Characterisation and spoilage potential of *Bacillus subtilis* complex group isolated from milk processing environment

By

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Submitted in partial fulfilment of the requirements for the degree Ph.D. (Food Science)

In the
Department of Consumer and Food Sciences
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DECLARATION

I, the undersigned, declare that this thesis, which I hereby submit for the degree Ph.D. (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.

_____

James Ayokunle Elegbeleye

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DEDICATION

To the God of all grace who saw me through

To my loving wife Grace Elegbeleye

To my sweet sisters Olufisayo Okoosi and Adeola Adebisi and their lovely families

To my father and brother Mr Ayodele Elegbeleye and Ayotomiloye Elegbeleye

To the loving memories my late mother Mrs Funmilayo Elegbeleye

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Abstract

Characterisation and spoilage potential of *Bacillus subtilis* complex group isolated from milk processing environment

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Supervisor: Prof. E. M. Buys
Department: Consumer and Food Sciences
Degree: PhD Food Science

Thermophilic *Bacillus* and their spores are important in dairy processing due to their ubiquity, resistance to high temperature, chemical inactivation and their biofilm forming potential. The presence of such microbial contaminants results in shelf-life reduction of processed foods serving as a potential hotspot of outbreaks and spoilage of post-processed milk. Therefore, this study seeks to characterise *Bacillus subtilis* and *Bacillus velezensis* from raw, pasteurised and packaged extended shelf-life (ESL) milk samples, determine their biofilm forming and spoilage potential as well as the effect of ultraviolet C (UVC) in the inactivation of their spores with the intent of mitigating their deleterious presence in the dairy processing plant.

The isolates were identified *B. subtilis* and *B. velezensis* with the potential of forming weak, moderate and strong biofilms with *B. velezensis* strain LPL-K103 (B44) with the most robust biofilm. All the isolates are novel sequence types (STs) using their multi-locus sequence type profile with the closest STs are 96 for *B. velezensis* and 128 for *B. subtilis* mostly isolated from the soil. The heat resistance profile indicated all 12 isolates are psychrotolerant as well as thermophilic with temperature ranges of 6 °C to 55 °C, 6 °C to 60 °C and 15 °C to 60 °C. All isolates can produce both lipolytic and proteolytic enzymes both in their planktonic and biofilm states.

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The maximum lethality rate after UVC exposure is 6.5 for *B. subtilis* strain SRCM103689 (B47) and highest percentage hydrophobicity was 54.9 % from the sample *B. velezensis* strain LPL-K103 (B44). Flow cytometry analysis of UVC treated spore suspensions showed a divergence into subpopulations unaccounted for by plate counting on growth media which are: inactivated, live spores, dormant, sub-lethally injured and an unknown subpopulation. The Raman spectroscopy identified *B. subtilis* CECT 4002 (B4002) spores as the isolate possessing the highest concentration of Ca-DPA.

The spoilage potential of the isolates was determined by quantifying the concentrations of proteolytic and lipolytic enzymes produced by the biofilm and planktonic cells by using azocasein and p-nitrophenol palmitate (p-NPP) assays. In the planktonic cells, B48 has the highest proteolysis with 1033.6 ρL/CFU while B50 has highest lipolysis of 34.5 ρL/CFU. For the biofilms, B168 has the highest proteolysis and lipolysis per cell with a mean 3706 ρL and 179.9 ρL. The result of this study indicated that the spoilage potential (proteolysis and lipolysis) both of biofilms and planktonic culture are strain-dependent and that there seems to be a relationship between the strength or complexity of the biofilms and spoilage potential of the isolates. The study presents the significance of thermophilic *B. subtilis B. velezensis* and possible reason for their perpetuation in the dairy processing plant. The result linked the isolates to the raw milk used in the production of ESL milk-fed into the downstream processing line suggesting the survivability of the isolates by adaptation to the processing condition either as spores or as a community as in biofilms. The quality of the raw milk is thereby compromised which in turn affect the shelf-life of the final product. The result highlighted the effect of UVC in the inactivation of the spores and spore surface hydrophobicity are heterogenous with some strain-to-strain variations at molecular level among the organisms used.

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1 INTRODUCTION

According to the report of the International Dairy Federation (International Dairy Federation, 2018), cow milk production represents more than 80 % of total milk production globally in 2017 and this amounts to about 700 million tonnes. South Africa produced 3.5 million tonnes of cow milk in 2015 alone with more than 70 % of its production processed into UHT, pasteurised and sterilised milk (FAOSTAT, 2017).

Milk is a highly nutritive food that is widely consumed by people of diverse cultures and age-groups. Besides this demand for milk, there is also a growing desire by consumers for fresh-like, safe and quality milk with an extended shelf-life during storage (Schmidt et al. 2012). The nutrient components of milk, its pH (6.5-6.7) and high water activity predispose milk to microbial contamination thereby causing spoilage of the milk and illnesses that can impact negatively on the health of the consumers (Carballo et al. 2015; Quigley et al. 2013). This propensity for microbial contamination of milk and demands by the consumer has propelled the creation of innovative solutions to address the challenges in the dairy industry with little or no alteration to the processed milk in terms of nutritional and sensory quality (Georget et al. 2015).

Milk processors in South Africa commonly combine bactofugation and pasteurisation to produce extended shelf-life (ESL) milk but the presence of aerobic spore-forming bacteria in the raw milk is of concern and pose a great threat during processing and in the final extended shelf-life (ESL) milk product. Apart from their ubiquity in the environment, they produce spores which are recalcitrant to processing conditions as well as thermostable enzymes which can withstand processing conditions such as heat, pressure and chemical substances used during cleaning-in-place (CIP) and as preservatives (Ostrov et al. 2016; Reverter-Carrión et al. 2018). The presence of hydrolytic enzymes and other metabolites in food result in spoilage and

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reduction of product shelf-life (Sadiq et al. 2016). Several studies implicated raw milk as the major source of aerobic spore formers which may later end up in the finished product (Burgess et al. 2010; Mugadza and Buys, 2017; Sara A Scott, John D Brooks, Jasna Rakonjac, 2007).

Apart from the production of heat-resistant spores by this group of microorganisms, they also form a surface-attached community of cells called biofilms (Costerton et al. 1995). Biofilm formation is one of the emerging challenges to food safety in the dairy industry where they develop on the dairy plant and equipment (Phillips, 2016). Biofilms generally defy all strategies and measures previously adopted in controlling spoilage and pathogenic contaminants in the milk processing plant such as cleaning-in-place (CIP) and high temperature (Corcoran et al. 2014). Biofilm often serves as a safe-haven for pathogenic and spoilage organisms and thereby constituting a public health challenge to the populace and economic loss to the industry (XinNa and XianMing, 2009). The interactions among the microbial population within biofilm confer some physiological and morphological functions and advantages on the biofilm which are entirely different from that of the planktonic cells (Henry et al. 2016).

Unlike the planktonic or free-living form, biofilms possess multifaceted mechanisms to resist several environmental stresses. This is exhibited in the failure of antimicrobials and other chemical agents to penetrate the extracellular polymeric substances (EPS) due to strong adaptive response and development of a phenotypic variant of microbial cells associated with the biofilm called persister cells (Knudsen et al. 2013). The presence of the persisters which is characterised by highly reduced metabolism increased the survival of the biofilm to some control measures put in place. They are responsible for the recalcitrant nature of some cells. As they neither grow nor die, they are difficult to inactivate as most antimicrobials target some pathways in metabolically active cells (Lewis, 2013).

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Apart from the important role they play in the attachment of pathogenic and spoilage organisms to food and food contact surfaces, biofilms have been proven to break down the membranes used in reverse osmosis, contaminate food during processing (Carpentier and Cerf, 1993), reduces the effectiveness of heating and cooling equipment and corrodes metal surfaces in processing factory (Sharma and Anand, 2002). This development has raised safety and quality concerns in the dairy industry and make control more imperative.

Milk provides nutrients for microorganisms to thrive upon in the dairy plant most especially the hard-to-reach parts which create a rich niche for the biofilm formation due to their inaccessibility to CIP chemicals (Jindal et al. 2018). The development of biofilms in the dairy plants constitutes a major challenge in the food industry because of the potential impact on quality, functionality, and safety of the food products thereby resulting in great economic losses to the food industry and health risk for consumers if not prevented or controlled (Galié et al. 2018). Therefore, it is important to prevent and control the formation of biofilm in the dairy industry to achieve quality and safe products with an extended shelf-life.

There is an ongoing effort in finding innovative processing strategy and techniques to mitigating the challenge of aerobic spore-forming bacteria in the food industry (Van Impe et al., 2018). Therefore, this study will characterise members of the *Bacillus subtilis* complex group isolated from dairy processing plant using multi-locus sequence typing (MLST) with the aim of source-tracking the *Bacillus* contaminants and to determine their biofilm-forming and spoilage potential in milk and evaluate the effects of ultraviolet C radiation on the spores of the bacterial isolates.

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2 LITERATURE REVIEW

This review aims to critically assess the significance of aerobic spore-forming bacteria belonging to the *Bacillus subtilis* complex group and the associated food spoilage or shelf life impact challenge. Insights into the ability of this bacterial group to produce spores and form biofilms on food contact surfaces will be provided. This entails an understanding of the mechanisms of the formation, structure, architecture, and resistance of the spores and biofilms to various processing conditions and chemicals used for the CIP regimes. Lastly, novel technologies and strategies used to mitigate or eradicate this food safety challenge will be evaluated for their effectiveness and shortfalls.

2.1 Presence of microorganisms in the food industry and the inherent challenges

Several factors conspire to make milk a very rich medium for the growth of microorganisms: it is rich in fats, proteins, carbohydrates, minerals, vitamins and provides suitable pH and water activity (Quigley et al. 2013). These same factors also satisfy the nutritional requirement of microbes for growth and development (Hu et al. 2017). Hence, strict microbiological guidelines are adopted and applied during production in the dairy industry to prevent contamination of the product by microorganisms and their spores which compromise safety, quality and functionality as a result of extracellular enzymes, toxins and other metabolites produced by contaminating microbes that gain entrance into food (Teh et al. 2011).

It was estimated that the global food loss annually is over 1.3 billion tons of food which represent half of the food produced for human consumption (Gustavsson et al. 2011). Though difficult to quantify in South Africa, microbial-associated contamination of food resulting in potential food-borne infection costed the food industry in New Zealand (with a population of

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about 4 million) was $88.8 million in 2000 (Scott et al. 2000). A large proportion of the losses is due to microbial spoilage and contamination such as in cases of outbreaks. The recent *Listeria monocytogenes* outbreak of 2017/2018 in South Africa resulted in 5,812 tons of ready-to-eat processed meat recalled with the cost expended in curtailing the crisis exceeding $1 million (Hunter-Adams et al. 2018)

Globally, there is an increasing awareness in consumers of the health-related risks of using preservatives in processed food hence the demand for minimally processed, fresh-like food products with no or little synthetic chemical preservatives added. This demand presents an immense challenge to the food industry as it puts them under strain to meet this demand as well as others from industry regulators and government (Egan et al. 2016).

Milk in the udder is sterile, but it can easily get contaminated from microbes on the hides of farm animals, milking equipment, feeds and the environment (VanderKelen et al. 2016). Other possible routes of contamination are during transportation, storage, and processing of the raw milk (Gopal et al. 2015). The accepted level of total bacterial count (TBC) in unpasteurised raw milk is less than 100,000 cfu/ml with the general assumption that these cells will be inactivated during pasteurisation (Pantoja et al. 2012; US Food and Drug Administration, 2009). The presence of aerobic spore-forming bacteria in raw milk during the processing of the product often undermine the pasteurisation process. This can lead to a high rejection rate of the product because of the spoilage potential and the possibility of causing illness (Reich et al. 2017). Aerobic spore formers of the Bacillaceae have gained notoriety in the past two decades as the primary contributors to the quality issue faced by the dairy industry (Huck et al. 2008; Kent et al. 2016).

The thermophilic contaminants in the dairy industry are divided into two categories: facultative and obligate thermophilic bacilli. The key members of the obligate thermophiles are

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Geobacillus stearothermophilus and *Anoxybacillus flavithermus* with an optimum growth temperature of 40-68 °C. The members of the genus *Bacillus* are facultative thermophiles and include *B. licheniformis, B. pumilus* and *B. subtilis*. They grow at both mesophilic and thermophilic temperatures (between 50 and 65 °C) and are generally non-pathogenic (Burgess, Lindsay and Flint 2010; Heyndrickx and Scheldeman 2008; Kent et al. 2016). Apart from their ability to grow at high temperature, they also produce resistant spores and form biofilms within the dairy processing plant which make it difficult for them to effectively controlled (Burgess, Lindsay and Flint 2010; Kumar 2017; Sadiq, Li, Liu, Flint, Zhang, Yuan, et al. 2016). They compromise the product quality and shelf-life of ESL milk by producing proteolytic and lipolytic enzymes and other secondary metabolites that can lead to the development of off-flavour of milk e.g. *B. subtilis* has been indicted to cause spoilage in canned product and UHT milk (Heyndrickx and Scheldeman, 2008).

2.2 The genus *Bacillus*

The genus *Bacillus* is a highly diverse one with a rich history in the discipline of microbiology. They consist of more than 300 members that are well described (Fan et al. 2017). They possess a unique characteristic of endospore formation and belong to the phylum Firmicutes. It was first observed by Ferdinand Cohn when he described what he saw as *Bacillus subtilis* meaning "*thin rod*." Members of the genus are for the most part motile, obligate aerobe or facultative anaerobe, rod-shaped bacteria with the ability to form endospores under stress or nutrient depletion in the environment (Alina et al. 2015). They are ubiquitous and the spores found in the soil, dust, water, air, aquatic sediment, food and food processing environment, gastrointestinal tract, animal feeds etc (Fakhry et al. 2008; Flint et al. 2017; Tam et al. 2006).

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Application of 16S rRNA in *Bacillus* taxonomy classified this genus into five distinct groups based on the sequence similarity. These are the major groups of the genus based on the 16S rRNA typing *B. subtilis, B. licheniformis, B. amyloliquefaciens, B. megaterium, B. cereus, B. anthracis, B. thuringiensis*. *B. subtilis* and *B. cereus* are the most popular and widely studied of the genus because of the industrial application and pathogenicity respectively (Harwood, Mouillon, Pohl, Jos and Arnau 2018; Mugadza and Buys 2017). It is rod-shaped, motile, possessing peritrichous flagella forming terminal or sub-terminal endospore which is released upon the lysis of the mother cell. They grow at a very low temperature of between 5 to 20 ^{o}C which qualifies them as potential spoilage organism of ESL milk under refrigeration condition. They can also grow at a thermophilic temperature between 44 and 55 ^{o}C and can survive pasteurisation especially in the form of spores (Sadiq, Li, Liu, Flint, Zhang and He, 2016).

Members of the *B. subtilis* complex group are very closely related species. They cannot be fully delineated phylogenetically using biochemical, phenotypic not even 16S rRNA sequencing. This is because of the 16S rRNA regions in the genus are highly homologous hence the need for a region of the genes that show some hypervariability (Rooney, Price, Ehrhardt, Swezey and Bannan 2009). The degree of relatedness using 16S rRNA is $\geq$ 99 % among members of the group for proper speciation and the definition of new taxa of the *B. subtilis* members, the use of multi-locus sequence typing (MLST) have been proposed (Cho et al. 2018; Connor et al. 2010). MLST makes use of seven house-keeping genes coding for some proteins that are specific for members of the group. This technique can be used in tracking the source of contamination in the dairy industry.

The members are well-known for their ability to produce some secondary metabolites that have antimicrobial properties against some bacteria and fungi such as antimicrobial peptides (AMP), polyketides, terpenes and siderophores that have industrial benefits (Harwood et al. 2018). The

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genus is reputed for their industrial production of important metabolites that are of commercial importance such as riboflavin, enzymes, vitamins and antibiotics (Contesini et al. 2018; Schallmey et al. 2004). These characteristics that endeared the members of the *Bacillus* genus in their adoption to produce commercial chemicals such as enzymes make them undesirable in the food industry, especially the dairy. The production the proteolytic and lipolytic as well as other metabolites enzymes in milk have a deleterious effect that leads to spoilage (Logan, 2010).

2.2.1 *Bacillus*: their spores and presence in the dairy industry

Bacterial spores are metabolically dormant structures generally induced by low nutrients in the environment or in response to stressors. Spores are generally resistant to heat, chemicals such as CIP solutions, radiation, extreme pH and dehydration (Tola and Ramaswamy 2014; Setlow 2006). This confers on them the propensity to perpetuate in the environment over a long time sometimes up to hundreds of years (Cano et al., 1995; Kennedy et al. 1994). Spores germinate into the vegetative form upon sensing the presence of nutrients or germinants and other favourable growth conditions in the environment which may lead to food spoilage and foodborne illnesses (Setlow, 2003). Besides this, spores have been shown to have a better attachment to food-contact surfaces than the vegetative cells after which they form a biofilm (Burgess, Lindsay and Flint 2010; Flint, Palmer, Bloemen, Brooks and Crawford 2001; Parkar, Flint, Palmer and Brooks 2001; Seale, Flint, McQuillan and Bremer 2008). The presence of spores in milk could be attributed to the presence of calcium ions in milk and other minerals which triggers the sporulation process through an adaptive response of the vegetative cell to these stimuli. (Oomes et al. 2009; Seale et al. 2008). There is an adjustment to the bacterial

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transcriptomes through a cascade of complex reactions in response to the external stimuli (Nicolas et al. 2012).

Sporulation in *B. subtilis* is one the best understood and widely studied among aerobic spore formers. An in-depth and detailed understanding of the sporulation process provides a knowledge on the persistence of spores and methods that can be applied in assuaging their presence in the food industry. The mechanism of spore resistance seems to be similar in all *Bacillus* species, especially among the *B. subtilis* complex group (Setlow 2006). This review will focus mainly on the mechanisms of spore formation and resistance of *B. subtilis* as the representative organism. This is so since the mechanisms of dormancy and germination of spores are similar in most endospore formers except for few differences in the initiation stage which are likely niche-specific (Errington, 2003).

2.2.2 Formation and structure of *B. subtilis* Spore

Sporulation is an energy-consuming and irreversible process which involves a commitment before its commencement among sub-populations of organisms in an environment. The cells either initiate the process of sporulation or resort to other alternatives such as cannibalism of isogenic sub-populations that have lost the competitive edge or form biofilms to avoid committing to the process (Claverys et al. 2007; González-Pastor, 2011). As nutrients from the cannibalised cells further deplete, the surviving sub-population then initiate sporulation.

Sporulation of *B. subtilis*, as well as other endospore-forming bacteria, is an intricate process that is initiated as a response to nutrient depletion in the environment triggered by master transcription regulon called Spo0A (Piggot and Hilbert, 2004). The Spo0A has direct

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regulatory control of 121 genes in *B. subtilis* and is also responsible for the formation of biofilm and K-state cells (Dubnau et al. 2016; Hamon and Lazazzera, 2002).

The first step in endospore formation begins with the asymmetrical division of the cell into two morphologically different but genetically similar compartments called the forespore (prespore) and mother cell (Henriques and Moran, Jr., 2007). The prespore grows into the spore while the mother cell is important in the development of a complete spore after which it undergoes programmed cell death or autolysis while discharging the spore into the environment (Driks, 2002). The compartments are divided by polar septum made up of peptidoglycan which is believed to be hydrolysed by some membrane-bound proteases (SpoIID, SpoIIM, and SpoIIP) during the second stage of the sporulation process called engulfment leaving a thin layer of peptidoglycan residue within the septum (Tocheva et al. 2013). This enables the membrane to migrate encircling the prespore in a process similar to endocytosis thus completely engulfing it producing double-membrane spherical structure within the cytosol of the mother cell (Abanes-De Mello, 2002). After engulfment, metabolic activity in the forespore declines but there is a material exchange through a channel where small molecules are conveyed from the mother cell to the prespore to aid expression of genes that are essential for the sporulation process (Camp and Losick, 2009).

Following the engulfment of the prespore is the ongoing process of spore maturation. Part of this involves the development of the outermost structure in *B. subtilis* called the spore coat that serves as an encasement protecting the spore from harsh environmental influences (Saggese et al. 2014). Figure 2-1 summarises all the different steps involved in the formation of *B. subtilis* spores from the vegetative state to the spore release from the mother cell.

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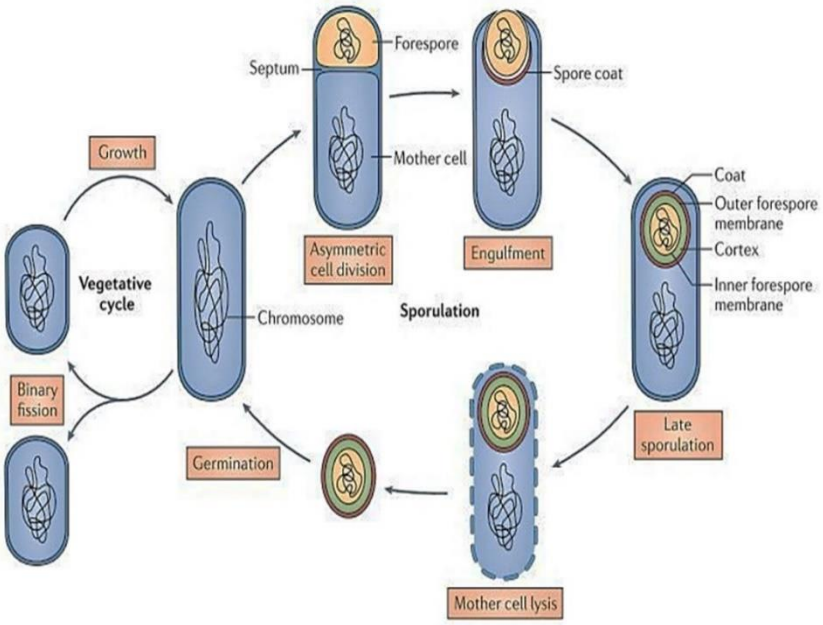

Figure 2-1: The cycle of spore formation and germination in *B. subtilis*. This commences when a sporangium splits asymmetrically to form two compartments: the mother cell and the prespore, which are separated by a septum. The mother cell engulfs the prespore and following membrane fission at the opposite pole of the sporangium, a double-membrane bound prespore is formed. Coat assembly commences just after the initiation of engulfment and continues throughout sporulation. The peptidoglycan cortex between the inner and outer forespore membranes is assembled during the late sporulation. In the final step, the mother cell lyses to release a mature spore into the environment. Spores are capable of quickly germinating and resuming vegetative growth in response to nutrients. Source (McKenney et al., 2013).

Structural studies indicated that the spore coat is morphogenetically derived from about 70 proteins among them is the s-protein (originally from the mother cell) and localised on the outer surface of the spore. The spore coat has been identified to possess 4 different layers: outer and inner coats, basement layer and the crust (Hobbs et al., 2011; McKenney et al. 2010). The outer coat is responsible for the resistance of the spore to low-pressure plasma, disruption, ultra-violet radiation, peroxide and other chemicals (Fiebrandt et al., 2016; McKenney et al. 2013). One of the various morphogenetic proteins, CotA, produces a pigment compound (laccase) structurally like melanin which may be responsible for their resistance to UV lysozyme and oxidising agents. The coat protects the spore against predation and regulates germination after

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a long period of dormancy through different surface receptors that respond to germinants, such as amino acid from the environment, and facilitate the dispersal to different ecological niches (Paredes-Sabja et al. 2010). In some species, such as *B. cereus* and *B. anthracis*, there is an additional structure outside the spore coat called exosporium which aids in their resistance and the site of various enzymes. It also facilitates the hydrophobic attachment of the spores to surfaces (Henriques and Moran, Jr., 2007).

The next concentric layer surrounding the spore is a cortex made from unique peptidoglycan similar to that in vegetative cells. This with the spore coat, shield the spore from heat and extreme dehydration (McKenney et al., 2013). The spore at this stage is highly dehydrated due to the uptake large of dipicolinic acid (DPA) mainly from Calcium ions and other divalent minerals replacing the water in the core (Rao et al., 2016). Vegetative cells are generally lacking in Calcium ions unlike their preponderance in spores. The cortex aids in the resistance of the spore to wet heat constituting roughly 10 % of the spore dry weight (Paidhungat et al. 2000). For germination to occur, there are lytic enzymes that break down the unique peptidoglycan releasing the outgrowing cell (Wells-Bennik et al. 2016). Figure 2-2 gives the detailed structure a bacterial spore.

Though the knowledge of bacterial spore formation with its cascading regulatory mechanisms is increasing, numerous grounds are still uncovered to fully comprehend the numerous interactions of the morphogenetic proteins and their expressions. This understanding will help in curtailing the presence of aerobic spore formers in the processing plant by applying it in designing more effective cleaning-in-place (CIP) chemical regimes and food contact surfaces that repel or prevent attachment of spores or their vegetative forms, thus nipping in the bud development of biofilms and guaranteeing safe products (En Ezech and Faille, 2018).

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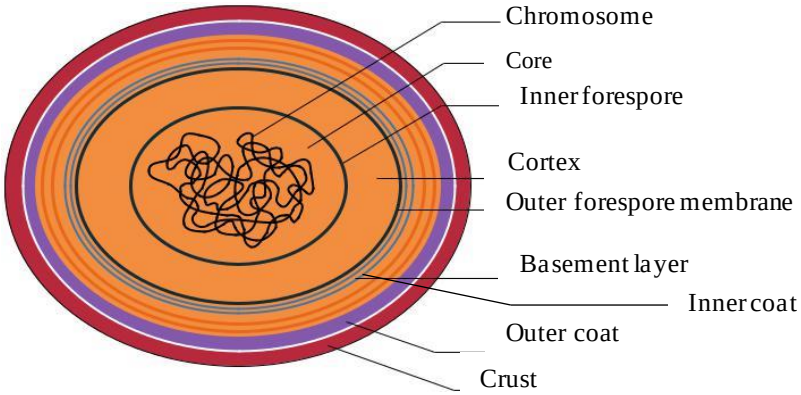

Figure 2-2: A typical *B. subtilis* spore showing the different concentric layers. Each layer serves to protect the genome, which is housed in the partially dehydrated central core. Source (McKenney et al. 2013).

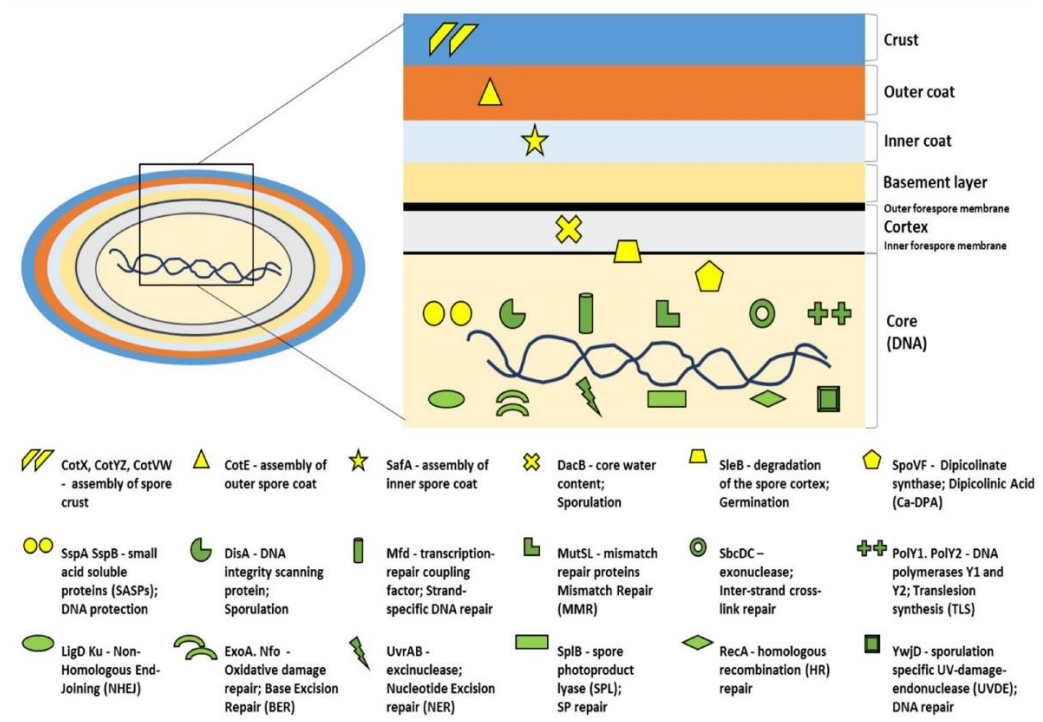

Figure 2-3: *B. subtilis* spore structure depicting the main resistance mechanisms. Each protection (in yellow) or DNA repair (in green) mechanism is represented by a symbol. Each symbol is coupled with a small description of the gene, the protein it codes for, followed by the main cellular event it is involved in. The location of the symbol corresponds to the main place of action within the spore (Cortesão et al. 2019).

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2.2.3 Mechanisms of spore resistance

Several collective mechanisms are responsible for the resistance of *Bacillus* spores to extreme physical and genotoxic conditions such as ultraviolet and gamma radiation, ultrahigh-pressure, desiccation, high temperature both as dry or wet heat, oxidising and alkylating agents as well as genotoxic chemicals (Reineke, Mathys, et al. 2013; Setlow, 2014; Tola and Ramaswamy, 2014).

The mechanisms of spore resistance can be broadly classified into damage prevention and damage repair (Cortesão et al. 2019). The structure of the spore with its concentric layers is intrinsically designed to protect the spores from environmental stresses, controls its adhesion to substratum through hydrophobic interaction as well as aids its response when the conditions are favourable leading to germination (Shuster et al. 2019; Wells-Bennik et al. 2016b). The spore coat, outer and inner membranes are the first layer of defence mounting a barrier against the entry of small-molecule biocides, such as those applied during CIP, that can inflict damage to the spores (Dong et al. 2019). The coat protects against damage by many oxidising agents, such as hypochlorite and hydrogen peroxide, serving as a chemical-sieve preventing the access of such agents to the core where most enzymes and DNA are entrapped (Leggett et al. 2012). There are specialised proteins and enzymes within the spore core that insulate the cells against biocides, some shielding the DNA from these potential threats. (Fiebrandt et al. 2016; Shuster et al. 2019). The outer membrane has some spore pigmentation mainly carotenoids that protect the spore from ionising radiation and perhaps low plasma sterilisation (Fiebrandt et al. 2016; Khaneja et al. 2009). The low water content of the core and the number of divalent metal ions in the core have been implicated as playing a functional role for resistance to wet heat (Rao et al. 2016). In a study by Dittmann et al., (2015), it was observed that the nucleoid of all *Bacillus* exists in the crystalline form within the core. This form shields it against the damaging effects

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of some genotoxic substances that permeabilise the spore from the environment. The crystalline nucleoid is highly stabilised by binding with some small acid-soluble proteins (SASP) which insulate the DNA against the damaging effect. Table 1 (below) highlights different sporicidal agents that are commonly used and the intrinsic factors that protect the spores against their debilitating effects.

