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EXTENDED SHELF LIFE MILK PROCESSING: EFFECT OF CLEANING-IN-PLACE (CIP) ON THE SURVIVAL OF *BACILLUS SUBTILIS*

By

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DECLARATION

I declare that the dissertation herewith submitted for the degree MSc Food Science at the University of Pretoria has not been previously submitted by me for a degree at any other university of institution of higher learning.

Nanamhla Adonis

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DEDICATION

I wholeheartedly dedicated this dissertation to my wonderful mother and number one fan Zukiswa Adonis

ABSTRACT

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Bacillus subtilis vegetative cells have been found to remain in dairy processing equipment such as filler nozzles and heat exchangers even after cleaning processes such as Cleaning in Place (CIP). The cells form biofilms on the stainless steel and continue to grow and spread, detach, and move to other processing areas and equipment further down the processing line, leading to cross-contamination and ultimately accelerated spoilage of Extended Shelf Life (ESL) milk during storage post-processing at refrigeration temperatures. The objective of this study was to determine the effect of simulated CIP on the physiological state of *B. subtilis* cells, their attachment and subsequent growth in understanding the effectiveness of CIP and subsequent survival of *B. subtilis* vegetative cells. Three *B. subtilis* strains previously isolated from packaged ESL milk and ESL milk stored at 4 and 7 °C were subjected to a simulated CIP procedure with cells then subjected to flow cytometry and scanning electron microscopy. Enzymatic analysis was performed to

determine the capability of the vegetative cells to produce proteolytic and lipolytic enzymes and the subsequent effect of these enzymes on the quality and shelf life of ESL milk. Flow cytometry results showed that approximately 98% of *B. subtilis* cells were physiologically dead after simulated CIP treatment, with 0.1% remaining viable. SEM revealed that the cells could reattach to stainless steel after simulated CIP treatment, and some cell multiplication was evidenced. The enzyme assays showed that all the *B. subtilis* strain cells continued to produce proteolytic enzymes after treatment, and only one strain could produce lipolytic enzymes. Over the 28 days of storage at 7 and 10 °C, the cells could grow in the milk. The results showed that simulated CIP treatment did not influence cell reattachment with bacterial growth evident 28 days at 7 and 10 °C after the treatment, accelerating the deterioration of the ESL milk. The industry must identify easier non-evasive methodologies of identifying biofilm formation and develop new food processing equipment coated surfaces that discourage the attachment of bacterial cells and spores.

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CHAPTER 1: INTRODUCTION AND PROBLEM STATEMENT

Recently, there has been increased consumer demand for minimally processed milk products with acceptable sensory properties coupled with extended shelf life. Consequently, the milk industry has developed innovative and safe processing techniques to deliver an array of shelf-stable milk products (Goff and Griffths, 2006, Koutchma and Barnes, 2013). This new development has indirectly benefited the dairy industry, culminating in decreased refrigeration storage space and energy costs. One such product is extended shelf life (ESL) milk, requiring processing temperatures of between 125-130 °C for approximately 2-5 s (Scott, 2008). Thus, ESL milk offers a longer shelf-life than pasteurised milk but retains similar essential sensory and nutritional characteristics as the latter. In recent times there has been the incorporation of filtration and bactofugation techniques in ESL milk processing to reduce heat processing temperatures whiles still maintaining shelf life and improving sensory properties. However, the most common processing method of ESL milk is indirect or direct heating temperatures of between 125-130 °C. Though these milk products are safe and shelf stable, the formation of biofilms on processing equipment remains a problem in the quality and safety of ESL and other thermal processing techniques of milk. Bacteria from biofilms can contaminate milk undergoing ESL processing. Biofilms are aggregated micro-colonies of bacterial cells which produce extracellular polymeric substances (EPS). These EPS play a protective role, acting as a barrier that restricts penetration of antimicrobial and cleaning agents (Donlan, 2001; Drenkard, 2003) during cleaning in place of industrial processing equipment.

By assessing filler nozzles of ESL processing equipment, Khoza (2015) hypothesised that post pasteurisation contamination of ESL milk could be attributed to the formation of biofilms on stainless-steel pipe surfaces. Consequently, that study confirmed that one of the reasons for post pasteurisation contamination of ESL milk was *Bacillus cereus* and *Micrococcus luteus* to form biofilms at the surfaces of filler nozzles and subsequently contaminating the milk. ESL milk should be stored at refrigeration temperatures, regardless of whether it is opened or unopened. However, *B. subtilis*, a psychrotolerant microbe, can grow at temperatures below 8 °C (Mugadza and Buys, 2014) and known to grow in ESL packaged milk actively. Thus, refrigeration may not be an effective hurdle for preventing the proliferation of *B. subtilis* vegetative cells in ESL packaged milk.

Additionally, *B. subtilis* is the most common spoilage species found in milk (Janštová et al., 2006; Svensson, 2000) and can form biofilms in equipment and storage tanks. Further, *B. subtilis* poses a health hazard and challenge to processing and cleaning regimes as it produces thermo-resistant spores which attach readily to surfaces such as stainless steel, glass, and rubber (Chmielewski and Frank, 2003; Svensson, 2000;). These bacterial spores can withstand high temperature treatment and subsequently contaminate heat treated milk, later germinate under favourable conditions, and affect the quality and safety of milk products. *Bacillus* spores in processed milk can lead to milk spoilage, and enterotoxins released by some species may pose a health risk (Marchand et al., 2012). Upon germination, these spores can cause milk spoilage by producing and releasing enzymes that affect the milk's integrity (Mugadza and Buys, 2014).

Bacillus spp. can produce extracellular thermostable enzymes, which affects ESL milk shelf life. These are proteolytic and lipolytic enzymes that promote the breakdown of essential milk proteins and fats responsible for milk's stability and quality (nutritional and sensory). This breakdown destabilises the milk composition and decreases its shelf-life (Janštová et al., 2006). The proteolysis of casein is of most significance, concerning the stability of α-casein, para-κ- casein and β-casein. These caseins are hydrolysed, resulting in coagulation of the milk proteins and whey separation, resulting in undesirable milk characteristics. Age gelation and free fatty acids (FFA) are other signs of enzymatic activity that affect the shelf life of ESL milk (Schmidt et al., 2011).

Extracellular polymeric substances (EPS) produced by the biofilm may also consist of extracellular enzymes that can affect the milk shelf life. Within the EPS, these enzymes are protected from heat degradation and may be released into the milk causing polymer degradation (Flemming et al., 2007). The objective of this study was to determine the effect of CIP on the growth and viability of *B. subtilis* vegetative cells within biofilms to improve the shelf life of ESL milk.

CHAPTER 2: LITERATURE REVIEW

2.1 Milk production in South Africa

Farming has been a very popular activity in South Africa, providing income for small scale farmers and jobs to thousands of people (DAFF, 2010). Over the past few years, there has been an increase in the demand for animal products such as meat and milk (Dovie et al., 2006), and the dairy industry has been working hard to meet this demand. Milk from cows and, to a small extent, goats and sheep are sometimes sold directly to consumers but largely to commercial dairy farmers for processing and onward sale to consumers (Dovie et al., 2006 and Lassen, 2012). Lassen (2012) reported that 98% of the milk produced in South Africa is sold formally to commercial processors, which is different to most other African countries where milk is mostly sold to smaller processors. However, in South Africa, there has been a rise in small scale milk processors informally selling milk and milk products to consumers.

As depicted in table 2.1, though the number of milk producers decreased from 3551 to 1253 (2009-2010), there was a 31% increase in production (2009-2018) (Lactodata, 2019), invariably leading to an 18% decrease in imported milk. The increased milk demand locally has meant that there is also a 7% drop in milk exports. The increase in milk production has been attributed to water and resource availability (Lassen, 2012), especially water being an important component of milk processing. However, the recent drought in the Western Cape is likely to impact milk production figures. The most recent production figures (2018) suggest that KwaZulu Natal, Western and Eastern Capes account for 85% of milk production in South Africa (Figure 2.1).

Table 2.1: Number of milk producers in South Africa over the last decade (2009-2019), adapted from LACTODATA 2019

Provinces	**Jan 2009**	**Jan 2011**	**Jan 2012**	**Jan 2014**	**Jan 2015**	**Jan 2016**	**Jan 2017**	**Jan 2018**	**Jan 2019**
Western Cape	795	683	647	529	533	502	481	419	402
Eastern Cape	387	314	283	264	262	251	244	212	201
Northern Cape	37	28	21	25	14	14	7	7	6
Kwa-Zulu Natal	373	323	322	281	267	253	247	221	212
Free State	884	601	535	389	328	280	249	206	165
North-West	540	386	352	233	222	181	165	135	117
Gauteng	217	127	126	109	100	97	98	84	83
Mpumalanga	286	201	164	117	94	93	87	69	56
Limpopo	32	23	24	14	14	12	15	12	11
Total	**3551**	**2686**	**2474**	**1961**	**1834**	**1683**	**1593**	**1365**	**1253**

Though the use and intake of fresh milk increased to 4.8% in 2018 from the previous year, UHT and pasteurised milk products still account for three-quarters of milk products on the SA market (Figure 2.2). Also, between 2011-2012, Lassen (2012) reported a slight increase in the demand for fresh milk and a 20% net increase for milk products (including soft cheeses and flavoured milk). This increased milk production mentioned earlier has resulted in new and increased milk products (Gertenbach, 2007). Increasing consumer demand for more innovative milk products will drive demand for milk and milk products in South Africa.

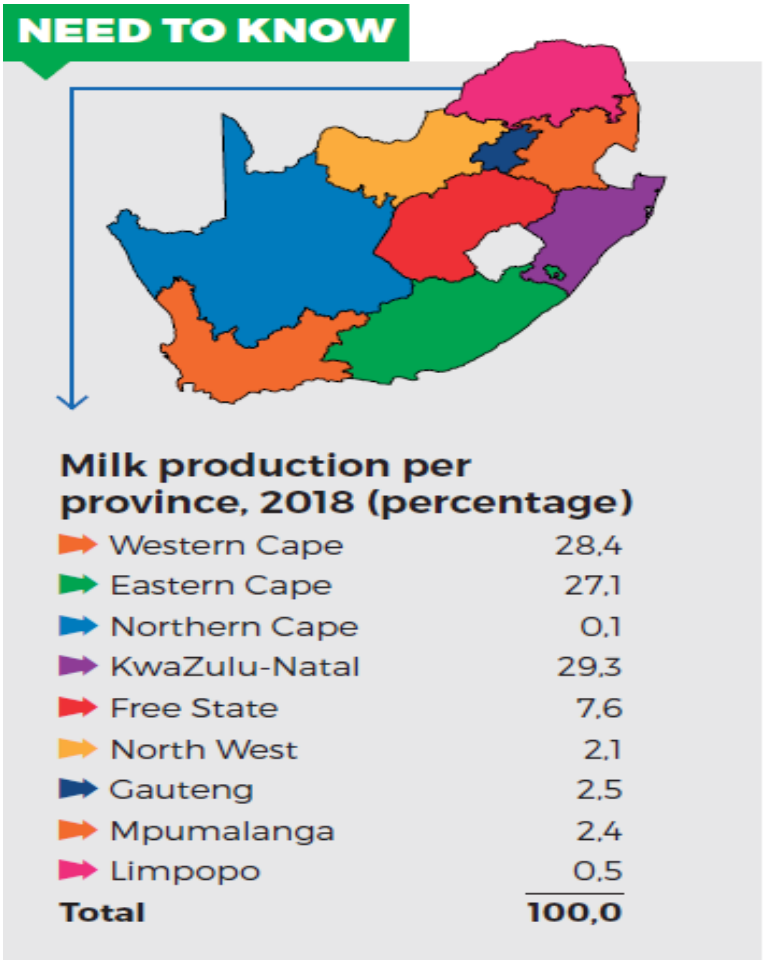

Figure 2.1: Milk production in South Africa per province between 2018-2019 (Lactodata, 2019)

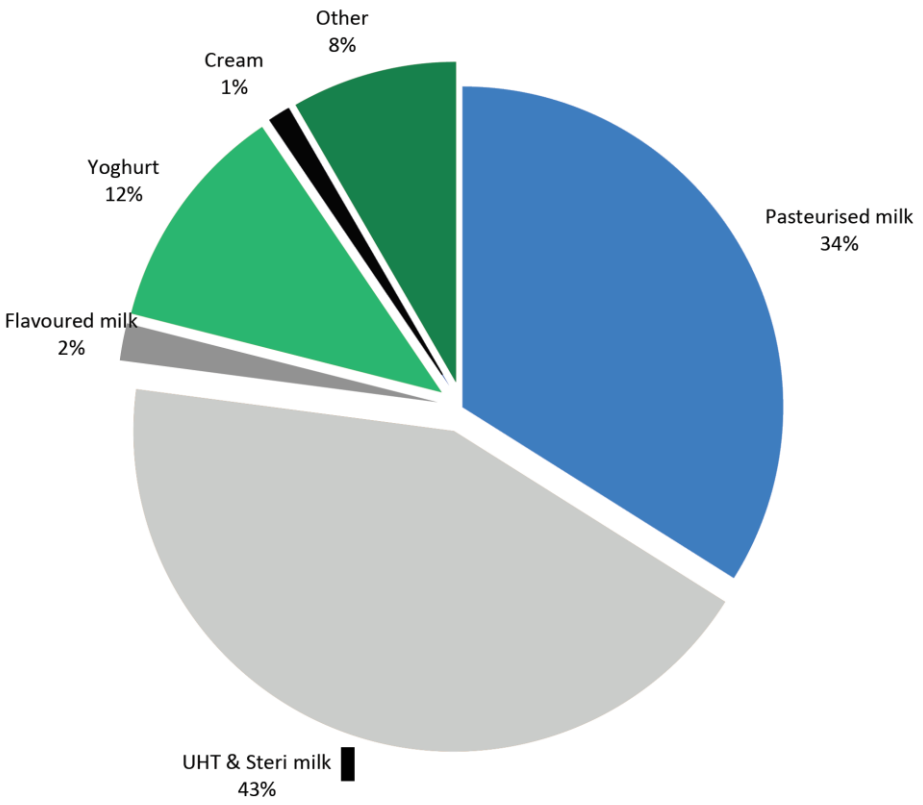

Figure 2.2: Composition of the South African liquid products market on a volume basis (Lactodata, 2018)

2.2 Dairy industry market in South Africa

Fresh milk is considered very desirable in parts of South Africa and indeed other parts of the world. Some individuals and small-scale farmers make cheese from fresh milk in SA due to the unique, pleasant taste and mouthfeel it imparts, which is considered a delicacy. However, fresh milk poses many safety hazards due to its high somatic cell count, susceptibility to bacterial contamination, and antibiotic residues. After collection and before processing, fresh milk is held at 24-28 h in

tanks at 5-7 °C, a condition which favours the growth of psychotropic bacteria (Buehler et al., 2018). As fresh milk is highly perishable, it is conveniently processed into products that increase its shelf-life, with most consumers showing a higher affinity for shelf stable milk products (Deeth, 2017; Gedam and Vijay, 2007). As such, the dairy industry has, over the past two decades, invested in the development of improved separation, standardisation, pasteurisation, homogenisation, and packaging techniques (Goff and Griffiths, 2006), with the view of producing milk products that offer convenience and variety. These improvements have been possible through research and development and knowledge of the functional properties of the different ingredients and components of milk and their effects on the structure and texture of the final product. Apart from extending the shelf life of milk, other improvements have been made in the continuously evolving dairy industry. Standardisation of milk has become faster and automated to receive results of milk analysis faster and with enhanced methods of adjusting the required milk fat content (Goff and Griffiths, 2006).

