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CONTROL OF THERMOPHILIC SPORES BY SPORICIDAL AGENTS AND THERMAL INACTIVATION

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ABSTRACT

In this study, the adhesion patterns of thermophilic spores of *Geobacillus thermodenitrificans* DSM 465^T, *Geobacillus thermoglucosidans* B84a, *Anoxybacillus kamchatkensis* subp. *asaccharedens* F81 and *Anoxybacillus flavithermus* DSM 2641^T, all of which are biofilm producers in dairy products, were investigated by epifluorescence microscopy on 6 different abiotic surfaces commonly used in the dairy industry. The spores of *G. thermodenitrificans* DSM 465^T and *A. kamchatkensis* subp. *asaccharedens* F81 were found to adhere mainly to rubber, polycarbonate, PTFE and stainless steel surfaces. In addition, the efficacy of sporicidal agents on the spores of these bacteria was investigated and only peracetic acid, cetylpyridinium chloride and formaldehyde were found to be the most effective of the sporicidal agents tested. Among the sporicidal agents that showed high efficacy against spores, peracetic acid and nitric acid were selected because they had the shortest contact time, low toxicity and cost. Binary combination effects were tested by determining the LD50 values of the selected agents and it was found that there was a synergistic effectbetween these two effective chemicals. In addition, the thermal resistance profiles of planktonic and sessile spores of *A. flavithermus* DSM 2641^T and *G. thermodenitrificans* DSM 465^T were evaluated.

Keywords: *Anoxybacillus*, Biofilm, Endospore, *Geobacillus*, Thermophilic bacteria, Sporicidal agent

INTRODUCTION

Thermophilic bacilli can develop and survive at high temperatures and are capable of forming spores and biofilms. These characteristics are common in some industrial environments, such as the dairy and fruit juice industries **(Burgess *et al.*, 2010)**. Species belonging to the genus *Geobacillus* and *Anoxybacillus* are mostly isolated from factories where dairy products are manufactured **(Murphy *et al.*, 1999; Flint *et al.*, 2001a,b; Ronimus *et al.*, 2003; Scott *et al.*, 2007; Burgess *et al.*, 2010; Palmer *et al.*, 2010)**. Thermophilic bacilli and their spores are commonly found on heat exchanger plates, cream separators, evaporator preheaters and evaporators **(Murhpy *et al.*, 1999; Refstrup, 2000; Scott *et al.*, 2007; Yuan *et al.*, 2012)**.
The spores of thermophilic bacilli can survive in heat treatment processes such as pasteurization and sterilization. The existence of the spores in such environments indicates poor hygiene **(Muir *et al.*, 1986; Burgess *et al.*, 2009)**. Since they can form biofilms in industrial environments with heat treatment and multiply in a short time, thermophilic bacilli can be easily transferred to final products, and the presence of thermophilic biofilms in these environments leads to continuous contamination **(Flint *et al.*, 1997)**. Because spores are inherently more hydrophobic than vegetative cells, they can adhere more readily to surfaces **(Husmark and Roenner, 1992)**. Spores adhering to surfaces can transform into vegetative forms under suitable environmental conditions, and these vegetative cells can develop into a thermophilic biofilm **(Scott *et al.*, 2007)**. If adequate cleaning application is not performed, this cycle causes continuous contamination in the environment where the products are processed. This continuous contamination leads to clogging of the hoses used in the production phase, prolongation of production time, corrosion of the surfaces, reduction of heat on the heat exchanger plates and indirect deterioration of the heat treatment used in sterilization **(Parkar *et al.*, 2004)**. Therefore, the removal efficiency of the chemical cleaning process is essential.
The antimicrobial and sporicidal properties of disinfectants and cleaning-in-places (CIP) procedures commonly used in the food industry have been demonstrated in studies on both thermophiles and mesophiles **(Sykes, 1970; Russell, 1990; Te Giffel *et al.*, 1995; Flint *et al.*, 1997; Hinton *et al.*, 2002)**. Spores are more difficult to remove with disinfectants than vegetative cells **(Sykes, 1970)**. Due to their more complex and durable structure, endospores prove to be more resistant to chemical and physical agents than vegetative cells **(Russell, 1998)**.

In this study, the thermal resistance of biofilms and spores of *Geobacillus* and *Anoxybacillus* was investigated. The resistance of their spores to various sporicidal-disinfectant agents was evaluated so comprehensively for the first time. The adhesion properties of these thermophilic bacilli spores were determined on surfaces made of 316L stainless steel, polycarbonate, polypropylene, polyvinyl chloride, polytetrafluoroethylene (PTFE) and rubber, all of which are commonly used in the food industry.

MATERIAL AND METHODS

Microbial strains

In this study, four endospore-forming thermophilic bacilli known to be potential biofilm formers in dairy environments were preferred **(Karaca *et al.*, 2019, Karaca and Cihan, 2020)**. These species are *Geobacillus thermodenitrificans* DSM 465^T, *Geobacillus thermoglucosidans* B84a, *Anoxybacillus flavithermus* DSM 2641^T and *Anoxybacillus kamchatkensis* subsp. *asaccharedens* F81.

Stimulation of sporulation

DSM1X (Nutrient Broth 8 g/L, KCl 1 g/L, $MgSO_4.7H_2O$ 0.25 g/L and 1 mL of 1 M $Ca(NO_3)_2$, 1 mL of 0.1 M $MnCl_2.H_2O$, 1 mL of 1mM $FeSO_4$, and 2 mL of 50% glucose solution for 1 liter of media), DSM2X, and TBL (K_2HPO_4 4.35 g/L, KH_2PO_4 3.4 g/L, tryptone 7.5 g/L, glucose 1 g/L) media were tested for stimulation of spore formation. Furthermore, these media contents were additionally supplied with L-glutamic acid, which plays a key role in the sporulation process **(Donnellan *et al.*, 1964)**. Twelve combinations of these three basic media were prepared with or without the addition of glucose and L-glutamic acid (TBL, TBL + glucose, TBL + L-glutamic acid, TBL + glucose+L-glutamic acid DSM 1X, DSM1X + glucose, DSM 1X + glucose, DSM1X + glucose + L-glutamic acid, DSM 2X, DSM2X + glucose, DSM 2X + L-glutamic acid, DSM2X + glucose + L-glutamic acid).
The strains were first inoculated into TSA (Tryptic Soy Agar) medium and then incubated under static conditions at 60 °C for 18 h. The strains were then incubated in TSA medium. After incubation, the culture was harvested and suspended in 0.85% NaCl to obtain an optical density between 0.2 and 0.4 at 600 nm. Finally, the suspended cultures were inoculated in 5 mL of sporulation medium at a ratio of 4% and then incubated at 60 °C for 24 and 48 h at 120 rpm. After incubation, 1

mL of each culture suspension was harvested (6000 rpm, 15 min, 4 °C) and the pellets were resuspended in 1 mL of isotonic solution. These suspensions were heat treated at 100 °C for 15 min to eliminate vegetative cells. The heat-treated suspensions were then diluted 10-fold and each dilution was counted on TSA plates (Tryptic Soy Agar, Merck, Germany) using the spread-plate method.

