Extended shelf life milk processing: Effect of simulated cleaning in place on the germination and attachment of *Bacillus cereus* spores

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Abstract

The effect of simulated cleaning in place (CIP) was determined on the structure, attachment and growth of *Bacillus cereus* spores isolated from raw milk and biofilms in filler nozzles from extended shelf life (ESL) milk processing lines. Simulated CIP treatment structurally affected >98% of *B. cereus* spores, while 0.1% remained intact. Following simulated CIP treatment, *B. cereus* spores were able to attach to stainless steel coupons and form biofilms. *B. cereus* spores were capable of germination and growth under refrigerated conditions for more than 28 days. Contamination with *B. cereus* spores may lead to a reduced shelf life and potentially be a safety risk in ESL milk with a prolonged shelf life.

Keywords: Extended shelf life milk; Cleaning in place; *Bacillus cereus* spores; Germination; Attachment

INTRODUCTION

Extended shelf life (ESL) milk is processed with the aim of obtaining a product encompassing the sensorial characteristics of fresh milk, but with a longer shelf life. To achieve these qualities, a sequence of processes is employed which may include pasteurisation, steam injection, bactofugation and ultra clean filling technologies. This yields a product with excellent bacteriological quality, but with minimal chemical changes (Deeth, 2017). Although the technology for ESL milk production is designed to prevent post-pasteurisation contamination (Kapadiya *et al*. 2001), recent studies by Mugadza and Buys (2017) have shown the occurrence of psychrotrophic spore formers in finished ESL milk products. The spores of *B. cereus* are a problem in the dairy industry as they are exceedingly resistant to heat as well as cleaning and disinfection processes (Faille *et al*. 2001). In ESL milk, *B. cereus* spores are of particular concern due to their heat resistance as well as the ability of certain strains to germinate and grow at refrigerated temperatures (Schmidt *et al*. 2012). ESL heat treatment may activate spore germination (Deeth 2017), and the long shelf life of ESL milk subsequently allows psychrotrophic *B. cereus* time to germinate and grow during storage. Additionally, the high bacteriological quality of ESL milk could result in *B. cereus* experiencing less competition by other microbes to obtain nutrients. Another challenge in ESL milk production is post-processing contamination caused by biofilms in filler nozzles (Deeth 2017). The ability of bacteria such as *B. cereus* to attach to stainless

steel and to form biofilms can be a primary cause of contamination in ESL milk (Khoza 2015). Contaminating bacteria in ESL milk possibly originate from biofilms in the machine filler nozzles and are then dispensed into the final ESL milk during the filling process (Mugadza *et al*. 2019). Minimising biofilms has become a challenge for dairy manufacturers (Flint *et al*. 2011). In South Africa, milk processors struggle to extend the shelf life of ESL milk beyond 14 days. A shorter shelf life than expected can lead to economic losses.

In the food industry, an adequate cleaning in place (CIP) programme is part of the process to eradicate microorganisms (Carpentier and Cerf, 1993; Gibson *et al*. 1999). The effectiveness of CIP procedures is, however, limited by the accumulation of microorganisms on equipment surfaces (Zottola and Sasahara, 1994). Moreover, the survival of heat resistant *B. cereus* spores during CIP further restricts the efficiency of CIP processes (Khoza 2015). In the processing facility, manufacturing costs are reduced by the recycling of caustic wash solutions, but by doing so, the risk of contamination with spores may be equally increased (Brent Seale *et al*. 2011). The success of CIP procedures against *B. cereus* biofilms has not been extensively reported. *B. cereus* spores surviving CIP processes may germinate upon favourable conditions and cause spoilage of the final ESL milk product which could lead to a reduced shelf life and potentially be a safety risk in ESL milk with a prolonged shelf life. It is, therefore, essential to determine the effect of the CIP process on the structure, attachment, growth and biofilm forming capability of *B. cereus* spores during ESL milk processing.

MATERIALS AND METHODS

Bacillus cereus isolates

B.cereus isolates used in this study were previously isolated and characterised by Khoza (2015) and Mugadza and Buys (2017). The three *B. cereus* strains used in this study are referred to as strains BC10, BC17 and BC24. Strain BC24 was isolated from raw milk meant for ESL milk processing, and strains BC10 and BC17 were isolated from biofilms in filler nozzles in an ESL milk processing line.

Bacillus cereus spore preparation

The procedure to isolate *B. cereus* spores was established after multiple trials of numerous methods, including a modification of a method by Rym *et al*. (2016). Spores were prepared by growing *B. cereus* strains BC10, BC17 and BC24 on brain heart infusion (BHI) agar (Oxoid, Hampshire, England) for 21 days at 35 °C. The agar plates were flooded with 6 mL sterile phosphate-buffered saline (PBS) (Oxoid). The PBS on the surface of the agar was scraped gently with a spatula taking caution not to damage the agar. The subsequent mixture of PBS (Oxoid) and spores (scraped from the surface of the agar) of each strain was transferred aseptically to sterile 2mL Eppendorf tubes. Suspensions in Eppendorf tubes were heated to 95 °C for 30 min to inactivate any residual vegetative cells. The spore suspensions were washed by centrifugation (Digicen 20; Ortoalresa, Madrid, Spain) at 155 RCF for 20 min and then washed three times at 9184 RCF for 3 min. Sample pellets were resuspended in 1 mL sterile PBS (Oxoid). The purity of spore suspensions was checked by spore staining as described by the Schaeffer–Fulton method with malachite green and light microscopy. Spores were visible as stained blue to green, and vegetative cells were stained pink to red.