High temperature short time (HTST) pasteurised milk, processed at 72 °C for 15 s, was one of the early innovations for increasing the shelf life of milk, with a shelf life of 10-14 days when refrigerated (Hoffmann et al., 2006; Lewis and Deeth, 2009; Schmidt et al., 2011). Another process used to increase the shelf life of milk further is ultra-high temperature (UHT) processing. UHT milk was introduced to form a product with a longer shelf life than HTST milk, with the former having a shelf life of 9 months at ambient temperature and processed at 135-140 °C for 1-2 s (Schmidt et al., 2011), using either direct heating, steam injection or infusion. UHT milk was mostly

produced to accommodate households with limited or inadequate refrigeration and a convenience product when travelling or baking (Goff and Griffiths, 2006).

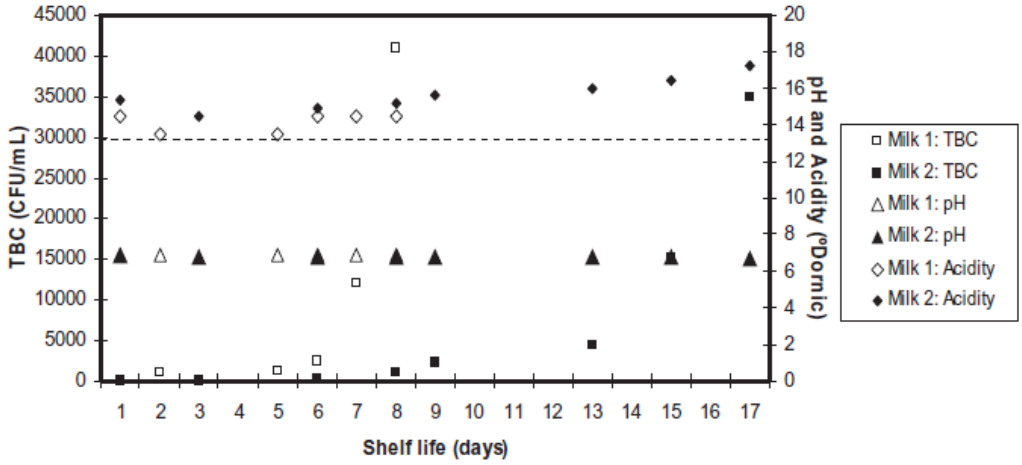

Figure 2.3: Titratable acidity, pH, and total bacterial count (TBC) of pasteurised and UHT milk throughout 17 days (García and Rodríguez, 2014)

However, there are consequences of using high heat processing techniques. Though consumers prefer its longer shelf life, they are not as enthusiastic about its undesirable cooked or caramelised flavour because of Maillard's browning. Maillard browning reactions are caused by a reaction between sugars and proteins in the milk and cause a slight browning of the milk due to the increased processing temperature (Goff and Griffiths, 2006; Melini et al., 2017; Schmidt et al., 2011). ESL milk has the advantage of shelf life of 21-28 days at 7 °C but with the non-existent burnt flavour of UHT milk and closer to the taste of fresh milk (Goff and Griffths, 2006; Melini et al., 2017).

Though ESL milk is also produced at high temperatures, it is followed by microfiltration (Goff and Griffiths, 2006). However, consumers still prefer UHT over ESL milk, with the latter preferred over HTST milk. Lorenzen (2011) confirms that UHT milk dominates the markets with a 70% market share in Germany, followed by ESL milk with 20-25% and HTST with 5-10% only. Also, because of consumer convenience and sustainability, there has been a revolution of milk packaging, shifting from the use of glass packaging to plastics and cardboard packages with re-sealable screw caps (Goff and Griffiths, 2006).

2.3 Production of Extended Shelf Life milk

In the production of ESL milk (Figure 2.4), fresh raw milk is exposed to temperatures of 123-127 °C for 5 s (Lorenzen et al., 2011; Schmidt et al., 2011), and subsequent microfiltration to further reduce the microbial load. Raw milk is first separated into skimmed milk and milk fat; the former is then microfiltered through ceramic membranes, with 0.8-1.4 µm pore diameter resulting in a spore reduction of 3-5 $\log_{10}$ (Hoffmann et al., 2006; Lorenzen et al., 2011). The resulting skim milk is then pasteurised at high heat. The milk fat and the bacteria-enriched retentate obtained from microfiltration of the skim milk are then subjected to UHT treatment (123-127 °C, 1-5 s) and added to the skim milk (Schmidt et al., 2011). Thus, though high temperatures are employed in ESL milk, temperatures used strikes a balance between the thermal inactivation of vegetative cells and spores and the preservation of organoleptic and nutritional characteristics. ESL milk can be produced by incorporating various techniques, including bactofugation, pulsed electric fields,

high-pressure processing and microfiltration, with the latter being the most common industrial method used.

Crossflow microfiltration is an additional bacterial reduction step used at low temperature and affects up to 4.5 $\log_{10}$ in bacterial spores (Tomasula et al., 2011). In this procedure, a filter with a 1.4 µm is used to exclude bacteria while allowing the milk through, together with components such as lactose, protein, and ash. However, due to the size of the milk fat globules, fouling of the filter occurs quickly as the fat globules are similar in size to the bacterial spores and thus forms a fatty layer on the membrane affecting the efficiency of milk filtration (Dat et al., 2014). Consequently, this fouling adds to increased costs as the filter needs to be cleaned regularly. As a result, cross-flow microfiltration is more applicable in the case of skim milk processing. Heat processing is applied after microfiltration to reduce microbial load further. Since lower temperatures are applied in this case instead of ultra-pasteurisation, this type of milk has much more favourable sensory attributes due to the lesser effects of Maillard browning.

2.4 Bacterial contamination of processed milk

Pasteurisation has always been thought of as an effective way of ensuring milk safety. However, despite this assertion, there have been instances of foodborne outbreaks associated with milk products. *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Campylobacter* are the most frequent potential pathogens associated with milk or dairy products in industrialised countries (Jakobsen et al., 2011). However, there has been a recent

increase in contamination of dairy products with spore-forming bacteria such *as B. cereus, B. subtilis, B. licheniformis* and *B. sporothermodurans* (Sepulveda et al., 2005). This increase is because of high heat processing techniques selecting for spores of these microbes (Deeth, 2017).

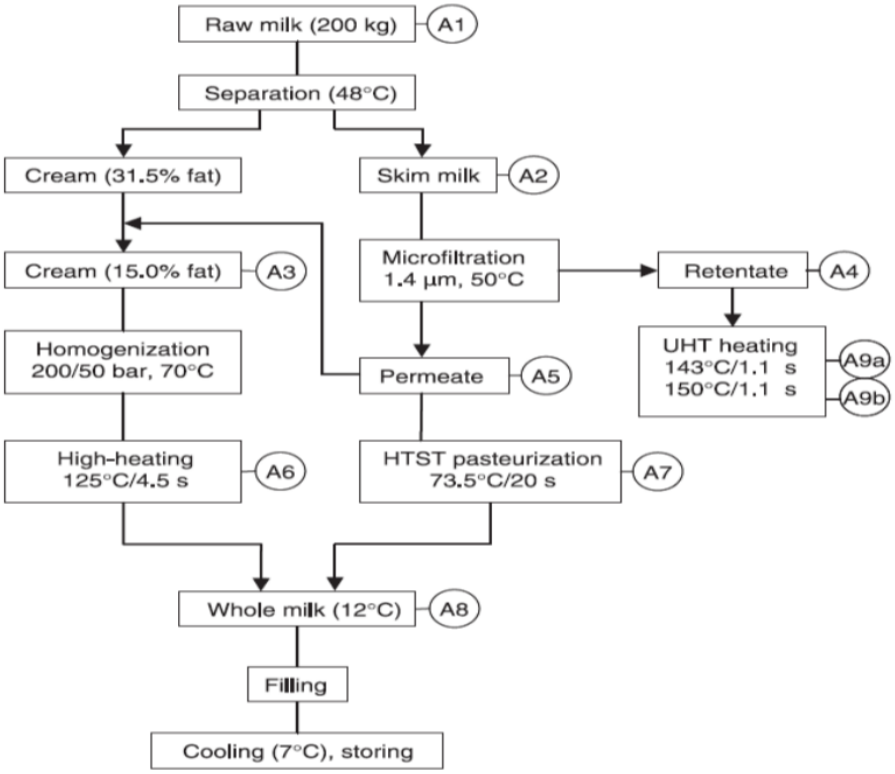

Figure 2.4: Typical processing of ESL milk from fresh raw milk (Hoffmann et al., 2006)

Thermoduric and thermophilic spore-forming bacteria are known to be the most common spoilage bacteria in ESL milk. They can survive ESL heat treatment mainly in the spore form and will often be found on equipment surfaces downstream of the processing line after the pasteurisation step. *Bacillus* spp. spores are of concern as they are more adhesive to stainless steel than vegetative cells

and can also germinate in manufactured products (Flint et al., 2001). Bacterial contamination of milk may arise from a high initial microbial load of raw milk, poor milk handling practices, unsanitary processing equipment arising from ineffective cleaning regimes and the formation of bacterial spores that later germinate into vegetative cells. Bacterial spores can be resistant to wet heat and thus may survive ESL processing conditions (Simmonds et al., 2003). Also, the spores are hydrophobic, which allows them to attach more firmly to stainless steel processing equipment (Simmonds et al., 2003), with the progressive growth of these spores to vegetative cells contributing to the formation of biofilms. *Bacillus* spp. and *Paenibacillus* are especially concerned about milk safety, and quality as their spores can survive pasteurisation temperatures and most importantly, germinate under refrigeration (Masiello et al., 2014). *Bacillus* spp. and *Paenibacillus* are ubiquitous and, as such, are found in the soil, plant surfaces, and the digestive tract of insects and mammals, as such easily contaminate milk during the milking process (Masiello et al., 2014). Criely et al. (1994) observed that *B. cereus*, *B. circulans*, *B. firmus*, *B. licheniformis*, *B. subtilis*, *B. coagulans*, *B. sphaericus*, and *B. mycoides* were more frequently isolated from raw bulk tanks. On the other hand, *Paenibacillus* spp. were mostly isolated from silage, dairy cow feed concentrates, and raw milk. In post-pasteurised milk stored at 6 °C, *Bacillus* spp. are known to be the principal genus up to day 7, however from day 17 onwards, *Paenibacillus* dominates (Masiello et al., 2014). These bacterial groups are also responsible for major milk spoilage and contribute to off-flavours, “sweet curdling” and “bitty cream” defects due to the activity of proteinases, lipases, and phospholipases (Schmidt et al., 2011).

B. cereus produces toxins that cause food poisoning, resulting in serious illness that may be fatal. *Paenibacillus* spp. and *Microbacterium* spp., on the other hand, cause spoilage in ESL milk by producing rancid and bitter off-flavours and an increase in free fatty acids. In a study by Schmidt et al. (2011), ESL milk spoilage was attributed mainly to *B. cereus* and *Paenibacillus* spp. and with the conclusion that the combined use of microfiltration and pasteurisation may eliminate other bacteria but not necessarily spore formers.

2.5 Biofilms

Antony van Leeuwehoek discovered biofilms in dental plaque in 1684 and subsequently in the 1940s by marine scientists studying marine organisms (Mogha et al., 2014). Biofilms are a complex aggregation of microbial cells naturally attached to a surface through the assistance of a slimy matrix composed of various macromolecules such as polysaccharides, proteins and nucleic acids (Simões et al., 2009; O'Toole et al., 2000; Kokare et al., 2007). These macromolecules aforementioned are collectively referred to as extracellular polymeric substances (EPS). Naturally, microorganisms tend to attach themselves to nutrient rich inanimate surfaces, such as tooth enamel, heart valves and processing equipment (stainless steel, as shown in Figure 2.5; thereby accelerating corrosion). They multiply and form microcolonies (Mogha et al., 2014; Kokare et al., 2007), creating biofilms in the process. Biofilms may compromise single or multiple microbial species (Kokare et al., 2007), albeit multi-species are predominant, the latter forming more stable biofilms (O'Toole et al., 2000; Mogha et al., 2014; Chmielewski and Frank, 2003). Biofilms may be single

or multi-layered (Kokare et al., 2007). Biofilm formation because of ineffective cleaning regimes is a threat to dairy processing, concerning the production of milk products with high safety and quality standards (Mogha et al., 2014).

During initial biofilm production, the presence of EPS aids in maintaining the structure and integrity, surrounding the bacterial cells, and anchoring them (Figure 2.6) within the complex (Branda et al., 2005). The effective cleaning and control of bacterial biofilms are essential to curtailing foodborne illnesses due to dairy products. It is thus important to constantly monitor the formation of biofilms on processing equipment surfaces and put in place monitoring mechanisms to ensure the gradual build-up of biofilms which will eventually contaminate the food, is curtailed (Simões et al., 2009). Aside from economic losses attributed to the discarding of contaminated food products, some losses can be attributed to the early destruction of food equipment because of corrosion attributed to sustained biofilm production. The corrosion process attributed to biofilm formation may subsequently affect the flow dynamics and reduction in heat transfer between the equipment and food product (Simões et al., 2009). There is also the loss of production time because of increased cleaning regime times, especially at biofilm prone areas, including the filler nozzles of milk producing equipment.