The other line of defence involves numerous mechanisms that are involved in the repair of DNA after a genotoxin has exerted its effects. This mainly involves homologous recombination, excision and mismatch repairs, integrity scanning of the core DNA, cross-link repair, error-prone translesion synthesis, among others. These mechanisms are kick-started during spore germination safeguarding the integrity of the vegetative cell (Lenhart et al. 2012; Moeller et al. 2012). These defence mechanisms fortify the bacterial spore against all sporicidal assaults perpetuating them in the processing plant (see Figure 2-3). The spores potentially may be lodged on the food contact surface through hydrophobic interaction and presence of conditioning films. It then germinates to form vegetative cells with the innate ability to form a biofilm (Jindal and Anand, 2018).

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Table 2-1: Protective factors important in spore resistance to various inactivating agents.

Type of agent	Protective factor	References
γ-Radiation	DNA saturation by α/β-type SASP; DNA repair during spore outgrowth	Fiebrandt et al. 2016; Setlow, 2006
UV radiation	DNA saturation by α/β-type SASP; DNA repair during spore outgrowth, low water contents, carotenoids in spore outer layer.	Fiebrandt et al. 2016; Setlow, 2006
Oxidizing agents	Spore coat protein; low permeability of spores, inner membrane; DNA saturation by α/β-type, SASP; detoxifying enzymes in spore's outer layers	Setlow, 2014, 2006; Van Impe et al., 2018
Plasma	Spore coat, DNA saturation by α/β-type SASP	Fiebrandt et al., 2016; Moisan et al., 2001; Van Impe et al., 2018
Disinfectants	Spore coats, perhaps cortex and inner membrane structure	Setlow, 2014, 2006; Young and Setlow, 2004
Acids and alkali	Not yet understood	den Besten et al. 2012; Setlow, 2014; van Melis et al. 2011
Desiccation	DNA saturation by α/β-type SASP; DPA	
Dry heat	DNA saturation by α/β-type SASP; DNA repair during outgrowth; DPA; perhaps divalent metal ion content	Hayrapetyan et al. 2016; Setlow, 2014, 2006; Watterson et al. 2014
Wet heat	DNA saturation by α/β-type SASP; DPA level; low core water content; sporulation conditions including temperature; divalent metal ion content; sporulation temperature optimum	Coleman et al. 2007; Coleman and Setlow, 2009; Hayrapetyan et al., 2016; Rao et al. 2016; Zhang et al. 2011
Genotoxic chemicals	The low permeability of spores' inner membrane; DNA saturation by α/β-type SASP; DNA repair during spore outgrowth; low core water content.	Setlow, 2014
High Pressure	Pressure-induced germination; release of DPA and other small molecules	Reineke et al. 2013, 2011; Soni et al., 2016; Van Impe et al. 2018
Dialdehydes	Spore coats	Setlow, 2014
Bacterivores	Spore coats	Klobutcher, 2005; Laaberki and Dworkin, 2008

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2.2.4 Spore germination and biofilm formation

Attachment of spores and vegetative cells on the equipment surface may lead to the formation of biofilm. Some bacterial spores show a propensity for adhesion to food contact surfaces compared with vegetative cells (Jindal et al. 2018). Since spores are more resistant to cleaning procedures and the effect of CIP is rather asymptotic, bacterial spores have better chances at forming biofilms within processing lines (Lelièvre et al. 2002). Despite the flow dynamics (mechanical stress and shear flow) of the chemical solutions during CIP regimes, detached spores contaminate the equipment surface further down the processing line (Le Gentil et al. 2010).

Microorganisms exist in nature as a complex community or aggregate attached non-specifically and irreversibly to the biotic or abiotic surface than free-living or planktonic form. This complex community is known as a biofilm (Sauer et al. 2007; Vlamakis et al. 2013). Biofilms exist either as a single or multi-species interaction with the cells embedded within an extracellular polymer matrix that provide stability and survival to the microbial community of the biofilm as well as mediate their adhesion to surfaces (Røder et al. 2015, 2016; Verran, 2002). There is a significant physiological variation between the phenotype of microbes in the biofilm and the planktonic cells. These phenotypic changes make them resistant to chemical sanitizers and antimicrobial agents when they contaminate the milk processing plant (Simões et al. 2010; Tan et al. 2014). Biofilms are of great importance to the dairy industry and their control is imperative. They have been studied and identified to possess increased genetic exchange, heightened resistance to both chemical and biological agents against microbes, high rate of metabolite production (such as enzymes and acids) which are responsible for the degradation of the components of the processed milk resulting in the spoilage (Chopra et al.

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2015; Lewis, 2007; Verstraeten et al. 2016). A study revealed biofilms to be up to a thousand times resistant to antibacterial agents than the planktonic counterpart (Stewart et al. 2001).

In the milk processing plant, several parts of the equipment provide ecological niches for a specific organism. The processing conditions select for a particular type of microorganisms e.g. the plate heat exchanger (Simões et al. 2010). The response of the microbes to some environmental stresses and chemical signalling or quorum sensing among the microbes then initiates the process of biofilm formation after the adhesion of the cells to food-contact surfaces (Chmielewski and Frank, 2003). Quorum sensing has been implicated in other microbial processes apart from biofilm formation such as toxin and bacteriocin production, the formation of dental plaque, spoilage of food, bioluminescence, among others. Contamination of product occurs when processed food comes in contact with such surfaces. This can result in spoilage of the food as a result of the production of enzymes and other metabolites or food-borne illness (Lindsay et al. 2002).

A phenotype commonly associated with biofilms is persister cells which are non-dividing dormant cells with high resistivity to sanitising and antibacterial agents. A study revealed that they show high resistance to an antibacterial agent by shutting down targets for such agent and the inhibition of persister cell formation is thus vital in the control of biofilms (Lewis, 2008). The persister cell phenotype has been suggested to be formed in response to the different number of stresses that some cells within the biofilm mass experienced such as nutrient limitations, oxygen-tensions, metabolite accumulation, etc. (Flint et al. 2015). Apart from sheltering some pathogens (e.g. *Salmonella* and *Listeria*) and reducing the shelf-life of ESL milk, biofilms are responsible for some conditions in milk processing plant such as a reduction in heat-cold transfer, food spoilage, corrosion and clogging in pipes and infection on the consumption of contaminated milk product (Flint et al. 2017; Tan et al. 2014).

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Aerobic spore formers are frequent contaminants in the dairy industry. Moreover, they can withstand high processing temperature and produce heat-resistant spores which are adaptive features that ensure their survival and persistence under harsh environmental conditions. Mesophilic spores are also heat resistant and have been found to resist heat treatment during processing (Sadiq, Li, Liu, Flint, Zhang, Yuan, et al. 2016). Sources of these microbial contaminants may include contact with contaminated sources on the farm such as air, hay, infected teats contaminated by soil, milking machine and bulk milk storage tank and transfer lines (Flint et al. 1997; Jessen and Lammert, 2003; Vacheyrou et al. 2011). Localised contamination also occurs on the dairy processing plant such as in pasteuriser and gaskets in milk processing lines due to improper cleaning-in-place (CIP) operation (Ostrov, Harel, Bernstein, Steinberg and Shemesh 2016; Sharma and Anand 2002). Mugadza and Buys (2017) identified filler nozzle of aseptic filling machines as critical points in the dispensing of microbes in ESL milk during production. The bacterial spores germinate due to the heat applied in processing and form biofilm in the processing plant which frequently detaches contaminating the stream of the product (Parkar et al. 2001; Setlow, 2003).

2.2.5 Formation, structure and composition of biofilm

There are essentially five main steps in the formation of biofilms. These are initial reversible interaction with the surface, irreversible attachment of bacterial cells or spores to the surface, cell replication and aggregation in multiple layers, maturation of the biofilm with the formation of microcolonies, cell dispersal from matured biofilm to initiate a fresh biofilm (Arciola, Campoccia, Speziale, Montanaro and Costerton 2012; Srey, Jahid and Ha 2013).

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The main factor influencing initial interaction with a surface is not clearly defined but several factors are thought to influence this interaction of the microbial cell or spore with the food contact surface as a result of different external or environmental prompts or cues (Mielich-Suss and Lopez, 2015; Romero et al. 2010). These cues may be biological, physical or chemical and may include:

- Hydrophobicity, chemistry (surface charge, functional group etc.) and microtopography of the surface (Abdallah et al. 2014; Donlan, 2002).
- Nutrient, pH, temperature and the availability of supporting or inhibiting organisms (Nilsson et al., 2011).Operational factors of the milk processing plant such as cleaning protocols, plant design, flow system, etc (Blanc et al. 2014; Jindal et al. 2018).
- Diverse processes the microbes use in their interaction with the surface as well as their surface structure such as fimbriae, pili, adhesion and flagella (Asadishad et al. 2014; Flint et al. 2015; Jindal and Anand, 2018; Welin et al. 2004). Figure 2-4 shows the interactions of diverse factors, both intrinsic and extrinsic, contributing to the formation of biofilms.

Microorganisms do not naturally go into the formation of biofilms because it is energy demanding and thus requiring several environmental cues for its initiation (Cairns et al. 2014). Such environmental cues may be oxygen, surface adherence, nutrient exhaustion or surplus of the same have been shown to impact on biofilm formation with variations among different species (Mielich-Suss and Lopez, 2015; Morikawa, 2006). At the molecular level, a lot of gene regulations and control of the transcriptional processes responsible for matrix production are put in place before the formation of biofilms. Hence, the key element in the formation of biofilm is mainly phosphorylation (activation) of the transcription factor (Branda et al. 2005).

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B. subtilis cells lose their motility and form long chains of cells that are firmly held together at the commencement of the biofilm process (Branda et al. 2006).

Molecules such as cAMP and bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) mediate between cues from the environment and regulation of genes apart from quorum sensing between cells (Toyofuku et al. 2016). Bacteria attach differently to a wide variety of materials from which the milk processing plant is made up of such as plastic, polyester or polyurethane from which conveyor belt is made, surface coatings of the equipment used in the processing plant and stainless-steel (Chaturongkasumrit et al. 2011). Attachment to food contact surface usually commences with the passive attachment of the cell to the surface as a result of diffusion, gravity, fluid dynamic, etc. (Chmielewski and Frank, 2003). An additional factor that might influence attachment is conditioning film this occurs when there is an alteration in the physicochemical characteristics of the food-contact surface as the product comes in contact with the material from which the equipment is made such as steel (Dat et al. 2014). In a dairy setting, butyric acid from the lipolysis of milk fat is one the cues triggering biofilm formation on the processing line and other surfaces (Pasvolsky et al. 2014).

Subsequently, the cell is irreversibly attached to the surface, it replicates producing microcolonies of the early colonisers and exopolymeric substance (EPS), extracellular DNA (eDNA), and may include proteins and lipids. The EPS is a deep-seated structural element of the matrix produced in response to stresses in the cells (Marvasi, Visscher and Casillas Martinez 2010). It is also implicated in the resistance and invasion of biofilms. It harbours quorum sensing molecules as well as digestive enzymes used in the degradation of organic matters (Nadell et al. 2015). Seven different types of EPS were suggested and these are structural, sorptive, surface-active, active, informative, redox-active and nutritive EPS (Flemming et al. 2007). The role of the eDNA has not been clearly defined but has been

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confirmed to be a vital part of the matrix and has been proposed to be involved in the initial attachment (Das et al. 2010; Flemming and Wingender, 2010). For *B. subtilis*, the matrix is mainly made up of EPS and proteins (Marvasi, Visscher and Casillas Martinez 2010). Two major structural proteins associated with the EPS, hydrophobicity and pellicle formation are the TasA and BslA. The master regulatory proteins SpoA controls the genes necessary for biofilm formation and sporulation in *B. subtilis* (Dogsa et al. 2013; Yan et al. 2016).

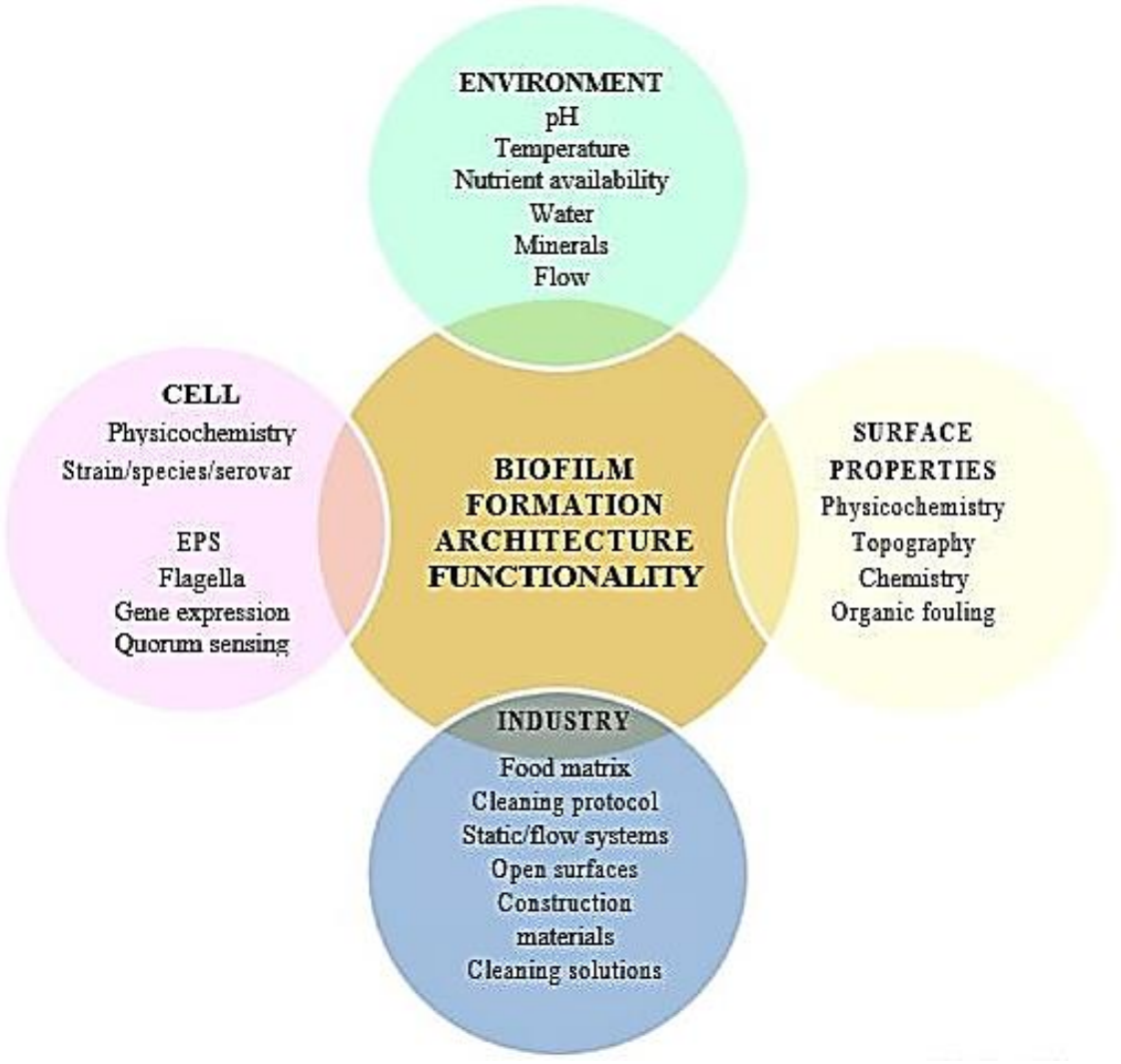

Figure 2-4: A multifaceted interactions of among several factors result in the formation of biofilm, architecture, and functions which are connected to the specific settings in food industry (Whitehead and Verran, 2015).

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There is a further development of the biofilm to produce microcolonies and later the production of macrocolonies through an increase in dimensions of the microcolonies. These macrocolonies then fuse to form a three-dimensional structure characterised by different and channels through which gases and nutrient diffuse (Poulsen, 1999). There are different interactions within the biofilm which shaped the features of the biofilm such as aggregation of cells, cell to cell signalling or quorum sensing, production of some metabolites in several folds beyond the amount that is obtainable in planktonic form, and production of EPS (Jakubovics, 2015). The different conditions within the biofilm can lead to cells that are phenotypically different and genotypically similar produced as a result of reactions to different signalling chemicals in the system (Mielich-Suss and Lopez, 2015). This phenotypic heterogeneity in sub-population is a necessary requisite for the structure of the colony (Vlamakis et al. 2008). The final step in the cycle of the development of biofilm is the propagation of the cells within the biofilm to another part of the ESL processing plant. This can be dispersal, sloughing off, seeding, or erosion depending on the number of cells involved in the propagation at a specific period. Bacterial spores and cells may gain entry into the product, therefore, contaminating it or reattach further down the plant to initiate another cycle of the biofilm (Le Gentil et al. 2010; Hinton et al. 2002). The different steps in the formation of biofilms are illustrated in Figure 2-5. Figure 2-6 is a scanning electron micrograph of a *B. subtilis* biofilm on stainless steel surface after 24 h incubation in reconstituted skim milk medium.

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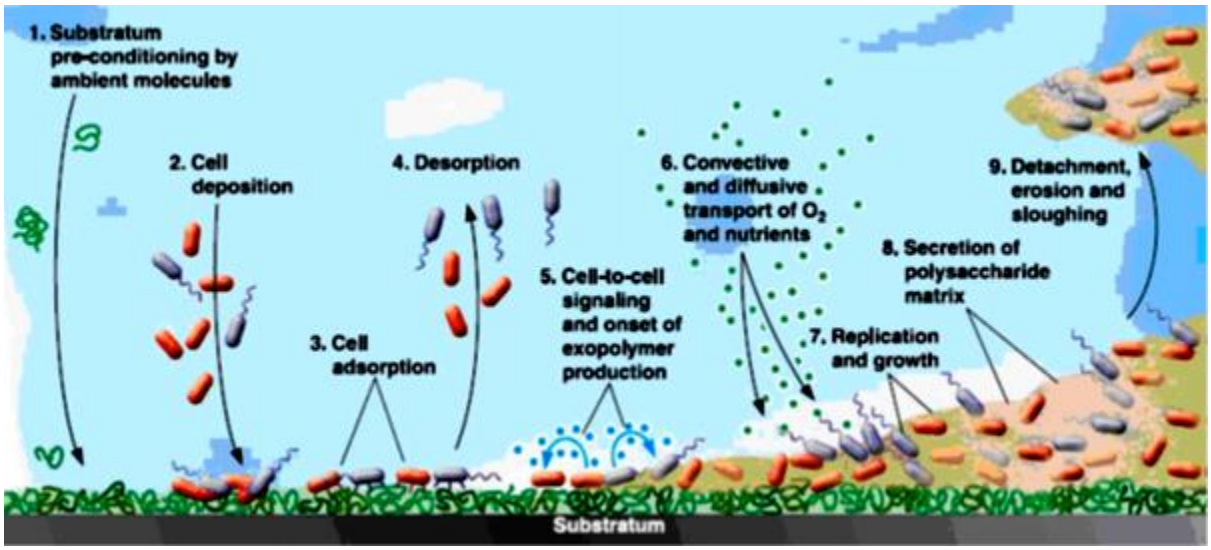

Figure 2-5: The steps in the formation of biofilm (Bryers and Ratner, 2004).

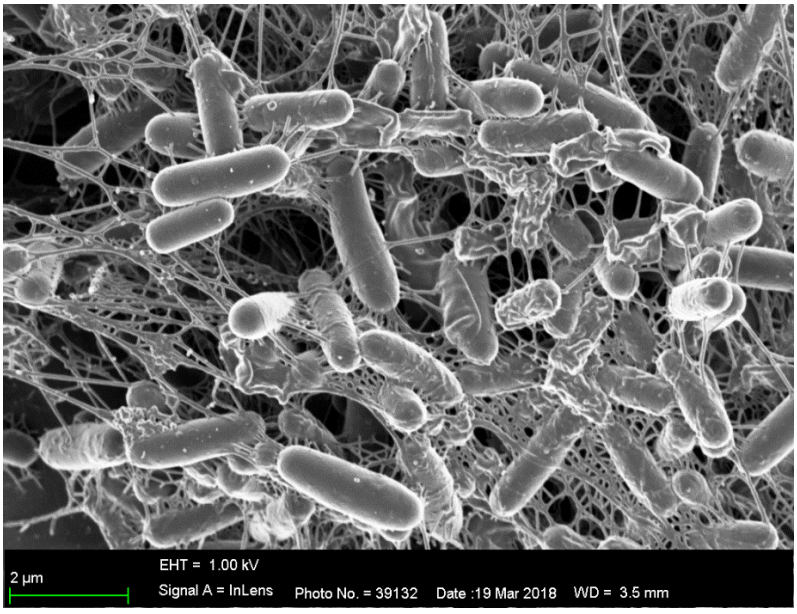

Figure 2-6: A classical Scanning Electron Micrograph (SEM) of *B. subtilis* biofilm on a stainless-steel substratum.

2.2.6 Strategy to control biofilm in the industry

Biofilms are unique in the sense that they are highly resistant to different antibiotics that are effective in inactivating a planktonic cell (van Meervenne et al. 2014). Resistance can be

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attributed to several factors such as the nature of the EPS, the presence of efflux system, poor penetration of antibiofilm agents, presence of persister phenotypes among other factors (Van Acker et al. 2014).

The key to controlling biofilm formation in the dairy processing plant is to reduce or eliminate the contamination of raw milk by aerobic spore-formers due to their resistance to CIP and processing conditions with their propensity to form biofilms and if presence prevents their adhesion or attachment to the food contact surface. Gram et al. (2007) observed that after CIP, 10 % of the bacterial population are unaffected and they show the potential of re-attaching to a different part of the processing line or end up in the packaged product. Therefore, any CIP regime that must be adopted in the industry must be timely applied, effective and prevent the re-attachment of any vegetative cell or spore (Meyer, 2003). Factors that play a crucial role in the efficacy of the CIP regime asides the type of chemical used include the quality of water, pH, contact time of CIP solution with the surface, temperature and presence of inhibitors and organic matter (Bremer et al. 2002; Kuda et al. 2008; Li et al. 2014).

The strategy of biofilm control can be divided into two: monitoring biofilm formation, prevention, and eradication of biofilm. Some biosensors can monitor real-time the presence of adhering bacteria and biofilm progression in the industry. This essentially involves the measurement of some physicochemical parameters (e.g. vibration, electric and electrochemical signals, capacitance, etc.) in the environment which are then allotted some calibrated values. Some materials used for the monitoring include a nanostructured electrode, nanoplasmonics microfluidic system, etc (Bruchmann et al. 2015, 2016; Funari et al. 2018; Sedki et al. 2019). Online-monitoring has the potential of reducing the cost incurred through cleaning, maintenance, and other operations unlike the traditional method of studying biofilm contamination in the industrial setting (Galié et al. 2018).

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There are different approaches in the prevention or eliminating biofilm formation in an industry setting and some have shown promises in their application. These approaches include chemical treatments such as the use of sanitiser during CIP, enzymatic disruption of the matrix, modification of food contact surface, quorum sensing quenchers, bacteriophage, biosurfactants, and bacteriocins etc (See table 2-2) (Galié et al. 2018; Lazar, 2011). H_2O_2 and Chlorine-derived sanitisers are widely used on food contact surfaces in the food industry because of their strong oxidising property. They are effective against some spores as well as biofilms of many microbes (Nam et al. 2014; Srey, Jahid and Ha 2013). Other chemical biocides used are ozone, quaternary ammonium, brominated furanones and salicylic compounds which are often combined (Rosenberg, Carbone, Römling, Uhrich and Chikindas 2008; Vestby et al. 2014). There has been increasing resistance to these chemical sanitisers by microbial biofilms especially after exposure to a sublethal concentration of such agents and repeated use over some time (Capita and Alonso-Calleja 2013; Techaruvichit et al. 2016).

Since a biofilm is made up of different macromolecules that can be hydrolysed by enzymes without leaving toxic residues, this has led to their application in the industry to combat and eliminate biofilms. Such enzymes used include proteases, DNases, glycosidases, amylases etc (Boels 2011; Coughlan, Cotter, Hill and Alvarez-Ordóñez 2016). They can be used as detergents or combined with other control measures in degrading biofilm structures. The commercial use of enzyme-based control of biofilm is limited because of the high cost involved and issues around patent rights .

Nanotechnology has found application in the fight against biofilm formation in the food industry. They have been used on food contact surfaces by modifying such surfaces using nanocomposites and nanoparticles for their antibacterial effect or prevention of bacterial adhesion (Ishwarya et al. 2018; Rai et al. 2016). They have the advantage of having a wider

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effect because of the large surface area and close contact with bacterial cells and absence of fear of possible resistance to any agent as the use of antibiotics or chemical sanitiser is completely non-existent. Table 2 summarises different antibiofilm strategies applied in the industry and the mechanisms of actions. Other antibiofilm strategies that show potential in the food industry are bacteriophage, bacteriocins, quorum sensing inhibition, essential oils, biosurfactants, and high-pressure processing though with different limitations and benefits. There is a need for more study before their general adoption in the industry.

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Table 2-2: Methods of controlling biofilms in the industry (Galié et al., 2018).

Methodology	Examples	Mechanism of Action	Reference
Photocatalysis		Bactericidal	Chorianopoulos et al. 2011; Ishwarya et al. 2018; Nica et al. 2017
Chemical treatments	Sanitisers (NaOCl, peracetic acid, NaOH, H_2O_2)	Cell structures oxidation	Bayoumi et al. 2012; Rosenberg et al. 2008; Ban and Kang, 2016; Nam et al. 2014; Yang et al. 2016; Møretrø et al. 2017
Enzymatic disruption	Cellulases	Extracellular matrix disruption	Coughlan et al. 2016; Stiefel et al. 2016; Wang et al. 2012; Boels, 2011; Chaignon et al. 2007; Huang et al. 2014; Oulahal-Lagsir et al. 2003
	Proteases		
	Glycosidases		
	DNAses		
Steel coatings	Nanoparticles (Ag_2C, Fe_3O_4, TiO2, ZnO, CuO, MgO)	Alteration of bacterial membrane	Alexander, 2009; Beyth et al. 2015; Rai et al. 2016; Campoccia et al. 2013; Gu et al. 2017; Jindal et al. 2016; Sandreschi et al. 2016; Swartjes and Veeregowda, 2015
	Repelling surfaces (monolayers, hydrogels, modified topography)	Inhibition of bacterial membrane	
	Functionalised surfaces (with lysozyme or nisin)	Bactericidal	
Biosurfactants	Lichenysin	Inhibition of bacterial adhesion	Coronel-León et al. 2016; Zhang et al. 2017; Zhao et al. 2017
	Surfactin		
Bacteriophages	P100	Cell lysis	Abedon, 2015; Chan and Abedon, 2015; Fister et al. 2016; Gutiérrez et al. 2016; Iacumin et al. 2016
Bacteriocins	Nisin	Cell membrane alteration	Chopra et al. 2014; Strempel et al. 2014
QS inhibition	Binding of inhibitors to QS receptors (lactic acid)	Downregulation of adhesion and virulence mechanisms	Amrutha et al. 2017; Brackman and Coenye, 2014; Coughlan et al. 2015; Rasmussen et al. 2005
	Enzymatic degradation of QS signals (paroxonases) sRNA post-transcriptional control inhibition of QS signals biosynthesis		Dong et al. 2001; Koh et al. 2013; Pérez-Martínez and Haas, 2011; Uroz et al. 2008; Yang et al. 2005; Adonizio et al. 2008; Al-Shabib et al. 2016; Chung et al. 2011; Zhu et al. 2015
	Furanones	Motility inhibition	Keskinen and Annous, 2011; Vestby et al. 2014
Essential oils	Citral	QS inhibition, motility inhibition	Shi et al. 2017
	Carvacrol	Bactericidal	Friedman, 2014
High hydrostatic pressure	H2O	Bactericidal (also endospores)	Evelyn and Silva, 2015; Santos et al. 2017
Non-thermal plasma	UV plus O_2, N_2, O3, H_2O and He		
Plasma		Bactericidal	Cui et al., 2016; Scholtz et al. 2015

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2.3 Ultraviolet C as a potential technology in combatting bacterial spores in the food industry

Ultraviolet light is a form of electromagnetic radiation occupying the region between on the spectrum between visible light and X-ray as illustrated in figure 2-7 below (Bintsis et al. 2000). Ultraviolet (UV) radiation has found some potential applications in the food industry apart from its conventional application in sterilising surfaces. There are three different types of UV based on the wavelength of the UV spectrum with each causing a specific type of injury to the DNA in an overlapping manner. These are ultraviolet A (UVA) with a wavelength of 320 nm to 400 nm; ultraviolet B (UVB) with a wavelength of 290 nm to 320 nm and ultraviolet C (UVC) with a wavelength of 100 nm to 290 nm (Baysal et al. 2013).

UV exerts its effects directly or indirectly on the DNA and RNA in microbial cells. The direct corresponds to the absorbance spectrum of the UV light with UVC possessing the maximum bactericidal effect on vegetative cells and spores of bacteria, especially pathogenic and spoilage bacteria, without residual toxins and alterations to the nutritional and sensory properties of the food unlike thermal processing (Gouma et al. 2015). The mechanisms of damage by directly exposing bacterial cells and spores to photons may be through the photodimerization between adjacent pyrimidines, cytosine photohydration, rate base adducts, cross-linking between proteins and DNA strands. The indirect effect is mostly due to the generation of reactive oxygen species (ROS) which cause DNA damage (Bintsis et al. 2000).

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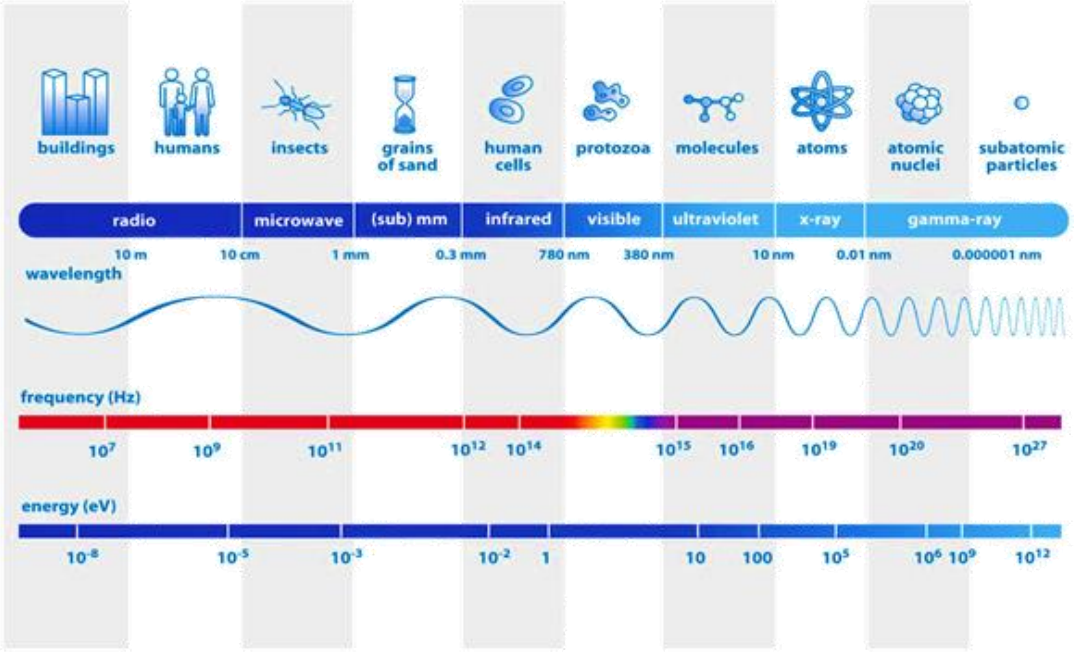

Figure 2-7: The spectrum of electromagnetic radiation with UV light between visible light and X-ray with the corresponding wavelengths, frequencies and energy levels (European Space Agency Medialab).