Simulated CIP treatment

The simulated CIP treatment procedure was modified from a method described by Khoza (2015). Isolated spores were standardised to 1.0 McFarland standard in sterile PBS (Oxoid) and centrifuged (Digicen20; Ortoalresa) for 3 min at 9184 RCF. The supernatant was removed, and simulated CIP treatment was carried out on the remaining pellets in the Eppendorf tubes of each strain. Spore cells were washed in Eppendorf tubes with 1 mL 0.5% Liquid Super Klenz solution (pH 12.46) (Ecolab, Johannesburg, South Africa) (Liquid Super Klenz comprises a mixture of 10%–30% sodium hydroxide and 1%–5% sodium hypochlorite (Ecolab Material Safety Data Sheet (MSDS)) for 10 min at 70 °C in a heated water bath (Labotec, model number 132, Cape Town, South Africa), followed by centrifugation at 9184 RCF for 3 min. The spore cells were washed with sterile PBS (Oxoid) and centrifuged at 9184 RCF for 3 min. Spore cells were washed with 1 mL 1% CIP acid (pH 0.90) (Ecolab) (CIP acid comprises a mixture of 30%–60% nitric acid (Ecolab MSDS)) solution for 30 min at 70 °C in a heated water bath. Spore cells were centrifuged at 9184 RCF for 3 min. The spore cells were washed with sterile PBS (Oxoid) and centrifuged at 9184 RCF for 3 min. Spore cells were washed with 1 mL 1% Super-San solution (Ecolab) at 70 °C for 5 min in a heated water bath followed by centrifugation for 3 min at 9184 RCF. One mL sterile PBS (Oxoid) was added to the remaining pellets. In this study, unless stated otherwise, *B. cereus* spores that have undergone simulated CIP treatment are referred to as treated, and nonsimulated CIP-treated spores (control) are referred to as untreated.

Flow cytometry analysis of *Bacillus cereus* spores

Flow cytometry (FCM) was used to determine the effect of simulated CIP treatment on the structure of *B. cereus* spores. FCM measures the fluorescence and light scattering ability of individual spores in a fluid stream (Cronin and Wilkinson, 2010). Thus, it reflects the physical changes in spores following simulated CIP treatment. The FCM method was derived from the method by Cronin and Wilkinson (2007). Controls used in FCM analysis included damaged spores (autoclaved spores) and intact (untreated) spores. Damaged spore controls were prepared by autoclaving spore suspensions at 121 °C for 20 min directly before FCM staining. In preparation for FCM, spores (controls and simulated CIP-treated spores) were diluted to a cell density of 10^8 cells/mL (0.5 McFarland Standard) in Eppendorf tubes. Of the diluted spore suspensions (controls and simulated CIP-treated suspensions), 100 µL was pipetted into sterile FCM tubes and stained under minimal light conditions with 1 µL of 0.5 mM SYTO™ 9 Green Fluorescent Nucleic Acid Stain (Molecular Probes Inc., Eugene, OR, USA). Directly after staining, spore suspensions were mixed in the FCM tubes using a vortex and were mixed again directly before FCM analysis commenced. Stained cells were protected from light and incubated at ambient temperature in the dark for 10 min before FCM analysis.

Flow cytometry analyses were performed by a BD Accuri™ C6 Plus analyser (BD Biosciences, Johannesburg, South Africa). The instrument was equipped with two lasers, two light scatter detectors and generated four fluorescence signals, a forward scatter (FSC) and side scatter (SSC). The instrument's blue laser was used, and green fluorescence was captured using the FL1 detector. Samples were analysed using fluidic's low setting, and the flow rate was 24 µL/min, with 10 000 events being acquired per sample. Accuri C6 Plus software files were exported in FCS 3.1 format for data import into the flow cytometry analysis program FlowJo™ software (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA).

Transmission electron microscopy analysis

Transmission electron microscopy (TEM) was used to observe structural damage of untreated and treated *B. cereus* spores. The method was carried out as described by Tabit (2010) with some modification, that is the centrifugation (Digicen20; Ortoalresa) of spores took place at 9184 RCF for 5 min, and pellets were resuspended in a solution containing 1 mL 2.5% glutaraldehyde (GA)/ formaldehyde (FA) for 1 h.