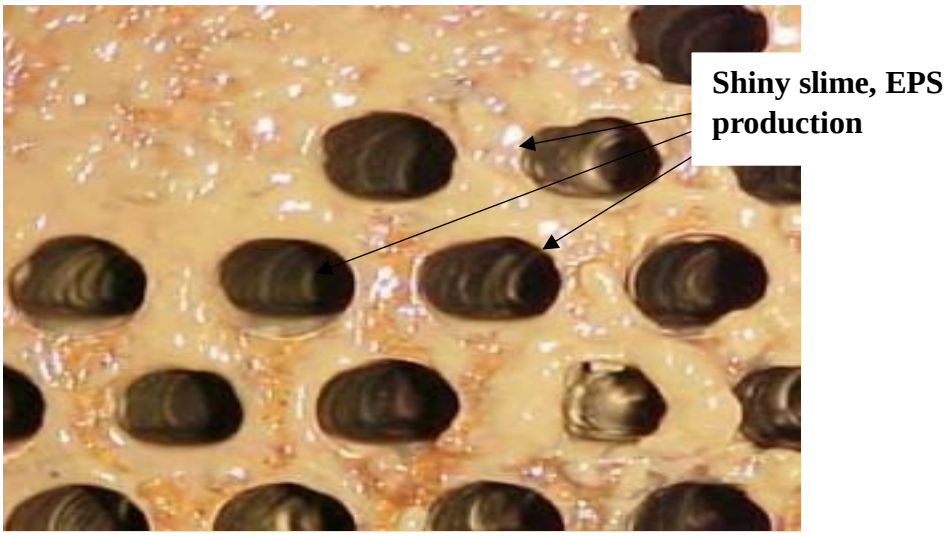

Figure 2.5: Biofilm formation on the grates of a heat exchanger and showing slimy EPS layer produced by the cells (http://amsainc.com/biofilm-basics/)

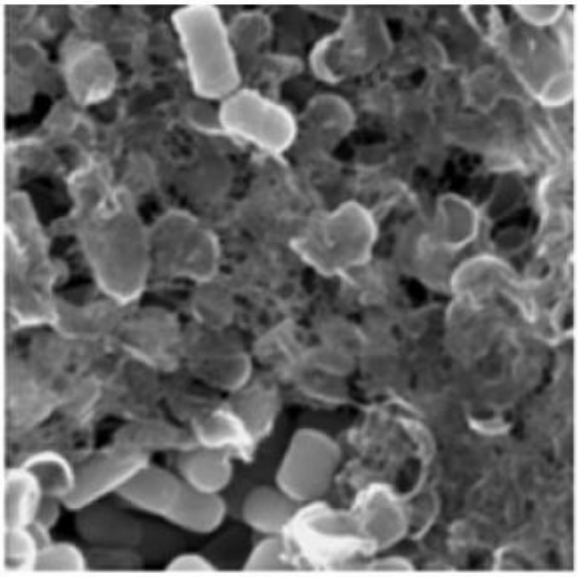

Figure 2.6: Mature biofilm showing bacterial cells attached to a surface with visible EPS (Branda et al., 2006)

2.5.1 Biofilm formation

The formation of biofilms occurs in various stages and may take several hours to weeks to form (Marchand et al., 2012). The formation rate depends on the biofilm's bacterial composition, the surface composition of equipment, and the composition of the food matrix (Chmielewski and Frank, 2003). Different mechanisms and stages are involved in the attachment, growth, and colonisation of bacteria on milk contact surfaces (Mogha et al., 2014). Biofilm formation will normally comprise the following stages: initial attachment, adhesion, growth, maturation, and release (Marchand et al., 2012), as illustrated in Figure 2.7.

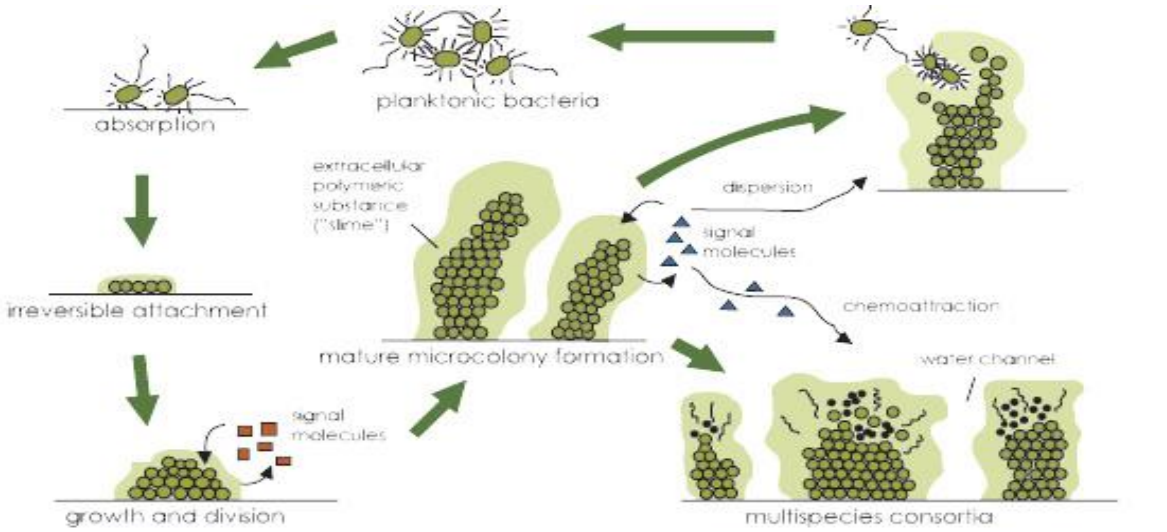

Figure 2.7: Biofilm formation showing the most common stages (http://www.becscreen.com/about/about-biofilms)

At the inception of biofilm formation, there is an initial attachment, followed by cell adhesion to the processing equipment's nutrient-rich surface (Mogha et al., 2014). The biofilm is relatively easier to clean at this initial stage as effective EPS is yet to form. After this stage, the gradual formation of EPS is normally seen as shiny slime on the equipment surface (shown previously in Figure 2.5) (Chmielewski and Frank, 2003). As EPS plays a protective role for the attached cells, cleaning regimes become less effective at this stage. The bacteria continue to grow in the next stage, and cell-cell signalling occurs (Chmielewski and Frank, 2003) to control gene expression activities. The next stage involves forming microbial colonies as the biofilm starts to mature, leading to the final stage where loosely attached cells are released, an action known to be physiologically controlled (Vlková et al., 2008). The released vegetative cells or spores are transferred to other parts of the equipment to restart the biofilm process again and result in chemical and biological reactions that accelerate corrosion of equipment surfaces and restrict material flow in heat exchangers and pipelines (Simões et al., 2009). This transfer of microbial material subsequently results in the contamination of the food matrix.

The microbial colonies mentioned earlier are maintained by forming water channels, which provide passage for waste, metabolites, and nutrients exchange (Marchand et al., 2012), thus keeping the biofilm as a functional life form. Aside from the water channels, the biofilm is maintained through cell to cell signalling by producing acyl homoserine lactones (AHL) which control gene expression, in a process referred to as quorum sensing. This process enhances microbial growth and the production of extracellular protease. However, AHL analogues can be used to degrade AHL, thus preventing

quorum sensing and the maintenance of the biofilm microbial community (Goff and Griffith, 2006).

2.5.2 Extracellular polymeric substances (EPS)

During the second stage of biofilm formation, the bacterial cells produce EPS made up of proteins, DNA, and polysaccharides (Donlan, 2001; Mogha et al., 2014). EPS acts as a bridge between bacteria and the substratum, helping to stabilise the colony against any changes, including cleaning regimes, increased temperatures, and food flow (Mogha et al., 2014). As a result of the protective function of EPS, antimicrobial agents may be ineffective, especially against a fully matured biofilm. The EPS restricts the antimicrobial agents to diffuse into the biofilm environment effectively. There are cases where EPS is known to react with the antimicrobial molecules making them less effective (Donlan, 2001) in inactivating microbial colonies in the biofilm environment.

The production rate in the biofilm environment is stimulated by cell density, nutrient availability, and environmental stress (Chmielewski and Frank, 2003). In static liquid media, *B. subtilis* forms a pellicle-like shape layer because of the movement of motile planktonic cells moving to the medium's surface. These cells grow as long non-motile parallel cells (Figure 2.8) which subsequently gives the biofilm its structure. Specific genes which carry out this cell transformation are referred to as bundling genes and assist with the synthesis of EPS. The *pgc*A gene encodes for an enzyme that catalyses the production of nucleotide sugars; whiles *eps*A-O produces biological material that uses nucleotide sugars to produce EPS (Brenda et al., 2005). Finally, the *Sin*R gene

is responsible for transitioning from a single motile to a non-motile bundle of cells in the extracellular matrix (Branda et al., 2005).

2.5.3 Enzyme production by *Bacillus* spp.

Bovine milk contains indigenous enzymes which influence milk shelf life. However, apart from these enzymes, microbial enzymes also play a part in ESL and UHT milk quality. Vegetative cells are known to produce thermostable enzymes, which affect the shelf life and integrity of ESL milk even after the cells themselves have been inactivated, with the thermostable enzymes remaining active during milk storage (Flach et al., 2014; Melini et al., 2017). *B. subtilis* produces multiple extracellular enzymes, including proteinases, lipases, phospholipases, and amylases, albeit mostly for industrial purposes (Degering et al., 2010). The proteolytic and lipolytic enzymes are known to be more detrimental to milk shelf life by producing off-flavours and causing age gelation (Chen et al., 2001; Melini et al., 2017). Proteinases have their sub-group of enzymes, namely serine proteinases (plasmin), cysteine proteinases (cathepsin B), aspartic proteinases (cathepsin D) and metalloproteinases (thermolysin). Proteinase production normally occurs maximally in the late exponential or stationary phase of growth (Chen et al., 2001).

Lipolysis in milk can be spontaneous or induced, with each enzyme type having its mechanism of action (Deeth et al., 2006). Lipases, one of two classes of hydrolases, are mainly active against water-insoluble substrates, such as triglycerides composed of long chain fatty acids (Kulkarni and

Gadre, 2002). The second class of hydrolases, carboxylesterases, act against simple esters and short chain triglycerides, with less than C6 (Kulkarni, 2002). Hydrolysis of triglycerides in milk increases in short chain fatty acids such as butyric acid, caproic acid and caprylic acid responsible for off-flavours and sour-like taste of milk. On the other hand, medium chain fatty acids such as capric and lauric acid give a soapy taste to milk (Montanhini and Bersot, 2013; Omar et al., 2017; Schmidt et al., 2011) identified that inoculated milk incubated for 106 h showed increased lipase activity with corresponding increased cell counts. However, fatty acid concentrations decrease, more so at higher storage temperatures.

2.6 Effect of microbial enzymes on ESL milk shelf life

Microbial enzymes inflict technological and flavour defects on milk production, such as “unclean flavour” and browning during heating due to proteolysis of amino acid products (Melini et al., 2017). Proteolytic enzymes are usually associated with a bitter flavour and gelatinisation of ESL milk after long storage periods due to the hydrolysis of peptide bonds. Lipolytic enzymes cause rancidity of milk that has undergone pasteurisation (Flach et al., 2014). Flach et al. (2014) found that cells in biofilms have higher proteolytic and lipolytic activities. Also, *Bacillus* spp. are generally considered to have a higher intracellular and extracellular proteolytic activity than other bacteria (Chen et al., 2001). Most proteinases from psychrotrophs firstly attack caseins then whey proteins, with β and *k* caseins more susceptible than αs-caseins (Chen et al., 2001). Proteases are the main cause of age gelation, which occurs in ESL milk (Montanhini, 2013), and though high

temperatures can inactivate proteases, the high temperatures are detrimental to milk functionality (Chen et al., 2001).

2.7 *Bacillus subtilis*

B. subtilis is a ubiquitous bacterium that exhibits varying genome diversity that allows it to be broadly adaptive. It is naturally found in soil and secretes enzymes to degrade substrates, allowing it to survive and adapt to changing environments. Being both thermoduric and psychrotolerant supports its ability to remain a huge spoilage organism in the food industry, especially the dairy industry. Like other Gram-positive spore-formers, *B. subtilis* are among the most abundant bacterial contaminants in processed milk (Figure 2.9). *B. subtilis* can grow at temperatures between 3-75 °C and thus can cause milk spoilage during refrigeration. Other cellular adaptations such as heat-stable DNA, proteins and membranes enable the bacteria to grow and survive in dairy processing equipment (Teh et al., 2015). *B. subtilis* does not produce endotoxins and is considered as GRAS (Generally Recognised as Safe) (Westers et al., 2004). Like other members of the genus *Bacillus*, *B. subtilis* can form highly resistant spores during nutrient and environmental stress (Earl et al., 2008) and is known to cause spoilage in milk and milk products (Montanhini and Bersot, 2013). When conditions become unfavourable, *B. subtilis* growth slows, and metabolic activity reduces whilst initiating sporulation (Weinstock, 2001). The onset of spore formation may occur at 44 °C depending on the strain and growth medium (Piggot, 2009) and may also occur in biofilms. Bacterial endospores, including those of *B. subtilis*, can survive multiple stresses such as chemicals, including those used in industrial cleaning regimes, UV radiation, and desiccation. *B.*

subtilis is a motile microorganism that uses peritrichous flagella, however during its exponential phase on nutrient-rich media, it grows in chains without flagella and thus are non-motile. At the end of the exponential phase, they break off into single or doublet morphology and can subsequently re-establish motility (Piggot, 2009). *B. subtilis* is the most common prokaryote used to produce recombinant proteins in the industry (Westers et al., 2004). During its transition state from active growth into the stationary phase, *B. subtilis* strains produce amylase, proteases, and a variety of antibiotics (Piggot, 2009). More than half of the commercially available enzymes are produced by *Bacillus* species, the majority being secreted naturally into the growth medium. The removal of some extracellular proteases can decrease the proteolytic activity of *B. subtilis* and lead to an increase in cell death (Westers et al., 2004). Subtilisin, the most common industrial protease produced by *B. subtilis*, has various applications, including use in detergents (Degering et al., 2010).