UVC is highly effective against pathogens and spoilage bacteria commonly associated with milk such as pathogenic *Escherichia coli* O157:H7 and non-pathogenic strains of the same species, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Salmonella typhimurium* and *Staphylococcus aureus* (Gabriel and Marquez 2017; Ochoa-Velasco et al. 2018).

UVC has been proven at the laboratory to be highly effective for inactivating fungal and bacterial spores in food as well as biofilms (Argyraki et al. 2017). As the spores of Gram-positive are peculiarly resistant to the UPHP, UVC is a proven alternative either as a stand-alone technology or in combination with other processes such as pressure or thermal (Reverter-Carrión et al. 2018). UVC is most effective at 254 nm due to the high energy photons which are absorbed by the DNA causing the formation of pyrimidine dimers thus preventing transcription and replication of the genetic material without any toxic residue (Unluturk et al. 2010). It also has the advantage of being cheaper in terms of low energy consumption when

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compared with thermal processing. The disadvantage of UVC is that they have a low penetration in milk or other coloured liquid food with large suspended particles for it to exert its biocidal effect (Bintsis et al. 2000). UVC treated milk was shown through a study done by Barnes (2015) to be significantly similar to heat-treated samples in terms of chemical composition (fatty acid composition, vitamin D and A and volatile components, lipid oxidation, to the proximate analysis the fatty acid profile, protein profile). Another study found that after 15 min of treating of milk, the level of the radical- scavenging activity antioxidant DPPH was the highest when compared with hydrostatic pressure and far-infrared treatment. Also, there was an overall digestibility of the alpha casein present in the milk therefore suggesting the potential health-promoting property of such product (Hu et al. 2017).

The application of non-thermal technologies on an industrial and small scale should be evaluated in relations to the economic feasibility in tandem with the promising application in ensuring safe and quality food. More work still needs to be done with the information integrated for the practical application during the processing of food especially in different food matrices with diverse physicochemical properties (Li and Farid, 2016). Another aspect of the application of the UVC reactor in the food industry that must be considered is the design of the reactor. This must be done in such a way that facilitates the maximum interaction between the UVC source (usually a lamp), creates turbulence, allows for several passes without irregularity in dosage delivery and capable of combination with other technologies that are not hindered by colour or suspended particles (Ramesh et al. 2016).

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2.4 Characterising aerobic spore-forming *Bacillus subtilis* complex group

Members of the *Bacillus subtilis* complex are dominant aerobic spore-forming contaminants in milk. They are a closely-related group of microbes and unique in the sense that they cannot be differentiated using traditional biochemical and phenotypic typing method. Members of the species show extreme diversity in their metabolic diversity hence the difficulty in using a biochemical method to classify them into taxonomic groups (Adelskov and Patel, 2016). Interestingly, the widely used routine 16S rRNA-based method lacks the discriminatory power in resolving this group of *Bacillus* into the different taxonomic groups (Rooney et al. 2009). A polyphasic approach combining Matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) with multi-locus sequence typing (MLST) is highly recommended in such cases where 16S rRNA sequencing is not sufficient to resolve the genetic variations in organisms.

2.4.1 Matrix-assisted laser desorption ionisation time of flight (MALDI-TOF)

The principle is a fast, reproducible, high throughput mass spectrometric method based on the analysis of cellular proteome applied in microbial profiling using cell extract or intact microbial cells. It involves ionising an analyte within an organic matrix, such as α-cyano-4-hydroxycinnamic acid (CHCA) or 2,5-dihydroxy benzoic acid (DHB), using a laser beam. The matrix co-crystallised with analyte generates protonated ions which migrated within a flight tube. They are separated using their mass to charge ratio which is measured by time of flight (TOF) analyser. The spectrum from the analyser called peptide mass fingerprints (PMF) is compared with the database to properly identify the specific organism or analyte. The PMF consists mainly of the ribosomal proteins present in the analysed organism. This aids in the identification up to the strain level for some organisms irrespective of the media used and

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physiological condition of the cells though an actively dividing culture of 24 h is advised. MALDI-TOF has the advantage of being fast, accurate to identify at least to the genus level and cheaper when compared with another method such as molecular method. The method cannot adequately distinguish members of the *B. subtilis* complex group hence the use of MLST using three or more house-keeping genes.

2.4.2 Multi-locus sequence typing (MLST)

MLST is a typing arrangement that directly catalogues the nucleotide sequence dissimilarity of multiple protein-encoding house-keeping genes within a population of microorganisms (Maiden, 2006). Multi-locus sequence typing (MLST) bridges the gap of inaccurate strain identification and poor reproducibility commonly associated with biochemical tests and molecular typing of bacteria commonly associated with outbreaks and for taxonomic purpose in bacterial systematics. It is built on the technique of multi-locus enzyme electrophoresis (MLEE) (Selander et al. 1986). It is essentially a non-specific typing method based on population genetics (Maiden et al. 1998). It is keyed on the variations that exist between multiple-loci within nucleotide sequences which can be used to construct evolutionary relatedness, especially over a short time-scale. The goal of the MLST is to amplify coding regions of several house-keeping genes of approximately 500-600 bp containing maximum polymorphic sites. Numbers are given to sequences or alleles and sequence type assigned based on a combination of several allelic profiles (genotype). Sequences from an unknown organism can then be compared with what is on the pub-MLST database (http://pubmlst.org). MLST has found application in strain characterisation, source-tracking, and relatedness between microbes. It was first used in typing *Neisseria meningitidis* with more than 80 different organisms (mostly bacteria) and phages (Yang et al. 2017).

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The steps involved a PCR amplification using degenerate primers designed from the internal consensus for the multi-loci specific for the organism of interest. For the *B. subtilis* complex group, the following house-keeping genes were used for (MLST): glycerol uptake facilitator (*glpF*); dihydroxy-acid dehydratase (*ilvD*); phosphotransacetylase (*pta*); phosphoribosylaminoimidazole carboxy formyl formyltransferase (*purH*); pyruvate carboxylase (*pycA*); RNA polymerase major sigma factor (*rpoD*, aka *sigA*); and triose phosphate isomerase (*tpiA*).

2.4.3 Flow cytometry

The flow cytometry (FCM) technique is widely used to gain deeper insights into the responses of cells/spores to various treatments at the molecular level especially among isogenic and sub-populations of bacterial cultures (Reineke, Ellinger, et al. 2013). The flow cytometer is highly sensitive equipment use to analyse and enumerate in real-time the different phenotypes or intermediate states of cells (e.g. non-culturable cells or spores) some of which may be incapable of growing on traditional media due to some physiological injuries. In contrast with the traditional plate count method, FCM can measure different parameters at the single-cell level in a population besides the cell viability. Thus, it has found a very useful application in rapidly determining the physiological states and heterogeneity of each bacterial cell within a population (Lv et al. 2019). The method involves the use of DNA-binding exclusion stains such as propidium iodide (PI) and SYTO 9. While PI only enters the cells when the integrity of the cell membrane is compromised which is indicative of a damaged membrane, SYTO only stains the intact cells indicating cell viability (Majeed et al. 2018). The flow cytometer operates by the principle of hydrodynamic focusing. It is made up three main parts which are: the fluidics system which transports the cells or particles to be analysed in an aqueous stream, the optics consisting the lasers, lenses, beam splitters, filters and detectors that interrogate with the pre-stained particle. The last

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part is the electronic systems which is responsible for the transformation of the light signals into an electronic signal that can be visualised on a screen using a computer algorithms (Tracy et al. 2010).

2.4.4 Conclusion

The growing resistance and bacterial biofilm challenge in the industry call for an innovative approach in facing this foe. Effort must be made to reduce to the barest minimum or eliminate the presence of bacterial spores in raw milk before processing and during processing. Bactofugation is a common processing method widely used among South Africa dairy processors but this is insufficient in mitigating the spore and biofilm challenge (Mugadza, 2017).

Bactofugation reduces vegetative cells approximately by 90 % and efficient for spores but it recontamination is a potential threat with this method (Hussain and Oh, 2017). The process of bactofugation involves the microfiltration after which the product is thermally pasteurised (Hoffmann et al. 2006). The effort is to tackle both aerobic spore formers, their spores and biofilm in a combinatorial approach without leaving any toxic residue in the food or contact surface. The traditional use of sanitisers or disinfectants during CIP regime has been shown to prove ineffective in eliminating bacterial spores and biofilms (Bremer, Fillery and McQuillan 2006). Therefore, there is the need to seek a cost-effective biocidal or technological method that will eradicate their presence on the food contact surface such as in pasteuriser or processing lines. This should inculcate the most resistant vegetative cells, spores and biofilms especially older biofilms which are often overlooked in most studies (Bremer, Fillery and McQuillan 2006).

There is a lot of promising possibilities ahead for the food industry though it may take a while to apply them on a commercial level due to hurdles of regulations that must be overcome before

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the adoption by the industry players. This is to ensure they are meeting the generally regarded as safe (GRAS) standard and chemicals used are food-grade meeting the demands of consumers.

3 HYPOTHESES AND OBJECTIVES

3.1 Hypothesis 1

Aerobic spore-formers such as the genus *Bacillus* will contaminate raw milk and survive pasteurisation and other processing conditions because of their preponderance in nature and presence in feeds, equipment, skin and udder of farm animals (Lücking et al. 2013). Their spores and vegetative cells are highly resistant structures to high temperature and pressure as well as cleaning-in-place (CIP) chemicals used during the processing of Extended Shelf-life (ESL) milk. The spores or vegetative cells will attach to the contact surface through hydrophobic interactions where they form biofilms thereby contaminating the downstream processing of milk (Faille, 2010; Jindal and Anand, 2018). Therefore, *Bacillus* in ESL milk will show genetic relatedness which is an indication of contamination from the source.

3.1.1 Objective 1

To characterise the spore-forming bacteria belonging to the *Bacillus subtilis* complex group isolated from raw milk, pasteurised milk and stored ESL milk using multi-locus sequence typing (MLST) with the aim of source-tracking their origin in raw, pasteurised and packaged ESL milk. To determine their biofilm-forming potential on stainless steel in reconstituted skim milk medium.

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3.2 Hypothesis 2

Ultraviolet C (UVC) will inactivate the spores of *B. subtilis* complex group in phosphate buffer because of the high penetration rate and low interference in a clear liquid such as phosphate buffer. UVC has a low penetration depth in opaque or turbid fluids such as milk (Cappozzo et al. 2015; Koutchma, 2009). It affects its biocidal property by penetrating the nucleic acid and causing the formation of pyrimidine dimers within a cell or spore (Reverter-Carrión et al. 2018). Therefore, spores will show a different level of resistance after the exposure to UVC possibly due to variations in spore characteristics such as surface microtopography of the spore, amount of UV-absorbing pigments, and dipicolinic acid (DPA) (Li et al. 2018).

3.2.1 Objective 2

To determine the effects of ultraviolet light (UVC) in the inactivation of spores of *B. subtilis* complex group and isolated from milk samples to mitigate their presence in liquid foods such as milk, fruit juice and the composition of spore influencing its resistance.

3.3 Hypothesis 3

Bacterial cells or spore will adhere to food contact surfaces through hydrophobic interactions in response to environmental cues such as conditioning film to produce biofilms. Biofilms will produce spoilage enzymes and other metabolites in amount exceeding the planktonic counterpart thereby causing spoilage of ESL milk (Sadiq, Flint and He 2018; Teh et al. 2012). This is because biofilms are better adapted to produce more metabolites when compared to the planktonic cells because of the phenotypic modification, growth and gene transcription within

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the cells making up the biofilms (Vyas et al. 2016). Therefore, ESL milk contaminated with biofilms of the same strain will have a shorter shelf-life in comparison to same product contaminated with planktonic cells.

3.3.1 Objective 3

To quantify the number of cells within the biofilms of *B. subtilis* complex group and determine their spoilage potential by quantifying the proteolysis and lipolysis within biofilms in comparison with the planktonic cells.

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4 RESEARCH CHAPTER

4.1 Molecular characterisation and biofilm formation potential of *Bacillus subtilis* and *Bacillus velezensis* in extended shelf-life (ESL) milk processing line

4.1.1 Abstract

This study aims to characterise *Bacillus subtilis* complex group from raw, pasteurised and packaged extended shelf-life (ESL) milk samples, to determine their biofilm potential and source-track the microbial contaminants to control their presence during processing. The isolates were characterised using multi-locus sequence typing (MLST) with seven housekeeping genes. The primers used were designed from the coding regions with the highest number of polymorphic sites. The heat resistance profile indicated all 12 isolates are psychrotolerant as well as thermophilic with temperature ranges of 6 to 55 °C (B43, B44, B52, B54, B55, B56, B57), 6 to 60 °C (B46, B47, B48) and 15 to 60 °C (B49, B50). A General Linear Model (GLM) two-way repeated measure ANOVA of the biofilm-forming potential of the isolates shows that there is a statistically significant difference across the time (6, 12, 18 and 24 h) of incubation ($P< 0.05$). All the isolates formed moderate to strong biofilms except 2 with B44 having the most robust biofilm formation (3.14 ±0.60). The scanning electron and confocal microscopy images reveal the strain-specificity of the biofilm structure. The MLST analysis identified all isolates to either belong to *B. subtilis* or *B. velezensis*. All the isolates are novel sequence types (STs) when compared to the PubMLST database but showed relatedness to those isolates in the raw milk that was processed. The closest STs are 96 for *B. velezensis* and 128 for *B. subtilis* mostly isolated from the soil. The study presents the significance of biofilms of thermophilic *B. subtilis* and *B. velezensis* as well as their possible perpetuation in

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the dairy processing plant. The information provided is a call for an innovative food contact surface or any other intervention that can minimise or prevent microbial adhesion in the processing plant which in turn impact negatively in ESL milk.

1

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4.1.2 Introduction

ESL milk has the inherent characteristics of a shelf-life that is longer than that of the HTST milk and lacks the burnt flavour commonly associated with the UHT (Mugadza and Buys 2017). Temperature regime that bridges the gap between high-temperature short-time (HTST) and ultra-high temperature (UHT) processing is employed in the processing of ESL milk (Lorezen et al. 2011). The challenge of post-process contamination of ESL milk during production was reported in a pilot study by Martin et al. (2018) which found the aseptic filler nozzle highly contaminated with bacteria especially Gram-positive such as *Bacillus* and *Paenibacillus* dominating the microflora.

Milk is a nutrient-rich medium widely consumed worldwide for its health and sensory benefits. Its neutral pH, lipid, sugar and protein contents make it a ready target for microbial spoilage (Hamadi et al. 2014; Srey, Jahid and Ha 2013). Presumably, milk is sterile when derived from a healthy udder and microbial contamination usually occurs through the contact of raw milk with the udder, teats and improperly sanitised equipment used in the milking process (Srey et al. 2013). Other sources of milk contaminants are animal feeds, water and soil (Machado et al. 2017). Processing equipment also acts as reservoirs for bacterial contaminants. The microbial flora of raw milk encompasses genera from both Gram-positive, Gram-negative bacteria and some fungi. The genera of Gram-positive commonly found in raw milk include those of *Bacillus, Clostridium, Corynebacterium, Microbacterium, Micrococcus, Staphylococcus,* with the lactic acid bacteria (LAB) such as *Lactococcus, Lactobacillus* and *Streptococcus* usually found in high numbers (Oliveira, Favarin, Luchese and McIntosh 2015; Quigley et al. 2013; Vithanage et al. 2016). Of both Gram-negative and positive genera, *Bacillus* and *Pseudomonas* are the dominant genera in milk because of their ability to sporulate and grow faster than other bacteria under low-temperature conditions respectively (Huck et al. 2008; Meng et al. 2017).

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Members of the genus *Bacillus* are of economic importance in the dairy industry due to the ability of the endospores produced by this group to survive desiccation, disinfectants and heat processing as well as ultra-high temperature processing (Huck et al. 2008; Lorezen et al. 2011). The unaffected spores germinate to form vegetative cells which in turn produce thermostable enzymes and acids causing unfavourable physiochemical changes in the milk (Chen et al. 2003; von Neubeck et al. 2015). The microbial peptidases and lipases hydrolyse the peptide bonds of the milk proteins and glycerol bonds in the milk fat triggering functional and sensory changes of extended shelf-life (ESL) milk especially under the condition of long storage (Baur et al. 2015; Lücking et al. 2013; Burgess, Lindsay and Flint 2010). Another factor of the economic importance of the genus is the ability to form biofilms on the surface of processing equipment and milking utensils (Sharma and Anand, 2002).

The natural mode of existence of bacteria is a surface-associated community of cells surrounded by exopolysaccharide substances (EPS) called 'biofilm' which is the association or aggregation of same or different species of microorganisms (Carpentier and Cerf, 1993; Chmielewski and Frank, 2003). *Bacillus* spp. have been shown selectively to attach to milk contact surface but predominate in the formation of process biofilm due to their ability to form resistant endospores (Seale et al. 2008). Several factors combined to influence the attachment of cells on a food contact surface. These factors comprise the roughness of the surface, presence of conditioning film, electrostatic charge, hydrophobicity of the substratum surface, and composition of the processed product (Araújo et al. 2010; Dat et al. 2014; Peña et al. 2014; Teh et al. 2014; Whitehead and Verran 2015). Wirtanen et al. (1996) observed that biofilms of some *Bacillus* spp. adhered firmly to stainless steel than on Teflon. Thus, the effect flowrate in detaching the cells from the substratum during processing is likely not to be a substantial factor.

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B. subtilis biofilms produce spores most, especially under low nutrient condition (Lindsay et al., 2006).

There is established evidence of the failure of 16S rRNA marker to delineate phylogenetically the members of the *B. subtilis* group into the respective taxa because of sequence similarity hence the suggestion of multi-locus sequence typing (MLST) in resolving this challenge in *Bacillus* systematics (Austin and Bergeron 1995; Rooney, Price, Ehrhardt, Swezey and Bannan 2009). In the dairy processing plant, contamination of products may come through various sources such as raw milk, processing environment, ineffective cleaning-in-place, and personnel. The growth of biofilms in downstream equipment used in food processing is a major challenge due to their persistence and resistance to cleaning regimes. Previous studies have investigated the adhesive ability of common thermophilic spore-formers such as *Geobacillus stearothermophilus* and *B. sporothermodurans* of dairy origin with little data in the molecular characterisation and diversity (Balaban et al. 2013; Jindal et al. 2018).

This study, therefore, focuses on tracking the sources of spore-forming *B. subtilis* and *B. velezensis* isolates from different parts of the dairy processing plant, raw milk, and packaged extended shelf-life milk using MLST technique and to quantitatively determine their potential-to produce biofilms under simulated dairy environment.

4.1.3 Materials and methods

4.1.4 Bacterial selection and identification

Twelve isolates of *Bacillus* spp. from raw milk, pasteurised milk, packaged ESL milk and ESL milk stored at 4 ^{o}C from the Gauteng province of South Africa were used in the study (Mugadza and Buys 2017; Khoza 2015). Sample collection was done as follows: 1000 mL of raw milk,

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1000 mL of pre-packaged pasteurised milk, 16 packaged ESL milk stored at 4 °C (250 mL), and 80 swabs taken from different nozzles of aseptic filling machines post-CIP. All samples were collected from the processing line in a total of four visits and plated out within 4 hours after collection. Matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectroscopy was used in the preliminary identification of bacterial isolates. Purified bacterial cultures were inoculated on nutrient agar (Oxoid, Basingstoke, UK) and incubated for 24 h. A single colony was picked with a sterile tip and placed in a MALDI Biotyper target plates in duplicate (Bruker Daltonics, Bremen, Germany). The typing was done using the protocol of Bittar et al., (2009). The output was analysed using MALDI Biotyper 3.0 software (Bruker Daltonics, Germany). The degree of spectral pattern matching is expressed as a logarithmic identification score and interpreted according to the manufacturer instruction. The results were expressed as logarithmic values from 0 to 3 levels. Scores ≥2.300 indicate species identification with a high level of confidence, ≥2.000 indicates species identification, 1.700 -1.999 indicates genus identification, and <1.700 indicates no identification (Croxatto et al. 2012).

4.1.5 Biofilm-forming potential

4.1.5.1 Adhesion to polystyrene

A slight adjustment to the protocols described by (Hussain and Oh, 2017) was used in the quantification of the biomass within the biofilms. 2 µl of an overnight bacterial culture grown in Luria broth was added to 200 µl broth of the same medium in 96-well polystyrene microtiter plates. For all experiments, the bacterial inocula were standardised to 1 MacFarland (approximately 3×10^8 cfu/mL) using a densitometer (Grants Instruments, Cambridgeshire, UK). The plates were incubated at 30 °C for 6, 12, 18 and 24 h. The medium was discarded

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from each well after incubation. The wells were then carefully washed three times by aspiration using 300 µl phosphate-buffered saline (PBS) (Oxoid, Basingstoke, United Kingdom) to remove unattached cells. The biomass was stained with 250 µl of 0.1 % crystal violet solution (Difco Lab, Michigan, US) for 30 min. Unbound crystal was discarded by washing each well three times with PBS. A 200 µl volume of 70 % ethanol was added to each well to release the bound crystal violet from the biofilm. The plate was covered with Parafilm (Sigma Aldrich, St. Louis, MO) and left to stand at room temperature for 30 min. The absorbance of the resulting crystal violet solution was measured at a wavelength of 590 nm on a microplate reader (Thermo Scientific, Waltham, MA). Isolate B168 (weak biofilm-former) was used as the negative control while B3610 (strong biofilm-former) was used as the positive control. The isolates were characterised based on their biofilm-forming potential into non-adherent, weakly, moderately, and strong adherent using the method of Stepanović et al., (2000) as given below:

$OD_{cut} = OD_{avg}$ of negative control + (3 × standard deviations of OD of negative control)

$OD \leq OD_{cut}$ = non-adherent

$OD_{cut} < OD \leq 2 \times OD_{cut}$ = weakly adherent

$2 \times OD_{cut} < OD \leq 4 \times OD_{cut}$ = moderately adherent

$OD > 4 \times OD_{cut}$ = strongly adherent

4.1.5.2 Adhesion on Stainless-steel

The adhesion to stainless-steel was performed by modifying the method of Teh et al. (2012). Overnight bacterial culture grown in tryptose soy broth (TSB) was standardised to 1.0 MacFarland (~3×10^8 CFU/ml). 1 ml of standardised inoculum was pre-incubated in 2 ml of reconstituted skim milk (RSM) for 1 h for each of the 12 test organisms and 2 standard strains

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used as controls. 4.5 ml of RSM in a centrifuge tube was inoculated with 0.5 mL of the pre-incubated cultures to make approximately 1000 cells in 5 ml. A stainless-steel coupon (316L-0.90 mm- 2B PVC; dimension: 50 mm x 13 mm) was semi-submerged in the tube for each sample and incubated for 24 h. After the incubation, the coupons were cleaned by dipping in sterile PBS 3 times to dislodge unattached cells. Samples were prepared for microscopic visualisation.

4.1.6 Microbial Adhesion to Hydrocarbon (MATH) assay

Surface hydrophobicity of the isolates was determined using microbial adhesion to hydrocarbon (MATH) assay as developed by Rosenberg et al. (1980). Overnight bacterial cultures for all the isolates were washed by centrifuging at 7000 × g for 10 min at 4 °C. The pelleted cells were suspended in sterile distilled water to an optical density at 600 nm (OD_{600nm}). 3 mL each of hexadecane and the cell suspension was added together followed by vigorous mixing on a vortex mixer at room temperature for 60 s and incubating at 30 °C for 10 min. After 10 min of incubation, the suspension was agitated on a vortex mixer for 2 min and allowed to stand for 20 min at ambient temperature (Chao, Guo, Fang and Zhang 2014; Elhariry 2011). The absorbance of the aqueous layer was measured at OD_{600nm} using a spectrophotometer (Spectronic 200, version 2.06; Thermo Fisher Scientific). The experiment was repeated three times in duplicates (n = 6). The relative hydrophobicity is the ratio of the final cell hydrophobicity post-incubation and the initial cell hydrophobicity taken pre-incubation expressed as a percentage. The percentage of cell surface hydrophobicity was calculated using the formula as provided:

$$\mathrm{RH} = \frac{OD\mathrm{initial} - ODresidual}{ODinitial} \times 100\%$$

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4.1.7 Heat resistance profile of isolates

To determine the temperature adaptation of the isolates, the minimum and maximum temperatures for growth were determined by streaking the isolates on nutrient agar (Oxoid, Basingstoke, United Kingdom) plates and incubated at various temperatures of 15 °C for psychrophiles, 25 °C for psychrotrophs, 32 °C for mesophiles, 55 °C for thermophiles and 65 °C for extreme thermophiles. The plates were incubated between 24 to 48 h except at 6 °C with incubation of 10 days. Working bacterial cultures were cryopreserved in 25 % glycerol at -80 °C (Duncan et al. 2004; Ivy et al. 2012; Lorenzen et al. 2011).

4.1.8 Electron microscopy

4.1.8.1 Biofilm structure and architecture

Approximately 1000 cells were inoculated into a centrifuge tube containing 4.5 mL of reconstituted skim milk media with pre-treated stainless-steel coupons in an upright position. The coupons were washed and processed for SEM and CLSM after 24 h incubation time. The coupons were washed using 0.075 M phosphate buffer. The buffer was removed, and 2.5 % glutaraldehyde/formaldehyde solution was used to fix the samples for 1 h. The fixative was removed, and samples were washed three times in buffer. 1 % osmium tetraoxide solution was later added and samples and post-fixed for 1 h. Samples were washed three times for 15 min and were centrifuged to get a pellet between each step. After the removal of the wash buffer, samples were dehydrated using a graded series of ethanol (30 %, 50 %, 70 %, 90 % and 100 %) for 15 min each with the last step carried out three times. Samples were left in the last 100 % ethanol for 30 min. Coupons were left in a 50:50 mixture of hexamethyldisilazane (HDMS)

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and 100 % ethanol for 1 h. The same process was repeated for HDMS only for another 1 h. Fresh HDMS was later added, and coupons were left to dry. The coupons were coated with carbon before mounting on the scanning electron microscope (Zeiss, Oberkochen, Germany).

4.1.8.2 Confocal Laser Scanning Microscopy (CLSM)

The stainless-steel coupons were dipped in distilled water three times to wash off unattached cells and residual milk. The coupons were later semi-submerged in PBS and stained with 500 µM of propidium iodide (PI) and 1.5 mM of SYTO 9 (Thermo Fisher). The coupons were left for 20 min in the buffer and observed under the microscope (Zeiss LSM 880, Oberkochen, Germany).

4.1.9 Multi-locus sequence typing (MLST) analysis

4.1.9.1 Primer design for MLST

MLST was done by using 7 house-keeping genes encoding for glycerol uptake facilitator (*glpF*); dihydroxy-acid dehydratase (*ilvD*), phosphotransacetylase (*pta*), phosphoribosylaminoimidazole carboxy formyl formyltransferase (*purH*), pyruvate carboxylase (*pycA*), RNA polymerase major sigma factor (*rpoD*), and triose phosphate isomerase (*tpiA*) according to the pubmlst scheme for *Bacillus* spp. The universal primers, which were employed in both amplification and sequencing, were designed to amplify a 500-600 bp fragment from the coding region of each gene that contained the most polymorphic sites (~100).

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4.1.9.2 DNA Extraction and Sequencing

Extraction of bacterial DNA was performed for all isolates using a bacterial DNA MiniPrep extraction kit (Zymo Research, Irvine, CA). We performed PCR amplification using the same primer set with a final volume of 20 µL, which consisted of 2 × 10 µL of master mix (EconoTaq PLUS, Lucigen Corp., Middleton, WI): [0.1 unit/µL of EconoTaq DNA polymerase reaction buffer (pH 9.0), 400 µ*M* each dNTP, 3 m*M* $MgCl_2$, and a proprietary mix of PCR enhancer–stabilizer and blue and yellow tracking dyes], 1 µL of gDNA (30 ng/µL), 1 µL of primer (10 µ*M*), and 7 µL of nuclease-free water. The thermal cycler program consisted of a pre-heating stage at 94 °C. For initial denaturation of the DNA, the reactions were incubated at 94 °C for 2 min. Denaturation was performed at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min for 35 cycles, with the final extension at 72 °C for 10 min. The PCR products were cleaned using ExoSAP (Thermo Fisher). The ExoSAP master mix was prepared by adding to a 0.6 mL microcentrifuge tube 50.0 µL of exonuclease I (20 U/µL) and 200.0 µL of shrimp alkaline phosphatase (1 U/µL). We added 10.0 µL of PCR mixture to 2.5 µL of the ExoSAP mix. The mixture was vortexed and incubated at 37 °C for 30 min. The reaction was stopped by heating the mixture to 95 °C for 5 min. Sequencing was then performed with the ABI v3.1 BigDye Kit (Thermo Fisher). The labelled products were then cleaned with the Zymo Seq Clean-Up Kit, after which the products were injected onto ABI 3500xL Analyzers (Thermo Fisher) with a 50 cm array, using POP-7.

4.1.9.3 Sequence Alignment and Phylogenetic Analyses

The sequences obtained for all isolates were cleaned, aligned and prepared for the MLST analysis using the Molecular Evolutionary Genetics Analysis software (MEGA-X, version

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10.0.05; https://www.megasoftware.net/). Each isolate was characterised based on the combination allelic profile. The allelic sequence was submitted to the PubMLST blast query for *B. subtilis* (https://pubmlst.org/bsubtilis/) sited at the University of Oxford (UK) to generate the information on the sequence types (ST) and phylogenetic analysis.

4.1.10 Statistical analysis

Statistical analysis was performed to determine whether the significant differences between bacterial adhesion at 6, 12, 18, and 24 h is more than expected by chance at a 95 % confidence interval. A 2-way repeated measure ANOVA was conducted to see whether statistically significant difference ($P < 0.05$) occurred in the adhesion to polystyrene among isolates across the periods (6 h, 18 h, and 24 h), using the general linear model (GLM). All analyses were performed using GraphPad Prism (version 8.0.2; GraphPad Software Inc., La Jolla, CA).

4.1.11 Results

4.1.12 Biofilm-forming potential

Comparing isolates visually with the negative control (*B. subtilis* ATCC 168) revealed differences in their aggregation sites after incubation for 24 h, such as the air-liquid interface and the liquid-solid interface. A noticeable pellicle developed at the top of each well, except for the negative control, as the biofilm matured. We found a substantial main effect for time $F_{1.058,11.64} = 24.73$, $P = 0.003$, and a significant main effect among the isolates $F_{3.079,33.87} = 55.01$, $P < 0.0001$, with all the isolates showing an increase in adhesion capacity across the 4 time periods. This is supported by the results of descriptive statistics with the means across the 4 time periods: 6 h (mean = 0.07174), 12 h (mean = 0.5929), 18 h (mean = 0.9787), and 24 h

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(mean = 1.468). A significant interaction was also observed between the bacterial isolates and time $F_{5.434,59.78} = 13.19$, $P < 0.0001$, with the isolates contributing the largest source of variation (28 %), followed by periods (26 %). The result of the adhesion to polystyrene microtiter plate using tryptose soy broth as a growth medium is summarized in Figure 4-1. Isolate B44 showed the greatest biofilm-forming potential at 12 h, 18 h, and 24 h, with a value of 3.14 ± 0.60 after 24 h of incubation at 32°C. The biofilm-forming potential of sample B44 was statistically significant ($P < 0.05$) and higher than all other isolates, including the positive control (*B. subtilis* ATCC 3610), which showed moderate biofilm-forming ability. Apart from B44, other isolates with strong abilities to form biofilm are B50, B52, and B54, with values of 2.50 ± 0.69, 2.41 ± 1.20, and 2.32 ± 1.41, respectively. Isolates B56 and B48 showed the lowest biofilm potential, with values of 1.25 ± 0.77 and 1.63 ± 0.58 (Table 4-1). Isolates 46 and 57 were non-biofilm formers, indicating that adhesion did not necessarily result in biofilm formation. The negative control, B168 (B. subtilis ATCC 168), was observed to have the best adhesive capacity at the incubation period of 18 h, with little or no difference from an incubation time of 24 h.