Evaluation of biofilm formation by germinated *Bacillus cereus* spores

Biofilm formation was assessed by quantifying the biomass of germinated *B. cereus* spores by means of optical density measurements. The assay was derived from a method by Hussain and Oh (2017). A 96-well microtiter plate was sterilised under UV light for 2 h. Wells containing 200 µL BHI broth (Oxoid) were inoculated with 5 µL of treated and untreated spore suspension (10^8 cells/mL (0.5 McFarland standard)) and incubated at 35 °C for 24 h. The medium was removed carefully from the wells using a micropipette to discard any unattached cells. Wells were washed three times with 200 µL sterile PBS (Oxoid). Biofilms were stained with 200 µL of 0.1% crystal violet for 30 min at ambient temperature during which the microtiter plate was covered with film to prevent wells from drying out. Crystal violet that failed to bind to biofilms was discarded by micropipetting, and wells were washed three times with 200 µL of PBS (Oxoid). Ethanol was used to release the biofilm bound by crystal violet by pipetting 200 µL of 70% ethanol into each well and incubating at ambient temperature for 30 min. The absorbance was measured at a wavelength of 595 nm on a Multiskan FC microplate reader (Thermo Fisher Scientific, Germiston, South Africa).

Scanning Electron Microscopy preparation and analysis of *Bacillus cereus* spores attached to stainless steel coupons

Scanning Electron Microscopy (SEM) was used to observe the attachment of untreated and treated *B. cereus* (ungerminated spores, germinated spores and/or vegetative cells) to stainless steel. The method was derived from methods by Khoza (2015) and Teh *et al*. (2012). Sterile conical polypropylene centrifuge tubes (15 mL) containing 9 mL sterile reconstituted skim milk (RSM) (Oxoid, Johannesburg, South Africa) were inoculated with 1 mL treated and 1 mL untreated spores. Stainless steel coupons, grade 316 (10 × 1 cm), were immersed into the suspension containing spores and RSM (Oxoid) and incubated at 36 °C for 24 h. To remove unattached cells on the stainless steel, the coupons were rinsed with phosphate buffer (20 mL $NaPO_4$ and 20 mL dH_2O) and were left to stand in 10 mL phosphate buffer for 15 min. In preparation for SEM, the coupons were fixed with 2.5% GA fixative (1 mL GA, 1 mL FA, 3 mL ddH_2O, 5 mL buffer). The coupons were washed three times in PBS (Oxoid) for 10 min and covered with osmium tetroxide for 45 min followed by washing three times in PBS (Oxoid) for 10 min. Coupons were gradually dehydrated in ethanol from 30% to 100% (10 min each for 30%, 50%, 70%, 90% and 100% with dehydration in 100% ethanol being repeated three times). Following dehydration, coupons were covered in 100% hexamethyldisilazane (HMDS) (Sigma-Aldrich, Johannesburg, South Africa). An additional 1–2 drops of HMDS were added to the coupons and allowed to dry overnight. Coupons were mounted and coated with carbon at least three times. SEM images of the stainless steel coupons were obtained using the Zeiss Ultra PLUS FEG-scanning electron microscope (Zeiss Microscopy, Durban, South Africa).

Growth of *Bacillus cereus* spores in milk over 28 days following simulated CIP treatment

The effect of simulated CIP treatment on the ability of *B. cereus* spores to germinate and grow under refrigerated conditions was determined. Each strain (1 mL (10^7 cells/mL) untreated and 1 mL treated spores) was inoculated into 250 mL sterile UHT milk. The milk was aseptically sealed and stored at 5 °C. Plate counts were done by plating serial dilutions of the milk onto BHI agar (Oxoid) every 14 days from the date of inoculation (day 0) until day 28.

Statistical analyses

A 2-way analysis of variance (ANOVA) with multiple comparisons using Tukey's HSD test was used to determine the effect of simulated CIP treatment on biofilm formation. A 3-way ANOVA with multiple comparisons was used to determine the effect of simulated CIP treatment, strain variations and period of incubation (days 0, 14 and 28) on the log counts of germinated *B. cereus* spores. All statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, California, USA) at $P < 0.05$ significance level. All experiments were assayed in triplicates and repeated at least twice.

RESULTS

FCM analysis of simulated CIP-treated and untreated *Bacillus cereus* spores

SYTO™ 9 (Molecular Probes Inc., Thermo Fisher Scientific, Germiston, South Africa) has an emission maximum rate of 520 nm and fluoresces green when the dye is bound to double-stranded deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). The fluorescence intensity was determined along the *x*-axis (Figure 1), that is with cell damage directly proportional to the intensity of the green fluorescence. Along the *y*-axis of the FCM-derived contour plot (Figure 1) is the SSC (Side Scatter) parameter. The SSC detector measures the degree to which light scatters from particulates or granules inside the cell. Thus, any laser light of the same wavelength of the laser (488 nm) that scatters from components inside the cell is collected and logged as SSC. FCM plots for controls were established to identify regions on the FCM plot represented by the physical state of a spore. Three regions were identified on the FCM plots (Figure 1). Each region represented a different physical state of the spores. FCM analysis (Figure 1) showed that simulated CIP treatment damaged at least 98.7% of spores and at least 0.11% of spores remained intact. The remainder of the cells consisted of spores unstained by SYTO 9 stain.

