The effect of calcium and milk formulations on biofilm formation of *Geobacillus stearothermophilus*

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Abstract

G. stearothermophilus contaminates milk powder products from bacteria spores released from biofilms on product contact surfaces in dairy manufacturing plants. The dairy industry has observed that calcium-reduced milk protein powder is associated with reduced spore contamination of thermophilic bacteria during milk protein powder manufacture. Calcium, as a major component of milk minerals, was previously found to affect *G. stearothermophilus* biofilms grown on stainless steel exposed to milk formulations. Additionally, *G. stearothermophilus* cells cultured with additional calcium showed an increased attachment to stainless steel. The current study investigated the effect of calcium and milk formulations on cell attachment, biofilm formation, spore production and spore heat resistance of *G. stearothermophilus* to pinpoint the potential factors contributing to the reduced thermophile contamination in the calcium-reduced milk protein powder.

The effect of calcium on biofilm formation of *G. stearothermophilus* dairy isolates A1, P3, and one reference strain 7953 in modified TSB media was studied to gain more insights into the role of calcium in biofilm formation of *G. stearothermophilus*. The presence of calcium increased biofilm cell numbers of strain A1, but reduced biofilm cell numbers of the reference strain and showed minimal effect on strain P3. Extracellular polymeric substances (EPS) quantity, in particular extracellular protein, varied between strains. Unlike the consistent biofilm promotive effect of calcium in milk formulations found in a previous study, the current findings suggest that a strain-to-strain difference exists in biofilm formation of *G. stearothermophilus* species in the presence of calcium.

Calcium plays an important role in cell attachment by changing cell surface properties, cell physiology and metabolism, modifying cell surface structure and bridging between bacteria and substrata. In the dairy industry, milk formulations with different cation profiles may affect the cell attachment of a common contaminant *G. stearothermophilus*. The current study investigated the effect of calcium on cell attachment of *G. stearothermophilus* strains A1, P3 and 7953 to stainless steel and polystyrene substrata and characterized the cell surface charge, hydrophobicity and cell surface polymers. In addition, cell attachment in milk formulations on stainless steel was characterized. The presence of calcium increased the cell attachment of dairy

isolates on polystyrene but not the reference strain, while calcium did not affect cell attachment of all strains on stainless steel. The presence of calcium changed the amount of cell surface polymers produced but not hydrophobicity. Although calcium affected the zeta potential, this did not correlate with the trend in cell attachment. It is assumed that the cell attachment of *G. stearothermophilus* is affected by the substrata, strain specific cell surface polymers, as well as calcium induced changes in cell surface polymers. Milk formulations (MF1 and MF2) with different cation profiles showed little effect on cell attachment of *G. stearothermophilus* on stainless steel, indicating a minimal effect of cell attachment to contamination levels of different cation-modified milk protein powder products in dairy manufacturing plant.

The effect of total calcium concentration, total sodium concentration and bacteria growth history were investigated on biofilm formation of *G. stearothermophilus* in MF1 and MF2. The numbers of culturable biofilm cells of *G. stearothermophilus* strain A1, P3 and 7953 were compared in MF1 and MF2 over 18 h. MF1 and MF2 have similar protein, lactose and fat content except for cation profiles, where MF2 has relatively high sodium and low calcium concentrations. Biofilm formation of all three strains was lower in MF2 than in MF1, but the inhibition of MF2 was conditional and was highly dependent on the growth history of bacterial inocula. The inhibition of MF2 on dairy isolates A1 and P3 were further investigated by cation supplementation. Supplementation of MF1 with sodium decreased biofilm formation at 18 h for A1 and P3. Supplementation of MF2 with either 2 or 26 mM calcium increased biofilm formation of A1 at 18 h, and P3 at 10 h. High sodium and low calcium concentrations in the milk formulation seem to be required to inhibit biofilm formation of *G. stearothermophilus.* However, it is not known if the inhibitory effect of MF2 was due to the direct effect of cations on biofilms or the change of milk protein structure on biofilms due to calcium removal.

Cations such as calcium and sodium were shown to affect sporulation and spore heat resistance of *G. stearothermophilus*. MF1 and 2 have distinctive cation profiles and therefore the sporulation and spore heat resistance of *G. stearothermophilus* A1, P3 and 7953 in milk

formulations were investigated. MF2 effectively reduced the total biofilm spore numbers of A1 and P3 over the 18 h culture period compared to MF1, but the effect was not observed in 7953. The sporulation percentage of A1 was higher in MF1 than MF2 at 14 and 18 h, and a similar effect was observed for P3 at 10 and 14 h. Supplementation of calcium to MF2 increased the sporulation percentage of A1 at 14 and 18 h, as well as P3 at 14 h. Supplementation of sodium to MF1 decreased the sporulation percentage of A1 at 18 h. No significant difference in spore heat resistance of A1, P3 and 7953 was observed between spores produced from MF1 and MF2.

Overall, the reduced biofilm formation and sporulation percentage of *G. stearothermophilus* in MF2 provided evidence to the reduced thermophile contamination in the calcium-reduced milk protein powder.

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Table of Contents

List of Figure Legends

List of Tables

List of Publications

Tianyang Wang, Steve Flint, Jon Palmer. (2019). "Magnesium and calcium ions: roles in bacterial cell attachment and biofilm structure maturation." Biofouling 35(9): 959-974.

Tianyang Wang, Steve Flint, Jon Palmer. (2021). "Heterogeneous response of *Geobacillus stearothermophilus* biofilms to calcium." International Dairy Journal: 104961.

List of Conference Presentations

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Chapter 1 Introduction

G. stearothermophilus is a common thermophilic bacilli contaminant in dairy manufacturing plants (Burgess et al. 2010, Carrascosa et al. 2021). *Geobacillus* spp. contaminate milk products by forming biofilms on the surface of process equipment and shedding cells and spores into the process flow (Burgess et al. 2014, Seale et al. 2015). The thermostable enzymes produced by thermophilic bacilli are thought to affect the quality of the recombined milk powder and the contamination of *G. stearothermophilus* in dairy products potentially leads to "flat-sour" spoilage (Chen et al. 2004, Burgess et al. 2010). Therefore, there is interest in characterising species diversity, enzyme production, biofilm formation and spore heat resistance of *G. stearothermophilus* (Chen et al. 2004, Burgess et al. 2014, Burgess et al. 2017, Wells-Bennik et al. 2019).

In the dairy industry, milk formulations with different cation profiles are produced via ion-exchange to improve the functional properties of the powder (Bhaskar et al. 2003, Bhaskar et al. 2004). A reduced thermophile contamination in calcium-reduced milk powder has been observed in industry settings. A reduced biofilm growth of *Geobacillus* spp. in a calcium-reduced milk formulation in comparison to the original milk formulation shown in a previous study could potentially explain the industrial observation (Somerton et al. 2015). Two mechanisms were proposed for this observation including the effect of electrostatic interaction between cations and biofilm matrix on biofilm structural integrity and the switch between planktonic and biofilm cells in response to different cation concentrations (Somerton et al. 2015). Among all the cations investigated, calcium was shown to affect both cell attachment and biofilm formation of *G. stearothermophilus* (Somerton et al. 2013, Somerton et al. 2015). In addition, calcium affects cell attachment and biofilm formation in several other bacterial species (Wang et al. 2019).

Biofilms represent a self-aggregated, stratified microbial community that usually constitutes bacteria and a self-produced matrix of extracellular polymeric substances (EPS) (Wang et al. 2019). Biofilm development consists of several stages including initial adhesion, growth, and maturation, and eventually detachment. Mechanisms of calcium ions in participating in bacterial cell attachment and biofilm formation include: (1) assisting in conditioning film

formation; (2) altering surface electrokinetic properties; (3) modifying cell surface polymers; (4) interacting with biofilm matrix; (5) regulating cell gene expression (Wang et al. 2019).

To understand the factors causing the reduced thermophile contamination in calcium-reduced milk powder in industry settings, the current study characterized the effect of calcium ions on cell attachment and biofilm formation of *G. stearothermophilus* in modified TSB media (mTSB) with varying calcium concentrations that can be correlated back to the total calcium levels in milk formulations used by Somerton (2013). Cell attachment and biofilm growth in milk formulations were also investigated to compare with the results obtained from mTSB. Sporulation and spore heat resistance were studied using milk formulations to provide further information on possible explanations for the industrial observation.

1.1 Research objectives and hypotheses

1.1.1 Objectives

- Characterize cell attachment and biofilm formation of *G. stearothermophilus* in the effect of calcium ion
- Investigate the effect of different milk formulations on cell attachment, biofilm formation and spore production/heat resistance of *G. stearothermophilus* to pinpoint potential factors contributing to the reduced thermophile contamination of the calcium-reduced milk powder in industry settings

1.1.2 Hypotheses

- Calcium affects biofilm biomass, cell numbers, and EPS production
- Calcium modifies cell surface polymers and it may affect the subsequent cell attachment
- Biofilm formation of *G. stearothermophilus* is inhibited in the calcium-reduced milk formulation
- Biofilm formation of *G. stearothermophilus* is affected by the cell growth history
- Spore production of *G. stearothermophilus* biofilms in the calcium reduced milk formulation is reduced
- Heat resistance of *G. stearothermophilus* spores is reduced when spores are produced from the calcium-reduced milk formulation

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Chapter 2 Literature review

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2.1 *G. stearothermophilus*-a problematic contaminant in the dairy industry

The *Geobacillus* genus is a group of Gram-positive, rod shaped spore forming thermophilic bacteria with the optimal growth temperature between 55-65 ℃ (Zeigler 2000). The spores of thermophilic bacilli are common contaminants of dry dairy products and dairy manufacturing plants (Burgess et al. 2010). The prevalence of *G. stearothermophilus* in milk powders has been repeatedly reported in previous studies (Chopra and Mathur 1984, Ronimus et al. 2003, Scott et al. 2007, Burgess et al. 2014). Specifically, *G. stearothermophilus* accounted for 64.7% of the total selected isolates derived from skim milk powder (Chopra and Mathur 1984). A sampling regime carried out by Ronimus et al. (2003) across New Zealand's milk powder factories showed that, among 1470 thermophilic isolates from milk powder, 98% were assigned to *G. stearothermophilus*, *Anoxybacillus flavithermus*, *Bacillus licheniformis* and *Bacillus subtilis* species. The most common and prevalent group of strains was closely related to *G. stearothermophilus* (DSMZ 22). Core genome sequence comparison of *G. stearothermophilus* species showed dairy strains isolated from the same manufacturing plant are closely clustered, and all dairy isolates were identified with a putative *lac* operon (Burgess et al. 2017).

Thermophilic bacteria show the propensity to form biofilms on the surface of stainless steel equipment commonly used in dairy manufacturing plants and the cells dislodging from biofilms can disperse into the processing flow and cause contamination in the final products (Seale et al. 2015). It is thought that *G. stearothermophilus* contaminates the dairy manufacturing equipment by attaching to surfaces and forming biofilms (Ronimus et al. 2003, Burgess et al. 2010). The biofilm forming ability of *Geobacillus* spp. on stainless steel surface in the dairy environment has been well studied (Burgess et al. 2014, Somerton et al. 2015, Karaca et al. 2019, Kumar et al. 2021). Scott et al. (2007) found that in a whole milk powder manufacturing plant, the predominant site of sporulation and vegetative cell growth was the preheat section of an evaporator and the evaporator, which ranged in temperature between 50 ℃ and 65 ℃. *A. flavithermus* and *Geobacillus* spp. were identified as the main contaminants. Burgess et al. (2014) isolated 33 thermophiles from evaporators of a milk powder plant, 29 of these isolates belonged to the *Geobacillus* spp., of which at least 10 isolates were identified as *G. stearothermophilus*. Although not pathogenic, the presence of thermophiles in milk could potentially cause sensory or functional defects (Hill and Smythe 2012), and high numbers of

thermophiles in dairy products indicate poor manufacturing hygiene and are unacceptable to customers (Burgess et al. 2010).

2.2 Properties of milk

2.2.1 The major components of milk

The major constituents of bovine milk are water, proteins, lipids, lactose, minerals (Bylund 2015). The major components of milk lipids are triglycerides, with small amount of Di- and monoglycerides, fatty acids, sterols, carotenoids, vitamins, cholesterol, and cholesteryl esters. They exist in small globules or droplets (Fox 2003, Bylund 2015). Milk protein can be classified into serum proteins and caseins. Casein constitutes around 80% of total proteins in milk and can be categorized into four main subgroups including α_{s1}-casein, α_{s2}-casein, κ-casein and β-casein. In milk, caseins are present in self-associated spherical colloidal particles called casein micelles. Since the micelles are of colloidal dimensions which are capable of scattering light, the white colour of skim milk can be largely attributed to this property (Bylund 2015). Serum proteins principally comprise α-lactalbumin, β-lactoglobulin and several minor proteins including serum albumin and immunoglobulins (Fox 2003, Bylund 2015). Lactose is a sugar uniquely found in milk and is the principle carbohydrate found in bovine milk. The other carbohydrates include glucose, galactose and oligosaccharides (Newburg and Neubauer 1995). The most important minerals are calcium, sodium, potassium and magnesium, which exist in forms of phosphates, chlorides, citrates and caseinates (Bylund 2015).

