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

By

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DECLARATION

I, Chané Kruger, declare that the dissertation, which I hereby submit for the degree MSc Food Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Chané Kruger

30 October 2018

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DEDICATION

This work is dedicated to my recently late grandfather, Jop Nagel, and most importantly, to the glory of God.

ABSTRACT

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

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Bacillus cereus spores are resistant to heat and chemicals and can attach and form biofilms on stainless steel surfaces, indicating that the spores may contribute to the contamination of extended shelf life (ESL) milk. Filler nozzles account for the most contamination in ESL milk. Ultimately, spores are dispensed into the final ESL milk and could germinate and multiply at refrigeration temperatures. The objective of this study was to determine the effect of simulated cleaning in place (CIP) on the structure, viability, growth and attachment of *B. cereus* spores with the aim of improving the shelf life and safety of ESL milk. In this study, three *B. cereus* strains isolated from biofilms from the filler nozzles of an ESL milk processing plant and raw milk were subjected to simulated CIP processes. Flow cytometry (FCM), epifluorescence microscopy and Transmission Electron Microscopy (TEM) were used to analyse the effect of simulated CIP treatment on spore structures. Following simulated CIP treatment, a biofilm assay was conducted to analyse biofilm formation and the attachment of *B. cereus* spores to stainless steel strips was analysed by Scanning Electron Microscopy (SEM). In addition, the growth kinetics of spores from *B. cereus* as well as the viability of *B. cereus* spores in milk over 28 days at refrigerated temperatures (5 °C) were determined. FCM and TEM revealed that CIP structurally damaged 92% of spores from *B. cereus* strains. However, 0.1% of spores survived and remained intact. A biofilm assay and SEM indicated that *B. cereus* strains were capable of forming biofilms and attaching to stainless steel strips following simulated CIP treatment. Furthermore, over 28 days, spores

were capable of germination and growth under refrigerated conditions. It was concluded that CIP has an effect on the structure of spores. However, CIP is not entirely adequate as some spores are able to survive CIP. CIP has no effect on the attachment of *B. cereus* spores to stainless steel or on the ability of spores to germinate and grow in milk over 28 days if stored at 5 °C. Variation among strains is evident as strains isolated from filler nozzles are more resistant to CIP. *B. cereus* strains that survive CIP are less susceptible to subsequent CIP. We have shown that these spores may attach to filler nozzles in dairy processing plants and germinate when favourable conditions arise and contaminate ESL milk. If ESL milk is stored for an extended period of time, *B. cereus* spores will germinate. This may lead to a reduced shelf life and potentially be a safety risk in ESL milk with a prolonged shelf life.

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1. INTRODUCTION AND PROBLEM STATEMENT

Although the technology for ESL milk production is designed to prevent post-pasteurisation contamination with the goal of extending the shelf life (Kapadiya *et al.*, 2001), recent studies by Mugadza and Buys (2017) have shown the occurrence of psychrotrophic spore-formers in finished ESL milk products. *B. cereus* has been recognised to be the leading spoilage organism in pasteurised milk, and besides causing spoilage, it can also produce a number of toxins (Mugadza & Buys, 2017). The spores formed by *B. cereus* are a problem in the dairy industry as they are resistant to heat as well as cleaning and disinfection processes (Faille *et al.*, 2001). *B. cereus* spores can firmly adhere to a wide variety of surfaces due to the exosporium being very hydrophobic. These spores are, therefore, often isolated from surfaces in dairy processing equipment during cleaning and disinfection processes (Te Giffel *et al.*, 1997; Faille *et al.*, 2010). In ESL milk, *B. cereus* spores are of particular concern due to their heat resistance as well as their capability of germinating and growing at refrigerated temperatures (Schmidt *et al.*, 2012).

Khoza (2015) found that bacteria in ESL milk possibly originate from the machine filler nozzles and are then dispensed into the final ESL milk during the filling process an indication that filler nozzles are contributing to ESL milk contamination.

A reduction in the shelf life of ESL milk may lead to economic losses and the attachment of bacteria to contact surfaces results in serious hygienic problems (Carpentier and Cerf, 1993; Zottola and Sasahara, 1994; Kumar and Anand, 1998). Moreover, the formation of biofilms containing *B. cereus* may result in product safety issues due to the ability of *B. cereus* to produce toxins (Flint *et al.*, 2011).

A biofilm is an accumulation of bacterial cells and their associated extracellular polymeric substances (EPS) actively attached to, growing and multiplying on a surface (Flint *et al.*, 1997). Biofilms can form on most surfaces in dairy plants. The development biofilms occur when microorganisms present in the milk or from the environment come into contact with the surface. Biofilms often comprise of single species that are well adapted to survive the extrinsic (heat, cooling) and intrinsic (pH, salt) factors associated with milk processing. Biofilms also provide mutual protection to bacteria against antimicrobial agents and environmental stress (Flint *et al.*, 2011). It is known that bacterial biofilms have an increased

resistance to antimicrobial treatments compared to individual cells grown in suspension (Kumar and Anand, 1998).

Minimising biofilms has become a challenge for dairy manufacturers (Flint *et al.*, 2011). In South Africa, ESL milk processors are still struggling to extend the shelf life longer than 14 days. According to Khoza (2015), the ability of bacteria such as *B. cereus* to attach to stainless steel and to form biofilms may be the primary cause of contamination in ESL milk. Biofilms tend to become a problem as a result of processing plants that are increasing in complexity which provides more niches for microbial growth. As processing durations lengthen, more time is allowed for bacteria to grow and for biofilms to develop. Bacteria attached to biofilms on food processing surfaces are more difficult to kill compared to free-living cells. After formation of the biofilm cells, spores may be released or pieces of the film are shed off, possibly contaminating the product stream (Flint *et al.*, 1997). Both vegetative cells and spores may reattach in downstream parts of the plant and initiate biofilm formation, completing the recontamination cycle (Brooks and Flint, 2008).

Usually, an adequate cleaning and sanitation program is part of the process in the food industry to inactivate and remove microorganisms from food processing surfaces to prevent the accumulation of microbial cells and biofilms (Carpentier and Cerf, 1993; Gibson *et al.*, 1999; Khoza, 2015). However, the limitation of CIP procedures is the ability to eliminate *B. cereus* spores on equipment surfaces (Zottola and Sasahara, 1994). It is therefore essential to determine the effect of the CIP process on the growth and biofilm forming capability of *B. cereus* spores which have been subjected to CIP treatment.

2. LITERATURE REVIEW

2.1 EXTENDED SHELF LIFE (ESL) MILK

Pasteurised milk's reasonably short shelf life of seven to ten days (given the product is kept at or below 6 °C) (Khoza, 2015) has led to the need and development of ultra-high temperature processed (UHT) milk which has a shelf life of up to 9 months unopened at room temperature (Fitzgerald, 2012; Khoza, 2015). According to Clare *et al.* (2005) and Zabbia *et al.* (2012), consumers don't always favour UHT milk because of its apparent cooked taste and smell. There was, therefore, a need for the extension of pasteurised milk's shelf life without the perceived flavour change that goes along with UHT milk, and this led to the development of ESL milk (Henyon, 1999; Rysstad and Kolstad, 2006).

ESL technology involves a high heat treatment of milk, which still gives the normal sensory characteristics of pasteurised milk, as well as ultraclean packaging that gets operated in a controlled filling environment with container sterilisation. This process will result in a reduced microbial load of the product beyond that of regular pasteurised milk, and ultimately the shelf life will be extended under refrigeration conditions. Fundamentally, ESL milk is a combination of packaging and processing (Rysstad and Kolstad, 2006) and has a shelf life of 18 to 40 days (Rysstad and Kolstad, 2006). Therefore, ESL milk products should have a better shelf life than pasteurised milk as long as there is no contamination post-pasteurisation through bacterial biofilms formed in the fillers (Dhillon, 2012; Khoza, 2015). Additionally, aseptic ESL filling machines provide greater protection against recontamination from the filling environment due to their hygienic design. Thus, ESL milk products are bound to have a reduced microbial load during storage and distribution when compared to pasteurised milk (Khoza, 2015).

2.2 ESL MILK PROCESSING

ESL milk consists of various processing steps to reduce the microbial load as much as possible. These processes include a heat treatment, bactofugation and microfiltration.

Pasteurisation is a processing step done intended to eliminate or reduce harmful or spoilage microorganisms in milk products to such a level that the organisms cannot cause significant harm. Pasteurisation aids in extending the shelf life of milk products and only causes minor sensory, chemical and physical changes (Lewis and Deeth, 2009; Khoza, 2015).

Pasteurisation is carried out by treating the milk with the combination of a specific temperature for a specific period of time. This temperature-time combination is usually a heat treatment of 72 °C for 15 seconds in a continuous pasteurisation process (Munsch-Alatossava et al., 2013; Khoza, 2015). For a shelf life of 3-6 weeks (at > 7 °C) the heat treatment for ESL milk processing includes heating at 123–127 °C for 1–5 seconds (Lorenzen *et al.*, 2011). In addition, ESL milk must be heat treated as microfiltration and bactofugation contribute to a 3 and 1-2 Log reduction in the spore count respectively, compared to high-temperature processed milk that can achieve an 8 Log spore reduction (Rysstad and Kolstad, 2006). Regulations relating to milk and dairy products (Regulation 1555) by the Department of Health (DOH), South Africa (1997), states that pasteurised milk with a standard plate count beyond 50 000 CFU/ml is not to be sold.

Bactofugation is a process where centrifugal force is used to remove bacteria and bacterial spores from milk as bacterial cells and spores are heavier and have a higher density compared to milk. Consequently, bactofugation is employed to improve the shelf life of the ESL milk product (Stack and Sillen, 1998).

Microfiltration is the process where bacterial cells and bacterial spores are mechanically removed from milk using a membrane or filter. This process has some challenges, i.e. whole milk cannot be microfiltered due to the particle size and distribution of bacterial cells and spores being similar to that of milk fat globules (Rysstad and Kolstad, 2006). Therefore, the milk must first undergo centrifugation so that only skim milk will be allowed through the filter membrane. Furthermore, the pore size has to be adjusted due to cells and spores having a similar particle size and distribution to that of casein micelles. Thus, to minimise the milk composition being compromised, ceramic membranes with 0.8-1.4 mm sized pores in diameter are commercially used (Rysstad and Kolstad, 2006). The milk subsequently needs to undergo pasteurisation as this pore size allows for a fraction of bacteria to go through the filter membrane.

2.3 BACTERIA IN ESL MILK

Factors essential for recontamination in milk products were studied, and according to Rysstad and Kolstad (2006), contaminating organisms were mapped using random amplification of polymorphic DNA (RAPD DNA). The results showed that filling machines were the main source of recontamination. Filler nozzles are of particular concern as Ralyea, Wiedmann and

Boor (1998) also identified filler nozzles as the primary source of contamination of dairy processing lines (Rysstad and Kolstad, 2006). Eneroth (2001) found that the same bacterial type (shown by RAPD-typing) may be present in the filling machines over an extended period of time. Heat resistant Gram-positive spore formers may contribute toward contamination throughout the processing line, including cracks, joints and crevices where the spores of *B. cereus* may attach to the surfaces due to their hydrophobic properties (Rysstad and Kolstad, 2006).

According to Khoza (2015) and Brent Seale *et al*. (2011), there was a link between hydrophobicity and the ability of bacteria to adhere to surfaces. The increase in attachment was possibly due to an increase in hydrophobic residues on the spore surface as a result of the solubilisation of the spore coat proteins by hydrolysis (Brent Seale *et al.*, 2011). Additionally, isolates taken from filler nozzles were also capable of forming biofilms on stainless steel strips and this suggests that there could well be a community of multiple species of bacteria adhered to the ESL filling nozzles (Khoza, 2015). Khoza (2015) further stated that the occurrence of *Bacillus* spp. in filler nozzles of ESL filling machines is due to their resistance to heat treatment during the CIP process as well as their adhesion capabilities to stainless steel. Khoza (2015) found that species belonging to *Bacillus* spp. (41.3% of test isolates) were identified to dominate the bacterial community of ESL filler nozzles. *B. cereus* (41% of test isolates) was present throughout the sampling period of four weeks. Not only were *B. cereus* spores found in filling nozzles, but the spores were also found to be in packaged ESL milk products which might suggest that the spores gained access into the final product through the aseptic ESL filling machines (Khoza, 2015).

Schmidt *et al*. (2012) found that due to the low concentration of bacteria remaining in newly processed ESL milk, bacteria are distributed in a stochastic manner during the ESL milk filling process. This could explain why, during cold storage, various populations were established between milk packages coming from the same batch. The ability of microorganisms to grow to high numbers up to the expiration date of the ESL milk might be due to the extended storage time and shelf life of ESL milk compared to the storage time of pasteurised milk. Additionally, *B. cereus* may reach counts of 6 Log_{10} CFU/ ml in ESL milk following storage at 8 °C for 14 days as a result of the lack of other competitive organisms in the ESL milk (Schmidt *et al.*, 2012).

2.3.1 *BACILLUS CEREUS*

B. cereus is a rod-shaped, facultatively anaerobic, Gram-positive, bacterium that occurs ubiquitously and can form spores (De Vries *et al.*, 2004). Once the spores are formed, they will germinate upon favourable conditions and convert into vegetative cells (Elhariry, 2011; Aijuka, 2014)

B. cereus is an important food poisoning organism that can produce enterotoxins. According to Peng *et al.* (2002), it is one of the most significant organisms blighting the quality and preservation of milk that has been pasteurised. Additionally, it has been reported to be responsible for sweet curdling and bitterness defects associated with milk. Moreover, spores from *B. cereus* can attach to stainless steel surfaces and form biofilms which is problematic for the food and dairy industry (Peng *et al.*, 2002). Although *B. cereus* is not the foremost organism in ESL milk, the fact that it can grow at 7 °C makes it an organism worthy of consideration in terms of the shelf life and safety of ESL milk (Mugadza and Buys, 2017).

Mugadza and Buys (2017) found a range of *B. cereus* strains not following any specific contamination pattern succeeding ESL milk processing. This was determined due to the concurrent presence of mesophilic, psychrotrophic as well as the simultaneous presence of at least two virulence genes among some of the isolates, indicating that the contamination of ESL milk by *B. cereus* followed different contamination sources. The study by Mugadza and Buys (2017) evidenced that raw milk and filler nozzles are substantial sources of contamination of ESL milk.

B. cereus produces enterotoxins which could lead to illness. The two toxins that are produced by *B. cereus* are a thermostable emetic enterotoxin which is formed in food (Wijman *et al.*, 2007), and a thermosensitive diarrhoeal type enterotoxin which is produced in the small intestine (Chorin *et al.*, 1997; Wijman *et al.*, 2007). Food-related gastrointestinal illnesses by enterotoxins are caused when *B. cereus* levels rise above 10^6 CFU/g (Elhariry, 2011). Since contamination caused by *B. cereus* does not substantially affect the sensorial properties of a food product, consumers may not suspect that the food has been contaminated (Chorin *et al.*, 1997).

Intoxication with *B. cereus* toxins is self-limiting, and the progression is moderate (Stenfors Arnesen *et al.*, 2008; Schmidt *et al.*, 2012), however, there have been reports on the emetic toxin causing severe illness with fatal consequences as a result of fulminant liver failure as

well as rhabdomyolysis (Schmidt *et al.*, 2012). According to Granum and Lund (1997), there have been several case studies regarding intoxication with the diarrhoeal type enterotoxin following the ingestion of contaminated milk, and therefore the incidence of the diarrhoeal toxins in milk are reported to be higher (Schmidt *et al.*, 2012).

2.3.2 *BACILLUS CEREUS* SPORES

According to Ali *et al.* (2017), endospores are said to be the most resilient living structures known due to their high resistance to heat, ultraviolet radiation and chemicals. Spores are a problem in the food industry due to the fact that not all spores are eliminated during general food processing (Ali *et al.*, 2017)and cleaning processes (Faille *et al.*, 2010). Spores are produced in response to stress such as starvation or when conditions in the environment become unfavourable (Brantner *et al.*, 2014).

The process whereby spores are produced is called sporulation and is generally set in as a response to stress, i.e. nutrient starvation. Spores can remain dormant for years until a proper germinant, such as nutrient availability, is encountered and then the spore will enter the germination stage. Germination is followed by outgrowth which entails the germinating spore being converted into a growing bacterial cell. Ironically, it is the germination process that causes the spore to lose its extreme resistance and ultimately be relatively easy to deactivate (Setlow, 2014).

Food spoilage occurs when spores germinate to produce vegetative cells (which could well lead to sporulation). Foodborne illness may result when foods containing pathogenic spores are consumed, arising in spores germinating and subsequently growing in the gut leading to diarrhoea. Illness could also be as a consequence of food poisoning when food containing germinated spores, that have already produced high levels of toxins in the food, was consumed (Wells-Bennik *et al.*, 2016). According to Wells-Bennik *et al.* (2016), *B. cereus* accounts for approximately 1.3% of cases of bacterial foodborne diseases.

B. cereus can contaminate milk at a very low infectious dose of 10^3 bacterial cells/g. Since pasteurisation is not able to eliminate all spores, spores can survive and grow in cold environments during storage after processing. This is due to the psychrotrophic nature of some strains (Ali *et al.*, 2017; Mugadza and Buys, 2018). Furthermore, the spores can persist during routine cleaning procedures due to their ability to adhere to vessels and pipes used in

the dairy industry. The treatment of these particular spores is often dealt with greater difficulty due to their heat and chemical resistance (Ali *et al.*, 2017).

Sporulation is initiated when one of the five sensor kinases is auto-phosphorylated followed by the initiation of the phosphorelay-pathway. During this dormant stage, bacterial cells are allowed to live for an extended period of time. Once the conditions become favourable, the endospores will germinate into vegetative cells, and it is the vegetative cells that are the culprits capable of producing toxins to cause food poisoning or food spoilage (Ali *et al.*, 2017).

The spore consists of numerous layers (Figure 1) that are responsible for the protection of the spore against heat. Additionally, it contributes to the resistance toward chemical inactivation of the spores by preventing diffusion into the spore core (Setlow, 2006; Brantner *et al.*, 2014). The layers include the exosporium, the spore coats, the inner membrane, the cortex and the core. The proteinaceous spore coat is made up of the outermost layers that contain proteins protecting the spore and restricting the access of potentially toxic molecules to the spore core, enabling resistance to lysozymes, oxidising agents and chemicals (Mckenney *et al.*, 2013; Wells-Bennik *et al.*, 2016). The exosporium surrounds the spore coat and is in direct proximity to the environment. The exosporium is a loose fitting layer that is considerably larger compared to the underlying spore (Brantner *et al.*, 2014). Following germination the germ cell wall becomes the cell wall of the vegetative cell, and this lies outside the inner membrane. The inner membrane below the germ cell wall is a significant permeability barrier against several potentially damaging chemicals and surrounds the core (Abhyankar *et al.*, 2013).

As a result of the low permeability of the inner membrane, the spore can remain more stable in an aqueous solution, enabling it to be more resistant to chemical inactivation (Brantner *et al.*, 2014). The inner membrane also contains proteins that are necessary for germination and the cortex is essential for developing resistance towards wet heat (Wells-Bennik *et al.*, 2016). Resistance against high temperatures is aided by the dehydration of the spore core which is facilitated by proper cortex formation. The cortex is positioned underneath the coat (Abhyankar *et al.*, 2013), outside the inner membrane and is surrounded by the outer membrane. The cortex encompasses spore-specific peptidoglycan which is uniquely

characterised by the low peptide cross-linking and the muramic-δ-lactam moiety (Wells-Bennik *et al.*, 2016).

According to Wells-Bennik *et al.* (2016), spores may survive treatments such as disinfectants or heat but may sustain DNA and protein damage. Dipicolinic acid (DPA) in the spore core contributes to the relatively low water content in the spore core which in turn aids in the resistance of the spore (Coleman *et al.*, 2010).

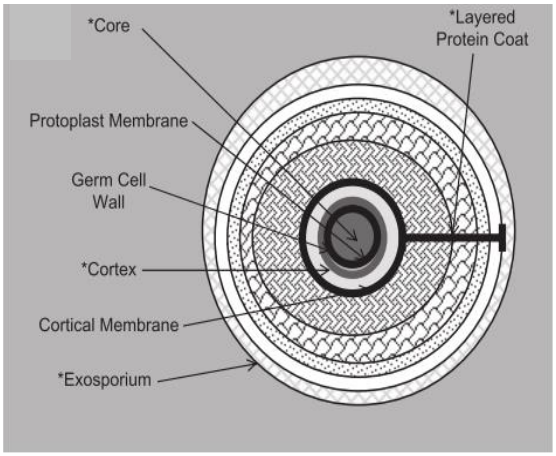

Figure 1. Typical *B. cereus* spore with various layers illustrated (Cronin & Wilkinson, 2007).

Small acid-soluble proteins (SASP) offer protection against DNA damage to genetic material in the spore. Due to the long dormancy of the spore, it is assumed that repair in the spore is unable to take place as a result of no metabolic activity. Ultimately, damage accrues until metabolic activity resumes and only then repair processes can become active (Wells-Bennik *et al.*, 2016). However, it is believed that wet heat targets the germination machinery of the spore, and thereby causes protein damage (Wells-Bennik *et al*., 2016). Furthermore, spores are armed with enzymes that carry out repair work. These enzymes are formed during endospore formation and enable rapid repair once germination commences (Wells-Bennik *et al.*, 2016).

Spores are damaged by wet heat causing the release of DPA which leads to spore hydration. The resultant spores could become damaged by protein denaturation (Coleman *et al.*, 2010). According to Coleman *et al*. (2010), spores that retain DPA can still germinate although the germination may be prolonged. Minimal heat treatment may lead to sublethally damaged spores (Postollec *et al.*, 2012; Wells-Bennik *et al.*, 2016). The ability of damaged spores to recuperate is to a large extent influenced by the food matrix and storage conditions, i.e. nutrients, water activity, pH, presence of antimicrobials, temperature, and the availability of oxygen. Furthermore, the survival, germination and ultimate outgrowth of spores could also be influenced by stresses that spores experience during food processing and storage (Warda

et al., 2015; Wells-Bennik *et al.*, 2016). Wells-Bennik *et al.* (2016) as cited by Van Melis *et al.* (2014) said that the germination and outgrowth of spores that underwent heat stress was not as uniform compared to spores that experienced no heat stress.

According to Khoza (2015), it has been demonstrated that appendages on the *B. cereus* spore surface improve adhesion by overcoming electrostatic repulsion forces. The appendages on the spore surface, as well as the hydrophobicity of spores, may enhance the attachment of spores to stainless steel (Faille *et al.*, 2010; Khoza, 2015). Khoza (2015) also found that isolates obtained from the aseptic ESL filling machine nozzles were, in fact, capable of attaching and forming biofilms on stainless steel strips and that there could be a community of multiple species of bacteria attached to the ESL filler nozzles.

Sub lethal heat treatments used in high-temperature short time pasteurisation induces spores to lose their dormancy leading to germination from where germinated spores can grow as vegetative cells, and ultimately proliferate to numbers that essentially spoil the milk (Setlow, 2014; Buehler *et al.*, 2018). Previous studies suggested that, as a result of psychrotolerant spore formers, over 50% of milk produced in New York contain bacterial levels surpassing 20000 CFU/ml throughout the milk's shelf life when stored at 6 °C (Buehler *et al.*, 2018).