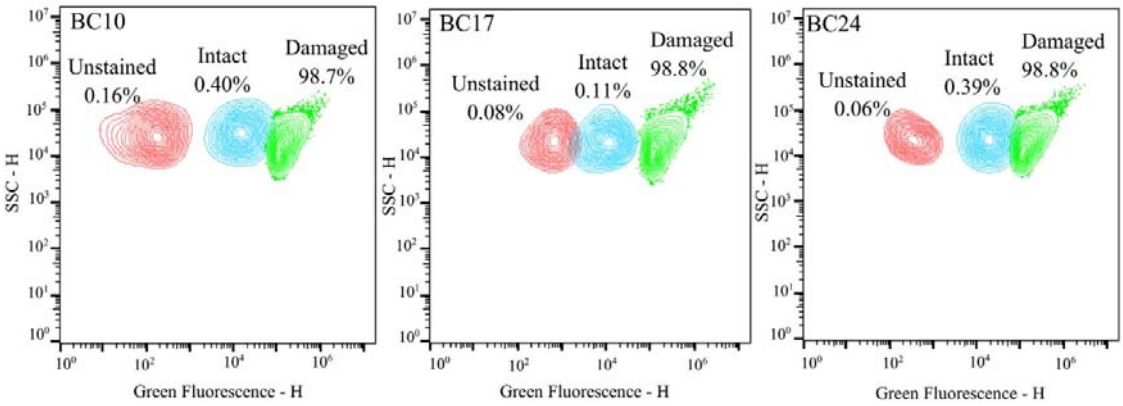

Figure 1. Flow cytometry (FCM)-derived contour plots of side scatter (SSC) versus green fluorescence intensities emitted by SYTO 9-stained intact and damaged *B. cereus* spores following simulated cleaning in place (CIP) treatment.

Effect of simulated CIP treatment on the structure of *Bacillus cereus* as observed by Transmission electron microscopy (TEM) analysis

Transmission electron microscopy (TEM) images of untreated spores showed intact spores compared to TEM images of *B. cereus* spores following simulated CIP treatment which showed damage to the spore structure, with evidence of leaked cell components (Figures 2-4).

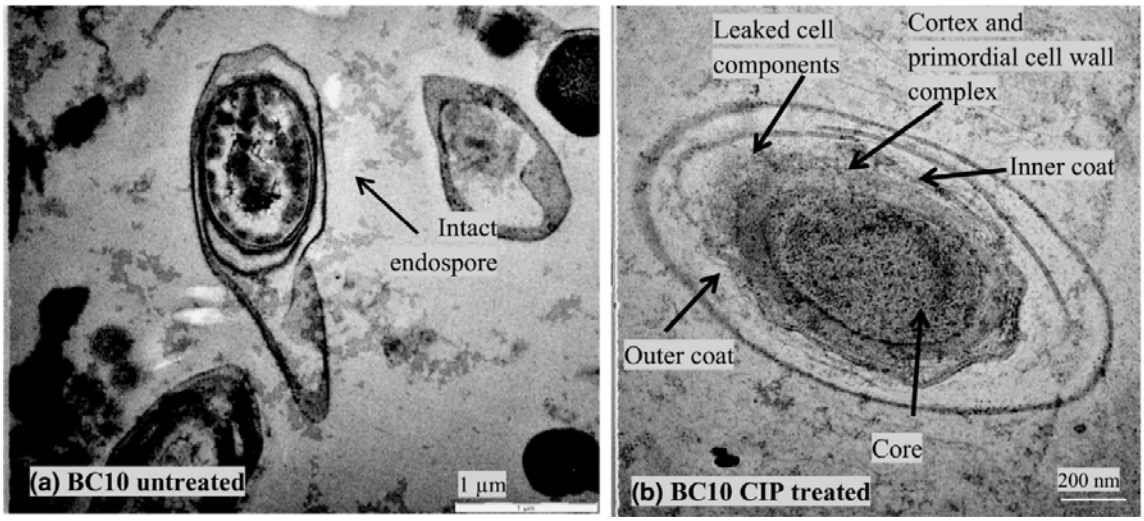

Figure 2. Transmission electron microscopy (TEM) images of *B. cereus* spores from strain BC10 isolated from filler nozzles of extended shelf life (ESL) milk processing lines showing (a) untreated intact spore and (b) damage to the spore layers following simulated cleaning in place (CIP) treatment.