2.2.2 Milk minerals

Minerals constitute around 8-9 g/L of milk (Gaucheron 2005). The principal cations in bovine milk are sodium, potassium, calcium and magnesium (Fox 2003b, Gaucheron 2005). They are either diffusible, or non-diffusible fractions that are mainly associated with colloidal casein micelles and aqueous casein molecules (Gaucheron 2005). The diffusible fraction is usually prepared by dialysing water against a much larger volume of skim milk through a semipermeable membrane (Holt 1985). Sodium and potassium exist mainly in the ionized state in the diffusible fraction of milk (Holt et al. 1981), and only a small portion is combined with

inorganic anionic groups or in colloidal forms (Fox 2003, Gaucheron 2005). Calcium, on the other hand, equilibrates between an ionized form, citrate, phosphate, chloride form and protein complex. Specifically, in the diffusible fraction, calcium exists as ionic calcium and a stable complex primarily associated with citrate, and subsequently with inorganic phosphate and with chloride (Gaucheron 2005, Mekmene et al. 2009). Holt et al. (1981) and Mekmene et al. (2010) also showed that most of the calcium and magnesium in milk diffusate are complexed to citrate. When present in casein micelles, calcium is bound directly to casein via phosphoserine residues or to the inorganic phosphate (Gaucheron 2005). Colloidal magnesium is also thought to be incorporated in micellar calcium phosphate or bound directly to casein (Holt 1985). Holt and Jenness (1984) commented that quantitatively, 66% of calcium, 33% of magnesium, 50% of the inorganic phosphorus and 10% of the citrate are bound to casein micelles in milk. Philippe et al. (2003) reported that in milk, from a total concentration of 32 mM of calcium, 22 mM is in a colloidal state and 10 mM is diffusible, with only 2 mM of this diffusible calcium as free ions. A detailed cation distribution can be seen in Table 2-1. At present there is no evidence that macro-elements such as calcium, magnesium, sodium or potassium, are bound to the fat globules and lactose in important amount (Gaucheron 2005).

Table 2-1 Macro-element amount and forms in bovine milk (Fox 2003, Oh and Deeth 2017)

Constituent	**Concentration (mg/L)**	**Soluble**		**Colloidal**
		Percentage	Form	
Sodium	500	92	Ionized	8
Potassium	1450	92	Ionized	8
Chloride	1200	100	Ionized	-
Sulphate	100	100	Ionized	-
Phosphate	750	43	10% bound to Ca and Mg	57
			51% H_2PO	
			39% HPO_4^{2-}	
Citrate	1750	94	85% bound to Ca and Mg	
			14% $Citr^{3-}$	
			1% $HCitr^{2-}$	
Calcium	1200	34	35% Ca^{2+}	66
			55% bound to citrate	
			10% bound to phosphate	
Magnesium	130	64	25.4% Mg^{2+}	36
			63.5% bound to citrate	
			11.1% bound to phosphate	

2.2.3 Effects of processing on partition of milk minerals

A number of operations including the use of additives, concentration by means of heating, decalcification and pH adjustment are used in the manufacture of dairy products (Holt 1985, Mekmene et al. 2012, Meena et al. 2017). The effects of salt supplementation, pH, concentration and temperature were found to have a significant effect on the mineral equilibrium of milk (Holt 1985), and this further address major impacts on the stability of milk proteins (Fox 2003).

2.2.3.1 Temperature

Temperature affects the solubility of complexed salt such as calcium phosphate in the diffusible fraction of milk and results in equilibrium change between the diffusible and non-diffusible fraction (Gaucheron 2005). Increasing temperatures decreases the solubility of calcium phosphate and leads to the transference of soluble calcium-phosphate to the colloidal phase, with the release of H^+ and a decrease in pH, and this change is reversible upon cooling (Fox 2003). In addition, the free calcium fraction also declines at elevated temperatures, but returns upon cooling (Holt 1985). Holt (1985) also reviewed that when milk was heated to 120 ℃ for 15 min, the soluble calcium level decreased, the pH became lower (6.3 rather than 6.6). Conversely, an increase in soluble calcium occurred during 30 ℃ storage. In response to heat treatment, calcium phosphate in the colloidal phase also transforms into a less soluble form at 115 ℃. The transformation to the less soluble form of calcium phosphate involves a conversion of the original micellar calcium phosphate and not just precipitation of diffusible calcium and phosphate (Holt 1985). The heat-induced modifications of equilibrium are considered reversible when the heat treatment is less than 95 ℃ for a few minutes. However, heating at 120 ℃ for 20 min can cause irreversible changes in casein micelles and salt partitioning (Gaucheron 2005).

2.2.3.2 pH

Acidification of milk progressively dissolves micellar calcium phosphate (Holt 1985, Mekmene et al. 2012), and further reduces casein-binding of calcium and magnesium (Holt

1985). The solubility of colloidal calcium phosphate (CCP) is also affected by pH. CCP dissolves completely and becomes soluble below approximately pH 4.9 (Fox 2003), which contrasts to the presence of CCP in casein micelles at the native pH of milk between 6.6-6.8. At pH lower than 4.9, the solubilization of calcium is the calcium directly associated with phosphoseryl residues of caseins (Ahmad et al. 2008). Acid-induced solubilization of CCP was also reported by Silva et al. (2013), characterization of an acidified milk suspension that had been dialyzed against milk ultrafiltrate showed that both total and colloidal calcium phosphate decrease progressively as a function of acidification. Because of demineralization-dissociation, the turbidity of milk suspensions decreases with decreasing pH (Silva et al. 2013). Not only CCP, but small amounts of magnesium and citrate associated with casein micelles are also dissolved when the pH of the milk is reduced (Gaucheron 2005). Silva et al. (2013) reported that magnesium shows a similar trend to calcium, with levels of total and colloidal magnesium decreasing as the pH decreases. Mekmene et al. (2010) showed that colloidal citrate is totally solubilized at pH 5.2 while the magnesium bound to colloidal citrate is linearly solubilized from natural milk pH to pH 5.2 and the magnesium directly bound to caseins is solubilized at about pH 3. Ahmad et al. (2008) also showed that the diffusible calcium and magnesium increase as a function of acidification. Conversely, calcium increasingly binds to casein as pH increases (Holt 1985). In addition, the concentration of free calcium decreases on raising pH, but the extent of fall is dependent on the type of alkaline additive used. For example, a smaller fall in free calcium was observed when trisodium citrate was used to raise the milk pH when compares to the use of Na_2HPO_4 (Holt 1985). Unlike the reversibility in mineral equilibrium upon temperature change, modifications of repartition induced by acidification are irreversible (Gaucheron 2005). One example of application is the production of calcium-reduced milk protein concentrate, in which the milk protein concentrate solution is acidified to pH 4.6-6 followed with dialysis and/or ultrafiltration and/or diafiltration (Bhaskar et al. 2003).

2.2.3.3 Addition of cations (especially calcium)

It is believed that addition of cations, such as $CaCl_2$, affects mineral equilibrium between aqueous and micellar phases and the pH of milk (Fox 2003). When calcium was added into milk, the concentrations of aqueous calcium, ionic calcium and casein associated calcium increases simultaneously (Mekmene et al. 2009). Similarly, when 10 mM calcium chloride was added to milk, 80 % of the calcium became associated with casein micelles and inorganic

phosphate and citrate in the diffusible fraction are converted to the micellar edifice (Gaucheron 2005). The increase in ionic strength induces decreased activity coefficients of ions, which results in dissociation of the ion pairs (Gaucheron 2005). The addition of cations will consequently increase the concentration of the free form of cations that were not added. For example, the addition of sodium chloride leads to a decrease in pH and an increase in free calcium in the diffusible fraction of milk (Gaucheron 2005). Mekmene et al. (2009) also showed that addition of sodium chloride results in an increase in aqueous and free calcium while decreasing the concentration of calcium that binds to casein.

2.2.3.4 Concentration and filtration process

Common dairy processing techniques such as ultrafiltration and evaporative concentration perturb the mineral equilibrium of milk. It is thought that evaporative concentration of milk increases ionic strength and the resultant lower ion activity allows more diffusible phosphate and calcium. The concentration of milk also increases the amount of free calcium and magnesium, but by less than the concentration factor (Holt 1985). Due to the low solubility and saturation nature of calcium phosphates in the diffusible phase, precipitation occurs during concentration. Tanguy et al. (2016) showed that calcium phosphate precipitates during the concentration of milk ultrafiltrate and microfiltrate while magnesium, sodium, potassium and chloride remain soluble. In addition, salt precipitation of tricalcium citrate or calcium phosphate also occurs during prolonged storage of concentrated milk products (Holt 1985). Increased precipitation during storage was also observed in ultrafiltration and microfiltration milk concentrates (Tanguy et al. 2016).

2.2.3.5 Ion-exchange and chelating agents

Ion-exchange and chelating process are commonly used to remove calcium from milk in the manufacture of milk protein concentrate and can decrease calcium concentrate by 30-100% as compared to a control milk protein concentrate. The removal of calcium from milk and milk concentrates is used to improve functionality such as solubility and product stability (Bhaskar et al. 2004, Meena et al. 2017). The preferred ion-exchange process involves the use of strong acid cation- exchange resin in the sodium form (Bhaskar et al. 2003). Food acidulants such as

citric acid and tartaric acid are preferred chelating agents in removing calcium and the chelating agents are used preferably in conjunction with dialysis and/or ultrafiltration and diafiltration (Bhaskar et al. 2003). Removing calcium from milk using ion-exchange leads to a structural change in the milk colloidal system including dissociation of casein micelles and colloidal calcium phosphate (Xu et al. 2016), and the application of calcium reduced milk protein concentrate in dairy beverage formulations shows some benefits such as improved heat stability.

2.3 Biofilm- a consortium of bacteria

2.3.1 Definition

It is now recognized that most bacteria live in self-aggregated cell bundles known as biofilms (Stoodley et al. 2002, Flemming et al. 2016). Biofilms represent a stratified microbial community that usually constitutes one or several kinds of bacteria and self-produced matrix of extracellular polymeric substances (EPS). The bacteria are embedded in the EPS matrix, which is composed primarily of polysaccharides, protein and nucleic acids (Flint et al. 1997, Teh et al. 2015, Flemming et al. 2016). Biofilms can grow on biotic or abiotic surfaces, or even as a floating form, commonly referred to as a pellicle or floc (Donlan and Costerton 2002). Biofilm development consists of several stages including initial adhesion, growth and maturation, and eventually detachment (Hall-Stoodley et al. 2004). Initial attachment can be sequentially divided into reversible and irreversible attachment, which originate from bacterial cells overcoming the initial electrostatic repulsion to the substratum by cell appendages, surface-associated polymers and characteristics like cell surface hydrophobicity, and then adhesion becomes irreversible due to production of extracellular polymeric substances (EPS) and adhesins that complex with the substratum (Cappitelli et al. 2014). Thereafter is biofilm maturation, during which biofilms develop into a mature morphology, which includes either a flat or three-dimensional structure depending on nutrient source, temperature, fluid flow in contact with biofilm and the bacterial species (Flint et al. 1997, Hall-Stoodley et al. 2004, Bremer et al. 2015). Eventually, cells or cell bundles start to detach from the biofilm, actively or passively, through internal processes such as endogenous enzymatic degradation or perturbations from the passing stream (Hall-Stoodley et al. 2004, Cappitelli et al. 2014, Sadiq et al. 2017).

2.4 Effect of divalent cations on cell attachment and biofilm formation with particular emphasis on calcium

As illustrated in Figure 2-1, the role of divalent cations in biofilm formation is multi-faceted. The presence of divalent cations is thought to affect the initial attachment as a function of assisting in conditioning film formation, bridging between molecules, modifying cell surface adhesins and reducing apparent surface charge and surface potential. Cations also influence biofilm formation via physiochemical interactions, gene regulation, signal transduction and functioning as a protein component. Due to the attraction between divalent cations and negatively charged functional groups of EPS (Higgins and Novak 1997, Lattner et al. 2003, Mangwani et al. 2014), this constitutes a positive role in biofilm structural stability. In contrast, the intricate roles of divalent cations on the physiological functions of bacterial biofilms are reflected by electrolyte composition such as cation concentration and cation type as well as the bacteria species and strains.