Production of the spore-stock

To obtain a large number of spores, the ideal sporulation medium was used. 100 mL of the appropriate sporulation medium was inoculated for each strain at the 4% inoculation rate. The culture suspensions were incubated at 60 °C, 120 rpm for 24 and 48 h, respectively. At the end of incubation, cultures were harvested at 6000 rpm for 15 min at 4 °C. The pellets were sonicated (40% amplitude, 0.6 cycles, 2 min) and then washed with physiological saline (6000 rpm, 15 min, 4 °C). The resulting pellets were again dissolved in 2 mL of physiological saline. The cell-spore suspensions were layered on 10 mL of 55% sucrose solution and centrifuged at 4000 rpm for 10 min to obtain cell-free spore stocks. The cell-free spores were stored and used for further studies. Spore counts were performed using the spread plate method, and the total number of spores was determined for each stock.

Spore adhesion assay on different abiotic surfaces

In this study, six abiotic surfaces were preferred to evaluate the spore adhesion properties of thermophilic bacilli. These surfaces are polypropylene, polycarbonate, polyvinyl chloride, stainless steel, PTFE (polytetrafluoroethylene or Teflon) and rubber (all in circular shape; r: 7 mm).

The previously prepared spore stocks were diluted in physiological saline to 10^7 spores/mL. Two pieces of 6 surfaces were exposed to 5 mL of the adjusted spore suspension for 30 min at room temperature. After incubation, the surfaces were washed with sterile distilled water and the unbound spores were removed. One of the surfaces was used for spore counting. Spore counting was performed for each surface examined microscopically and the results were converted to $\log_{10}/cm^2$. The other surface was soaked in 0.001% acridine orange solution (prepared in 100 nmol phosphate buffer, pH: 7.2; Sigma-Aldrich, USA, 235474-5G, excitation/emission values-RNA; 460/650 nm, DNA; 500-526 nm) for 3 min. The stained surfaces were washed 3 times with sterile distilled water to remove excess stain, analyzed at 20× magnification in a Zeiss MTB 2004 epifluorescence microscope, and photographed with a Zeiss Axiocam MRc5 camera. AxioVision Release 4.3 software (11-2004) was used to acquire the images.

Effects of sporicidal agents

The sporicidal agents used are listed in Table 1. The sporicidal activity test was carried out in 96-well microtiter plates (LP Italiana, Italy) to evaluate the effect of the sporicidal agents on thermophilic spores. A maximum treatment time of 30 min was selected as contact time for each agent (1, 2, 5, 10, 15, 20, 25 and 30 min). The concentration values used in the experiment were doubled in cases where the recommended concentrations did not result in effective removal of spores. However, in the cases where the efficacy of the agent could not be established at increased concentration, no further increase in concentration was tested and it was assumed that the agent evaluated was not sufficient to remove the spores of the bacteria tested.

Table 1 Sporicidal agents used in this study

Sporicidal Agents	Concentrations used in the literature	Concentrations used in this study	References
Chlorocrezol (4-chloro-3-methylphenol)	0.4% (w/v)[a]	0.4%, 0.8%, 1.2%	**Russell, 1990**
Crezol (m-cresol)	0.5%	0.5%, 1.0%, 1.5%	**Russell, 1990**
Phenol	0.5%	0.5%, 1.0%, 1.5%	**Russell, 1990**
Chlorhexidine diacetate	0.05%	0.05%, 0.1%, 0.15%	**Russell, 1990**
Cetylpyridinium chloride	0.05%	0.05%, 0.1%, 0.15%	**Russell, 1990**
Glutaraldehyde	2.0%	2.0%, 4.0%, 6.0%	**Russell, 1990**
Formaldehyde	4.0%-8.0%	0.8%, 1.6%, 2.4%	**Russell, 1990**
Hypochlorite	20 ppm[b]	20 ppm, 40 ppm, 60 ppm	**Russell, 1990**
Nitric Acid (HNO_3)	1.0%	1.0%, 2.0%, 3.0%	**Marchand *et al*., 2012**
Iodine	12.5–25.0 ppm	12.5 ppm, 25 ppm, 37.5 ppm	**Banner, 1995**
Peracetic acid	4.0%-15.0%	4.0%, 8.0%, 12.0%	**Banner, 1995**
Chlorine dioxide	100–200 ppm	200 ppm, 400 ppm, 600 ppm	**Banner, 1995**

Legend:[a]weight/volume; [b]parts per million

Finally, 10^5 spores/mL of spore stock were produced from each bacterium prior to testing. Based on the recommended concentrations of the evaluated sporicidal agents, test wells were prepared on the plates for each contact time (1, 2, 5, 10, 15, 20, 25 and 30 min). 100 µL of the active ingredient content was added to these wells, followed by 10 µL of each of the spore suspensions. At the end of each contact time, 100 µL of the contents were removed from the wells and neutralization was performed according to the chemical tested. These contents were subjected to serial dilution (10-fold), and 100 µL of the dilutions were inoculated onto TSA plates. The plates were incubated at 60 °C for 24 h and spore counts were performed. Log reductions (LR; log reduction) were calculated by subtracting the final counts from the initial spore counts. Percent log reduction was calculated using the following formula: $(1\text{-}10^{-LR}) \times 100$ (LR; log reduction).

Combinative treatment of the most effective sporicidal agents

In this study, two highly effective sporicidal agents were selected within the scope of the previous study. Two reference strains (*G. thermodenitrificans* DSM 465^T and *A. flavithermus* 2641^T) displaying high resistance to the sporicidal agents were evaluated in this assay. The study was conducted using two strategies based on the selected sporicidal agents.