Buehler *et al.* (2018) developed a predictive model to improve predictions of the shelf life of milk and to assess approaches in controlling spore-forming psychrotolerant bacteria in milk processing. By lowering the amount and by controlling these bacteria, the quality and shelf life of milk will be improved. Psychrotolerant spore-forming bacteria can influence the shelf life of milk in various ways. These include the initial spore concentration in raw milk, the frequency of spore formers in raw milk, and the corresponding growth rates of the psychrotolerant spore formers. In a study by Buehler *et al.* (2018), maximum growth rate had the most significant effect on the predicted concentrations of psychrotolerant spore formers in milk.

Buehler *et al.* (2018) cited Vissers *et al.* (2007) stated that the modelled concentration of spores from *Bacillus* spp. in raw milk was based on management decisions at farm-level, and the results suggested that the initial sources of spores including soil and feed had the highest influence on the predictions of models. In a study by Buehler *et al.* (2018), the lag phase did not have a significant effect on model predictions. However, analyses by Buehler *et al.* (2018) indicated that storage temperature at 4 °C had a substantial impact on lowering the

mean concentration of psychrotolerant spore formers in milk where the average shelf life of ESL milk (spoilage at >20,000 CFU/mL) will be extended by nine days if the storage temperature is lowered from 6 to 4 °C. However, maintaining these temperatures from farm to fork is challenging as domestic refrigerator storage temperatures vary greatly. Thus, consumers need to be well informed about the implementation of proper refrigeration temperatures (Buehler *et al.*, 2018).

2.4 BIOFILMS

A biofilm constitutes an accumulation of microbial cells as well as extracellular polymeric substances (EPS), which are attached to and actively grow on a surface (Figure 2).

The film contains 90–97% water and the size of the film ranges from a couple of micrometres to several millimetres thick. Mixed species biofilms exist in which bacteria are accompanied by EPS, eukaryotes, enzymes, proteins, bacteriocins and nucleic acids that may be present due to cell lysis (Brooks and Flint, 2008). Contamination from bacteria in biofilms is possible at any stage during milking by way of transportation, storage and processing, even though milk produced by a cow is supposedly sterile (Flint *et al.*, 1997).

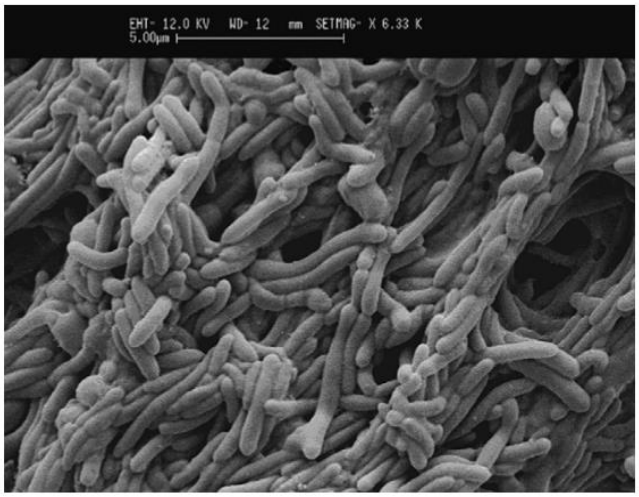

Figure 2. Scanning electron micrograph illustrating the formation of a *B. cereus* biofilm on stainless steel.
x 6330: bar = 5µm (Simoẽs *et al*., 2010).

2.4.1 BIOFILM FORMATION AND DEVELOPMENT

Biofilms develop on surfaces that come into contact with aqueous environments. The rate and degree to which the biofilms build up are administered by various processes, such as adsorption to surfaces and subsequent growth (Flint *et al.*, 1997).

There are various ways in which microorganisms may come across a surface, adhere to the surface, engage in cell-cell interactions (such as quorum sensing), and ultimately grow as an intricate biofilm structure. The formation of a biofilm entails a number of phases. Figure 3 shows the initiation of biofilm formation which firstly includes the surface being preconditioned by macromolecules in the medium (i.e. milk). Thereafter follows the transfer of planktonic cells to the surface from the liquid, the irreversible adhesion of bacterial cells to the surface, the production of molecules used for cell-cell communication, the conveyance and metabolism of substrates to and within the biofilm structure, and the transfer of products from the biofilm (Bryers and Ratner, 2004).

Generally, attachment of bacterial cells will occur more readily on rougher, hydrophobic surfaces, and surfaces coated by surface conditioning films. Characteristics of the bacterial cell surface, such as extra-cellular appendages and interactions during cell-cell signalling, as well as the production of EPS, are essential for the development and establishment of biofilms (Simões *et al.*, 2010).

2.4.2 SURFACE ATTACHMENT

Factors that affect the attachment of bacteria to stainless steel surfaces involve the viability of the bacteria, the growth phase of the bacteria, how long they are in contact with the surface, the temperature of the medium in which the bacteria are, surface roughness, flow rates and the hydrophobicity of the surface (Austin and Bergeron, 1995; Flint *et al.*, 1997).

Weak electrostatic and van der Waals' forces aid in the initial attachment of bacterial cells which occurs rapidly. After that, gene expression gets altered and the biofilm commences growth succeeding the physical attachment of cells to the surface using complex polysaccharides (Zottola and Sasahara, 1994; Brooks and Flint, 2008).

Before microbial adhesion can occur, a macromolecular conditioning film must be formed on a surface, and the adsorption depends on the state of this conditioning film (Brooks and Flint, 2008).

Conversely, it has been demonstrated that *Bacillus* spp. attach to stainless steel without a conditioning film (Brooks and Flint, 2008).

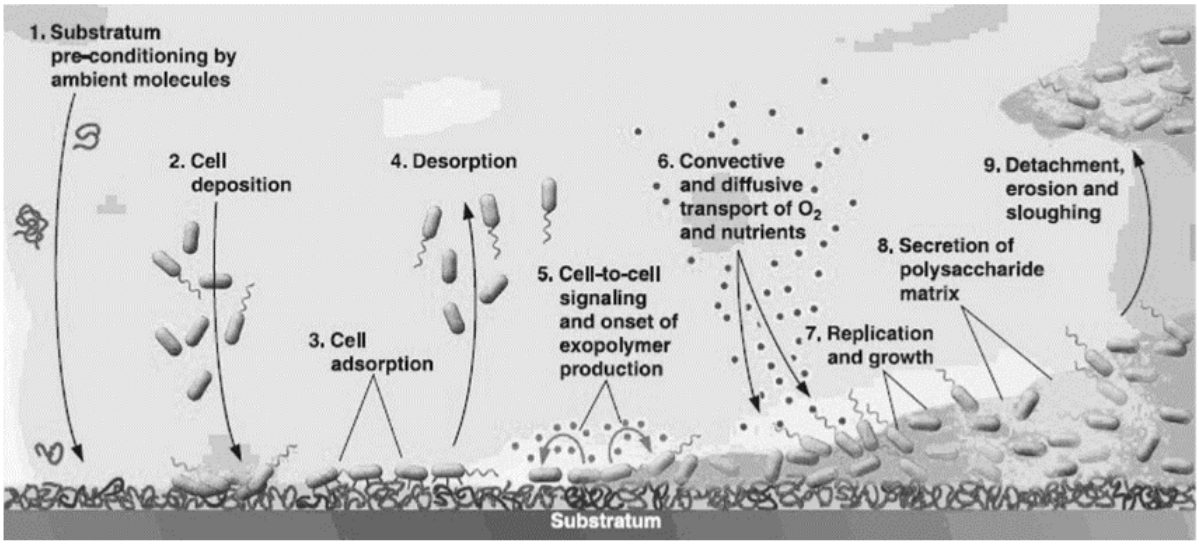

Figure 3. Processes involved in biofilm formation (Bryers & Ratner, 2004).

The microbial attachment may be influenced by cell surface hydrophobicity as well as the presence of extracellular filamentous appendages on cell surfaces. With surfaces being increasingly non-polar, hydrophobic interactions tend to intensify. Therefore, the hydrophobicity of the cell surface is of importance in adhesion (Peng *et al.*, 2001; Marchand *et al.*, 2012; Khoza, 2015). To some extent, the interaction of hydrophobic domains on cell surfaces affects the capability of bacteria to attach to surfaces and to one another (Drenkard and Ausubel, 2002). Extracellular filamentous appendages are also produced by many cells, and these appendages may play a part in the attachment procedures.

2.4.3 EXTRACELLULAR POLYMERIC SUBSTANCES (EPS)

EPS bind cells together (cohesion), and bind cells to surfaces (adhesion). Generally, bacterial EPS consists of polysaccharides, nucleic acids, proteins, lipids and phospholipids (Sutherland, 2001). A gel phase is formed by biofilms where microorganisms exist (Sutherland, 2001; Tsuneda *et al.*, 2003; Simões *et al.*, 2010).

EPS protect microorganisms in biofilms against hostile conditions, most likely due to EPS acting as a barrier by delaying or preventing sterilising and cleaning chemicals from reaching microorganisms in the biofilm (Mah and O'Toole, 2001; Simões *et al.*, 2010). In the biofilm matrix molecules that are necessary for vital cell-cell signalling could rise to levels high enough to be effective (Sutherland, 2001; Simões *et al.*, 2010).

2.4.4 CELL–CELL SIGNALLING

Cell-cell communication plays an important role in the adhesion and detachment of cells in biofilms (Daniels *et al.*, 2004). Bacteria naturally form colonies and demonstrate intricate systems of intercellular connections and communications to facilitate their adaptation to changing and challenging environments (Fuqua and Greenberg, 2002). The success of this adaptation depends on the ability of bacteria to sense and ultimately respond to the environment and accordingly adjust gene expression (Daniels *et al.*, 2004). Quorum sensing is built on autoinduction and is a tool for the regulation of microorganisms (Parsek and Greenberg, 2005). It is an environmental sensing system that permits bacteria to observe and react to their population densities. The bacteria produce a diffusible organic signal (autoinducer molecule) during growth which will accumulate in the environment (Fuqua and Greenberg, 2002). Higher cell densities will effect in high signalling concentrations which will encourage the expression of certain genes, ultimately, physiological changes will occur in adjacent cells (Parsek and Greenberg, 2005).

2.5 PROBLEMS CAUSED BY BIOFILMS

A significant source of contamination in dairy manufacturing plants originates from the presence of biofilms. Among other places, biofilms are established in pipelines in processing equipment and on product contact surfaces. Biofilms become problematic as a result of processing plants that are developing in complexity which provides an increased amount of niches for the growth of bacteria. More time is permitted for bacterial cell growth with lengthening manufacturing hours (Flint *et al.*, 2011).

Bacillus spp. are more tolerant of heat and can endure heat treatment, or consequently, heat-resistant spores may be formed. *B. cereus* spores originating from raw milk could stay behind in heat exchange equipment, eventually germinate and grow, which may lead to the contamination of the product (Te Giffel *et al.*, 1997). Brooks and Flint (2008) showed that cleaning and disinfection did not remove all spores.

Bacteria attaching to food contact surfaces may lead to hygienic issues and economic losses as a result of food spoilage (Carpentier and Cerf, 1993). The restraint of cleaning in place (CIP) processes is the attachment and accumulation of bacteria on the surfaces of equipment leading to biofilm formation (Brooks and Flint, 2008). Inferior quality products, a shorter production run-time, an increase in time needed to clean and control fouling, lower heat

transfer coefficients and product losses are some of the main issues caused by biofilm formation. Also, biofilm formation may necessitate higher pumping energy to uphold the required flow rates (Flint *et al.*, 2011).

During a particular stage following biofilm formation, cells and spores may be released. In addition, pieces of the biofilm are scraped off which results in the contamination further down the production line (Flint *et al.*, 1997). This could be due to bacteria and spores initiating biofilm formation as they have reattached to surfaces in downstream parts of the plant (Brooks and Flint, 2008).

2.5.1 RESISTANCE OF BACTERIA IN BIOFILMS TO ANTIMICROBIAL SUBSTANCES

Reducing biofilm growth is challenging for dairy manufacturers as biofilms provide mutual protection to bacteria from chemicals and environmental stresses. Compared to free-living cells, bacteria attached to surfaces are more challenging to eradicate. Contamination from biofilms could potentially reduce the quality and moreover, the safety of dairy products (Flint *et al.*, 2011).

It is eminent that bacteria in biofilms have higher resistance to antimicrobials compared to cells grown in suspension media (Kumar and Anand, 1998). This resistance is attributed to reduced diffusion through the biofilm, physiological changes due to reduced growth rates as well as enzymes that degrade antimicrobial compounds. Combinations of these mechanisms cause resistant populations and not necessarily a single mechanism. The 3-dimensional structure of the biofilm is involved in antimicrobial resistance which will be lost the moment this structure is damaged (Kumar and Anand, 1998). Deeply located cells could be protected by high amounts of exopolysaccharides produced by bacteria during biofilm formation and growth. Exopolysaccharides bind with antimicrobial compounds thereby reducing their effectiveness as they diffuse through the biofilm (Kumar and Anand, 1998).

Some studies indicated that disinfectants such as peracetic acid and formaldehyde did not influence biofilms (Carpentier and Cerf, 1993). The reduced efficacy of such agents against the biofilms may be due to the insufficient penetration of the biofilm by chemicals as well as the variety in environmental conditions on food contact surfaces (Huang *et al.*, 1995; Kumar and Anand, 1998). Antimicrobials have a greater effect against actively growing cells, thus, the most effective disinfectant for planktonic cells may not be as suitable against biofilm cells

(Holah *et al.*, 1990; Kumar and Anand, 1998). This suggests that the bacteria in the biofilm do not show the same pattern physiologically, and display oxygen and nutrient gradients through the biofilm. Additionally, bacteria in the biofilm receive a smaller amount of oxygen and fewer nutrients in comparison to bacteria at the surface of the biofilm (Kumar and Anand, 1998). Thick biofilms are formed in serious biofouling cases, and these biofilms may also include various dead or metabolically dormant cells such as bacterial spores. In this case, cells might be in a different physiological state and have an altered growth rate which results in amplified resistance to antimicrobials (Kumar and Anand, 1998).

Nutrient deficiency is evident in biofilms of mixed species due to microbes having to compete for nutrients. This also contributes to the higher resistance of bacteria in biofilms to antimicrobials (Kumar and Anand, 1998). Older biofilms (>24 h old) with foodborne bacteria tend to have an even greater resistance against disinfectants compared to younger biofilms (Anwar *et al.*, 1990; Kumar and Anand, 1998). Furthermore, bacteria produce antibiotic-degrading enzymes which contribute to the resistance of bacteria toward antimicrobials. As antibiotics permeate the envelope of the cells, they get degraded and inactivated by enzymes. Enzymes become concentrated as they get entrapped in the biofilm matrix. According to Kumar and Anand (1998), changes of molecular targets of antibiotics contribute to the increased resistance by biofilms.

An EPS matrix and cells in the outside layers of the biofilm surround bacterial cells within a biofilm, thereby protecting them from harsh environments. This results in bacteria within biofilms being more resistant to cleaning agents and other antimicrobials (Peng *et al.*, 2002).

Brent Seale *et al.* (2011) found that caustic wash caused a 2 Log reduction in spore viability, however, spores that survived were able to attach to stainless steel in even higher numbers. This may be as a result of increased hydrophobic residues on the spore surface because of the spore coat being solubilised (Brent Seale *et al.*, 2011).

2.5.2 *BACILLUS CEREUS* SPORES IN BIOFILMS

The resistance of endospores against heat and disinfectants as well as their hydrophobic properties enable them to attach to processing equipment easily, and subsequently survive cleaning processes (Andersson *et al.*, 1995). Typically, pasteurisation does not effectively inactivate endospores due to their heat resistance. This limits the possibilities of extending the

shelf life of pasteurised products or manufacturing minimally processed food (Lücking *et al.*, 2013).

Important to consider is the structure of EPS that acts as a diffusional barrier to antimicrobial penetration. The composition and structure of EPS are said to influence both diffusional resistance and an oxidising chemical demand. The resistance of spores is likely due to several interrelated factors, including diffusion barriers, differential metabolic activity, and the cell-wall ultrastructure (Mittelman, 1998).

Concerns are apparent over emerging heat-resistant spores that can withstand ultrahigh temperature processing during the production of products that are commercially sterile. Further concerns have been whether contaminated ingredients in combination with harsh food processing conditions increase the adaptation of highly resistant spore producers (Postollec *et al.*, 2012). The occurrence of spore-forming bacteria in dairy products can deleteriously affect the quality and safety of the product. Some spore-formers produce toxins and may cause food poisoning. *B. cereus* - an aerobic spore-former - has the potential to cause emetic and diarrhoeal type food poisoning by the production of the heat-stable cereulide and heat-labile enterotoxins (Lücking *et al.*, 2013).

Furthermore, food contaminated with spore-forming bacteria could result in microbial growth and consequently, early spoilage of the products. Microbial enzymes are produced which include lipases, phospholipases and proteases, that can incite textural changes leading to structural defects and off-flavours (Huis In't Veld, 1996).

Food spoilage and failed food preservation can result in significant economic losses or reputational damage to food companies may be the consequence. By improving the control measures for the occurrence of spore-forming bacteria, food loss due to microbial spoilage might be reduced (Lücking *et al.*, 2013).

2.5.3 THE ADHESION OF *BACILLUS CEREUS* SPORES

One of the main reasons why it is so difficult to control *B. cereus* spores is because of their ability to adhere easily to different surfaces. The adhesion of *B. cereus* spores is mainly attributable to the moderately high hydrophobicity, the low surface charge of the spore and the morphology of the spore. Adhesion is promoted by long appendages on the *B. cereus* spores. Adhesion of spores to hydrophobic materials is particularly high as a result of the relatively high hydrophobicity of spores (Andersson *et al.*, 1995).

2.6 METHODS TO DETECT BIOFILMS

Two methods are generally used in a manufacturing plant to detect biofilms. The first method is by swabbing the surface and subsequently plating out cells that have been recovered onto agar plates. The second method is by taking samples and culturing clean water that has flushed through the plant. Manufacturers need rapid detection techniques for biofilms to enable the swift assessment of hygiene in manufacturing plants. Detecting bacteria in biofilms may include the detection of bacterial proteins, polysaccharides, or ATP present in water that has been flushed through the lines or on surfaces. These fast techniques will aid in monitoring how effective cleaning processes are (Flint *et al.*, 1997).

The intercycle period (the period between cleaning and when processing equipment is in use) affects bacterial levels because extensive bacterial growth can take place during this time of inactivity. Therefore, it is preferable to sanitise equipment directly before it is put into use or to keep sanitiser inside the equipment for the time duration of the intercycle period. A reverse flow cleaning system can reduce the downstream recontamination of bacterial cells that dislodge from surfaces as a result of the passage of cleaning products (Flint *et al.*, 1997).

It is probable that in a processing plant the growth of a biofilm will advance with each subsequent batch of the product going through the plant, more likely so if the CIP process is lacking. Therefore, a biofilm could be detected within a shorter period of time (Flint *et al.*, 1997).

2.7 BIOFILM PREVENTION AND CONTROL

When preventing biofilm formation, the type of surface and the type of cleaning regimes are controllable factors that can be modified. A possibility could be to physically or chemically change surfaces (e.g. electropolishing stainless steel surfaces) so that the attachment of microorganisms is reduced. Adhesion may be reduced and cleanability increased if modifications to the surface charge are brought about (Flint *et al.*, 1997).

2.8 CLEANING AND DISINFECTION

The operations for cleaning and disinfection play a vital role in the production of milk. How efficiently these operations are performed has a significant effect on the quality of the end product. Following an ineffective cleaning procedure the biofilm matrix residue on a surface is not appropriately penetrated by disinfectants, and thus not all the biofilm living cells are

eradicated. This emphasises the importance of the cleaning step to enhance the sanitation of equipment used during processing. There is a need to develop new control tactics due to the increased resistance of biofilms to conventional treatments (Simões *et al.*, 2010).

Cleaning chemicals commonly include surfactants or alkali products that decrease the surface tension, thereby suspending and dissolving food residues by emulsifying fats, and by denaturing proteins. These cleaning chemicals are often used in combination. The use of acid cleaners is required in dairy manufacturing plants for surfaces that are soiled with minerals, for instance, milk stone (Simões *et al.*, 2010).

Mechanical action is very useful in eliminating biofilms. For a cleaning procedure to be effective, it must disrupt and dissolve the EPS matrix in the biofilm to allow disinfectants access to living cells. An effective cleaning process won't necessarily kill bacteria, but it can remove 90% of bacteria on the surface. Bacteria can dislodge from the biofilm and reattach at other locations (Simões *et al.*, 2010).

Disinfectants are required to be safe and easy to use without leaving toxic residues that might affect consumer health and sensory attributes of the final product. Additionally, disinfectants should easily be rinsed from surfaces, and importantly, they should be effective (Simões *et al.*, 2010). Susceptible bacterial cells will be deactivated if a biofilm is confronted by sufficient concentrations of antimicrobials. However, some cells are resistant and could have physiological plasticity, or they could mutate and undergo genetic exchange to obtain these properties (Simões *et al.*, 2010).

2.9 CLEANING IN PLACE (CIP)

CIP is the cleaning of the inner surfaces of pipelines, filters, vessels, and processing equipment without dismantling the equipment. Cleaning solutions and chemicals involved in the CIP process include different kinds of detergents, sanitisers and disinfectants (Thomas and Sathian, 2014).

The efficiency of the CIP process is determined by factors such as chemicals used, the mechanical power involved, temperatures employed as well as the contact time of the treatment (Wirtanen and Salo, 2003; Thomas and Sathian, 2014). Any remaining organic contamination, milk and water plaque have to be removed. Therefore, the CIP sanitation program is set to assure the removal of organic and inorganic contamination, as well as the disinfection of 99% of surface microorganisms (Vlková *et al.*, 2008; Thomas and Sathian,

2014). Usually, CIP comprises either the spraying of surfaces or the circulation of the chemical cleaning solutions through the plant at a certain temperature accompanied by specific flow rates. CIP systems aid in shortening the required time for cleaning, while recovering cleaning solutions in the CIP system. Due to the CIP system being automated, it supposedly allows for safe and reproducible results as well as an economically optimal process (Thomas and Sathian, 2014).

2.9.1 CIP ACTIVE INGREDIENTS

The chemicals used in the CIP process may comprise of alkali or acid detergents. Sodium hydroxide (caustic soda), potassium hydroxide (caustic potash) and sodium carbonate (soda ash) are among the most commonly used alkali detergents during the CIP process. The most widely used acid detergents include hydrochloric acid, nitric acid, phosphoric acid and citric acid (Thomas and Sathian, 2014). Caustic soda is excellent at removing fatty oils and protein-rich soils by means of saponification. Milk stones present in pipelines are eradicated by hot caustic soda which breaks up protein into water-soluble units (Lewis and Deeth, 2009; Thomas and Sathian, 2014). Caustic soda, a strong alkali, is relatively cheap and concentrations of 0.15 – 1% at a temperature of 70-80 °C for 10 to 30 minutes is typically used (Lewis and Deeth, 2009). If surfaces are very soiled or fouled, concentrations of 4% caustic soda can be used (Thomas & Sathian 2014). Acid detergents in CIP are used to get rid of proteins and salts in the inner surfaces of equipment. The required flow rate is beyond 1.5 m per second to reach the mechanical force that is necessary to stop biofilms from forming (Wirtanen and Salo, 2003; Thomas and Sathian, 2014). An acid detergent wash ensues following the alkaline wash. This step with acid boosts the draining and drying of pipelines and also effects in bacteriostatic conditions. Furthermore, acid detergents aid in eliminating minerals such as milk stone that deposit against food contact surfaces. Nitric acid is the more commonly used acid detergent in CIP and is normally used at a concentration of 0.5-1.0% at a temperature of 55 °C for 5 to 20 minutes (Thomas and Sathian, 2014).