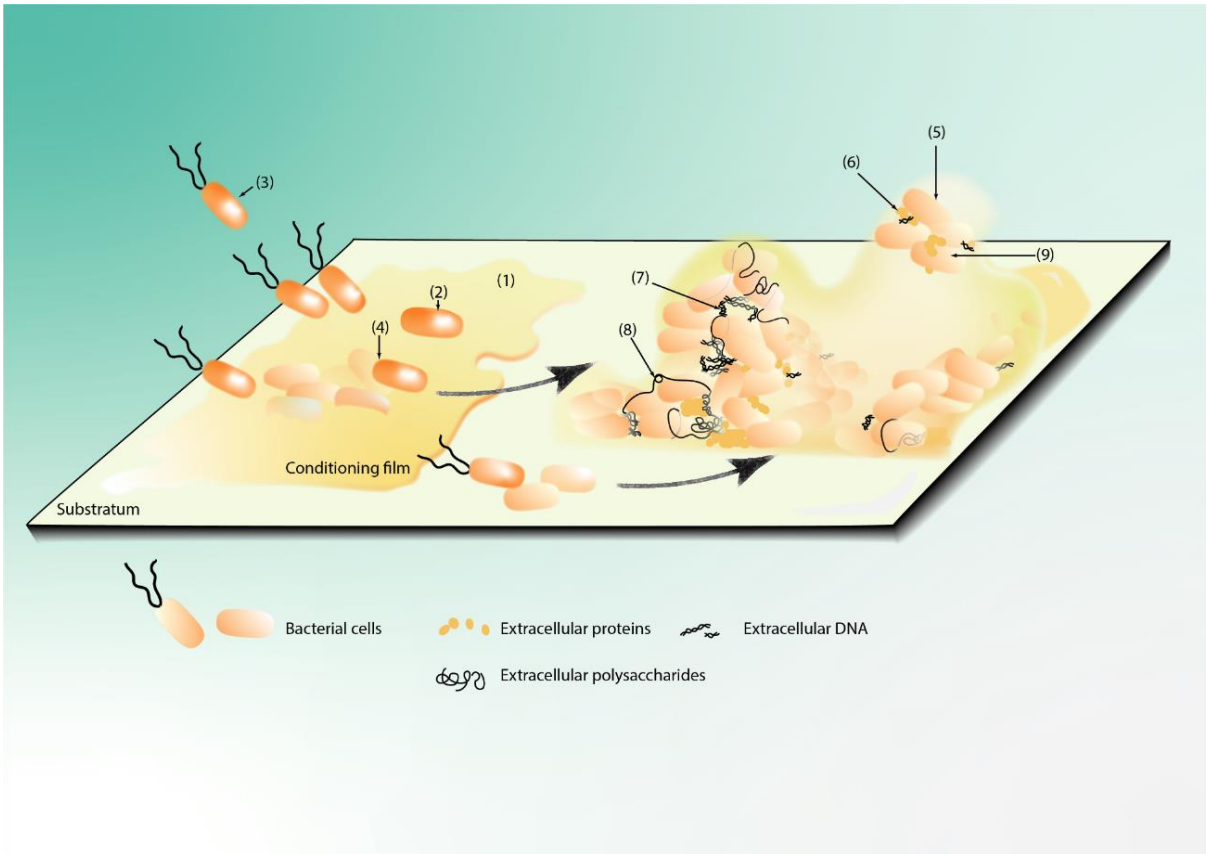

Figure 2-1 Mechanisms of divalent cations in participating bacterial cell attachment and biofilm formation.(1) to (4) indicate roles in initial cell attachment, (4) to (9) indicate roles in biofilm formation. (1) Assist in conditioning film formation; (2) Alter cell surface electrokinetic properties; (3) Modify cell surface adhesins; (4) Bridge molecular functional groups of cell surface. (5) Modify cell surface proteins; (6) Modify extracellular proteins; (7) Crosslink extracellular DNA; (8) Crosslink extracellular polysaccharides; (9) Regulate cell gene expression

2.4.1 Cell attachment

By interacting with ligands on the bacterial cell surface, divalent cations could reduce cell surface charge and potential, bridge molecules or rearrange cell surface structures, and consequently, alter the ability of bacteria to attach to a surface. Venegas et al. (2006) reported that increasing cell-hydroxyapatite particle aggregation is accompanied by an elevated calcium concentration in a dose-dependent manner for *Lactobacillus salivarius*, *Lactobacillus fermentum* and *Streptococcus mutans*, possibly due to the charge reversal and bridging through functional groups of hydroxyapatite particles and bacterial surfaces. Likewise, the adhesion kinetics of *Burkholderia cepacia* and *Halomonas pacifica* on a quartz surface were found to be markedly affected by calcium and the adhesion efficiency increases over calcium concentrations of 10 mM to 316 mM for *B. cepacian*. For *H. pacifica*, an increase in adhesion efficiency to a quartz surface is seen between calcium concentrations of 31.6 mM to 56 mM.

However, there are limits to the effect of increasing the ionic strength of calcium on adhesion for both bacteria. Chen and Walker (2007) suggested that the adhesion properties are sensitive to solution ionic strength, ionic valence and an electrosteric interaction associated with solution chemistry and cell surface polymers also participate in the process. The importance of Ca^{2+} for bacterial adhesins, reveals a structural function that upon binding to Ca^{2+}, protein structure is strengthened and this may facilitate the extension of protein from the cell surface to the substratum. Vance et al. (2014) proposed that upon Ca^{2+} binding to acidic residues, the rod-like structure of Region II of *Marinomonas primoryensis* antifreeze protein is strengthened and projects the ice-binding domain away from the bacterial cell surface to bind to ice. Ca^{2+} binding also influences the adhesin SiiE from *Salmonella enterica*, a rod-like habitus that reaches out beyond the lipopolysaccharide layer, to promote adhesion to epithelium host cells (Griessl et al. 2013).

Although, evidence indicates that the presence of calcium assists in bacterial attachment to surfaces, too much calcium may occasionally be detrimental to bacterial attachment. For example, Chen and Walker (2007) found that adhesion efficacy on quartz slightly decreases for both *B. cepacia* and *H. pacifica* when the ionic strength of calcium is approximately 1000 mM and it was suggested that repulsive steric interaction occurred at high ionic strength. Venegas et al. (2006) reported the aggregation between *Lactobacillus casei* cells and hydroxyapatite particles does not increase at high calcium concentration, as opposed to the observation in *S. mutans*, *L. salivarius* and *L. fermentum*. The inherent hydrophilic surface of *L. casei* may play a key role in this difference (Venegas et al. 2006).

Different theories have been used to describe the cell-substratum interaction such as Derjaguin-Landau-Verwey-Overbeek (DLVO) and extended Derjaguin-Landau-Verwey-Overbeek (XDLVO) theories (Hermansson 1999). The classic DLVO theory accounts for two interactions, which are Lifshitz- van der Waals force and electrostatic double layer interactions (Hermansson 1999). The Lifshitz-van der Waals represents attractive forces when two molecules are in proximity. The electrostatic double layer interaction describes the repulsive forces between charged colloidal particles. Briefly, the surface charge of a colloidal particle is balanced by a stern layer adjacent to its surface that is comprised of counterions via chemical interactions. Outside the stern layer, counterions attracted by a not fully neutralized surface

form a diffuse layer that asymptotically reaches the ion concentration of bulk solution as a function of distance. Generally, the electric double layers of the particles generate the repulsive force when the double layers incline to merge with each other as many colloidal particles and surfaces are negatively charged in the natural state. In addition to the classic DLVO theory, Van Oss (1991) completed the theory by adding acid-base interaction. Specifically, the acid-base interaction includes hydrophobic attraction and hydrophilic repulsion. Several researchers have used this theory to study the forces involved in the cell-substratum interaction qualitatively or quantitatively. Albeit XDLVO can be used reasonably to assess bacterial attachment, a lack of accuracy is observed (Subramani and Hoek 2008, Thwala et al. 2013), which is partially due to the fact that the theory was commonly used to characterize the colloidal system in which the colloidal particle surface is homogeneous. In fact, cell surface is a highly dynamic surface in response to changes in ionic strength, pH and the presence of macromolecules (Palmer et al. 2007).

Therefore, mechanisms inherent in the initial attachment of bacteria in the presence of calcium are complex. As reviewed by Geesey et al. (2000), charge neutralization, surface hydrophobicity, surface polymer production and structural effects, as well as bridging effects are all contributing factors in bacterial attachment. Consequently, it is important to consider these factors when analysing bacterial attachment.

Depending on the cation type and concentrations, divalent cations can also assist in forming conditioning films on a surface in the presence of biopolymers and in return facilitate bacterial attachment. De Kerchove and Elimelech (2008) tested the adhesion of *Pseudomonas aeruginosa* on quartz or an alginate-coated quartz substratum. As one of the most extensively studied extracellular polysaccharides in biofilms of mucoid *P. aeruginosa* strains (Davies and Geesey 1995, Tielen et al. 2005), alginate was used as the conditioning polymer. They noted that *P. aeruginosa* adhesion is facilitated by cation bridging interactions between high affinity sites on bacterial surface and substrata for non-motile cells. Alginate conditioning of a surface was observed to increase adhesion at low concentrations (less than 1 mM) for Ca^{2+}. Higher adhesion was observed on alginate-conditioned quartz than clean quartz. For motile bacteria, the adhesion onto an alginate-conditioned quartz was dependent on the structure and viscoelastic properties of alginate film in the presence of Ca^{2+}. Specifically, the layer thickness

of alginate conditioning film increases with increasing Ca^{2+} concentrations from 1 mM until 10 mM. These results suggest that calcium can organise structural changes in the alginate layer on the substrata that increase the adhesion of motile bacterial cells. This also highlights that cations not only affect bacterial surfaces but also affect a conditioning film on substrata. Conditioning films formed by protein and other extracellular polysaccharides are also thought to influence bacterial attachment (Palmer et al. 2007), but the interaction between Ca^{2+} and the conditioning films is less studied. Further research is required to investigate the effect of calcium on bacterial attachment in the presence of the conditioning film formed by protein, extracellular DNA and extracellular polysaccharide.

2.4.2 Biofilm formation

Different theories have been proposed to describe the cation effects on biofilm formation after initial cell attachment. The alginate theory was proposed to explain the role of cations in bioflocculation of activated sludge where Ca^{2+} may help to maintain the three-dimensional exopolymer matrix of a sludge floc by cross-linking alginate like exopolymers (Bruus et al. 1992). The mechanism of the structure maintenance has been extensively studied that the dimerization of two alginate chains is mediated by interaction between Ca^{2+} and guluronate residues of alginate. This structure is referred to as the egg-box model (Figure 2-2 (A)) (Sikorski et al. 2007). Unlike the specific binding of Ca^{2+} in the alginate theory, the divalent cation bridge theory refers to a non-specific binding. According to the divalent cation bridge theory, the divalent cations bind to the negatively charged functional groups within or between the EPS chains, which assists in aggregation of EPS and bacteria (Sobeck and Higgins 2002). Additionally, as a major component of EPS, various proteins have been found to participate in biofilm formation in the presence of calcium. Figure 2-2 illustrates the different methods adopted by bacteria in assembling biofilm structures.