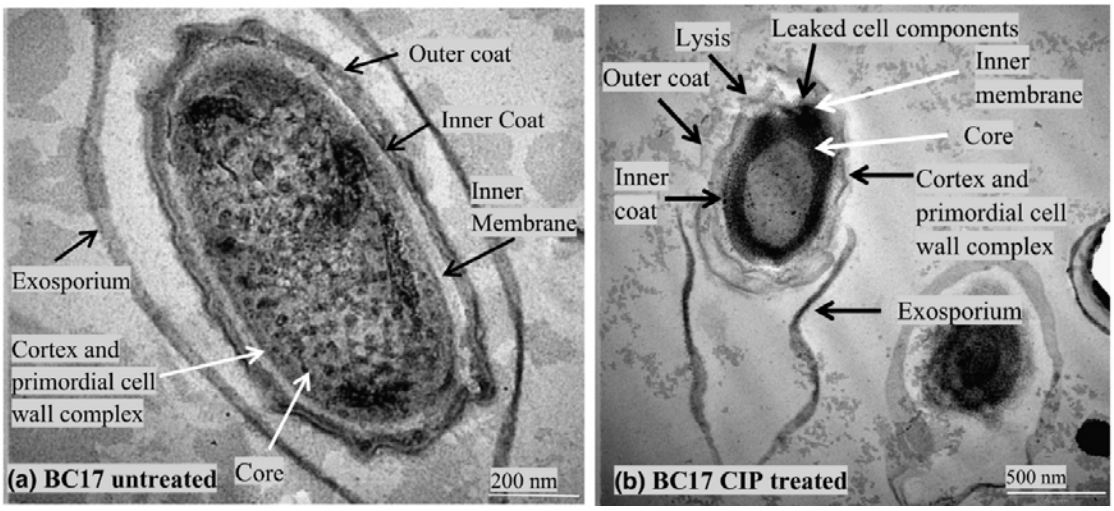

Figure 3.
Transmission electron microscopy (TEM) images of *B. cereus* spores from strain BC17 isolated from filler nozzles of extended shelf life (ESL) milk processing lines showing (a) untreated intact spore and (b) damage to the spore layers following simulated cleaning in place (CIP) treatment.

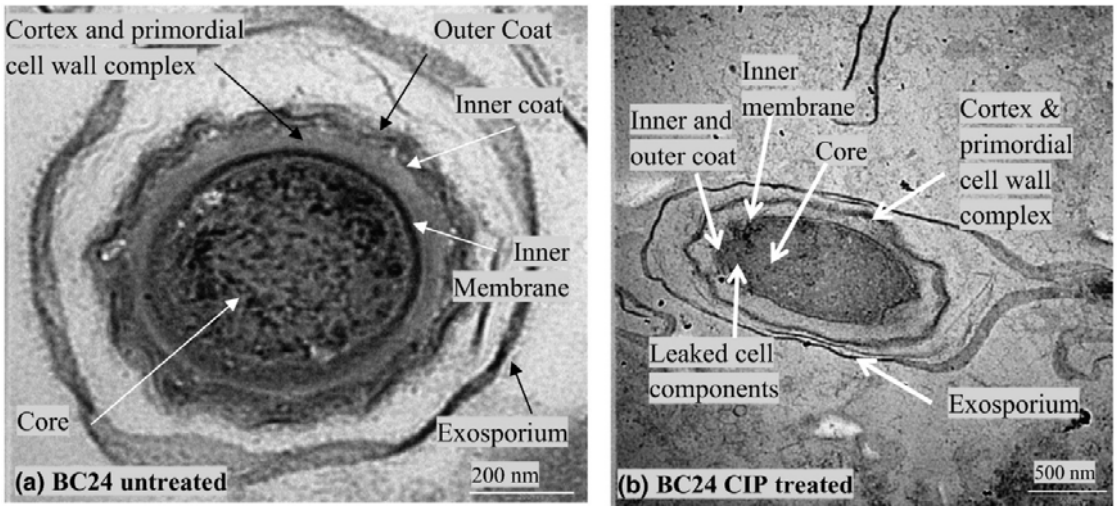

Figure 4. Transmission electron microscopy (TEM) images of *B. cereus* spores from strain BC24 isolated from raw milk showing (a) untreated intact spore and (b) damage to the spore structure following simulated cleaning in place (CIP) treatment.

Growth of *Bacillus cereus* spores in milk at 5 °C over a period of 28 days

No noticeable difference was observed in the log counts of untreated and treated spores for strains BC10 (Figure 5) and BC24 (Figure 7) after 28 days in milk at a storage temperature of 5 °C. Strain BC17, however, had a noticeable ($P \leq 0.05$) increase in the log counts of treated spores compared to untreated spores throughout the 28 days of shelf life at a storage temperature of 5 °C (Figure 6).

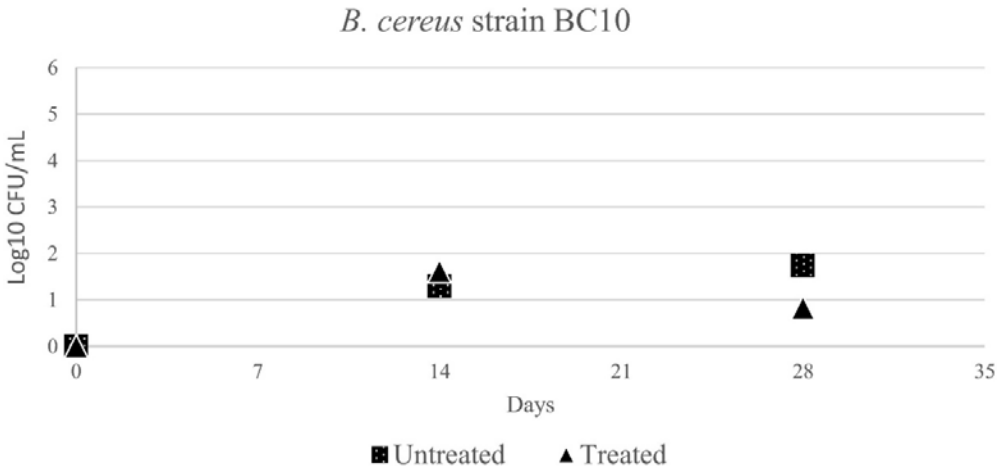

Figure 5. Growth over 28 days at 5 °C of untreated and treated (simulated cleaning in place (CIP) treated) spores from *B. cereus* strain BC10 isolated from filler nozzles of extended shelf life (ESL) milk processing lines.