In the food processing facility, the last step of the CIP process involves a disinfectant. Disinfectants are oxidising agents which include hypochlorite, hydrogen peroxide, ozone and peracetic acid. Sanitizers containing per oxygen can eradicate spores, but this is only possible at high concentrations and temperatures, and under such conditions, these detergents can become corrosive and toxic (Wirtanen and Salo, 2003; Thomas and Sathian, 2014).

Challenges do arise during CIP processes. Le Gentil *et al.* (2010) studied the likely recontamination of food processing line surfaces during the CIP process and found significant surface contamination depending on the specific properties of the spores. Furthermore, *Bacillus* spores, which were dislodged from surfaces contaminated during the CIP process, were, in fact, able to recontaminate surfaces further down in the plant. It was observed that the most contaminated surfaces during the CIP process included areas situated next to bends as well as sophisticated equipment (Le Gentil *et al.*, 2010). To achieve the most effective CIP results, the production process, as well as the CIP components and circuits, need to be designed simultaneously, enabling equal attention to production and cleaning requirements (Flickinger *et al.*, 2010; Thomas and Sathian, 2014).

2.9.2 EFFECT OF CHEMICALS AND HEAT ON SPORES

In a study by Brent Seale *et al.* (2011), it was found that spores exposed to caustic wash (1% NaOH at 65 °C for 30 minutes) were reduced from 7 to 5 Log_{10} CFU/ml and that the hydrophobicity of these spores was six times higher than that of untreated spores. Additionally, caustic treated spores showed peeling and cracked spore coats. Caustic treated spores had an increased negative charge possibly due to the partial hydrolysis of the spore coat (Brent Seale *et al.*, 2011).

According to Tabit (2010), a more compact spore core is indicative of advanced dehydration which is vital in spore heat resistance. Furthermore, Tabit (2010) observed structural damage within the cortical membrane region when spores were heated at 130 °C for 4 minutes. After 8 minutes' heat treatment cortical materials were also lost which led to the core being less dense. Upon further heat treatment for 12 minutes the exosporium and cortex were extremely damaged and the core density reduced. Additionally, the spore surface layers collapsed, and most of the contents of the spore core were washed away (Tabit, 2010).

Mustafa *et al.* (2010) did a study on the effect of wet heat treatments on spores and found that heat treatment caused morphological changes in *B. cereus* endospores and, excluding the exosporium, endospores shrank significantly. However, there was some variation among strains with regards to changes. Heat treated endospores had fewer uniform cores due to more electron dense aggregates (ribosome-containing cytoplasm and DNA-SASP) that fragmented into tiny black spots which were visible in the core (Mustafa *et al.*, 2010). Treated spores showed thicker cortices that were enlarged by up to 75%. However, some strains' cortices decreased by 28%. Following heat treatment, the endospores appeared more tortuous than

untreated spores. Mustafa *et al.* (2010) further explained the exosporia of the treated spores were damaged and somewhat destructed or fragmented.

Additionally, the exosporium became quite loose following heat treatment which indicated the endospore volume decreased. Furthermore, the straight pili of endospores appeared bent around the spore, and the pili tended to clump into a net-like shape following heat treatment. Mustafa *et al.* (2010) reported that changes in the spore core suggest that the spore core was the site of damage in spores when exposed to wet heat due to heat damaging the membranes, which ultimately caused protein denaturation which led to the detriment of DNA strands (Russell, 2003).

In a study by Ali *et al.* (2017), green tea polyphenols weakened the spore coat which destroyed the structural integrity leading to germination inhibition. Furthermore, green tea polyphenol-treated spores clumped together and the surface of the spores were rough and irregularly shaped, and cell lysis was also observed (Ali *et al.*, 2017).

The spore core is surrounded by the inner membrane and protects the spore against chemicals (Setlow, 2003; Griffiths and Setlow, 2009; Wells-Bennik *et al.*, 2016). Spores may be able to survive chemical treatments, but damage to DNA and proteins could be the result (Wells-Bennik *et al.*, 2016). In a review by Wells-Bennik *et al.* (2016), wet heat supposedly damages proteins by aiming at the germination mechanisms of the spore.

3. HYPOTHESIS AND OBJECTIVES

3.1 HYPOTHESIS

B. cereus spores isolated from filler nozzles and raw milk during ESL milk processing will be damaged by simulated CIP processes, but a fraction of the spores will survive simulated CIP treatment, germinate, grow and consequently attach to stainless steel surfaces and form biofilms. The proteinaceous spore coat and inner membrane restrict the access of potentially toxic molecules to the spore core, enabling resistance to oxidising agents and chemicals (Abhyankar *et al.*, 2013). Spore resistance to high temperature is aided by the dehydration of the spore core which is facilitated by proper cortex formation (Abhyankar *et al.*, 2013). The occurrence of *B. cereus* spores in the filler nozzles of ESL filling machines is due to their resistance to heat treatment during the CIP process as well as their adhesion capabilities to stainless steel, which may result in biofilm development (Wijman *et al.*, 2007; Khoza, 2015). *B. cereus* spores are very hydrophobic which enhances their ability to attach to various surfaces (Rysstad and Kolstad, 2006; Brent Seale *et al.*, 2011; Khoza, 2015). Since pasteurisation is not able to eliminate all spores, spores can survive and grow in cold environments during storage after processing due to the psychrotrophic nature of some strains (Ali *et al.*, 2017; Mugadza and Buys, 2018). Moreover, the ability of microorganisms to grow to high numbers up to the expiration date of the ESL milk might be due to the extended storage time of ESL milk compared to the shelf life of pasteurised milk, as well as the lack of competing microorganisms due to the extensive processing of ESL milk (Schmidt *et al.*, 2012).

3.2 OBJECTIVES

1. To determine the effect of simulated CIP treatment on the structure of *B. cereus* spores isolated from raw milk and filler nozzles from ESL milk processing lines.

2. To determine the effect of simulated CIP treatment on the attachment, viability and growth of *B. cereus* spores isolated from raw milk and filler nozzles from ESL milk processing lines.

4. RESEARCH

4.1 INTRODUCTION

This study was divided into two phases that aimed at determining the effect of simulated CIP treatment on the structure, attachment and viability and growth of spores from *B. cereus* strains isolated from filler nozzles from ESL milk processing lines and raw milk.

Phase 1 (4.2) involved determining the effect of simulated CIP treatment on the structure of *B. cereus* spores. Phase 2 (4.3) involved determining the effect of simulated CIP treatment on the attachment and viability and growth of *B. cereus* spores.

4.2 EFFECT OF CIP ON THE STRUCTURE OF SPORES FROM *BACILLUS CEREUS* STRAINS ISOLATED FROM FILLER NOZZLES FROM ESL MILK PROCESSING LINES AND RAW MILK

4.2.1 ABSTRACT

ESL milk is a product in between UHT and pasteurised milk in terms of processing temperature and time, and it is generally anticipated that ESL milk products should have a longer shelf life compared to conventionally pasteurised milk. However, this may be difficult due to post-pasteurisation contamination. Contamination of ESL milk involves raw milk and filler nozzles. CIP is used in the dairy industry to clean processing lines and equipment and ought to be able to remove any organic contamination that may account for post-pasteurisation contamination. *B. cereus* spores are incredibly resilient and may potentially survive CIP treatment. Therefore, in this study flow cytometry, epifluorescence microscopy and transmission electron microscopy were used to analyse the effect of simulated CIP treatment on the structure of spores from *B. cereus* strains isolated from raw milk and filler nozzles during ESL milk processing. Flow cytometry indicated that simulated CIP treatment damaged 92% of the spore population studied. Epifluorescence microscopy and TEM revealed that spores were structurally affected and damaged by CIP chemicals. It was apparent that the strain isolated from raw milk during ESL processing was more susceptible to CIP treatment compared to the strains isolated from filler nozzles. It was concluded that CIP affects the structure of spores; however, it is not entirely effective in eliminating *B. cereus*. Spores that survive CIP will possibly germinate and contaminate the final ESL milk. This could lead to a reduced shelf life and moreover, be a safety risk for ESL milk consumers.

4.2.2 INTRODUCTION

ESL milk products are expected to have a longer shelf life than normal pasteurised milk unless there is post-pasteurisation contamination (Dhillon, 2012; Khoza, 2015). A study by Mugadza and Buys (2017) indicated that sources of contamination of ESL milk included raw milk and filler nozzles and Khoza (2015) identified *B. cereus* as the dominant bacteria attached to ESL filler nozzles and that these bacteria were resistant to heat treatment and cleaning agents that were used in the cleaning regime. Consequently, these bacteria were dispensed into the final ESL milk during the filling process (Khoza, 2015) and ultimately, they could multiply at refrigeration temperatures and during the distribution chain.

In the dairy industry processing lines undergo cleaning-in-place (CIP) procedures. CIP involves the cleaning of the inner surfaces of pipelines, vessels, filters and processing equipment without dismantling the equipment and consists of several steps which include a caustic wash, an acid wash and lastly a sanitising step (Thomas and Sathian, 2014).

CIP is used to assure the elimination of organic and inorganic contamination as well as the disinfection of 99% of surface microorganisms (Vlková *et al.*, 2008), and whilst CIP may be effective in killing vegetative cells, *B. cereus* spores are extremely resilient to many food processing conditions and cleaning regimes (Faille *et al.*, 2010), such as CIP.

B. cereus can contaminate milk at a very low infectious dose of 10^3 bacterial cells/g (Ali *et al.*, 2017). The fact that *B. cereus* spores may potentially survive CIP treatment compromises the food safety and shelf life of ESL milk products. This study was aimed at improving the shelf life and safety of ESL milk by studying the spore structure of *B. cereus* spores associated with ESL filler nozzles and raw milk following CIP treatment.

4.2.3 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) (Table 1). The three *B. cereus* strains used in this study will be referred to as strains BC10, BC17 and BC24. Strains BC10 and BC17 were isolated during ESL milk processing from biofilms in filler nozzles after the CIP process, and strain BC24 was isolated from raw milk during ESL milk processing.

Table 1. Characteristics of the *B. cereus* strains used in this study (BC10, BC17 and BC24) isolated during ESL milk processing (Khoza 2015; *Mugadza & Buys 2017).

***B. cereus* strain**	**Isolate origin**	**Cluster group allocation***	**Presence of 16s psychrotrophic signature**	**Pathogenicity genes identified**
BC10	ESL filler nozzles	Group 1	Negative	cer, nheA, hblA
BC17	ESL filler nozzles	Group 1	Negative	cer, nheA, hblA
BC24	Raw milk	Group 2	Negative	nheA, hblA

*Polymerase chain reaction (rep-PCR fingerprinting) using the (GTG)5 primer. The *B. cereus* strains were clustered into groups at 95% similarity level.

Bacillus cereus spore preparation

Spores were prepared by growing the *B. cereus* strains, BC10, BC17 and BC24 on brain heart infusion (BHI) agar (Oxoid, Hampshire, England) for 21 days at 35 °C. Agar plates were flooded with 6 ml sterile phosphate buffered saline (PBS) (Oxoid) and mixed gently with a spatula taking caution not to damage the agar. The mixture of each strain was transferred aseptically to sterile 2 ml Eppendorf tubes. Samples in Eppendorf tubes were heated to 95 °C for 30 minutes to inactivate any residual vegetative cells after which Eppendorf tubes were washed once by centrifugation at 1300 rpm for 20 minutes and then washed another three times at 10000 rpm for 3 minutes. Samples were resuspended in sterile PBS (Oxoid). The purity of spore suspensions was checked by spore staining with malachite green and light microscopy.

Spore staining

Spore staining was executed as described by the Schaeffer-Fulton method (Schaeffer and Fulton, 1933). A loopful of bacterial spores was heat fixed onto glass microscopy slides and covered with absorbent paper. Steam was applied to the slides for 10 minutes during which the absorbent papers were stained with malachite green enough times to ensure the absorbent paper did not dry out. The slides were removed from the steam and left to cool down. A counterstain safranin-O was applied to the slides for a minute whereafter the slides were rinsed and allowed to dry. The slides were viewed under oil immersion with a light microscope. Spores were visible as stained blue to green and vegetative cells were stained pink to red.

Preparation of spore suspension

Spores from the *B. cereus* strains were standardised in sterile 2 ml Eppendorf tubes to 1.0 McFarland standard in sterile PBS (Oxoid) and centrifuged for 3 minutes at 10000 rpm. The supernatant was removed whereafter simulated CIP treatment commenced.

Simulated CIP treatment

Following preparation of spore suspension cells were washed in the 2 ml Eppendorf tubes with 1 ml 0.5% Super Klenz liquid (pH 12.46) (Ecolab, South Africa) solution for 10 minutes at 70 °C in a heated water bath, followed by centrifugation at 10000 rpm for 3 minutes. The supernatant was removed and cells were washed in the 2 ml Eppendorf tubes with sterile PBS (Oxoid) and centrifuged at 10000 rpm for 3 minutes. The supernatant was removed and cells were washed with 1 ml 1% CIP-acid (pH 0.90) (Ecolab, South Africa) solution for 30 minutes at 70 °C in a heated water bath. Cells were centrifuged at 10000 rpm for 3 minutes. The supernatant was removed, and cells were once again washed in the 2 ml Eppendorf tubes with sterile PBS (Oxoid) and centrifuged at 10000 rpm for 3 minutes. Lastly, cells were washed with 1 ml 1% Super-San (Ecolab, South Africa) solution at 70 °C for 5 minutes in a heated water bath followed by centrifugation for 3 minutes at 10000 rpm. The supernatant was removed, and 1 ml sterile PBS (Oxoid) was added to the remaining pellet in the 2 ml Eppendorf tubes.

Flow cytometry (FCM)

Bacterial spores were stained preceding flow cytometry analysis. Two staining regimes were used. The first regime of staining involved SYTO™ 9 Green Fluorescent Nucleic Acid Stain (Molecular Probes Inc., Eugene, Oregon, USA), which has an emission maximum of 520 nm and fluoresces green when the dye is bound to double-stranded DNA or RNA. The second staining regime was carried out directly after the first and involved propidium iodide (PI) (Molecular Probes Inc., Eugene, Oregon, USA) which has an emission maximum of 617 nm and fluoresces red when the dye is bound to nucleic acid.

For both staining regimes, spores were taken from the same harvest of *B. cereus* strains BC10, BC17 and BC24, respectively and divided into controls and treated (simulated CIP treated) groups. Controls used in FCM analysis included damaged spores (autoclaved spores) and intact (untreated (non-simulated CIP treated)) spores. Damaged spore controls were prepared by autoclaving spore suspensions at 121 °C for 20 minutes directly before FCM staining. Intact spores were left untreated following *B. cereus* spore isolation. The treated spores involved spores being subjected to the simulated CIP process directly before FCM staining.

Staining of endospores in preparation for FCM

Two sets of spores (one set for SYTO 9 (Molecular Probes Inc.) staining and one set for PI (Molecular Probes Inc.) staining) from *B. cereus* strains BC10, BC17 and BC24 were prepared for FCM. These sets of spores included samples for controls and simulated CIP treated spores. Spores (controls and simulated CIP treated spores) were diluted to a cell density of 10^8 cells/ ml (0.5 McFarland Standard) in separate Eppendorf tubes. 100 µl of the diluted spore suspensions from each sample (controls and simulated CIP treated suspensions) was pipetted into a sterile flow cytometry tube. In each respective flow cytometry tube, 100 µl of diluted spore suspensions was stained under dark conditions with 1 µl of a final concentration of SYTO 9 (Molecular Probes Inc.) of 0.5 mM and 20 mM of PI (Molecular Probes Inc.) respectively. Samples were mixed in the flow cytometry tubes using a vortex directly after staining and again directly before FCM analysis. Stained cells were protected from direct light and incubated at room temperature in the dark for 10 min before FCM analysis.

FCM of *Bacillus cereus* spores

Flow cytometric analyses were derived from methods described by Cronin and Wilkinson (2007). A BD Accuri™ C6 Plus analyser (BD Biosciences, Johannesburg, South Africa) was

used to perform the FCM analyses. The instrument was equipped with two lasers, two light scatter detectors and generated four fluorescence signals as well as forward scatter (FSC), and side scatter (SSC). The instrument's blue laser was used, and green fluorescence (from SYTO 9 (Molecular Probes Inc.)) was captured using the FL1 detector, and red fluorescence (from PI (Molecular Probes Inc.)) was captured using the FL3 detector. Samples were analysed using fluidic's low setting, and the flow rate was 24 µl/min, with 10000 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.

Epifluorescent microscopy

Epifluorescent microscopy was carried out using a Zeiss Inverted Fluorescence microscope with high-NA bright field and DIC optics. 1.6 x 6.3 magnification was applied. In this analysis controls included untreated (non-simulated CIP treated) spores from *B. cereus* strains isolated from ESL filler nozzles (strains BC10 and BC17) and raw milk (strain BC24). 50 µl of SYTO 9 (Molecular Probes Inc.) stained endospore suspensions of each of the simulated CIP treated and untreated spore suspensions were transferred to clean microscopy slides for trapping and observation. A number of fields per slide were observed and recorded.

Transmission electron microscopy (TEM)

TEM was used to observe structural damage to *B. cereus* spores following CIP treatment. In this analysis, control included untreated (non-simulated CIP treated) spores from *B. cereus* strains isolated from ESL filler nozzles (strains BC10 and BC17) and raw milk (strain BC24). Untreated spores and spores following simulated CIP treatment were prepared in the following manner: Spores in 2 ml Eppendorf tubes were centrifuged (at 10000 rpm for 5 min) whereafter pellets were resuspended in a solution containing 1 ml 2.5% GA/FA for 1 h. Samples were centrifuged and washed three times with PBS (Oxoid) for 15 min. Samples were continuously vortexed between steps to loosen pellets. Samples were centrifuged, and osmium tetroxide was added to the pellets and left for 1 h. Samples were centrifuged and washed three times with PBS (Oxoid) for 15 min and once again followed by centrifugation. The pellets were dehydrated by washing once in 30, 50, 70, and 90% ethanol for 15 min and subsequently three times in 100% ethanol for 15 min, centrifuged and left overnight. After 24 h the ethanol was replaced with fresh 100% ethanol and resin (29.8% quetol, 44.6% nadic methyl anhydride, 16.6% dodenyl succinic anhydride, 2% araldite RD2, 1% 2-dimethylaminoethane), was added in a 50:50 mixture to the ethanol in the Eppendorf tubes.

Samples were left to spin slowly for 30 min. A 100% resin was added and left for 4 h. After 4 h the resin mixture was added to the moulds and sample numbers were allocated to each sample. Moulds were put in an oven at 60 °C for 48 h to polymerize. Samples were removed from the moulds and further cut with an ultramicrotome. Thin sections of resin-polymerised samples were placed on grids and stained with uranyl acetate for 5 min and lead citrate for 2 min and then allowed to dry. Micrographs were taken using the Philips EM301 transmission electron microscope (Eindhoven, Netherlands).

4.2.4 RESULTS

4.2.4.1 Flow Cytometry

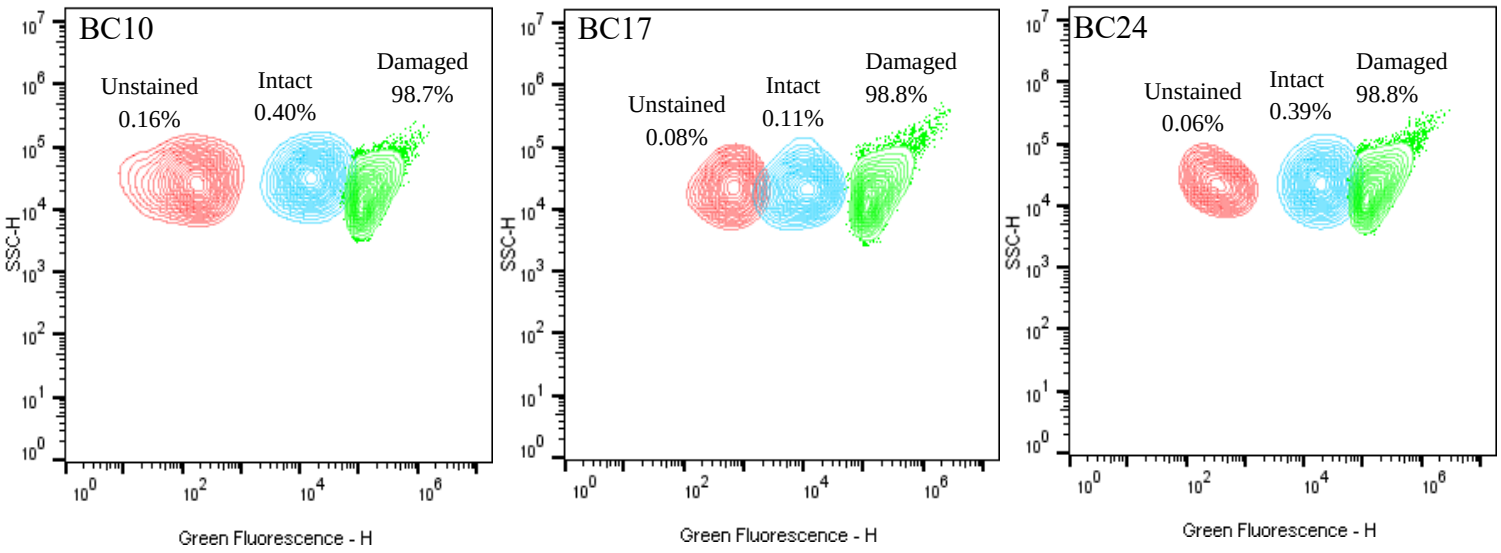

Figure 4. FCM-derived contour plots of the green fluorescence versus side scatter (SSC) intensities of simulated CIP treated SYTO 9-stained *B. cereus* spores isolated from filler nozzles (strains BC10, BC17) from ESL milk processing lines and raw milk (strain BC24) showing regions for unstained spores, intact spores and autoclaved spores.

The fluorescence intensity was determined along the X-axis (Figure 4), i.e. the more the cell was damaged, the higher the intensity of the green fluorescence. Along the Y-axis of the FCM-derived contour plot (Figure 4) 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 (488nm) that scatters from components inside the cell is collected and logged as SSC.

Control FCM plots were used to identify what each region represented regarding the physical state of the *B. cereus* spore. For all three *B. cereus* strains, three regions were visible on the FCM plots (Figure 4). Each region represented a different physical state of the *B. cereus* spore. FCM analysis (Figure 4) showed that simulated CIP treatment damaged at least 98.7% of spores. Furthermore, following simulated CIP treatment at least 0.11% of spores remained intact.