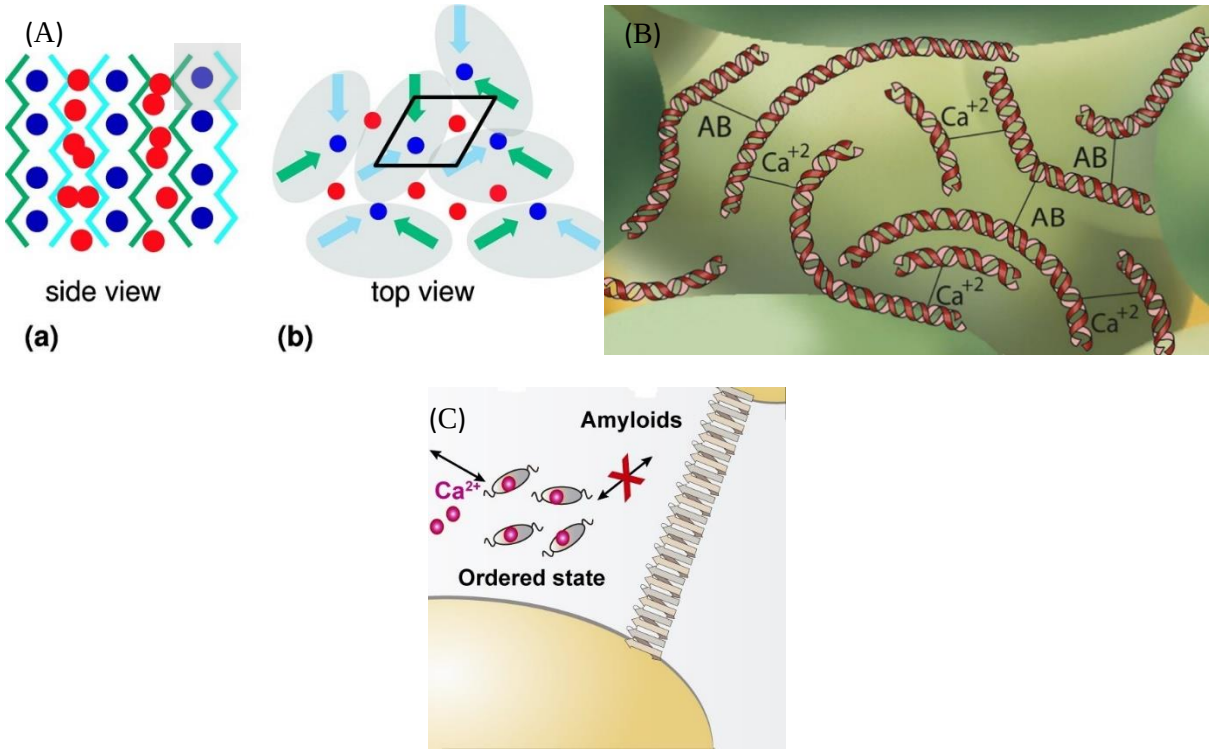

Figure 2-2 (A) Hypothesized model for the interaction between Ca^{2+}, Na^{+} and ***α***-L-guluronic acid (G)-rich alginate (based on egg-box model). Green and blue serrated chains (a) and arrows (b) represent G-rich alginate respectively. Blue dots and red dots represent $Ca^{2+}$and Na^{+} respectively. Egg-box-like dimers are indicated by light grey ellipses. A junction zone is indicated by light grey square. The junction zone in (A) represents the dimerization of random pairs of polymer chains, one Ca^{2+} cation is coordinated by four guluronate residues (two from each chain). Interactions between dimers are mediated by Na^{+}, Ca^{2+}, water molecules and hydrogen bonding and they are disorderly featured (Sikorski et al. 2007). Reprinted with permission from [Sikorski, P., F. Mo, G. Skjåk-Bræk and B. T. Stokke (2007). "Evidence for egg-box-compatible interactions in calcium− alginate gels from fiber X-ray diffraction." Biomacromolecules 8(7): 2098-2103.] Copyright [2007] American Chemical Society; (B) Cell aggregation as a function of acid-base interaction (AB) and calcium bridging effect of eDNA (Das et al. 2014). The green oval-shaped objects indicate cells. Adapted by permission from [PLOS]: [PLOS] [*PLOS ONE*] [Influence of calcium in extracellular DNA mediated bacterial aggregation and biofilm formation, Das, T, S. Sehar, L. Koop, Y. K. Wong, S. Ahmed, K. S. Siddiqui, M. Manefield], [2014]; (C) In the presence of calcium ions, Bap undergoes conformational change and stabilizes at a state that is unable to self-assemble into amyloid (spring- shaped object) and fails to mediate multicellular behaviour (Taglialegna et al. 2016). The beige oval-shaped objects indicate cells. Adapted by permission from [PLOS]: [PLOS] [*PLOS Pathogens*] [Staphylococcal Bap proteins build amyloid scaffold biofilm matrices in response to environmental signals, Taglialegna, A., S. Navarro, S. Ventura, J. A. Garnett, S. Matthews, J. R. Penades, I. Lasa, J. Valle], [2016]

2.4.3 Phenotypic characterization

Cation-induced phenotypic changes in bacterial biofilm can be reflected by changes in colony morphology, biofilm 3-D dimensional structure, total biomass, biofilm cell numbers and adhesion between biofilms and substratum, as well as cell aggregation.

In *Vibrio* species, Ca^{2+} (0-9.9 mM) increases the colony polysaccharide phase variation, a switch from one colony morphotype to another, and also promotes the formation of more biofilm biomass for all phase variant types of *Vibrio vulnificus*, Ca^{2+} may serve a dual function in biofilm formation of *V. vulnificus* by acting as a signal switch to a more biofilm-proficient phase and as a structural component of the resulting biofilm (Garrison-Schilling et al. 2011).

In *Pseudomonas mendocina*, 20 mM Ca^{2+} significantly increases the average thickness of the biofilm, roughness coefficient and surface area of biomass, increased EPS production in the presence of calcium is thought to be responsible for the enhancement in biofilm in the presence of Ca^{2+} (Mangwani et al. 2014). In *Pseudomonas fluorescens* biofilms, 1.5 to 15 mM of Ca^{2+} lead to more bio-volume and higher surface coverage. Atomic Force Microscope (AFM) probes, measuring the forces involved as an AFM probe touches and then moves away from the biofilm showed larger adhesive values at the surface of biofilm at 5 and 15 mM compared to 0 mM Ca^{2+} concentration. These trends are consistent with production of more EPS with the increasing Ca^{2+} concentration (Safari et al. 2014). At 0.5 mM of Ca^{2+} biofilms of *Shewanella oneidensis* demonstrated increased biofilm coverage, whereas roughness decreases accordingly (Zhang et al. 2019). Calcium is also found to increase the biofilm biomass of *Pectobacterium carotovorum* subsp. carotovorum (Haque et al. 2017), and biofilm cell numbers of *Geobacillus* spp. (Somerton et al. 2015). Somerton et al. (2015) reported a reduced biofilm formation of three *Geobacillus* spp. in a calcium-reduced milk formulation compared to that in milk formulation with higher amount of total calcium and lower amount of sodium. Supplementation of $CaCl_2$ to a calcium reduced milk formulation significantly increased the biofilm formation of *Geobacillus* spp., and the underlying mechanism was thought to be the electrostatic interactions of cations on biofilm structure or physiological effects of calcium ions on bacterial cells (Somerton et al. 2015).

Calcium can also shape biofilm structure in urinary catheters by forming a crystalline insoluble deposit. The formation of the crystalline biofilm in indwelling urinary catheters is closely associated with the Ca^{2+} and Mg^{2+} in the urine (Morris et al. 1999). Urease producing bacteria, such as *Proteus mirabilis*, are able to elevate the pH of the urine by enzymatic hydrolysis of the urea, which induces the formation of struvite (ammonium magnesium phosphate

hexahydrate) and the poorly crystalline form of calcium phosphate, hydroxyapatite (Morris et al. 1999). Aggregates of this deposit accumulate in the biofilm that develops on the catheter surfaces (Stickler 2008). Aggregates of microcrystals were also found to facilitate biofilm formation by forming a crystalline foundation layer on the catheter surface in early stages of biofilm formation where bacterial colonization takes place (Stickler and Feneley 2010). A similar biological process is found in biomineralization of *B. subtilis* biofilm, where calcium carbonate formation is biologically induced and controlled in biofilms (Reddy 2013, Keren-Paz et al. 2018). When colonies of *B. subtilis* were grown on calcium supplemented medium agar, the calcium carbonate layer spreads and covers more of the colony area as the colony ages and the layer limits the diffusion of fluorescein (Keren-Paz et al. 2018). Hence, the mineral scaffolds lead to complex biofilm morphology.

Although, the presence of Ca^{2+} generally enhances biofilm formation, Ca^{2+} has been reported to be inhibitory to biofilm formation, but this inhibitory effect can be concentration dependent or bacterial growth-stage dependent. Zhou et al. (2013) found that biofilm biomass, determined by crystal violet staining, of *Enterobacter cloacae* is enhanced in the presence of 32, 64 and 128 mM Ca^{2+}. However, at 256 mM of Ca^{2+}, the biofilm biomass was reduced to levels observed when *E. cloacae* biofilms are grown in the presence of 0-4 mM of Ca^{2+}. Ye et al. (2015) studied the effect of Ca^{2+} on 23 strains of *Cronobacter sakazakii* and showed $CaCl_2$ (concentration 0.25-1.5%) initially increases biofilm formation at low concentrations but inhibits biofilm formation of most strains at 1.5% concentration. El Khatib et al. (2017) studied *Providencia stuartii* and reported that Ca^{2+} (1 mM to 50 mM) has an inhibitory effect on both cell attachment of the stationary phase cells (20 mM to 50 mM) and biofilm genesis (biofilm induction) of the lag phase cells (20 mM to 50 mM), but results in a consolidation (the increase of cell viability) of the pre-formed biofilm (1 mM to 50 mM).This suggests the role of Ca in bacterial attachment and biofilm growth and maintenance of *P. stuartii* may be dependent on the growth phase of the bacteria. Therefore, the effect of Ca^{2+} ions depends on the bacterial species or strain and concentration of Ca^{2+}. It would be interesting to quantify the strain variability in biofilm formation under different concentrations to fully understand the effect of calcium on the heterogeneity and diversity of biofilm.

2.4.4 Extracellular polysaccharide mediated biofilm formation

By interacting with negatively charged functional groups in polysaccharides, divalent cations help to interlink polysaccharides and stabilize biofilm structure (Wloka et al. 2004, Sarkisova et al. 2005). In addition, Ca^{2+} can act specifically with negatively charged cell associated polysaccharides to induce cell aggregation in biofilm formation (Kierek and Watnick 2003).

Ca^{2+} has been found in biofilm structures in various bacterial species. Analysis of the extracellular polysaccharide composition of *Halomonas* spp. TG39 showed that it is intrinsically-associated with Ca^{2+} (Gutierrez et al. 2012). Exopolysaccharides extracted from *S. mutans* and *Lactobacillus rhamnosus* cultures show high binding affinity towards calcium (Astasov-Frauenhoffer et al. 2017). The binding could be mediated by cations between bound extracellular polysaccharide (polysaccharides that are bound with cells) or soluble polysaccharides (polysaccharides that are weakly bound with cells or dissolved into solutions). Kierek and Watnick (2003) suggested that Ca^{2+} may be interacting directly with the negatively charged phosphate groups in *O*-antigen polysaccharides that are anchored on the outer cell membrane of *Vibrio cholerae*, and maybe involved in the construction of salt bridges between cells or within cells.

The cation-extracellular polysaccharide interaction has been studied using gel rheology and binding specificity of alginate. The structure of *P. aeruginosa* EPS can be explained using rheology. The EPS tends to form a weakly cross-linked temporary network which is stabilized by mechanical interference caused by entanglements between polymer molecules. The addition of calcium ions, results in more stable cross-linking points formed by coulomb or electrical interactions, stabilizing biofilm networks, which can immobilize bacteria in such a system (Wloka et al. 2004). A ^{13}C-NMR study of the interaction between alginate and divalent cations revealed that Ca^{2+} preferentially binds to mannuronate-guluronate (MG) pairs, within the alginate structure. The bridging effect of Ca^{2+} (0 mM to 2.6 mM) between MG of adjacent alginate chains may result in gelation of alginate (Lattner et al. 2003). An in vitro study consolidated the role of Ca^{2+}-alginate interaction in supporting biofilm structure. Sarkisova et al. (2005) studied the effect of Ca^{2+} on the extracellular matrix of *P. aeruginosa* biofilms, using an alginate overproducing (mucoid) isolate, and observed that the addition of Ca^{2+} (1 mM and

10 mM) results in biofilms that are 10-fold thicker and concluded that the addition of Ca^{2+} affects the structure and extracellular matrix composition of a mucoid strain of *P. aeruginosa*.

2.4.5 Protein and extracellular DNA mediated biofilm formation

The roles of Ca^{2+} in bacterial macromolecules are not only in extracellular polysaccharides but also in proteins and eDNA, which are also important in biofilm formation (Flemming and Wingender 2010). Upon binding cations, biofilm associated proteins often undergo conformational changes that result in a functionality switch in EPS matrix formation or biofilm cell aggregation.

Various researchers have observed a protein mediated inhibitory effect caused by Ca^{2+} in *Staphylococcus aureus* biofilms. In *S. aureus*, biofilm formation is inhibited at low concentrations of Ca^{2+} (0.1-2.0 mM) (Lee et al. 2016). Similar observations were observed by Arrizubieta et al. (2004), where biofilm formation of Bap (biofilm-associated protein) containing *S. aureus* is supressed by the addition of 6-10 mM Ca^{2+} to the growth medium. The addition of Ca^{2+} results in a conformational change in the Bap protein, which compromises its ability in assisting biofilm formation, suggesting the Bap protein is highly dependent on calcium. Upon calcium binding, Bap undergoes tertiary conformation rearrangement, which impedes the self-assemblage of protein molecules and further impairs biofilm development (Figure 2-2 (C)) (Taglialegna et al. 2016). Apart from Bap, other protein-mediated mechanisms have been found to be inhibited by calcium. Abraham et al. (2012) observed that for specific strains of *S. aureus*, a thicker biofilm is formed in the presence of Ca^{2+}-chelating agents possibly because protein clumping factor B (ClfB) activity is inhibited when it binds to Ca^{2+}. The inhibitory effect of $Ca^{2+}$was also observed in *P. fluorescens*. Ca^{2+} binding is required for the activity of a periplasmic cysteine protease LapG in *P. fluorescens* and the active LapG cleaves the adhesion factor LapA resulting in loss of ability to form a biofilm (Boyd et al. 2012).