In the first approach, the application of the two most effective agents for both strains (*G. thermodenitrificans* DSM 465^T and *A. flavithermus* 2641^T) was performed sequentially at their ideal concentrations. First, 100 µL of the first agent was transferred into 96-well microtiter plate wells and the spores were treated according to the ideal contact time. After the first application, the second agent was applied to the wells at its effective concentration and the spores were treated again during the appropriate contact time. At the end of the second treatment, the contents were neutralized and serially diluted 10-fold. Finally, each dilution was inoculated onto the TSA plates. The plates were incubated for 24 h at 60 °C. The colony forming unit was calculated and the log reductions were calculated according to the initial concentration.

In the second approach, the Chou-Talalay method was preferred to determine possible synergistic, antagonistic and additive effects in different combinations considering the ideal concentrations of sporicidal agents **(Chou, 2010)**. The Chou-Talalay method developed for the evaluation of active ingredient combinations is based on the "median effect" equation, which originates from the "mass-action" principle. The combination indices (CI) determined according to the Chou-Talalay theory allow the quantification of possible additive, synergistic and antagonistic interactions between 2 or more active substance combinations (CI: 1, additive effect; CI < 1, synergism, CI > 1, antagonism). First, a series of dilutions (1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128) were made considering the previously determined ideal concentrations of both agents (Tab 2). In this experimental setup, the ideal concentration of each agent was determined in µg. 10 µL of the spore suspension of each strain was added to the wells of the plate, and then 100 µL of the agent with the corresponding concentrations was added to the wells. At the end of the contact periods, the previously mentioned steps were repeated and spore counts were performed. Based on the spore counts, Probit analysis was performed and LD50 values were calculated for both agents (SPSS 22.0, USA).

Table 2 Nitric acid and peracetic acid concentrations in the test setup

Dilution	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Nitric acid (µg)	4239 (3.0%)	2119.5	1059.75	529.88	264.94	132.47	66.23	33.12
Peracetic acid (µg)	4600 (4.0%)	2300	1150	575	287.5	143.75	71.88	35.94

In preparing different combinations, seven dual combinations were tested to consider the LD50 values of each agent (1st agent-2nd agent: 1:1, 1:2, 2:1, 1:3, 3:1, 1:4, 4:1; each ratio corresponds to the multiple of the LD50 of the agent). The reaction mixtures were prepared with the indicated combination ratios, and spore counts were performed at the end of the application as explained previously. As a result of the counting, the response effect values of the combined doses were

calculated by considering the decrease in spore number (XLSTAT, 2015). Finally, isobolograms were generated according to the dose-response curves and reference lines of dual combinations and possible additive, synergistic and antagonistic interactions were determined using Compusyn software.

Thermal inactivation of thermophilic spores

To study the thermal inactivation profiles of spores in both biofilm and planktonic phases, boiling at 100 °C for 30 and 60 min, sterilization at 121 °C for 15 min (autoclave), and processes partially corresponding to dry heat treatment (130 °C and 140 °C treatment for 1, 5, 15, 30 and 60 min) were applied to thermophilic spores. After the heat treatment applications at the indicated times, the samples were immediately cooled in ice. Moist heat uses water, so unlike dry heating methods, it does not cause dehydration. It also penetrates deeper into cells, making it far more effective than dry heat. This study was again carried out with the reference strains *A. flavithermus* DSM 2641^T and *G. thermodenitrificans* DSM 465^T due to their high resistance to sporicidal agents. Planktonic spores and the spores in biofilm samples not subjected to heat treatment were designed as positive control groups.

For thermal inactivation of planktonic spores, the spore stocks prepared at concentrations of ~10^7 spores/mL were used. 1 mL of these suspensions were transferred to sterile glass tubes and the tubes were subjected to the indicated heat treatments. After the heat treatments, a series of dilutions were prepared and inoculation was performed on the TSA plates using the spread plate method. The plates were incubated at 55 °C for 24 h and then spore counting was performed. Based on the log count results, the log reduction (LR; log reduction) was calculated as a function of the initial spore concentration.

316L stainless steel surfaces (2.5 × 0.8 cm), commonly used in the dairy industry, were preferred for sporulation in the biofilm samples. 6-well cell culture plates were used for biofilm sampling. Biofilm sampling and inoculation preparation were performed based on the method proposed by Karaca *et al*. (2019). Two of the sterile steel surfaces were transferred to each well of the microtiter plate. Then, 5 mL of the milk samples previously mixed with 4% active cultures were transferred into the wells. The plates were incubated at 60 °C for 48 h. At the end of 24 h, the milk content was renewed and the coupons were inverted. After the incubation period, the coupons were removed under aseptic conditions and washed twice with sterile saline to remove loosely adhering bacteria. After washing, the coupons of the control group (samples not subjected to any treatment) were transferred to Falcon tubes containing 4.5 mL of sterile saline and 10 g of glass beads (R: 3 mm). The tubes were vortexed at maximum intensity for 2 min. The prepared biofilm suspensions were boiled for 15 min to eliminate the vegetative cell forms of the spores. A series of 10-fold dilutions were then performed and 100 µL of the diluted samples were inoculated into TSA plates. At the end of the 24 h incubation at 55 °C, the colonies were counted and converted to a logarithmic scale. These results are intended as a control for the number of spores in biofilm samples.

Stainless steel surfaces containing biofilm samples were transferred to glass test tubes containing 4.5 mL of sterile saline for boiling and autoclaving processes. The tubes were boiled for 30 and 60 min and autoclaved for 15 min at 121 °C. After boiling and autoclaving, the physiological saline and coupons in the tubes were transferred to new Falcon tubes containing 10 g sterile glass beads. The tubes were vortexed at maximum intensity for 2 min. Final enumeration was performed by a dilution procedure from these contents.

Stainless steel coupons containing biofilm samples were also transferred to glass tubes containing 4.5 mL of sterile saline after washing, and the tubes were then held in a dry heat block at 130 and 140 °C for 1, 5, 15, 30, and 60 min. At the end of the heat treatment, the contents of the tubes were transferred to Falcon tubes containing glass beads and vortexed. The biofilm suspensions were subjected to an additional boiling process to eliminate possible viable vegetative cell forms. After this process, the dilution and counting processes were performed. All spore count results were converted to a logarithmic scale.

RESULTS AND DISCUSSION

Determination of the ideal sporulation medium

After combination experiments for different sporulation media, the best sporulation medium and incubation conditions for the thermophilic bacilli in each case are given in Table 3. Under these conditions, spore counts of ≤ 10^9 spores/mL were achieved for all thermophilic endospore formers required for further experiments. It was also noteworthy that the ideal sporulation media for 4 of the thermophilic species were all different from each other during a 48 h incubation period.