4.1.13 Microbial Adhesion to Hydrocarbon (MATH) assay

Hydrophobicity of all the isolates was expressed as hydrophobicity percentage as shown in Figure 4-2. When the isolates were compared with the positive control reference strain (*B. subtilis* ATCC 3610), there was a significant difference among the means for all isolates ($P < 0.05$). Four of the isolates had a significantly higher percentage hydrophobicity compared to the positive control strain. These isolates are B44 ($P < 0.0001$), B47 ($P < 0.0133$), B48 ($P < 0.0035$) and B49 ($P < 0.0001$). B44 had the highest mean percentage hydrophobicity ($M = 41.2$) followed by B49 ($M = 40.4$) with B52 as the lowest ($M = 12.8$). In summary, all isolates

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demonstrated better hydrophobicity to hexadecane than the reference strain (*M* = 20.2) except B43 (*M* = 13.5), B50 (*M* = 15.4), B52 (*M* = 12.8) and the negative control reference strain *B. subtilis* ATCC 168 (*M* = 15.3).

4.1.14 Heat resistance profile

Isolates demonstrated the ability to grow across all the temperature ranges: psychrophilic (6 °C), psychrotrophic (15 °C), mesophilic (32 °C) and thermophilic (55 °C) temperatures (Table 4-1). Three of the isolates (B46, B47 and B48), all belonging to the species *B. subtilis*, were able to grow within a temperature range 6 to 60 °C with the rest of the isolates growing at 6 to 55 °C except B49 and B50.

4.1.15 Microscopy

From Figure 4-3, all isolates adhered to the coupons in varying degree and attachment strength, biovolume, thickness and biofilm architecture. Spatial stratification can be seen in most of the biofilms except in B168 (negative control) and B46. The structure ranges from multiple-layer biofilm with honeycomb-like channels in most of the isolates as seen in B49 and the B3610 to a flat structure with low biovolume as observed in B46. Extracellular polymeric substances (EPS) linking one cell to the other at the surface are visible in most of the images. The CLSM shows the distributions between dead and living bacterial cells. The red-stained dead cells are seen in the centre of the biofilm structure with green-stained living cells around the biofilm. Generally, there seems to be better biofilm formation on the stainless-steel coupons compared with a polystyrene microtitre plate.

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Table 4-1: The adhesive capacity, biofilm potential, cell surface hydrophobicity, heat resistance profile of *B. subtilis* and *B. velezensis* from milk samples (ESL, raw, pasteurised and packaged milk samples from raw milk, packaged ESL milk, pasteurised milk tank, stored ESL milk). Isolate B44 (*B. velezensis* strain LPL-K103) has the highest % hydrophobicity as well as biofilm formation on polystyrene with the ability to grow at a temperature range 6 °C-55 °C.

Isolates	Adhesion to polystyrene (Mean/Std)	Biofilm forming potential	Hydrophobicity (%)	Growth range (°C)	Source of isolation
***B. velezensis* strain LPL-K103 (B43)**	1.89 ± 0.78	Moderate	13.5±2.30	6°C -55 °C	Raw milk
***B. subtilis* str. SRCM101392 (B46)**	0.35±0.16	None	25.6±1.31	6°C -60 °C	Raw milk
***B. subtilis* strain SRCM103689 (B47)**	1.74±0.90 *	Moderate	28.8±1.27	6°C -60 °C	Raw milk
***B. subtilis* subsp. *subtilis* str. NCIB 3610 (B48)**	1.63±0.58 **	Moderate	30.0±1.19	6°C -60 °C	Raw milk
***B. velezensis* strain LPL-K103 (B52)**	2.41±1.20	Strong	12.8±1.05	6°C -55 °C	Raw milk
***B. subtilis* strain ATCC 11774 (B49)**	2.06±0.80 ***	Moderate	40.4±4.07	15°C -60 °C	ESL milk stored at 7°C
***B. subtilis* strain ATCC 11774 (B50)**	2.50±0.69	Strong	15.4±1.23	15°C -60 °C	ESL milk stored at 5°C
***B. subtilis* strain SRCM103637 (B54)**	2.32±1.41	Strong	25.6±1.47	6°C -55 °C	Packaged ESL milk
***B. velezensis* strain LPL-K103 (B57)**	0.54±0.45	None	26.0±3.06	6°C -55 °C	Packaged ESL milk
***B. velezensis* strain LPL-K103 (B44)**	3.14±0.60 ***	Strong	41.2±1.70	6°C -55 °C	Packaged ESL milk
***B. velezensis* strain LPL-K103 (B55)**	2.03±1.40	Moderate	25.3±0.63	6°C -55 °C	Pasteurised milk tank
***B. velezensis* strain LPL-K103 (B56)**	1.25±0.7708	Moderate	26.3±0.99	6°C -55 °C	Pasteurised milk tank
***B. subtilis* ATCC 168 (Negative Control)**	0.19±0.13	None	15.3±1.77	NA	Culture collection
***B. subtilis* ATCC 3610 (Positive Control)**	2.00±0.84	Moderate	20.2±2.84	NA	Culture collection

*NA = data not available for the reference strains. For the temperature profile, isolates were incubated at 6 °C (days), 10 °C (10 days), 15 °C (4-6 days), 55 °C (48 h) and 60 °C (48 h). Growth at 6 °C was observed to be very slow and small. * Asterisks indicate significant difference among means (*=$P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$ and ****= $P \leq 0.0001$).

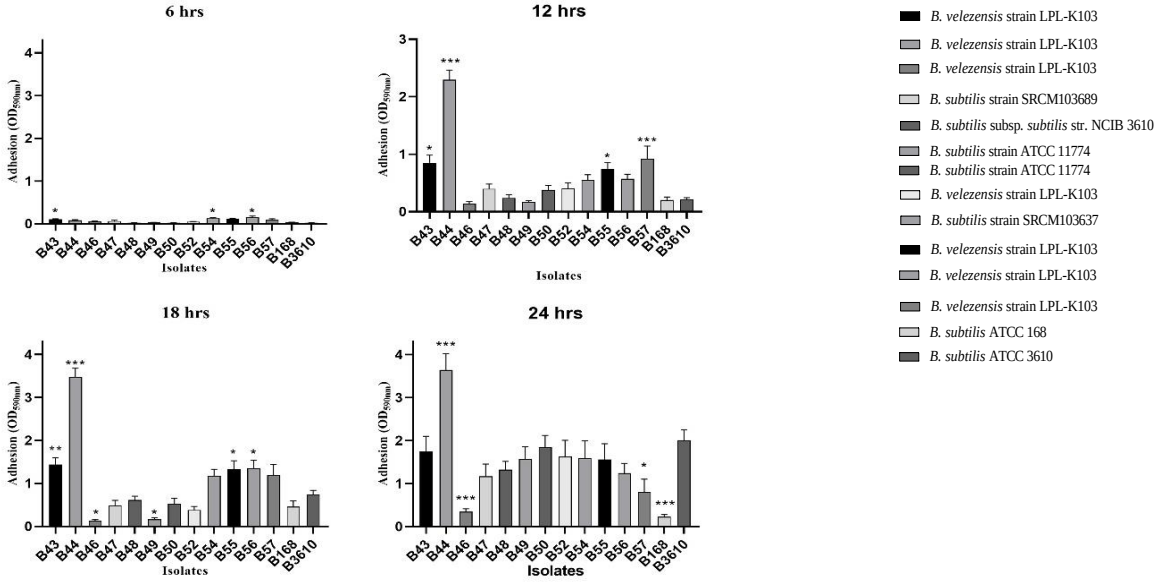

Figure 4-1: Total biomass and the number of bacterial cells in biofilms formed in formed in 96-well polystyrene microtiter plates with TSB at 32 °C for 6 h (A), 12 h (B), 18 h (C) and 24 h (D). Total biomass formation was determined using the crystal violet assay at OD_{590}. Error bars indicate standard error of means (SEM). Differences in biofilm-forming potential among isolates was done using one-way ANOVA and Tukey's post hoc test ($P < 0.05$). Dunnett multiple comparison was used to compare isolates with the positive control (*B. subtilis* ATCC 3610). Samples with asterisks (*) indicate significant difference among means (*=$P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$ and ****= $P \leq 0.0001$). *B. subtilis* ATCC 168 serves as the negative control.

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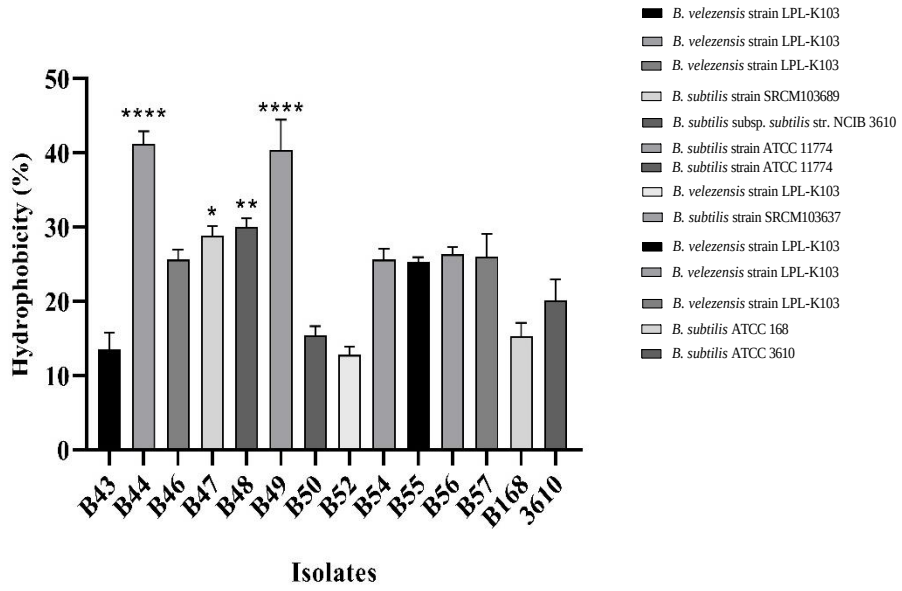

Figure 4-2: Results of microbial adhesion to hydrocarbon (MATH) assay, showing percentage hydrophobicity of all the isolates (*B. subtilis* and *B. velezensis*). Error bars indicate SEM. Adhesive capacity was determined using one-way ANOVA and Dunnett multiple comparison for the post hoc test ($P < 0.05$), comparing all the isolates with the positive control (*B. subtilis* ATCC 3610). Asterisks indicate significant differences among means ($*P \leq 0.05$, $**P \leq 0.01$, $****P < 0.0001$).

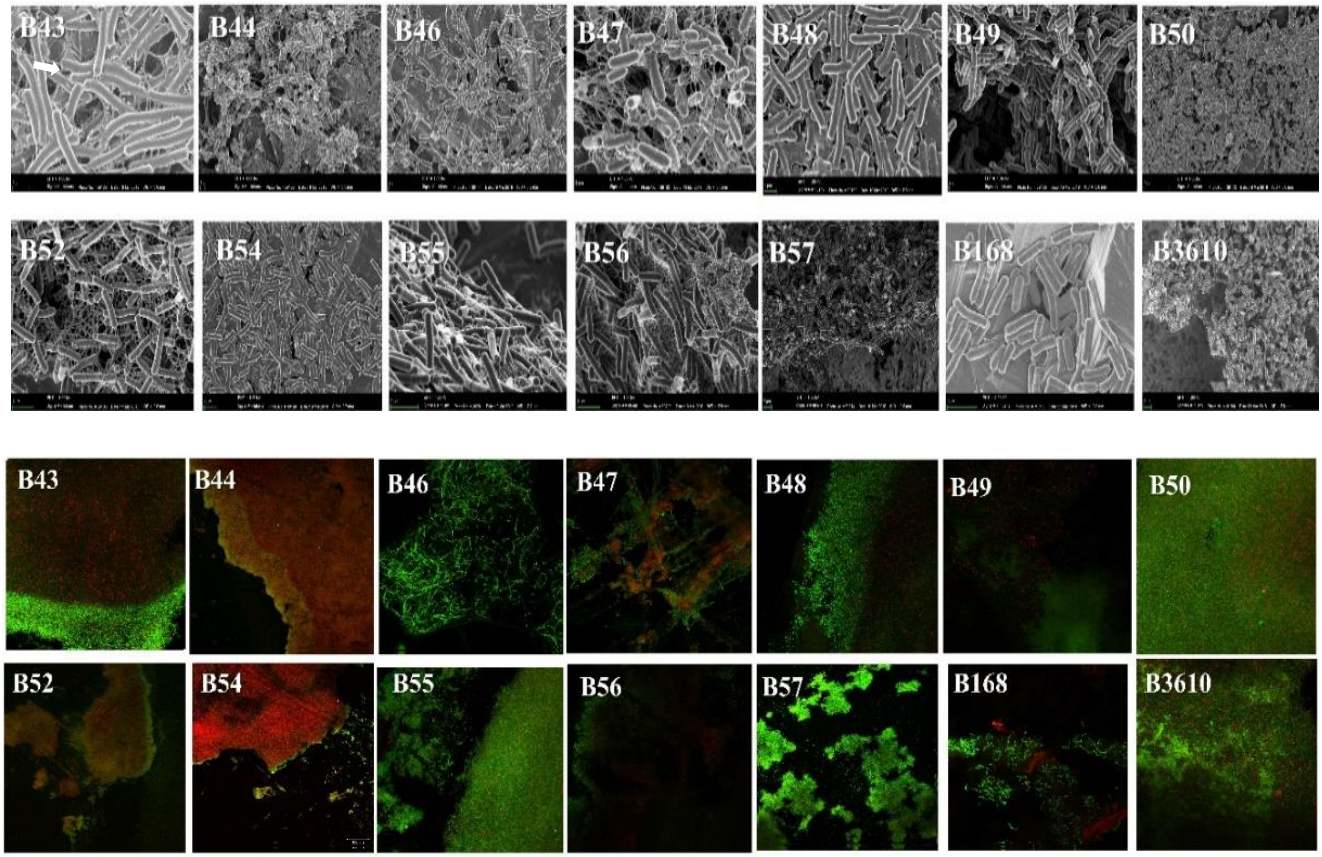

Figure 4-3: Scanning electron micrographs and CLSM images adhesion on stainless steel coupons semi-submerged in reconstituted skimmed milk at 32 °C. The arrows indicate the presence of mesh-like extracellular polymeric substances (EPS) between cells within the biofilm matrix. Spatial stratification in the architecture can be seen in some of the isolates such as B44, B49, B55, and the positive control B3610.

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4.1.16 Multi-locus sequence typing (MLST)

The MLST analysis was done by comparing the sequences from the isolates with the PubMLST database for *B. subtilis* (https://pubmlst.org/bsubtilis/). All isolates were identified as new sequence types as shown in Tables 4-2 and 4-3. Closely related sequence types are provided based on the degree of similarity in the alleles. There are nine closely related sequence types based on the output: ST1, ST3, ST105, ST69, ST96, ST151, ST40, ST161 and ST128 with ST96 as the most abundant (*n*=5). The result suggests that some of the isolates with related STs have the same source of contamination despite their isolation from the different part of the processing equipment. Most of the STs were isolated from soil from where they can quickly spread along the food-chain such as in the contamination of raw milk and subsequence survival of processing condition. According to Table 4-2, other sources of their isolation are from corn starch, rhizosphere, naturally fermented feed and faecal matter of pig and human.

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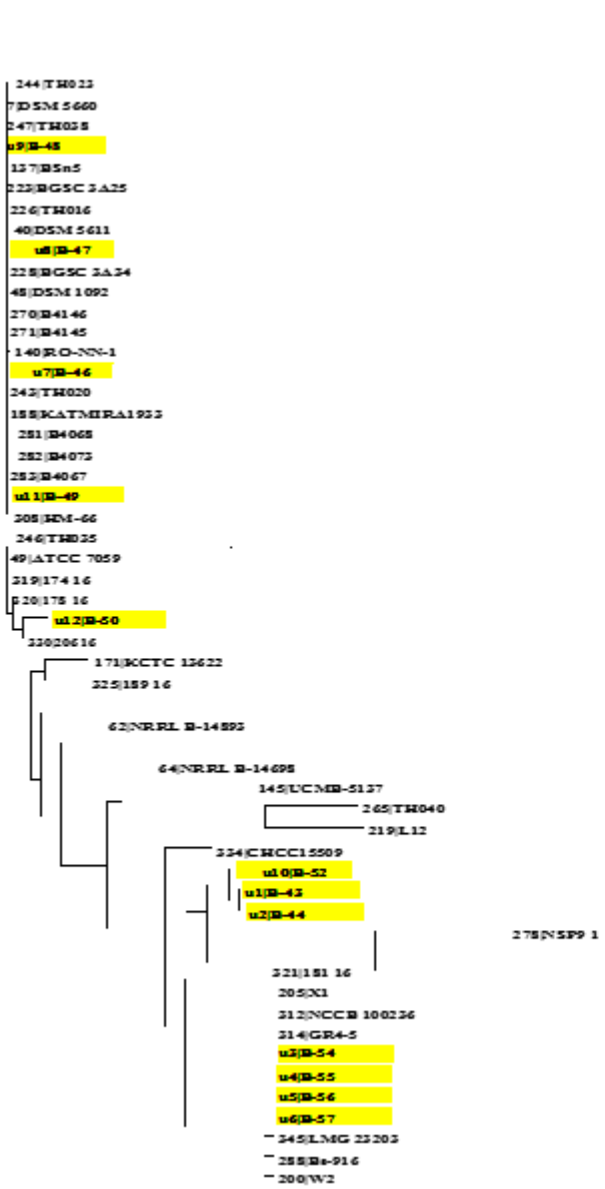
Table 4-2: Multi-locus sequencing of *B. subtilis* complex group from milk samples (ESL, raw, pasteurised and packaged milk samples from raw milk, packaged ESL milk, pasteurised milk tank, stored ESL milk) showing matching alleles (in red), differences in the genes, country of origin and sequence types from the database that is the closest to the isolates. All isolates have at least two allelic differences from the ones available on the database. Isolates B54, B55, B56 and B57 have no matching alleles to the database hence some uniqueness to their allelic profiles. Similar alleles are indicated with a red numbering.

Isolates	*glpF*	*ilvD*	*pta*	*purH*	*pycA*	*rpoD*	*tpiA*	Matching alleles		Culture collection no		Countries	Sub-species	*glpF*	*ilvD*	*pta*	*purH*	*pycA*	*rpoD*	*tpiA*	*ST*
B48	1	1	36	1	26	1	1	5					*Bacillus subtilis* *subtilis*	1	1	1	1	1	1	1	1
B47	3	3	2	66	2	1	1	6	40	DSM 5611	NRRL B-360	USA, corn starch	*Bacillus subtilis* *subtilis*	3	3	2	3	2	1	1	3
B46	61	64	4	63	40	68	3	2	228	BGSC 3A34	HU58	Unknown, human faeces	*Bacillus subtilis* *subtilis*	3	64	44	66	2	3	3	105
B44	87	28	64	96	40	31	44	2	165	SQR9		China	*Bacillus velezensis*	48	50	53	58	50	31	44	69
								2	205	X1		China, soil	*Bacillus velezensis*	57	42	64	69	50	44	44	96
								2	312	NCCB 100236	CBMB205; KACC 13105	South Korea, rhizosphere	*Bacillus velezensis*	57	42	64	69	50	44	44	96
								2	314	GR4-5		South Korea, soil	*Bacillus velezensis*	77	87	87	96	81	62	44	151
B57	87	28	64	96	81	31	44	3	314	GR4-5		South Korea, soil	*Bacillus velezensis*	77	87	87	96	81	62	44	151
B56	87	28	64	96	81	31	44	3													
B55	87	28	64	96	81	31	44	3													
B54	87	28	64	96	81	31	44	3													
B52	87	28	64	69	55	62	28	2	127	Companion	BGSC 3A37; GB03	Unknown	*Bacillus velezensis*	27	28	30	36	30	2	28	40
								2	205	X1		China, soil	*Bacillus velezensis*	57	42	64	69	50	44	44	96
								2	297	5B6	22740678	South Korea	*Bacillus velezensis*	27	28	30	36	30	2	28	40
								2	312	NCCB 100236	CBMB205; KACC 13105	South Korea, soil	*Bacillus velezensis*	57	42	64	69	50	44	44	96
								2	338	CHCC15540		Denmark, pig faeces	*Bacillus velezensis*	87	95	47	105	30	2	28	161
B50	75	56	35	69	79	3	1	4	308	HM-66		China, naturally fermented feed	*Bacillus subtilis* *subtilis*	75	64	35	83	79	56	1	128
B49	61	56	35	83	79	3	1	4	308	HM-66		China, naturally fermented feed	*Bacillus subtilis* *subtilis*	75	64	35	83	79	56	1	128
B43	87	28	64	96	40	31	44	2	165	SQR9		China	*Bacillus velezensis*	48	50	53	58	50	31	44	69
								2	205	X1		China, soil	*Bacillus velezensis*	57	42	64	69	50	44	44	96
								2	312	NCCB 100236	CBMB205; KACC 13105	South Korea, rhizosphere	*Bacillus velezensis*	57	42	64	69	50	44	44	96
								2	314	GR4-5		South Korea, soil	*Bacillus velezensis*	77	87	87	96	81	62	44	151



Bacillus subtilis	*subtilis*	104	soil	USA	TH023	
Bacillus subtilis	*subtilis*	2		Unknown	DSM 5660	
Bacillus subtilis	*subtilis*	2	soil	USA	TH038	
					B-48	
Bacillus subtilis	*subtilis*	47	Amorphophallus konjac tissue	China	BSn5	
Bacillus subtilis	*subtilis*	100	forest soil	India	BGSC 3A25	
Bacillus subtilis	*subtilis*	102	soil	USA	TH016	
Bacillus subtilis	*subtilis*	3	cornstarch	USA	DSM 5611	
					B-47	
Bacillus subtilis	*subtilis*	105	human feces	Unknown	BGSC 3A34	
Bacillus subtilis	*subtilis*	5	fermented soybeans (natto)	Unknown	DSM 1092	
Bacillus subtilis	*subtilis*	125	mayonnaise	The Netherlands	B4146	
Bacillus subtilis	*subtilis*	125	cereals	The Netherlands	B4145	
Bacillus subtilis	*subtilis*	49	soil	USA	RO-NN-1	
					B-46	
Bacillus subtilis	*subtilis*	103	soil	USA	TH020	
Bacillus subtilis	*subtilis*	83	yogurt-flavored beverage	USA	KATMIRA1933	
Bacillus subtilis	*subtilis*	130	curry cream	The Netherlands	B4068	
Bacillus subtilis	*subtilis*	130	curry soup	The Netherlands	B4073	
Bacillus subtilis	*subtilis*	131	food	The Netherlands	B4067	
					B-49	
Bacillus subtilis	*subtilis*	128	naturally fermented feed	China	HM-66	
Bacillus subtilis	*subtilis*	6	fermented soybean (natto)	Japan	ATCC 7059	
Bacillus subtilis			Tobacco isolate	Spain	174_16	
Bacillus subtilis	*subtilis*		Tobacco isolate	Spain	178_16	
					B-50	
Bacillus amyloliquefaciens			Tobacco isolate	Spain	206_16	
Bacillus tequilensis		57	2000-year old shaft tomb	Mexico	KCTC 13622	
Bacillus subtilis	*inaquosorum*		Tobacco isolate	Spain	189_16	
		22			NRRL B-	
Bacillus vallismortis			soil	USA	14893	
					NRRL B-	
Bacillus mojavensis		24	soil	USA	14698	
Bacillus atrophaeus		52	grass rhizosphere	Ukraine	UCMB-5137	
Bacillus benefactor		121	soil	USA	TH040	
Bacillus sonorensis		97	fermented food (Gergoush)	Sudan	L12	
Bacillus amyloliquefaciens		157	Pig faeces	Germany	CHCC15509	
					B-52	
					B-43	
					B-44	
Bacillus sp.			salt crystallizer pond	India	NSP9.1	
Bacillus velezensis			Tobacco isolate	Spain	181_16	
Bacillus velezensis		96	soil	China	X1	
Bacillus velezensis		96	rhizosphere soil	South Korea	NCCB 100236	
Bacillus velezensis		151	soil	South Korea	GR4-5	
					B-54	
					B-55	
					B-56	
					B-57	
Bacillus velezensis		168	Water	Spain	LMG 23203	
Bacillus velezensis		137	rice roots	China	Bs-916	
Bacillus mojavensis		92	soil, saffron field	India	W2	

Table 4-3: Maximum likelihood phylogenetic comparison of *Bacillus subtilis* complex group from milk samples (ESL, raw, pasteurised and packaged milk samples from raw milk, packaged ESL milk, pasteurised milk tank, stored ESL milk) with other known isolates from the MLST database with the sequence type, source of isolation, country of origin and culture collection number. Isolates used in the study are highlighted in yellow.

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4.1.17 Discussion

B. velezensis has not been associated with any challenge of biofilm and contamination of dairy processing equipment and product in the past. On the other hand, the previous study by Duanis-Assaf et al. (2016) reported quorum sensing and biofilm formation in *B. subtilis* to be dose-dependent to the presence of the sugar lactose that is present in milk. We observed the ability of all the isolates in forming moderate to strong biofilms on polystyrene microtitre plate except for two which are B46 and B57. In contrast to the microtitre plate assay, all isolates seemed to have a better biofilm-forming ability on stainless-steel from observation of the SEM and CLSM images. This variation in the adhesion to the different substrata (microtitre and stainless-steel) can be attributed to the physicochemical characteristics of the surfaces such as surface charge, hydrophobicity, nanotopography and the presence of conditioning film (Legeay et al. 2010). Apart from the stereo-specific interaction of the bacterial cells in the adhesive strength of the isolates, the surface characteristics also play an essential role. The present result indicated that bio-adhesive and biofilm-forming potential is strain-specific for the members of the *B. subtilis* complex group used in the study. Environmental stresses have been suggested to be responsible for the quantitative variation in the potential of the strains to form a biofilm (Elhariry, 2011). This result on differences in biofilm formation among strains is consistent with other similar studies such as in the observed variation in the strains of *Candida albicans* (Barak et al. 2007; Li, 2003).

The result of the MATH assay revealed that isolate B44 possesses both strong biofilm-forming and adhesion capacity to hydrophobic interaction to substratum such as stainless-steel surface which is commonly used in the food industry. Despite the ability of B43 to form a moderate biofilm, it has very low adhesion to the hydrocarbon used in the MATH assay (hexadecane). We can safely conclude that hydrophobic interaction of bacterial cells to a substratum despite

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playing a critical role in the initiation of biofilm-forming process, other intrinsic and extrinsic factors such as the growth rate of the contaminating bacteria, pH, temperature, nutrient composition of the medium, quorum sensing, presence of other bacteria, etc are equally important in the overall biomass and structure of the biofilm (Bohinc et al., 2016; Tirumalai, 2015). As in the result of the biofilm assay, we observed that bacterial cell surface hydrophobicity is strain-specific with all the isolates having percentage hydrophobicity of less than 50 % (or 0.5) hence, they can safely be classified as hydrophilic than hydrophobic based on their cell surface property.

From the SEM and CLSM images of some of the isolates, all isolates produced microcolonies on stainless-steel surface using reconstituted milk medium 24 h producing biofilm with different adhesive ability except in isolates B48 and B168 that are not well-defined. The structure or biofilm architecture seems to be strain-dependent just as in the case of the adhesion ability on polystyrene surface. Spatial stratification and EPS can be conspicuously seen in all the isolates but in varying degree, producing a mesh-like connection between one cell and another and to the substratum. This type of multi-layered stratification in a biofilm is capable of driving phenotypic heterogeneity and morphological differentiation of cells within biofilm matrix and formation of spores within the processing plant which can perpetuate the problem of contamination and spoilage of product being processed within the processing equipment (Soni et al. 2016). Diverse phenotypes or cell-types have been identified within the *B. subtilis* biofilm specialised for the production of metabolites such as surfactin, matrix, exoproteases as well as spores and motile cells (Lopez et al. 2009; Marlow et al. 2014; Veening et al. 2008). From the confocal images, the non-viable or older cells stained orange/red while the green cells are the metabolically active viable cells. We can infer that the biofilm expands from within the centre of the structure outward with actively dividing cells surrounding the structure. Since the

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isolates are spore forming bacilli, it can be concluded that centre of the biofilm structure is the hot-bed of spore production and a probable source of bacterial recontamination in the plant and resistance to the cleaning-in-place regime (CIP). The production of spores is not limited only to this site within the cluster of bacterial cells but can also be distributed throughout the biofilm as well as in patches as observed by (Faille et al., 2014). The heat resistance profile showed that all the isolates were able to grow at a temperature ranging from psychrotolerant to thermophilic on nutrient agar. Specifically, all the isolates can be divided into three categories according to their heat-resistance profile: 6 °C to 55 °C (B43, B44, B52, B54, B55, B56, B57), 6 °C to 60 °C (B46, B47, B48) and 15 °C to 60 °C (B49, B50). The implication of this is that all the isolates may be capable of surviving pasteurization conditions as well as survive storage and refrigeration temperature as vegetative cells. The ability to survive pasteurization may be influenced by the strength of the biofilms the strains can form besides sporulation that occur within the biofilms. This means the eradication of the isolates may prove to be a challenge as they can form process biofilms, spores and vegetative cells which can survive the high temperature employed during the pasteurization process.

MLST analysis identified the strains of species of the *Bacillus* contaminants as *B. subtilis* and *B. velezensis*. Six of the isolates were identified as strain *B. velezensis* LPL-K103 after a BLAST analysis of the concatenation of all the seven housekeeping genes used. The strain was isolated from the raw milk, pasteurised milk tank and packaged ESL milk suggesting a possible adaptation of the strain to pasteurisation conditions. Such adaptive features include spore and biofilm production, cell-surface hydrophobicity, heat-resistance to the heat of spores and vegetative cells as well as a good attachment strength to stainless-steel surface. The study justifies the isolates of possessing at least some of these properties. Other strains of *B. subtilis* identified are *B. subtilis* ATCC 11774 (ESL milk at 5 and 7 °C), *B. subtilis* subsp. *subtilis* str.

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NCIB 3610 (raw milk), *B. subtilis* str. SRCM101392 (raw milk), *B. subtilis* strain SRCM103689 (raw milk) and *B. subtilis* strain SRCM103637 (packaged ESL milk). The result of a similar study with a focus on *B. cereus* using the same milk samples implicates the aseptic filler-nozzle as a possible reservoir for these thermophilic spore-formers during ESL milk processing (Mugadza et al. 2019).