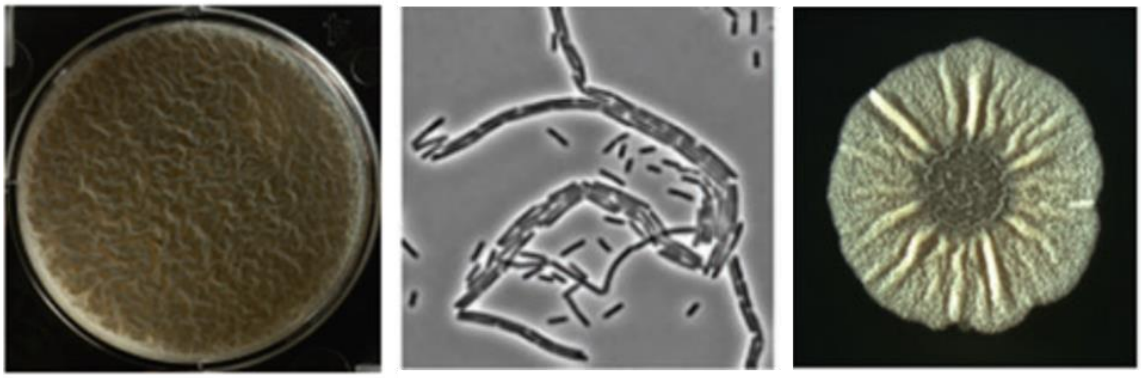

Figure 2.8: *B. subtilis* showing the formation of pellicles and biofilms (Branda et al., 2006)

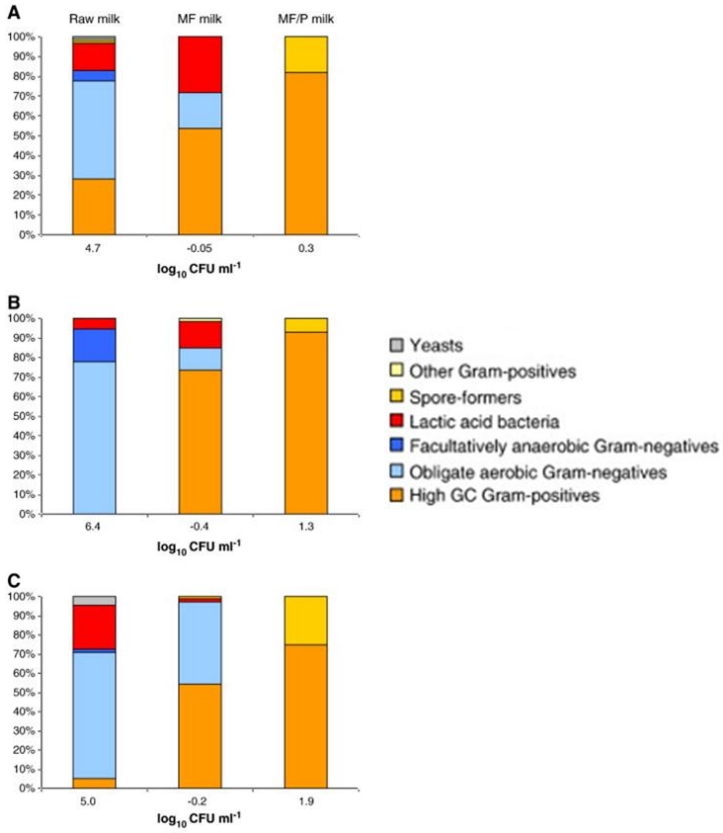

Figure 2.9: Occurrence of microorganisms isolated from raw milk, microfiltered milk (MF) and microfiltered and pasteurised milk (MF/P) in 3 batches (A, B and C) of ESL milk (Schmidt et al., 2011)

2.8 Effect of cleaning-in-place (CIP) on *Bacillus subtilis*

Milk is a nutrient-dense food, making it a good medium for bacterial contamination. Thus, heat processing of milk or other bacterial inactivation techniques is critical to producing milk products with long shelf life. Pasteurisation and UHT are the most common heat treatments used in milk processing, and though they are predominately effective at reducing initial microbial load or

sterilising milk products, they can still be ineffective in cases where biofilms form in processing equipment (Rysstad and Kolstad, 2006).

The effective cleaning of milk processing and storage equipment is very important in preventing the build-up of biofilms which in turn can cause bacterial contamination and lead to safety and quality issues. Ineffective cleaning regimes will result in the multiplication of bacterial cells and the development of biofilms, the latter providing a source of contamination of subsequent production runs (Lombardia, 2009). Cleaning of equipment should include chemicals, heat, and physical action and should comprise pre-rinse, chemical action, and post rinse phases. The time to complete each phase differs, with the phase involving the chemical action requiring a longer time to act on and break down any biofilms that may be present (Simões et al., 2009).

As milk moves through the production equipment, surface conditioning of the processing equipment occurs. Surface conditioning involves coating the substratum/abiotic surface with a layer of molecules from the food matrix's movement through the processing equipment. Surface conditioning provides a good environment for bacteria to adhere to and form biofilms. Various food products have different surface conditioning properties, with whey protein known to especially promote the adhesion of bacteria (Dat et al., 2010). Also, during milk processing, pH is important as acidic pH conditions cause the precipitation of the milk onto the processing surface as the proteins coagulate.

CIP is the preferred method for cleaning dairy processing equipment and should be regular as bacteria require short contact times to attach to processing surfaces and form biofilms. Despite the

short time required to form biofilms; effective CIP regimes are crucial in reducing subsequent contamination in future production runs. CIP regimes involve treatments with varying temperatures and chemical concentrations and normally using a combination of acidic and alkali solutions. The balance of pH involved in CIP chemicals is important as low pH may make bacterial attachment easier (Dat et al., 2010). A typical CIP treatment regime for milk processing lines may include the following 1; a pre-rinse with cold water to remove any residue 2; circulation of detergent to remove major and any remaining minor residues 3; cold water rinses to remove detergent used 4; circulation of disinfectant to kill remaining microorganisms 5; final cold water rinses to remove any remaining detergent and disinfectant (Simões et al., 2009).

Despite the importance of CIP treatment regimes in reducing food contamination in processing equipment, there can still be residues of microbial cells, especially in cases where there is extensive biofilm formation. As a result, *B. subtilis* and other spore formers have been isolated at various stages of milk processing, from raw milk storage tanks, filler nozzles to final packaged products. Subtyping of these bacterial contaminants has been proven to have originated from raw milk storage tanks and other parts of the processing equipment because of biofilm formation and inadequate CIP regimes (Masiello et al., 2014). *B. subtilis* spores can survive CIP treatments due to their durable outer spore coat structure and germination during favourable conditions and subsequently contaminating the food product. *B. subtilis* vegetative cells may also survive CIP albeit at low numbers, but can then multiply, forming colonies and subsequently biofilms (Simmonds, 2003).

2.9 Hypotheses

1. *B. subtilis* vegetative cells isolated from ESL milk and forming part of biofilms will survive CIP treatment due to the protection offered by the EPS barrier, thus assisting in their survival to the cleaning regimes. Industrial CIP cleaning conditions may leave behind active vegetative cells protected in biofilms (Peng et al., 2002). The EPS layer acts as a barrier against antimicrobial agents and chemicals, making it more difficult for CIP chemicals to diffuse into and reach cell residues (Donlan, 2001). As a result, *B. subtilis* vegetative cells surviving CIP may enter sporulation and germinate, multiply, and consequently contaminate milk products when conditions are favourable, affecting the milk shelf life.

2. The extracellular proteolytic and lipolytic enzymes released by *B. subtilis* biofilms will contribute to the accelerated spoilage of ESL milk. Extracellular proteolytic and lipolytic enzymes produced by *B. subtilis* are heat stable and remain active even after heat treatment. Proteinases and lipases are hydrolytic, heat stable enzymes that break down milk components (casein and milk fats, respectively) and affect the shelf life of the milk due to loss of stability provided by the casein and milk fats. Hydrolysis of casein leads to milk gelation and sweet curdling (Champagne et al., 1994; Schmidt et al., 2011). Hydrolysis of milk fats increases free fatty acids and results in a bitter and rancid taste (Champagne et al., 1994; Samaržija et al., 2012; Sørhaug et al., 1997).

2.10 Objectives

1. To determine the effect of simulated and non-simulated CIP treatment on *B. subtilis* vegetative cells isolated from Extended Shelf Life (ESL) milk to ascertain the effectiveness of a simulated CIP regime and its contribution to the shelf life of ESL milk.

2. To determine the effect of controlled and simulated CIP treatment of *B. subtilis* vegetative cells on bacterial growth dynamics to determine the spoilage trajectory of ESL milk over 28 days.

CHAPTER 3: MATERIALS AND METHODS

3.1 *Bacillus subtilis* isolates and growth conditions

Three *B. subtilis* strains (Table 3.1) were used in the study. The strains were isolated and characterised by Khoza, 2015 and Mugadza and Buys, 2017 (Figure 3.1).

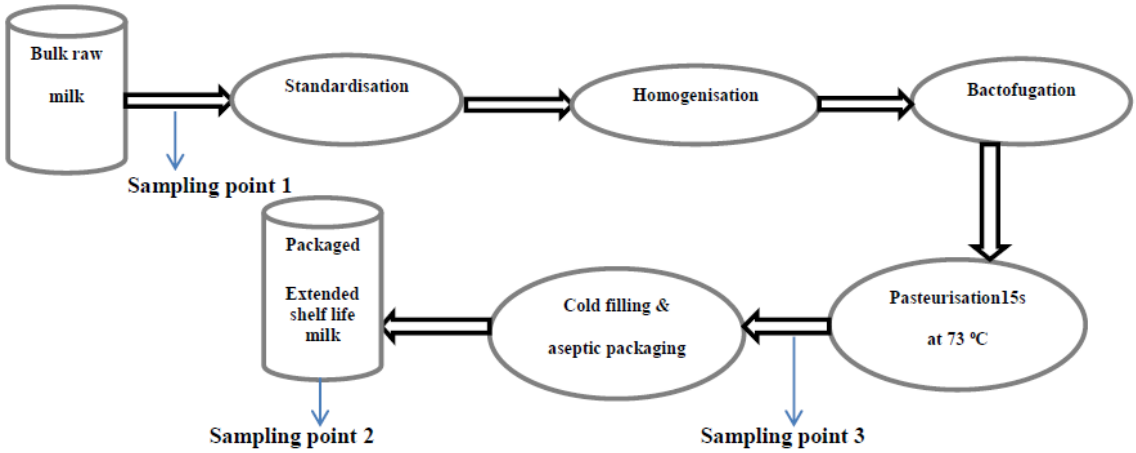

Figure 3.1: Sampling process followed by Mugadza (2017) to obtain strains used in this study

Table 3 .1: *Bacillus subtilis* strains used in the present study and indicating their source of isolation (Khoza, 2015; Mugadza, 2017)

Strain	Source
B. subtilis 49	ESL milk stored at 7 °C
B. subtilis 50	ESL milk stored at 4 °C
B. subtilis 54	Packaged ESL milk
B. subtilis ATCC 3610 (positive control)	Culture collection
B. subtilis ATCC 168 (negative control)	Culture collection

All *B. subtilis* strains BS49, BS50 and BS54 were cultivated on brain heart infusion (BHI) agar (Oxoid, Basingstoke, UK) at 30 °C for 24 h.

3.2 Simulated CIP treatment

Bacterial cells were washed with 1 mL 0.5% Super Klenz solution (pH 12.51) (Ecolab Johannesburg, RSA) in 2 mL Eppendorf tubes for 10 min in a water bath at 70 °C. The cell suspension was centrifuged at 10000 rpm for 2 min. After removing the supernatant, cells were washed with sterile phosphate-buffered saline (PBS) (1x, pH 7.4) (Oxoid) and centrifuged at 10000 rpm for 2 min. The supernatant was removed, and the cells subsequently washed with 1 mL CIP acid solution (1%, pH 0.90) (Ecolab) for 30 min in a water bath at 70 °C. Cell suspensions were centrifuged at 10000 rpm for 3 min, the supernatant removed and washed with sterile PBS (Oxoid) in 2 mL Eppendorf tubes. Cell suspensions were centrifuged at 10000 rpm for 2 min, washed with 1 mL Super-San solution (1%) (Ecolab) for 5 min in a water bath at 70 °C. Suspensions were centrifuged for 3 min at 10000 rpm. After the supernatant was removed, 1 mL sterile PBS (Oxoid) was added to the pellet in the 2 mL Eppendorf tubes. Controls including damaged cells (autoclaved at 121 °C for 15 min) and intact (not exposed to simulated CIP treatment) were incorporated in the study.

3.3 Fluorescent labelling and microscopy of *Bacillus subtilis* cells

The simulated CIP treated cells were centrifuged, and the remaining pellets were washed twice with sterile PBS (Oxoid, UK) (pH ±7.4). The treated cells were standardised to 0.5 MacFarland (10^8 /mL), and 100 µL of the standardised cell suspensions were pipetted into sterile flow cytometry tubes. Each 100 µL cell suspension was stained with 1 µL propidium iodide (PI) and carboxyfluorescein diacetate (cFDA) (Thermo Fisher Scientific) (Shen et al., 2009). Cell suspensions were thoroughly mixed with a vortex after stain addition and immediately before FCM analysis. The non-fluorescent cFDA diffuses into the cytoplasm of the cells and is converted by cytoplasmic esterase-catalysed hydrolysis to carboxyfluorescein (cF) and two molecules of acetate; the cF interacts with cellular amines, which generate and emit an intense impermeant green fluorescence. A stock solution of 0.78 mM cFDA and 0.748 mM PI was prepared in distilled water. After, 500 µL of the cell suspension was stained with 5 µL of cFDA and PI. Fluorescence microscopy using a Zeiss Inverted Fluorescence microscope with high-NA bright field and DIC optics (Carl Zeiss Microscopy, USA) was used to confirm stain absorption and subsequent dyes' emission before FCM. Live cells appeared green under the microscope, and dead cells appeared red; injured cells took up both dyes and appeared both green and red under the microscope (Williams et al., 1998). *B. subtilis* controls were included as mentioned in section 3.2 above.