Table 3 Ideal sporulation media and incubation conditions

Reference strains/Isolates	Medium/Incubation conditions	Number of spores per mL
A. flavithermus DSM 2641^T	DSM[a] 2X/60 °C, 48 h	10^9 spores/mL
A. kamchatkensis subsp. *asaccharedens* F81	TBL + L-Glutamic acid/60 °C, 48 h	10^9 spores/mL
G. thermodenitrificans DSM 465^T	DSM 1X[b], 60 °C, 48 h	10^{10} spores/mL
G. thermoglucosidans B84a	DSM 2X[c] + Glucose+L-Glutamic acid, 60 °C, 48 h	10^9 spores/mL

Legend:[a]DSM; Difco Sporulation Medium; [b]1X: one-fold concentrated; [c]two-fold concentrated

The thermal resistance of bacterial spores can vary depending on the minerals and water activity in their environment **(Beaman *et al*., 1982)**. In addition, conditions such as temperature and pH of the environment in which sporulation occurs affect spore resistance **(de Vries *et al*., 2004)**. Minerals such as magnesium, calcium and potassium also play an essential role in this process. These minerals are essential for the development of mature spores and promote sporulation **(Vinter, 1970)**. Calcium accumulates in the core part of the spore at the beginning of spore formation. Calcium also increases the expression of genes that initiate sporulation **(Oomes *et al*., 2009)**. Mineral salts are commonly used in sporulation media **(Cooney and Freese, 1976; Kaul and Singh, 1982; Warriner and Waites, 1999)**. Since these minerals are also present in dairy products, they promote sporulation of thermophilic bacilli. The time required for sporulation in thermophilic bacilli varies among species and genera. For instance, while some thermophilic bacilli can be fully sporulated within a week in media supplied with minerals, some Geobacillus isolates can be sporulated for 12 to 18 h in standard Tryptic Soy Broth (TSB) supplied with minerals ($CaCl_2$, $MnSO_4$, $FeSO_4$, or $MgCl_2$) (10^5-10^7 spores/mL) **(Seale *et al*., 2008)**. In this study, TBL, DSM1X and DSM2X media were used to promote sporulation of thermophilic bacilli. Among these media, TBL medium consists of $MgSO_4$, $MnSO_4$, $ZnSO_4$, $FeSO_4$ and $CaCl_2$. These salts are commonly used in sporulation media to promote sporulation. DSM1X and DSM2X supplied with $Ca(NO_3)_2$, $MnCl_2$ and $FeSO_4$ were also investigated to promote sporulation. It was found that the requirements for sporulation differed according to the thermophilic species tested. Also, the high amount of calcium stimulated sporulation of 4 of the endospore formers. Furthermore, in contrast to the study by **Seale (2008)**, all the thermophilic bacilli used in that study were found to be highly sporulated only after 48 h of incubation (Tab 3).

Adhesion of thermophilic spores on abiotic surfaces

Adhesion patterns of thermophilic bacilli on six abiotic surfaces are shown in Supplementary File (Figure 1-6). As can be seen in fluorescence microscopy analysis, excessive irradiation was observed in the fluorescence spectrum of acridine orange especially on surfaces like polypropylene, polyvinyl chloride, polycarbonate (autofluorescence). Therefore, for such surfaces as polyvinyl chloride, the filter set20 Transmitter R Red filter (excitation; ≈546 nm, emission; ≈640 nm) of the epifluorescence microscope was used during imaging and for all other abiotic surfaces the filter set10 Transmitter G Green filter (excitation; ≈450-490 nm) emission ≈515-565 nm) was used (Kepner and Pratt, 1994).

Based on these results, while the spores of *G. thermodenitrificans* DSM 465^T mainly adhered to the rubber and polycarbonate surfaces, the spores of *G. thermoglucosidans* B84a mostly preferred Teflon and stainless steel surfaces. In addition, the spores of *A. kamchatkensis* subsp. *asaccharedens* F81 mostly adhered to Teflon and polyvinyl chloride surfaces, while the spores of *A. flavithermus* DSM 2641^T mostly adhered to polycarbonate and stainless steel surfaces. The spores observed under epifluorescence microscope with diameters of about 1-2 µm produced a bright fluorescence image of single spores or spore clusters depending on the wavelength of light. The spore numbers also correlated with the microscopy images, and fluorescence illumination was also observed more intensely on these surfaces (Supplementary File, Figure 1-6). According to these results, the surfaces preferred by thermophilic spores were found to be different depending on the bacterial species.

Spores differ significantly from the attachment behavior of vegetative cells to surfaces because they are not metabolically active and immobile **(Setlow, 2007)**. The adhesion properties of thermophilic bacteria to the surface have been determined in some previous studies **(Flint *et al*., 1997; Seale, 2008)**. In related studies, surface hydrophobicity was reported to increase spore adhesion to surfaces. Adhesion of vegetative cells and spores to surfaces is a process involving many factors. Factors affecting the adhesion of bacteria to surfaces include the properties of the surface, the growth phase of the bacteria, the contact time with the surface, the temperature of the environment, the roughness of the surface, the chemical signaling between cells, and the cleanliness of the surface **(Stickler, 1997; Flint *et al*., 1997)**. Cell surface electrical charge and hydrophobicity also influence bacterial adherence **(Hood and Zottola, 1995)**. Spores with hydrophobic surface properties tend to adhere more strongly to stainless steel than vegetative cells. Proteins in milk cause both vegetative cells and spores to adhere more

strongly to surfaces. In addition, the electrical charge and wettability of the surface can affect the ability of spores to adhere **(Parkar *et al*., 2001)**. The relationship between surface topography and bacterial attachment has been studied over the last 45 years **(Flint *et al*., 1997)**. Whitehead and Verran, (2007) have shown that the attachment pattern depends on surface topography and not chemistry. However, contrary to the findings of **White and Verran's (2007)** study, some studies have emphasized that surface chemistry significantly affects the attachment of spores and vegetative cells. Most of the surface materials (polycarbonate, polyvinyl chloride, polypropylene) preferred in dairy industry are mostly in polymeric structure. Although most of these polymeric materials are thermally, chemically and biologically stable, they are highly hydrophobic and accelerate protein absorption **(Turkiewicz *et al*., 2006)**. Absorption of milk proteins on these surfaces increases hydrophobicity and facilitates attachment of bacterial cells and spores **(Brooks and Flint, 2008)**. **Palmer *et al.* (2010)** also found that the adhesion patterns of vegetative cells and spores of *A. flavithermus* vary depending on surface type and chemistry.

