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The effect of phosphate on the heat resistance of spores of dairy isolates of *Geobacillus stearothermophilus*

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ABSTRACT

In this study, we show that phosphate decreases the spore heat resistance by accelerating the rate of loss of cations from spores. Heat resistance of spores of *Geobacillus stearothermophilus* A1, D1, P3 and ATCC 12980 were determined in distilled water containing varying concentrations (0.1, 1 and 2% *w/v*) of di-sodium phosphate. The average decimal reduction times (D value) for strains A1, D1, P3 and ATCC 12980 in distilled water were 5.8, 6.8, 5.7 and 9 min at 110 °C respectively. On the addition of 0.1, 1 and 2% *w/v* of di-sodium phosphate, the average D_{110} values of all the strains in distilled water were lowered by 50, 61 and 70% respectively. Addition of 0.05% *w/v* of Na-EDTA to distilled water resulted in lowering of the average D_{110} value of all the strains by 55%. Heat resistance of spores of A1, D1, P3 and ATCC 12980 was found to be associated with the Dipicolinic Acid (DPA) content whose concentrations were 0.25, 0.30, 0.27 and 1.6 pg per spore respectively. Analysis by atomic absorption spectroscopy revealed that the phosphate present in the heating medium causes excess release of calcium from spores with 2% *w/v* phosphate being highly effective, thus confirming the chelating effect of phosphate. This study provides insight into the heat resistance and the increased heat sensitivity of spores of *G. stearothermophilus* A1, D1 and P3 in the presence of phosphate, which can be used in the design of Cleaning in Place (CIP) systems involving phosphate based cleaning agents to combat biofilms and spores in the dairy industry.

1. Introduction

Thermophilic bacteria are prevalent during dairy powder manufacture where elevated temperatures (40–65 °C) prevail (Cempírková, 2007). These bacteria are capable of forming heat resistant spores in dairy processing facilities and their presence is an indication of poor plant hygiene (Burgess et al., 2010). Germination of spores upon attachment to stainless steel surfaces leads to biofilm formation (Scott et al., 2007). Upon maturation of the biofilm, vegetative cells and spores can slough off and contaminate the processing line and end product (Seale et al., 2010).

Heat resistance of bacterial spores is attributed to a variety of factors including the outer coat of the spore and the relatively impermeable inner membrane (Cortezzo and Setlow, 2005; Nicholson et al., 2000; Sonenshein et al., 2002). In addition, the spore's central region of the core contains DNA bound with soluble acid soluble proteins (SASP) which protects the DNA from heat, desiccation and chemicals (Setlow, 1995; Sonenshein et al., 2002). The core of the spore apart from hosting the DNA contains high levels (5 to 15% of dry weight) of DPA, which is found as a 1:1 chelate with metal cations predominantly calcium (Setlow, 2006). DPA, present within the core is responsible for maintaining the low core water content which plays a vital role in the heat resistance of spores to wet heat (Paidhungat et al., 2000). During initial stage of spore germination, DPA is lost and is replaced with water which leads to rehydration of the core and revival of spore metabolism (Setlow, 2003).

In dried milk powder samples, the three commonly isolated spore formers are *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*), *Anoxybacillus flavithermus* and *Bacillus* species (Scheldeman et al., 2006). Owing to their high heat resistance, spores of *G. stearothermophilus* are commonly used as a biological indicator to validate the efficiency of sterilization processes (Gonzalez et al., 1995). The heat resistance of spores is influenced by several factors including sporulation temperature, pH of the heating medium and spore recovery conditions (Cook and Gilbert, 1968; Fernandez et al., 1994; Gonzalez et al., 1995). According to Cook and Gilbert (1968), the heat resistance of spores of *G. stearothermophilus* is reduced when the pH of the heating medium is lowered. In addition, they concluded that phosphate present

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in the heating medium might lower the heat resistance of spores. Although, phosphate is known to lower the heat resistance of spores of *G. stearothermophilus* (López et al., 1996b), only a few studies have reported on the possible mechanism of action of phosphate. Amaha and Ordal (1957) suggested that the effect of phosphate might be that of a chelating agent, wherein phosphate may accelerate the rate of loss of cations from within the spore which might contribute towards the heat resistance of the spore. The role of phosphate as a chelating agent was hypothesised but was not studied further.

In this study, the key aim is to validate the previously suggested hypothesis on the role of phosphate as a chelating agent in lowering the heat resistance of spores of *G. stearothermophilus* A1, D1 and P3 isolated from a milk powder manufacturing plant.