3.4 Flow cytometry (FCM) of bacterial cells

The cell suspensions in the tubes were analysed with a flow cytometer, BD Accuri™ C6 Plus (BD Biosciences, SA), to determine the physiological state of the cells after simulated CIP treatment (dead, injured or alive) and including the presence of structural cell damage. The BD Accuri™ C6 PLUS flow cytometer were equipped with two lasers, and two light scatter detectors. The blue laser was used, with the green fluorescence (cFDA) for capture by the FL1 detector and the red (PI) for capture by the FL3 detector. The forward scatter (FSc), sideward scatter (SSc), green and red fluorescence were measured for each cell and converted into digital signals for later processing. Logarithmic amplification was used to collect signals. Samples were analysed at a flow rate of 24 µL/min with 10000 events and in duplicates. Data were analysed using the flow cytometry analysis program FlowJo™ software (Bunthof et al., 1999; Garcia-Betancur et al., 2012).

3.5 Biofilm assay

The *B. subtilis* strains (BS49, BS50, BS54) consisting of simulated CIP treated and untreated cells and two *B. subtilis* reference strains, ATCC 3610; wild strain with robust biofilm formation and ATCC 168; non-biofilm former were used in this assay. The biofilm assay was derived from a method by Hussain & Oh (2017). A 96-well plate microtiter plate sterilised under UV light for 2 h was filled with 200 µL BHI broth (Oxoid) into individual wells. After, 5 µL of cell suspension (0.5 McFarland standard) was inoculated into the individual wells containing the 200 µL of BHI

broth. Four replicates were undertaken for this assay. To prevent the microtiter plates from drying out, they were covered with parafilm and subsequently incubated at 30 °C for 24, 48 and 72 h.

After incubation, the remaining growth medium was carefully pipetted out, and wells washed three times with 200 µL sterile PBS to remove any unattached cells. After washing, wells (containing proposed biofilms or otherwise) were stained 200 µL (0.1%) crystal violet solution at room temperature for 30 min. As done previously, microtiter plates were covered with parafilm to prevent dehydration of well solution. Excess crystal violet solution that did not bind to biofilms was pipetted out. Wells were again washed three times with 200 µL sterile PBS. Afterwards, 200 µL of 70% ethanol was pipetted into each well and incubated for 30 min at room temperature to allow for biofilm release, bound to crystal violet. The resulting solutions in the microtiter plates were administered into a Multiskan FC microplate reader (Thermo Scientific, USA) for absorbance determination at 595 nm wavelength. A blank sample consisting of double distilled water was incorporated into the assay.

3.6 Attachment of *Bacillus subtilis* cells to stainless steel

B. subtilis strains BS49, BS50 and BS54 were used in this assay, with each isolate incorporating a control (no CIP treatment). Bacterial suspensions (1 mL) with 10^8 cells/ mL density were inoculated into 2 mL sterile UHT milk and incubated for 1 h at 30 °C. Afterwards, 1 mL of the UHT milk containing the bacterial cells was inoculated into 40 mL Schott Duran bottles containing 9 mL sterile UHT milk. Sterile preconditioned stainless-steel coupons (grit:120) were inserted

into the bottles and incubated in a rotating water bath, 65 rpm at 30 °C for 24 h. After incubation, the metal coupons were removed and washed with sterile PBS solution and subsequently with double distilled water to remove any unattached bacterial cells. Bacterial cells attached to the stainless-steel coupons were fixed with 2.5% glutaraldehyde for 1 h in preparation for scanning electron microscopy.

3.7 Scanning electron microscopy (SEM)

SEM was used to observe the attachment of bacterial cells to the stainless-steel coupons from section 3.6. Before SEM, the stainless-steel coupons were fixed with 2.5% glutaraldehyde (GA)/formaldehyde (FA) and afterwards washed three times in PBS for 10 min. The steel coupons were covered with osmium tetroxide for 45 min, and after washed three times in PBS for 10 min and dehydrated with 30, 50, 70, 90 and 100% ethanol concurrently. Dehydration in 100% ethanol was repeated three times and left to air dry. Steel coupons were then covered in 100% hexamethyldisilazane (HMDS) and allowed to dry overnight. Coupons were then mounted and coated with carbon three times. A Zeiss Ultra Plus FEG-scanning electron microscope (Carl Zeiss Microscopy, SA) was used to capture images.

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3.8 *Bacillus subtilis* growth in UHT milk following CIP treatment

CIP and non-CIP treated *B. subtilis* cells were grown in BHI broth and standardised to 0.5 MacFarland (10^8/ mL). UHT milk (250 mL) was inoculated with a 10^3/mL concentration of study

strains. After 15 min the *B. subtilis* count in the milk was determined on BHI agar plates incubated at 30 ˚C for 24 h. The remaining inoculated milk was stored for 28 days at 5 ˚C and 10 ˚C. The *B. subtilis* count was determined for each milk sample every 7 days and up to 28 days.

3.9 Proteolytic and lipolytic enzyme activity

The enzyme activity of proteases and lipases was determined using skim milk agar and tributyrin agar (Oxoid, UK). The plates were incubated at 30 ˚C for 24 h, and the characteristic clear zones measured for each isolate (Carrazco-Palafox et al., 2018 and Chantawannakul et al., 2002).

3.10 Statistical Analyses

A three-way analysis of variance (MANOVA) with multiple comparisons was used to determine the effect of *B. subtilis* (BS49, BS50 and BS54); CIP treatment (control vs CIP), storage time (0, 7, 14, 28 days) and storage temperature (4 and 7 ˚C). Principle component analysis (PCA) was used to determine the variance-covariance correlation of the variables (treatment. strain, treatment time and storage temperature). All statistical analyses were done using GraphPad Prism version 7.0 (GraphPad Software, CA, USA) and SPSS version 26.0 (IBM Corp, NY, USA) 22 at $p < 0.05$ significance level.

CHAPTER 4: RESULTS

4.1 Effect of simulated CIP treatment on *Bacillus subtilis* strains by FCM analysis

Control FCM plots were used to identify the various regions representing the state of cell damage of the *B. subtilis* vegetative cells (Figure 4.1). FCM imagery using FlowJo™ depicts the intensity of green fluorescence increasing along the X-axis depicting live cells and the intensity of the red fluorescence depicting dead cells increasing along the Y-axis.

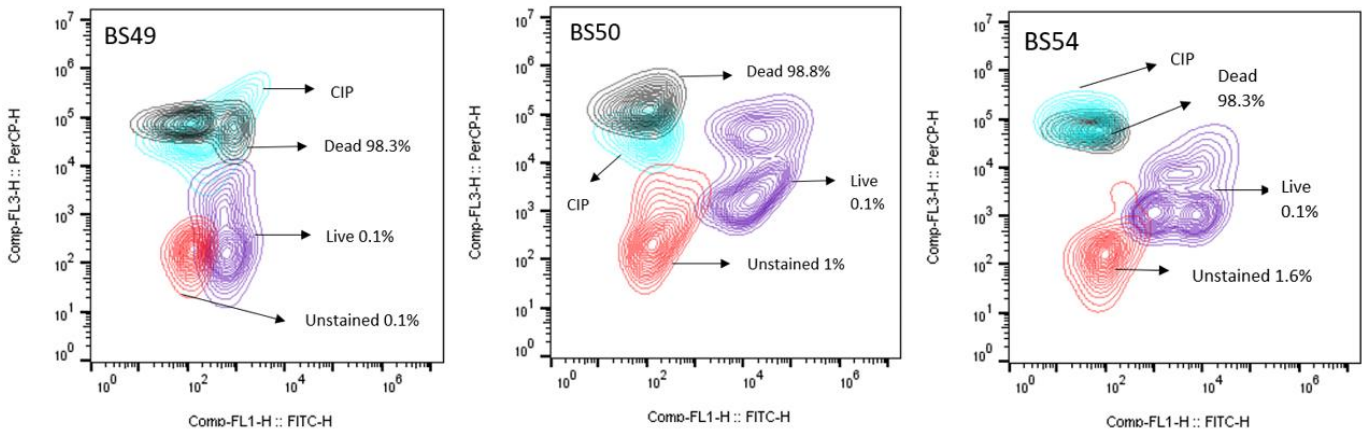

Figure 4.1: Contour plots of green fluorescence (cFDA) versus red fluorescence (PI) of *B. subtilis* vegetative cells treated with simulated CIP

FCM contour plots were compiled from the laser and scatter detectors and generated four fluorescence signals. The regions depicting unstained, live, and dead were visible on the plots for each of the three strains, indicating the physiological state of the cells. The contour plots (Figure 4.1) indicated that simulated CIP treatment worked effectively on all three strains and that 98.3%

of the cells were dead after simulated CIP treatment. The plots further showed that 0.1% of the cells remained intact, thus considered live for each *B. subtilis* strains.

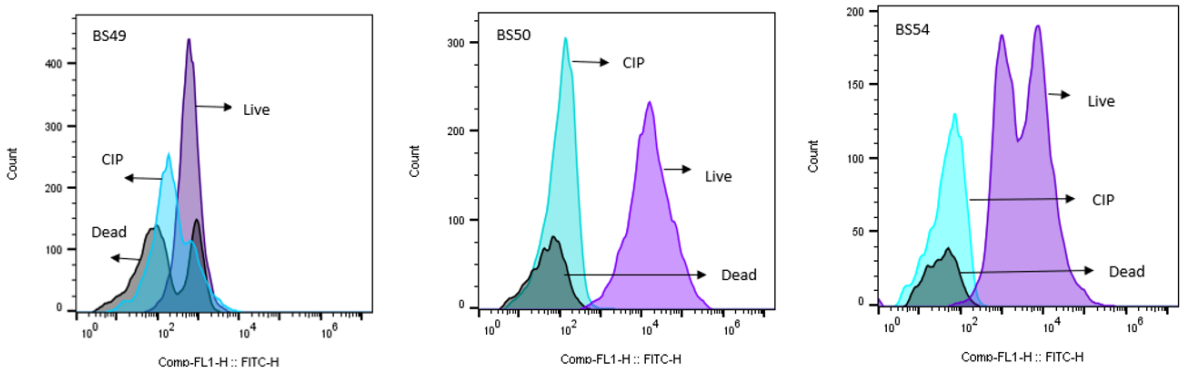

Figure 4.2: Histogram plots of green fluorescence (cFDA) versus red fluorescence (PI) of *B. subtilis* vegetative cells for strains BS49, BS50 and BS54, treated with simulated CIP

Histogram plots (Figure 4.2) indicate that all three strains showed significant overlap between the dead regions and the CIP treated cell regions, indicating that PI was absorbed by all the strains, indicating that the cells were dead. Further, Figure 4.2 depicts that there is also an absorbance overlap between the CIP treated regions of the study strains with the live regions and especially so for strain BS49.

4.2 Epifluorescence microscopy

Epifluorescence microscopy was used to confirm the absorption of cFDA and PI dyes before simulated CIP treatment to check the suitability and effectiveness of both dyes at staining live and intact cells from dead or inactive cells. Red fluorescence (Figure 4.3a) corresponds to the

absorption of PI, indicating inactive and dead cells, whilst green fluorescence (Figure 4.3b) indicates the absorption of cFDA, thus metabolically active cells. Bacterial cells from strain BS49 were used to confirm the use of PI and cFDA as stains. Therefore, when a cell is dead, it has no metabolic activity and cannot convert the non-fluorescent cFDA into the fluorescent cF. The intensity of the fluorescence of cF is often said to be a measure of the degree of metabolic activity of a cell. PI cannot penetrate the intact membrane of viable cells; however, for damaged cells, PI is absorbed and binds to the nucleic acids to form complexes that emit red fluorescence.

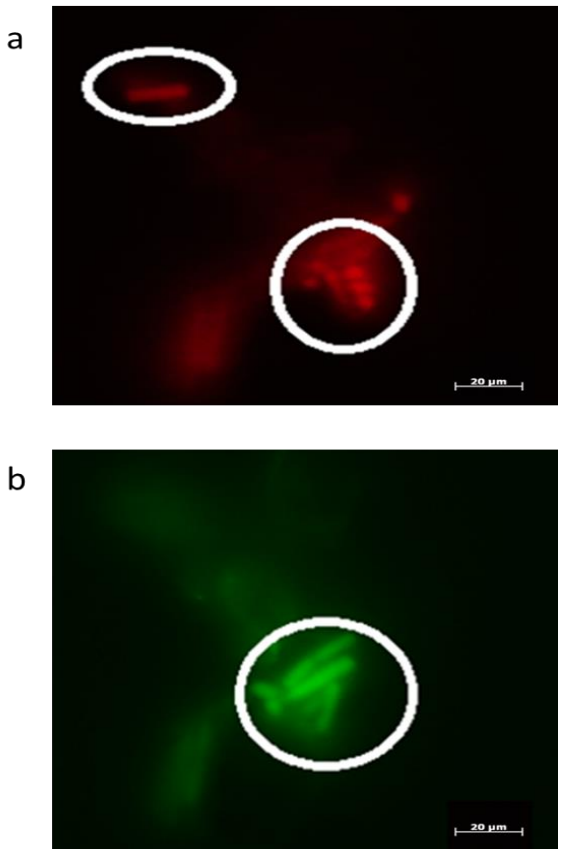

Figure 4.3: Confirmation of absorption and emission of PI and cFDA stains of *B. subtilis* vegetative cells by Epifluorescence microscopy. (a) cell absorption of PI indicating inactive cells

post simulated CIP treatment: (b) cell absorption of cFDA by metabolically active cells post simulated CIP treatment.

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

SEM micrographs of all *B. subtilis* strains before and after simulated CIP treatment (Figures 4.4 – 4.6) showed attachment of intact cells. This indicates that intact cells surviving simulated CIP could still attach to stainless steel. Some of the cells were embedded in what may be extra polymeric substances (EPS) (Figures 4.4a & 4.6b), secreted by the bacteria and subsequently contributing to the structural integrity of biofilms. Figure 4.6b (strain BS54) shows the presence of appendages on the cells, helping cells be further secured to food manufacturing surfaces and/or EPS. No visible damage was identified in any of the bacterial cells attached to the steel coupons, especially in those having undergone simulated CIP treatment. This suggests that these intact cells may likely multiply and cause further contamination along the processing line. Figure 4.4b (strain BS49) show a cell undergoing sporulation. The SEM images also show that most of the cells that have attached are intact.