In this study, the adhesion patterns of 4 different thermophilic bacilli spores on 6 surfaces were determined by examination under epifluorescence microscope. It was found that *A. flavithermus* DSM 2641^T had the highest adhesion rate for all the surfaces tested. According to these results (Supplementary File, Figure 1-6), the spores of *A. flavithermus* DSM 2641^T were found to be highly hydrophobic. While the spores of *G. thermoglucosidans* B84a had the highest adhesion rate on the rubber surface, the spores of *G. thermodenitrificans* DSM 465^T had the highest adhesion rate on the Teflon surface. Based on these results, the adhesion rates of thermophilic spores on surfaces with different physicochemical properties vary at the species level. Based on the high adhesion capacity of *A. flavithermus* DSM 2641^T (4.8-6.1 logCFU/cm^2) and *G. thermodenitrificans* DSM 465^T (4.0-5.7 logCFU/cm^2) on all surfaces, it can be assumed that the spores of these species can transform and develop as vegetative form, forming biofilms and thus causing continuous contamination of products in the dairy industry. The lowest adhesiveness was observed for the spores of *A. kamchatkensis* subsp. *asaccharedens* F81 (3.3-4.1 logCFU/cm^2) (Supplementary File, Figure 1-6).

Effects of sporicidal agents on thermophilic spores

The efficacy of some sporicidal agents in the food industry was tested according to the recommended concentrations and exposure times in the respective study (Tab1). On evaluation of the results, it was found that sporicidal agents such as chlorine dioxide, phenol and iodine eliminated only *G. thermoglucosidans* B84a spores. Chlorocresol eliminated the spores of *A. kamchatkensis* subsp. *asaccharedens* F81. In addition, nitric acid eliminated all the spores of *A. flavithermus* DSM 2641^T, *G. thermoglucosidans* B84a and *A. kamchatkensis* subsp. *asaccharedens* F81 and 99.998% of the spores of *G. thermodenitrificans* DSM 465^T. Chlorhexidine diacetate eliminated 100% of the spores of *A. flavithermus* DSM 2641^T, *G. thermoglucosidans* B84a, and *A. kamchatkensis* subsp. *asaccharedens* F81, and 99.993% of the spores of *G. thermodenitrificans* DSM 465^T within the concentrations and contact times indicated. Complete removal of all thermophilic spores was achieved only by treatment with peracetic acid, cetylpyridinium chloride and formaldehyde.

Cumulative evaluation of the efficacy of the sporicidal agents against spores showed that the spores of strains *G. thermodenitrificans* DSM 465^T and *A. flavithermus* DSM 2641^T were much more resistant than the spores of *G. thermoglucosidans* B84a and *A. kamchatkensis* subsp. *asaccharedens* F81.

Of the sporicidal agents, only peracetic acid, cetylpyridinium chloride and formaldehyde had an absolute effect on the spores of 4 thermophilic species. However, cetylpyridinium chloride, a quaternary ammonium compound used as a drug in some cases, is known to be toxic in high doses and is a very expensive chemical. Formaldehyde is also a toxic chemical in liquid form and can be carcinogenic. In this regard, peracetic acid can be considered as the least harmless agent among these 3 agents as it acts on the spores of 4 thermophilic species. When chlorhexidine diacetate and nitric acid, which are 100% effective against all spores, were compared, it was found that chlorhexidine diacetate will be more costly for clean-in-place (CIP) procedures in industrial environments. 4.0% peracetic acid and 3% nitric acid formulations effective against the spores of 4 thermophilic species were preferred due to their low toxicity to human and environmental health and low cost (Supplementary File, Tab 5-8).

Combinative treatment of ideal sporicidal agents

In the first strategy where nitric acid and peracetic acid were treated consecutively, all *A. flavithermus* DSM 2641^T, and *G. thermodenitrificans* DSM 465^T resistant spores were eliminated. In the second strategy, an experimental design was performed to determine possible synergetic, additive, and antagonistic interactions between the 2 sporicidal agents. Accordingly, a series of dilutions were made first considering the previously determined ideal concentrations of both sporicidal agents [(3.0% Nitric acid solution; 100 µL in the reaction mixture: 4239 µg), (4.0% Peracetic acid solution; amount in 100 µL reaction mixture: 4600 µg)], (Tab 2).

Based on the concentration values indicated in Table 2 and the contact times of both sporicidal agents, LD50 values were determined by Probit analysis for *G. thermodenitrificans* DSM 465^T and *A. flavithermus* DSM 2641^T spores (Tab 4 and Tab 5). A treatment design was performed in 7 binary combinations, considering the LD50 values of each sporicidal agent (30 min contact time of nitric acid), (Nitric acid-Peracetic acid; 1:1, 1:2; 2:1, 1:3, 3:1, 1:4, 4:1; each ratio corresponds to the multiples of the LD50 of the active substance). Reaction mixes were prepared with the combination ratios, and spore counts were performed at the end of the treatment, as explained previously. As a result of the counting results, the dose responses of evaluated agents were calculated considering the decreases in the spores (XLSTAT, 2015), (Tab 6).

Table 4 Effects of different nitric acid and peracetic acid concentrations on *A. flavithermus* DSM 2641^T spores, and their LD50 values (Probit analysis)

Nitric acid concentrations (µg)	Initial spore concentration log(spore/mL)	Spore counts after treatment log(spore/mL)	Reduction per mL log(spore/mL)	
33.12	5	4	4.9542	
66.23	5	3.954	4.9590	
132.47	5	3.875	4.9661	LD50[a]
264.94	5	3.531	4.9850	1.801 µg
529.88	5	3.079	4.9948	
1059.75	5	1.940	4.9996	
2119.5	5	1.536	4.9998	
4239	5	1.342	4.9999	
Peracetic acid concentrations (µg)	**Initial spore concentration log(spore/mL)**	**Spore counts after treatment log(spore/mL)**	**Reduction per mL log(spore/mL)**	
4600	5	4.3617	4.88649	
2300	5	4.0792	4.94448	
1150	5	2.5185	4.99856	LD50
575	5	1.9085	4.99965	18.88 µg
287.5	5	1.7404	4.99976	
143.75	5	1.1761	4.99993	
71.88	5	0.9031	4.99997	
35.94	5	0.4771	4.99999	

Legend:[a]LD50: lethal dosage 50

Table 5 Effects of different nitric acid and peracetic acid concentrations on *G. thermodenitrificans* DSM 465 ^{T} spores and their LD50 values (Probit analysis)