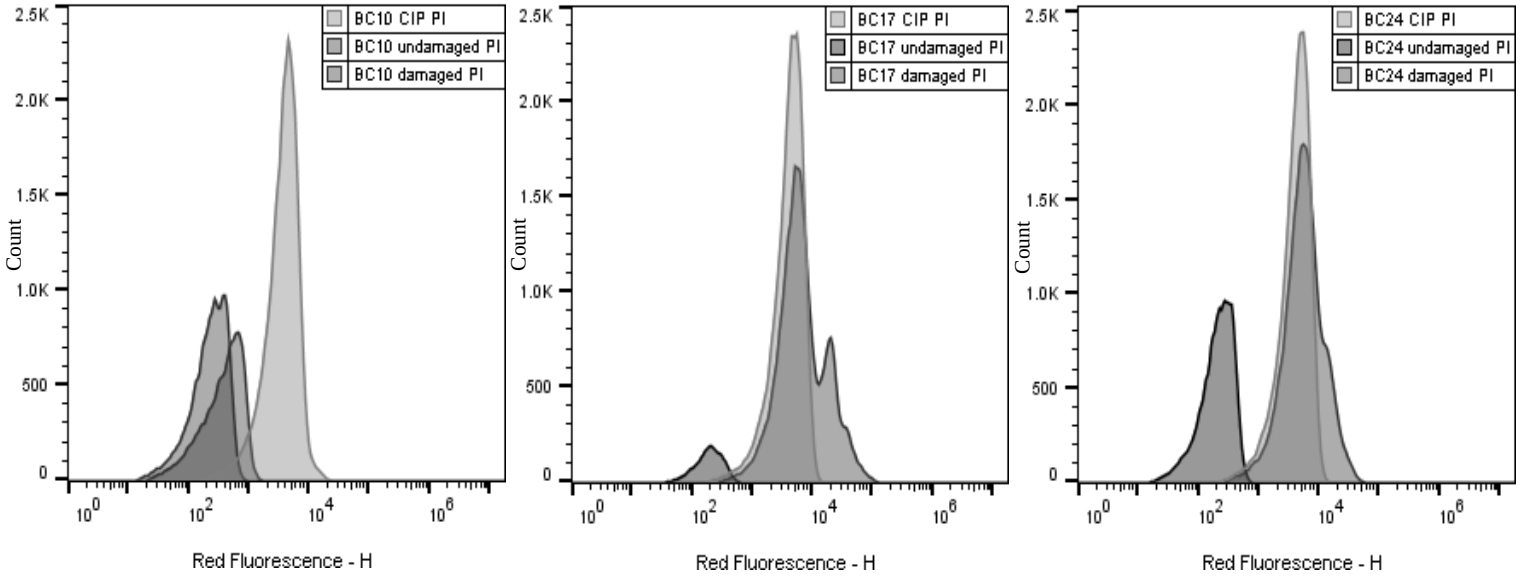

Figure 5. FCM-derived contour plots of the green fluorescence versus side scatter (SSC) intensities of simulated CIP treated SYTO 9-stained *B. cereus* spores isolated from filler nozzles (strains BC10, BC17) from ESL milk processing lines and raw milk (strain BC24) showing regions for unstained spores, intact spores and autoclaved (damaged) spores.

91% of spores from strain BC10 and 92% of spores from strains BC17 and BC24 were stained with PI following simulated CIP treatment. Strain BC24 had the most damaged spores as well as undamaged spores of all the strains and strain BC17 had the least, according to the FCM analysis (Figure 5). The peaks on the PI-stained FCM plot for simulated CIP treated spores overlapped with the peaks for the damaged spore control confirming that simulated CIP treated spores had been damaged.

4.2.4.2 Epifluorescent microscopy

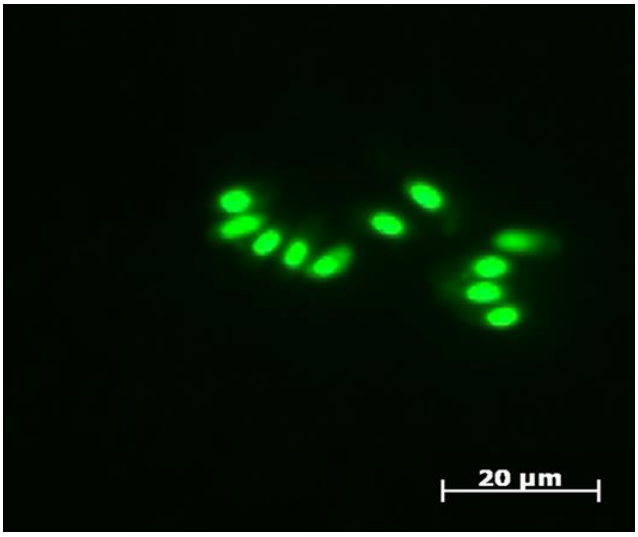

Figure 6. Epifluorescence micrograph of typical SYTO 9 stained (green fluorescent), untreated (non-CIP treated) spores from *B. cereus* strains isolated from filler nozzles from ESL milk processing lines and raw milk.

The effect of simulated CIP treatment was also examined using epifluorescence microscopy. The micrograph (Figure 6) showed typical SYTO 9 stained (green fluorescent) intact spores that did not undergo simulated CIP treatment.

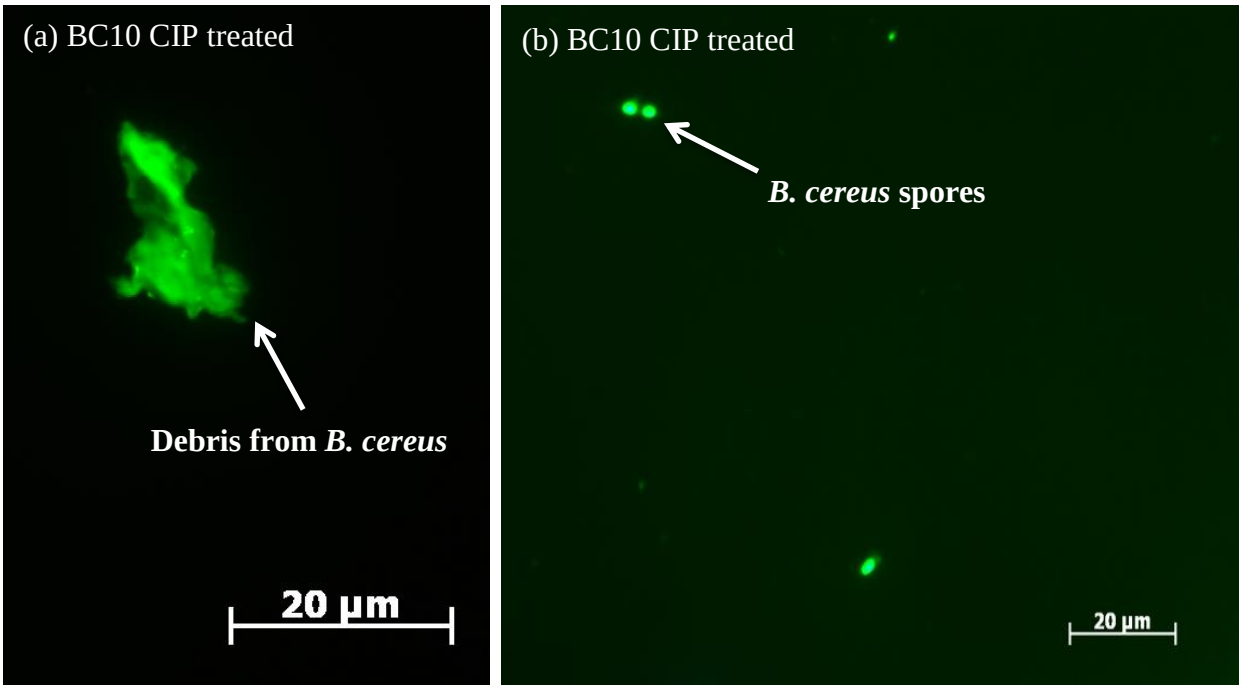

Figure 7. Epifluorescence micrographs of green fluorescent SYTO 9 stained spores from *B. cereus* strain BC10 isolated from filler nozzles from ESL milk processing lines showing (a) debris from damaged *B. cereus* and (b) undamaged *B. cereus* following simulated CIP treatment.

Epifluorescence microscopy indicated that following simulated CIP treatment strain BC10 had debris from damaged *B. cereus* (Figure 7a) and intact (Figure 8b) *B. cereus*. No distinct spore-like structures could be observed among the green fluorescing debris in Figure 7a).

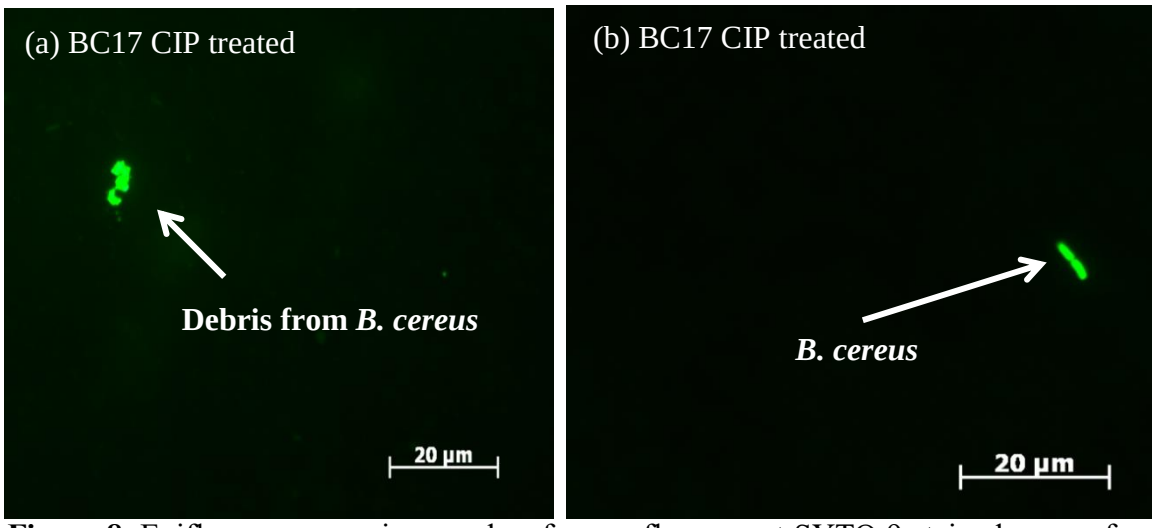

Figure 8. Epifluorescence micrographs of green fluorescent SYTO 9 stained spores from *B. cereus* strain BC17 isolated from filler nozzles from ESL milk processing lines showing debris from damaged *B. cereus* (a) and (b) undamaged *B. cereus* following simulated CIP treatment.

Epifluorescence microscopy indicated that strain BC17 had damaged (Figure 8a), as well as intact (Figure 8b) spores following simulated CIP treatment which corresponded with results obtained from strain BC10 (Figure 7a and b). Similarly, the damaged spores had a strong green fluorescence, and no distinct spore-like structures were observed among the green fluorescing debris-like structures (Figure 8a).

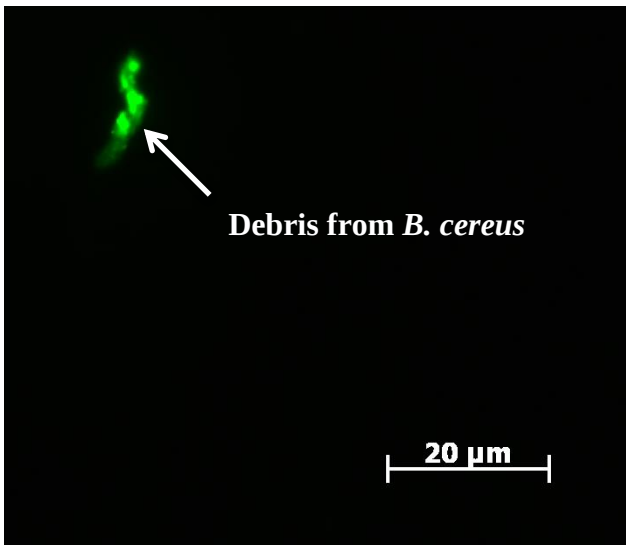

Figure 9. Epifluorescence micrograph of SYTO 9 stained (green fluorescent) *B. cereus* strain BC24 isolated from raw milk during ESL milk processing showing debris from damaged *B. cereus* following simulated CIP treatment.

Compared to strains BC10 and BC17 no visible intact spores could be identified in the epifluorescence micrographs from strain BC24 following simulated CIP treatment. However, prominent green fluorescent debris was evident (Figure 9).

4.2.4.3 TEM Analysis

(a) BC10 Untreated (b) BC17 Untreated (c) BC24 Untreated

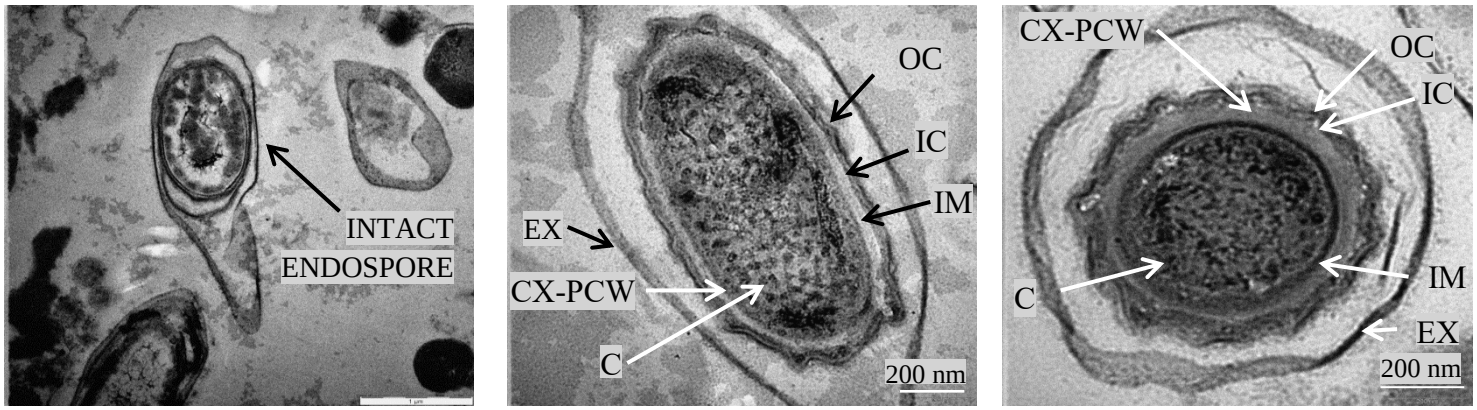

Figure 10. TEM images of untreated *B. cereus* spores isolated from filler nozzles (strains BC10, BC17) from ESL milk processing lines and raw milk (strain BC24).

*Intact Exosporium (**EX**);

Outer coat (**OC**);

Inner coat (**IC**);

Cortex and primordial cell wall complex (**CX-PCW**);

Inner membrane (**IM**); and

Core (**C**).

TEM micrographs (Figure 10a, b and c) for untreated *B. cereus* spores showed intact spores.

The various spore layers including the exosporia, outer and inner coats, cortex, inner membrane and core were clearly illustrated.

Intact exosporia (EX), outer coats (OC), inner coats (IC), cortex and primordial cell walls (CX-PCW), inner membranes (IM) and cores (C) were visible in all three *B. cereus* strains (Figure 10a, b and c).

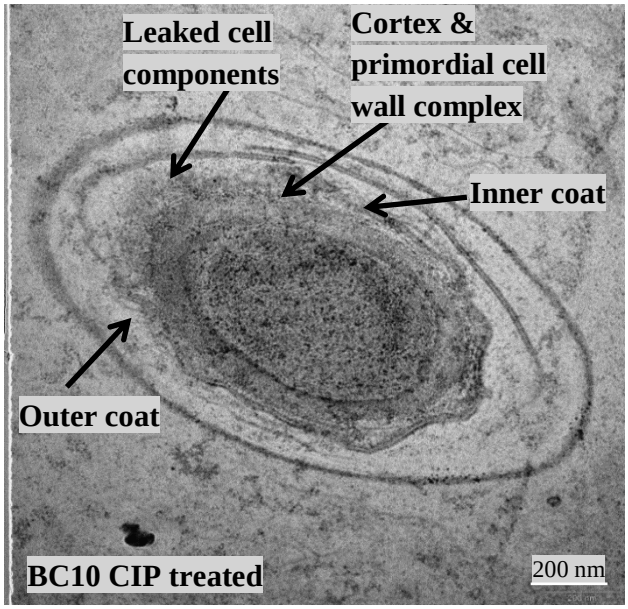

Figure 11. TEM image of a spore from *B. cereus* strain BC10 isolated from filler nozzles from ESL milk processing lines showing damage to the spore layers following simulated CIP treatment.

The TEM micrographs of all three strains were similar following simulated CIP treatment. The TEM micrograph of *B. cereus* strain BC10 following simulated CIP treatment (Figure 11) showed damage had occurred to the spore.

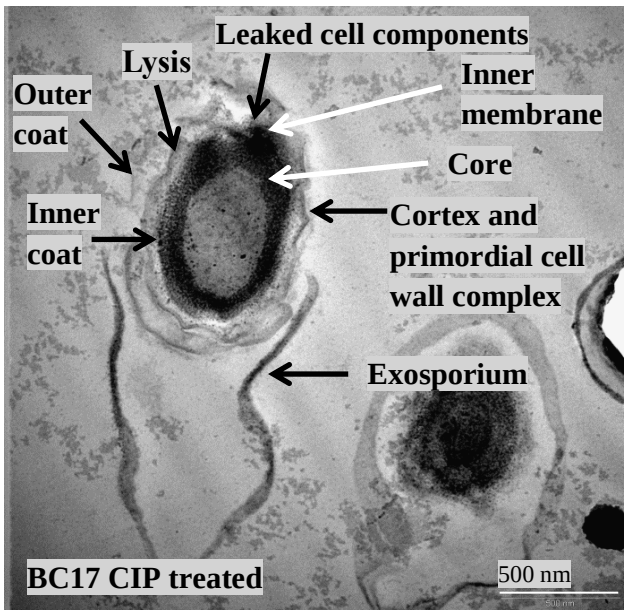

Figure 12. TEM image of spores from *B. cereus* strain BC17 isolated from filler nozzles from ESL milk processing lines indicating damage to the spore structure following simulated CIP treatment.

The TEM image of strain BC17 (Figure 12) showed damage to the spore structure similar to that of strain BC10 (Figure 11).

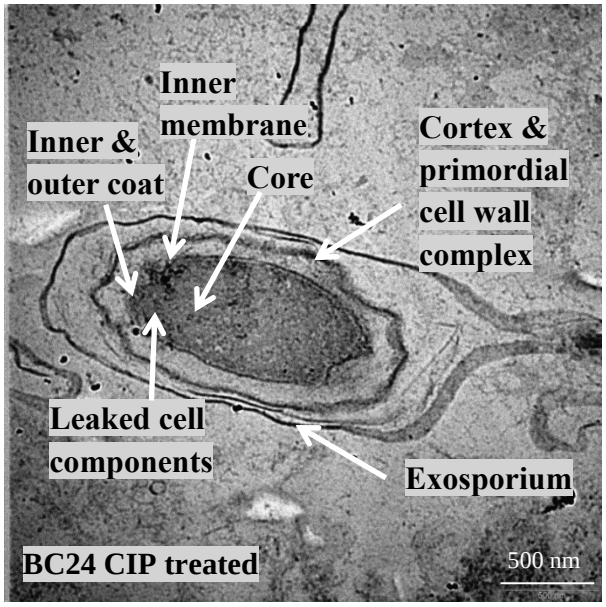

Figure 13. TEM image of a spore from *B. cereus* strain BC24 isolated from raw milk during ESL milk processing indicating damage to the spore structure following simulated CIP treatment.

The TEM image following simulated CIP treatment for *B. cereus* strain BC24 (Figure 13) indicated that the spore structure was damaged as leaked cell components and damaged layers were evident.

4.2.5 DISCUSSION

The physical effects of simulated CIP treatment on spores from *B. cereus* strains isolated from filler nozzles and raw milk during ESL milk processing was determined using FCM analyses, epifluorescent microscopy and TEM.

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. PI, a red fluorescent nucleic acid-binding stain, does not permeate the cell membrane and nonviable, dead germinated endospores stain with PI (Cronin and Wilkinson, 2007). PI is therefore not taken up by healthy cells (Stocks, 2004), thus, PI only penetrates spores with damaged membranes.

This study found that following simulated CIP treatment at least 91% of spores were stained with PI during FCM analysis, indicating that CIP treatment damaged the permeability

barriers of *B. cereus* spores which enabled PI to bind with the nucleic acid in the spore. When bound to DNA, SYTO 9 emits green fluorescence allowing the fluorescence intensity to be determined. Flow cytometric analyses with SYTO 9 revealed that simulated CIP treatment damaged at least 98% of spores from the three *B. cereus* strains included in this study. This could be due to CIP chemicals disrupting spore coat layers, enabling SYTO 9 to bind with cellular components. According to Cronin and Wilkinson (2008), the disruption of the permeability barrier (possibly the inner membrane) causes treated endospores to release DPA which may result in the increased accessibility of DNA to dyes such as SYTO 9.

When spores are damaged, DPA is released which causes the spore core to become hydrated. This may result in more rapid protein denaturation in the spore core ultimately, damaging the spores (Coleman *et al.*, 2010). Spores that retain DPA may still germinate, although germination could take longer compared to untreated spores (Coleman *et al.*, 2010). CIP treatment may lead to the inactivation of glucose-6-phosphate dehydrogenase, an essential metabolic enzyme, which may affect the inactivation of *B. cereus* spores (Coleman *et al.*, 2010).

Germinating or undamaged germinated endospores stain brightly with SYTO 9 and intact ungerminated undamaged endospores only exhibit marginal staining that increases in fluorescence upon germination (Cronin and Wilkinson, 2008). The region on the FCM plots representing intact spores showed a lower SSC and a weaker green fluorescence compared to the damaged spores, meaning less SYTO 9 could bind to the intact spores that were unaffected by simulated CIP treatment. Also, outgrowing endospores probably accounted for the FCM region with a less intense green fluorescence compared to germinating spores, which according to Cronin and Wilkinson (2007) might be as a result of the activity of cellular efflux pump systems with the commencement of metabolism.

Furthermore, this could explain why the regions to the far left of the FCM plots displayed a lower green fluorescence compared to the greater green fluorescence of the damaged spores. Cronin and Wilkinson (2007) suggested that injured endospores, which were permeable to SYTO 9, had high SSC values, which corresponds with the FCM results obtained in this study where spores in the damaged region on the FCM plot had higher SSC values compared to intact or unstained spore regions. Additionally, in the study by Cronin and Wilkinson (2007) the permeability of cells to SYTO 9 increased upon lethal heat treatment, but they

indicated that one region might not exclusively represent a specific state of the spore, i.e. being damaged or intact.

FCM showed that not all spores that underwent simulated CIP treatment were stained by PI indicating they were still intact due to the PI not being able to gain access to the nucleic acid in the cells. As a result, CIP was not entirely effective in causing damage to 100% of spores. This corresponds with results obtained from staining spores with SYTO 9 where 0.1% of spores remained intact following simulated CIP treatment.

Epifluorescent microscopy showed that simulated CIP caused some structural damage to spores of all the *B. cereus* spore strains investigated in this study. Following simulated CIP treatment damaged spores and spore debris had strong green fluorescence suggesting CIP chemicals disrupted permeability barriers of *B. cereus* spore membranes leading to leaked DNA components binding strongly to the SYTO 9 stain (Cronin and Wilkinson, 2008). Since DPA and other smaller molecules comprise 5 to 15% of the dry weight of the spore, once DPA is released following damage to the spore, it is expected that the spore structure could collapse following simulated CIP treatment (Perkins *et al.*, 2004). This could explain why *B. cereus* spores had been damaged and displayed a green fluorescence after exposure to the simulated CIP process.

TEM was used to determine the extent of structural damage caused by simulated CIP treatment on spores. As was seen with FCM and epifluorescent microscopy, the TEM micrographs of spores from all *B. cereus* strains following simulated CIP treatment revealed damage to several layers of the spores ranging from being slightly damaged to being severely damaged. Similar to results found by Ali *et al.* (2017), we observed evident lysis of the exosporia following simulated CIP treatment. The outer coat, inner coat, inner membrane, cortex and primordial cell wall were damaged. Leaked cell components were apparent due to the integrity of the cortex and primordial cell wall being lost. CIP chemicals may have caused the leakage of contents of the exosporium (Tabit, 2010), as well as potentially weakened the spore coat which led to the destruction of the structural integrity of the spore.