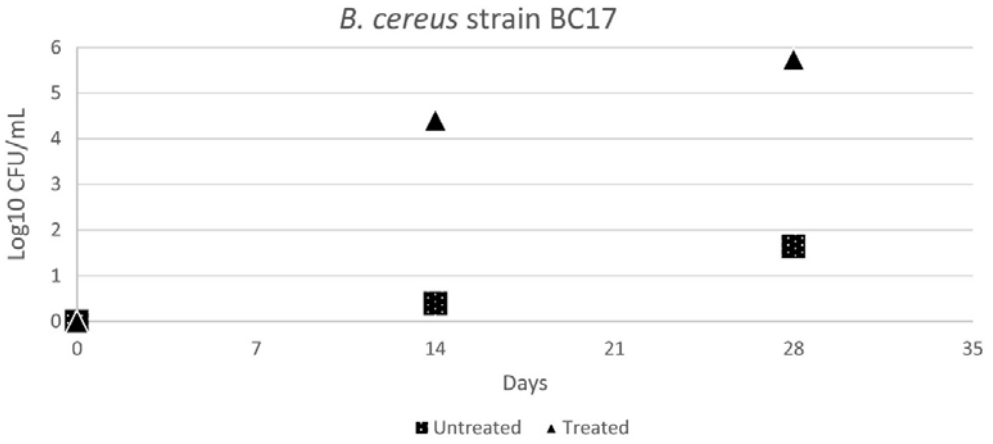

Figure 6. Growth over 28 days at 5 °C of untreated and treated (simulated cleaning in place (CIP) treated) spores from *B. cereus* strain BC17 isolated from filler nozzles of extended shelf life (ESL) milk processing lines.

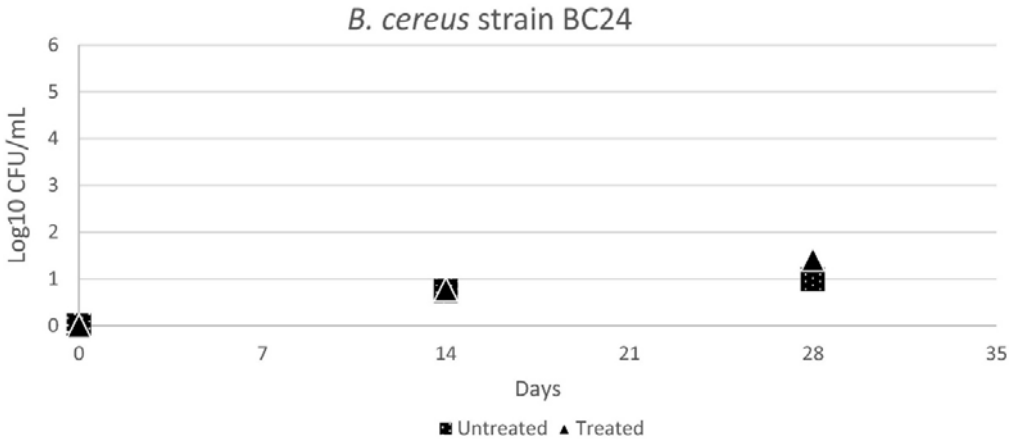

Figure 7. Growth over 28 days at 5 °C of untreated and treated (simulated cleaning in place (CIP) treated) spores from *B. cereus* strain BC24 isolated from raw milk.

There was significant ($P \leq 0.05$) growth of spores in milk stored at 5 °C between days 1 and 28, regardless of being treated or not, when only considering the effect of days on the growth over 28 days. However, there was no significant difference in the growth due to effects of the strain, simulated CIP treatment or any interaction of strain, treatment and/or day on the growth of spores. Besides strain and treatment factors having no significant effect on the growth of spores, multiple comparisons of strain responses at each day indicated that strain BC17 had unusual growth behaviour compared to strains BC 10 and BC 24 over 28 days at a storage temperature of 5 °C.

Biofilm formation evaluation: Biomass quantification of germinated *Bacillus cereus* spores following simulated CIP treatment

Strain BC10 showed significant difference between simulated CIP-treated and untreated spores with regard to biofilm formation ($P \leq 0.05$). However, simulated CIP treatment did not affect the biofilm formation of strains BC17 and BC24. After 24 h of growth, untreated *B. cereus* spores from strain BC10 germinated to form a biofilm with greater absorbance (1.99 Au) in comparison with strains BC17 and BC24 (0.51 and 0.28 Au, respectively). The biofilm formed by treated spores from the strain isolated from raw milk (BC24) showed lower absorbance (0.058 Au) compared to strains BC10 and BC17 isolated from filler nozzles (0.66 and 0.24 Au, respectively) (Figure 8).