Other bacteria, including *Rhizobium leguminosarum, S. oneidensis, Streptococcus mutants* and *Pseudomonas putida*, were also found to adopt a protein-mediated method in assembling biofilms. However, unlike *S. aureus*, the presence of Ca^{2+} induces biofilm formation in *R. leguminosarum, S. oneidensis* and *P. putida* (Theunissen et al. 2010, Martinez-Gil et al. 2012,

Abdian et al. 2013). Ca^{2+} accelerates the biofilm development of *P. putida* and increases the total biomass of biofilm. The C-terminal region of LapF protein, a large secreted protein involved in microcolony formation and biofilm maturation in *P. putida*, undergoes multimerization by binding Ca^{2+}, which is localized, mainly between the biofilm cells. The addition of a calcium chelator EGTA dissolves the aggregates (Martinez-Gil et al. 2012). In the presence of calcium ions, RapA2, a protein from *R. leguminosarum*, folds from a molten globule state into a compact structure that recognizes and binds exopolysaccharides (Abdian et al. 2013), resulting in the development of a biofilm matrix on the roots of plants. Various other calcium-binding proteins that are involved in biofilm formation have being reported and are listed in Table 2-2.

Table 2-2 Calcium binding proteins involved in bacterial biofilms.

Bacteria	Protein	References
Salmonella	BapA	(Guttula et al. 2019)
S. mutants	GbpC	(Mieher et al. 2018)
S. oneidensis	BpfA	(Theunissen et al. 2010)
Streptococcus	AspA	(Hall et al. 2014)
Vibrio vulnificus	CabA	(Park et al. 2015)
S. aureus	SdrD	(Roman et al. 2015)

In addition, to cementing biofilm structures, calcium ions act cooperatively with eDNA to modulate biofilm structures. eDNA promotes aggregation of *Aeromonas hydrophila, S. aureus* and *Enterococcus faecalis*. For example, Das et al. (2014) demonstrated that in the presence of 1 mM calcium and exogenous DNA, *A. hydrophila*, *S. aureus* and *E. faecalis* exhibited increased cell aggregation. However, limited research investigated the interaction between eDNA, calcium and biofilm formation. Therefore, more insight is needed to explain the interaction between divalent cations and eDNA in supporting biofilm structure.

Research addressing the interaction between cations and components of the EPS matrix provides invaluable insights on how the most frequently encountered environmental factors affect the biofilm matrix. Manipulating the cation concentration, in combination with other strategies, could offer new methods in control biofilm development.

2.4.6 Signal transduction and gene regulation

As a biofilm is a biological system, cations can also play a regulatory role in tuning bacterial physiological functions. For example, a positive correlation was established between the extracellular Ca^{2+} (0-2.5 mM) with *Xylella fastidiosa* biofilm formation and cell adhesion force (2 mM Ca^{2+}). Surprisingly, when *X. fastidiosa* cells are inactivated by pre-treating with tetracycline, the adhesion forces in Ca^{2+}-supplemented medium and non-supplemented medium show no significant difference (Cruz et al. 2012). These results indicate that Ca^{2+} could induce a physiological change in the cell and alter the biofilm potential of *X. fastidiosa*. The effect of Ca^{2+} and Mg^{2+}can be explained by signal transduction and gene regulation in the presence of cations. *P. aeruginosa* is an opportunistic pathogen that causes chronic biofilm infections (Jennings et al. 2015). It has been used to study bacterial biofilms, arguably due to its clinical relevance (Vlamakis et al. 2013). Calcium chelation was also found to trigger gene expression in *P. aeruginosa* biofilms. For example, calcium chelation by alginate triggers the Type III secretion system (T3SS, a conserved virulence mechanism for cytotoxicity and immune evasion in Gram-negative bacteria) (Horsman et al. 2012). The effects of Ca^{2+} on various other bacteria in regulating biofilm formation are outlined in Table 2-3.

The knowledge of how calcium ions exert their physiological function in biofilm may enable researchers to understand biofilm formation mechanisms and suggest potential control strategies

Table 2-3 The effect of calcium ions on the bacterial biofilm physiology

Bacteria	Cations	Concentrations	Effects on representative genes, regulatory proteins and functional proteins	References
V. vulnificus	Ca^{2+}	0-10 mM	Ca^{2+} regulates *brp* expression via a feed-forward signalling circuit.	(Chodur et al. 2018)

Table 2-3 continue

Bacteria	Cations	Concentrations	Effects on representative genes, regulatory proteins and functional proteins	References
Aggregatibacter actinomycetemcomitans	Ca^{2+}	0-1 mM	*pga* gene expression is increased.	(Hisano et al. 2014)
P. aeruginosa	Ca^{2+}	0-10 mM	PhoPQ and PmrAB two-component systems were downregulated by Ca^{2+}.	(Guragain et al. 2016)
P. aeruginosa	Ca^{2+}	0-10 mM	Proteins involved in iron acquisition, post-translational modification and proteins associated with cellular stress are differentially expressed.	(Patrauchan et al. 2007)
Vibrio fischeri	Ca^{2+}	0-10 mM	Sensor Kinases BinK, RscS, SypF, HahK and response regulator SypG are involved in the calcium-dependent biofilm formation.	(Tischler et al. 2018)
X. fastidiosa	Ca^{2+}	0-4 mM	266 genes are differentially expressed over time.	(Parker et al. 2016)
Pseudomonas syringae	Ca^{2+}	0-2 mM	Cvs inversely regulated biofilm formation in a Ca^{2+}-dependent manner.	(Fishman et al. 2017)

Table 2-3 Continue

Bacteria	Cations	Concentrations	Effects on representative genes, regulatory proteins and functional proteins	References
Citrobacter werkmanii	Ca^{2+}	0-1000 mM	48 proteins are differentially expressed under 12.5 mM Ca^{2+} in biofilm, while in the presence of 400 mM Ca^{2+}, 58 and 45 proteins are differentially expressed in biofilm around the wall and in the bottom respectively.	(Zhou et al. 2016)
***Pseudoalteromonas* sp.**	Ca^{2+}	0.25-10 mM	Calcium affects gene expression of 39 proteins including biopolymer transport protein and outer-membrane protein	(Patrauchan et al. 2005)
V. cholera	Ca^{2+}	0-10 mM	A new two-component regulatory system, CarRS, was identified and it negatively regulates biofilm formation to an external Ca^{2+}.	(Bilecen and Yildiz 2009)

Table 2-3 Continue

Bacteria	Cations	Concentrations	Effects on representative genes, regulatory proteins and functional proteins	References
S. mutans	Ca^{2+}	0-0.25 mM	VicK of a two-component system was suggested to be responsible for sensing calcium ions. Calcium ions enhances cell lysis and eDNA release and subsequent biofilm formation.	(Jung et al. 2017)
Flavobacterium columnare	Ca^{2+}	0-4.5 mM	441 genes are differentially expressed due to combined effect of live stage and calcium.	(Cai et al. 2019)

Note: the concentrations are relative concentrations compare to control groups

2.4.7 Practical implications

The effects of calcium in cell attachment prompted researchers to explore potential applications such as facilitating probiotic settlement in the gut and counteracting cell attachment of dental caries related species by metal chelating agents. Probiotics research has shown beneficial effect of divalent cations in facilitating intestine attachment of probiotic bacteria. The presence of 10 mM Ca^{2+} ions significantly increase the binding of lactobacilli cells to IPEC-J2 cells. Although the adhesion mechanism is not explained, the fact that non-viable cells also show similar levels of adhesion in the presence of calcium, indicates that calcium-dependent adhesion is not related to signal transduction (Larsen et al. 2007). As an adhesin-mediated model of *Salmonella* cells on intestinal epithelium was suggested (Griessl et al. 2013), a similar calcium-dependent surface adhesin may also participate in lactobacilli adhesion. The effect of calcium has been extensively studied in dentistry as biofilms formed on the teeth surfaces cause dental caries

(Selwitz et al. 2007). Badihi Hauslich et al. (2013) tested the adhesion of several oral bacteria on the surface of Ti implants in the presence of Ca^{2+} and EGTA and noted Ca^{2+} pre-conditioned Ti surfaces increase cell attachment of *S. mutans* and *Fusobacterium nucleatum* but not *Porphyromonas gingivalis*. In addition, EGTA causes a significant decrease in the adhesion of *S. mutans*. This indicates that Ca^{2+} may serve as a bridging agent in the adhesion of bacteria to Ti surfaces, possibly mediated by cell surface protein.

In biological wastewater treatment, pollutants are removed by bacterial aggregation. Calcium is involved in this process by bridging negatively charged functional groups on extracellular biopolymers or microbes. The addition of Ca^{2+} to water treatment system was found to be an effective approach in improving performance. Ca^{2+} was shown to increase the biomass attachment at 20 mg/L in an aerobic granular activated sludge and flocculent activated sludge (Hao et al. 2016). Liu et al. (2015) found that the total biomass increases with increasing Ca^{2+} concentration and the total protein content of biofilm also increases accordingly. Similarly, biofilm from hybrid constructed wetland (a system for waste water treatment) forms a more stable structure in the presence of Ca^{2+} (Sehar et al. 2016).When monitoring biofilm in denitrifying fluidized bed bioreactors, biofilm in a synthetic municipal wastewater with a Ca^{2+} concentration higher than 120 mg/L shows significantly lower detachment rates than that in 20 mg/L and 60 mg/L, as reflected by the amount of biomass leaving the reactor effluent per total amount of attached biomass in the reactor. With lower Ca^{2+} concentrations, the specific denitrification rate drops by 20-40% (Eldyasti et al. 2013). Biofilm detachment rate is lower in wastewater that has high Ca^{2+} concentration, indicating improvement in physical strength of biofilm (Eldyasti et al. 2014). In reverse osmosis, the EPS conditioning film secreted by wild type *P. aeruginosa* increases both the adsorption and rigidity of biofilm in the presence of Ca^{2+}, whereas no change was detected for EPS from the Δ*psl* mutant. This is possibly due to the high amounts of positively charged Pel polysaccharides in EPS compared to the wild type strain (Ferrando et al. 2018). When performing wastewater treatment using forward osmosis, water flux decline is more severe because of a thicker, denser biofilm formed on the membrane when wastewater contains high Ca^{2+}. The characterization of the biofilm revealed that extra Ca^{2+} does not increase the cellular bio-volume (space taken up by cells in a biofilm), thus the changes in biofilm are mainly due to the EPS (Xie et al. 2015). These findings support the equivocal role of Ca^{2+} in water treatment, in which the biofilm aggregation is either favoured or undesired. The results of these findings could be applied to a real situation and improve

performance efficiency of water treatment and reduce the cost. By studying the effect of divalent cations on activated sludge performance, the results suggest that the divalent cation bridge theory is a useful tool in assessing activated sludge performance, thus assisting in evaluating chemical-addition strategies (Higgins et al. 2004).

The presence of EPS within a biofilm structure provides a diffusion barrier for antimicrobial molecules, which enables the biofilm to be more resilient in unfavourable environments (Donlan and Costerton 2002). Therefore, disrupting the biofilm by removing divalent cations weakens the intact EPS structure and disrupts the biofilm. For example, Cavaliere et al. (2014) found that the addition of 25 mM EDTA to preformed biofilm results in a thinner and less structured biofilm in both static and flowing systems. In addition, the presence of EDTA enhanced the efficacy of antibiotic treatment. Akpolat et al. (2003) reported that in the presence of EDTA, the slime production of *Staphylococcus epidermidis* strains is significantly reduced, this could be explained by chelation of free divalent cations in the environment. In an attempt to inactivate *P. aeruginosa* biofilm, Banin et al. (2006) found that EDTA increases the efficacy of gentamicin, possibly by chelation of divalent cations within biofilm and increasing cell permeability by chelating magnesium associated with LPS.

2.5 The effect of calcium on bacterial spores

The spores of thermophilic bacilli are common contaminants of dry dairy products and dairy manufacturing plants (Burgess et al. 2010). And cations, including calcium, were reported to affect spore production (Wells-Bennik et al. 2019), and spore heat resistance of *G. stearothermophilus* (Bender and Marquis 1985).

2.5.1 Definition

Bacterial endospores, or spores, are metabolically dormant life forms that enable bacteria to survive unfavourable environmental conditions such as heat, chemicals and irradiation (Seale et al. 2015). Among hundreds of Gram-positive spore-forming species, *Geobacillus* is a sporeformer of significance in foods and of particular research interest in the dairy industry (Seale et al. 2015, Setlow and Johnson 2019).