2. Materials and methods

2.1. Production of spores

G. stearothermophilus A1, D1 and P3 were previously isolated from the evaporator section of a New Zealand milk powder manufacturing plant and the genotypic, phenotypic analyses revealed that these three strains varied in their ability to produce biofilm and spores (Burgess et al., 2014). In this study, *G. stearothermophilus* ATCC 12980 was used as a reference strain. The strains used in this study were obtained from −80 °C stocks. Tryptic soy agar (plates) and tryptic soy broth (BD diagnostic systems, Germany) were used as growth media unless mentioned otherwise. Serial dilutions were performed using 0.1% (*w/v*) buffered peptone water (Difco, Germany). *G. stearothermophilus* A1, D1, P3 and ATCC 12980 were first grown in 250 mL conical flasks containing 25 mL tryptic soy broth supplemented with 0.2% soluble starch (hBARSCI, USA). The mixture was shaken (150 rpm) for 24 h at 55 °C. 200 μL of the 24 h culture was spread onto sporulation agar (final pH 7.2) consisting of 40 g/L tryptic soy agar supplemented with the following minerals (/L) $MgSO_4{\cdot}7H_2O$, 0.51 g; KCl, 0.97 g; $CaCl_2{\cdot}2H_2O$, 0.2 g; $MnSO_4.H_2O$, 0.003 g; $FeSO_4.\ 7H_2O$, 0.55 mg. The plates were then incubated at 55 °C for 5 days. Presence of spores was visually confirmed through staining with malachite green and observed through a light microscope (Olympus Bx41, Japan). Spores were harvested with cold sterile distilled water (5 mL) by scraping the entire surface of the sporulation agar using sterile hockey spreaders and were then subjected to three centrifugation washings (9000 × *g*, 10 min, 23 °C). After washing, spore suspensions were stored at 4 °C in sterile water until experimental use. Spore numbers were enumerated using heat treatment at 90 °C for 30 min in a water bath to eliminate vegetative cells then subsequently diluted using buffered peptone water and plated on tryptic soy agar plates and incubated for 24 h at 55 °C.

2.2. Estimation of DPA content of spores

DPA concentration of spores was measured using a terbium complexation method (Jamroskovic et al., 2016). Known concentrations of DPA (0.005, 0.05, 0.5 μM) (Sigma-Aldrich, USA) were suspended in sterile distilled water containing terbium chloride ($TbCl_3$) (Sigma-Aldrich, USA) at a final concentration of 30 μM. Photo luminescence was measured using a spectro fluorimeter (Perkin Elmer LS55, USA) with the following settings: excitation wavelength: 276 nm, emission wavelength: 546 nm, slit width: 15 nm and scan speed: 500 nm/min. A 420 nm long pass filter was used in front of the emission monochromator throughout the analysis to eliminate second order diffraction effects. A sample containing 30 μM $TbCl_3$ was used as a blank.

Spore suspensions of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 containing 10^8 spores/mL were autoclaved at 121 °C for 30 min to ensure the complete release of DPA. After autoclaving, the spore suspension was centrifuged and the resulting supernatant was diluted five times in sterile distilled de-ionized water. $TbCl_3$ was then added to each dilution at a final concentration of 30 μM and the photo-luminescence was measured in triplicate. DPA concentration per millilitre of spore suspension containing 10^8 spores was determined and used to calculate the DPA content per spore.

2.3. Heat resistance of spores

2.3.1. Heat treatment of spores

Spores were subjected to three centrifugal washings (2500 × *g*, 4 min, 23 °C) before every heat treatment. Microscopic observation of the washed spore suspensions revealed the presence of vegetative cells which were inactivated by heating at 90 °C for 30 min in a water bath. The heat resistance of spores was estimated using the capillary tube method (Sadiq et al., 2016). 50 μL of spore suspension was aseptically transferred into a soda glass capillary tube (Globe Scientific, USA) with an inner diameter of 1 mm and the ends of the tube were heat-sealed. Heat treatments were carried out at 110 °C in a thermostat controlled oil bath containing silicone oil. Each tube was completely submerged in the oil for a selected time interval. After heating, tubes were immediately transferred to cold water (temperature < 4 °C) for 30 s, then washed with 70% ethanol, cut aseptically and serial diluted using 0.1% peptone water. Serial dilutions were then plated on tryptic soy agar and incubated at 55 °C up to 24 h. The minimum detection limit was 1.7 log CFU/mL. Decimal reduction time (D value) was calculated through the negative inverse of the slope of the regression line plotted with values of the survival curve. All heating experiments were carried out as triplicate experiments involving three individual spore suspensions.

2.3.2. Role of the heating medium

To study the effect of pH of the heating medium on the heat resistance of spores, citrate phosphate buffer of pH 4, 7 and 8 were prepared as described previously (McIlvaine, 1921) by mixing 7.7, 16.5 and 19.5 mL of 0.2 M solution of di-sodium phosphate (Sigma- Aldrich, USA) with 12.3, 3.5 and 0.5 mL of 0.1 M solution of citric acid (Sigma-Aldrich, USA) respectively.

Di-sodium phosphate solution of varying concentrations (0.1, 1 and 2%) (*w/v*) were prepared in sterile distilled water and were used as the heating medium for experiments as described below. The pH of the 0.1, 1 and 2% di-sodium phosphate solutions before heating were 8, 8.2 and 8.6 respectively. The effect of phosphate on the heat resistance of spores was further studied in the presence of an equimolar concentration of calcium acetate (Sigma-Aldrich, USA) and di-sodium phosphate (0.07 M) as the heating medium. The effect of EDTA on the heat resistance of spores was studied in the presence of 0.05% of EDTA (Sigma-Aldrich, USA) in distilled water as the heating medium.

