dairies: thermal resistance in milk. Dairy Sci. Technol. 96 (4), 569–577.
Tamime, A., 2009. Cleaning-in-Place: dairy, food and beverage operations. In: Cleaning-in-Place: Dairy, Food and Beverage Operations, third ed. .
Veening, J.W., Smits, W.K., Kuipers, O.P., 2008. Bistability, epigenetics, and bet-hedging in bacteria. In: Annual Review of Microbiology, vol. 62. pp. 193–210.
Watterson, M.J., Kent, D.J., Boor, K.J., Wiedmann, M., Martin, N.H., 2014. Evaluation of dairy powder products implicates thermophilic sporeformers as the primary organisms of interest. J. Dairy Sci. 97 (4), 2487–2497.
Wedel, C., Wunsch, A., Wenning, M., Dettling, A., Kayser, K.H., Lehner, W.D., Hinrichs, J., 2018. Thermal treatment of skim milk concentrates in a novel shear-heating device: reduction of thermophilic spores and physical properties. Food Res. Int. 107, 19–26.
Witthuhn, M., Lücking, G., Atamer, Z., Ehling-Schulz, M., Hinrichs, J., 2011. Thermal resistance of aerobic spore formers isolated from food products. Int J Dairy Technol 64 (4), 486–493.
Yuan, D.-D., Liu, G.-C., Ren, D.-Y., Zhang, D., Zhao, L., Kan, C.-P., Yang, Y.-Z., Ma, W., Li, Y., Zhang, L.-B., 2012. A survey on occurrence of thermophilic bacilli in commercial milk powders in China. Food Control 25 (2), 752–757.
Zhao, Y., Caspers, M.P.M., Metselaar, K.I., De Boer, P., Roeselers, G., Moezelaar, R., Groot, M.N., Montijn, R.C., Abee, T., Korta, R., 2013. Abiotic and microbiotic factors controlling biofilm formation by thermophilic sporeformers. Appl. Environ. Microbiol. 79 (18), 5652–5660.