Spores are produced through sporulation, which has been extensively studied based on a model organism of *B. subtilis* (Setlow and Johnson 2019). The first morphological change observed during sporulation is the creation of a forespore compartment and the larger mother cell compartment. The forespore is then engulfed by the mother cell and eventually the mother cell lyses (Seale et al. 2015, Setlow and Johnson 2019). The spatiotemporal expression of genes present during sporulation occurs at locations of the mother cell and forespore (Setlow and Johnson 2019). This expression of sporulation genes acts through a phosphorylation cascade to activate several sigma factors and a master transcription factor, *spo0A* (Seale et al. 2015). As illustrated in Figure 2-3, a multi-layered structure is observed in bacterial spores. The outermost layer of a spore is the exosporium. The exosporium is composed of several macromolecules including lipids, carbohydrates and proteins. The emporium acts to exclude large molecules including antibodies, influencing spore surface adhesion and playing a role in spore pathogenesis. For example, exosporium proteins of *Clostridium difficile* spores such as cytosolic elongation factor and α-enolase are possibly involved in pathogenesis that aid in the evasion of spores from host's innate immunity (Díaz-González et al. 2015). It is known that *Geobacillus* spores of dairy isolates possess an exosporium (Seale et al. 2010). Underneath the exosporium are spore coats, which are primarily composed of proteins (Leggett et al. 2012). These proteins are predicted or known to act as enzymes, facilitate nutrient germinant access to receptors, and play a role in the assembly of another protein in the coat (Setlow and Johnson 2019). The major protective effects of spore coats include acting against lytic enzymes and chemicals (Setlow and Johnson 2019). The sensitivity of *B. subtilis* spores with coat defects increases when exposed to lysozyme (Klobutcher et al. 2006). Underlying the spore coat is the outer spore membrane, this structure is thought to play an essential role in spore formation, during which the outer prespore membrane functions as a localization site for protein-protein interactions (Piggot and Hilbert 2004). However, the function of the outer spore membrane remains elusive (Leggett et al. 2012). The inner structures are sequentially the cortex and the germ cell wall. Both structures are composed of structurally distinctive peptidoglycan (Setlow and Johnson 2019). In comparison to vegetative-cell peptidoglycan, the spore cortex peptidoglycan lacks teichoic acids from the *N*-acetylmuramic acid residues, and the shortage of peptide side chains of *N*-acetylmuramic acid residues prevents the formation of peptide cross-links (Leggett et al. 2012). Unlike peptidoglycan in the cortex, the germ cell wall peptidoglycan appears to be identical to that of the vegetative-cell peptidoglycan (Setlow and

Johnson 2019). The innermost structure, the spore core is covered by a layer of inner membrane (Leggett et al. 2012). The inner membrane is impermeable to most molecules and protects the spore core (Seale et al. 2015, Setlow and Johnson 2019). The spore core contains DNA, calcium dipicolinate (CaDPA) and small acid-soluble proteins. CaDPA replaces much of the water of the spore core and renders the core as the most dehydrated region in the spore (Seale et al. 2015, Setlow and Johnson 2019).

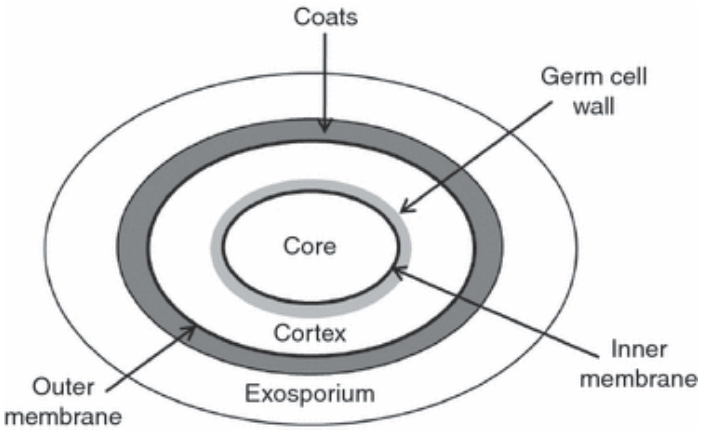

Figure 2-3 A representative structure of bacterial spore. Core is surrounded by inner membrane, germ cell wall, cortex, outer membrane and coat. The presence of exosporium is species specific. The relative proportions between structures do not represent real scale (Leggett et al. 2012)

2.5.2 Effect of calcium on sporulation and spore heat resistance

Cazemier et al. (2001) showed that *Bacillus* spores produced from medium containing Ca^{2+} (1.36 mM), Mg^{2+}, Mn^{2+}, Fe^{2+} and K^{+} are more heat resistant than those from medium containing Mn^{2+} only. The difference in spore heat resistance can be correlated to water content in the spore protoplast, with lower water content resulting in greater heat resistance. In addition, lower water content was observed in spores from medium with a mixture of ions of Ca^{2+} (1.36 mM), Mg^{2+}, Mn^{2+}, Fe^{2+} and K^{+} (Cazemier et al. 2001). High spore heat resistance conferred by Ca^{2+} (1 mM), Mg^{2+}, Mn^{2+} and K^{+} was also observed in strains of *G. stearothermophilus* (Wells-Bennik et al. 2019). It is thought that higher core mineralization leads to decreased core water content, and results in stronger wet heat resistance (Setlow and Johnson 2019). Ca^{2+} confers a higher wet heat resistance in spores than other cations (Leggett et al. 2012). However, the

correlation between calcium concentration of sporulation media and spore heat resistance of *G. stearothermophilus* is still unknown.

2.5.2.1 Sporulation

In the production of spores, an increased spore yield and higher sporulation rate were observed in the presence of calcium for *Clostridium botulinum* and *Clostridium sporogenes*. The addition of 10-, 100- and 200-fold increases in calcium concentration to a defined minimal medium slightly increased the sporulation rate of *C. botulinum* (Lenz and Vogel 2014). Spore yield (the total amount of spores) also benefits slightly from the addition of calcium. The addition of $CaCl_2$ (0.5%) to a basal medium results in a slight but not significant increase in spore numbers of *C. sporogenes* within three weeks of incubation compared with the addition of $MgCl_2$ and KCl (Mah et al. 2008). Wells-Bennik et al. (2019) tested the sporulation of *G. stearothermophilus* on several different media and found the highest spore yield is obtained on two cation supplemented media, namely nutrient agar supplemented with 1.13 mM $CaCl_2$ and 1 mM $MnSO_4$ or nutrient agar supplemented with 0.13 mM $MnSO_4$, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 13 mM KCl. In contrast, calcium deficiency in sporulation medium markedly reduces the spore formation of *B. subtilis* (Minh et al. 2011). Slepecky and Foster (1959) revealed that lack of calcium in a chemically defined liquid medium diminishes sporulation of *Bacillus megaterium*. Minh et al. (2011) also showed that excluding $CaCl_2{\cdot}2H_2O$ (0.1 g/L) from a medium extends the sporulation time of *B. subtilis* and results in a reduced final spore number compared to the standard medium. In conclusion, the effect of calcium in promoting spore yield and sporulation rate of various spore-forming bacteria has been established by previous studies. However, the effect of calcium on sporulation within a biofilm is unknown.

2.5.2.2 Heat resistance

The heat resistance of spores is a problem for the food industry (Setlow and Johnson 2019). Heat resistance is often quantified by *D* value, which is expressed as the time needed to inactivate 90 % of spore population at a specific temperature (Setlow and Johnson 2019). A positive correlation has been established between available calcium during sporulation and spore heat resistance. Slepecky and Foster (1959) observed that the heat resistance of spores of *B. megaterium* can be related to the increase in calcium concentration (from 0.02 mg/50 mL to

36.1 mg/50 mL) in sporulation media, and spores with high calcium content showed high heat resistance. Levinson and Hyatt (1964) observed that supplementation of a sporulation base medium containing approximately 0.25 mM calcium with 0.5, 1 or 2.5 mM $CaCl_2$ increases the total calcium content of spores of *B. megaterium*. A similar correlation between calcium content of sporulation media and the total calcium content of spores was also observed in a synthetic medium. Spores produced on calcium supplemented basal media show higher heat resistance than those produced on non-supplemented medium. Similarly, Fleming and Ordal (1964) showed that the addition of 50 mM calcium to sporulation medium causes greater uptake of calcium in spores and heat resistance of *B. subtilis*. Surprisingly, the addition of calcium was also found to entirely or partially compensate for lowered heat resistance due to NaCl supplementation. Bender and Marquis (1985) produced demineralized spores (H forms) using acid titration that converted demineralized spores to various desired salt forms (Na, K, Mg, Mn and Ca forms) by back titration. A consistency was found between *B. megaterium* and *B. subtilis niger* in that H forms are more heat sensitive than native forms, while remineralization of spores with various base solutions results in an increase of heat resistance except for the Na forms. Ca forms confer the highest heat resistance among various salt forms. Additionally, there is a progressive increase in heat resistance with increasing calcification of spores (Bender and Marquis 1985). Evidence accumulated by these studies suggests that calcium present during sporulation of several *Bacillus* species seems to accumulate in spores during spore formation. As a result, the highly mineralized spores exhibit high heat resistance. A similar effect of calcium on spore heat resistance was also observed in *G. stearothermophilus* and *C. botulinum* type E spores (Bender and Marquis 1985, Lenz and Vogel 2014). Therefore, contrary to supplementing calcium during sporulation, calcium deficiency during sporulation sensitizes spores of *B. subtilis* to heat (Minh et al. 2011).

Hitherto, limited information is known regarding the effect of milk formulations and cations on spore production and spore heat resistance of *G. stearothermophilus* dairy isolates. Therefore, addressing this potential effect could provide evidence to explain the reduced thermophile contamination in calcium-reduced milk protein powder observed in industry settings.

2.6 Overall summary

Calcium has been reported to play a role in bacterial attachment (Somerton et al. 2013), biofilm formation (Somerton et al. 2015), spore production (Lenz and Vogel 2014), as well as spore heat resistance (Levinson and Hyatt 1964). However, our current knowledge of the role of calcium in milk formulations is insufficient to explain the industrial observation that calcium-reduced milk protein powder shows less thermophilic spore-former contamination than normal milk protein powder. Thus, research needs to be done to investigate the effect of calcium and milk formulations on different stages of biofilm development to pinpoint the possible reasons for the industrial observation.

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Chapter 3 Heterogeneous response of *G. stearothermophilus* biofilms to calcium

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3.1 Introduction

Thermophilic bacilli are common contaminants in dairy manufacturing plants that may contaminate milk powders (Burgess et al. 2010). A sampling regime carried out by Ronimus et al. (2003) across several New Zealand's milk powder factories showed that, among 1470 thermophilic isolates from milk powder, 98% were assigned to *G. stearothermophilus*, *A. flavithermus*, *B. licheniformis* and *B. subtilis*. Burgess et al. (2014) isolated 33 thermophiles from an evaporator of a milk powder plant, 29 of these belonging to the genus *Geobacillus*. Sadiq et al. (2017b) examined the biofilm forming capacity of thermophilic bacteria on stainless steel in skim milk and found dairy isolates of *G. stearothermophilus* showed a strong propensity to form biofilms. *G. stearothermophilus* also forms biofilms on polystyrene surfaces (Cihan et al. 2017). During biofilm formation and within mature biofilms in skim milk powder plants, sporulation occurs and leads to large numbers of spores present within the biofilm (Burgess et al. 2014). Spores of some *G. stearothermophilus* isolates were found to withstand treatment in alkaline and acid solutions, which are commonly used for cleaning-in-place in dairy manufacturing plants (Wedel et al. 2019). As a result, *G. stearothermophilus* biofilms on the surface of stainless steel equipment can disperse into the process flow and cause contamination of the final product (Seale et al. 2015). A typical example is foulant on the surface of milk powder plant. Scott et al. (2007) examined the foulant on the surface of a direct steam injection unit and evaporator and the isolates from foulant samples were identified as *Geobacillus* spp.

Bacteria live predominantly in a sessile life form, known as biofilm (Stoodley et al. 2002, Cappitelli et al. 2014). Biofilms represent a community of microorganisms that attach to, grow and multiply on a surface, comprising of microbes, self-generated extracellular polymeric substances such as polysaccharides, DNA, lipids and proteins (Flint et al. 1997, Bremer et al. 2015, Jakobsen et al. 2017, Sadiq et al. 2017a).

Calcium is naturally present in the milk (Fox 2003), and the effect of calcium has been shown on both sessile and motile forms of *G. stearothermophilus* cells. Somerton et al. (2013) showed that *G. stearothermophilus* cells grown in calcium supplemented media have higher numbers of attached cells on stainless steel than cells grown in medium without calcium

supplementation. Compared to *G. stearothermophilus* biofilms cultured in normal reconstituted milk protein concentrate, lower cell numbers were found in biofilms cultured in a cation-modified counterpart, in which calcium was exchanged for sodium (calcium reduced milk protein concentrate). In return, the addition of calcium to the calcium reduced milk protein concentrate increased the biofilm cell numbers (Somerton et al. 2015). However, the underlying mechanism of the effect of calcium on *G. stearothermophilus* biofilm formation remains elusive.