2.4. Effect of phosphate on the release of calcium

Atomic absorption spectroscopy was used to determine the influence of di-sodium phosphate on the release of calcium from within spores. Spore suspensions containing 10^8 spores/mL were centrifugally washed three times at 9000 × *g* in deionized distilled water prior to the heat treatment step to get rid of cell debris. The procedures employed were adopted from those described previously (Rotman and Fields, 1969; Bulgarelli and Shelef, 1985). In short, release of calcium from spores exposed to 0.1, 1 and 2% w/v of di-sodium phosphate was determined before and after incubation of spores at 110 °C for 5 min. After heat treatment, 1 mL of spore suspension was filtered through a 0.45 μm syringe filter to eliminate intact spores. The filtrate was then transferred to Pyrex glass tubes (Corning, USA) and were subjected to dry ashing at 500 °C overnight. The ash was then dissolved in 1 mL of 6 M ion free sulphuric acid and 5 mL of 5000 ppm lanthanum chloride solution was added (Udoh, 2000). The solution was then warmed carefully to boiling and contents were transferred quantitatively to a

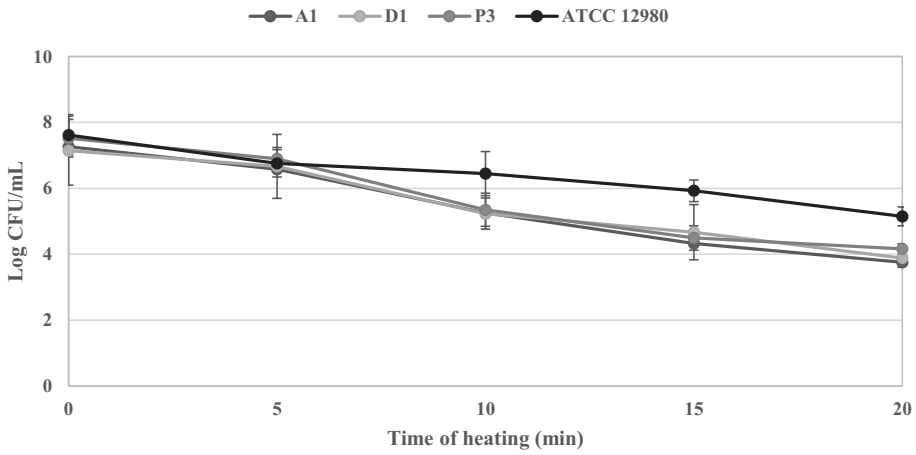

Fig. 1. Survival curves of spores of *Geobacillus stearothermophilus* A1, D1, P3 and ATCC 12980 heated at 110 °C in distilled water. Error bars represent standard deviation of triplicates.

10 mL volumetric flask and made to volume using deionized distilled water. An Xplor AA atomic absorption spectroscope (GBC Scientific Equipment, Australia) was used in all analyses. A nitrous oxide- acetylene flame was used to minimize chemical interference by phosphate. The amount of calcium released was determined through the differences in content of control and heat-treated samples. Total calcium content of spores after autoclaving the spores at 121 °C for 15 min was also determined through atomic absorption spectroscopy. Values obtained were used to calculate the percentage release of calcium from spores. All data in the study represents the average of triplicates.

3. Results and discussion

3.1. Heat resistance of spores

Spore suspensions obtained by plating of cultures of each strain for 5 days were heated in distilled water at 110 °C and plated to determine viability. Heat survival curves of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 were generated for each strain (Fig. 1). Regression lines were established for each strain studied and the D_{110} values were obtained as the negative inverse of the slope of the regression line.

D_{110} value of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 in distilled water were between 5.7 and 9.0 min (Table 1). The D_{110} value of the reference strain ATCC 12980 was the highest whereas the dairy strain P3 exhibited the lowest D_{110} value among all the strains that were studied. Strain variability of the heat resistance of spores between the dairy strains and the reference strain is notable. Variation in the spore heat resistance among different strains of *G. stearothermophilus* has been reported to be caused by strain variability and cultivation media conditions during sporulation (Wells-Bennik et al., 2018). In this study, the spore cultivation conditions were kept uniform for all strains, which suggests that the observed variability in the spore heat resistance is due to strain variability.

Table 1
Comparison of the D_{110} value and the dipicolinic acid content of spores of *Geobacillus stearothermophilus* A1, D1, P3 and ATCC 12980.

Strain	DPA content per spore (pg)	Estimated D_{110} value in distilled water (min)
A1	0.25 ± 0.07	5.8 ± 1.9
D1	0.30 ± 0.1	6.7 ± 2.7
P3	0.27 ± 0.08	5.7 ± 1.5
ATCC 12980	1.6 ± 0.05	9.0 ± 2.1

Strains isolated from the dairy environment are indicated in **bold**.