The leakage of cell components suggested that damage may have been caused to the contractile cortex mechanism (Mustafa *et al.*, 2010) by which, according to Lewis *et al.* (1960), the low degree of crosslinking enables the spore to expand and contract in response to changes in the environment. This is detrimental to the spore as the cortex is essential for controlling the water content of the spore (Mustafa *et al.*, 2010), and this could contribute to

the inhibition of spore germination (Ali *et al.*, 2017). We found that contents of the exosporium, cortex and some cortical membranes were lost, rendering the spores less dense. As was seen in a study by Mustafa *et al.* (2010), the spore cores appeared to have a lower density of ribosome-containing cytoplasm and DNA-SASP compared to the cores of untreated (non-simulated CIP treated) spores. The lower density of the spore could be attributed to cortical materials being lost as a result of the effect of CIP chemicals on the spore structure (Tabit, 2010).

Moreover, following simulated CIP treatment the spore coat structures pulled away from the cortex within the cortical membrane (Tabit, 2010) and the endospore shrank in volume (Mustafa *et al.*, 2010), which resulted in the exosporium appearing loose. TEM micrographs of simulated CIP treated spores revealed fewer uniform cores due to more dense electron clumps fragmenting into tiny black spots distributed throughout the spore core (Mustafa *et al.*, 2010).

Damage to the spore core, such as protein denaturation and breakage in DNA strands, adversely affects spore viability. According to Tabit (2010), a compact spore core could indicate more thorough dehydration which is an imperative aspect in the heat resistance of the spore, but this aspect was lacking in most of the observed simulated CIP treated spores indicating CIP damaged the structure of the spores severely.

Moreover, the two strains (BC10 and BC17) isolated from filler nozzles from ESL milk processing lines indicated that perhaps the strains isolated from filler nozzles have gained increased resistance to CIP components compared to the strain isolated from raw milk during ESL milk processing (strain BC24). The inner membrane surrounding the core contains various proteins that protect against chemicals (Setlow *et al.*, 2002). These proteins are also necessary for germination (Wells-Bennik *et al.*, 2016).

TEM observations indicated that most of the spore structures from the *B. cereus* strains used in this study were damaged by simulated CIP treatment. However, there is a possibility that the fraction of spores in the sample which were viewed under the TEM contained intact spores (unaffected by simulated CIP treatment) that were not seen. Intact spores might have been present elsewhere in the sample, but were not observed due to the small surface area covered by the TEM.

Despite FCM indicating that the majority of the spore structures were damaged following simulated CIP treatment, we found that the structures of a fraction (0.11 – 0.40%) of the spore population (stained with SYTO 9) remained unaffected by simulated CIP treatment and may still germinate once conditions become favourable. 8% of spores did not stain with PI after simulated CIP treatment indicating these spores were intact and thus impermeable to PI. The resistance of *B. cereus* spores toward simulated CIP treatment could be attributed to the relatively low amount of water in the spore core which aids in high spore resistance (Paidhungat *et al.*, 2000; Coleman *et al.*, 2010). Many factors contribute to the low water content of the spore core, of which one is the accumulation of DPA with the associated displacement of water (Setlow, 2006; Coleman *et al.*, 2010).

Furthermore, spore resistance to CIP treatment could be due to the dehydration of the spore core which is facilitated by the formation of a thick layer of peptidoglycan underneath the coat, namely the cortex (Wells-Bennik *et al.*, 2016). The cortex, proteinaceous spore coat and inner membrane restrict the access of potentially damaging molecules into the spore core, enabling resistance to potentially toxic chemicals (Setlow *et al.*, 2002). In addition to FCM, epifluorescent microscopy showed that a fraction of the spores from *B. cereus* strains BC10 and BC17 were intact and unaffected by simulated CIP which implied that these spores were resistant and that simulated CIP treatment was not effective in damaging all the *B. cereus* spore structures. These results were in agreement with results obtained by FCM in this study.

Despite the minimal structural resistance observed by *B. cereus* strains BC10 and BC17, no intact *B. cereus* could be seen in epifluorescence micrographs for strain BC24 following simulated CIP treatment which suggested that perhaps this strain (isolated from raw milk during ESL milk processing) was more sensitive towards simulated CIP treatment compared to the strains isolated from biofilms in filler nozzles during ESL milk processing. The lack of exposure to CIP chemicals in raw milk as opposed to the recurring exposure to CIP experienced by biofilms in filler nozzles during ESL milk processing could explain the possible susceptibility of strain BC24 toward CIP chemicals. In other words, strains BC10 and BC17 had previously been exposed to CIP chemicals multiple times before, and thus, these two strains have conceivably developed increased resistance to the CIP process.

According to a study by Mustafa *et al.* (2010), the resistance of *B. cereus* spores could be explained by the high protein content of the exosporium which enhances resistance through protein hydrophobicity as well as the exosporium being a multi-layered physical barrier. The

destruction of endospore membranes by CIP chemicals is therefore imperative as the membranes are responsible for protecting the spore core (Mustafa *et al.*, 2010).

Although this study determined that *B. cereus* spores may be vulnerable to CIP treatment, damage to DNA and proteins may still be the result, and this could inhibit the spore from ultimately germinating when conditions become favourable. However, according to Wells-Bennik *et al.* (2016), simulated CIP treatment can cause a spore to become sublethally damaged and that spores could potentially be supplied with repairing enzymes that facilitate rapid repair once germination commences. As a result, damaged spores may still recover themselves and ultimately germinate to grow into a vegetative cell (Wells-Bennik *et al.*, 2016).

Overall in this study FCM, epifluorescent microscopy and TEM showed that simulated CIP treatment caused damage to the structure of spores from the three *B. cereus* strains. The effect of simulated CIP treatment could have an inhibiting effect on the germination of *B. cereus* spores which may negatively affect the viability of spores in ESL milk. Despite the positive impact shown by simulated CIP treatment, FCM and epifluorescent microscopy also revealed that a small percentage of the spores investigated were possibly not affected by simulated CIP treatment which may still germinate upon favourable conditions. Epifluorescent microscopy indicated that the spores of the *B. cereus* strain isolated from raw milk might be more susceptible and sensitive toward simulated CIP treatment compared to strains which were isolated from biofilms in filler nozzles during ESL milk processing. The effect of simulated CIP treatment on the viability of *B. cereus* spores will be discussed in the next chapter of this study.

4.2.6 CONCLUSIONS

This study indicates that CIP treatment has an effect on the structure of *B. cereus* spores isolated from raw milk and filler nozzles from ESL milk processing lines. The *B. cereus* spores surviving CIP treatment may germinate upon favourable conditions and cause spoilage of the final ESL milk product which could lead to reduced shelf life and potentially be a safety risk to consumers.

4.3 EFFECT OF CIP ON THE ATTACHMENT, VIABILITY AND GROWTH OF SPORES FROM *BACILLUS CEREUS* STRAINS ISOLATED FROM RAW MILK AND FILLER NOZZLES FROM AN ESL MILK PROCESSING PLANT

4.3.1 ABSTRACT

The processing and shelf life of ESL milk is in between that of pasteurised and UHT milk in terms of the processing temperature-time parameter. The shelf life of ESL milk dramatically depends on the quality of raw milk. *B. cereus* isolated from filler nozzles from an ESL milk processing plant attached to stainless steel strips and formed biofilms. Furthermore, psychrotolerant *B. cereus* strains exist in dairy processing facilities and may also produce toxins. In a dairy processing plant, CIP is used to clean organic and inorganic contamination from surfaces within processing equipment and filler nozzles. Although CIP is effective in removing vegetative cells, the challenge with *B. cereus* is that it produces exceptionally resistant spores that are potentially able to withstand the CIP process. This could lead to spores attaching to surfaces of processing equipment resulting in the contamination of the final ESL milk product. In this study, the effect of simulated CIP treatment on the attachment and viability of *B. cereus* spores isolated from raw milk and filler nozzles from an ESL milk processing plant were studied. SEM was used to analyse the effect of simulated CIP treatment on the attachment of *B. cereus* spores and growth kinetics as well as biofilm forming capabilities. Additionally, the viability of CIP treated *B. cereus* spores in milk over 28 days at refrigeration temperatures (5 °C) was studied. Results indicated that following simulated CIP treatment spores were capable of forming biofilms, although the strain isolated from raw milk had the weakest biofilm of all the three strains. Furthermore, SEM revealed that simulated CIP treated spores were able to attach to stainless steel. CIP did not affect the viability and growth of spores from *B. cereus* in milk over a prolonged shelf life of 28 days at 5 °C. It was concluded that CIP may affect the structure of *B. cereus* spores but has no effect on the attachment ability of spores to stainless steel or on the ability of spores to germinate and grow in milk over 28 days at 5 °C. It is also apparent that there is variation between strains as strains isolated from filler nozzles are more resistant to CIP. Spores surviving CIP may attach to filler nozzles in dairy processing plants and consequently contaminate the final ESL milk. This could lead to ESL milk with a shortened shelf life and may pose a food safety risk to consumers.

4.3.2 INTRODUCTION

ESL milk is a dairy product in between UHT and pasteurised milk with regards to the processing temperature-time parameter and shelf life (Mugadza and Buys, 2018). The shelf life of ESL milk is dependent on the quality of raw milk as well as storage conditions (Schmidt *et al.*, 2012). Khoza (2015) demonstrated that *B. cereus* originating from ESL filler nozzles were capable of attaching to stainless steel strips and forming biofilms. These findings reflect that potentially there are biofilms present in the ESL filler nozzles and that these bacteria may act as a potential source of contamination which could lead to ESL milk with a limited shelf life (Khoza, 2015).

Although *B. cereus* is considered to be mesophilic, psychrotolerant strains of *B. cereus*, do exist. Although this microorganism may not be the most competitive organism in ESL milk, it should be noteworthy as it can germinate and multiply at temperatures as low as 7 °C (Stenfors and Granum, 2001; Mugadza and Buys, 2018). *B. cereus* is also capable of producing toxins, i.e. the non-haemolytic enterotoxin, the single protein cytotoxin and the emetic toxin cereulide (Schmidt *et al.*, 2012; Mugadza and Buys, 2017) which is a food safety concern.

In a dairy manufacturing plant processing lines undergo CIP to clean equipment without dismantling. This process includes several steps including a caustic wash, an acid wash and lastly a sanitising step (Thomas and Sathian, 2014). CIP is used to assure the removal of organic and inorganic contamination (Vlková *et al.*, 2008). Aside from the efficiency of CIP treatment against vegetative cells, the challenge with *B. cereus* spores is its resistance to cleaning regimes such as CIP (Faille *et al.*, 2010).

Spores may attach to a surface within the ESL milk processing line and once favourable conditions arise they will germinate and ultimately biofilms could be formed (Khoza, 2015). Consequently, this could lead to contamination of the final ESL milk. This study was aimed studying the viability, growth and attachment of *B. cereus* spores associated with filler nozzles and raw milk of from ESL milk processing plant following simulated CIP treatment.

4.3.3 MATERIALS AND METHODS

Bacillus cereus isolates

B. cereus isolates from *B. cereus* strains isolated from filler nozzles from an ESL milk processing plant (strains BC10 and BC17) and raw milk (strain BC24) were prepared as previously described in section 4.2.3.

Bacillus cereus spore preparation

B. cereus spores were prepared as previously described in section 4.2.3.

Simulated CIP treatment

Simulated CIP treatment was carried out as previously described in section 4.2.3.

Bacillus cereus spore viability and growth in milk over 28 days following simulated CIP

Spores from the three *B. cereus* strains were standardised to 0.5 McFarland standard in sterile PBS (Oxoid). The cultures were diluted in buffered peptone water (Oxoid) (0.1%) to a standard cell concentration of 10^7. Each strain (1 ml control and 1 ml simulated CIP treated spores respectively) 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.

Biofilm: biomass quantification

The biofilm assay was derived from a method by Hussain & Oh, (2017). Simulated CIP treated and untreated (non-simulated CIP treated control) spores from strains isolated from filler nozzles (strains BC10 and BC17) and raw milk (strain BC24) during ESL milk processing were used in this analysis. A 96-well microtiter plate was sterilised under UV light for 2 h, thereafter 5 µl of spore suspension containing a density of 10^8 cells/ ml (0.5 McFarland standard) was inoculated into wells containing 200 µl BHI broth (Oxoid, South Africa) and incubated at 35 °C for 24 h. Following incubation, 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). Following washing, biofilms were stained with 200 µl of 0.1% crystal violet for 30 min at ambient temperature. Microtiter plates were covered with film to prevent wells from drying out. Crystal violet that failed to bind to biofilms was discarded by micro pipetting and wells were once again washed three

times with 200 µl of PBS (Oxoid). Thereafter 200 µl of 70% ethanol was pipetted into each well and incubated at room temperature for 30 min to release the biofilm bound by crystal violet. The absorbance of the resulting crystal violet solution in the microtiter plate was measured at a wavelength of 595 nm on a Multiskan FC microplate reader (Thermo Scientific, South Africa).

Optical Density Growth Curves

A 96-well microtiter plate, as described in the method for the biofilm assay, was prepared where simulated CIP treated and untreated (non-simulated CIP treated control) spores were inoculated into wells containing 200 µl BHI broth (Oxoid). The microtiter plate was covered with plastic film and incubated at 35 °C for 24 h during which the optical density reading was taken every 2 h from 0 h up until 24 h. The optical density was measured at a wavelength of 595 nm on a Multiskan FC microplate reader. Plots were obtained where optical density was plotted against exposure time. The curve fitting of Baranyi's equation was done using DMFit 2.0 program, and the model of Baranyi and Roberts Growth curves were obtained by DMFit (Pla *et al.*, 2015).

Attachment of *Bacillus cereus* to stainless steel

1 ml of spores from each *B. cereus* strain, with a spore suspension density of 10^8 cells/ ml (0.5 McFarland standard), was used in this analysis. Each of the three strains had an untreated (control – non-simulated CIP treated spores) as well as a simulated CIP treated spore suspension. The suspensions were inoculated into 2 ml sterile reconstituted skim milk (RSM) (Oxoid) and incubated for 1 h at 36 °C, thereafter 1 ml of the inoculated RSM (Oxoid) was inoculated into 15 ml conical sterile polypropylene centrifuge tubes containing 9 ml sterile RSM (Oxoid). Thereafter, stainless steel coupons (grade 316, 10 x 1 cm) were immersed in the 15 ml conical sterile polypropylene centrifuge tubes. The centrifuge tubes were incubated at 36 °C for 24 h. After 24 h the stainless steel coupons were removed from the RSM (Oxoid) and rinsed with phosphate buffer (20 ml $NaPO_4$ and 20 ml dH_2O) to remove unattached cells on the stainless steel. The stainless steel strips were then placed in 15 ml centrifuge tubes containing 10 ml phosphate buffer (20 ml $NaPO_4$ and 20 ml dH_2O) for 15 minutes. Thereafter, the stainless steel strips were immediately fixed with 2.5% glutaraldehyde fixative (1 ml glutaraldehyde (GA), 1 ml formaldehyde (FA), 3 ml ddH_2O, 5 ml buffer) in preparation for electron microscopy.

Scanning Electron Microscopy (SEM)

SEM was used to observe the attachment of *B. cereus* (ungerminated spores, germinating spores, and/or vegetative cells) to stainless steel of untreated (non-simulated CIP treated control) and simulated CIP-treated spores from the *B. cereus* strains. Preceding SEM observations, stainless steel coupons were fixed in 2.5% GA/FA followed by washing three times in PBS (Oxoid) for 10 minutes. The coupons were covered with osmium tetroxide for 45 minutes, thereafter coupons were washed three times in PBS (Oxoid) for 10 minutes. Coupons were gradually dehydrated in ethanol from 30 to 100% (10 minutes 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). 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, South Africa).

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 and strain variation on the rate of growth, lag phase duration and biofilm formation. A 3-way ANOVA with multiple comparisons was also used to determine the effect of simulated CIP treatment, strain variations and period of incubation (day 0, 14 and day 28) on the Log counts of germinated *B. cereus* spores. All statistical analyses were done using GraphPad Prism version 7.0 (GraphPad Software, CA, USA) at a significance level of 0.05. All experiments were repeated at least two times, and each experiment was done in duplicates.

4.3.4 RESULTS

4.3.4.1 *Bacillus cereus* spore viability of untreated (non-simulated CIP treated) and simulated CIP treated *Bacillus cereus* in milk over 28 days at a storage temperature of 5 °C

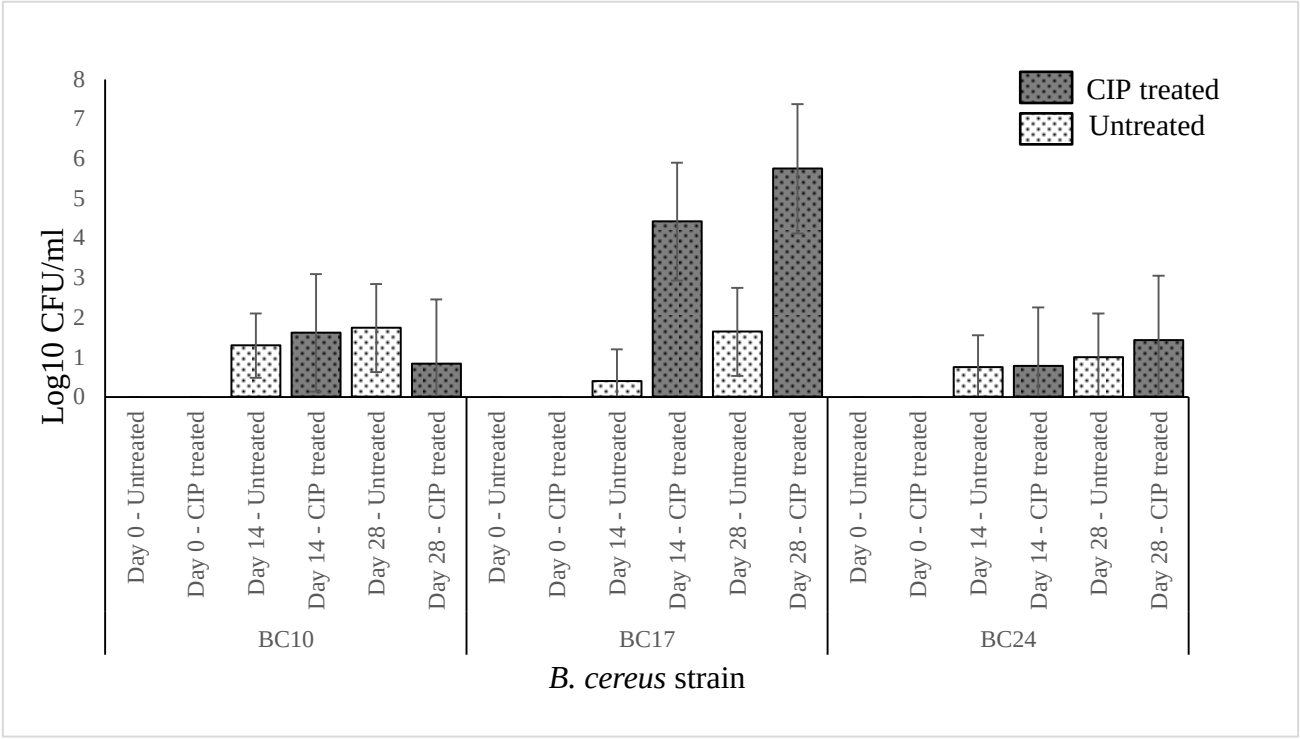

Figure 14. Viability and growth of untreated (non-simulated CIP treated control) and simulated CIP treated spores from *B. cereus* strains isolated from filler nozzles from ESL milk processing lines (strains BC10, BC17) and raw milk (strain BC24) at 5 °C.

There was no noticeable difference in the Log counts between untreated (non-simulated CIP treated) and simulated CIP treated spores for strains BC10 and BC24 after 28 days in milk at a storage temperature of 5 °C (Figure 14). Strain BC17, however, had a noticeable increase in the Log counts of simulated CIP treated spores compared to untreated spores throughout the 28 days of shelf life at a storage temperature of 5 °C (Figure 14).

Table 2. Analysis of variance (3-way) indicating the effect of strain, simulated CIP treatment and interaction on the viability of untreated (non-simulated CIP treated control) and simulated CIP treated spores from *B. cereus* strains isolated from filler nozzles from ESL milk processing lines (strains BC10, BC17) and raw milk (strain BC24) in milk over 28 days at a storage temperature of 5 °C (n=6).

ANOVA	DF	p-value
Strain	2	0.135
Day	2	0.017*
Treatment	1	0.134
Strain x day	4	0.593
Strain x treatment	2	0.097
Treatment x day	2	0.554
Strain x treatment x day	4	0.609

*Significant (p≤0.05)

Table 3. Tukey's multiple comparisons test for factors day, strain within simulated CIP treatment, simulated CIP treatment and simulated CIP treatment within strain (Log counts) for untreated (non-simulated CIP treated control) and simulated CIP treated spores from *B. cereus* strains isolated during ESL milk processing from filler nozzles (strains BC10, BC17) and raw milk (strain BC24) in milk over 28 days at a storage temperature of 5 °C.

Comparisons factor		p-value
Day	Day 0 vs. Day 14	0.092
	Day 14 vs. Day 28	0.744
	Day 0 vs. Day 28	0.017*
Strain within simulated CIP treatment	BC17 vs. BC24	0.032*
	BC17 vs. BC10	0.039*
	BC10 vs. BC24	0.997
Simulated CIP treatment	CIP treated vs. Untreated	0.135
Simulated CIP treatment within strain	CIP treated vs. Untreated (BC10)	0.847
	CIP treated vs. Untreated (BC17)	0.011*
	CIP treated vs. Untreated (BC24)	0.88

*Significant (p≤0.05)

There was a significant (p≤0.05) difference in the Log counts regarding the 'days' factor (Table 2). However, there was no difference in the Log counts concerning strain, treatment or any interaction of strain, treatment and/or day (Table 2). Both simulated CIP treated and untreated spores germinated and significantly (p≤0.05) multiplied in milk over 28 days of storage at 5 °C.

With regards to day, when day 0 and day 28 were compared, there was a significant (p≤0.05) difference in the Log counts, but there was no difference when day 0 and day 14 or day 14 and day 28 were compared (Table 3). There was a significant (p≤0.05) difference in the Log counts within simulated CIP treatment when strains BC17 and BC24 were compared (Table 3). Furthermore, there was a significant (p≤0.05) difference in the Log counts within simulated CIP treatment when strains BC17 and BC10 were compared (Table 3). There was no significant difference in the Log counts when simulated CIP treated and untreated spores were compared (Table 3). In terms of treatment within strain, there was a significant (p≤0.05) difference when the Log counts between untreated and simulated CIP treated spores in milk over 28 days were compared for strain BC17. However, there was no difference between the Log counts of untreated and simulated CIP treated spores for strains BC10 and BC24 in milk over 28 days at a storage temperature of 5 °C (Table 3).

Table 4. Tukey's multiple comparisons test for factor day (day 0, 14, 28) within *B. cereus* strains isolated from filler nozzles from ESL milk processing lines (strains BC10, BC17) and raw milk (strain BC24) Log counts in milk over 28 days at a storage temperature of 5 °C.