The limitation in the study is that the biofilm-forming potential of the strains was carried out under static condition, unlike the flow condition that is at play during the processing of ESL milk. Although it is possible that the ESL processing conditions pre-selected the isolates due to their ability to produce spores and tolerate thermophilic temperature (above 50 °C), biofilms naturally exist in a consortium of multi-species and not mono-species biofilms assumed in this study. Nonetheless, the study proves that isolates of *B. velezensis*, hitherto unknown to prove itself a challenge during milk processing, may be important in ESL milk processing as well as other well-known *Bacillus* species.

This study uses MLST to track the sources of contamination of *B. subtilis* and *B. velezensis* in ESL processing plant. From all indications, the isolates are linked to the raw milk used in the production of ESL milk since they are clonally related. They seem to be well adapted to survive the selective pressure created during processing either as a vegetative cell or spore. Although *B. subtilis* and *B. velezensis* are generally non-pathogenic, this study demonstrates their biofilm-forming ability and the potential to constitute a nuisance in a processing plant where they compromise the quality of the ESL milk during extended storage after processing. This challenge may become aggravated in the scenario where CIP is ineffective against spores of the organisms and the plant is shut down before the commencement of another processing round. This study is a part of a cohort with the specific aim of characterising spore-forming bacilli in extended shelf-life milk and their intrinsic resistance to CIP as well as other control

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measures. The goal is to mitigate or eradicate the challenge of thermophilic spore formers and their potential impacts on both the dairy industry and consumers.

4.1.18 Conclusions

The quality of raw milk used in the production of ESL milk is crucial. Based on our results, the source of contamination seems to be from the raw milk used. From this standpoint, it imperative to devise at the farm level actions designed at minimising the risk of bacterial contaminants. However, the hygiene condition of the dairy processing environment, bulk milk tank, tanker used during transport of raw milk, the processing equipment as well as personnel must be prioritised to avoid the persistence challenge of biofilms of thermophilic spore formers.

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4.2 Ultraviolet-C inactivation and hydrophobicity of *Bacillus subtilis* and *Bacillus velezensis* spores isolated from extended shelf-life milk

4.2.1 Abstract

Bacterial spores are important in food processing due to their ubiquity, resistance to high temperature and chemical inactivation. This work aims to study the effect of ultraviolet C (UVC) on the spores of *B. subtilis* and *B. velezensis* at a molecular and individual level to guide in deciding on the right parameters that must be applied during the processing of liquid foods. The spores were treated with UVC using phosphate buffer saline (PBS) as a suspension medium and their lethality rate was determined for each sample. Purified spore samples of *B. velezensis* and *B. subtilis* were treated under one pass in a UVC reactor to inactivate the spores. The resistance pattern of the spores to UVC treatment was determined using dipicolinic acid (Ca-DPA) band of spectral analysis obtained from Raman spectroscopy. Flow cytometry analysis was also done to determine the effect of the UVC treatment on the spore samples at the molecular level. Samples were processed for SEM and the percentage spore surface hydrophobicity was also determined using the Microbial Adhesion to Hydrocarbon (MATH) assay to predict the adhesion strength to a stainless-steel surface. The result shows the maximum lethality rate to be 6.5 for *B. subtilis* strain SRCM103689 (B47) and highest percentage hydrophobicity was 54.9 % from the sample *B. velezensis* strain LPL-K103 (B44). The surface hydrophobicity for all isolates was statistically significant ($P<0.05$) when compared with the control strain B4002. Flow cytometry analysis of UVC treated spore suspensions showed a divergence into sub-populations unaccounted for by plate counting on growth media. The Raman spectroscopy identified B4002 as the isolate possessing the highest concentration of Ca-DPA as well as a pattern between Ca-DPA concentration and lethality rate. The study justifies the critical role of Ca-DPA in spore resistance and the possible sub-

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populations after UVC treatment that may affect product shelf-life and safety. UVC shows a promising application in the inactivation of resistant spores although there is a need to understand the effects at the molecular level to design the best parameters during processing.

2

[2] Ultraviolet-C inactivation and hydrophobicity of *Bacillus subtilis* and *Bacillus velezensis* spores isolated from extended shelf-life milk. James A. Elegbeleye, Ramon Gervilla, Artur X. Roig-Sagues, Elna M. Buys. International Journal of Food Microbiology (*accepted*).

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4.2.2 Introduction

Bacterial spores are dormant and adaptive structures able to cope with potential and destructive stresses and to survive harsh environmental conditions. This adaptation enables them to withstand a high-temperature, salt, extreme pH, radiation, low nutrient and desiccation. The germination of contaminating microbial spores in processed food result in spoilage or foodborne illness depending on the type of bacteria (Logan, 2012; Mugadza et al. 2019; Wells-Bennik et al. 2016). A spore is a multi-layer, highly dehydrated and dormant structure encapsulating different chemical compounds that offer resistance against environmental pressure. There is the presence of α/β-type small acid-soluble protein (SASP) and dipicolinic acid (DPA). The former protects the spore against DNA damage while the latter raises the spore wet heat resistance thus protecting core proteins against inactivation (Setlow et al. 2006). The various layers of spores such as the coat are designed to protect against toxic chemicals, lysozyme, desiccation and protozoan ingestion (McKenney et al. 2013). All these attributes conspire to perpetuate spores in the environment and eventual contamination of food. The spread of spores in the food chain is often inevitable thus making them a food safety challenge, especially in the dairy industry.

While the conventional pasteurisation of milk might be sufficient to eradicate most vegetative cells, the application of high temperature such as ultra-high temperature (UHT) is often required to inactivate bacterial spores present in food (Bressuire-Isoard et al. 2018). This results in an undesirable heated milk flavour due to Maillard reactions (Mehta, 1980). Microfiltration followed by pasteurisation has been observed by Schmidt et al. (2012) to decrease bacterial counts by 5–6 Log_{10} units to lower than 1 CFU/mL but cannot eliminate some spore formers especially in the case of low quality of the raw milk used and post-process contamination (Germain et al. 2013; Huck et al. 2008; Svensson et al. 2000, 1999).

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Adhesion of these surviving spores to the wall of the processing equipment poses a big threat to the dairy industry due to the high resistance nature to processing conditions than what vegetative cells are susceptible to. They have been observed to be more hydrophobic with greater adhesive ability to food contact surface such as stainless steel than vegetative cells. The attached spores may then germinate, form biofilms and sporulate therefore contaminating the processing line with the consequential reduction in shelf-life of the processed product (Harimawan et al. 2013). This higher hydrophobicity of spores also, presumably, makes them be of a greater food safety concern than vegetative cells (Seale et al. 2008). Some of these spores can grow at both psychrotrophic and mesophilic temperatures as observed by Mugadza and Buys (2017) thus making their control indispensable during processing.

Non-thermal processing generally preserves the sensorial and nutritional qualities of food maintaining the fresh-like taste that is desirable to the consumers. The application of these emerging technologies to foods have been proven to exceed that of the thermally treated products in terms of quality, safety and shelf-life (Jermann et al. 2015). Such non-thermal processing technologies include ultra-high pressure homogenisation (UHPH) and ultraviolet C (UVC). They have shown a considerable lethal action against the spores of spoilage and pathogenic organisms in liquid foods with no known toxic residue. However, Gram-positive bacteria and their spores, such as *Bacillus* spp., are generally more resistant to UHPH than Gram-negative which can present a setback in its adoption (Smelt, 1998; Wuytack et al. 2002).

Ultraviolet (UV) radiation is a form of non-ionising, electromagnetic radiation occupying the region between visible light and X-ray (Bintsis et al. 2000). There are three different types of UV based on the wavelength of the spectrum with each causing a specific type of injury to the DNA in an overlapping manner. These are ultraviolet A (UVA) with a wavelength of 320nm to 400nm; ultraviolet B (UVB) with a wavelength of 290nm to 320nm and ultraviolet C (UVC)

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with a wavelength of 100nm to 290nm (Handan Baysal et al. 2013). UVC is approved by the United States Department of Agriculture (USDA) in the processing of liquid foods such as milk because it can inactivate a wide range of microbial contaminants in the food and contact surfaces such yeasts, viruses, bacteria, moulds etc. (Gunter-Ward et al. 2018; Hu et al. 2017; Jennifer A Crook et al. 2015; Van Impe et al. 2018).

UVC exerts its effects through photons of light penetrating the cells/spores and causing a cross-linking of pyrimidine bases making them unable to replicate which results in the death of vegetative cells (Jennifer A. Crook et al. 2015). In bacterial spores, UVC exposure causes the formation of a spore photoproduct (5-thyminyl-5,6-dihydrothymine), cyclobutene pyrimidine dimers and breakage of single and double strands (Delorme et al. 2020). UVC effectiveness is influenced by several factors such as the type of the microorganisms, retention time, concentration of cells/spores, number of passes of product in the UVC reactor, product characteristics (Fan et al. 2017). Such intrinsic characteristics of the product that may affect UVC efficacy include turbidity, UV absorbance, presence of colour compounds and solid matters.

Although the application of UVC has been validated to be about ten thousand times cheaper than thermal processing making it economically and commercially viable, nonetheless, some grey areas need further study before it gains wide acceptance (Rodriguez-Gonzalez et al. 2015). The disadvantage of UVC is that the effect decreases in liquid foods that are opaque or turbid hence the advice to design the UVC reactor to run in thin-film with a capacity for multiple passes (Koutchma, 2009). Besides, extreme UVC treatment may lead to significant changes in some properties of the food such as oxidation of protein and lipid components of processed milk. Hence, the need to apply UVC treatment in moderation during the treatment of liquid foods (Fernández et al. 2014).

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This study seeks to determine the effect of ultraviolet C (UVC) on the inactivation of spores of *B. subtilis* and *B. velezensis* in phosphate buffer saline (PBS), the hydrophobicity of the spore surface and resistance pattern to UVC using dipicolinic acid (Ca-DPA) from Raman spectral analysis as chemical features. Also, the different sub-populations of spores will be determined after UVC exposure. This will guide in determining the right parameters that must be applied during the processing of liquid foods. *Bacillus* spp. was used in the study because of their ability of members to perpetuate within the processing environment through sporulation thereby causing enzymatic spoilage of pasteurised milk and illness in some cases (Caplan and Barbano, 2013; Wells-Bennik et al. 2016).

4.2.3 Materials and methods

4.2.3.1 Selection of *Bacillus* strains

Five *Bacillus* strains isolated from ESL processing plant in the Gauteng province of South Africa were used in the study (see section 4.1). Sample collection was done as using 1 L of raw and packaged ESL milk stored at 5 ˚C. All samples were collected from the processing line in a total of four visits and plated out within 4 hours after collection. Matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectroscopy was used in the preliminary identification of bacterial isolates. The strains were selected based on their ability to form good biofilms on the stainless steel surface and produce proteolytic activity on skim milk agar. The strains are *B. velezensis* strain LPL-K103 (raw milk), *B. velezensis* strain LPL-K103 (ESL milk), *B. subtilis* strain SRCM103689 (raw milk), *B. subtilis* strain ATCC 11774 (ESL milk), *B. velezensis* strain LPL-K103 (raw milk) and a control organism *B. subtilis* CECT 4002 from the Spanish Type Culture Collection (CECT, Universidad de Valencia, Spain). The

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spore of the control organism *B. subtilis* CECT 4002 is known for its resistance to UVC. The isolates were maintained in peptone broth (Merck, Darmstadt, Germany) containing 20% (v/v) glycerol at −80 ˚C.

4.2.3.2 Preparation and purification of spores

Overnight culture from a nutrient agar plate was inoculated into 10 mL volume of tryptone glucose broth (Oxoid Ltd., Hampshire, UK) and incubated for 24 h at 30 ˚C ±1. 1mL aliquot from the broth was spread over the surface of Campden Sporulation Agar (60 mL) in a Roux flask. The flasks were then incubated at 30 ˚C for 4 weeks. The sporulation process was monitored using phase-contrast microscopy. The spores were harvested after maturation to inactivate all vegetative cells by adding 40 mL of deionized water into each Roux flask. The surface of the agar-containing spores was scratched gently with wire loop with the liquid collected into a centrifuge tube. The centrifuge tube was heated in a water bath at 90 ˚C for 30 min and cooled in a cold water bath. The spore suspension was washed three times by centrifuging at 12000× *g* for 15 min at 4 ˚C. The pellet was resuspended in 20 mL deionised water and centrifuged again for 10 min and standardised to obtain a spore concentration approximately 1×10^8 (≥ 90% purity) after which it was stored at 4 ˚C for later use (López-Pedemonte et al. 2003).

4.2.3.3 UVC treatment of spores in PBS

The treatment was carried out by running the spore suspension through a UVC reactor as described by (Reverter-Carrión et al. 2018) using 10 mL of inoculum in 1L of PBS. For the

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control (A_1), the spore suspension was run through the reactor without switching on the UVC lamp while it was switched on for the UVC treated spore suspension (A_2). The UVC parameters consisted of a single lamp of radiance 41 mW/cm2 with a retention time of 10 seconds which corresponds to a dose in the matrix of 410 mJ/cm2 or 3.9 J/mL) under 1 pass (20 ± 1 ºC). The other parameters are flow rate (25.2 L/h), length of UVC lamp (765 mm), gap size between lamp and quartz sleeve (2 mm) cold air, gap size between the quartz sleeve and spiral (1 mm) with the volume of the reactor calculated to be 70 mL. Since the matrix was PBS buffer, it is assumed that the absorbance by the PBS is negligible and theoretically all the energy generated by the reactor has a strong effect on the bacterial spores. Treated and untreated samples were collected aseptically in sterile bottles inside a laminar flow cabinet (Mini-V PCR cabinet Telstar, Terrassa, Spain) fitted with a stainless steel socket connected at the exit of the equipment.

4.2.3.4 Plate counting of UVC-treated and untreated spore samples

The lethality was determined by taking the logarithmic difference between the UVC treated and untreated (control) spore suspension for all the isolates. 1 mL aliquot of the treated and untreated spore suspension was plated in trypticase soy agar (TSA) supplemented with 0.6% glucose (Merck, Darmstadt, Germany) using the pour plate method. The plates were incubated at 30 ˚C for 24 h to 48 h after which the germinated spores were enumerated. The inactivation of the spore samples by UVC was expressed as a lethality rate using the formula given below:

$$\text{Lethality rate} = \log_{10} \frac{A_1}{A_2}$$

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Where A_1 is the number of untreated spores and A_2 the number of UVC-treated spores respectively.

4.2.3.5 Adhesion of intact spore to hydrocarbon (hexadecane)

The surface hydrophobicity of the untreated spore samples was determined by adapting the microbial adhesion to hydrocarbon (MATH) assay as developed by (Rosenberg et al. 1980; Wiencek et al. 1990). Bacterial spore suspension (in distilled water) was adjusted to an optical density between 1.2 to 1.4 at 600 nm (OD_{600} nm). 3 mL each of hexadecane and suspension of spores was added together followed by vigorous mixing on a vortex mixer at room temperature for 60 s and then incubated at 30°C for 10 min. After 10 min of incubation, the suspension was agitated on a vortex mixer for 2 min and allowed to stand for 20 min at ambient temperature. The optical density of the aqueous layer was measured at OD_{600} nm using a spectrophotometer (Thermo Scientific, MA, US). The experiment was repeated three times in duplicates ($n=6$). The percentage of cell surface hydrophobicity was calculated using the formula as provided:

$$\text{RH} = \frac{OD\text{initial} - ODresidual}{ODinitial} \times 100\%$$

4.2.3.6 Flow cytometry analysis of UVC-treated spore suspension

Flow cytometry was done on the UVC-treated spore suspension using 500 µL of the treated and control samples (used for gating) with a cell concentration of approximately $10^{\%}$ cfu/mL. The analysis was done using FACS Calibur flow cytometer (Franklin Lakes, NJ, USA) equipped with a 15 mW air-cooled 488 nm argon-ion laser and a 633 nm red diode laser. Side-angle-scatter (SSC-H), green fluorescence (FL1-H detector, 530/30 filter) and red

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autofluorescence (FL3-H detector, 670LP filter) were used to quantify the spores into different sub-populations. Double-distilled water was used as the sheath fluid (Coulter Corporation, Miami, FL, USA). Propidium iodide and SYTO 9 (Invitrogen, Mount Waverley, Australia) were added to 500 µL of the spore suspension to make a final concentration of 15 µM and 500 nM respectively (Park et al. 2013; Smelt et al. 2008). The stained suspensions were mixed and incubated in the dark at room temperature for 15 min before analysis. Data acquisition was done on a logarithmic scale set at a nominal flow rate of 1000 events s−1. To discriminate the spores, the side scatter (SSC) threshold level was adjusted manually to 307 mV. The green emission from SYTO 9 (indicative of cortex hydrolysis) was collected through a 530 nm band-pass filter, and the red fluorescence from propidium iodide-staining (indicative of damage to the inner membrane) was collected through a 585 nm band-pass filter. The detected signals were amplified logarithmically, and a gate generated in the dot plot of the forward scatter versus side scatter to differentiate spores from artifacts. (Borch-Pedersen et al. 2017; Mathys et al. 2007; Stiefel et al. 2015). Gates were drawn using purified spore sample that was untreated and heat treated sample at 121 ˚C for 1 h and 121 ˚C for 15 min. This was done in triplicate with either stained with SYTO 9, PI or SYTO 9+ PI. The operating software was BD CellQuest Pro (version 4.0.2; BD) (Mathys et al. 2007).

4.2.3.7 Morphology of spores using scanning electron microscopy

To visualise the morphology of purified spore, two preparations were made, and these are spores attached to a contact surface (stainless steel) and spores suspended in distilled water. The first preparation was done by adding the spore samples to centrifuge tubes containing PBS with stainless-steel (316L-0.90 mm-2B PVC; dimension: 50 mm x 13mm) semi-submerged in

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the liquid. The tubes were then incubated at 30 ˚C for 24 h. After incubation, the stainless coupons were dipped three times to remove unattached spores and prepare for microscopy. The second preparation was done by adding some spore suspension into Eppendorf tubes which was then washed in 0.075M phosphate buffer. The buffer was removed, and 2.5 % glutaraldehyde/formaldehyde solution was used to pre-fix the samples for 1 h (Reineke et al. 2013). The fixative was then removed after which the samples were washed 3 times in buffer. 1 % osmium tetraoxide solution was later added to the samples and post-fixed for 1 h. Samples were washed 3 times for 15 min then centrifuged to get a pellet from each step. After the removal of the wash buffer, the spore samples were dehydrated using a graded series of ethanol (30 %, 50 %, 70 %, 90 % and 3×100 %) for 15 min each. Samples were left in the last 100 % ethanol for 30 min. Coupons were left in a 50:50 mixture of hexamethyldisilazane (HDMS) and 100 % ethanol for 1 h. The same process was repeated for HDMS only for another 1 h. After the addition of an aliquot of fresh HDMS, coupons were left to dry. The coupons were coated with carbon before mounting on the Scanning Electron Microscope (Asahi et al. 2015; Jing et al. 2019; Rozali et al. 2017).

4.2.3.8 Raman spectroscopy

This analysis is a non-destructive and label-free imaging technique. 100 µL of purified spore suspension in distilled water was dispensed on microscopic coverslips. The samples were dried under vacuum (Thermo Scientific, USA). The analytes (spores) on the glass coverslip were subjected to the spectral analysis by using 1.5 mW of 532 nm laser excitation and 0.9 and 100× magnification. The acquisition time of 13 min was used to obtain a high-quality vibrational spectrum appropriate for the analysis. The spectral acquisition was done acquired using a

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confocal Raman spectroscopy (WITec alpha300R, Germany) with a slight modification to the method used by Kong et al. (2012).

4.2.3.9 Statistics

All experiments were performed three times in duplicate ($n=6$). Analysis of variance (ANOVA) was done using GraphPad Prism (version 8.0.2) using Dunnette multiple comparison hypothesis testing to determine significant differences between the treatments ($P \leq 0.05$). FlowJo (version 10 CL) software was used in analysing the flow cytometry data. Further analysis of the Raman spectra was done using the software OriginPro 2019 (version 9.6).

4.2.4 Results and discussion

4.2.4.1 Lethality rate of spore samples after UVC treatment

From our results, we confirmed the ability of UVC to either inactivate or injure *B. subtilis* and *B. velezensis* spores as proven by other observations (Martinez-Garcia et al., 2019; Thi Tuyet Nhung et al., 2012; Zhang et al., 2014). The UVC treatment showed that the difference between the spore samples with a maximum lethality rate of 6.51 and minimum lethality rate of 3.38 is statistically significant (Figure 4-3). There was an overall significant difference at the $P < 0.05$ level in the lethality rate of spores $F(5,6) = 44$, $P = 0.0001$ with mean lethality rate and standard deviations for samples given as follows: B43 (M = 4.60, SD = 0.12), B44 (M = 4.17, SD =0.04), B47 (M =6.51 , SD = 0.49), B50 (M = 3.38, SD =0.28), B52 (M =5.04 , SD =0.05) and B4002 (M = 3.71, SD =0.07). Three of the samples have (B43, B47 and B52) had

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significant higher lethality rate when compared with the positive control whereas the rest of the samples exhibited no difference. The spores of B50, from observation, proved to be the most resistant among the samples with the lowest lethality rate of 3.38 in PBS followed by the positive control (B4002) with 3.71. Lethality of spores generally increases with increasing dose of UVC applied during processing and decreased with the presence and type of pigmented compounds in spores or the suspending medium (Khaneja et al. 2009).

The absorbance coefficient of a medium such as PBS is quite low with a high penetration unlike in food matrices such as milk or fruit juices, therefore, the effect of the UVC is expected to be greater in PBS. Factors responsible for the low penetration and high absorption coefficient include organic matter, solutes and colour complexes present in the food which is absent in this present study (Guerrero-Beltrán and Barbosa-Cánovas, 2004; Sauceda-Gálvez et al. 2019). Consequently, to obtain treated products that possess the intended shelf-life under prolonged storage condition will require the processing of the product with UVC not as a stand-alone technique but in combination with other novel technologies. Another way to ensure the total inactivation of spores is to apply the UVC treatment at a dosage that guarantees maximum effect without alteration to the physicochemical and sensorial properties of the processed food. Besides, the UVC reactor can be equipped with an arrangement that creates a turbulent flow of product to ensure homogenous distribution creating a maximum exposure of the spores to the lethal effect of UVC during processing. Although UVC treatment is effective against the spores of diverse strains of *B. subtilis* complex group used, there is a variation in the effectiveness. What accounts for the differences in resistance or lethality may be the relative amount of Ca-DPA present in each spore which is a factor of genetics and other environmental factors such as growth medium used for the cultivation of the spores.

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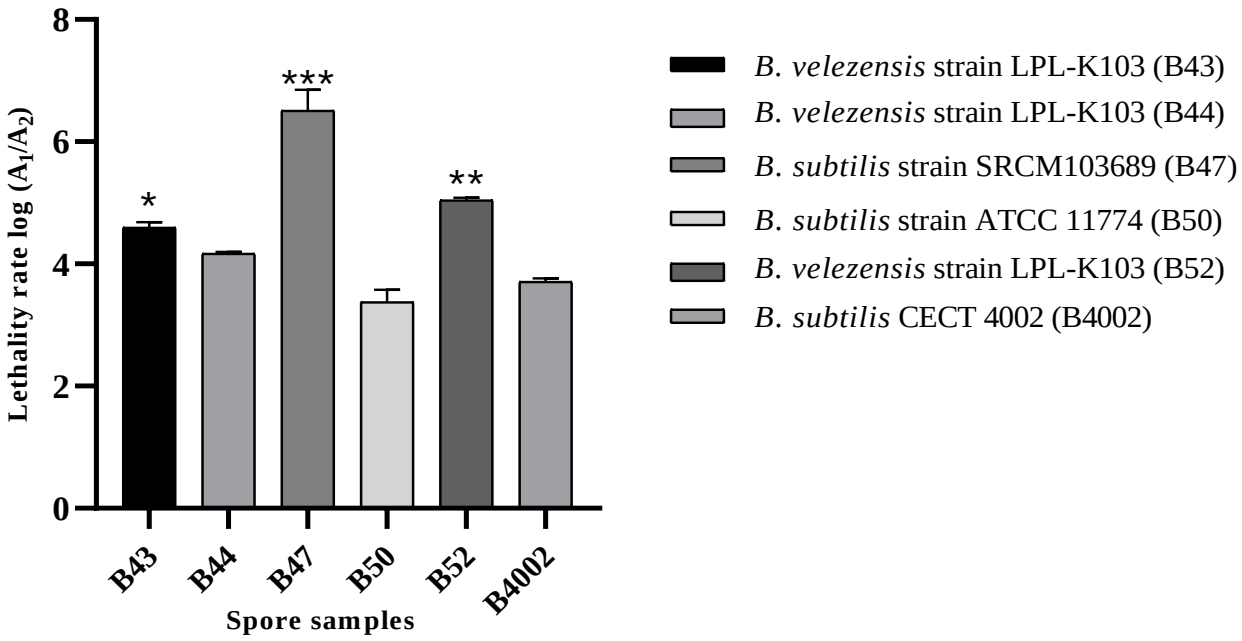

Figure 4-4: The lethality rate of *B. subtilis* and *B. velezensis* spores with phosphate buffer (PBS) as a suspending medium after treatment with UVC radiation (410 mJ/cm2) for 10s at 20 ˚C. The treatment was performed three times and plated out in triplicate (n=9). Differences in the lethality rate were determined using one-way ANOVA ($P< 0.05$). Asterisks (*) indicate significant difference among means (*=$P\leq 0.05$, **= $P \leq 0.01$, ***= $P\leq 0.001$).

It may be practically challenging for UVC processing either as a stand-alone or in a combinatorial system to completely inactivate all spores present in food during processing based on the complexity of the food and presence of pigments. Nonetheless, a desirable level of commercial sterility can be achieved that is in tandem with all regulatory standards without compromising safety.

Previous study using the same strains besides the control organism showed their ability to grow at 6 ˚C although slowly (section 4.1). Some of the spores, though sub-lethally injured after exposure to UVC radiation, may have the possibility of germinating in the post-processed food such as extended shelf-life (ESL) milk thereby compromising the safety of such product. This may occur especially under long storage or in any part of the food chain resulting in spoilage and illness in the case the spores are from pathogenic microorganisms. This potentially viable but non-culturable (VBNC) sub-population is not likely to be picked up using the traditional

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plate counting. Hence, the application of flow cytometric technique to account for the different sub-populations of the post-treated spores thus enabling the visualisation of the physiological diversity of the samples.

Flow cytometry analysis provided better clarity into the heterogeneity of spore sub-populations at single-cell levels. Dual-parameter (FL3 versus FL1) dot plots was constructed by plotting the parameters from the red autofluorescence detector (FL3-H/PI) against the green fluorescence detector (FL1-H/SYTO 9). Five distinct sub-populations were observed in the spores post-UVC treatment based on their uptake of the stains (PI/SYTO 9) and staining characteristics. The different heterogeneous sub-populations are presumably inactivated spores, live spores, dormant, sub-lethally injured spores and an unknown group whose physiological state cannot be readily deciphered.

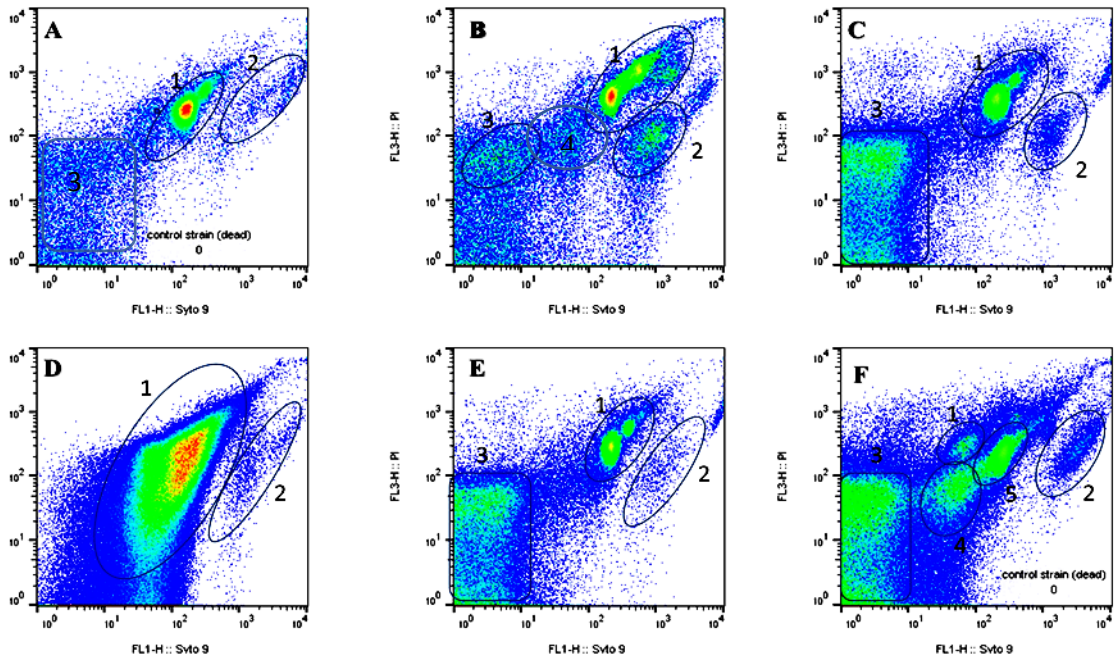

Figure 4-5: Flow cytometric analysis of UVC-treated *B. subtilis* and *B. velezensis* spores. All isolates are from raw milk except B50 and B44 which were isolated from packaged ESL milk stored at refrigeration temperature. Note the different clusters indicating the heterogeneity of the sub-populations after UVC treatments using density plots. (A) *B. velezensis* strain LPL-K103 (B43), (B) *B. velezensis* strain LPL-K103 (B44), (C) *B. subtilis* strain SRCM103689 (B47), (D) *B. subtilis* strain ATCC 11774 (B50), (E) *B. velezensis* strain LPL-K103 (B52), (F) *B. subtilis* strain CECT 4002 (B4002). The numbers represent different spore sub-populations 1. inactivated spores 2. live spores 3. dormant 4. unknown 5. sub-lethally injured spores. The live sub-population may be because of induced germination by UVC.

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Observable responses of the spore samples to the UVC dosage appeared to be strain-specific and varies from sample to sample as shown in Figure 4-4. The gating strategy was done using controls stained with either PI or SYTO 9 or the two at the same time on untreated and wet-heat inactivated spore samples as previously described.

The red fluorescent stain, PI, only enters the spore in the case of damage while SYTO 9 can permeate the inner membrane (IM) of the spore in the absence of any damage or rupture of the spore. The degree of the fluorescence intensity to either one or both stains was used to classify the spores into the sub-populations. The intensity of the cytometric analysis using the dual-staining method of the spores reflected the degree of damage inflicted by the UVC radiation on the spore cortex as well as the outer membrane. This made all observations or sub-populations to be between PI positive or SYTO 9 positive. All spore samples have sub-populations that are inactivated, live and dormant in various degrees except sample B50 which is dominated by the inactivated and live sub-populations. Meanwhile, B44 had an unknown sub-population whose state cannot be clearly defined besides the inactivated and live sub-populations. The live sub-population is possibly a group of spores with UVC-induced germination. There is a no evidence of UVC-induced germination of spores but there are evidence of high pressure-induced germination of *Bacillus* spores which can happen with or without the presence of nutrients (Reineke et al. 2012; Shigeta et al. 2007; Wuytack et al. 2002).