3.2. DPA content of spores

DPA content per spore is not constant and varies depending on the size of the spore with a larger spore containing a higher DPA content and vice versa (Huang et al., 2007). In this study, DPA concentration per spore was calculated assuming that all spores are uniform in size. Our results indicate that spores from dairy and non-dairy environments exhibit a considerable difference in their DPA content (Table. 1).

DPA content per spore was the highest among spores of ATCC 12980. Spores of the dairy strains exhibited lower DPA content per spore in comparison with the reference strain. On comparing the D_{110} values of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 suspended in distilled water with their respective DPA content per spore, we suggest that the D value of the spores may be associated to their respective DPA content. Our findings are in accordance with previous findings, discussing the role of DPA on the heat resistance of bacterial spores (Magge et al., 2008; Setlow et al., 2006).

3.3. Effect of pH of the heating medium on the heat resistance of spores

Survival curves of spores of *G. stearothermophilus* A1, D1, P3, ATCC 12980 in citrate-phosphate buffer at pH 4, 7 and 8 are shown in Fig. 2. D_{110} values of spores of A1, D1, P3 and ATCC 12980 at pH 4, 7 and 8 are shown in Table A.1. Maximum heat resistance of spores of A1, D1, P3 and ATCC 12980 was obtained at pH 7. Heat resistance was lowered at pH 8 and at pH 4 in comparison with the heat resistance at pH 7 for all the strains.

Moreover, we observed that the D_{110} values of spores in citrate-phosphate buffer were lower than the values obtained in distilled water. Our findings are in agreement with Cook and Gilbert (1968), who found that spores of *Bacillus stearothermophilus* (now *Geobacillus*) were more heat resistant when heated in water than in citrate-phosphate buffer. López et al. (1996a) found that the heat resistance of spores of *B. stearothermophilus* was at a maximum when the pH of the heating medium was 7 and decreased as the pH of the heating medium was altered. Variation among heat resistance of spores is known to be affected by the pH, the strain and the composition of the heating medium (Berendsen et al., 2015; López et al., 1996a). Demineralization of spores leading to the lowering of the heat resistance of spores under acidic pH conditions has also been reported previously (Alderton et al., 1976).

3.4. Effect of phosphate in lowering the heat resistance of spores

Based on the result from the previous experiment, we observed that the heat resistance of spores suspended in citrate phosphate buffer was lower in comparison with distilled water.

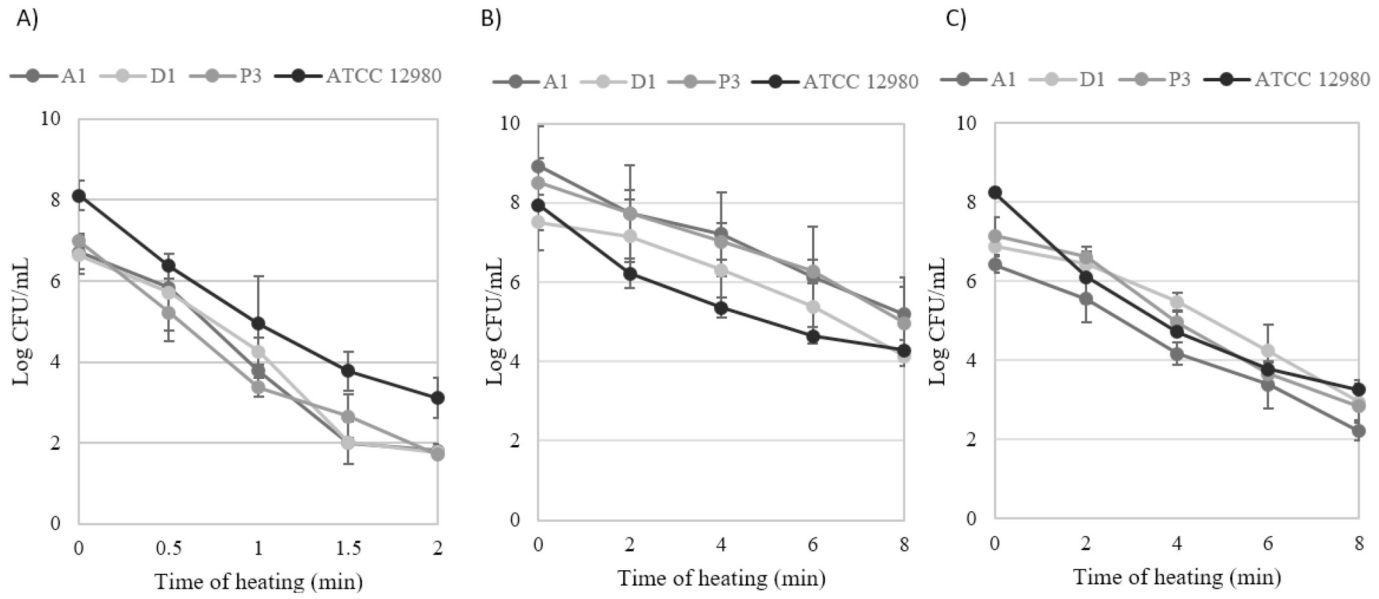

Fig. 2. Survival curves of *Geobacillus stearothermophilus* A1, D1, P3 and ATCC 12980 in citrate-phosphate buffer at pH 4 (A), pH 7 (B) and pH 8 (C). Error bars represent standard deviation of triplicates.