***B. cereus* strain**	**Comparison factor (Day)**	**p-value**
BC10	Day 14 vs. Day 0	0.474
	Day 14 vs. Day 28	0.99
	Day 28 vs. Day 0	0.556
BC17	Day 14 vs. Day 0	0.013*
	Day 14 vs. Day 28	0.553
	Day 28 vs. Day 0	0.139
BC24	Day 14 vs. Day 0	0.594
	Day 14 vs. Day 28	0.929
	Day 28 vs. Day 0	0.813

*Significant (p≤0.05)

Apart from the absence in the overall effects of strain and treatment factors on the Log counts, multiple comparisons of strain responses at each day indicated that strain BC17 had unusual growth behaviour compared to strains BC10 and BC24. Strain BC17 showed a significant ($p \leq 0.05$) difference in its Log counts between day 0 and day 28 (Table 4). The day factor did not have an effect on the Log counts of strains BC10 and BC24 in milk over 28 days at a storage temperature of 5 °C (Table 4).

4.3.4.2 Growth curves of untreated (non-simulated CIP treated) and simulated CIP treated *Bacillus cereus*

The effect of simulated CIP treatment on viability and growth was determined by growth curves and subsequent analysis of the lag phase and growth rate of germinated *B. cereus* spores using growth data and curve fittings of the Baranyi model as discussed in section 4.3.3. The control used in this analysis was untreated (non-simulated CIP treated) for all three strains (BC10, BC17 and BC24).

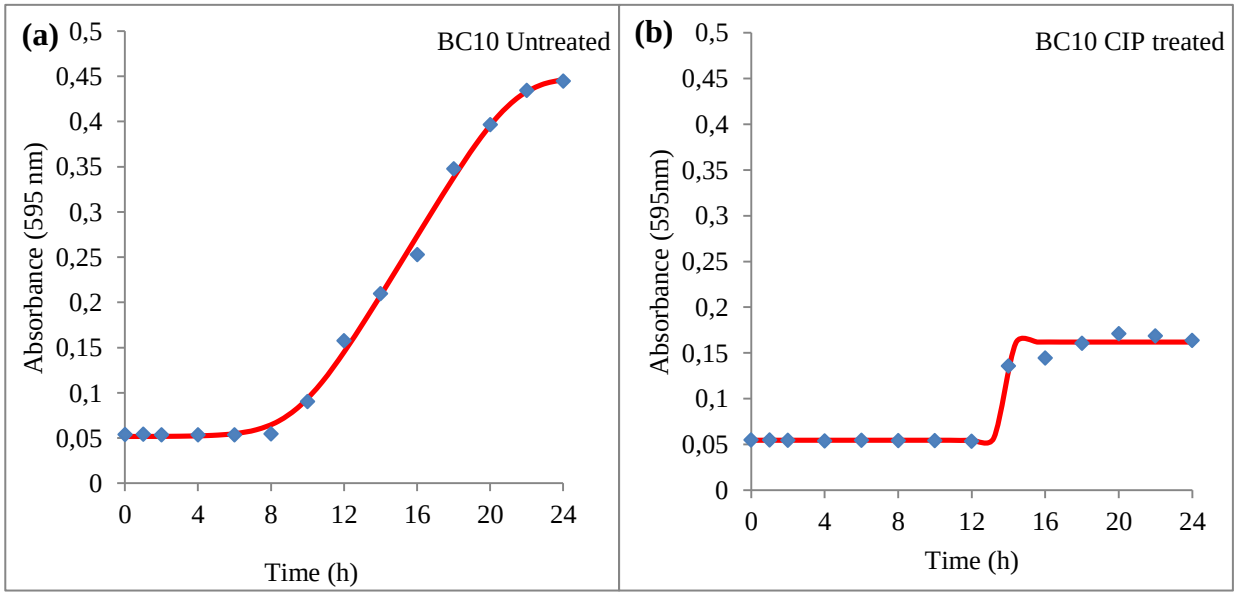

Figure 15. Growth curves for untreated (non-simulated CIP treated) (a) and simulated CIP treated (b) spores from *B. cereus* strain BC10 isolated from filler nozzles from ESL milk processing lines.

Untreated (non-simulated CIP treated) spores from *B. cereus* strain BC10 had a lag phase duration of 6 h before the exponential phase commenced (Figure 15a), whereas the simulated CIP treated strain BC10 had a lag phase double the length at a duration of 12 h (Figure 15b). The exponential phase begins once the initial population has doubled, and during exponential

growth, the rate at which cells increase in the culture is proportional to the number of cells present at any particular time. The exponential phase for strain BC10 was longer (Figure 15a) compared to the simulated CIP treated strain BC10 (Figure 15b). However, this could easily be the germination phase of viable CIP treated spores and not necessarily an exponential phase. Simulated CIP treated strain BC10 reached the stationary phase earlier at approximately 18 h (Figure 15b) compared to the untreated strain BC10 that reached the stationary phase at 22 h (Figure 15a).. The last phase of the growth curve is the death phase, which is characterised by a net loss of culturable cells. Simulated CIP treated strain BC10 reached the death phase earlier (Figure 15b) compared to untreated strain BC10 (Figure 15a), however, due to the growth curve being measured by optical density the onset of the death phase might not be readily apparent. One could say viable spores had just started growing and that the experiment was terminated too soon, but the experiment was limited to 24 h so as to represent the length of time the spores would have to potentially germinate in a factory environment before CIP typically takes place.

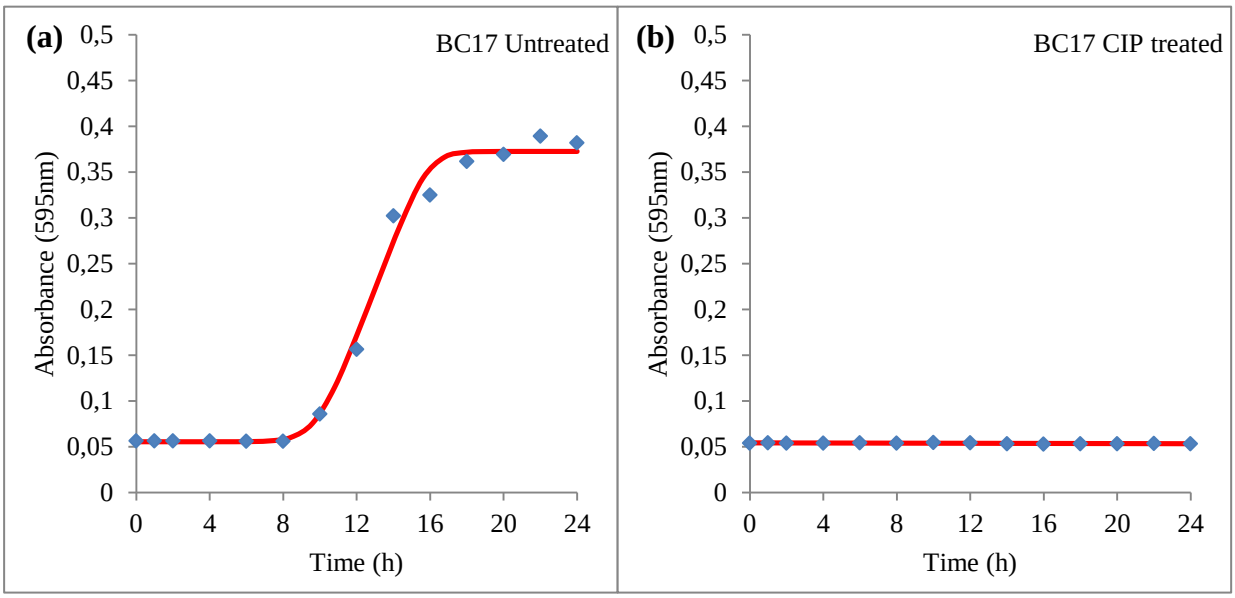

Figure 16. Growth curves for untreated (non-simulated CIP treated) (a) and simulated CIP treated (b) spores from *B. cereus* strain BC17 isolated from filler nozzles from ESL milk processing lines.

The untreated (non-simulated CIP treated) sample of *B. cereus* strain BC17 had a lag phase duration of 8 h before the exponential phase commenced (Figure 16a) which was to some extent longer compared to the lag phase duration of the untreated strain BC10 (Figure 15a). Untreated strain BC17 had a shorter exponential phase compared to untreated strain BC10 (Figure 15a). Untreated strain BC17 reached the stationary phase earlier at approximately 18

h (Figure 16a) compared to the untreated strain BC10 that reached the stationary phase at 22 h (Figure 15a). Following the stationary phase, the death phase ensued for untreated strain BC17 (Figure 16a). There was no noticeable increase in absorbance for strain BC17 following simulated CIP treatment (Figure 16b).

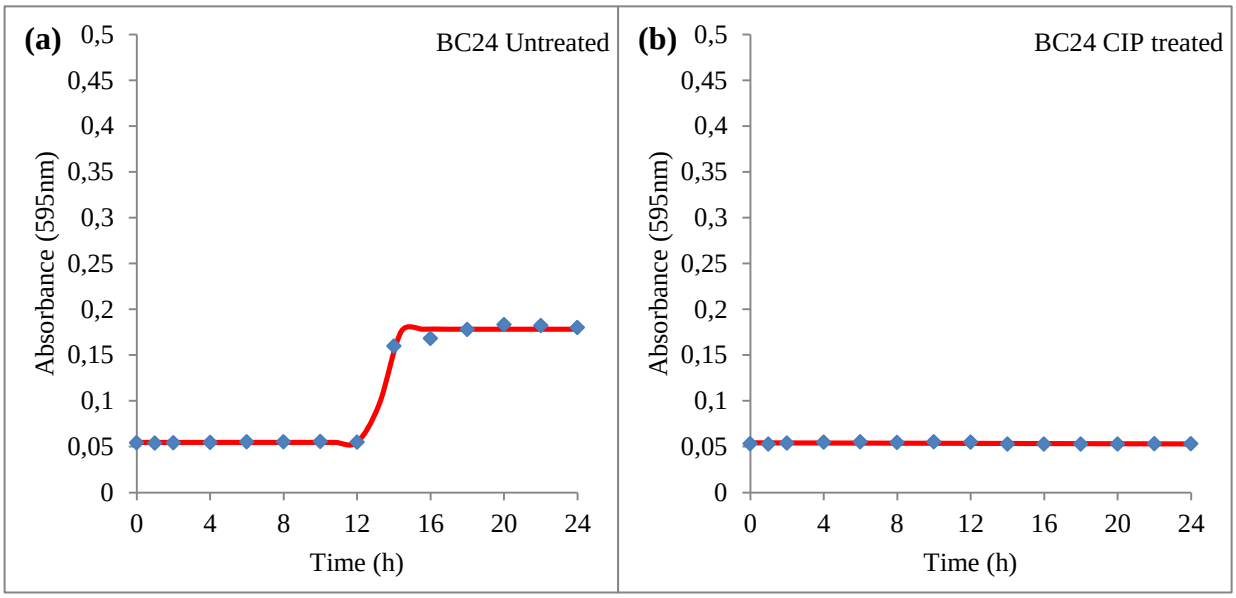

Figure 17. Growth curves for untreated (non-simulated CIP treated) (a) and simulated CIP treated (b) spores from *B. cereus* strain BC24 isolated from raw milk during ESL milk processing.

The untreated (non-simulated CIP treated) sample of *B. cereus* strain BC24 had a lag phase duration of 12 h before the exponential phase commenced (Figure 17a) which was longer compared to the untreated lag phase durations of strains BC17 (Figure 16a) and BC10 (Figure 15a). The exponential phase of untreated strain BC24 was noticeably shorter (Figure 17a) at a duration of approximately 2 h compared to the exponential phases of both untreated strains BC17 (Figure 16a) and strain BC10 (Figure 15a). Untreated strain BC24 reached the stationary phase earlier at approximately 14 h (Figure 16a) compared to the untreated strain BC10 that reached the stationary phase at 22 h (Figure 15a) as well as untreated strain BC17 (Figure 16a). Following the stationary phase, the death phase ensued for untreated strain BC24 (Figure 17a). Similar to strain BC17 (Figure 16b), there was no noticeable change in the growth of strain BC24 following simulated CIP treatment (Figure 17b).

Table 5. Analysis of variance (2-way) indicating the effect of strain, simulated CIP treatment and interaction on the growth rate, lag and biofilm formation of spores from *B. cereus* strains isolated from filler nozzles (strains BC10, BC17) from ESL milk processing lines and raw milk (strain BC24) during ESL milk processing (n=6).

Source of variation		**p-value**		
	DF	**Growth rate**	**Lag**	**Biofilm**
Strain (BC10, BC17 & BC24)	**2**	0.0282*	0.0001*	<0.0001*
Treatment	**1**	0.5242	0.2375	<0.0001*
Interaction (Strain x treatment)	**2**	0.0005*	<0.0001*	0.0017*

*Significant (p≤0.05)

Strain (BC10, BC17 and BC24) had a significant effect on the growth rate, lag phase and biofilm formation of *B. cereus* spores. Simulated CIP treatment had no effect on growth rate and lag of the *B. cereus* spores (Table 5). However, simulated CIP treatment had a significant (p≤0.05) effect on the biofilm formation of strains BC10, BC17 and BC24 (Table 5). The interaction between strain and treatment showed to have a significant (p≤0.05) effect on the growth rate, lag and biofilm formation of strains BC10, BC17 and BC24 (Table 5). This implied that the CIP treatment was dependent on the strain to have an effect on the growth rate, lag and biofilm formation of *B. cereus*. This could suggest that biofilm formation, growth rate and lag phase will differ depending on the specific strain in combination with simulated CIP treatment.

Table 6. Tukey's multiple comparisons test for untreated (non-simulated CIP treated control) and simulated CIP treated spores from *B. cereus* strains isolated from filler nozzles (strains BC10, BC17) from ESL milk processing lines and raw milk (strain BC24).

Comparisons factor	**p-value**		
	***B. cereus* strain**		
	BC10	**BC17**	**BC24**
Biofilm formation	<0.0001****	0.4765	0.5846
Lag phase	0.0332*	0.5778	<0.0001****
Growth rate	0.0354*	>0.9999	0.0021**

*Significant (p≤0.05)
Level of significance indicated by amount of *.

Concerning treatment, there was no difference in lag phase of spores from *B. cereus* strains used in this study (Table 5). There was, however, a significant ($p \leq 0.05$) difference with regards to strain effect and interaction between strain and simulated CIP treatment (Table 5). Interaction accounted for 47.99% of the total variation and strain effect was responsible for 33.42% of the total variation.

Strain BC10 showed significant ($p \leq 0.05$) difference between simulated CIP treated and untreated spores with regards to biofilm formation (Table 6). However, simulated CIP treatment did not affect the lag phase of strain BC17 (Table 6).

4.3.4.3 Lag phase of untreated (non-simulated CIP treated) and simulated CIP treated *Bacillus cereus* spores

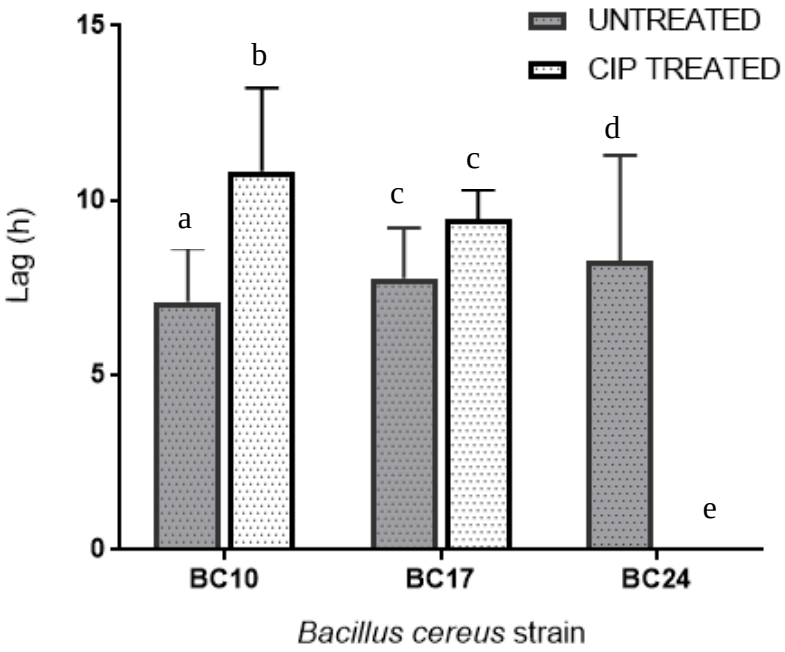

Figure 18. Lag phase of untreated (non-simulated CIP treated control) and simulated CIP treated spores from *B. cereus* strains isolated from filler nozzles (strains BC10, BC17) from ESL milk processing lines and raw milk (strain BC24) as estimated by the Baranyi model.
*Bars with different letters for each strain indicate significant difference.

Following simulated CIP treatment, the lag phase was noticeably longer for strain BC10 (Figure 18). Strain BC24 showed no growth following simulated CIP treatment.

4.3.4.4 Growth rate of untreated (non-simulated CIP treated) and simulated CIP treated *Bacillus cereus* spores

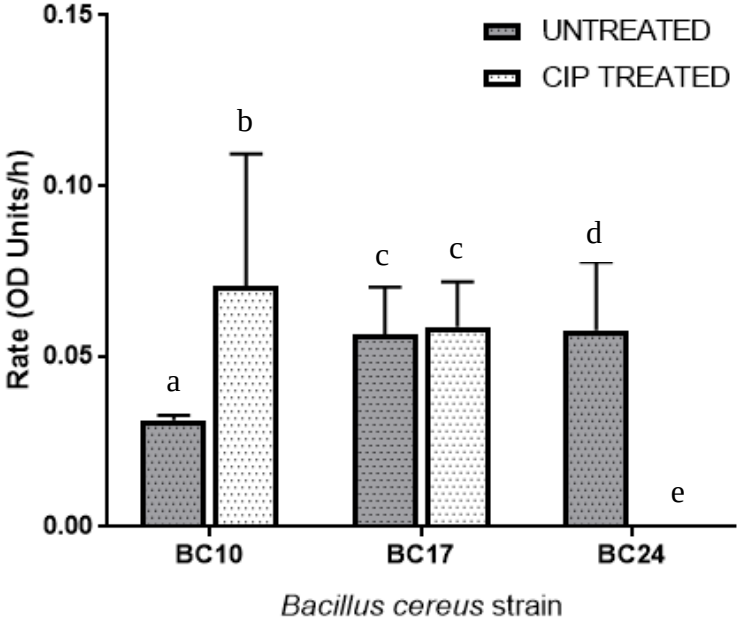

Figure 19. Germination and growth rate of untreated (non-simulated CIP treated control) and simulated CIP treated spores from *B. cereus* strains isolated from filler nozzles (strains BC10, BC17) from ESL milk processing lines and raw milk (strain BC24) as estimated by the Baranyi model.
*Bars with different letters for each strain indicate significant difference.

Following simulated CIP treatment, there was an evident increase in the growth rate of strain BC10 indicating simulated CIP treatment increased the growth rate whereas simulated CIP treatment markedly decreased the growth rate of strain BC24 (Figure 19). There was no difference in the growth rate regarding treatment effect (Table 5). There was, however, a significant ($p \leq 0.05$) difference in the growth rate of *B. cereus* strains with regards to strain effect as well as the interaction between strain and treatment (Table 5), and this interaction accounted for the highest variation at 48.26% of the total variation of the growth rate. Strain effect represented 17.41% of the total variation of the growth rate. Therefore, although treatment by itself showed no difference, the combination of strain and treatment showed to have a significant ($p \leq 0.05$) effect on the growth rate of the *B. cereus* strains. When the growth rate of simulated CIP treated and untreated spores were compared there was a significant ($p \leq 0.05$) difference in the growth rates of strains BC10 and BC24 (Table 6), thus their growth rate was strongly influenced by simulated CIP treatment. However, there was no difference between the growth rates of the simulated CIP treated and untreated spores of strain BC17 (Table 6).

4.3.4.5 Biofilm formation of untreated (non-simulated CIP treated) and simulated CIP treated *Bacillus cereus* spores

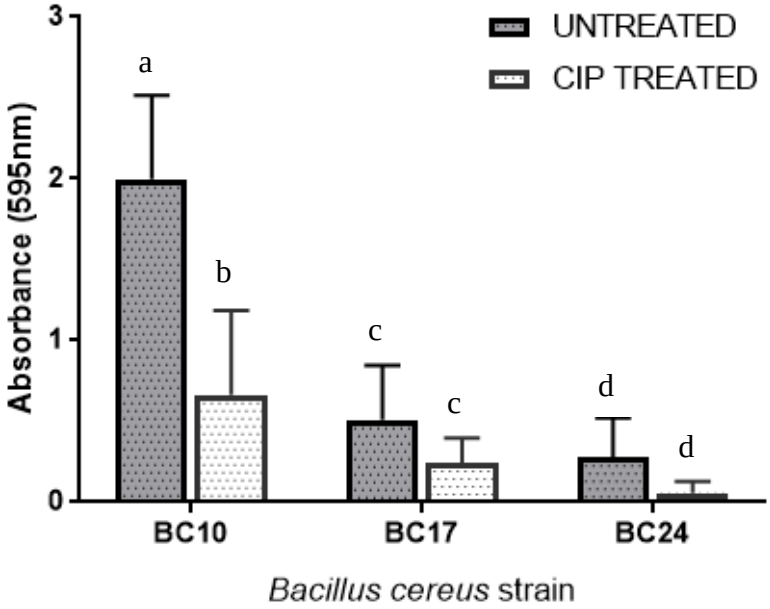

Figure 20. Biofilm growth of untreated (non-simulated CIP treated control) and simulated CIP treated spores from *B. cereus* strains isolated from filler nozzles (strains BC10, BC17) from ESL milk processing lines and raw milk (strain BC24).
*Bars with different letters for each strain indicate significant difference.

B. cereus strain BC10 formed a stronger (highest absorbance value) biofilm compared to the untreated biofilms of the other two strains, and strain BC24 formed a noticeably weaker (low absorbance value) biofilm following simulated CIP treatment compared to the strains isolated from filler nozzles (Figure 20).

Strain, treatment and interaction showed to have a significant ($p \leq 0.05$) effect on biofilm formation (Table 5). Strain effect represented 60.15% of the total variation. Treatment effect accounted for 23.92% of the total variation and interaction between strain and treatment made up 15.35% of the total variation. Strain effect was the most significant ($p \leq 0.05$) with regards to biofilm formation.

There was a significant ($p \leq 0.05$) difference between the biofilm formation of simulated CIP treated and untreated spores for strain BC10 (Table 6). However, simulated CIP treatment did not affect the biofilm formation of strains BC17 and BC24 (Table 6).