In all, the flow cytometry analysis accounted for sub-lethally injured, inactivated, live, dormant and an unknown sub-population. The dormant and sub-lethally injured spore sub-populations are the potential risks in food because they are likely to germinate and contaminate post-processed product under storage and favourable growth conditions. In addition to this, both sub-populations are mostly undetected by the traditional enumeration method hence

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complicating spore quantification during sterilization test (Zhang et al. 2019). Although, sample B50, which is the most resistant because it has the lowest lethality rate, may likely exert its potential spoilage faster than B47 with a large percentage of dormant spores.

The sub-lethally injured sub-population presumably has it cortex damaged by UVC but within its intact inner membrane is an undamaged DNA allowing for a partial displacement of the stain SYTO 9 by PI. A probable explanation for the unknown sub-population according to Mathys et al. (2007) is that there was indeed an uptake of both PI and SYTO 9 through the UVC-permeabilised membrane though the spores are generally non-viable unlike in the case of the injured spores. The simultaneous uptake of the two dyes (PI and SYTO) within a spore or cell is known as fluorescence energy transfer (FRET) (Manoil et al. 2014).

4.2.4.2 Analysis of Ca-DPA domain by Raman spectroscopy

Raman scattering plays a role in revealing compositional variability and relative concentrations of functional groups that promote resistance of the spores to UVC treatment. A spectral range of 0 to 2000 cm^{-1} was used as observed in figure 4-5. The background noise was modelled and subtracted during the spectral analysis. Cauchy-Lorentzian distribution of the data identified the following vibrational features dominating the analytes. Table 4-4 summarises the Raman shifts and band assignments from major studies on the endospores of some microorganisms to be 661, 824, 1017,1395, 1555 and 1582 cm^{-1}.

The observed spectra are 216, 284, 394,495, 599, 659, 817 and 1300 cm^{-1} with some differences in samples B43, B44 and B4002. B44 lacks the 817 cm^{-1} and 659 cm^{-1} spectra that are common in the other samples besides B47. A comparison of the peak heights of the

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vibrational spectra of each analyte reveals that B4002 has a unique spectral signature with the maximum intensity among the spore samples which is an indication of the abundance of functional groups, especially of the Ca-DPA bands. This means that the relative concentrations of the compounds in B4002 (positive control), well known for its resistance to UVC, is more than the rest of the samples. Calcium dipicolinic acid (Ca-DPA) is one of the notable compounds within a spore structure. It is a pyridine-2,6-dicarboxylic ring chelated to a divalent Calcium cation in 1:1 ratio (Magge et al. 2008). The characteristic Raman bands for the Ca-DPA domain are in the range of 824, 1017, 1395, 1446 and 1572 cm^{-1} in some *B. subtilis* and 662, 824, 1017, 1395, 1450, and 1572 cm^{-1} in some dormant spore of B. cereus as observed by Kong et al. (2017).

The intensities (a.u.) of the Ca-DPA bands of samples B50 and the control B4002 are higher than the average observed in other spores. Comparing the peak intensities of the Ca-DPA bands of samples B43 and B4002 reveals that there are relatively no significant differences in most of the bands except in the 1395 cm^{-1} spectrum with an intensity of 2984 a.u. and 5964 a.u. in B43 and B4002 respectively. However, there seem to be other contributors to the resistance of spores besides the Ca-DPA signature when a comparison is made between the samples B43 and B44 especially in the 200 to 700 cm^{-1} spectra. The other contributors adding to the peak intensities are likely compounds such as the amino acids e.g. tyrosine and phenylalanine. Juxtaposing the observations of samples B43 and B44 in figures 4-3 and 4-5 easily reveals this supposition. Despite B43 having a slight intensity in its Ca-DPA band especially in the 1395 cm^{-1} spectrum with an intensity of 2984 a.u., sample B44 has a lower intensity with 2074 a.u. but with significant higher intensities. At positions 218 cm^{-1}, 310 cm^{-1}, 400 cm^{-1} and 615 cm^{-1}, the intensities of B43 were 1732, 1882, 1712 and 1327 respectively whereas for at the same positions B44 has 2980, 3000, 2035 and 1617as the peak intensities.

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B47 has its highest intensity as 1666 a.u which appeared to be the lowest intensity of Ca-DPA among all the samples. The position of the Ca-DPA in B47 is also different from the rest of the samples. While the rest of the samples have the highest concentration at wave number 1395 cm^{-1} B47 has its highest concentration at 1582 cm^{-1}. The differences in the band positions of Ca-DPA observed in the strains is linked to the physicochemical forms the compound takes within the spore structure (Jamroskovic et al. 2016). Besides Ca-DPA concentration, another unique thing about B47 is the relatively abundance of phenylalanine at wave number 992 cm^{-1}. Other peaks found in the sample besides the ones mentioned are 65 cm^{-1}, 123 cm^{-1}, 512 cm^{-1} and 670 cm^{-1} which mostly compounds such as amino acids present in different forms within the spore.

From all indications, we can conclude that the relative concentration of Ca-DPA in the spore core is a possible indicator of the level of resistivity exhibited by a specific spore to the deleterious effects of processing conditions such as UVC, moist heat, high pressure. Apparently, besides the presence of pigmentation in the spore and presence of other compounds, the concentration of Ca-DPA plays the most important role in the lethality or susceptibility of a spore to UVC exposure (Khaneja et al. 2009). There is no evidence of the correlation between the physicochemical state of Ca-DPA and its resistance to UVC.

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Table 4-4: Putative band assignments of the Raman bands in the spectrum of *B. subtilis and B. velezensis* spores

Raman shift (cm^{-1})	Band assignments
527	S-S stretch (cysteine in spore coat)
622	Proteins (phenylalanine)
638	C-S stretch (cysteine in spore coat)
661	Ca-DPA
725	DNA
780	DNA
824	Ca-DPA
864	C–C stretch (Proteins)
1004	Phenylalanine
1017	Ca-DPA (pyridine ring vibrations)
1155	Proteins (C–N, C–C) Carotenoids
1200–1244	Protein amide III
1336	DNA
1395	Ca-DPA(Carboxyl group: O–C–O symmetrical stretching)
1448	Proteins, Lipids and Ca-DPA (pyridine ring vibrations)
1485	DNA
1555	Ca-DPA, Proteins (alanine, glycine), Lipids, Amide II
1582	Ca-DPA, Proteins (phenylalanine, tyrosine)
1602	Lipids
1616-1624	Proteins (phenylalanine, tyrosine)
1645–1674	Protein amide I, Lipids

*Band assignments are from previous studies (De Gelder et al., 2007; Nelson et al., 2004; Noothalapati et al., 2016; Romano et al., 2018)

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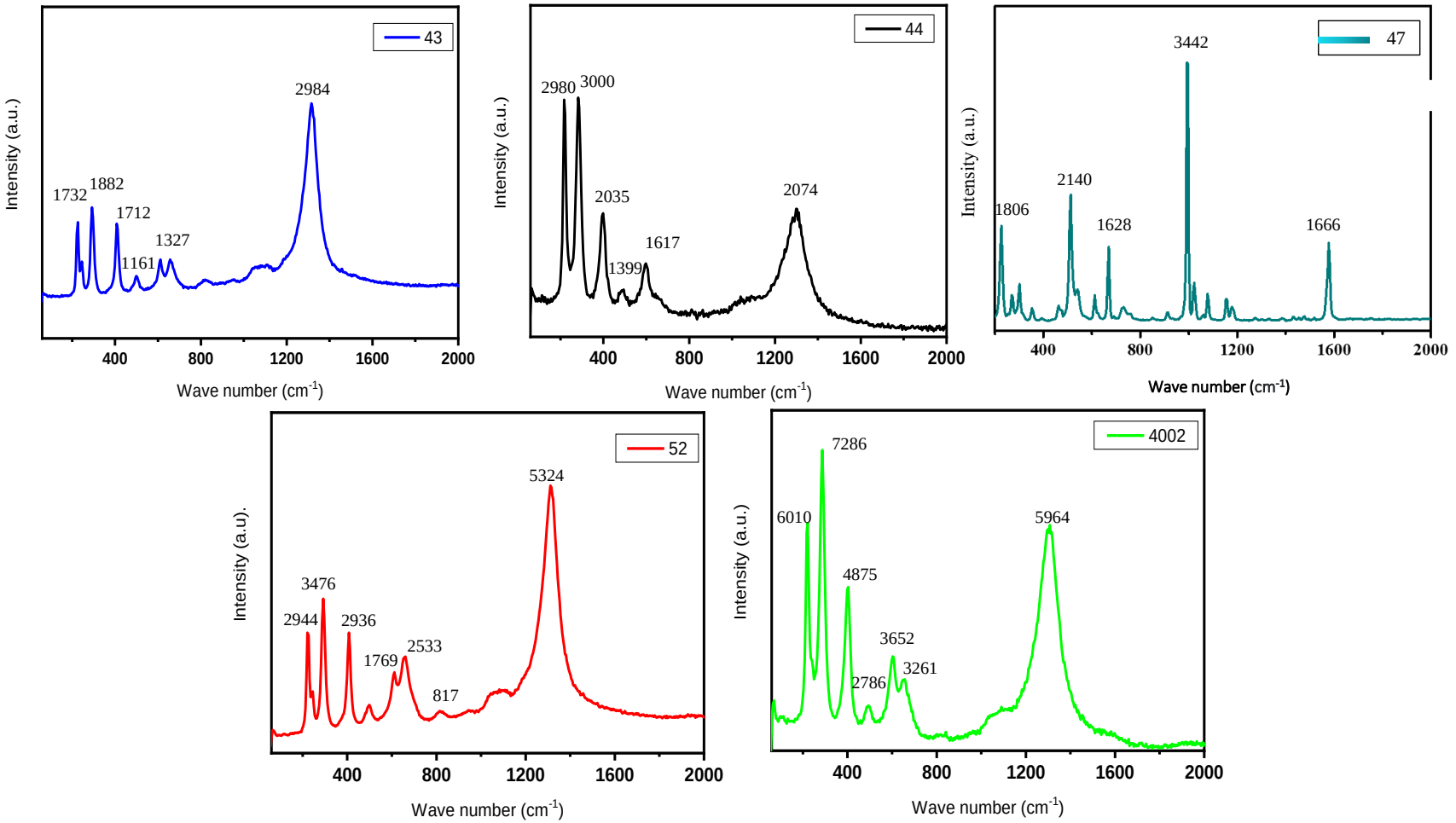

Figure 4-6: Raman spectroscopic analysis of vacuum-dried *B. subtilis* and *B. velezensis* spores with various peaks and intensities. The peak intensities indicate the relative abundance of some chemical compounds or signatures within the spore. (A) *B. velezensis* strain LPL-K103 (B43), (B) *B. velezensis* strain LPL-K103 (B44), (C) *B. subtilis* strain SRCM103689 (B47), (D) *B. subtilis* strain ATCC 11774 (B50), (E) *B. velezensis* strain LPL-K103 (B52), (F) *B. subtilis* strain CECT 4002 (B4002). Note the height of the peaks in samples B52 and B4002 which corresponds in a level to the result of their lethality rate post-UVC treatment.

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4.2.4.3 Adhesion potential of spore on a food contact surface

A one-way between-groups analysis of variance was conducted to determine the differences in the percentage (%) hydrophobicity of the spores by comparing the control strain with the test samples. There was a statistically significant difference at $P < 0.05$ in the % hydrophobicity of spores $F(5, 40) = 105$, $P = 0.0001$. Post-hoc analysis was done using the Dunnett multiple comparison hypothesis testing. The mean % relative hydrophobicity for the spore samples are given as follows: B43 (M = 15.43 %, SD = 2.72), B44 (M = 54.86 %, SD = 1.68), B47 (M = 47.26 %, SD = 2.20), B50 (M = 35.20 %, SD =2.16), B52 (M = 27.3 %, SD =0.93) and B4002 (M = 38.11 %, SD =9.53). All isolates significantly differ in their % hydrophobicity when compared with the control (B4002) except sample B50 (see Figure 4-6). B44 has the highest % hydrophobicity with B43 having the lowest. The result implies that B44 is capable of greater adhesiveness to food contact surfaces such as stainless steel. Though both B43 and B44 are typed and identified to be *B. velezensis* strain LPL-K103, we observed that surface hydrophobicity is type-specific just like in the case of UVC treatment. Microbial spores are generally more hydrophobic with a greater zeta potential than the vegetative counterpart and thus adheres better to food contact surfaces. The surface characteristic of spores are influences by the nature of the media and incubation environment during the sporulation process (Bressuire-Isoard et al. 2018). Since spores are generally more resistant to processing, such as thermal or high-pressure processing, their attachment on surfaces can readily initiate the development of biofilm consequently contaminating the downstream processing (Sadiq et al. 2018).

Apart from being a hydrophobicity assay, MATH also takes into consideration other interactions at play such as the van der Waals and electrostatic forces. These spore surface

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properties of adhesion and hydrophobicity are influenced by certain morphogenic protein components of the spore crust (Seale et al. 2008; Shuster et al. 2019). Processing conditions such as thermal processing is reported to increase surface hydrophobicity of surviving spores and hence promotes adhesion to food contact surface such as stainless-steel (Wiencek et al. 1990). It has also been reported that the variation in hydrophobicity of the stainless-steel substratum is also affected by prior chemical treatment (Boulange-Petermann et al. 1993). Though there are no reported cases of UVC increasing the hydrophobicity of surviving spores after processing, the rule of the thumb is to apply UVC in combination with other novel processing technologies such as high pressure or with inhibitory compounds such as nisin.

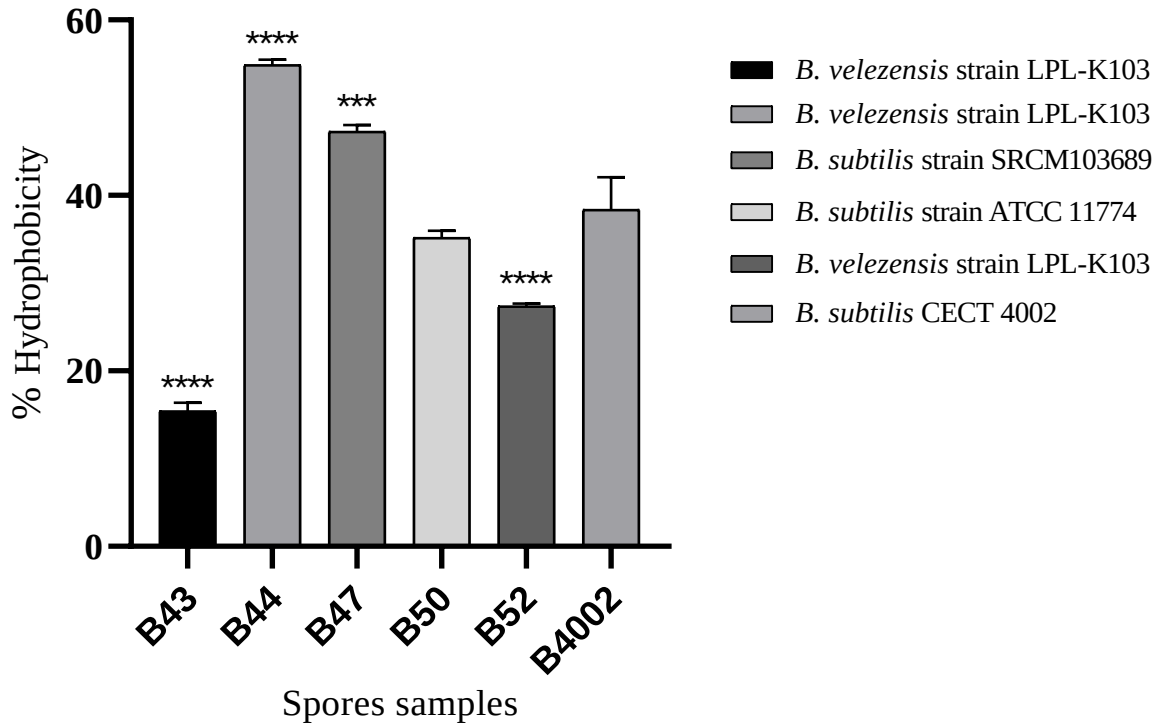

Figure 4-7: The adhesion of *B. subtilis* and *B. velezensis* spore spores samples to hexadecane (hydrocarbon) shown as % hydrophobicity. All isolates are from raw milk except B50 and B44 which were isolated from packaged ESL milk stored under refrigeration temperature. Statistical differences were done using Dunnett multiple comparisons for the post-hoc test ($P< 0.05$) by comparing all the spore samples with the positive control (*B. subtilis* CECT 4002). *B. velezensis* strain LPL-K103 (B44) has the highest % hydrophobicity with 54.9 % and *B. velezensis* strain LPL-K103 (B43) with 15.4 %. Samples with asterisks

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(*) indicate significant difference among means (*=$P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$). Error bars indicate standard error of means of the population (SEM).

Figure 4-8 reveals the morphological heterogeneity among the strains in reference to their sizes and shapes under an electron microscope at a magnification of 2 µm in length. Though the differences in size are not conclusive, all samples have approximately the same size and a common elliptical shape associated with most bacilli except B50 (*B. subtilis* strain ATCC 11774) which is spherical-shaped with a bunch of grapes in appearance. Apart from variation in shape, sample B50 also has the smallest size with an average size of 1 µm in length in comparison with other samples except for the reference control which is slightly larger than sample B50. The rest of the samples are larger than the average spore size which is typically 1.2 µm in length and 0.8 µm in width with none of the samples possessing an exosporium (Chada et al. 2003). The limitation of previous work is the failure carry out genetic typing of the sporulating organisms used in UVC study. The present work indicates that the response of bacterial spores to UVC and adhesion to food contact surface are likely strain-dependent. Nonetheless, the result of UVC-induced inactivation of *Bacillus* spores is consistent with other studies despite the application of different parameters.

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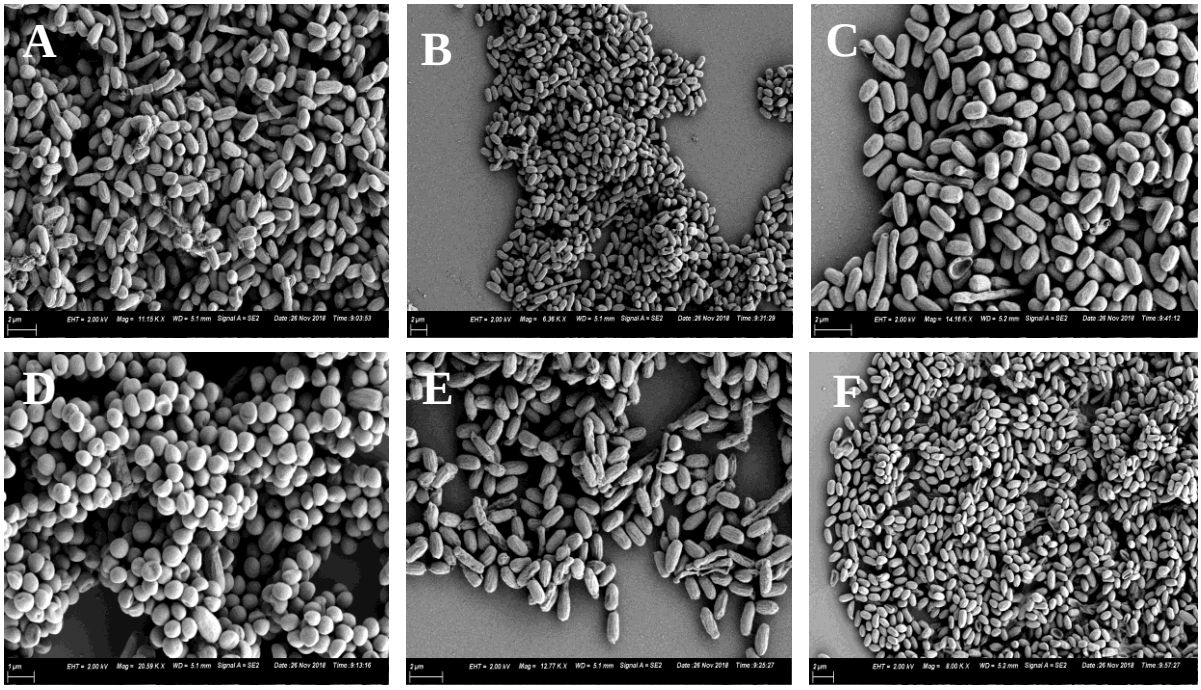

Figure 4-8: Scanning electron micrographs (SEM) images of strains of *Bacillus subtilis* and *Bacillus velezensis* on (glass) coverslips at a magnification of 2 µm. Note the heterogeneity in spores morphology A (*B. velezensis* strain LPL-K103 B43), B (*B. velezensis* strain LPL-K103 B44), C (*B. subtilis* strain SRCM103689 B47), D (*B. subtilis* strain ATCC 11774 B50), E (*B. velezensis* strain LPL-K103 B52) and (f) *B. subtilis* strain CECT 4002**.** Note the spherical shape of sample D and its bunch of grape appearance which is different compared to other samples including the control. All samples (A, B, C, E and F) have the characteristic shape of a rod except sample D which has the shape of a bunch of grapes. All isolates are from raw milk except B50 and B44 which were isolated from packaged ESL milk stored at 5 ^{o}C.

The spores can be seen under an electron microscope to be firmly attached to stainless-steel surface pre-treated by increasing the surface roughness as shown in figure 4-8. The attached spores can be differentiated from the vegetative cells and maturing biofilms as in figures 4-8A and 6C. The adhesion was initiated by dispensing aqueous suspension of the spores into centrifuge containing 4.5 mL PBS and stainless-steel coupons (316L-0.90, 2B PVC; dimension: 50 × 13 mm) semi-submerged and incubated vertically at 30 °C for 24 h. Germination of the attached spores was induced by some residual milk on the surface of the coupons though in a limiting way to allows for differentiation of vegetative cells and their biofilms from the attached spores.

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Adhesion of spores or their vegetative cells to an abiotic surface such as stainless steel or glass is a complex process with diverse interwoven environmental factors playing pivotal roles in the process such as surface roughness, surface charge, strain-type and other environmental factors (Bohinc et al. 2016). Although the type of organism has been linked to the attachment strength of bacterial spores, there is no evidence of the shape and size of a bacterial spore as a contributor to its attachment on substratum especially food contact surface (Faille et al. 2002).

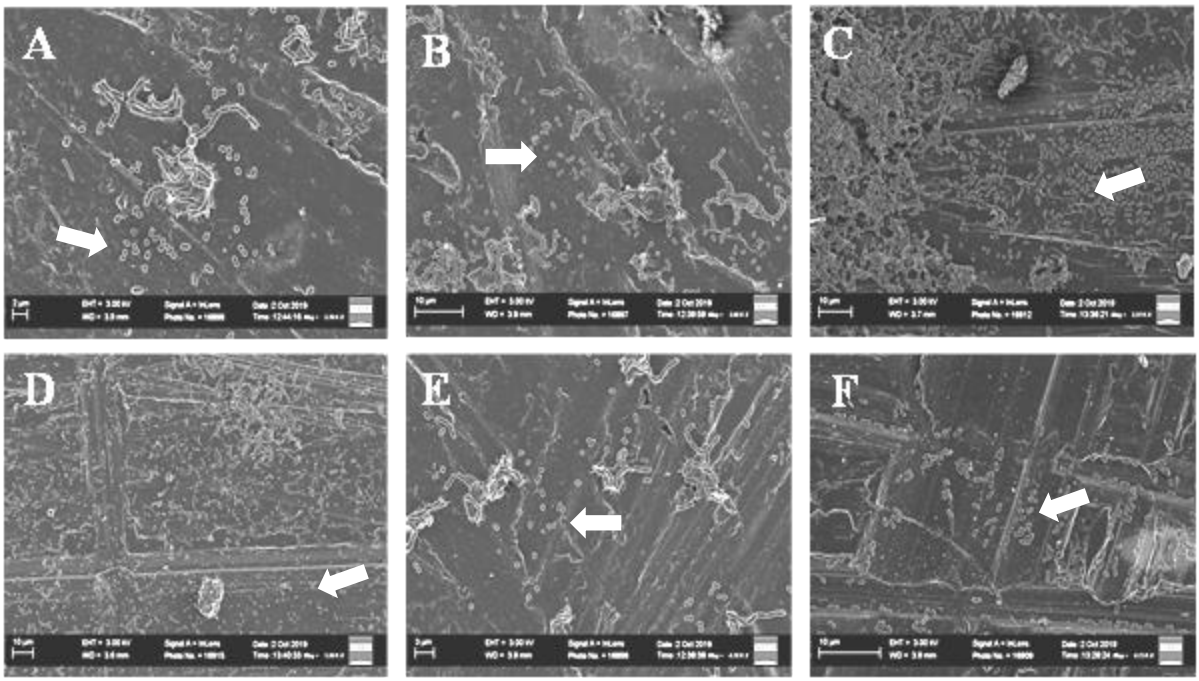

Figure 4-9: Scanning electron micrographs (SEM) of *B. subtilis* and *B. velezensis* spores attached to stainless-steel (grade 316L, 2B finish). Attachment of spores to the food contact surface is mainly influenced by factors such as hydrophobicity of the spore and nanotopography of the food contact surface. A (*B. velezensis* strain LPL-K103 B43), B (*B. velezensis* strain LPL-K103 B44), C (*B. subtilis* strain SRCM103689 B47), D (*B. subtilis* strain ATCC 11774 B50), E (*B. velezensis* strain LPL-K103 B52) and (f) *B. subtilis* strain CECT 4002**.** Arrows point to the attached spores as differentiated from vegetative cells whose germination was induced by residual nutrient and conditioning film.

The result of this study seems to suggest a strong correlation between the lethality rate and the concentration of Ca-DPA, especially around the wavenumber 1395 cm^{-1} for most of the

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samples. Though the resistance of spores to UVC is presumably multifactorial, there is a trend that can be observed between the lethality rate and the result of the Raman spectral analysis. Lethality rate seems to increase with increasing intensity or concentration of Ca-DPA. For the lethality rate, the samples followed this order from the most resistance to the least B50, B4002, B44, B43, B47 (excluding B52) and for Ca-DPA concentrations the order is given from the most concentrated to the least as B50, B4002, B44, B43, B47. This observation is supported by a similar study in which *B. subtilis* spores were exposed to UV from solar radiation (Slieman and Nicholson, 2001). The heterogeneity in the content may be attributed to the size of the spore than the shape. It is safe to assume that the bigger the spore the more the concentration of Ca-DPA and hence the more the resistance to UVC (Huang et al. 2007).

Overall, B50 and B44 hypothetically have the greatest threat during processing. Specifically, B50 is the most resistant with a high concentration of Ca-DPA and a relatively good % hydrophobicity among all samples. This means more of the spores of B50 can potentially survive UVC exposure under the same conditions used in this study with good adhesion to the food contact surface. The implication of this is that the spores may germinate in the processed food thereby causing its spoilage during storage. The adhered spores may also form biofilms on food contact surface which may contaminate the processed food if their development is not properly handled using effective cleaning-in-place (CIP) regime. Sample B44 also has the potential of causing the same damage though with less severity compared to B50.

Despite the observation in the resistance of the spore samples, their presence can be mitigated and further inactivated by either increasing the retention time in the UVC reactor or having another run of the suspending medium. These are highly recommended especially in the case the food to be processed is milk due to the high turbidity and low penetration of the UV

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radiation in such medium. Besides the increased retention time and a second run of the processed food in the reactor, a mild agitation may also be incorporated in the design of the reactor to facilitate effective mixing and exposure of contaminating microflora to the lethal power of UVC. There is a need to further investigate the recovery ability of the dormant and injured sub-populations after UVC exposure, the process parameters that can potentially inactivate all vegetative cells and spores without any alteration to the nutritional and sensory properties of different liquid foods with which UVC processing may find application.

4.2.5 Conclusions

Although UVC may have a promising application in the inactivation of spores in the processing of liquid foods, there is a need to understand the effects and responses at the molecular and individual level. The outcome of this study suggests that the adhesion of spore to hydrocarbon, UVC treatment and Ca-DPA spectral from the Raman analysis are mostly heterogeneous with some strain-to-strain variations as observed from the spectral characteristics. This factor must be taken into consideration when designing a UVC regime. UVC regime must be designed in such a way that almost all the spores and vegetative cells are inactivated without undesirable impact or alteration to the food. Apart from the interference of pigments to UVC penetration of a spore, the concentration of Ca-DPA in the spore possibly plays an important role in the lethality rate or resistance of spores to UVC. This assumption, though supported by the high concentration or intensity of Ca-DPA band in the positive control, is not conclusive. There is a necessity to further study the roles of Ca-DPA in the resistance of a bacterial spore to UVC. We proposed the utilisation of other methods (e.g. flow cytometry) besides plate counting on agar plates to analyse the response of spores to UVC. The information from the study will guide

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in deciding on the right parameters that can be applied during the processing of liquid foods. The study justifies the critical role of Ca-DPA in spore resistance and the possible sub-populations after UVC treatment that may compromise product shelf-life and safety.

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4.3 Spoilage potential of biofilms and planktonic cells of *Bacillus subtilis* and *Bacillus velezensis* in extended shelf-life milk

4.3.1 Abstract

Thermophilic bacilli pose a threat in the dairy industry because of their inherent ability to survive pasteurisation either as planktonic cells or process biofilms. This contamination results in the spoilage of extended shelf-life (ESL) milk due to the production of proteolytic and lipolytic enzymes by the bacteria. In this study, we aimed to quantify and compare biofilm-induced proteolysis and lipolysis of *B. subtilis* and *B. velezensis* with that of the planktonic cells. To grow the cells, 0.5 mL and 0.1 mL of pre-incubated bacterial inocula were added to a centrifuge tube containing 4.5 mL of UHT for the biofilms and planktonic cells, respectively. Stainless-steel coupon was placed in each of the centrifuge tube containing the inoculated UHT milk as a substratum for biofilm formation except for planktonic cells. All the tubes were incubated for 24 h at 30 °C. Both planktonic and biofilm cells were stained and enumerated in a flow cytometer after incubation. The concentrations of proteolytic and lipolytic enzymes produced by the submerged biofilm and planktonic cells of the isolates were quantified using azocasein and p-nitrophenol palmitate (p-NPP) assays, respectively. In the planktonic cells, sample B48 has the highest proteolysis with 1033.6 ρL/CFU while B50 has the highest lipolysis of 34.5 ρL/CFU. For the biofilms, B168 has the highest proteolysis and lipolysis per cell with a mean 3706 ρL and 179.9 ρL. The result of this study indicated that the spoilage potential (proteolysis and lipolysis) both of biofilms and planktonic culture are strain-dependent and that there seems to be a relationship between the strength or complexity of the biofilms and spoilage potential of the isolates. The implication to the industry is that weak biofilm formers have better spoilage potential than the strong biofilm formers in spore forming bacilli.