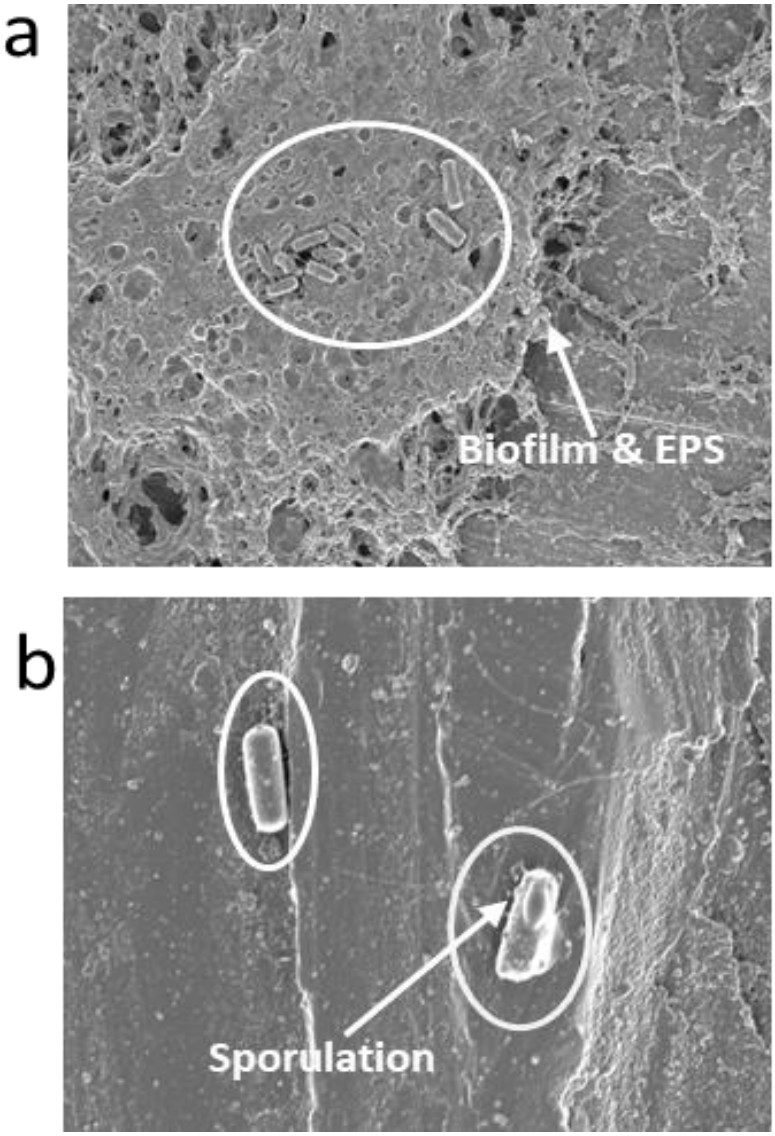

Figure 4.4: SEM micrographs of *B. subtilis* strain BS49 vegetative cells isolated from ESL milk and showing attachment to the stainless-steel surface. (a) CIP treated strain BS49 (b) simulated CIP treated strain BS49 cells. a) x: 7040 bar 2 µM b) x: 17140 bar 2 µM (Simoẽs et al., 2010)

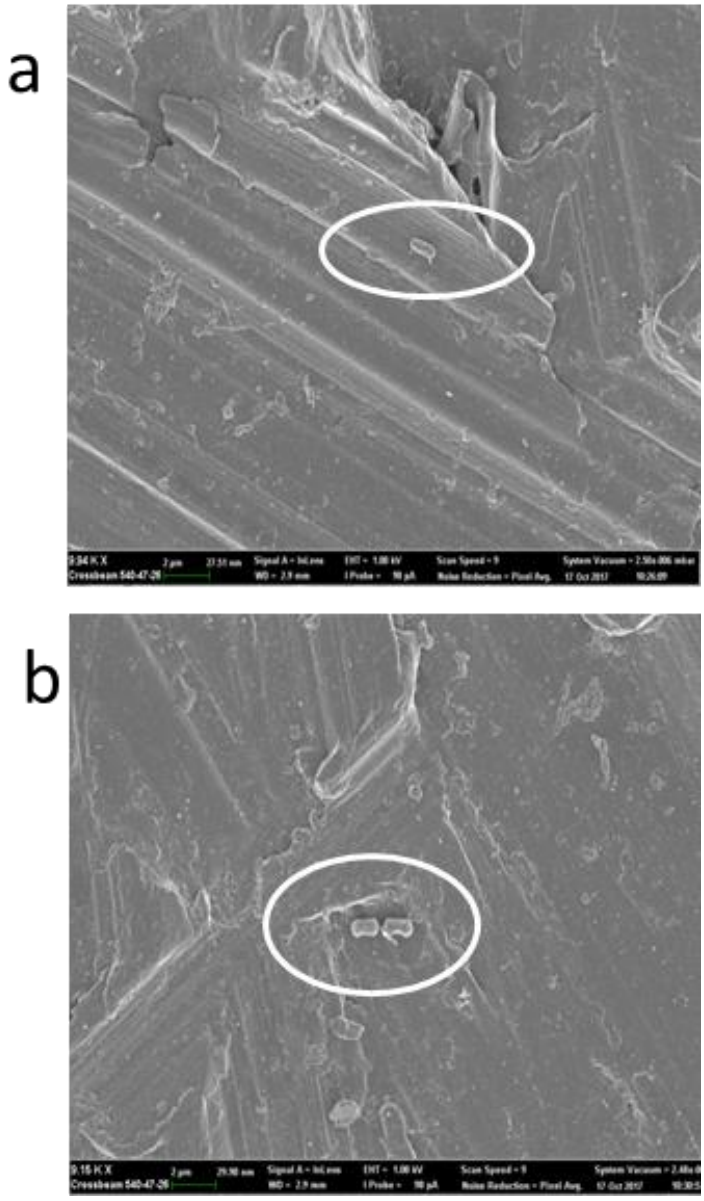

Figure 4.5: SEM micrographs of *B. subtilis* strain BS50 vegetative cells isolated from ESL milk and showing attachment to the stainless-steel surface. (a) BS50 control cells; x: 9.940 bar 3 µM (b) simulated CIP treated strain BS50 cells x: 9150 bar 3 µM

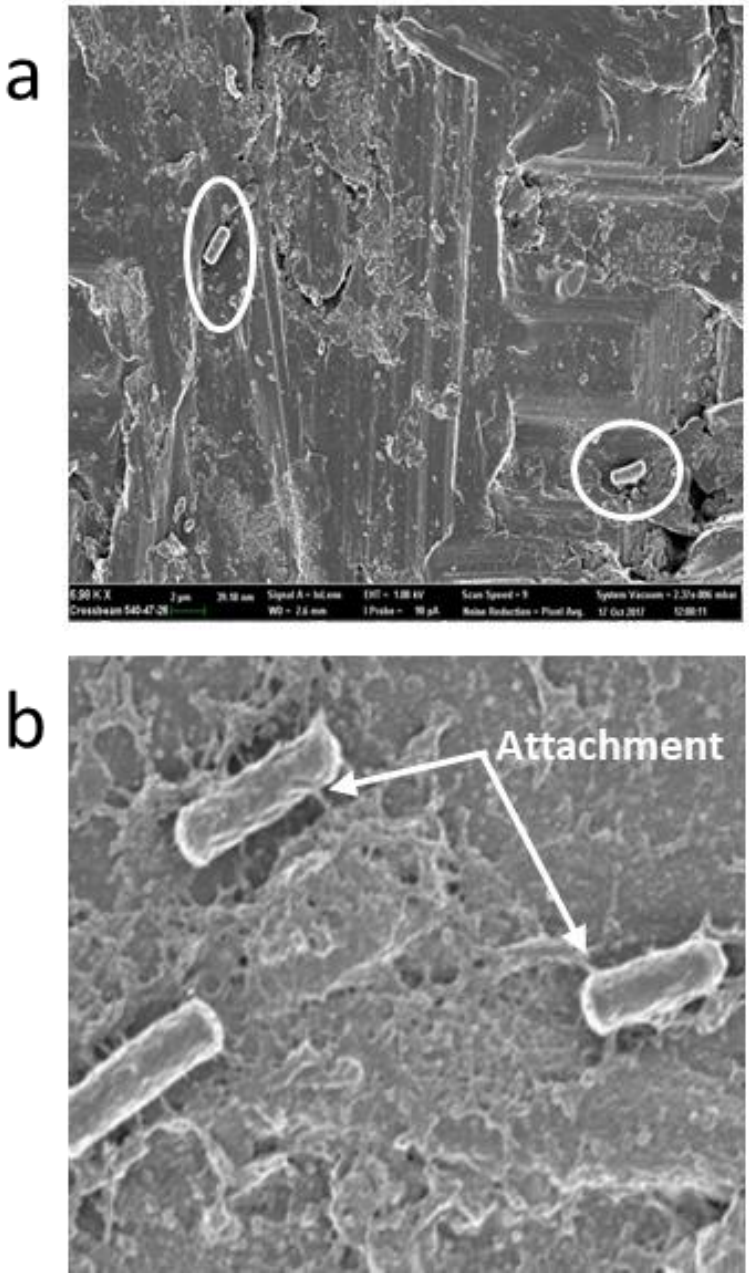

Figure 4.6: SEM micrographs of *B. subtilis* strain BS54 vegetative cells isolated from ESL milk and showing attachment to the stainless-steel surface. (a) BS54 control cells x: 6980 bar 3 µM; (b) simulated CIP treated strain BS54 cells biofilm, EPS, and appendages for attachment to stainless steel x: 9690 bar 1 µM

4.4 Biofilm formation: Biomass quantification of *Bacillus subtilis* following simulated CIP treatment

B. subtilis strains were assessed for their biofilm formation capabilities over (24, 48 and 72 h), with *B. subtilis* ATCC 3610 and ATCC 168 used as positive and negative controls, respectively (Figure 4.6 and 4.7). *B. subtilis* ATCC 3610, characteristic for strong biofilm formation, compared to *B. subtilis* ATCC 168, initially had a significantly higher absorbance reading after 24 h. However, after 48 h, ATCC 3610 had a reduction in biofilm formation while ATCC 168 had 3 times as much biofilm as compared to *B. subtilis* ATCC 3610. At 72 h, *B. subtilis* ATCC 168, not known as an extensive biofilm former, had on average 4 times as much biofilms formation compared to the study strains isolated from ESL milk. A significant difference ($p < 0.05$) between biofilm formation for simulated and non-simulated CIP treatment for the study strains isolated from ESL milk. At 48 h of cell growth, strains BS49 and BS50 had significantly higher biofilm formation for the simulated CIP treated vegetative cells than the untreated cells. At 72 h of cell growth, all study strains had significantly ($p < 0.05$) lower biofilm formation than at 24 h of cell growth. At 72 h, the cells may be at the last stage of biofilm formation, where they break away into the surrounding medium. Analysis by a three-way analysis indicated interactions between strain type, simulated and non-simulated CIP treatment and treatment duration (Table 4.1) concerning biofilm formation.

Table 4.1: Analysis of variance (MANOVA) indicating strain, CIP treatment, and time effects on the biofilm formation of simulated and non-simulated CIP treated vegetative cells of *B. subtilis* strains BS49, BS50 and BS54 isolated from ESL milk

Parameter	**Df**	**p-value**
Strain	2	0.0000
Treatment (CIP/Non-CIP)	1	0.0000
Treatment time (Hours)	2	0.0000
Strain x Treatment	2	0.0000
Strain x Treatment time	4	0.0000
Treatment x Treatment time	2	0.0000
Strain x Treatment x Treatment time	4	0.0000

*Significant ($p \leq 0.05$)

Table 4.2: Biofilm growth of simulated and non-simulated CIP treated vegetative cells of *B. subtilis* strains BS49, BS50, and BS54 isolated from ESL milk

Strain	Treatment time (Hours)	Treatment	Absorbance (OD)	
			Control	Simulated CIP
BS49	24	CIP	$0.2^{abc}\pm0.1$	$0.2^{abc}\pm0.1$
BS49	48	CIP	$0.1^{abc}\pm0.1$	$0.2^{bc}\pm0.1$
BS49	72	CIP	$0.1^{ab}\pm0.0$	$0.1^{ab}\pm0.0$
BS50	24	CIP	$2.0^{d}\pm1.0$	$0.1^{abc}\pm0.1$
BS50	48	CIP	$0.1^{abc}\pm0.0$	$0.3^{c}\pm0.1$
BS50	72	CIP	$0.1^{ab}\pm0.1$	$0.1^{ab}\pm0.0$
BS54	24	CIP	$0.2^{bc}\pm0.1$	$0.2^{abc}\pm0.0$
BS54	48	CIP	$0.1^{abc}\pm0.1$	$0.2^{abc}\pm0.1$
BS54	72	CIP	$0.1^{ab}\pm0.0$	$0.1^{a}\pm0.0$

Superscripts of different letters for each strain indicate a significant difference ($p\leq0.05$)

Table 4.2 indicates no significant difference ($p\leq0.05$) in the absorbance and hence biofilm formation of BS49 between simulated and non-simulated CIP treatment regimes for 24 and 48 h, however not for 72 h treatment. This trend mentioned above was repeated for strain BS54. For strain BS50, there was a significant difference in the biofilm formation of non-simulated CIP treated cells at 48 h treatment time, and simulated CIP treated cells at 72 h.