4.3.4.6 SEM analysis of untreated (non-simulated CIP treated) and simulated CIP treated *Bacillus cereus*

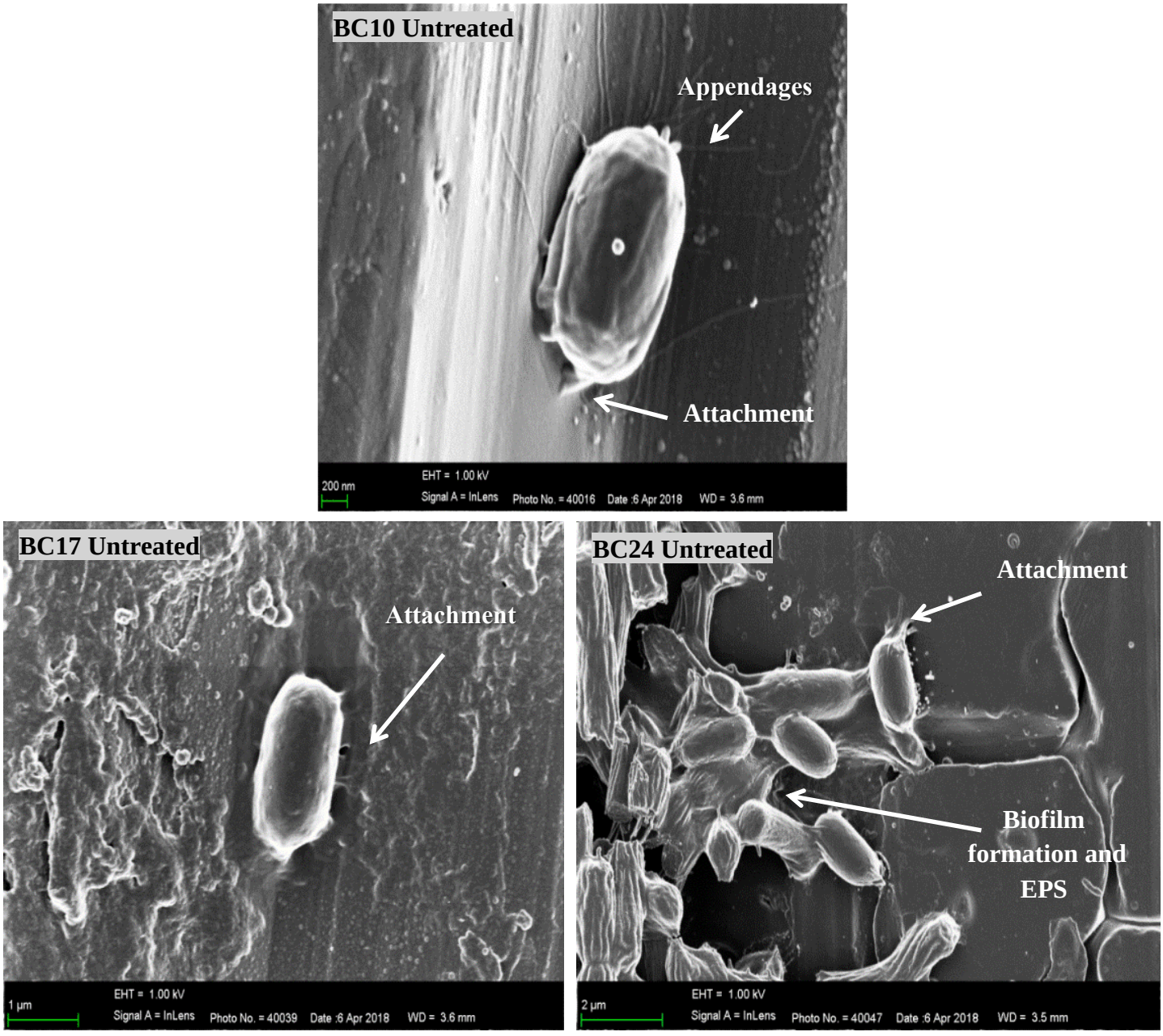

Figure 21. SEM micrographs of untreated (non-simulated CIP treated control) *B. cereus* strains isolated from filler nozzles (strains BC10, BC17) from ESL milk processing lines and raw milk (strain BC24) during ESL milk processing attached to a stainless steel surface.

SEM micrographs of untreated spores from all three *B. cereus* strains showed attachment of intact spores to the stainless steel surface (Figure 21). Bacterial pores 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 possible spores on the micrographs in order to distinguish between spores and vegetative cells. The spores had a layer covering them which could be the conditioning film. The conditioning film could consist of some milk components such as protein as well as EPS that are secreted by the bacteria into the environment. The EPS contribute to the functional and structural integrity of biofilms. Long appendages enable great adherence and are visible in the SEM micrographs (Figure 21).

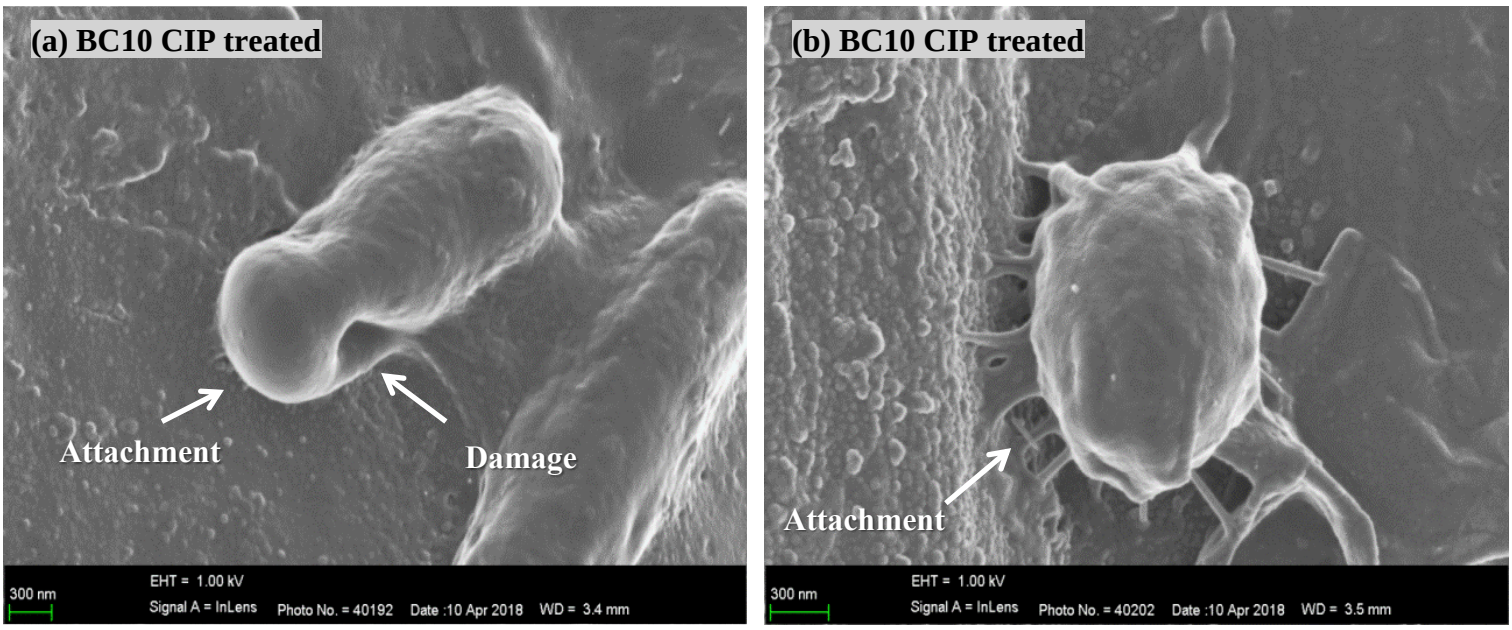

Figure 22. SEM micrograph showing the attachment on steel by *B. cereus* strain BC10 emanating from spores isolated from filler nozzles which have been subjected to CIP treatment.

SEM micrographs of strain BC10 from filler nozzles showed attachment of *B. cereus* to the stainless steel surface following simulated CIP treatment (Figure 22a and b). Some damage to the outer layer of the spore was visible (Figure 22a); however, intact spores were also detected following simulated CIP treatment (Figure 22b). Nonetheless, these micrographs (Figure 22a and b) indicated that strain BC10 was able to attach to stainless steel and form a biofilm following simulated CIP treatment.

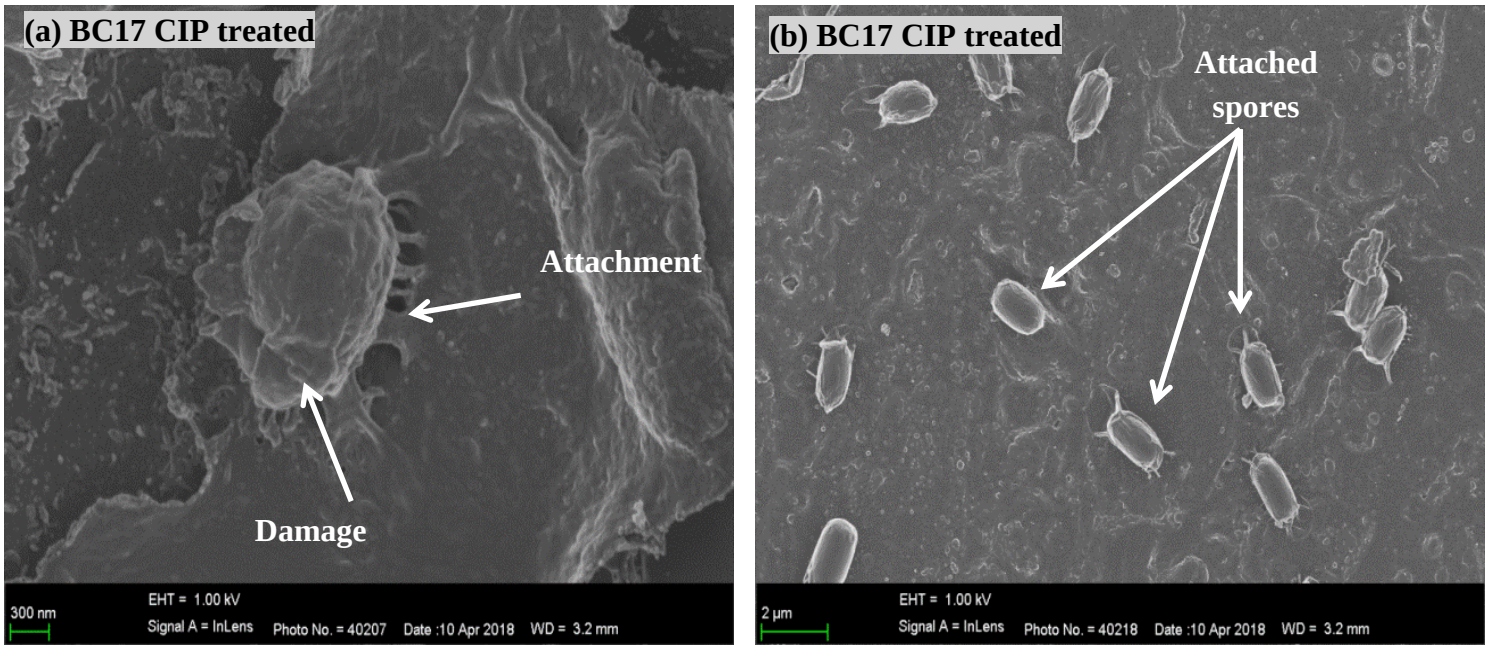

Figure 23. SEM micrograph showing the attachment on steel surface by *B cereus* strain BC17 emanating from spores isolated from filler nozzles and which have been subjected to CIP treatment.

SEM micrographs showed the attachment of spores from *B. cereus* strain BC17, isolated from filler nozzles, to the stainless steel surface (Figure 23a and b) as was seen with strain BC10 (Figure 22a and b). The outer layer of the spore appeared to be more tortuous (Figure 23a) compared to strain BC10 (Figure 22a and b). There was damage evident to the spores due to shrinking and twisting (Figure 23a). However, some spores did not show any visible damage and were still capable of adhering to the stainless steel (Figure 23b).

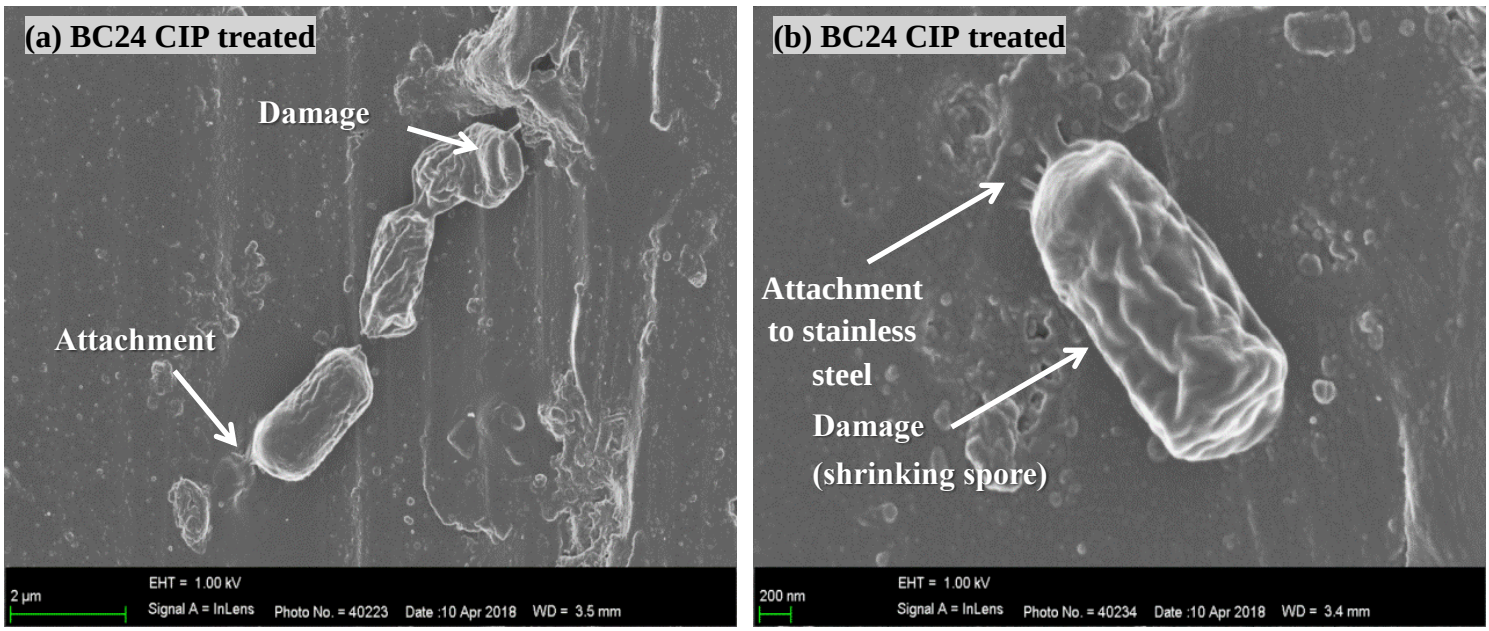

Figure 24. SEM micrograph showing the attachment on steel surface by CIP treated *B cereus* strain BC24 emanating from spores isolated from raw milk during ESL milk processing.

The SEM micrographs, for the *B. cereus* strain from raw milk, following simulated CIP treatment showed visible attachment of spores to the stainless steel surface (Figure 24a and b). Similar to strain BC17 (Figure 23a and b), the spore was more tortuous compared to strain BC10 (Figure 22a and b). Most of the spores appeared to be damaged (Figure 24a) and correspondingly to strains BC10 (Figure 22a and b) and strain BC17 (Figure 23a and b), spores from strain BC24 were still capable of attachment following simulated CIP treatment (Figure 24b).

4.3.5 DISCUSSION

Milk can be contaminated by *B. cereus* at a low infective dose of 10^3-10^4 bacterial cells/g (Ali *et al.*, 2017). Pasteurisation is not sufficient to eradicate all spores. Additionally, the spores may thrive in cold environments due to their inherent psychrotrophic nature, and the appendages on the spore surface allow for adherence to the surfaces of dairy processing equipment, therefore, spores may persist throughout routine cleaning procedures (Ali *et al.*, 2017).

Simulated CIP treatment had no apparent effect on the survival of strains BC10 and BC24 after 28 days at a storage temperature of 5 °C. Strain BC17, however, had a noticeable increase in the Log counts following simulated CIP treatment compared to untreated spores (non-simulated CIP treated). The increase in growth for strain BC17 following CIP treatment could be as a result of a stress response or its inherent emerging ability to grow at lower temperatures. Strain BC17 was isolated from biofilms in filler nozzles during ESL milk processing, and this may also have contributed to its apparent resistance toward CIP.

Stresses during storage may also influence the survival of the spores as well as their germination and ultimate outgrowth (Wells-Bennik *et al.*, 2016). While *Bacillus* spp. are generally mesophilic it has been reported that psychrotolerant strains do exist (Mugadza and Buys, 2018). Moreover, some *B. cereus* strains are capable of germinating and growing under refrigerated conditions (Schmidt *et al.*, 2012). According to Mugadza and Buys (2018), certain mesophilic bacteria may possess the 16S psychrotrophic signature which could enable them to display psychrotrophic characteristics during cold storage at 7 °C (Von Stetten *et al.*, 1998), and that the minimum growth temperature for *Bacillus* spp. is between 10 and 20 °C, but psychrotolerant strains have been isolated that grew at 6 °C (De Vos *et al.*, 2009).

In this study over 28 days, the day factor only had an effect on the Log counts of spores from *B. cereus* strain BC17. However, the day factor overall had a significant effect on all the *B. cereus* strains. Consequently, if the milk is stored for a prolonged period of time at 5 °C, Log counts would potentially increase. This implies that regardless of CIP treatment spores can germinate and multiply in contaminated milk to significant levels the longer the milk is stored at 5 °C.

After 28 days of storage at 5 °C, there was a significant difference in the Log counts as opposed to only 14 days. This could be due to the prolonged shelf life of ESL milk providing *B. cereus* spores adequate time to germinate and grow to significant numbers. The ability of spores from strain BC17 to germinate and grow, even under refrigeration, may be a consequence of the extended shelf life of ESL milk. ESL milk has a prolonged shelf life which is three times the length of that of pasteurised milk. This enables bacteria to grow to high numbers in ESL milk until the expiration date is reached (Schmidt *et al.*, 2012). The ability of bacteria to grow to significant numbers could indicate a possible food safety risk with regards to *B. cereus* spores, especially psychrotolerant strains.

Naturally associated microbiota are less densely concentrated in ESL milk which results in fewer competitors which possibly contributes to a single species gaining dominance in the ESL milk (Schmidt *et al.*, 2012). In a study by Schmidt *et al.* (2012), mesophilic strains of *B. cereus* grew to counts of 6 Log_{10} CFU/ ml following 14 days of storage at 8 °C likely due to a low presence or absence of competitive bacteria in ESL milk.

In this study, the difference in Log counts was significant ($p \leq 0.05$) within simulated CIP treatment when strains BC17 and BC24 were compared. Similarly, differences in the Log counts within simulated CIP treatment were significant ($p \leq 0.05$) when *B. cereus* strains BC10 and BC17 were compared. There was no difference in the Log counts when simulated CIP treated and untreated spores were compared indicating that simulated CIP treatment had no effect on the overall Log counts of *B. cereus* strains BC10, BC17 and BC24 in milk over 28 days at a storage temperature of 5 °C.

There was no difference when Log counts of untreated and simulated CIP treated spores for strains BC10 and BC24 in milk were compared throughout 28 days at a storage temperature of 5 °C, demonstrating that simulated CIP treatment did not affect the growth of spores in milk over 28 days when stored at 5 °C. In a study by Brent Seale *et al.* (2011), spores and

vegetative cells treated with 1% caustic had an increase in their relative hydrophobicity, possibly contributing to the resistance of the spores. The resulting increase in the hydrophobicity is conceivably due to the hydrolysis of the spore coat as a result of the spore coat being solubilised (Brent Seale *et al.*, 2011).

Apart from the absence in the overall effects of strain and treatment factors on the Log counts, multiple comparisons of strain responses at each day indicated that strain BC17 had unusual growth behaviour compared to strains BC10 and BC24. Strain BC17 showed a significant ($p \leq 0.05$) difference in its Log counts between days 0 and 28. The difference in the bacterial load may be as a result of the quality of the raw milk, water and equipment quality as well as the season of production and the storage conditions (Schmidt *et al.*, 2012; Mugadza and Buys, 2018). A difference in counts in the early days is most likely attributed to differences in survival and recovery after CIP.

Good quality raw milk and good hygienic processing conditions contribute to the low levels of *B. cereus* present in milk even though *B. cereus* does not compete well. According to Mugadza and Buys (2018), *B. cereus* still poses a safety risk in spite of the organism being isolated in low quantities.

Furthermore, it has been shown that psychrotrophic *B. cereus* spores can germinate at temperatures lower than their minimum temperature of 4 °C (Larsen and Jorgensen, 1999; Mugadza and Buys, 2018). Overall, despite being treated with simulated CIP, spores from *B. cereus* strain BC17 showed an increase in the Log counts following simulated CIP treatment. Despite this fact, taking into consideration the length of the storage time (28 days), all strains (BC10, BC17 and BC24) demonstrated growth at a storage temperature of 5 °C. However, results suggested that strain BC17 (isolated from filler nozzles during ESL milk processing) might inherently handle stresses differently compared to strains BC10 and BC24.

The resistance towards simulated CIP treatment demonstrated by strain BC17 might be attributed to the fact that strain BC17 had been exposed to CIP chemicals multiple times before, as it was isolated from filler nozzles during ESL milk processing. In the study by Mugadza and Buys (2017), results suggested that strain BC17 did not possess the psychrotrophic 16S signature; however, in this study results indicated that strain BC17 could potentially have psychrotrophic tendencies; therefore, the inherent phenotypic psychrotrophic nature of this strain should be studied further. Moreover, the resistance of strain BC17 could imply that although strain BC10 did not show the same response as strain BC17, it may still

potentially build up resistance to CIP chemicals upon increased exposure to CIP. The seeming low tolerance of strain BC24 towards stresses could be due to this strain never being exposed to CIP chemicals before as this strain was isolated from raw milk during ESL milk processing, and it had not yet built up resistance towards CIP chemicals.

Therefore, even though *B. cereus* may not be the most highly competitive organism in ESL milk, it should be noteworthy as it can potentially grow at 7 °C (Mugadza & Buys, 2017). *B. cereus* is also capable of producing food poisoning toxins, i.e. the non-haemolytic enterotoxin, the single protein cytotoxin and the emetic toxin cereulide. Thus, apart from spoilage, *B. cereus* spores could be a food safety concern as these strains could potentially germinate at temperatures as low as 4 °C and be able to grow at temperatures under 10 °C (Dufrenne *et al.*, 1994; Mugadza and Buys, 2018).

Growth curves were used to determine the effect of simulated CIP on the germination of spores from *B. cereus* strains isolated during ESL milk processing from filler nozzles (strains BC10 and BC17) and raw milk (strain BC24). The lag phase is the length of time necessary for a bacterial population to commence exponential growth after transitioning to a new environment (Maier, 2009), for instance after CIP treatment.

The stationary phase in a batch culture can be defined as a state of no net growth, meaning growth is merely balanced by an equal number of cells dying (Maier, 2009). Following simulated CIP treatment, the lag phase of spores from strains BC10, BC17 and BC24 were longer compared to the lag phase of untreated spores, indicating that simulated CIP contributed to the increased lag phase duration.

The extended lag phase can be attributed to CIP components (caustic soda and acid wash combined with heat) inhibiting the germination and subsequent outgrowth of spores. However, spores from *B. cereus* strains isolated from filler nozzles survived simulated CIP treatment, whereas growth from the strain isolated from raw milk was not substantial following simulated CIP treatment as this strain (BC24) remained in the lag phase. The spores from *B. cereus* strains BC10 and BC17 survived simulated CIP possibly due to the exosporium and spore coat providing protection against chemicals (Setlow, 2014). The inner membrane surrounding the core contains various proteins that protect against chemicals (Setlow *et al.*, 2002). These proteins are also necessary for germination (Wells-Bennik *et al.*, 2016), and the proteins may have been denatured by simulated CIP treatment leading to the extended lag phase duration.