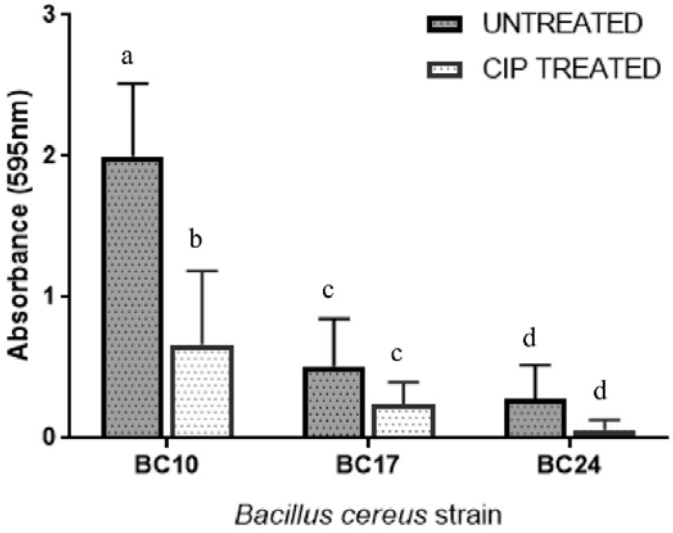

Figure 8. Biofilm growth of untreated and simulated cleaning in place (CIP) treated spores of *B. cereus* strains after 24 h. Strains were isolated from raw milk (strain BC24) and filler nozzles (strains BC10, BC17) of extended shelf life (ESL) milk processing lines. *Bars with different letters for each strain indicate significant difference.

Effect of simulated CIP treatment on the attachment of *Bacillus cereus* to stainless steel coupons by SEM analysis

Scanning electron microscopy micrographs of untreated spores showed attachment of intact *B. cereus* spores and vegetative cells to the stainless steel surface (Figure 9). Bacterial spores are typically 1.55 ± 0.16 µm long and 1.55 ± 0.16 µm wide (Zandomeni *et al*. 2005). These parameters were used to estimate the size of bacterial cells on the micrographs to distinguish

between spores and vegetative cells, since the possibility existed that some spores germinated into vegetative cells. SEM micrographs showed attachment of treated and untreated *B. cereus* spores and from all three strains to the stainless steel surface. A layer, which could be the conditioning film, covered some of the spores, and long appendages were visible (Figure 9). Damage to treated spores was visible, and outer layers of treated spores from strains BC17 and BC24 appeared to be more tortuous compared to strain BC10. There was evident damage to treated spores due to shrinking and twisting (Figure 9). However, intact spores were also detected with SEM following simulated CIP treatment, and some spores were still able to attach to stainless steel and form a biofilm.

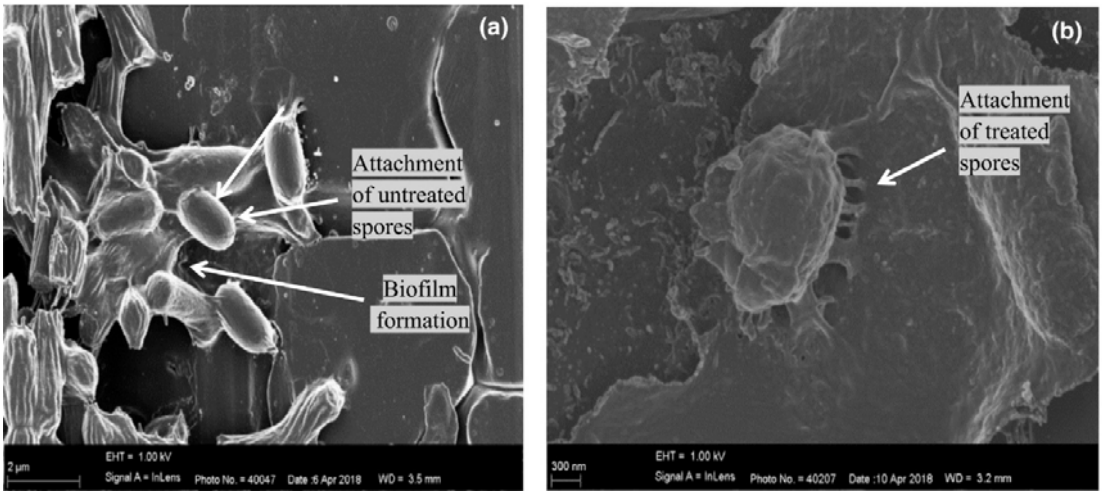

Figure 9. Scanning electron microscopy (SEM) images showing the attachment of untreated *B. cereus* spores (a) and the attachment of *B. cereus* spores after cleaning in place (CIP) treatment (b) to stainless steel. Strains were isolated during extended shelf-life milk (ESL) milk processing.

DISCUSSION

Flow cytometry and TEM analyses demonstrated that simulated CIP treatment structurally damaged *B. cereus* spores which may inhibit spore germination. However, a fraction of the *B. cereus* spore population under investigation was capable of surviving and was consequently able to attach to stainless steel, germinate and grow in milk under refrigerated conditions over a prolonged period.