The lowering of the heat resistance of spores in the presence of phosphate in the heating medium has been reported previously (Amaha and Ordal, 1956; El-bisi and Ordal, 1956; Williams and Hennessee, 1955). El-bisi and Ordal (1956) hypothesised that this was due to the carryover of phosphate into the plating medium that influenced results. Results from preliminary experiments conducted in our laboratory suggest that the reduction in the heat resistance of spores is not caused by the carryover of phosphate into the plating medium (data not shown). Amaha and Ordal (1957) offered an alternative explanation that phosphate present in the heating medium acts as a chelating agent for divalent cations present within the spore thereby lowering the heat resistance of spores through enhancing the rate at which divalent cations are removed from the heated spores. It was of interest, therefore, to determine whether phosphate added to the heating medium would affect the heat resistance of spores. To validate this hypothesis, we determined the heat resistance of spores in the presence of varying concentrations of di-sodium phosphate in the heating medium.

Survival curves of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 suspended in distilled water containing varying concentration of di-sodium phosphate (0.1, 1 and 2% *w/v*) are shown in Fig. 3. D_{110} values of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 in distilled water containing 0.1, 1 and 2% *w/v* di-sodium phosphate are shown in Table A.2.

We observed that the heat resistance of spores was maximum when suspended in distilled water and decreased as the phosphate concentration in the heating medium increased. 2% di-sodium phosphate was highly effective in reducing the heat resistance of spores whereas 0.1% of di-sodium phosphate had the least effect. Reduction in the heat

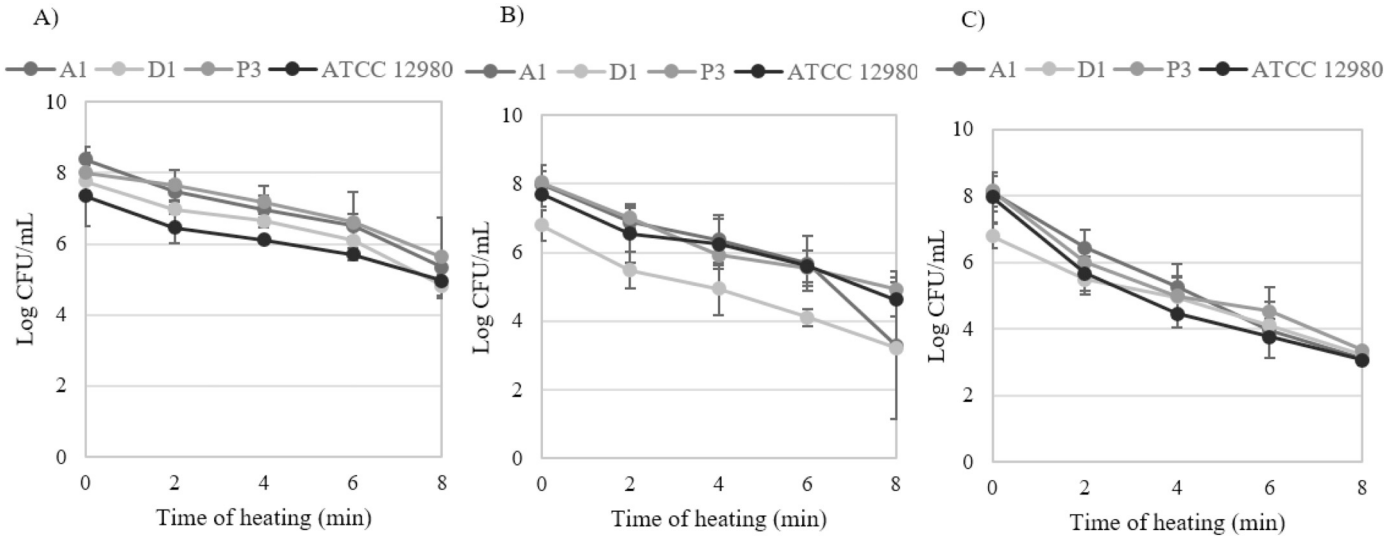

Fig. 3. Survival curves of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 heated at 110 °C in distilled water containing 0.1% (A), 1% (B) and 2% (C) (*w/v*) of di-sodium phosphate. Error bars represent standard deviation of triplicates.