Nitric acid concentrations (µg)	Initial spore concentration log(spore/mL)	Spore counts after treatment log(spore/mL)	Reduction per mL log(spore/mL)	
33.12	5	4.322	4.898	
66.23	5	3.362	4.990	LD50
132.47	5	2.778	4.997	10.47 µg
264.94	5	2.572	4.998	
529.88	5	1.623	5.000	
1059.75	5	0.000	5.000	

2119.5	5	0.000	5.000	
4239	5	0.000	5.000	
Peracetic acid concentrations (µg)	**Initial spore concentration log(spore/mL)**	**Spore counts after treatment log(spore/mL)**	**Reduction per mL log(spore/mL)**	
4600	5	4.097	4.942	
2300	5	2.708	4.998	
1150	5	2.223	4.999	LD50
575	5	1.000	5.000	15.92 µg
287.5	5	0.000	5.000	
143.75	5	0.000	5.000	
71.88	5	0.000	5.000	
35.94	5	0.000	5.000	

Table 6 Dose response effects of nitric acid, peracetic acid and nitric acid-peracetic acid combinations on spores of *A. flavithermus* DSM 2641^T and *G. thermodenitrificans* DSM 465^T strains

Nitric acid concentrations (µg)	**Nitric acid dose-response effect**	**Peracetic acid concentrations (µg)**	**Peracetic acid dose-response effect**	**Nitric acid-Peracetic acid combinations**	Combined dose-response effect
A. flavithermus DSM 2641^T					
33.12	0.900	35.94	0.770	1:1	0.954
66.23	0.910	71.88	0.880	1:2	0.968
132.47	0.925	143.75	0.997	2:1	0.943
264.94	0.966	287.5	0.999	1:3	0.917
529.88	0.988	575	0.999	3:1	0.931
1059.75	0.999	1150	1.000	1:4	0.880
2119.5	1,000	2300	1.000	4:1	0.790
4239	1.000	4600	1.000		
G. thermodenitrificans DSM 465^T					
33.12	0.790	35.94	0.875	1:1	0.984
66.23	0.977	71.88	0.995	1:2	0.989
132.47	0.994	143.75	0.998	2:1	0.990
264.94	0.996	28.50	1.000	1:3	0.990
529.88	1.000	575	1.000	3:1	0.992
1059.75	1.000	1150	1.000	1:4	0.990
2119.5	1.000	2300	1.000	4:1	0.993
4239	1.000	4600	1.000		

When the dose-response curve is evaluated, the slope of the curve obtained with the dual treatment of agents for *A. flavithermus* DSM 2641^T spores is closer to peracetic acid, which shows that peracetic acid is more effective on spores of this strain (Figure 1a). The points in figure 1b indicate each combination that shows significant interaction. The fact that these points are on the left side of the reference line (CI; combination index <1) indicates the synergism. In the elimination of *A. flavithermus* DSM 2641^T spores, there is a clear synergetic interaction in the combination ratios, except for 1:3, and 4:1 ratios (near the additive effect), (Figure 1b).

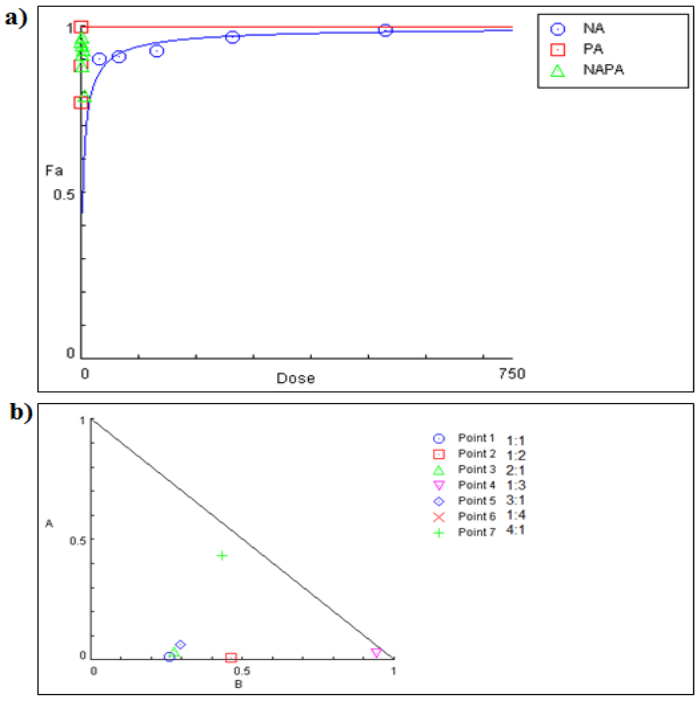

Figure 1 a) Dose response combination curve of Nitric acid, peracetic acid and nitric acid- peracetic acid (*A. flavithermus* DSM 2641^T) NA; nitric acid, PA; peracetic acid; NAPA; Nitric acid and peracetic acid combination. **b)** Isobologram of nitric acid-peracetic acid combinations. (*A. flavithermus* DSM 2641^T)

When evaluating the dose-response curve for *G. thermodenitrificans* DSM 465^T, peracetic acid was again found to be more effective in eliminating spores with a combination of dual agents. The combined treatment appears to be closer to the peracetic acid slope (Figure 2a). For *G. thermodenitrificans* DSM 465^T, it was revealed that there is a synergetic interaction in the sporicidal agent combinations (except 1:4, 1:3). The points in the graph indicate each combination that shows significant interaction. The fact that these points are on the left side of the reference line (CI; combination index < 1) that displays the synergism. The 1:4 combination indicates a close additive effect, while the 1:3 combination shows a slight antagonistic interaction (Figure 2b).