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4.3.2 Introduction

According to the 2014 report by USDA, about one-third of processed fluid milk in the United States is lost during the processing with an estimated value of $6.4 billion (Buzby et al. 2014; Ishangulyyev et al. 2019). Milk is a nutrient-rich diet widely consumed by humans of all age categories. These nutrient compounds also make it a potential medium for microbial contaminants to thrive causing some alterations in the sensorial property of the milk that is usually detrimental. The bacterial contaminants are usually from animal hides, feeds, air, soil, milking equipment (Quigley et al. 2013). Spoilage of milk can also occur as a result of post-pasteurisation contaminated (PPC) due to bad hygiene practice or by process biofilms especially around the filler nozzle and other parts of the processing equipment (Alles et al. 2018). The consequence of these contaminants is a reduction in the shelf-life of the product because of microbial spoilage which can be humongous on an economic and industrial-scale (González-Rivas et al. 2018; Zhao et al. 2018).

We have demonstrated from previous study the potential of *B. subtilis* and *B. velezensis* to contaminate processing line and form moderately to strongly adherent biofilms on stainless-steel surface (see section 4.1). Bacterial quorum sensing and its sub-system called self-sensing contribute significantly to the formation of biofilms and the expression of genes of the microbial cells within the biofilms through the production of chemical signals called autoinducers (Bareia et al. 2018). Signal molecules, such as N-butanoyl-L-homoserine lactone, have been implicated in modulating the production of proteases within biofilms of *Aeromonas hydrophila* (Khajanchi et al. 2009; Yi et al. 2018). Biofilms have been implicated as a reservoir for enzymes produced by actively metabolising cells populating the community thus providing a microenvironment for such enzymatic activity (Khajanchi et al. 2009; Rosche et al. 2009). These enzymes are associated with the periplasm of the microbial cells or secreted into the

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microenvironment either as inducible enzymes or through autolysis of cells populating the biofilms (Wang and Chen, 2009).

Apart from its structural role, the biofilm matrix entraps enzymes as well as other metabolites facilitating the hydrolysis of complex molecules such as lipids and milk protein causing the spoilage and reduction of shelf-life of processed milk (Fysun et al. 2019). It is generally believed that such production of enzymes (per cell) is commonly higher within biofilms than in the planktonic cells of the same isogenic strain. This assumption has been confirmed by a study of a mono-species biofilm of *B. licheniformis* R4 and *Pseudomonas fragi* BC5 under a submerged condition in milk. The result revealed that proteolysis was higher in biofilms than in the planktonic cells (Teh et al. 2012). In another study, a similar pattern was observed for lipolysis in the mono-species biofilm *Staphylococcus aureus* SF01 (Teh et al. 2013).

Therefore, this study hypothesised that the biofilms of *B. subtilis* and *B. velezensis* will produce proteolysis and lipolysis within their biofilms exceeding that of their planktonic cells. The objective is to quantify the number of cells as well as the concentrations of the spoilage enzymes in the intact biofilms and planktonic cells of the isolates and to determine the proteolysis and lipolysis per cell. This is very significant to the dairy industry because of the propensity of *Bacillus* spp. to contaminate raw milk and perpetuate in the processing line either as resistant biofilms or endospores thereby compromising the quality and shelf-life of ESL milk.

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4.3.3 Materials and methods

4.3.3.1 Selection of *Bacillus subtilis* and *Bacillus velezensis* isolates

Sample collection was done in the ESL milk processing line as previously described in Chapter 4.1. The reference strains used were obtained from the *Bacillus* Stock Centre of Ohio State University. These reference strains are *B. subtilis* 168 (poor biofilm-former) a wild-type *B. subtilis* 3610 (moderate biofilm-former).

4.3.3.2 Enzymatic screening of *Bacillus subtilis* and *Bacillus velezensis* on skim milk agar

B. subtilis and *B. velezensis* isolates were screened using skim milk agar (2 % agar, 10 % skim milk, 0.25 g yeast extract, 0.1 % glucose in water). The media was autoclaved for 5 min at 121 ^{o}C after which 0.028 % of bromocresol green was added. 100 µL suspension of an overnight bacterial culture was dispensed on plates containing the skim milk agar to form concentric circles of approximately 1 cm in diameter. The plates were incubated for 24 h and the diameter of the zone of hydrolysis for each bacterial isolate was measured in cm.

4.3.3.3 Growth of *Bacillus subtilis* and *Bacillus velezensis* planktonic cells

The planktonic cells were prepared using 18 h bacterial culture grown in TSB (Oxoid). The bacterial culture for each isolate was standardised to 1.0 MacFarland. 0.5 mL of standardised inoculum was pre-incubated in 4.5 mL of UHT milk for 1h for each of the twelve test organisms and the two reference strains. 0.1 mL of the pre-incubated culture was dispensed in another

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centrifuge tube containing UHT milk and incubated for 24 h at 30 °C after which the total number of cells in the culture was enumerated. The same process was repeated for the proteolytic and lipolytic assays.

4.3.3.4 Growth of *Bacillus subtilis* and *Bacillus velezensis* biofilms

To grow the submerged biofilms, 0.5 mL of the pre-incubated culture for each isolate was dispensed in a centrifuge tube containing 4.5 mL of the UHT milk. A modified stainless-steel coupon (316L-0.90, 2B PVC; dimension: 50 × 13mm) was semi-submerged in the centrifuge tube containing UHT milk as a substratum for the biofilm. The length of the semi-submerged coupon was 25 mm within the centrifuge tube containing the milk. The tubes were incubated for 24 h at 30 °C after which the cells within the biofilms quantitatively determined using a flow cytometer. An un-inoculated coupon in a centrifuge tube was used as a control.

4.3.3.5 Enumeration of cells within biofilms and planktonic culture using flow cytometer

For the enumeration, the stainless-steel coupon containing adhered biofilm was removed from the centrifuge tube containing UHT milk. The coupon was dipped three times in sterile distilled water to wash off unattached cells. The cells within the biofilms were detached according to the method of Wahlen et al. (2018). Each coupon was transferred into a centrifuge tube containing 9 mL phosphate buffer, some pieces of 6 mm glass beads. This was followed by a series of 30 s five steps of vortexing and sonicating in that order. The sonication was done at

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40 kHz (Grant Instruments, UK). 2 mL of the samples were stained with SYTO 9 to a concentration of 500 nM and allowed to stand for 15 min and the cells were later enumerated using flow cytometer (Accuri, BD Biosciences, NJ, USA). This was done for the biofilm and planktonic samples. The enumerated cells were expressed as $\log_{10}$ CFU/cm^2 and $\log_{10}$ CFU/mL for biofilms and planktonic cells respectively (Winkelströter et al., 2014).

4.3.3.6 Spoilage potential of biofilms and planktonic cells of *Bacillus subtilis* and *Bacillus velezensis*

4.3.3.6.1 Proteolysis

The proteolysis assay was performed using the azocasein method of Bussamara et al. (2009). A 3 % azocasein solution (Sigma-Aldrich, St. Louis, USA) was prepared in 5 mM phosphate buffer solution, pH 7.5 with 0.1 % sodium azide (Sigma-Aldrich, St. Louis, USA) and 0.1 mg chloramphenicol/mL (Sigma-Aldrich,). After the incubation of the biofilm and planktonic cells, the stainless-steel coupons were transferred from the centrifuge tube containing UHT milk into 9 mL of the 3 % azocasein solution while the planktonic cultures were centrifuged at 10,000 g for 5 min. 0.1 mL of the supernatant was transferred into 0.9 mL of the 3% azocasein solution in another centrifuge tube which was then incubated at 40 °C for a period of 24 h. After the incubation, 0.4 mL of the azocasein solution was mixed with 0.8 mL of 20 % trichloroacetic acid (Merck, Darmstadt, Germany) to stop the reaction process. The mixture was centrifuged at 10,000 g for 5 min. A volume of 0.15 mL of the supernatant was transferred into microtitre plate wells (Thermo Scientific, Massachusetts, US) in six replicates and the absorbance was read at 405 nm (Thermo Scientific,). The same thing was done with the control which is the uninoculated centrifuge tube containing UHT milk.

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The amount of proteolysis was determined by comparing the absorbance value of the samples with the absorbance value of the standard curve of the proteolysis by *Streptomyces griseus* (3.5 units/mg; Sigma-Aldrich, Auckland, New Zealand). The estimated concentration of the proteolysis produced was divided by the number of bacterial cells colonising the stainless-steel surfaces or in the planktonic cultures. The data were expressed as picolitre of proteolysis per CFU (ρL/CFU) for proteolysis and lipolysis assays.

4.3.3.6.2 Lipolysis

The amount of lipolytic enzyme produced by the isolates was determined by using p-nitrophenol palmitate (p-NPP) assay. This involves the utilization of 0.05 % solution of nitrophenol palmitate as described by Teh et al. (2013). This solution was prepared by adding 5 mg of p-NPP in 1 mL of isopropanol and 9 mL of 50 mM Tris–HCl solution, pH 8 containing 40 mg of Triton X-100 and 0.2 mg of arabic gum with 0.1 % sodium azide and 0.1 mg chloramphenicol/mL (Sigma-Aldrich, St. Louis, USA). The stainless-steel coupons containing biofilms were dipped three times in sterile distilled water to wash off unattached cells after incubation. The coupons were then transferred into another set centrifuge tubes containing 9 mL each of the 0.05 % p-NPP solution while 0.1 mL of the planktonic cell suspension was added to 0.9 mL of the same solution in an Eppendorf tubes. Both the centrifuge tubes containing the coupons and Eppendorf tubes with the cell suspension were further incubated for 8 h for the enzyme-subtrate reaction to occur. After the incubation, 0.6 mL of 96 % ethanol was added to 0.6 mL of the 0.05 % p-NPP solution to halt the reaction. The resulting solution was centrifuged at 10 000 g for 5 min and the absorbance read at 405 nm in a microtitre plate

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reader (Thermo Scientific). The amount of lipolysis per cell was estimated as described for the proteolysis assay.

4.3.3.7 Statistical analysis

All experiments were carried out independently and repeated at least three times. Analysis of variance (ANOVA) was done using GraphPad Prism (version 8.0.2) and the Dunnett multiple comparison hypothesis testing was used to determine significant differences between the isolates and concentration of enzymes produced per cell for the biofilm and planktonic cells respectively with a critical probability of $P \leq 0.05$. FlowJo (version 10 CL) software was used in analysing the flow cytometry data. The result of the enzyme assay was expressed as picolitre of proteolysis/lipolysis per CFU (ρL/CFU) for both biofilm and planktonic cells.

4.3.4 Results

4.3.4.1 Extracellular protease production on skim milk agar

All bacterial isolates produced zone of proteolysis on skim milk agar in varying degrees. This was observed as a transparent clearing in the middle of the bluish cloud of the skim milk agar. The 0.028 % of bromocresol green dye made the media blue which turned greenish with increasing incubation time. The dye allowed for a marked distinction of the clearing of the milk proteins from the surrounding medium which made the measurement easier. In all, there were significant differences in the extracellular production of protease among the isolates ($P < 0.05$) with F (13, 112) = 48.82, $P > 0.0001$ after the incubation time of 24 h on skim milk agar at 30

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˚C. The characteristic cloudiness of milk within the media disappeared to give rise to a dark blue clearing. From the result, B50 has the highest mean zone of hydrolysis of 2.10 mm ± 0.11 while the negative control, B168, has the lowest with 1.27 mm ± 0.06 (see figure 4-9). The mean zones of hydrolysis for the other isolates are given as follows: B43 ($\bar{x}$ = 1.81 mm, σ = ± 0.05), B44 ($\bar{x}$ = 2.00 mm, σ = ± 0.20), B46 ($\bar{x}$ = 1.32 mm, σ = ± 0.11), B47 ($\bar{x}$ = 1.90 mm, σ = ± 0.04), B48 ($\bar{x}$ = 1.87 mm, σ = ± 0.09), B49 ($\bar{x}$ = 1.50 mm, σ = ± 0.14), B52 ($\bar{x}$ = 1.80 mm, σ = ± 0.17), B54 ($\bar{x}$ = 1.81 mm, σ = ± 0.05), B55 ($\bar{x}$ = 1.56 mm, σ = ± 0.05), B56 ($\bar{x}$ = 1.56 mm, σ = ± 0.05), B57 ($\bar{x}$ = 1.52 mm, σ = ± 0.06), B3610 ($\bar{x}$ = 1.66 mm, σ = ± 0.08).

4.3.4.2 Enumeration of biofilms and planktonic cells

Enumeration of all the cells, regardless of their physiological and morphological states within the biofilms and planktonic cells, is given as density plots (figure 4-18). All *Bacillus* isolates used in this study produced biofilms *in vitro* with stainless-steel coupons as the substratum. All isolates exhibited different biofilm-forming potential at 30 ºC after the incubation time of 24 h with UHT milk as the growth medium. There was an overall significant difference at the $P < 0.05$ level in the log counts of cells within the biofilms on the stainless-steel surface $F_{(13,14)} = 23.4$, $P < 0.0001$. From figure 4-10, the total number of colonising bacterial cells retrieved from the coupon surface from the stainless-steel surfaces enumerated using a flow cytometer ranged from 5.6 $\log_{10}$ CFU/cm^2 (σ = ± 0.08) for B168 to 7.4 $\log_{10}$ CFU/cm^2 (σ = ± 0.08) for B48 respectively.

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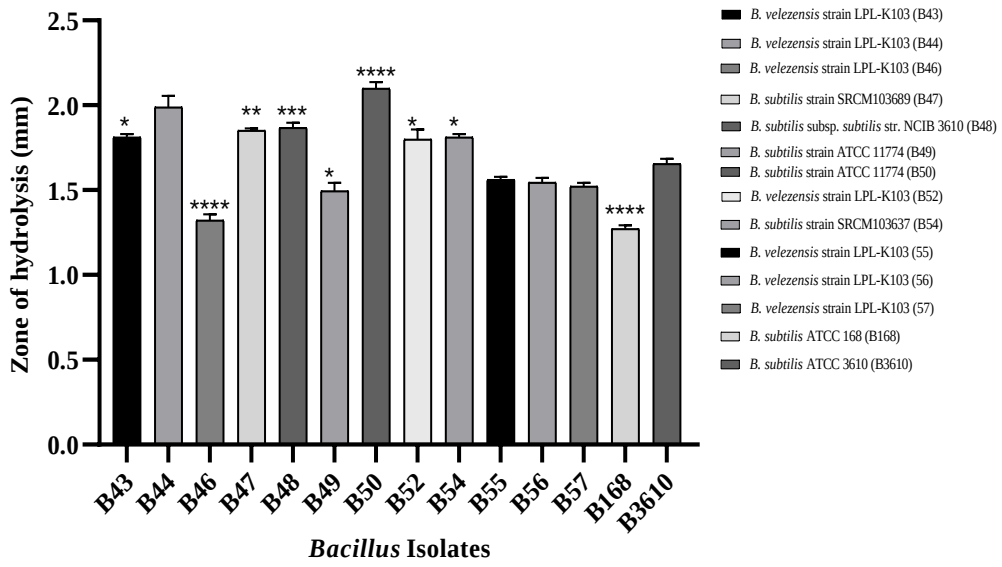

Figure 4-10: The zone of hydrolysis in millimetres originating from 100 µL overnight bacterial culture standardised to 0.5 MacFarland (1 cm in diameter) on skim milk agar. Samples with asterisks (*) indicate significant differences among means, $P \leq 0.05$ (Dunnett hypothesis testing). The asterisk (*) means that *=$P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$ and ****= $P \leq 0.0001$. All experiments were done in duplicates with at least three independent experiments. Error bars show standard error of means (SEM) of the bacterial population.

The mean log counts for the other isolates are given as follows: B43 ($\bar{x}$ = 7.1 $\log_{10}$ CFU/cm^2, σ = 0.15), B44 ($\bar{x}$ = 6.2 $\log_{10}$ CFU/cm^2, σ = ± 0.44), B46 ($\bar{x}$ = 6.6 $\log_{10}$ CFU/cm^2, σ = ± 0.08), B47 ($\bar{x}$ = 7.2 $\log_{10}$ CFU/cm^2, σ = ± 0.06), B49 ($\bar{x}$ = 7.1 $\log_{10}$ CFU/cm^2, σ = ± 0.04), B50 ($\bar{x}$ = 6.8 $\log_{10}$ CFU/cm^2, σ = ± 0.02), B52 ($\bar{x}$ = 6.7 $\log_{10}$ CFU/cm^2, σ = ± 0.2), B54 ($\bar{x}$ = 6.6 $\log_{10}$ CFU/cm^2, σ = ± 0.2), B55 ($\bar{x}$ = 6.8 $\log_{10}$ CFU/cm^2, σ = ± 0.05), B56 ($\bar{x}$ = 7.0 $\log_{10}$ CFU/cm^2, σ = ± 0.04), B57 ($\bar{x}$ = 6.9 $\log_{10}$ CFU/cm^2, σ = ± 0.06), B3610 ($\bar{x}$ = 5.9 $\log_{10}$ CFU/cm^2, σ = ± 0.03).

For the planktonic samples, the enumeration of the cells was expressed as $\log_{10}$ CFU/mL. There was no significant difference in the total number of cells at $P < 0.05$, $F_{(13,14)} = 44$, $P = 0.45$. The mean log counts and standard deviations for the isolates are given as follows: B43 ($\bar{x}$ = 7.6 $\log_{10}$ CFU/mL, σ = 0.15), B44 ($\bar{x}$ = 7.6 $\log_{10}$ CFU/mL, σ = ± 0.15), B46 ($\bar{x}$ = 7.6 $\log_{10}$

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CFU/mL, $\sigma = \pm 0.15$), B47 ($\bar{x}$ = 7.3 $\log_{10}$ CFU/mL, $\sigma = \pm 0.31$), B48 ($\bar{x}$ = 7.2 $\log_{10}$ CFU/mL, $\sigma = \pm 0.33$), B49 ($\bar{x}$ = 7.6 $\log_{10}$ CFU/mL, $\sigma = \pm 0.15$), B50 ($\bar{x}$ = 7.3 $\log_{10}$ CFU/mL, $\sigma = \pm 0.32$), B52 ($\bar{x}$ = 7.6 $\log_{10}$ CFU/ mL, $\sigma = \pm 0.13$), B54 ($\bar{x}$ = 7.3 $\log_{10}$ CFU/mL, $\sigma = \pm 0.33$), B55 ($\bar{x}$ = 7.6 $\log_{10}$ CFU/mL, $\sigma = \pm 0.13$), B56 ($\bar{x}$ = 7.3 $\log_{10}$ CFU/mL, $\sigma = \pm 0.32$), B57 ($\bar{x}$ = 7.3 $\log_{10}$ CFU/mL, $\sigma = \pm 0.33$), B168 ($\bar{x}$ = 7.6 $\log_{10}$ CFU/mL, $\sigma = \pm 0.15$), B3610 ($\bar{x}$ = 7.3 $\log_{10}$ CFU/mL, $\sigma = \pm 0.30$). The highest population mean for the planktonic culture was $\log_{10}$ 7.6 CFU/mL (B52) and the lowest was $\log_{10}$ 7.2 CFU/mL (B48) (see figure 4-11).

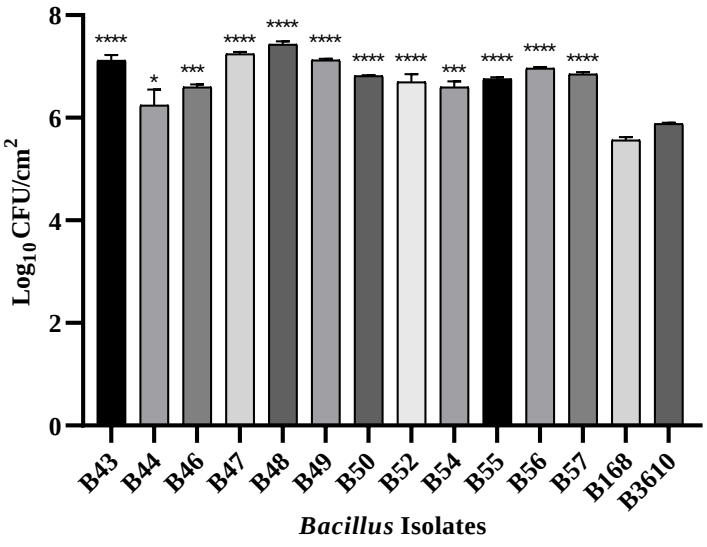

Figure 4-11: The number of cells within *in vitro* biofilms formed on stainless steels semi-submerged in UHT milk grown at 30 ˚C. The highest population means for the biofilm model 7.4 $\log_{10}$ CFU/cm^2 for B48, and the minimum was 5.6 $\log_{10}$ CFU/cm^2 for B168. Post-hoc analysis was done using Fisher's LSD test with a critical probability of P≤0.05. The asterisk. Samples with asterisks (*) indicate significant differences among means, $P \leq 0.05$ (*) means that *=$P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$ and ***= $P \leq 0.0001$. All experiments were done in duplicates with at least three independent experiments. Error bars show standard error of means (SEM) of the bacterial population within the biofilm.

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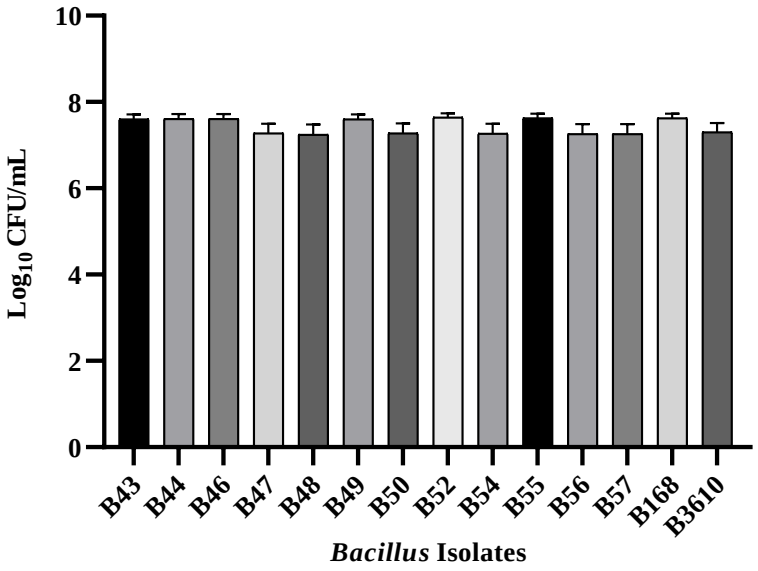

Figure 4-12: The number of cells within planktonic cultures grown in UHT milk grown at 30 ˚C. The highest population mean for the planktonic culture was 7.6 $\log_{10}$ CFU/mL (B52), and the lowest was 7.2 $\log_{10}$ CFU/mL (B48). Post-hoc analysis was done using Fisher's LSD test with a critical probability of $P \leq 0.05$. All experiments were done in duplicates with at least three independent experiments. Error bars indicate standard error of the means (SEM) of the planktonic culture.

4.3.4.3 Proteolysis in biofilm and planktonic cells

All the *Bacillus* isolates produced some degrees of proteolysis in the biofilms and planktonic cells with both results expressed as picolitre of proteolysis per CFU (ρL/CFU). For the planktonic cells, the result of the proteolytic assay showed that there was an overall significant difference at $P < 0.05$ level in the proteolysis per planktonic cell $F_{(13,14)} = 4001$, $P < 0.0001$. From figure 4-12, the observed highest and lowest means of proteolysis among isolates range from 1034 ρL/CFU± 11.37 in B48 and 178 ρL/CFU± 0.45 in B52, respectively. The mean proteolysis for the other isolates are given as follows: B43 ($\bar{x}$ = 190.2 ρL/CFU, σ = 0.52), B44 ($\bar{x}$ = 195.2 ρL/CFU, σ = 0.52), B46 ($\bar{x}$ = 194.3 ρL/CFU, σ = 0.52), B47 ($\bar{x}$ = 908.9 ρL/CFU, σ = 11.4), B49 ($\bar{x}$ = 227.8 ρL/CFU, σ = 0.62), B50 ($\bar{x}$ = 892.0 ρL/CFU, σ = 11.28), B54 ($\bar{x}$ = 928.0 ρL/CFU, σ = 12.0), B55 ($\bar{x}$ = 196.3 ρL/CFU, σ = 0.52), B56 ($\bar{x}$ = 953.5 ρL/CFU, σ =

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12.5), B57 ($\bar{x}$ = 960.2 ρL/CFU, σ = 12.8), B168 ($\bar{x}$ = 200.7 ρL/CFU, σ = 0.53) and B3610 ($\bar{x}$ = 860.3 ρL/CFU, σ = 10.1).

Unlike in the planktonic culture, there is an observable little amount of proteolysis produced within the biofilms of the isolates . There was an overall significant difference at $P < 0.05$ level in the proteolysis per cell within the biofilms $F_{(13,14)} = 306.1$, $P < 0.0001$. From figure 4-13, the biofilm of the B168 produced the highest amount of proteolytic enzyme per cell which is conspicously higher than in its planktonic form. The mean proteolysis (within biofilms) per cell for the other isolates are given as follows: B43 ($\bar{x}$ = 148.2 ρL/CFU, σ =22.8), B44 ($\bar{x}$ = 402.9 ρL/CFU, σ =2.8), B46 ($\bar{x}$ = 341.3 ρL/CFU, σ = 28.3), B47 ($\bar{x}$ = 81.8 ρL/CFU, σ = 2.1), B48 ($\bar{x}$ = 26.2 ρL/CFU, σ = 1.4), B49 ($\bar{x}$ = 162.4 ρL/CFU, σ = 28.3), B50 ($\bar{x}$ = 169.8 ρL/CFU, σ = 7.1), B52 ($\bar{x}$ = 202.2 ρL/CFU, σ = 2.1), B54 ($\bar{x}$ = 262.4 ρL/CFU, σ = 14.1), B55 ($\bar{x}$ = 274.2 ρL/CFU, σ = 14.1), B56 ($\bar{x}$ = 231.1 ρL/CFU, σ = 1.4), B57 ($\bar{x}$ = 145.7 ρL/CFU, σ = 13.5), B168 ($\bar{x}$ = 3706 ρL/CFU, σ = 284.5) and B3610 ($\bar{x}$ = 1873 ρL/CFU, σ = 99.3).

Overall, the planktonic cell produced significantly more proteolysis per cell than cells within the biofilms under a condition of nutrient abundance for most of the isolates except for B168 and B3610 which are the negative and positive control strains.

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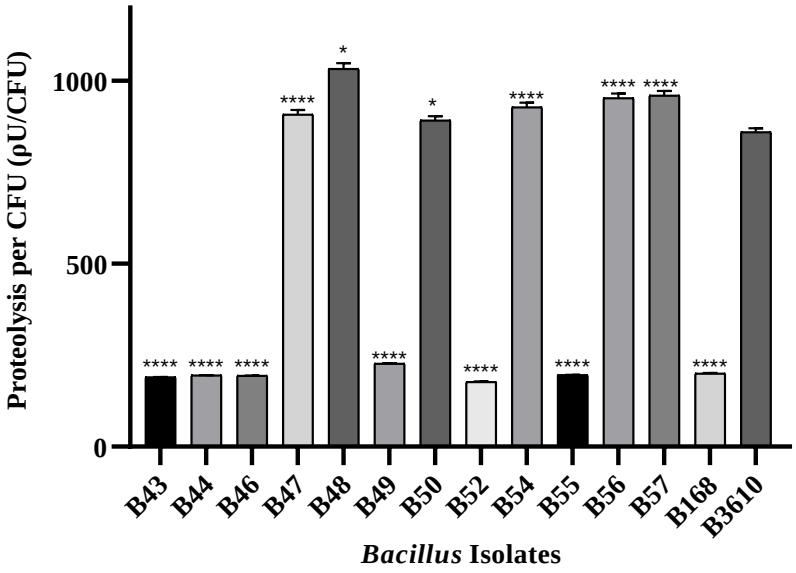

Figure 4-13: The proteolysis of planktonic culture in UHT milk grown at 30 ˚C as picolitre of proteolysis per CFU (ρL/CFU). The highest and lowest mean proteolysis was observed in B47 (1034 ρL/CFU) and B52 (178 ρL/CFU) respectively. Post-hoc analysis was done Dunnett multiple comparisons with a critical probability of $P \leq 0.05$. Error bars indicate the standard deviation of the means (SD). Asterisks (*) indicate significant difference among means (*=$P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$ and ****= $P \leq 0.0001$).

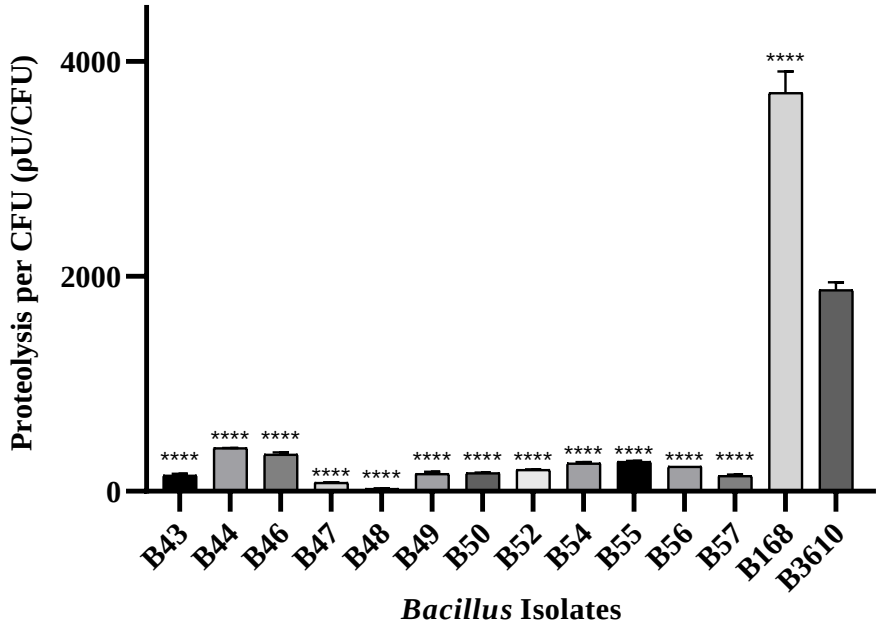

Figure 4-14: The proteolysis within the biofilms on stainless-steel substratum in UHT milk grown at 30 ˚C expressed as picolitre of proteolysis per CFU (ρL/CFU). The highest and lowest mean proteolysis was observed in B168 (3706 ρL/CFU) and B48 (27 ρL/CFU) respectively. Post-hoc analysis was done Dunnett multiple comparisons with a critical probability of $P \leq 0.05$. Error bars indicate the standard deviation of the means (SD). Asterisks (*) indicate significant difference among means (*=$P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$ and ****= $P \leq 0.0001$).