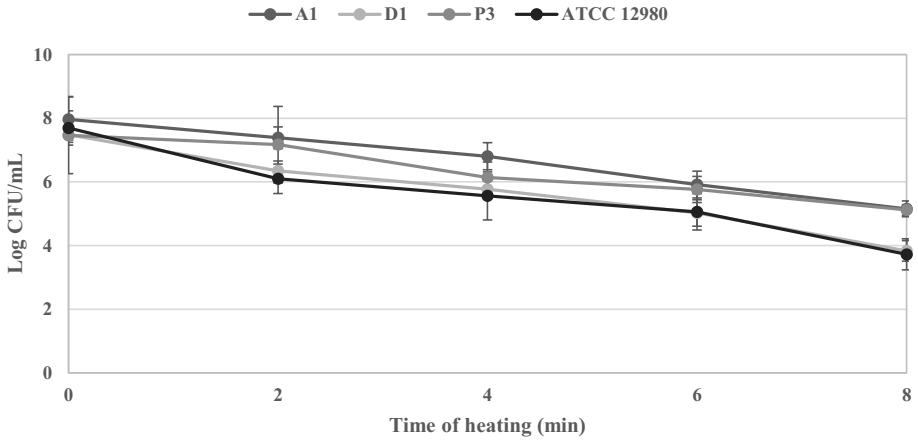

Fig. 4. Survival curves of spores of *Geobacillus stearothermophilus* A1, D1, P3 and ATCC 12980 heated at 110 °C in distilled water containing 0.05% w/v Na-EDTA. Error bars represent standard deviation of triplicates.

Table 2
Effect of phosphate on the percentage release of calcium from spores of *Geobacillus stearothermophilus* A1, D1, P3 and ATCC 12980.

Strain	Percentage release of Ca[a] in distilled water containing concentration of phosphate (% *w/v*)			
	0	0.1	1	2
A1	11 ± 0.9	30 ± 2.1	31 ± 1.4	36 ± 3.6
D1	24 ± 1.6	24 ± 3.3	30 ± 5.7	56 ± 2.6
P3	10 ± 1.9	12 ± 3.6	16 ± 1.5	44 ± 1.7
ATCC 12980	4.2 ± 0.4	8.7 ± 0.9	9.9 ± 0.8	11 ± 1.8

Incubation time for all samples was 5 min at 110 °C.
All values are based on average of triplicates ± standard deviation.
Strains isolated from the dairy environment are indicated in **bold**.
[a] Expressed as percent of average Ca concentration of 10^8 inactivated spores (Table A.4).

resistance varied between strains. Amaha and Ordal (1957) suggested that the rate of loss of cations from within spores should be greater in the presence of more powerful or more specific chelating agents. To determine whether the heat resistance of spores was altered in the presence of a strong chelating agent, we determined the heat resistance of spores suspended in 0.05% *w/v* of EDTA (Fig. 4).

D_{110} values of spores of *G. stearothermophilus* A1, D1, P3 and ATCC 12980 in the presence of 0.05% w/v Na-EDTA are shown in Table A.3. We observed that the heat resistance of spores in the presence of 0.05% w/v of EDTA in the heating medium (Fig. 4) was lower than the heat resistance in distilled water (Fig. 1). Our finding is in accordance with Santos and Zarzo (1997), who concluded that EDTA when present in the heating medium lowers the heat resistance of spores.

3.5. Effect of phosphate on the release of calcium

In order to validate our previously mentioned hypotheses, we estimated the release of calcium from within the spore in the presence of 0.1, 1 and 2% w/v of di-sodium phosphate through atomic absorption spectroscopy. Percentage release of calcium following the heat treatment of spores at 110 °C in the presence of 0.1, 1 and 2% w/v of di-sodium phosphate in the heating medium is summarised in Table 2. The amounts released were calculated as the difference in concentration between the control and heat-treated samples. It was observed that the release of calcium from spores increased with increasing concentration of di-sodium phosphate in the heating medium. Maximum release of calcium was obtained in the presence of 2% w/v of di-sodium phosphate whereas the minimum release of calcium was obtained in the absence of di-sodium phosphate in the heating medium.

Dairy strains exhibited a higher percentage release of calcium than the reference strain irrespective of the concentration of di-sodium phosphate in the heating medium. The reason for this is unknown and needs further investigation. The release of calcium is closely associated with the loss of spores heat resistance (Vries, 2004). Results of this study primarily point towards the effect of phosphate in accelerating the release of calcium, thus contributing towards the lowering of the heat resistance of spores as observed previously in our heat resistance studies.

4. Conclusion

In conclusion, this study indicated that phosphate lowers the heat resistance of spores of *G. stearothermophilus* by accelerating the release of calcium from within the spore. The reason behind the variation in the percentage release of calcium between the dairy isolates and the reference strains is unknown and needs further investigation. In addition, the D values of the spores were found to be associated with their respective DPA content. The outcome of this study can be beneficial in the design of thermal processing steps involved in the manufacture of commercially sterile dairy products. Cleaning in place (CIP) method of cleaning currently employed in the dairy industry to combat biofilms and spores involves the use of acid or alkali based detergents (Thomas and Sathian, 2014). The increased heat sensitivity of spores in the presence of phosphate as shown in this study suggests the use of a phosphate based cleaning agent in the future.