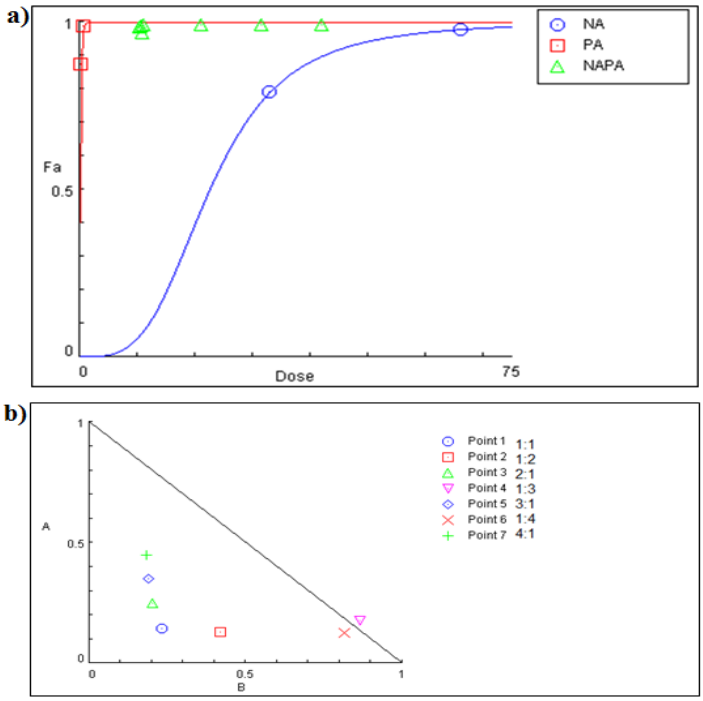

Figure 2 a) Dose response combination curve of Nitric acid, peracetic acid and nitric acid- peracetic acid (*G. thermodenitrificans* DSM 465^T) NA; nitric acid, PA; peracetic acid; NAPA; Nitric acid and peracetic acid combination. **b)** Isobologram of nitric acid-peracetic acid combinations (*G. thermodenitrificans* DSM 465^T)

Bacterial spores are structures that are resistant to chemical agents and physical treatments **(Russel, 1990)**. Examining the studies conducted in the literature, it can be seen that more studies have been conducted on the resistance of mesophilic bacterial spores than thermophilic ones to chemical agents, especially sporicidal agents. For example, **Sykes (1990)** studied the effects of phenol, chlorhexidine digluconate, glutaraldehyde, hydrochloric acid, chlorine gas releasing compounds and iodine on *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus stearothermophilus*. **Briggs and Yazdany (1974)** found that chlorocresol and benzalkonium chloride were insufficient to remove *B. pumilus* and *B. stearothermophilus* spores. In a study by Young and **Setlow (2003)**, a 3 log decrease in *B. subtilis* spores was observed when treated with hypochlorite and chlorine dioxide. They also demonstrated that chlorine dioxide has a stronger lethal effect on *B. subtilis* spores than chlorine. **Beuchat *et al.* (2004)** also demonstrated that oxidation reactions caused by disinfectants are influenced by organic material in the environment. Moreover, sporicidal agents in the study were tested only on the spores of the mesophilic bacteria according to the literature **(Borick, 1968; Briggs and Yazdany, 1974; Russell, 1990; Knott *et al*., 1995; Sagripanti and Bonifacino, 1996; Young and Setlow, 2003; Beuchat *et al*., 2004; Maillard, 2011)**.
According to the spore elimination tests, the spores of *G. thermodenitrificans* DSM 465^T and *A. flavithermus* DSM 2641^T are much more resistant to the chemical agents compared to the spores of *G. thermoglucosidans* B84a and *A. kamchatkensis* subsp. *asaccharedens* F81. Of these agents tested, only peracetic acid, cetylpyridinium chloride and formaldehyde were effective on 4 of the thermophilic bacilli spores. Peracetic acid seems to be one of the ideal sporicidal agents as it was a highly active sporicidal agent besides its economic and health benefits. For example, when comparing chlorhexidine diacetate and nitric acid, which kills all thermophilic spores except spores of *G. thermodenitrificans* DSM 465^T, chlorhexidine diacetate was found to be costly for clean-in-place procedures. It was found that 4.0% peracetic acid, which acts on the spores of 4 thermophilic species, and 3% nitric acid, which eliminates all spores except *G. thermodenitrificans* DSM 465^T spores (99.998%), are ideal agents due to their lowest contact time and concentration. Peracetic acid allows for time and cost savings as it is degradable, non-toxic and does not require rinsing after treatment **(Block, 2001)**. Alternatively, nitric acid is the most commonly used acid in clean-in-place systems, especially in the dairy industry **(Bremer *et al*., 2006)**. Moreover, in this study, the exact effective doses of these two sporicidal agents were determined by testing them on 4 thermophilic spores belonging to different species. It was found that the combined application of peracetic acid and nitric acid in the dairy industry is an ideal solution for the elimination of thermophilic spores, considering the recommended application concentrations.

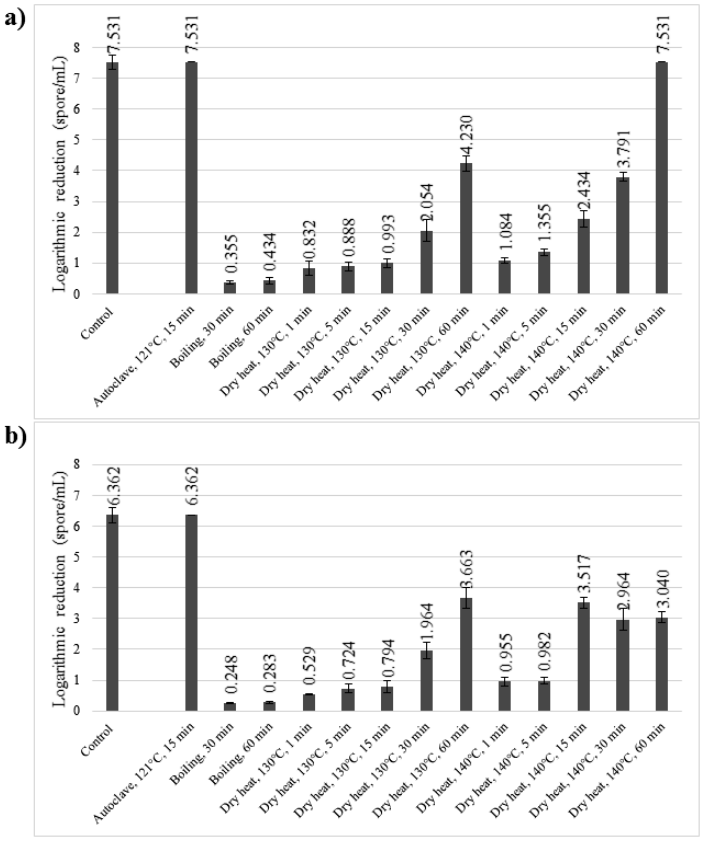

Figure 3 a) Thermal inactivation of *A. flavithermus* DSM 2641^T planktonic spores **b)** Thermal inactivation of *A. flavithermus* DSM 2641^T biofilm spores. Bars represented as logarithmic reduction

Thermal inactivation of thermophilic spores

The effects of thermal inactivation on the spores of *A. flavithermus* DSM 2641^T and *G. thermodenitrificans* DSM 465^T in both the planktonic phase and biofilm are shown in figure 3 and 4. Complete elimination of *A. flavithermus* DSM 2641^T biofilm spores was only possible by autoclaving. Dry temperature treatment (60 min) at 140 °C was able to eliminate all planktonic spores. According to these results, it was revealed that planktonic spores of this strain were more resistant to thermal treatments as much as spores in biofilm (Figure 3). Spores of *G. thermodenitrificans* DSM 465^T could be completely removed only after sterilization in autoclave, as was found for *A. flavithermus* DSM 2641^T. The planktonic spores of this strain have a lower thermal resistance than the planktonic spores of *A. flavithermus* DSM 2641^T, because the planktonic spores of *G. thermodenitrificans* DSM 465^T can be eliminated during dry temperature treatment (60 min) at 130°C. Results in figure 3 and 4 were illustrated as logarithmic reduction.