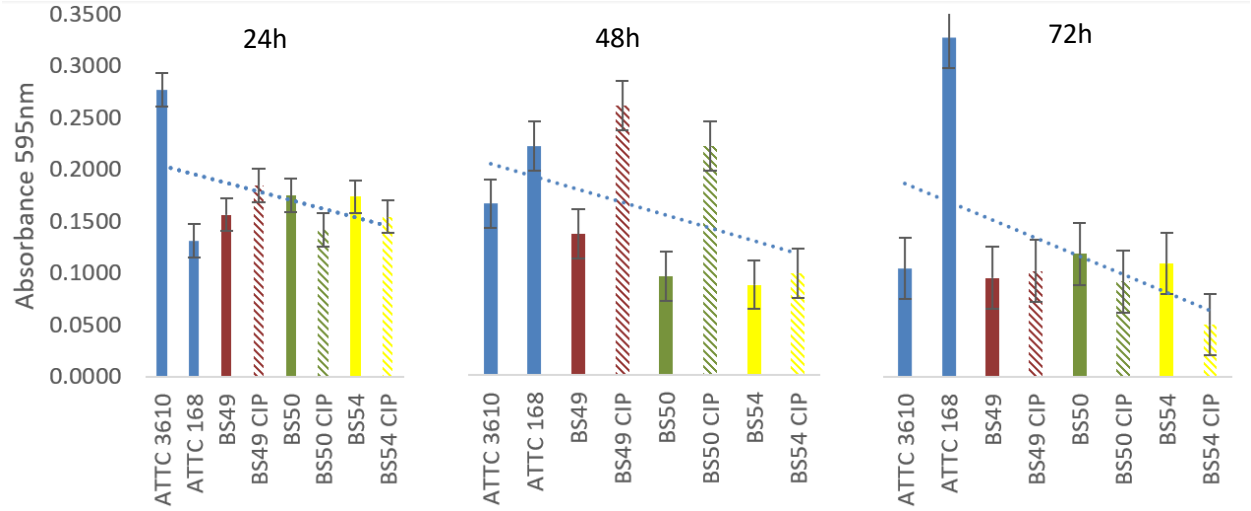

Figure 4.7: Effect of CIP treatment on biofilm formation expressed as absorbances of *B. subtilis* control strains ATCC 3610 and ATCC 168 in relation to simulated and non-simulated CIP treated cells of *B. subtilis* strains BS49, BS50 and BS54 isolated from ESL milk. Absorbance readings were taken after 24, 48 and 72h of cell growth (n=6)

4.5 Enzyme activity: Lipase and protease activity of *Bacillus subtilis* after CIP treatment

All three *B. subtilis* study strains at 0.5 McFarland concentration were plated on skim milk and tributyrin agars to determine milk deterioration and quality effects. All study strains showed observable protease activity with stains BS49 and BS50 having halos $0 \geq 3$ mm, while strain BS54 indicated 3 mm $\geq$ 6 mm. In the present study, only strain BS54 showed observable lipase activity.

Table 4.3: Lipase and protease activity of *B. subtilis* strains BS49, BS50 and BS54 isolated from ESL milk

***B. subtilis* strain**	**Protease activity**	**Lipase activity**
BS49	+	-
BS50	+	-
BS54	++	+

+ indicate enzyme activity 0 ≥ 3 mm halo size; ++ enzyme activity 3 mm ≥ 6 mm halo size; - indicate no observable enzyme activity

4.6 *Bacillus subtilis* growth dynamics – Effect of simulated CIP on the growth of *Bacillus subtilis* vegetative cells

There was a significant difference in the log counts between simulated and non-simulated CIP treated cells for all three study strains at the various storage times and temperatures. The non-simulated CIP treated vegetative cells' log counts were significantly higher than simulated CIP treated cell log counts for strains BS50 and BS54 for all storage and time parameters, except BS54 (21 days: 10 °C). Strain BS49 exhibited the exact reverse for all but one storage and temperature combination (7 d and 5 °C).

In general, a higher storage temperature of 10 °C resulted in a higher bacterial log count for the study strains (Figures 4.8 – 4.10). Strains BS50 and BS54 of simulated CIP treated bacterial cells had a longer lag time before the onset of the exponential phase (Figures 4.9 and 4.10). Also,

strains BS49, BS50 & BS54 for simulated CIP treated cells at 10 °C had a higher microbial growth rate in the exponential phase than the control. Except for the control of strain BS50, all study strains, regardless of CIP treatment, had a low initial growth rate at 5 °C.

A 3-way ANOVA, CIP treatment, storage time, and storage temperature all significantly affected the $\log_{10}$ bacterial counts. There was no interaction between CIP treatment and storage time and, similarly, between CIP treatment and storage temperature (Table 4.4). A 4- way ANOVA (Table 4.5) involving the strains, CIP treatment, storage time and storage temperature had a significant effect ($p \leq 0.05$) and had significant interactions amongst all study factors.

Table 4.4: MANOVA comparing CIP treatment, storage time (0, 7, 14, 21 & 28 days) and storage temperature (5 & 10 °C) on the $\log_{10}$ bacterial colony counts of *B. subtilis* strains BS49, BS50 and BS54 (n=3)

MANOVA	Df	p-value
Strain (BS49, BS50 and BS54)		
Treatment (CIP treatment)	1	0.0000*
Day (0, 7, 21, 28)	4	0.0000*
Temperature (5 & 10° C)	1	0.0001*
Treatment x day	4	0.3681
Treatment x temperature	1	0.2931
Day x temperature	4	0.0213*
Treatment x day x temperature	4	0.4142

*Significant ($p \leq 0.05$)

Table 4.5: Multiple factor (4-way analysis of variance) analysis showing *B. subtilis* strain type (BS49, BS50, BS54), the effect of CIP treatment (control and simulated), storage time (0, 7, 14, 21 & 28 days) and storage temperature (5 & 10 °C) on the $\log_{10}$ bacterial colony counts of *B. subtilis* strains BS49, BS50 and BS54 (n=3)

MANOVA	Df	p-value
Strain	2	0.0000*
Treatment	1	0.0000*
Day	4	0.0000*
Temperature	1	0.0000*
Strain x treatment	2	0.0000*
Strain x day	8	0.0000*
Strain x temperature	2	0.0000*
Treatment x day	4	0.0000*
Treatment x temperature	1	0.0000*
Day x temperature	4	0.0000*
Strain x treatment x day	8	0.0000*
Strain x treatment x temperature	2	0.0000*
Strain x day x temperature	8	0.0000*
Treatment x day x temperature	4	0.0000*
Strain x treatment x day x temperature	8	0.0000*

*Significant ($p \leq 0.05$)

Table 4.6: Colony count of *B. subtilis* strain BS49 cells before and after CIP treatment throughout 28-day storage (n=4)

Strain	LCC CIP (log CFU/ml)	LCC (log CFU/ml)	Storage time (days)	Storage temperature (ºC)
BS49	1.1±0.1	2.1±0.0	7	5
BS49	1.4±0.1	2.1±0.0	14	5
BS49	1.9±0.1	2.3±0.0	21	5
BS49	5.5±0.0	3.3±0.1	28	5
BS49	4.7±0.0	3.2±0.1	7	10
BS49	8.9±0.1	6.8±0.0	14	10
BS49	7.0±0.1	7.8±0.1	21	10
BS49	0.9±0.2	7.11±0.0	28	10

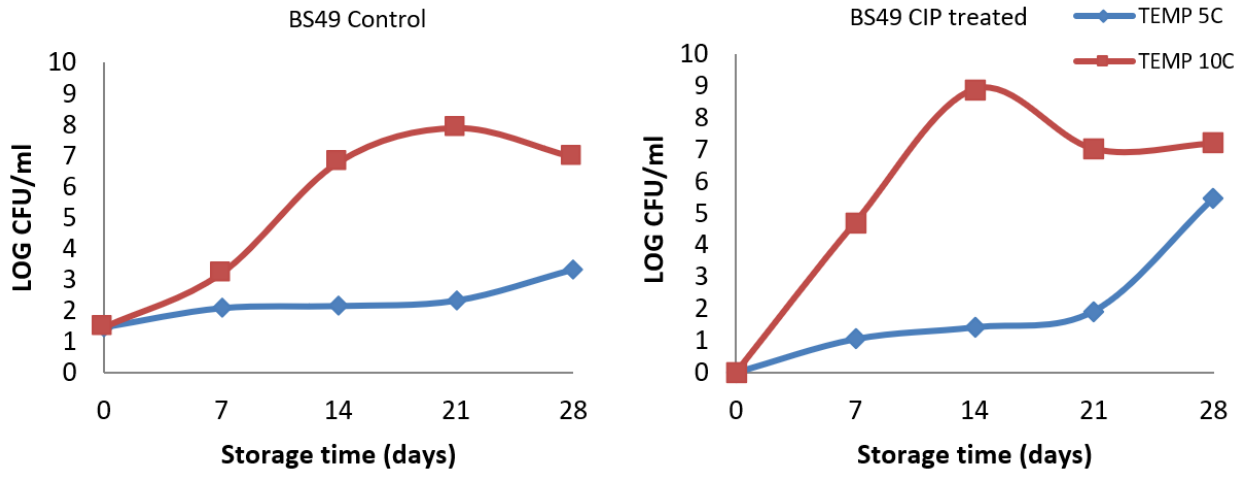

Figure 4.8: Effect of storage temperature (5 and 10 °C) and time in days (0, 7, 14, 21, 28) on the growth of simulated and non-simulated CIP treated *B. subtilis* strain BS49 isolated from ESL milk stored at 7 °C (n= 4)

Table 4.7: Colony count of *B. subtilis strain* BS50 cells before and after CIP treatment throughout 28-day storage (n=4)

Strain	LCC CIP (log CFU/ml)	LCC control (log CFU/ml)	Storage time (days)	Storage temperature (ºC)
BS50	0.5±0.3	5.9±0.1	7	5
BS50	0.9±0.1	8.8±0.1	14	5
BS50	1.1±0.2	6.8±0.1	21	5
BS50	1.2±0.1	6.9±0.1	28	5
BS50	1.1±0.1	7.2±0.0	7	10
BS50	1.1±0.1	3.2±0.0	14	10
BS50	5.7±0.1	7.2±0.0	21	10
BS50	0.9±0.2	7.1±0.0	28	10

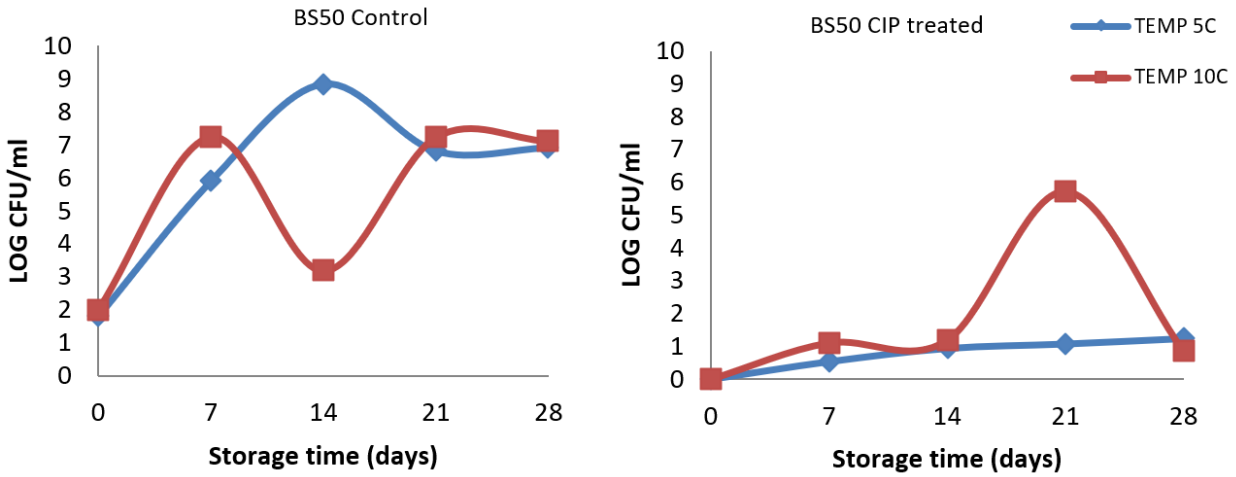

Figure 4.9: Effect of storage temperature (5 and 10 °C) and time in days (0, 7, 14, 21, 28) on the growth of simulated and non-simulated CIP treated *B. subtilis* strain BS50 isolated from ESL milk stored at 4 °C (n=4)

Table 4.8: Colony count of *B. subtilis* strain BS54 cells before and after CIP treatment throughout 28-day storage (n=4)

Strain	LCC CIP (log CFU/m)	LCC control (log CFU/ml)	Storage time (days)	Storage temperature (ºC)
BS54	0.5±0.3	5.9±0.1	7	5
BS54	0.8±0.1	1.4±0.1	14	5
BS54	1.3±0.0	2.0±0.1	21	5
BS54	1.4±0.0	4.5±0.0	28	5
BS54	0.9±0.1	1.9±0.0	7	10
BS54	0.8±0.1	2.1±0.0	14	10
BS54	6.2±0.0	4.4±0.0	21	10
BS54	0.7±0.3	5.0±0.0	28	10

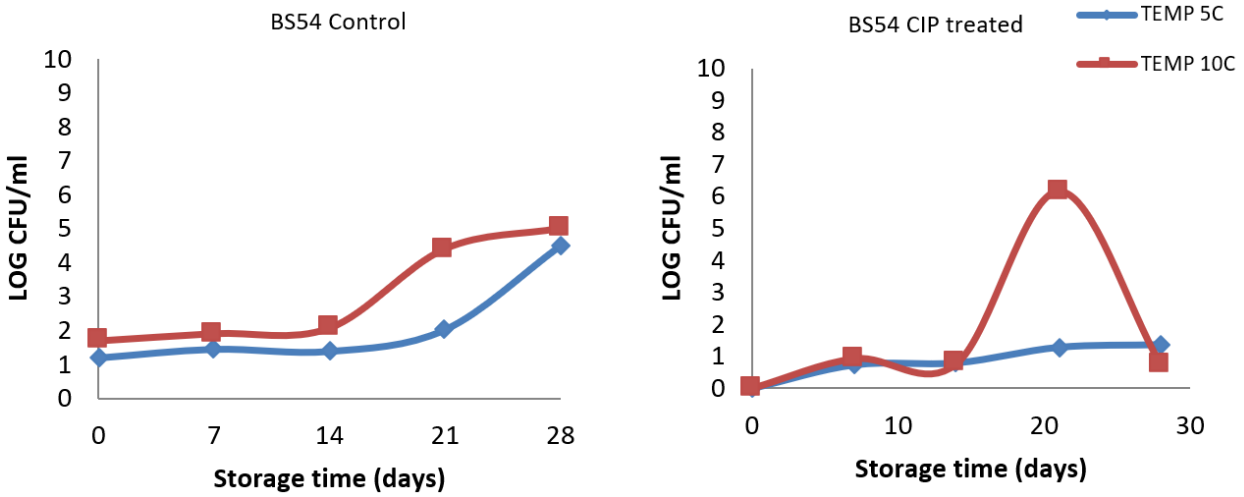

Figure 4.10: Effect of storage temperature (5 and 10 °C) and time in days (0, 7, 14, 21, 28) on the growth of simulated and non-simulated CIP treated *B. subtilis* strain BS54 isolated from ESL packaged milk (n=4)