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Appendix A

Table A.1
Heat resistance of spores of *Geobacillus stearothermophilus* A1, D1, P3 and ATCC 12980 in citrate phosphate buffer at pH 4, 7 and 8.

Strain	D value (min) in citrate phosphate buffer at		
	pH 4	pH 7	pH 8
A1	0.37 ± 0.01	2.4 ± 0.48	1.9 ± 0.02
D1	0.38 ± 0.07	2.4 ± 0.29	2.0 ± 0.34
P3	0.38 ± 0.02	2.2 ± 0.2	1.7 ± 0.13
ATCC 12980	0.4 ± 0.02	2.2 ± 0.09	1.6 ± 0.14

All values are average of triplicates ± standard deviation.
Strains isolated from the dairy environment are indicated in **bold**.

Table A.2
Heat resistance of spores of *Geobacillus stearothermophilus* A1, D1, P3 and ATCC 12980 in distilled water containing 0.1, 1 and 2% *w/v* di-sodium phosphate.

Strain	D value (min) in distilled water containing concentration of phosphate (% w/v)		
	0.1	1	2
A1	2.9 ± 0.33	2.1 ± 0.97	1.6 ± 0.07
D1	3.0 ± 0.48	2.7 ± 0.66	2.4 ± 0.57
P3	3.7 ± 1.1	2.6 ± 0.1	2.0 ± 0.11
ATCC 12980	3.7 ± 0.75	2.8 ± 0.04	1.8 ± 0.35

All values are average of triplicates ± standard deviation.
Strains isolated from the dairy environment are indicated in **bold.**

Table A.3
Heat resistance of spores of *Geobacillus stearothermophilus* A1, D1, P3 and ATCC 12980 in distilled water containing 0.05% w/v Na-EDTA.

Strain	D value (min)
A1	2.9 ± 0.47
D1	2.3 ± 0.29
P3	3.9 ± 1.7
ATCC 12980	2.3 ± 0.49

Strains isolated from the dairy environment are indicated in **bold**.

Table A.4
Total calcium content of spores of *Geobacillus stearothermophilus* A1, D1, P3 and ATCC 12980.

Strain	D value (min)
A1	1.8 ± 0.5
D1	1.0 ± 0.34
P3	1.9 ± 0.03
ATCC 12980	6.4 ± 1.0

All values are average of triplicates ± standard deviation.
Strains isolated from the dairy environment are indicated in **bold**.

References

Alderton, G., Ito, K.A., Chen, J.K., 1976. Chemical manipulation of the heat resistance of *Clostridium botulinum* spores. Appl. Environ. Microbiol. 31, 492–498.

Amaha, M., Ordal, Z.J., 1956. The effect of certain sporulation conditions on the thermal death rate of *Bacillus coagulans* var. *thermoacidurans*. J. Bacteriol. 71, 1–9.

Amaha, M., Ordal, Z.J., 1957. Effect of divalent cations in the sporulation medium on the thermal death rate of *Bacillus coagulans* var. *thermoacidurans*. J. Bacteriol. 74, 596–604.

Berendsen, E.M., Zwietering, M.H., Kuipers, O.P., Wells-Bennik, M.H.J., 2015. Two distinct groups within the *Bacillus subtilis* group display significantly different spore heat resistance properties. Food Microbiol. 45, 18–25.

Bulgarelli, M.A., Shelef, L.A., 1985. Effect of Ethylenediaminetetraacetic Acid (EDTA) on growth from spores of *Bacillus cereus*. J. Food Sci. 50, 1–4.

Burgess, S.A., Lindsay, D., Flint, S.H., 2010. Thermophilic bacilli and their importance in dairy processing. Int. J. Food Microbiol. 144, 215–225.

Burgess, S.A., Flint, S.H., Lindsay, D., 2014. Characterization of thermophillic bacilli from a milk powder processing plant. J. Appl. Microbiol. 116, 350–359.