Flow cytometry analyses with SYTO 9 revealed that simulated CIP treatment damaged at least 98% of *B. cereus* spores likely due to CIP chemicals disrupting spore permeability barriers. This may have caused the release of dipicolinic acid (DPA) in damaged spores which resulted in the increase in accessibility of DNA to SYTO 9 stain (Cronin and Wilkinson, 2008). When bound to DNA, SYTO 9 emits green fluorescence allowing fluorescence intensity to be determined on the FCM plot. Damaged spores emitted more intense green fluorescence compared to intact spores. Cronin and Wilkinson (2007) suggested that injured spores also had high SSC values on the FCM plots. In contrast, the region on the FCM plots representing intact spores showed lower SSC and weaker green fluorescence, indicating less SYTO 9 bound to intact spores.

Scanning electron microscopy analysis showed that simulated CIP treatment damaged *B. cereus* spores; however, spores remained capable of attaching to stainless steel. When

B. cereus spores adhere to filler nozzles, it may initiate biofilm formation which can lead to cross-contamination of ESL milk products (Mugadza *et al*. 2019). Brooks and Flint (2008) described that routine CIP processes in the dairy industry may not always be sufficient to remove all bacterial cells that have attached to a surface, a notion corroborated by Khoza (2015). In a review article by Deeth (2017), cleaning and sterilisation systems did not always suffice and may in fact activate *B. cereus* spores.

While cells within a biofilm are more resistant to chemical cleaning agents compared to planktonic cells (Dhillon 2012; Silva *et al*. 2018), the results from the biofilm assay in this study indicated that simulated CIP-treated *B. cereus* spores were still capable of forming biofilms, possibly due to certain strains being more resistant to CIP processes. The continuous exposure of bacteria to antimicrobials can result in decreased susceptibility, most likely resulting from phenotypic adaptations or mutations (Forbes *et al*. 2014). This study also indicated that inherently some strains could be better biofilm formers than others. The adaptation to stress caused by heat treatment and antimicrobials used in CIP processes may reveal modifications in physiological attributes such as the ability to form biofilms (Forbes *et al*. 2014).

Regardless of bactofugation and microfiltration significantly reducing spore formers in ESL milk, an increased amount of spore formers in the final product may indicate post-processing contamination most likely from filler nozzles (Eneroth *et al*. 2001; Mugadza and Buys, 2018). This could be problematic as we showed in the viability assay that *B. cereus* spores survived simulated CIP treatment and had the potential of growing under refrigerated conditions over a prolonged period, that is at least 28 days. A concern is that increased exposure to cold environments results in adaptation (Mugadza and Buys, 2017), which could lead to the emergence of psychrotrophic *B. cereus* strains, such as strain BC17. Additionally, increased consumer risk is possible if the storage temperature of packaged ESL milk is abused in households and especially if the ESL milk product is consumed beyond its expiration date with levels of *B. cereus* exceeding 10^5 cells/mL of milk.

In this study, we found that *B. cereus* strains isolated from filler nozzles during ESL milk processing proved to be more resistant towards simulated CIP treatment as opposed to the strain isolated from raw milk. CIP treatment could possibly initiate an adaptive stress response, which might induce the phenomenon of cross-protection. This may result in increased cell resistance which could challenge hurdle technology that is meant to improve preservation and guarantee microbial safety (Den Besten, 2010). Bacteria may become more resistant to subsequent hurdles after being exposed to a first hurdle such as CIP. The frequent exposure of spores to CIP treatment in a processing plant may explain why spores in this study that survived simulated CIP treatment seemingly acquired increased resistance compared to unexposed strains from raw milk. The correct combination of hurdles could be more effective in controlling bacteria compared to individual hurdles that may not inhibit bacterial growth adequately (Silva *et al*. 2018). The benefits of hurdle technology may, however, be void due to the ability of bacteria adapting to stressful environments (Den Besten, 2010). The fact that *B. cereus* spores may endure stresses such as CIP processing, as was shown in this study, is problematic because exposure to mild stresses over long and short periods was shown by Den Besten (2010) to prompt cross-protection against the more lethal stresses, and this could also have an effect on the virulence of pathogenic bacteria.

Our studies showed that strain BC17 could survive simulated CIP treatment, attach to surfaces and grow under refrigerated conditions over a long period of time. *B. cereus* spores

may contribute to the spoilage of ESL milk and could potentially be a food safety risk since the CIP process used in a dairy processing plant could allow certain strains of *B. cereus* to survive and ultimately thrive in ESL milk. Essential care must be taken to guarantee raw milk quality and good sanitation practices in the processing facility. Filler nozzles need to be cleaned more efficiently, and control measures for the effectiveness of the CIP process should be in place.

CONCLUSIONS

Simulated CIP treatment affects the structure of *B. cereus* spores isolated from raw milk and filler nozzles of ESL milk processing lines. However, simulated CIP treatment was less effective against some *B. cereus* strains from filler nozzles and did not affect the ability of *B. cereus* spores to attach to stainless steel or germinate and grow in milk. Consequently, *B. cereus* spores that survive CIP may be less susceptible to subsequent CIP treatment and attach to filler nozzles in dairy processing plants, thereby contaminating ESL milk.

CONFLICT OF INTEREST

The authors report no conflict of interests and are solely responsible for the content and writing of the article.

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