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4.3.4.4 Lipolysis in biofilm and planktonic states

All the isolates produced lipolysis but in smaller amount when compared with the proteolysis. The result shows there were significant differences between the lipolysis produced within the biofilms and the planktonic cells except in some isolates such as B47, B48, B49 and B56. In the planktonic cells, there was an overall significant difference at $P < 0.05$ level in the lipolysis per cell $F_{(13,14)} = 7001$, $P < 0.0001$ with the observed highest and lowest mean of lipolysis being samples B168 (1.13 ρL/CFU± 0.002) and B50 (34.5ρL/CFU± 0.44). The mean lipolysis for the other isolates are given as follows: B43 ($\bar{x}$ = 8.49 ρL/CFU, σ = 0.02), B44 ($\bar{x}$ = 1.23 ρL/CFU, σ = 0.003), B46 ($\bar{x}$ = 5.99 ρL/CFU, σ = 0.02), B47 ($\bar{x}$ = 27.4 ρL/CFU, σ = 0.34), B48 ($\bar{x}$ = 14.35 ρL/CFU, σ = 0.20), B49 ($\bar{x}$ = 18.74 ρL/CFU, σ = 0.05), B52 ($\bar{x}$ = 6.74 ρL/CFU, σ = 0.02), B54 ($\bar{x}$ = 7.06 ρL/CFU, σ = 0.09), B55 ($\bar{x}$ = 2.16 ρL/CFU, σ = 0.006), B56 ($\bar{x}$ = 14.18 ρL/CFU, σ = 0.19), B57 ($\bar{x}$ = 5.69 ρL/CFU, σ = 0.08) and B3610 ($\bar{x}$ = 4.29 ρL/CFU, σ = 0.05) respectively (see figure 4-14).

Hydrolysis of the p-NPP substrate was greater within the biofilms compared to the planktonic cultures in some of the isolates. An example is found in isolate B168 with a quantifiable lipolysis within its biofilms that is more than hundred and fifty times greater than its planktonic cell as observed in figure 4-15. For the rest of the isolates, the mean lipolysis produced within their biofilms are: B43 ($\bar{x}$ = 17.52 ρL/CFU, σ = 5.79), B44 ($\bar{x}$ = 121.0 ρL/CFU, σ = 1.41), B46 ($\bar{x}$ = 12.82 ρL/CFU, σ = 2.12), B47 ($\bar{x}$ = 17.6 ρL/CFU, σ = 1.14), B48 ($\bar{x}$ = 11.07 ρL/CFU, σ = 1.14), B49 ($\bar{x}$ = 12.7 ρL/CFU, σ = 1.05), B50 ($\bar{x}$ = 159.0 ρL/CFU, σ = 9.19), B52 ($\bar{x}$ = 49.7 ρL/CFU, σ = 7.07), B54 ($\bar{x}$ = 78.9 ρL/CFU, σ = 7.78), B55 ($\bar{x}$ = 24.7 ρL/CFU, σ = 3.53), B56 ($\bar{x}$ = 12.6 ρL/CFU, σ = 1.14), B57 ($\bar{x}$ = 21.81 ρL/CFU, σ = 2.94), B168 ($\bar{x}$ = 179.9 ρL/CFU, σ = 35.17) and B3610 ($\bar{x}$ = 63.0 ρL/CFU, σ = 3.34) respectively.

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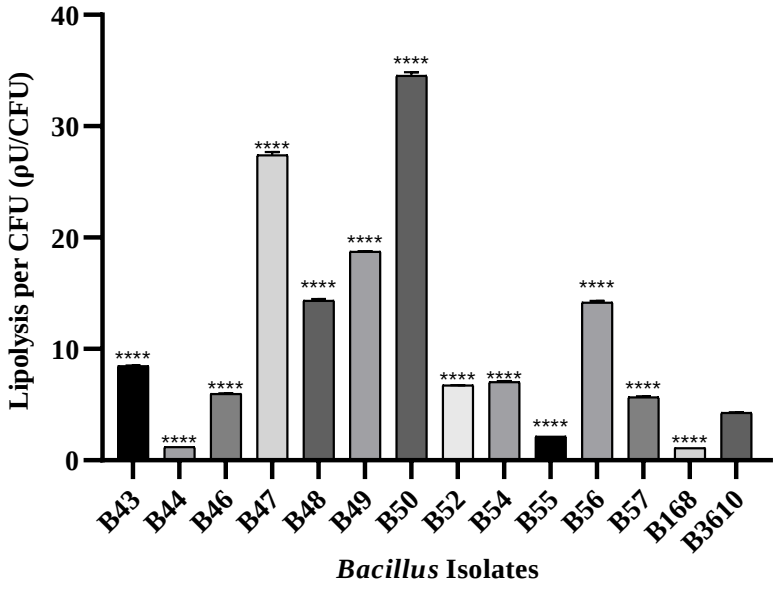

Figure 4-15: The lipolysis of planktonic culture in UHT milk grown at 30 ˚C expressed as picolitre of proteolysis per CFU (ρL/CFU). The highest and lowest mean proteolysis was observed in B50 (34.54 ρL/CFU) and B168 (1.13 ρL/CFU) respectively. Post-hoc analysis was done Dunnett multiple comparisons with a critical probability of $P \leq 0.05$. Error bars indicate the standard deviation of the means (SD). Asterisks (*) indicate significant difference among means (*= $P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$ and ****= $P \leq 0.0001$).

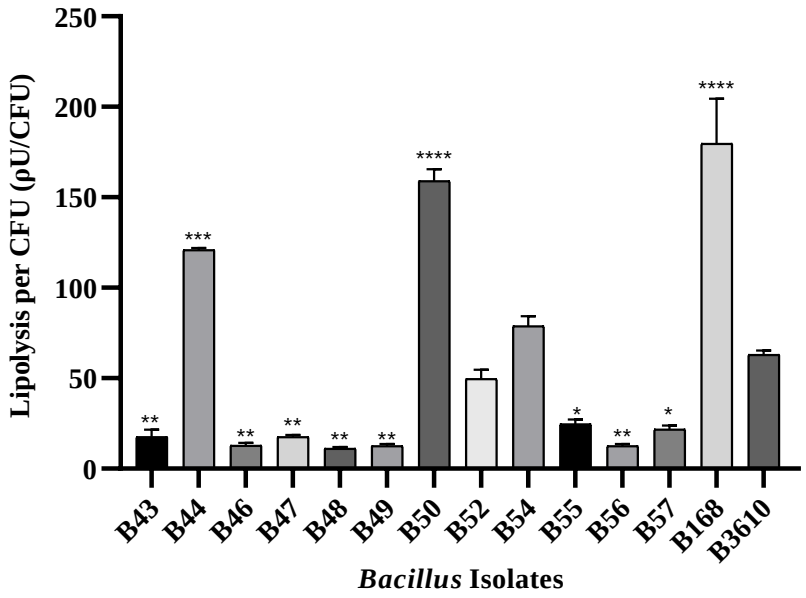

Figure 4-16: The lipolysis within the biofilms on stainless-steel substratum in UHT milk grown at 30 ˚C expressed as picolitre of proteolysis per CFU (ρL/CFU). The highest and lowest mean proteolysis was observed in B168 (179.70 ρL/CFU) and B48 (11.07 ρL/CFU) respectively. Post-hoc analysis was done Dunnett multiple comparisons with a critical probability of $P \leq 0.05$. Error bars indicate the standard deviation of the means (SD). Asterisks (*) indicate significant difference among means (*= $P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$ and ****= $P \leq 0.0001$).

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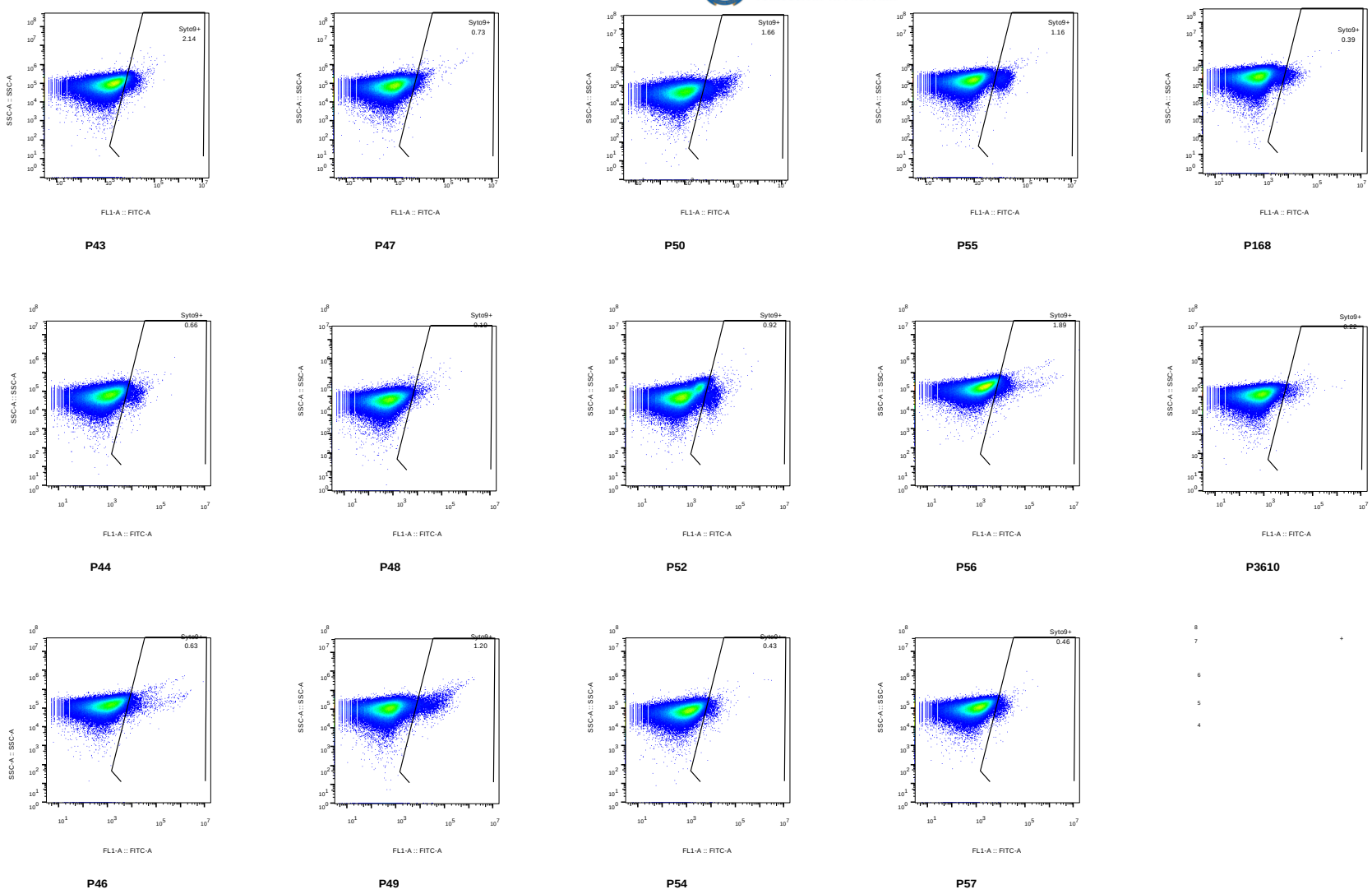

Figure 4-17: Flow cytometric analysis of planktonic cells grown in sterile UHT milk. Total bacterial count for all strains was done by staining cells in milk with SYTO 9 for both dead and living cells to be counted. SYTO 9 can bind the DNA of both living and dead bacterial by entering the cell through the semi-permeable membrane. The highest count was 7.6 $\log_{10}$ CFU/mL (B52), and the lowest was 7.2 $\log_{10}$ CFU/mL (B48).

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4.3.5 Discussion

This study is consistent with previous ones on the ability of planktonic cells as well as biofilms to produce lipolysis and proteolysis which may impact on the quality of processed milk. However, the result under the assay conditions revealed that proteolysis and lipolysis in the planktonic and biofilms states may be strain-dependent as well as the biofilm-forming potential. We compared the quantity of proteolysis and lipolysis within intact biofilms and planktonic cells of *B. subtilis* and *B. velezensis* contaminants of milk at 30 ˚C. Previous studies mostly evaluated the crude enzymes that were produced within the intact biofilms and planktonic in a temperature-dependent manner using the plate count method to enumerate the cells. This study employed the use of flow cytometer to enumerate the cells within the biofilm and planktonic culture to prevent the challenge of under-enumeration that is often associated with the plate count method. These ensure that all possible sub-populations of cells within the biofilms are enumerated using the flow cytometer. Such subpopulations may include viable but non-culturable (VNBC) cells such as persister cells and dormant spores which may not be able to grow and enumerated on the conventional growth media. This approach allows for the enumeration of cells that may be damaged during the extrication of cells within the biofilms using vortexing (with beads) and mild sonication. The DNA binding stain (SYTO 9) used for the flow cytometry analysis can bind the vegetative and damaged cells as well the spores making it more sensitive than plate counting used in the other study.

All isolates were able to grow and attached to the stainless-steel substratum under a submerged condition producing biofilms with variations in the total number of cells enumerated. There was no significant difference in the log count of the isolates in the planktonic culture unlike in the biofilms. This shows that the growth rate across the isolates is somewhat similar. In contrast, there were significant differences in the number of cells recovered from the biofilms.

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This outcome implies that B168 has a weak biofilm forming potential and presumably with the highest spread on the stainless-steel surface. This potentially makes the biofilms structurally and architecturally less complicated than in other samples.

We observed that the spoilage potential (proteolysis and lipolysis) of both biofilms and planktonic cells are strain-dependent at the conditions applied in this study. There seems to be a relationship between the strength or complexity of the biofilms and spoilage potential of the isolates as observed in B168 which is a reference strain of *B. subtilis* with a very weak biofilm forming potential on stainless-steel as stated in our previous study (see section 4.1). We observed that sample B168, in the biofilm state, has the highest mean proteolysis and lipolysis per cell. The sample also has the lowest number of cells enumerated within its biofilm. Thus, one can safely deduce that under the assay condition, the less complicated the biofilm is, the less complicated the structure is on a substratum, and the higher its spoilage potential. The reason for this can be adduced from the fact that the stronger a biofilm is, the more the complicated the structure becomes. The complex structure allows for the different gradient of nutrients and oxygen, resulting in the formation of different microenvironments within the biofilm matrix. These gradients lead to the heterogeneity of cells within the biofilms and such a situation may lead to the formation of dormant cell forms like persister cells and spores in the case of sporulating bacteria like *Bacillus* spp. Both persister cells and spores are metabolically inactive which they are not secreting enzymes like the metabolically active cells which may account for the low lipolytic and proteolytic enzymes in some of the cases.

The formation of biofilms and spores has been observed to occur simultaneously in sporulating bacteria with spore population as high as 10-50 % within the 8 h of attachment to the substratum according to the study by Burgess et al. (2009). Since B168 is a weak biofilm former, it implies that the biofilm lacks the three-dimensional complexity of a model biofilm with no gradient in

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oxygen or nutrient that allows for the formation of metabolically dormant structures. This typically means there will be more metabolically active cells present in a sample with low biofilm-forming potential to secrete enzymes that will, in turn, act on the substrates in the medium.

The reverse scenario is also true in the case of moderate or strong biofilm former with a more considerable heterogeneity, complexity and likelihood of producing spores within its biofilm. The implication of this to the industry is that the biofilms of the isolates used in the study pose a challenge during the processing and storage of milk. While the weak biofilm formers have better spoilage potential than the strong biofilm formers, the latter are hot-beds for the production of heat resistant spores which can withstand the processing condition and further contaminate the downstream process as well as the ESL milk.

Generally, the metabolites produced within mature biofilms are several-folds higher than the ones produced within the planktonic cells of the same strain. A similar study to the current one by Teh et al. (2013) corroborated the assertion that lipolysis was higher by ten-fold in biofilms than the planktonic cells of *Staphylococcus aureus*, *Streptococcus uberis Pseudomonas fluorescens* and *Serratia liquefaciens* isolated from milk. The same trend was observed in the result of our study of the lipolysis within biofilms.

From the result of the planktonic cells, B48 has the highest mean proteolysis with 1033.6 ρL/CFU while B50 has mean proteolysis of 892.0 ρL/CFU the highest mean lipolysis of 34.6 ρL/CFU which is about thirty times higher than the mean lipolysis of the reference strain B168 with 1.13 ρL/CFU. This implies that in its planktonic state, B50 poses the greatest risk to the milk requiring shorter time to cause the development of rancidity as well as a breakdown of casein micelles resulting in deterioration of the product and change in its sensorial quality.

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All the isolates produced more of the proteolytic than lipolytic enzymes in both the planktonic and biofilm states in no specific order under a submerged condition. The submerged method best simulates the situation of planktonic culture than the semi-quantitative agar diffusion method. Though the agar diffusion method is faster in generating a result, it can only be applied in screening isolates for their ability to secrete extracellular enzymes on the agar surface containing the appropriate substrate. Just as in the planktonic cells, B50 has the maximum mean proteolysis of 2.10 ± 0.11 while the negative control, B168, has the lowest with 1.27 ± 0.06. This suggests a correlation between the two methods and the ease at which the agar assay lends itself as the first-in-line profiling of potential spoilage microorganisms of milk.

The limitation in the present study is that it did not take into consideration the presence of enzymes that may be presence in the milk before the introduction of the test microorganisms which may influence the observation. Some bacterial lipases possess the ability to survive pasteurisation even in the absence of the bacteria producing them and effect the hydrolysis of lipids in the milk sample. The effect is thought to be minimal and was also mitigated by using the same UHT milk sample for all experiments. However, it is advisable to measure the initial proteolytic and lipolytic enzymes that may be present in the milk sample before any experiment. The assays used in this study can also be complemented with other methods such as chromatography and other chemometric strategies that can be optimised and automated on an industrial scale.

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4.3.6 Conclusions

B. subtilis and *B. velezensis* have the potential of producing proteolytic and lipolytic enzymes in planktonic and biofilm states. These enzymes degrade the protein and lipid components in milk which can potentially alter the sensorial and nutritional properties of the milk, especially under storage condition. An innovative strategy to mitigate the contamination of milk by thermophilic *Bacillus* spp. either as biofilms or planktonic cells cannot be over-emphasised.

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5 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

This section critically reviews some of the methodologies applied in this research, discusses the importance of the findings and provide some recommendation on the characterisation and spoilage potential of *B. subtilis* and *B. velezensis* isolated from the milk processing environment. Lastly, future research proposing the use of the multi-omics approach in accessing deeper insights into the subject matter to mitigate and control the presence of thermophilic bacilli during downstream processing will be discussed.

5.1 General discussion

This study highlighted the importance of source-tracking bacterial thermophilic bacilli during the processing of dairy products specifically ESL milk, the effects of UVC on their spores in aqueous suspension as well as their biofilm-forming and spoilage potential in UHT milk. We discovered that most of the intrinsic properties of the bacterial samples that were measured are strain-dependent. Such properties are biofilm-forming and spoilage potential, the hydrophobicity of the vegetative cells and spores, the response of the spores to UVC exposure and in the concentrations of Ca-DPA within the spores. This information is relevant in designing a cleaning-in-place (CIP) regime and inactivation parameter for ESL milk processing, especially when using non-thermal processing such as UVC.

Most of the strains were observed to have the ability to grow at elevated temperature. This temperature ranges from the psychrotolerant to a thermophilic temperature of between 6 °C to 55 °C in their vegetative form. Their ability to grow at this temperature range potentially means most of the strains can survive pasteurisation temperature either as spores or vegetative cells,

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form biofilms on the food-contact surface, contaminate the final product and cause spoilage, especially during prolonged cold storage. There are other extrinsic factors, of course, that may influence the ability of these strains to survive and effect their spoilage on the product. These factors are the initial microbial load in the raw milk, the temperature during bulk milk transportation and storage, pasteurisation temperature/time combination, the efficacy of the CIP regime and the type of technology used for processing and whether the processing is done in a stand-alone or combination with other technologies. It is highly recommended to initiate any strategy that minimises or reduce the initial microbial load in the raw milk, especially during the milking process on the farm as well as during transportation and storage. In South Africa, a bactofugation or microfiltration step is often used to reduce the microbial load before the pasteurisation of the milk during ESL milk processing. This microfiltration step makes the pasteurisation process better at inactivating the contaminants and prevent the growth of potential biofilms. It is recommended to install an online/real-time biosensor where it is possible to monitor the formation of biofilms or aggregation of cells attached to the processing line alongside the development of an effective CIP strategy that can mitigate against the formation of biofilm and inactivate all cells that may be present. The control of the thermophilic contaminants becomes rather tricky if they can attach to the contact surface and established a process biofilm for two reasons: the increased sporulation process within the biofilms as revealed in the CLSM images and the potential increased production of metabolites such as spoilage enzymes within the biofilm structure. We observed from the result of the study that the production of either spores or enzymes within the biofilm structure is likely dependent on several factors. Such factors are the strength of the biofilm that is produced besides other factors such as nutrient limitations and oxygen gradients within the structure. The more complex the structure, the likely the higher sporulation process. Conversely, the weaker the biofilm, the less

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the sporulation but the higher the production of spoilage enzymes. This is because most of the biofilm structure may likely be in contact with the substrates available on the substratum.

The challenge of the contamination may become more aggravated if CIP is done after the sporulation process within the biofilms, especially in the case of *Bacillus* spp. contamination. This implies that the flow caused by the CIP solution may disrupt the biofilms and potentially inactivate all vegetative cells without much damage to the spores. Some of the spores may attach to other parts of the processing line initiating the biofilm-forming process after attachment, thereby aggravating the problem. We observed that for six of the strains, the % hydrophobicity was on the average 25 % more in the spore samples than the vegetative cells and potentially more challenging to inactivate during pasteurisation. This observation that hydrophobicity or attachment strength is higher in bacterial spores than vegetative cells supported by many similar studies. The application of non-thermal processing, such as UVC is more effective in inactivating bacterial spores, as indicated in the current study. However, we also observed that there are spore population from the flow cytometry analysis that may not be able to grow on the traditional agar plate but may potentially germinate and cause spoilage, especially during extended storage. Hence the suggestion to develop a UVC strategy and optimise it for various liquid foods such as milk in a way that will not affect the nutritional and sensorial properties of the food being processed and also combine it with other technologies such as high-pressure processing.

The limitations of this study include the following: the biofilms are formed under static conditions which do not correctly simulate what happens in the processing plant. Also, the UVC inactivation was done in aqueous suspension and not in liquid food and lastly the lethally injured sub-population after the UVC exposure is not further analysed for their ability to grow in a food system such as milk and agar plate. Nonetheless, this limitations in any do not

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discredit the result of the study and the potential threat the thermophilic bacilli present in a processing plant. All the limitations highlighted here are grounds that can be further explored in a future study.

5.2 Methodological considerations

In this study, the isolates are characterised using multi-locus sequence typing (MLST) after preliminary identification with MALDI-TOF MS. This decision was informed by the fact that there is no selective media for *Bacillus subtilis* and *Bacillus velezensis* and because of the failure to distinguish members of *Bacillus* species solely based on their phenotypic characteristics. Besides, there is the failure of 16S rRNA to phylogenetically delineate the members of the *Bacillus subtilis* complex into species due to the highly conserved nature of the gene. Therefore, there is a need to use multiple protein-coding loci for identification as proposed by Rooney et al. (2009).

The simulated biofilm-forming potential of the strains was carried out using a microtiter plate and by staining the adhered cells with crystal violet. This microtitre plate assay was supported by growing the biofilm on stainless-steel (Grade 316L, 2B) coupon which is a standard food contact surface used in the industry. Though these methods are used for biofilms under a static condition and there are an alternating flow and static situations at play during the processing of ESL milk. Another shortcoming of the microtitre plate biofilm assay is that it does not entirely reflect or capture the behaviour of the cells during attachment regarding the hydrophobicity of a stainless-steel surface as applied to the dairy processing plant. Besides, the use of DNA-specific dyes for the enumeration of the biofilms is recommended rather than a non-specific crystal violet which binds to other non-cellular matters and thus can lead to a false-positive result. Nonetheless, the methods used hypothesised the prevailing condition that

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exists after processing and hard to clean recesses of the plant where adhering spores or cells are aided by the presence of conditioning films. The result of the biofilm-forming potential is further enhanced by visualising them under a scanning electron microscope (SEM) and confocal laser scanning microscope (CLSM) which reveal detailed structure and architecture of the biofilms.

Enumeration of surviving spores after UVC exposure was done tryptose soy agar (TSA) supplemented with 0.6 % glucose using the pour plate method. This method ensures that all cells are trapped at the sub-surface of the medium for correct enumeration since the colonies from the isolates tend to spread on the surface making enumeration difficult. Generally, standard plate counting using growth medium falls short because it fails to account for viable but non-culturable spores which are mostly sub-lethally injured by the exposure to UVC radiation.

Flow cytometry (FCM) method possesses the ability to bridge this gap of reliable enumeration of spores/cells at different physiological conditions. The method is highly sensitive and accurately quantifying cells/spores regardless of their heterogeneity with the use of appropriate stains. In this study, the spores after UVC treatment and bacterial cells within the biofilms of the isolates were quantified using live/dead staining kits containing SYTO 9 and propidium iodide (PI). These are dyes with an affinity for DNA with PI having the ability to bind DNA in the case where the cell membrane is highly permeabilised especially in the case of lethal or sub-lethal injury whereas SYTO 9 can easily move into a cell through an intact cell membrane. Hence, for total viable cell enumeration (both dead and living cells) within biofilms, only SYTO 9 was used. This result of such data is more reliable, sensitive and faster than the traditional plate counting using growth media (Zhang et al., 2020).

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The enzyme assay (proteolysis and lipolysis) as used in the study by Teh et al. (2012) and Teh et al. (2013) was designed to measure both the extracellular peptidases and lipases which are secreted in response to the presence of the respective substrates. The enzyme quantification was achieved by incubating the isolates, either in biofilm or planktonic form, for a specified time with the substrates. These substrates are azocasein solution for the proteolytic assay and p-nitrophenol palmitate with both substrates containing added sodium azide and chloramphenicol. The sodium azide and chloramphenicol serve as bacteriostatic agents used to stop the growth of the isolates and hence halt the secretion of enzymes for accurate quantification of secreted enzymes after the incubation time. This method is more sensitive than the enzyme hydrolysis on agar plates which is rather qualitative than quantitative though only extracellular enzymes are quantified since the stage where the cell wall is lysed for the liberation of the endoenzymes is absent.

5.3 Conclusions and Recommendations

This study essentially adopted the application of MLST to track the possible sources of contamination of *B. subtilis* and *B. velezensis* during the processing of extended shelf-life (ESL) milk. The result linked the isolates to the raw milk used in the production of ESL milk-fed into the downstream processing line suggesting the survivability of the isolates by adaptation to the processing condition either as spores or as a community as in biofilms. While isolates are generally non-pathogenic, this study indicates the readiness to produce biofilm in most part, thereby potentially constituting a nuisance during downstream processing of ESL milk and compromising its quality. This presence of thermophilic bacilli in the dairy plant calls for the development of effective cleaning regime targeted at their vegetative cells, biofilms as well as their spores. Innovative strategies such as the application of nanotechnology or other

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mechano-sensing devices in the development of biosensors specific for the detection of biofilm and spores- the two most resistant structures of thermophilic bacilli are highly recommended.

Although it is possible that the ESL processing conditions pre-selected the isolates due to their ability to produce spores and tolerate thermophilic temperature (above 50 °C), biofilms naturally exist in a consortium of multi-species and not mono-species biofilms as assumed in this study. Nonetheless, the study proves that isolates of *B. velezensis*, hitherto unknown to prove itself a challenge during milk processing, may be significant in ESL milk processing as well as other well-known *Bacillus* species.

Furthermore, the result highlighted the agency of UVC in the inactivation of thermophilic spores during the processing of liquid foods such as ESL milk and the importance of understanding the action at the molecular level. It was also confirmed that the hydrophobicity and effect of UVC treatment of spores are mostly heterogeneous with some strain-to-strain variations. These variations must be taken into consideration to create an effective UVC regime suitable for both vegetative cells and spores of thermophilic bacilli associated with raw milk without an undesirable change in sensory and nutritional properties.

Essentially, the quality of the raw milk used in the downstream processing of ESL milk is very critical to the quality of the final product. It is suggested that strategies to minimise contamination and growth of intrinsic microflora at farm level and during the transportation of bulk milk is implemented.

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5.4 Future Research

This study characterised the biofilm-forming and spoilage potential of *Bacillus subtilis* and *Bacillus velezensis* isolated from the dairy processing plant and the means of inactivating their spores using UVC. There is a need for an intensive investigation on the responses of the spores to UVC using milk as the suspension medium rather than phosphate buffer as used in this study. Such sub-populations of spores derived from UVC exposure must be further investigated for their ability to germinate when cultivated in milk or other growth media. This can be achieved by sorting out the different sub-populations of spores from the flow cytometer and growing in an appropriate medium, analysing the different sub-populations for structural damage and quantifying the amount of Ca-DPA pre and post-UVC exposure for possible leakage of spore contents. Notable among the sub-populations that should be sorted out is the sub-lethally injured spore sub-population which may likely require more than the 24 to 48 hours incubation used in the traditional plate counting method. The potential of such sub-population to resuscitate when inoculated into a food system such milk must be verified especially during storage.

Since the isolates demonstrated the ability to grow over a wide temperature that is optimum to psychrophiles to thermophiles, there is a need to analyse them for their enzymes production and metabolites at these extreme temperatures. This analysis may further provide information on the possible effects of these enzymes during and after pasteurisation and find a biotechnological application in other fields. The use of multi-omics approach will be highly recommended in this area for the possible discovery of novel metabolites and optimal result. This may involve some proteogenomic and metabolic characterisation of the isolates which aids in the prediction of their metabolic capacity and stress response to various processing conditions either in the planktonic or biofilm state.

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An investigation of the interactions occurring within the co-culture or multi-species biofilms of these strains is highly recommended. Since bacteria do not exist as single-cell planktons in nature, this will present better insight closest to the physiological variations between mono and dual-species biofilms and their interactions with a substratum such as stainless-steel. Such information can be applied in the development of nano-sensor and cleaning regimes which can be used in the prevention and control of thermophilic biofilms during the downstream processing of liquid foods such as extended shelf-life (ESL) milk.

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7 Publications and presentations from this work

Scientific paper

Elegbeleye, J.A., Buys, E.M., 2020. Molecular characterization and biofilm formation potential of *Bacillus subtilis* and *Bacillus velezensis* in extended shelf-life milk processing line. *Journal of Dairy Science*. 103, 1–13. https://doi.org/10.3168/jds.2019-17919

James A. Elegbeleye, Ramon Gervilla, Artur X. Roig-Sagues, Elna M. Buys, 2020. Ultraviolet-C inactivation and hydrophobic potential of *Bacillus subtilis* and *Bacillus velezensis* spores isolated from Extended shelf-life milk. International *Journal of Food Microbiology* (Accepted).

Elegbeleye, J.A., Buys, E.M., 2020. Spoilage potential of biofilms and planktonic cells of *Bacillus subtilis* and *Bacillus velezensis* in extended shelf-life milk. *Journal of Food Control.* (Submitted).

Posters

Effects of Ultraviolet C Processing on the Spores of *Bacillus subtilis* and *Bacillus velenzensis* and their Chemical Analysis Using Raman Spectroscopy. J. A. Elegbeleye, E. M. Buys, A. X. Sagues. Presented at the ASM Microbe 2020 online conference (June 18-July 22, 2020).

What has light got to do with milk? (2019). James A. Elegbeleye, Elna M. Buys, Ramon Gervilla, Artur X. Roig-Sagues. Presented at 23rd South African Association for Food Science

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(SAAFoST) Biennial International Congress and Exhibition (September 1-4, 2019) Birchwood Hotel and Conference Centre, Johannesburg, South Africa.

Molecular Typing, Biofilm Formation and Proteolytic Activity of *Bacillus* Isolates from Extended Shelf-Life Milk. Presented at 22nd South African Association for Food Science (SAAFoST) Biennial International Congress and Exhibition (September 3-6, 2017) Century City Conference Centre, Cape Town, South Africa.