The total calcium content in original and calcium reduced reconstituted milk protein concentrate is 35 mM and 7 mM respectively with the identical amount of free calcium that is 0.4 mM (Somerton et al. 2015). Total calcium content in other dairy products is close to 35 mM. The total calcium content in milk can vary between approximately 36 mM to 43 mM and is dependent upon the cows' diet (Akkerman et al. 2019). Commercial dairy products such as skim milk and UHT milk contain approximately 28 mM and 30 mM total calcium respectively (Ünal et al. 2005, Camara et al. 2019). As the total solids typically increase from 10% to 50% during evaporation in milk powder processing (Verdurmen and de Jong 2003), the calcium concentration increases accordingly.

This study examines the biofilm formation of *G. stearothermophilus* isolates under different calcium concentrations by investigating biofilm cell numbers and EPS production and provides new information on the response of *G. stearothermophilus* biofilms to calcium.

3.2 Materials and methods

3.2.1 Bacterial strains and culture conditions

Five *G. stearothermophilus* strains were used in this study. *G. stearothermophilus* A1, P3, 183 and F75 are dairy isolates, ATCC 7953 was used as a reference strain. One aliquot of frozen culture was streaked onto a TSA plate (40 g/L) (BD Biosciences) and incubated at 55 ℃ for 22-24 h. One colony from a TSA plate was picked and resuspended in 1 mL of 0.5 % buffered peptone water (BPW, Merck GranuCult), and 10 µL of resuspended culture was transferred into 10 mL modified TSB medium (mTSB, Table 3-1) containing 0.2 mM total calcium, 10 mL mTSB supplemented with 7 mM or 40 mM of $CaCl_2$. Due to calcium induced media acidification, the pH of all media was adjusted to pH 7.2 using 1 M HCl. The concentrations were chosen based on the approximate total calcium content in milk formulations from 10% (w/w) reconstituted milk protein concentrate and calcium-reduced milk protein concentrate used in Somerton, et al. (2015). Cultures were incubated at 55 ℃ for 10 h to achieve stationary phase cultures (Supplementary materials Figure S3-14 (B)), centrifuged and washed twice in 0.5% BPW. The cell pellet was resuspended and adjusted to a desired OD_{600nm} value (approximate 0.7, 0.2, 0.2, 0.5 and 0.9 for 183, A1, P3, F75 and 7953 respectively), the OD_{600nm} adjusted suspension of 183, F75 and 7953 were further diluted 100 times. The adjusted suspension was used to inoculate fresh mTSB and calcium-supplemented mTSB (0.1% or 1% inoculation level) to make the initial cell concentration at approximately 3 log CFU/mL for biofilm assay and 4 log CFU/mL for planktonic growth measurement.

Table 3-1 The composition of mTSB medium

Composition	**Concentration**
pancreatic digest of casein (BBL™ Trpticase™ Peptone, BD Biosciences)	17 g/L
papaic digest of soybean meal (BBL™ Phytone™ Peptone, BD Biosciences)	3 g/L
glucose (LabServ™, Thermo Fisher Scientific New Zealand Ltd.)	2.5 g/L
MOPS buffer (pH 7.30, 478 mM)	39 % (v/v)

MOPS buffer was made by dissolving 100.04 g MOPS (MOPS, Free Acid, ULTROL® Grade, EMD Millipore Corp.) in 1 L deionized water, the pH was adjusted to pH 7.3 with 10 M NaOH.

3.2.2 Culture storage

Bacterial isolates were grown in tryptic soy broth (BD Bioscience) to mid-log phase (approximately 8 h; growth curves of the selected strains are in supplementary materials Figure S3-14 (A)) and mixed homogenously with additional glycerol (10%, v/v). The mixture was stored at -80℃ until use.

3.2.3 Biofilm assay

A 96-well plate (Falcon 353072) biofilm assay was modified from He et al. (2016). Briefly, 150 µL of mTSB and calcium-supplemented mTSB inoculated as described above, was dispensed into each well in quadruplicate. The plates were wrapped in a resealable plastic bag and incubated at 55 ℃ for 10h. After incubation the plates were emptied by inverting and biofilm formed in each well was washed three times with deionized water and stained with 185 µL 0.1 % (w/v) crystal violet (CV) for 30 min at 30 ℃. Subsequently, biofilm was washed three times with water. The attached CV was dissolved in 200 µL 95 % ethanol. The absorbance of each well was measured at 570 nm using a spectrophotometer (Spectrostar Nano, BMG Labtech). To determine the biofilm on coupons, polystyrene coupons (RD128-PS) (Biosurface Technologies Corporation, Bozeman, MT) were ultrasonically cleaned in 1 M NaOH solution for 30 min and washed three times with deionised water. Coupons were soaked in 70% ethanol for more than 2h, rinsed three times in sterilized deionized water and dried overnight at 55 ℃. Stainless steel coupons (Part #RD128-316) (Biosurface Technologies Corporation, Bozeman, MT) were soaked in 1 M NaOH solution at 50 ℃ for 30 min and washed three times with deionized water, then coupons were soaked in acetone for 15 min at room temperature and washed three times with deionized water, autoclaved and dried overnight at 55℃. Coupons were transferred into a 24-well plate (Falcon) and placed horizontally. One millilitre inoculated mTSB or calcium-supplemented mTSB culture was dispensed into each well and coupons were kept submerged in the medium. The plate was incubated at 55 ℃ for 10 h. Coupons were then removed from the wells using sterilised forceps and washed three times in 0.85 % saline by gently dipping into the saline water with a new batch of saline used after each dipping. Washed coupons were then used for further examination.

3.2.4 Biofilm cell enumeration

To enumerate the biofilm cells on coupons, washed coupons were transferred into glass tubes, each containing 12 g sterile glass beads with a diameter of 6.35 mm (catalogue number 11079635; Biospec Products, Inc., Bartlesville) and 5 mL 0.5 % BPW. Glass tubes were vigorously mixed by vortex for 1 min followed by serial 10-fold dilutions in 0.5 % BPW. The drop-plate method based on Herigstad et al. (2001) was used to enumerate cell numbers on TSA plates.

To enumerate the biofilm cells attached to the 96-well plate, the wells were washed three times with 200 µL 0.85 % saline to remove unattached cells. Every five wells were swabbed to detach the biofilm cells, the swab was mixed by vortex in 0.5 % BPW and the cells were enumerated by plating as described previously.

3.2.5 Microscopic analysis

To observe biofilm polysaccharide, fluorescence microscopy, following staining with calcofluor white, which specifically stains the beta-1,3 and beta-1,4 linkages of polysaccharides, was used to stain biofilms on polystyrene and stainless steel coupons (Mulcahy and Lewenza 2011, Philips et al. 2017). Washed coupons were air dried in a fume hood, calcofluor white stain (Sigma 18909, calcofluor white M2R 1g/L and evans blue 0.5g/L) was dispensed on the surface of each coupon and stained for 1 min at room temperature. Excess stain was washed away by dipping each coupon into deionized water three times with a new batch of water used after each dipping and air drying. Coupons were observed using a fluorescence microscope (Olympus BX53) with filter 1 (excitation 340-390 nm, emission 410 nm). Each coupon surface was imaged at five different spots and the experiment was repeated on at least two different occasions.

To observe damaged cells in resuspension, eight-hour stationary phase cultures in mTSB and calcium-supplemented mTSB of 7953 were centrifuged at 10000×g for 1 min and resuspended with BPW in half the volume. A volume of 3 µL of 10 mM propidium iodide (Sigma) solubilized in DMSO was added to each 1mL of resuspended culture and cell suspensions were

mixed by vortex followed by incubation in the dark for 15 min at room temperature. After incubation, 5 µL of stained sample were trapped between a cover slip and a slide, and the sample was observed by fluorescence microscopy using filter 3 (excitation 530-560 nm, emission 570 nm). Each coupon surface was imaged at three different spots and the experiment was repeated on two different occasions.

For scanning electron microscopy (SEM), polystyrene coupons were incubated in fixative (3 % glutaraldehyde and 2% formaldehyde in 0.1 M phosphate buffer, pH 7.2) for at least 24 h at room temperature. Coupons were then washed three times in 0.1 M phosphate buffer (pH 7.2) and dehydrated in a graded ethanol series, after which the coupons were critical-dried using liquid CO_2. Coupons were mounted onto aluminium stubs and sputter-coated with gold and observed using a SEM (FEI Quanta 200; FEI Co., Hillsboro, OR, USA). The coupon surface was imaged for at least three different spots with varied magnifications, and the experiment was repeated on at least two different occasions.

3.2.6 EPS extraction and quantification

EPS was extracted based on a method described in Liu and Fang (2002) and D'Abzac et al. (2010) with some modifications. Ten washed polystyrene coupons of each condition were transferred into 10 mL saline. Biofilms on coupons were detached ultrasonically in a sonication water bath (Ultrasonic Cleaner 010T) for 5 min. The biofilm solution with coupons was mixed by vortex for 1 min. Coupons were picked out of the biofilm solution with sterilized forceps. 0.06 mL formaldehyde (36.5%) was added to 10 mL biofilm solution and the solution was incubated at 4 °C for 14 h. 4 mL 1M NaOH was added into biofilm solution and the solution was incubated at 4 °C for 3 h, after which the solution was centrifuged at 20000 $\times g$ at 4 °C for 20 min. The biofilm solution was then filtered through a 0.2 µm membrane and the filtrate (EPS solution) was concentrated to 1.3 mL at 10000 $\times g$ for 15 min using GE concentrators (Vivaspin 6) with a cut-off value of 3000 Dalton and desalted using milliQ water three times. The protein and polysaccharide content were then determined. Protein concentrations were measured using the Bio-Rad protein assay and measured at 595 nm. Polysaccharide concentrations were measured using a phenol-sulfuric acid method based on He et al. (2016). with some modification. Briefly, 100 µL 6% phenol solution was added into 200 µL EPS

concentrate, and 500 µL 98% H_2SO_4 was immediately added. Samples were incubated at room temperature for 20 min and optical densities were measured at 490 nm. Concentrations of protein and polysaccharide were calculated based on the standard curves generated using either bovine serum albumin (Sigma) or glucose (supplementary materials Figure S3-12 and Figure S3-13). Extracellular protein production per 10^8 culturable biofilm cells was calculated to check the effect of calcium on extracellular protein synthesis within biofilms.

3.2.7 Planktonic growth

Ten millilitres of mTSB, 7 and 40 mM calcium-supplemented mTSB medium inoculated with A1, P3 and 7953, were incubated at 55 ℃ for 8 h to research stationary growth phase (Supplementary materials Figure S3-10). At 8 h, 200 µL culture of each condition was transferred into a 96-well plate and OD_{600nm} was measured. The drop-plate method was used to enumerate total culturable cells at 8 h on TSA plates after serial dilution with BPW. Spores of 7953 in mTSB and calcium-supplemented mTSB 8 h cultures were enumerated by heating the cultures at 90 ℃ for 30 min to inactivate vegetative cells following serial dilution and plating on TSA.

3.2.8 Statistical analysis

All data presented were means with standard deviations from three biological replicates unless stated otherwise. Significant differences were determined by one-way ANOVA with Tukey post-hoc test using Minitab Statistical Software (Minitab Version 18, State College, Pennsylvania, USA) at a 95% confidence (P<0.05). The mixed effects model of Minitab Statistical Software was used to assess the effect of strain difference on biofilm cell numbers and calcium was incorporated as a factor.

3.3 Results and discussion

3.3.1 Biofilm formation

Ten hours of growth was chosen as the biomass of most strains started to decrease after 10 h (supplementary materials Figure S3-9). *G. stearothermophilus* strains 183 and 7953 demonstrated lower CV staining as the concentration of calcium increased (Figure 3-1). However, no significant difference was observed in P3 and F75 under different calcium concentrations (Figure 3-1). A1 showed a significantly higher (P<0.05) biofilm formation under 7 mM calcium but not 40 mM calcium (Figure 3-1). The varied strain to strain response to cations in biofilm formation is observed in other species such as *Bacillus cereus* to 250 μM $FeCl_3$ (Hayrapetyan et al. 2015). However, a consistent response of different strains to calcium and magnesium (0-1.5%, w/v) is observed in *C. sakazakii* isolates (Ye et al. 2015). Consequently, bacterial species, strain variation and cation concentrations could all be key determinants affecting biofilm formation as determined by CV staining.