Table 4.9: Contribution (%) of active variables to the shelf life of *B. subtilis* vegetative cells in ESL milk over 28-day storage (n=3)

Active variables	F1 (%)	F2 (%)	F3 (%)	F4 (%)
Storage time (Days)	14.3	53.4	15.3	17.1
Temperature (°C)	23.1	35.9	0.0	40.9
CIP	33.6	4.5	23.7	38.2
Non-CIP	28.9	6.3	61.0	3.8

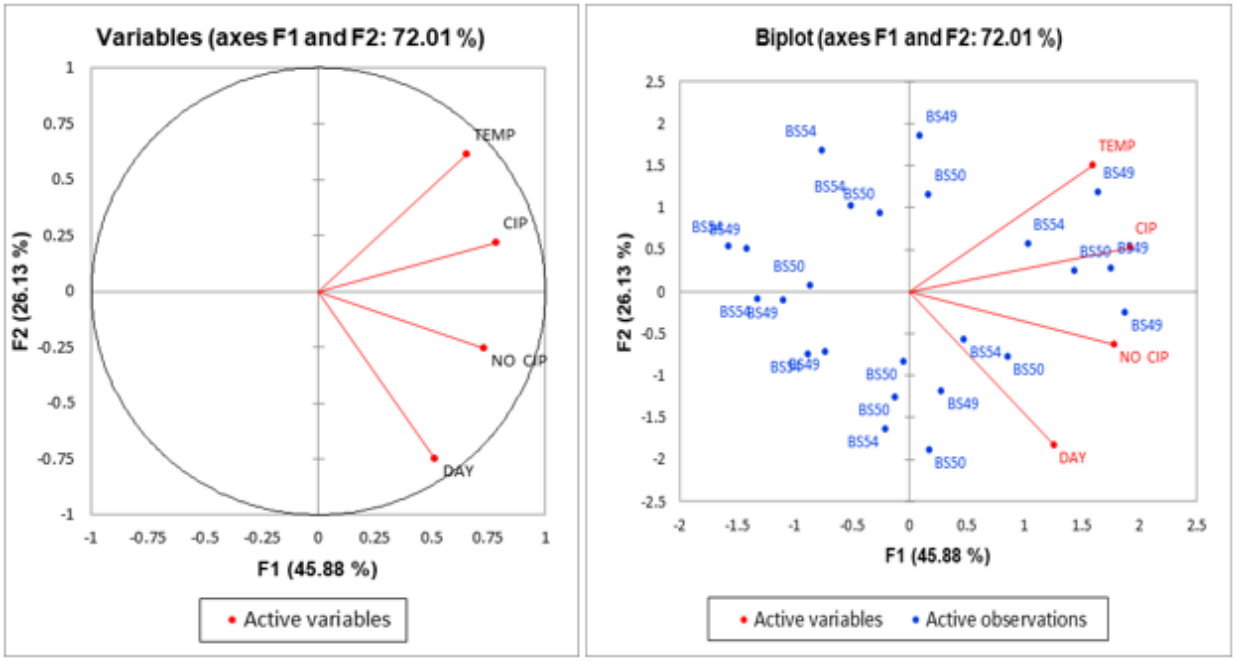

Figure 4.11: PCA correlation circle and biplot of *B. subtilis* strains BS49, BS50 and BS54 against study variables (temperature, day, CIP treatment, No CIP treatment)

Table 4.9 shows the percentage distribution of the active variables to the shelf life of ESL milk, obtained from Figure 4.11. The temperature has the most contribution to F2 (53.4%), and CIP treatment has the least (4.5%), whereas CIP treatment (33.6%) contributes the most to F1 and storage time (14.3%) contributes the least. Figure 4.8 shows all the active variables, including storage time and days, CIP and non-CIP treatment positively correlate with each other. Thus, both longer storage time and higher temperatures result in increasing bacterial log counts, including simulated and non-simulated CIP treated cells. *B. subtilis* strain BS49 from packaged ESL milk stored at 7 °C showed the most bacterial log count increase with increasing temperature for both simulated and non-simulated CIP treatment regimes. *B. subtilis* strain BS54 showed the least positive correlation of all the study strains.

4.7 Discussion

Biofilm formation on food processing equipment surfaces is of concern to the food manufacturing sector. These biofilms may harbour harmful microbes and lead to bacterial contamination of food products that encounter these processing surfaces. Furthermore, embedded microbes can survive thermal processing and subsequently survive cleaning regimes applied to remove bacterial and food residues in processing equipment. Recent studies have suggested the formation of biofilms by *Bacillus* vegetative cells under various nutrient and processing conditions (Lindsay et al., 2006).

In the present study, *B. subtilis* strains isolated from ESL milk in a previous study are subjected to a simulated chemical CIP cleaning regime to ascertain cleaning effectiveness and its effects on the structural integrity of *B. subtilis* vegetative cells. FCM analysis shows evidence of the removal

and inactivation of *B. subtilis* cells by simulated CIP cleaning with 98.3-98.8% of bacterial cells inactivated by the cleaning regime. However, SEM micrographs also show that the remaining fraction of bacterial cells can survive the simulated CIP treatment and attach to stainless steel surface to form biofilms. This indicates the probable survival and further contamination of dairy products by *B. subtilis* after CIP cleaning regimes used in the dairy industry. This study also shows that the simulated CIP treated *B. subtilis* vegetative cells can germinate and grow subsequently in ESL milk under refrigeration conditions. Spore-forming heat resistant organisms such as *B. subtilis* embedded within biofilm matrixes can form heat resistant spores resulting in long-term persistent contamination (Jindal et al., 2016).

In the present study, there is evidence through SEM of a vegetative cell undergoing sporulation. The enlarged SEM image of a vegetative cell undergoing sporulation in the present study seems to reflect the engulfment stage in the *B. subtilis* sporulation process (Higgins & Dworkin, 2012). The formation of spores by vegetative cells can lead to cross contamination of ESL milk products, as evidenced by Khoza (2015) with *B. cereus* spores. Although Khoza (2015) proved that CIP treatment is effective in processing plants because of low initial microbial loads, there is evidence that biofilms that harbour vegetative cells or spores will form in processing equipment regardless (Mogha et al., 2014). The formation of spores by *B. subtilis* during nutrient-limited growth conditions in processing equipment has been confirmed by Lindsay et al. (2006) and by Khoza (2015) in the filler nozzles of ESL milk machinery.

In the present study, the CIP procedure involved the use of an alkaline detergent containing sodium hypochlorite. The use of alkaline detergents with or without sodium hypochlorite and acid detergents have proven successful in inducing more than a 5.0 $\log_{10}$ CFU/ml reduction of cell numbers (Peng et al., 2002). The effectiveness of CIP systems may depend on the initial microbial load of raw milk as it depends on the actual effectiveness of the CIP regime itself concerning cleaning chemicals and process dynamics.

Simulated CIP treatment significantly reduced the bacterial numbers of *B. subtilis* strains BS50 and BS54 but not in *B. subtilis* strain BS49. SEM micrographs of strain BS49 in the present study show extensive biofilm and EPS production. Thus, cells of strain BS49 were protected by the extensive biofilm and EPS network cells from effective cleaning were protected by the simulated CIP regime applied in this study. Biofilm production in *B. subtilis* is known to be a combination of the TasA protein and EPS (Diehl et al., 2018), with Quorum sensing (QS) known to enable the resilience of biofilm communities. Also, mutations in certain genes involved in surfactin production, including the *sfp*, *epsC*, *swrA* and *degQ* genes, affect biofilm production in *B. subtilis* (McLoon et al., 2011). The deviation from the expected result of much more robust biofilm production from the wild strain; *B. subtilis* strain 3610 maybe because of mutations occurring during long-term storage and continuous sub-culturing due to domestication. However, this assertion can only be proven by gene expression studies. From this study, the CIP regimes are not full proof, and hence other technologies could be included to remove biofilms effectively. Quorum quenching (QQ) is a process of disrupting the quorum sensing mechanism using QQ driving

molecules (Paluch et al., 2020), including QS receptor auto inductors, and could be incorporated in CIP regimes.

Absorbance readings of CIP treated *B. subtilis* strain BS49 were marginally higher at the 24 – 48 h period. According to Peng et al. (2002), biofilm cells of *B. cereus* were more resistant to CIP than attached single cells and planktonic cells regardless of the CIP regime used. SEM micrographs in the present study show *B. subtilis* strain BS49 as attached single cells embedded in biofilms instead of existing as planktonic cells.

B. subtilis can grow at temperatures from 5 to 20 °C and up to 45-55 °C (Heyndrickx and Scheldeman, 2008). Concerning ESL milk, recommended storage temperature is a maximum of 8 °C for a shelf life of 21 – 30 days, though < 6 °C was recommended by Rysstad and Kolstad, (2006). However, based on the present study and another carried out by Deeth (2017), temperatures ≤ 4 °C is highly recommended. The present study shows an increase in the *B. subtilis* strains from day 0 to 28, albeit higher at 10 °C in most cases. Though *B. subtilis* is characterised as mesophilic (Heyndrickx and Scheldeman, 2002), there was on average a 3 $\log_{10}$ increase in this study, suggesting psychrotrophic capabilities and suggesting that ESL milk contaminated with *B. subtilis* cells will experience a gradual increase in microbial numbers even at refrigeration temperatures. Although Mugadza and Buys (2017) suggested that *B. subtilis* poses a bigger problem as a pre-processing microbial contaminant rather than growth at refrigeration temperatures, that study still experienced a gradual increase in bacterial numbers of *B. subtilis* under refrigerated conditions. Yagoub et al. (2007) indicated significant growth of *B. subtilis* cells at 7 °C resulting in the shelf life of 5 days of sterile milk and samples stored at higher temperatures offering 1-4 days shelf

life. Thus *B. subtilis* can significantly reduce the shelf-life of sterile milk products at recommended ESL storage temperatures if previously contaminated. As expected, non-CIP treated *B. subtilis* cells had higher log CFU numbers as the CIP treated cells become inactive or injured during cleaning regimes. However, CIP treated *B. subtilis* cells still reproduced at 5 and 10 °C though at a lesser rate in the former.

Enzymes play an important role in food systems, where they can either increase or decrease the value of food commodities (Teh et al., 2003). Biofilms are an emerging source of spoilage enzymes because of harbouring vegetative cells and spores in this protective matrix. All study strains exhibited some proteolytic activity, indicating *B. subtilis* as potentially one of the main causes of bacterial spoilage through proteolysis in ESL and other milk products (Mugadza & Buys, 2018). However, only one *B. subtilis* strain showed minimal lipolytic activity, consistent with a previous study (Mugadza & Buys, 2018) where *B. subtilis* isolates did not exhibit lipolytic activity. This indicates that the potential for *B. subtilis* to contribute to lipolytic spoilage of milk is not likely.

CHAPTER 5: GENERAL DISCUSSION

5.1 Critical review of methodology

The contamination of stainless-steel processing equipment has been a persistent problem in the dairy industry, affecting the quality and integrity of ESL milk and other long-term storage products. This study aimed to determine if CIP treatment cleaning process used in industry is an effective way to remove and inactivate *B. subtilis* in biofilms on stainless steel processing equipment and if biofilms can still produce enzymes that contribute to the degradation of milk hence affecting shelf life.

The simulation of the CIP process provided some difficulties as it happened on a small scale in an Eppendorf tube, and the excess chemicals and water used for washing the cells had to be continuously decanted after each step with a pipette with care to prevent decanting some of the cells in the pellet. A loss of cells in the pellet meant that the visuals of the flow cytometry images would not show the characteristic intense dark colouration due to low cell count. The process of the CIP simulation also took quite a few hours due to the decanting process and having to centrifuge with each step. The alternative method of executing this simulation currently would prove to be difficult even in a pilot plant because the equipment would have to be fouled with *B. subtilis*, and the chemicals needed would have to be of larger volumes. However, for large scale CIP treatments to closely mimic industrial type versions concerning water pressure, miniaturised models of processing equipment and CIP systems will need to be built. That, however, will add high costs to lab scale CIP studies.

The determination of the *B. subtilis* growth curve in ESL milk required significantly higher media preparation every week, and the number of plates needed to be increased every week due to the increase of the *B. subtilis* concentration in the milk leading to a need to increase the dilution for plating to achieve single colonies within 30-300 colonies counting range.

Biofilm studies in the present study utilised the crystal violet assay in microtiter plates using BHI. Other studies (Kwon et al., 2017) have used Tryptic soy broth (TSB) instead of BHI, the former known to form larger biofilms than the latter. Also, plastic microtiter plates were used in this study as opposed to glass or stainless-steel materials. Kwon et al. (2017) showed that stainless steel surfaces produced more biofilms than plastic and glass surfaces. Thus, these variations in surface type and temperature of treatment and type of carbon source can have significant differences in biofilm formation.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

Albeit a minute fraction, some *B. subtilis* vegetative cells can survive CIP treatment and attach to dairy processing equipment such as heat exchangers and filler nozzles which lead to cross contamination of final ESL milk products. Refrigerated storage of the contaminated final milk product leads to further proliferation of the *B. subtilis* cells, which will cause serious illness to the consumer; during this time, the cells also produce enzymes that affect the milk quality. CIP treatment does not influence the attachment of *B. subtilis* to stainless steel; the cells can also sporulate under these conditions until conducive conditions return of which can germinate.

6.2 Recommendations

It would be interesting to study further the environmental and strain factors affecting the sporulation of *B. subtilis* vegetative cells in a biofilm and studying the genes responsible for these actions.

A study on injured *B. subtilis* cells obtained through flow cytometry analysis using its cell sorter would show us a different perspective on what happens to those physically damaged cells but are still metabolically active with the possibility of being able to recover and subsequently grow.

Also, gene changes that occur within *B. subtilis* vegetative cells for enzyme secretion before and after CIP treatment could be a future recommended study.

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