In this study strain largely influenced the lag phase. The combination of strain and treatment (simulated CIP treatment) effect was significant, but treatment alone had no effect. This indicated that without the effect of strain, simulated CIP would be ineffective. The growth of germinated spores that survived simulated CIP treatment was more rapid in spores from strains isolated from filler nozzles during ESL milk processing compared to the growth rate of untreated *B. cereus* spores. The *B. cereus* strain isolated from raw milk during ESL milk processing remained in the lag phase. In this study, the growth rate and lag phase of *B. cereus* depended on the strain as well as CIP treatment. The growth rate was largely influenced by strain variation and the interaction of strain and simulated CIP treatment which caused a decrease in the growth rate, and the lag phase lengthened. However, simulated CIP treatment did not affect the growth rate.

The CIP acid used during the simulated CIP treatment in this study could have induced an acid stress response during which the growth rate resumed after the initial delayed lag phase. The subsequent resistance towards CIP chemicals may be ascribed to the ability of *B. cereus* cells to repair damage during the lag phase leading to the increase in internal pH and ATP concentration before growth commences. When exposed to an acidic environment it may lead to heightened protection once cells are consequently exposed to more fatal stress such as heat.

Consequently, the result could be amplified protection once bacteria are exposed to milder stress – this is often called cross-protection (Mols and Abee, 2011). As a result of the response to acid stresses, and the phenomena of cross-protection, the consequences for controlling *B. cereus* in food processing equipment may be severe. In a study by Den Besten (2010), genes in *B. cereus* that are involved in stress response were upregulated upon exposure to acid conditions. Such genes included protein repair chaperones and heat stress regulators. These stresses can be put into use as biomarkers for bacterial toughness (Den Besten, 2010).

The growth between *B. cereus* strains for simulated CIP treated and untreated spores differed and this could be attributed to the fact that within a bacterial population, even in a homogenous environment, there could be heterogeneity in the manner in which cells manage stress. This implies that a fragment of the bacterial population could be more resistant to stress leading to a stress response consisting of multiple phases (Den Besten, 2010). Following the lag phase bacterial growth may resume as a result of a population section

recovering or due to a small subpopulation being extremely resistant, therefore being able to adapt to a stressful environment and ultimately growing exponentially (Den Besten, 2010). Several strategies are employed by bacteria to adapt to stressful conditions, leading to bacteria becoming increasingly robust. In order to guarantee the microbial safety of food, it is essential to understand the stress responses of bacteria to gain a better understanding of improving food processing methods (Den Besten, 2010). In a study by Den Besten (2010), when cells in the exponential phase were exposed to 5% salt, the result was an initial lag phase right after the change in salinity, thereafter growth commenced without an increase in viable counts.

In a study by Mols and Abee (2011) the germination of spores from *B. cereus* were not affected by a mildly acidic pH of 5.5, however, there was a delay in the germination and a decrease in the outgrowth of cells (Mols and Abee, 2011; Van Melis *et al.*, 2011). The inefficient germination following simulated CIP treatment in this study might be due to the hydrophobicity of sorbic acid leading to the accumulation of sorbic acid in the inner membrane of the spore which ultimately inhibits the signalling action that is necessary for germination mediated by germinant receptors (Mols and Abee, 2011).

According to Mols and Abee (2011), transcriptome analyses showed that *B. cereus* spore germination and outgrowth occurs via a tightly controlled spore outgrowth programme regardless of acidic conditions. In contrast, we found that certain strains, from raw milk, were affected by conditions involving CIP acid. Furthermore, Mols and Abee (2011) found metabolic rearrangements in *B. cereus* following exposure to low pH which are associated with acid resistance and genes encoding for enzymes were induced following acid shocks. Consequently, bacterial enzymes were activated upon acid exposure which contributed to pH homeostasis (Mols and Abee, 2011).

B. cereus uses electron transport to transfer protons across the membrane of the cell thereby generating excessive protons outside the cell, subsequently creating a proton motive force. Protons are prevented from leaching into the cell and are also be pumped out of the cell. By producing alkaline products, i.e. ammonia, cells may counteract the cytoplasm being acidified (Mols and Abee, 2011).

Regardless of CIP treatment, biofilm formation varied between strains. It was evident that *B. cereus* strains from filler nozzles formed more substantial biofilms than the strain from raw milk which formed the weakest biofilm. Furthermore, simulated CIP treated spores formed

considerable biofilms, demonstrating that simulated CIP treatment had an inhibiting effect on biofilm formation.

The slow growth rate of bacterial cells within a biofilm could be as a result of a general stress response and not necessarily due to limiting nutrients. A stress response causes physiological changes to protect the cells from the stresses in the environment such as temperature shock, pH changes or chemical agents (Mah and O'Toole, 2001). The cell could become stressed by organic acid permeating the cell. The permeation results in protons being imported into the cell causing a depressed cytoplasmic pH as well as concentrated organic anions in the cytoplasm in proportion to the transmembrane difference. Consequently, the cell counteracts this by expending ATP to extrude protons or to break down organic acid molecules that have not been dissociated (Mah and O'Toole, 2001).

SEM was used to observe the effect of simulated CIP treatment on the attachment of *B. cereus* spores isolated during ESL milk processing from filler nozzles and raw milk. In this study it was found that untreated *B. cereus* spores attached to the stainless steel surface. Long appendages present on the surface of the spore permit great adherence by overcoming electrostatic repulsive forces (Marchand *et al.*, 2012; Khoza, 2015), and appendages were visible for both simulated CIP treated spores and untreated spores. Surrounding the spores was a layer which could be a conditioning film consisting of some milk components as well as EPS that are secreted by the bacteria. The EPS contribute to the functional and structural integrity of biofilms (Khoza, 2015).

Following simulated CIP treatment, *B. cereus* spores from all the strains were also able to attach to stainless steel suggesting that CIP does not affect the attachment apparatus of spores. However, the outer layer of the spore appeared to have been damaged by the CIP components. The weakened spore coat directs to the structural integrity being compromised which could result in the inhibition of endospore germination (Ali *et al.*, 2017). Brent Seale *et al.* (2011) reported that viable planktonic *Bacillus* spp. spores attached to stainless steel were reduced by 40% following treatment with 1% caustic soda, and that spores attached to a surface were more resistant towards CIP processes compared to planktonic spores (Faille *et al.*, 2001; Brent Seale *et al.*, 2011). Some species of bacteria tend to form filaments upon exposure to stressful conditions (Den Besten, 2010).

Khoza (2015) confirmed that isolates originating from ESL filler nozzles were able to attach and form biofilms on stainless steel, supporting the results obtained from SEM analysis in

this study. In a study by Brent Seale *et al*. (2011), it was found that the growth of spores exposed to caustic wash (1% NaOH at 65 °C for 30 minutes) were reduced by 2 Log counts and that these spores' hydrophobicity was six times higher than that of untreated spores. In addition, caustic treated spores had an increased negative charge possibly due to the partial hydrolysis of the spore coat. In addition, spores surviving caustic treatment attached to stainless steel surfaces in higher numbers compared to untreated spores (Brent Seale *et al.*, 2011).

4.3.6 CONCLUSIONS

CIP treatment has an effect on the structure of *B. cereus* spores. However, *B. cereus* is capable of attaching to stainless steel. CIP treatment has no effect on the attachment of *B. cereus* spores to stainless steel or on the ability of *B. cereus* spores to germinate and grow in milk over 28 days if stored at 5 °C. CIP treatment is less effective against *B. cereus* strains from filler nozzles than raw milk. *B. cereus* strains that survive CIP may be less susceptible to subsequent CIP treatment. Furthermore, *B. cereus* spores can survive CIP treatment and subsequently attach to filler nozzles in dairy processing plants, thereby contaminating final ESL milk products. If ESL milk is stored for an extended length of time, *B. cereus* spores will germinate, and this could pose a food safety risk to the consumer.

5. GENERAL DISCUSSION

5.1 CRITICAL REVIEW OF METHODOLOGY

This study was aimed at determining the effect of simulated CIP treatment on the structure, attachment and viability of spores from *B. cereus* strains isolated during ESL milk processing from filler nozzles and raw milk.

Although spore isolation techniques from previous studies were followed, we discovered that spores evidently differ between and within species and therefore the method used for spore isolation in this study had to be modified using multiple trials. Challenges experienced during the first set of trials of *B. cereus* spore isolation were that the spore incubation period was not adequate (minimum 21 days), which led to the agar not drying properly, and in turn it was difficult to scrape cells (mostly spores) from the agar without scraping agar with the cells. An inefficient incubation period also led to too many viable cells still growing on the agar, meaning centrifugation took much longer to separate spores from vegetative cells. Eventually, the method proved to be successful. Overall, it was incredibly challenging working with spores as they are extremely unpredictable and resilient.

In this study, CIP treatment was simulated in a lab setting and had to be carried out as realistically as possible to the CIP process used in a processing facility. In a processing facility, the CIP process is accompanied by flow rates and raised temperatures, which increases the efficiency of the process. In this study the exact flow rates could not be imitated, though the closest alternative was to create pressure utilising a micropipette tip whereby spores were flushed with CIP chemicals in an Eppendorf tube, which in effect created turbulent flow. The exact temperatures at which CIP takes place in a processing facility was imitated by floating Eppendorf tubes in a temperature-controlled water bath at the specified temperature for the specified length of time.

FCM, epifluorescent microscopy and TEM were used to determine the effect of simulated CIP treatment on the structure of the *B. cereus* spores. An advantage with using FCM is that it can measure multiple parameters for an individual cell in a bacterial population. It thus determines the physiological condition of individual spores, as well as the heterogeneity of the bacterial population in one sample. Advantageously, FCM is a rapid method in which a large amount of spores can be measured simultaneously (Cronin and Wilkinson, 2007). One

disadvantage with FCM is that it is costly, and it is not yet a common analysing technique. Consequently, FCM equipment is not always freely available and accessible. Also, FCM requires some training to run the analysis, as well as on the interpretation of results following FCM analysis.

Furthermore, care should be taken when handling FCM dyes as they are potentially carcinogenic. The dyes used in this study, SYTO 9 and PI, proved to work well when analysing the spores. SYTO 9 was effective due to its green fluorescence intensity determining the physical state of the spore. However, SYTO 9 was especially costly and not readily available. PI was an easy dye to work with as it only binds to damaged spores. An advantage with PI compared to traditional colony-forming unit counts is that it can report viable but nonculturable cells as being live, which correctly recognises them as being potentially harmful (Stocks, 2004).

In our study, due to the potential pathogenicity of *B. cereus* and the resistant nature of spores, it was difficult to get a hold of a cell sorting flow cytometer. This would have been useful to sort damaged spores following simulated CIP treatment from undamaged spores so that they can be further analysed. If SYTO 9 and PI are combined in a cell, SYTO 9 will be displaced as PI has a high affinity for nucleic acid. Thus, the cells would fluoresce red. However, some strains have efflux pumps that can actively eradicate PI from the cell (Stocks, 2004). Therefore, the combination of SYTO 9 and PI would not be recommended for future studies.

The *B. cereus* spore structures were further analysed with epifluorescent microscopy and TEM. Epifluorescent microscopy was disappointing due to it being time-consuming and the resulting images not being as clear as was anticipated. However, visualising the intensity of the green fluorescence of damaged spores made it worthwhile. Although the preparations for TEM were rather tedious, it proved to be a better analysis compared to epifluorescent microscopy due to the more detailed and highly magnified images obtained. However, for the CIP treated spores, TEM did not reveal any intact spores as was seen in FCM and epifluorescent microscopy. This may have been due to the fact that TEM permits the observation of individual spores. Nevertheless, it is impossible to analyse each and every single spore in the population. Thus, possibly there were intact spores somewhere in the population, they were just not observed by the TEM.

Growth curves were fitted using the Baranyi model which was carried out by the DMFit 2.0 program. Primary growth predictive models assist food microbiologists to predict the growth

of bacteria, and this method is also much less time-consuming and tedious in comparison to the plate count method (Pla *et al.*, 2015). According to Pla *et al.* (2015), compared to other predictive models, the Baranyi model gave the best-fitted growth curves and was the most capable model to fit optical density data. Furthermore, similar results were obtained when the microbial growth was measured by plate counts (Pla *et al.*, 2015). It may seem as though the growth experiment ended too soon at 24 h; however, extending the incubation time beyond 24 h for this experiment would not have been representative of a typical factory scenario where spores would have less than 24 h to germinate and grow before the next cycle of CIP would take place.

Biofilm formation of germinated *B. cereus* spores was assessed by quantifying biomass by employing optical density measurements. According to Hussain and Oh (2017), numerous studies such as the crystal violet assay, cell enumeration and sporulation can be used to analyse biofilm formation physiology. In this study, the crystal violet assay was used. In a study by Hussain and Oh (2017), the crystal violet assay correlated with the amount of immobile cells and spores during the early stages (first 24 h). Because the biofilm is incubated for a more extended period of time, i.e. 72 h, EPS will typically increase which may lead to a decrease in the correlation between the crystal violet assay and the amount of spores (Hussain and Oh, 2017). Results by Hussain and Oh (2017) showed no correlation between the concentration of planktonic cells and the crystal violet assay. Hussain and Oh (2017) further stressed the importance of using correlation between cell enumeration and the crystal violet assay when describing a biofilm phase related to sporulation. Crystal violet binds to EPS and immobile cells in the biofilm. Thus the crystal violet assay is considered to be a high data output method for the estimation of biofilm development (Hussain and Oh, 2017).

SEM was a useful tool in assessing the physical state and the attachment of *B. cereus* spores to stainless steel. During the first trial, *B. cereus* spores were inoculated into full-fat milk with stainless steel strips and incubated for 24 h in a shaking water bath as was described in the method used by Khoza (2015). This method was less successful in our study as milk constituents such as fat and protein covered the spores on the stainless steel strips which meant no spores were visible upon SEM observation. The absence of spores on the stainless steel strips during the first observation may have been due to the shaking water bath causing too much agitation; possibly leading to the spores not being able to settle and attach to the stainless steel strips. The method was revised, and during the second trial *B. cereus* spores

were inoculated into fat-free milk and incubated in a non-shaking incubator for 36 h. The improved method was successful as spores were attached to stainless steel strips and were clearly visible by SEM.

During the biofilm assay (biomass quantification assay) spores from *B. cereus* strains BC17 and BC24 did not form substantial biofilms after being incubated for 24 h, whereas with SEM observations, all three strains were capable of attaching and forming biofilms after 36 h of incubation. This is in favour of factory cleaning regimes, because CIP should be run at least once within 24 h. Therefore, biofilms that develop from germinated spores may not have enough time to become established in processing equipment.

The first trial of the viability assessment of *B. cereus* spores in milk following simulated CIP treatment was unsuccessful due to the initial concentration of the spore inoculum (20 spores/ml) being too low and subsequently, spore germination and growth was insufficient. The second trial proved to be more successful as the adjusted inoculum concentration (200 spores/ ml) sufficed, but this trial was also unsuccessful as the dilution factor to be plated out had been underestimated. The resulting plate counts were too numerous to count, and thus some data was missing. During the third and final trial the dilution factor was revised and the necessary amount of media plates was prepared. This method was challenging due to the extensive amount of media required for the analysis (cultures had to be plated out up until a dilution factor of 10^{-12} (in duplicate)). The study was essential in determining the viability and importance of *B. cereus* spores in ESL milk and how it affects the shelf life and safety of ESL milk.

5.2 RESEARCH FINDINGS

FCM analysis, epifluorescence microscopy and TEM demonstrated that CIP treatment structurally damaged *B. cereus* spores which may lead to the inhibition of the germination of spores. 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 of time.

SEM analysis showed that CIP damaged spores from *B. cereus* strains isolated from filler nozzles and raw milk during ESL milk processing to some extent, but that spores were still 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 (Khoza, 2015). Khoza (2015) noted that the CIP process used in the processing plant was effective due to low total plate counts (TPC) from the ESL filler nozzles. On the contrary, according to Mogha *et al.* (2014), biofilms developed on the sides of gaskets regardless of CIP procedures (Khoza, 2015). As yet, there are no CIP standards available to determine the effectiveness of the CIP process in the dairy industry (Khoza, 2015). Although the TPC were low in the study by Khoza (2015), it indicated that the bacteria isolated from the filler nozzles during ESL milk processing were capable of surviving the sanitisers and disinfectants used in the cleaning programme since bacterial cells were found in the ESL filler nozzles. This supported Brooks and Flint (2008) who said that in the dairy industry routine CIP processes may not always be sufficient to remove all bacterial cells that have attached to a surface (Khoza, 2015).

While cells within a biofilm are more resistant to chemical cleaning agents compared to planktonic cells (Dhillon, 2012; Khoza, 2015), the results from the biofilm assay in this study indicated that planktonic *B. cereus* spores treated with simulated CIP were still capable of forming biofilms. Although simulated CIP treatment affected the ability of *B. cereus* spores to form biofilms, it was still possible regardless of exposure to simulated CIP treatment. This study also indicated that inherently some strains could be better biofilm formers than others.

The study by Schmidt *et al.* (2012) indicated that the application of microfiltration and pasteurisation during ESL milk processing might result in an increased risk for the onset of final ESL milk spoilage. According to Eneroth *et al.* (2001), regardless of ESL processes such as bactofugation and microfiltration significantly reducing the number of spore formers, the amount of spore formers increased in the final product as a result of post-processing

contamination most likely due to 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 in due course had the potential of growing under refrigerated conditions over a prolonged period, i.e. 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. If the storage temperature of packaged ESL milk is abused in a domestic household, and the final ESL milk product is consumed past its expiration date with levels of *B. cereus* exceeding 10^5 cells/ml milk, it could put the health of consumers in danger.

CIP treatment could possibly initiate a stress response, and this may induce the phenomenon of cross-protection which could challenge hurdle technology aimed at improving preservation and guaranteeing microbial safety. The correct combination of hurdles may be more effective in controlling bacteria compared to individual hurdles that may not be sufficient for inhibiting bacterial growth. However, the benefits of hurdle technology may be void due to the ability of bacteria adapting to stressful environments. The fact that *B. cereus* may endure stress such as CIP processing, as was shown in this study, is problematic as exposure to mild stresses over long and short periods has shown to prompt cross-protection against the more lethal stresses, and this could also have an effect on the virulence of pathogenic bacteria. Safety margins for food processing conditions might be affected by the heterogeneity of the bacterial population and the subsequent adaptive stress response thereof (Den Besten, 2010).

Mugadza and Buys (2017) and Khoza (2015) indicated that contamination in ESL milk mainly originates from ESL filler nozzles but that contamination could originate from multiple sources. Moreover, the quality of raw milk is hugely relevant to the ultimate quality of the finished ESL milk quality. Therefore, seeing as though the CIP process may allow certain strains of *B. cereus* to survive and ultimately thrive in ESL milk, essential care must be taken to guarantee good raw milk quality and good sanitation practices in the processing facility. The contamination of ESL milk can be as a result of microorganisms that adhere to stainless steel surfaces due to ineffective cleaning processes (Khoza, 2015). According to Khoza (2015), spore-forming bacteria that attach to ESL filler nozzles are capable of growing at refrigeration temperatures and could be the hurdle that is limiting the shelf life of ESL milk past 14 days. Our studies confirmed that certain strains of *B. cereus*, such as strain BC17, are capable of surviving CIP treatment, attaching to surfaces, as well as growing under refrigerated conditions over a long period of time.

In this study, we found that *B. cereus* strains isolated from filler nozzles during ESL milk processing proved to be more resistant toward simulated CIP treatment as opposed to the strain isolated from raw milk. These spores survived simulated CIP treatment because of their extreme sturdiness and possible built-up of resistance due to the continued exposure to CIP treatment in the processing area compared to unexposed strains in raw milk. As a result, *B. cereus* spores may contribute to the premature spoilage of ESL milk and could potentially be a food safety risk. Filler nozzles need to be cleaned more efficiently, and control measures for the effectiveness of the CIP process should be in place. In the processing facility manufacturing costs are reduced by the recycling of solutions of caustic wash, but the risk of contamination with spores may be equally increased (Brent Seale *et al.*, 2011).

6. CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

This study indicates that CIP treatment has an effect on the structure of *B. cereus* spores isolated during ESL milk processing from raw milk and filler nozzles. However, CIP treatment is less effective against *B. cereus* strains from filler nozzles, and CIP treatment does not affect the ability of *B. cereus* spores to attach to stainless steel or the ability of *B. cereus* spores to germinate and grow in milk over 28 days if stored at 5 °C. Consequently, *B. cereus* spores in biofilms that have survived CIP can be less susceptible to subsequent CIP treatment and may attach to filler nozzles in dairy processing plants. *B. cereus s*pores surviving CIP treatment may germinate upon favourable conditions and cause spoilage of the final ESL milk product which may lead to a reduced shelf life and potentially be a safety risk in ESL milk with a prolonged shelf life.

6.2 RECOMMENDATIONS

A further flow cytometric analysis with a cell sorting flow cytometer would be interesting to determine the viability of *B. cereus* spores that were deactivated by the simulated CIP process and those that have survived the CIP process. This would further justify the ability of certain spores to grow following CIP. Furthermore, determining the mechanisms of spores surviving CIP would be remarkable.

B. cereus spores already attached to stainless steel should be subjected to simulated CIP treatment and subsequently be analysed by SEM to determine how effective CIP is in removing attached spores as well as biofilms on stainless steel.

It would also be interesting to observe the effect of CIP treatment on the different stages of *B. cereus* spore germination. Additionally, a subsequent study should include the effect of each CIP chemical (i.e. caustic, acid and sanitiser) on *B. cereus* spores respectively.

Genotypic analysis of strains should be carried out to determine specific traits with regards to emerging psychrotrophic tendencies as well as the inherent increasing resistance towards CIP chemicals.

A model to predict the growth of *B. cereus* exceeding the shelf life of 28 days should be developed to predict the growth of pathogens and spoilage organisms in ESL milk.

Further studies should be carried out on strain BC17 to determine its potential emerging psychrotrophic nature, its resistance toward cleaning chemicals as well as its ability to form food poisoning toxins.

Potential control measures that will improve the efficiency of CIP in removing or inactivating *B. cereus* spores from filler nozzles and from the surface of processing equipment should be further investigated.

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8 RESEARCH OUTPUT FROM THIS WORK

Kruger, C., Buys, E.M. 2017. Effect of Cleaning In Place (CIP) on the germination of *Bacillus cereus* spores isolated from Extended Shelf Life milk. Academic poster presented at the 50th annual SASDT (South African Society for Dairy Technology) Symposium, Kievitskroon, Pretoria, South Africa.

Kruger, C., Buys, E.M. 2017. Effect of Cleaning In Place (CIP) on the germination of *Bacillus cereus* spores isolated from Extended Shelf Life Milk. Oral presentation at the annual SASDT student evening 2017, University of Pretoria, Pretoria, South Africa.

Kruger, C., Buys, E.M. 2017. Effect of Cleaning In Place (CIP) on the germination of *Bacillus cereus* spores isolated from Extended Shelf Life Milk. Oral presentation at the 22nd biennial SAAFoST (South African Association for Food Science and Technology) International Congress and exhibition, Century City Conference Centre, Cape Town, South Africa.

Kruger, C., Buys, E.M. 2018. Effect of Cleaning In Place (CIP) on the germination of *Bacillus cereus* spores isolated during Extended Shelf Life Milk processing. Oral presentation at the 51st annual SASDT Symposium, Kievitskroon, Pretoria, South Africa.