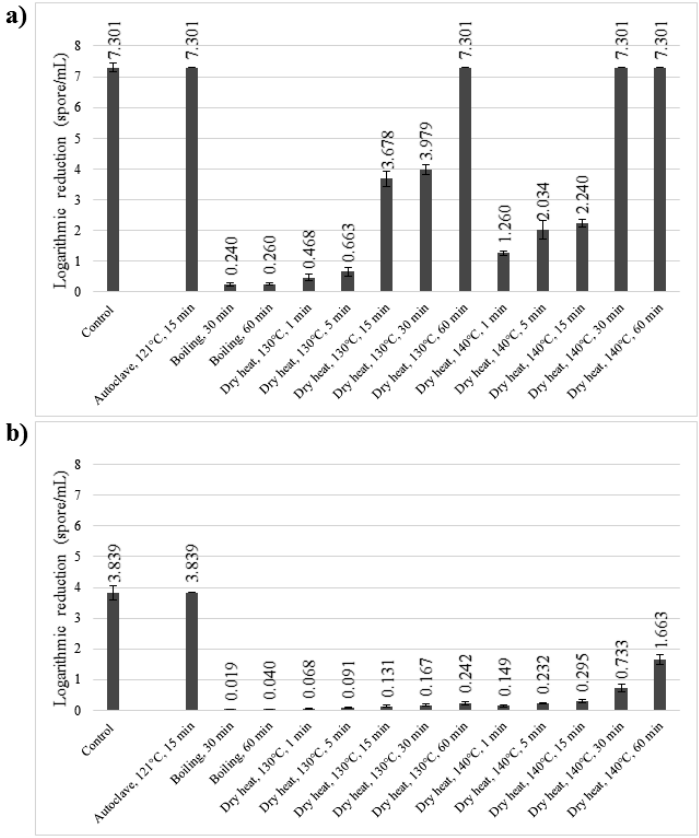

Figure 4 a) Thermal inactivation of *G. thermodenitrificans* DSM 465^T planktonic spores **b)** Thermal inactivation of *G. thermodenitrificans* DSM 465^T biofilm spores. Bars represented as logarithmic reduction

Recently, many studies have been conducted to define the thermal inactivation kinetics of bacterial spores **(Witthuhn *et al*., 2011; Gómez-Jódar *et al*., 2016; Stoeckel *et al*., 2016; Den Besten *et al*., 2018)**. Thermophilic spores can be effectively eliminated by UHT (> 135 °C). However, this heat treatment reduces the valuable content of whey proteins (Elliott et al., 2005). Another increasing problem is the coagulation of concentrated milk or whey due to protein aggregation **(Havea *et al*., 2009; Dumpler and Kulozik, 2015)**. The k-casein in milk and dairy products breaks down into concentration factors at high temperatures **(Anema, 1998)**. In concentrated whey products, cross-linking between proteins occurs depending on the pH of the medium and the mineral composition **(Havea *et al*., 2002)**. When casein micelles interact with denatured whey proteins, gel formation occurs at high temperatures, and this viscous content makes it difficult to eliminate bacterial spores. **Wedel *et al*. (2018)** showed that heat treatment for *A. flavithermus* at 110 °C for 30 min is sufficient to inactivate all spores. However, they observed that another strain of *A. flavithermus* used in the same study remained alive after exposure to 125 °C for 30 min. Thus, it was clear that thermal resistance also varied at the strain level. When the results of the current study are compared with those of **Wedel *et al*. (2018)**, it is clear that strain *A. flavithermus* DSM 2641^T was found to be more resistant to prolonged exposure to high temperatures. In a study by **Sadiq *et al*., (2016)**, 1.5 log decrease was observed at 110 °C for 30 min. However, the exposure of thermophilic spores to heat treatment was performed only in water. **Wells-Bennik *et al*., (2018)** suggested that food ingredients such as proteins and minerals and acidity or alkalinity of food are effective in thermal spore inactivation. In another study with *A. flavithermus* and *G. stearothermophilus*, the spore concentration decreased from 6.36-log to 3.72-log at 121 °C for 15 sec. Since *G. thermodenitrificans* DSM 465^T strain evaluated within the scope of this study was removed only by autoclaving process at 121 °C for 15 min, and dry heat treatment at 140 °C (30 and 60 min), it has been understood that the spores of this strain were more resistant to heat treatment than other bacilli evaluated in the literature. The fact that the *A. flavithermus* and *G. thermodenitrificans* spores evaluated in the study cannot be eliminated even at the temperatures used in UHT technology proves why thermophilic bacilli cause continuous contamination in the dairy industry. Another striking result in the study is that the biofilm spores of thermophilic bacilli in the presence of milk residues can only be removed by autoclaving. This makes it impossible to eliminate these spores in dairy environments by thermal treatment.

CONCLUSION

In this study, the recommended sporicidal treatments for the elimination of bacterial spores posing a risk in the dairy industry were comparatively evaluated. It was found that simultaneous treatment with widely used agents such as peracetic acid and nitric acid gave much more effective results against these extremely difficult to eliminate spores. It was revealed that these two agents, which are also widely used in the dairy industry, have synergistic interactions due to their low cost and health safety, and can be used at much lower doses through a combination treatment. In this study, it became clear that there are synergistic interactions between widely used sporicidal agents. Therefore, combined treatment with sporicidal agents can be considered as an alternative for the elimination of thermophilic spores in the dairy industry. It has also been recognized that thermophilic spores cannot be eliminated even at the processing temperatures used in the dairy industry, especially if they are in biofilm structures.

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