Cempírková, R., 2007. Contamination of cow's raw milk by psychrotrophic and

mesophilic microflora in relation to selected factors. Czech Journal of Animal Science 52, 387–393.
Cook, A.M., Gilbert, R.J., 1968. Factors affecting the heat resistance of *Bacillus stearothermophilus* spores. International Journal of Food Science & Technology 3, 295–302.
Cortezzo, D.E., Setlow, P., 2005. Analysis of factors that influence the sensitivity of spores of *Bacillus subtilis* to DNA damaging chemicals. J. Appl. Microbiol. 98, 606–617.
El-bisi, H.M., Ordal, Z.J., 1956. The effect of sporulation temperature on the thermal resistance of *Bacillus coagulans* var. *thermoacidurans*. J. Bacteriol. 71, 10–16.
Fernandez, P.S., Ocio, M.J., Sanchez, T., Martinez, A., 1994. Thermal resistance of *Bacillus stearothermophilus* spores heated in acidified mushroom extract. J. Food Prot. 57, 37–41.
Gonzalez, I., Lopez, M., Mazas, M., Gonzalez, J., Bernardo, A., 1995. The effect of recovery conditions on the apparent heat resistance of *Bacillus stearothermophilus* spores. J. Appl. Bacteriol. 78, 548–554.
Huang, S.S., Chen, D., Pelczar, P.L., Vepachedu, V.R., Setlow, P., Li, Y.Q., 2007. Levels of Ca2+ −dipicolinic acid in individual *Bacillus* spores determined using microfluidic Raman tweezers. J. Bacteriol. 189, 4681–4687.
Jamroskovic, J., Chromikova, Z., List, C., Bartova, B., Barak, I., Bernier-Latmani, R., 2016. Variability in DPA and calcium content in the spores of *Clostridium* species. Front. Microbiol. 7, 1–11.
López, M., González, I., Condón, S., Bernardo, A., 1996a. Effect of pH heating medium on the thermal resistance of *Bacillus stearothermophilus* spores. Int. J. Food Microbiol. 28, 405–410.
López, M., Mazas, M., González, I., González, J., Bernardo, A., 1996b. Thermal resistance of *Bacillus stearothermophilus* spores in different heating systems containing some approved food additives. Lett. Appl. Microbiol. 23, 187–191.
Magge, A., Granger, A.C., Wahome, P.G., Setlow, B., Vepachedu, V.R., Loshon, C.A., Peng, L., Chen, D., Li, Y., Setlow, P., 2008. Role of Dipicolinic acid in the germination, stability and viability of spores of *Bacillus subtilis*. J. Bacteriol. 190, 4798–4807.
McIlvaine, T.C., 1921. A buffer solution for colorimetric comparison. J. Biol. Chem. 49, 183.
Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., Setlow, P., 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiology and Molecular Biology Reviews: MMBR 64, 548–572.
Paidhungat, M., Setlow, B., Driks, A., 2000. Characterization of spores of *Bacillus subtilis* which lack dipicolinic acid. J. Bacteriol. 182, 5505–5512.
Rotman, Y., Fields, M.L., 1969. A study on the sporulation of rough and smooth variants of *Bacillus stearothermophilus*. J. Food Sci. 34, 346–349.
Sadiq, F.A., Li, Y., Liu, T.J., Flint, S., Zhang, G., Yuan, L., Pei, Z., He, G.Q., 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.
Santos, M., Zarzo, J., 1997. The effect of ethylenediaminetetraacetic acid on heat resistance and recovery of *Clostridium sporogenes* PA 3679 spores treated in HTST conditions. Int. J. Food Microbiol. 34, 293–305.
Scheldeman, P., Herman, L., Foster, S., Heyndrickx, M., 2006. *Bacillus sporothermodurans* and other highly heat-resistant spore formers in milk. J. Appl. Microbiol. 101, 542–555.
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, 109–117.
Seale, R.B., Bremer, P.J., Flint, S.H., McQuillan, A.J., 2010. Characterization of spore surfaces from a *Geobacillus* sp. isolate by pH dependence of surface charge and infrared spectra. J. Appl. Microbiol. 109, 1339–1348.
Setlow, B., Atluri, S., Kitchel, R., Koziol-Dube, K., Setlow, P., 2006. Role of dipicolinic acid in resistance and stability of spores of *Bacillus subtilis* with or without DNA-protective α/β-type small acid-soluble proteins. J. Bacteriol. 188, 3740–3747.
Setlow, P., 1995. Mechanisms for the prevention of damage to DNA in spores of *Bacillus* species. Annu. Rev. Microbiol. 49, 29–54.
Setlow, P., 2003. Spore germination. Curr. Opin. Microbiol. 6, 550–556.
Setlow, P., 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J. Appl. Microbiol. 101, 514–525.
Sonenshein, A.L., Hoch, J.A., Losick, R., 2002. *Bacillus subtilis* and its Closest Relatives: From Genes to Cells. 1 ASM Press, Washington.
Thomas, A., Sathian, C.T., 2014. Cleaning-in-place (CIP) system in dairy plant- review. Journal of Environmental Science, Toxicology and Food Technology 8, 41–44.
Udoh, A., 2000. Atomic absorption spectrometric determination of calcium and other metallic elements in some animal protein sources. Talanta 52, 749–754.
Vries, Y.P.D., 2004. Minireview the role of calcium in bacterial spore germination. Microbes Environ. 19, 199–202.
Wells-Bennik, M.H.J., Janssen, P.W.M., Klaus, V., Yang, C., Zwietering, M.H., den Besten, H.M.W., 2018. Heat resistance of spores of 18 strains of *Geobacillus stearothermophilus* and impact of culturing conditions. Int. J. Food Microbiol. 291, 161–172.
Williams, O.B., Hennessee, A.D., 1955. Studies on heat resistance.VII. The effect of phosphate on the apparent heat resistance of spores of *Bacillus stearothermophilus*. J. Food Sci. 21, 112–116.