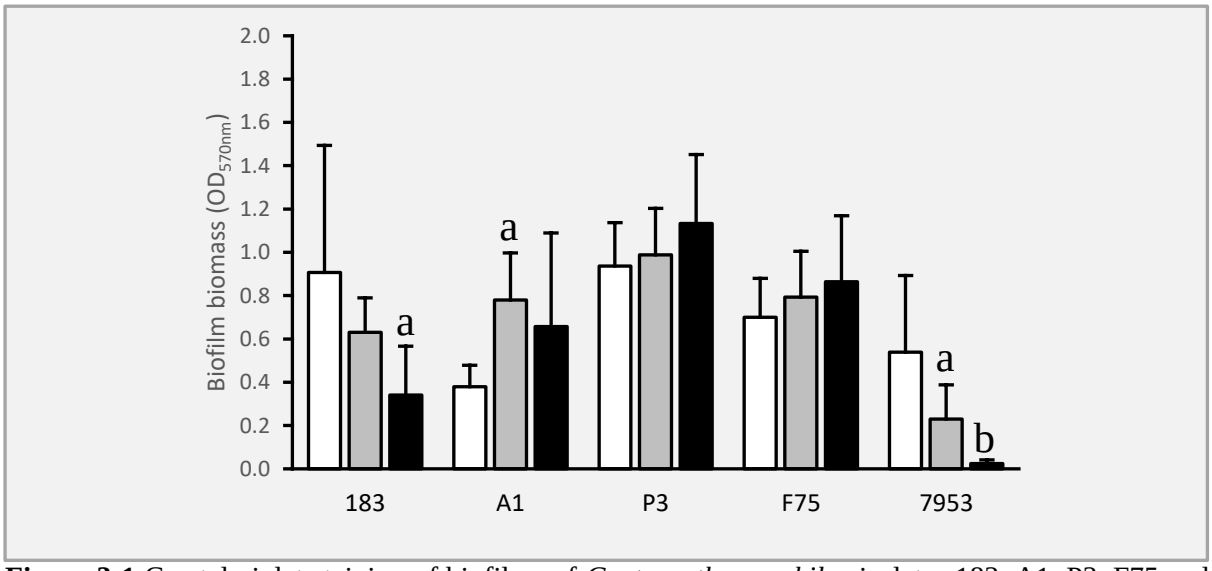


Figure 3-1 Crystal violet staining of biofilms of *G. stearothermophilus* isolates 183, A1, P3, F75 and 7953 after incubation at 55 °C for 10h in different concentrations of calcium. White bars represent control (mTSB without calcium supplementation), grey bars represent 7 mM calcium and black bars represent 40 mM calcium. Statistical difference was performed with ANOVA followed by post-hoc test (P<0.05), data bars with different letters within each strain represent significant difference to control and between each other

3.3.2 Biofilm cell enumeration

To check the biofilm cell numbers in the presence and absence of calcium, strains A1, P3 and 7953 were selected based on the response of either increasing, remaining constant or decreasing, respectively in the biofilm CV assay (Figure 3-1).

A strain specific response in biofilm cell numbers was observed under different concentrations of calcium (Figure 3-2). The presence of 7 mM and 40 mM calcium significantly increased the biofilm cell number of A1 (P<0.05) (Figure 3-2), while no significant difference was observed for P3 (P>0.05) (Figure 3-2). In contrast was the dose-dependent inhibition effect of calcium on 7953. Both 7 mM and 40 mM calcium significantly decreased the biofilm cell number of 7953 (P<0.05) (Figure 3-2).

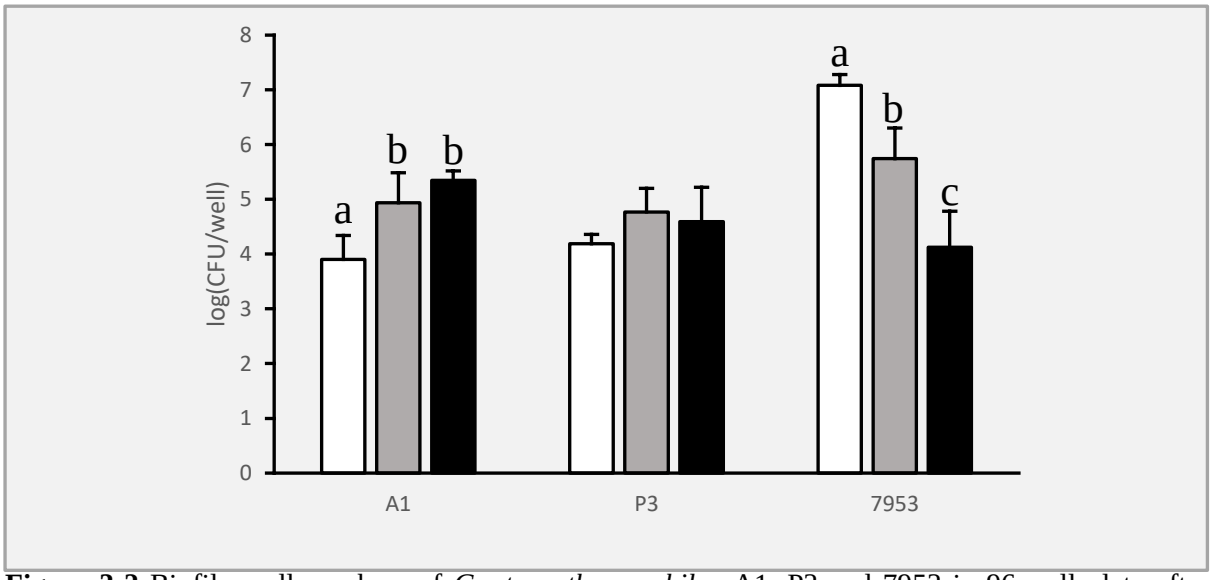


Figure 3-2 Biofilm cell numbers of *G. stearothermophilus* A1, P3 and 7953 in 96-well plate after incubation at 55 °C for 10h in control (mTSB medium without calcium supplementation, white bars), 7 mM (grey bars) and 40 mM (black bars) calcium supplemented mTSB. Statistical difference was performed with ANOVA followed by post-hoc test (P<0.05), data bars with letters stand for significant difference to each other for each strain

To compare biofilms formed on different substrata under the effect of calcium, polystyrene and stainless-steel coupons with the same shape and surface area were used.

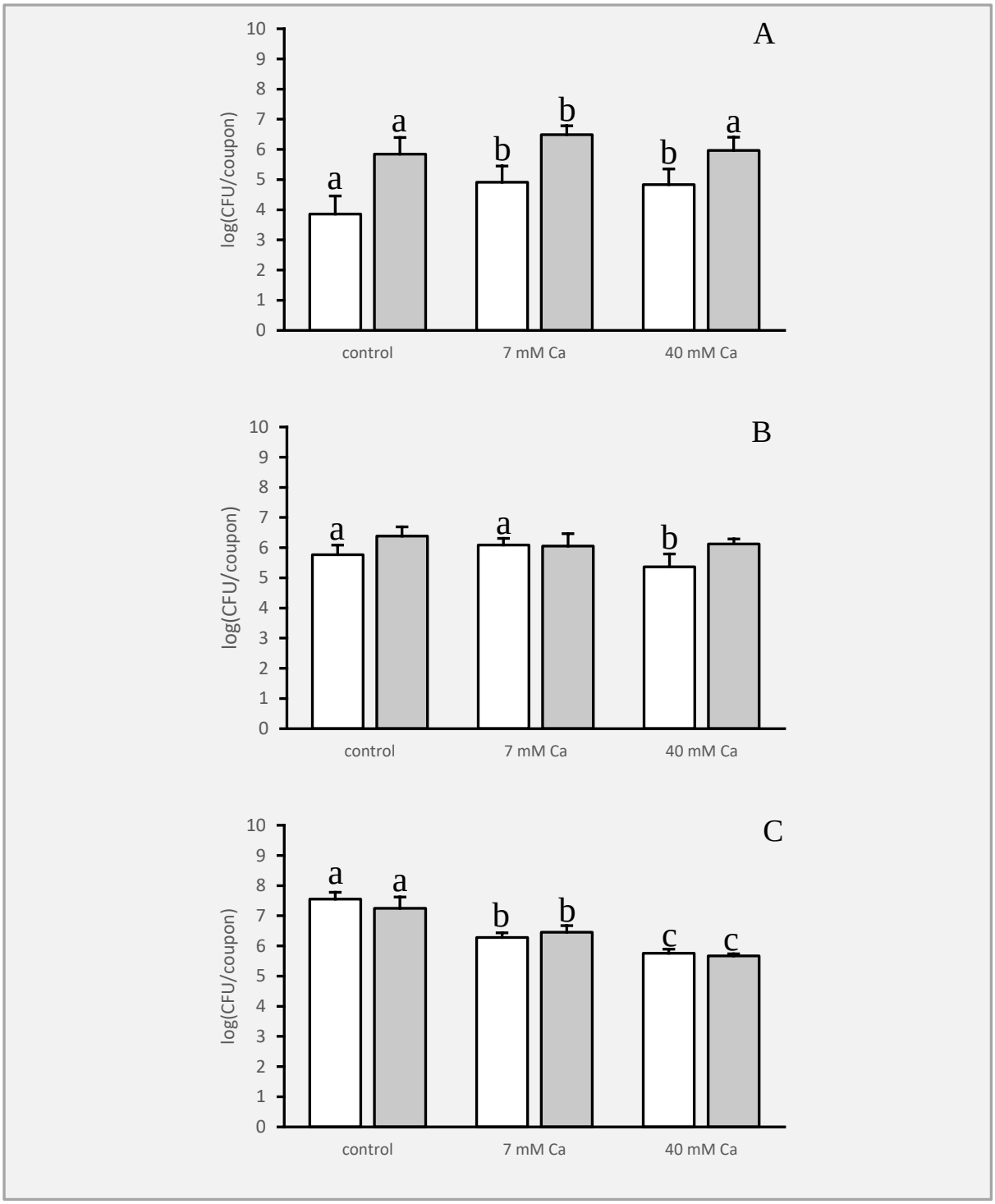


Figure 3-3 Biofilm cell numbers of *G. stearothermophilus* A1 (A), P3(B) and 7953 (C) on stainless steel (white bars) and polystyrene (grey bars) coupons after incubation at 55 °C for 10h in different concentrations of calcium in mTSB medium. Statistical difference was performed with ANOVA followed by post-hoc test (P<0.05), data bars with letters stand for significant difference to each other for each strain on the same surface

A different response to calcium in biofilm formation was observed between the stainless-steel and polystyrene for A1 and P3. While 7 mM and 40 mM calcium significantly increased the biofilm cell numbers of A1 on stainless steel, only 7 mM calcium was able to increase the biofilm cell number of A1 on polystyrene ($P<0.05$) (Figure 3-3A). Additionally, biofilm cell numbers of A1 on stainless steel were generally lower than on polystyrene. For P3, 40 mM calcium significantly decreased the biofilm cell number on stainless steel ($P<0.05$). However, no significant decrease was shown on polystyrene in the presence of 7 mM and 40 mM calcium ($P>0.05$) (Figure 3-3B). The difference in biofilm formation on stainless steel and polystyrene was also reported for *B. cereus* and *Listeria monocytogenes* (Hayrapetyan et al. 2015, Poimenidou et al. 2016). In contrast, a consistent decrease in biofilm cell number for 7953 was shown in the presence of 7 mM and 40 mM calcium on both surfaces ($P<0.05$) (Figure 3-3C). The effect of strain difference on biofilm cell number in the presence of calcium on either stainless steel or polystyrene was further assessed using the mixed effects model. Strain difference had a significant effect ($P<0.05$) on biofilm cell numbers in the control, 7 mM and 40 mM calcium culture conditions. However, the effect was only shown on the stainless steel. When comparing the biofilm forming ability between different dairy isolates, P3 consistently showed significantly higher biofilm cell numbers than A1 in both the control and 7 mM calcium conditions on stainless steel ($P<0.05$). At 40 mM, approximately 0.5 log higher biofilm cell numbers were observed in P3 compared with A1. Burgess et al. (2014) also reported that strain P3 produced higher biofilm cell numbers on stainless steel compared with strain A1.

Surprisingly, these current results are inconsistent with the observations from Somerton et al. (2015), where increasing calcium concentration in calcium-reduced milk formulation significantly increases biofilm cell numbers of *G. stearothermophilus* dairy isolates. Three potential explanations are proposed. First, although the calcium concentrations used in this study are close to that used in Somerton et al. (2015), the amount of free calcium ion was not the same in the current study. As the base medium used in current study contains buffer, glucose, amino acids and peptides, it is likely that most of the supplemented calcium is available to the bacteria. In addition, the amount of 0.2 mM calcium inherently present in mTSB media was minimal compared to the amount of supplemented calcium. The amount of free calcium in both the original and calcium-reduced milk formulation is 0.4 mM (Somerton et al. 2015), and is different from the available free calcium in mTSB media. As free calcium is believed to have the most biological significance to bacteria (Aranha et al. 1986), it is possible that differences

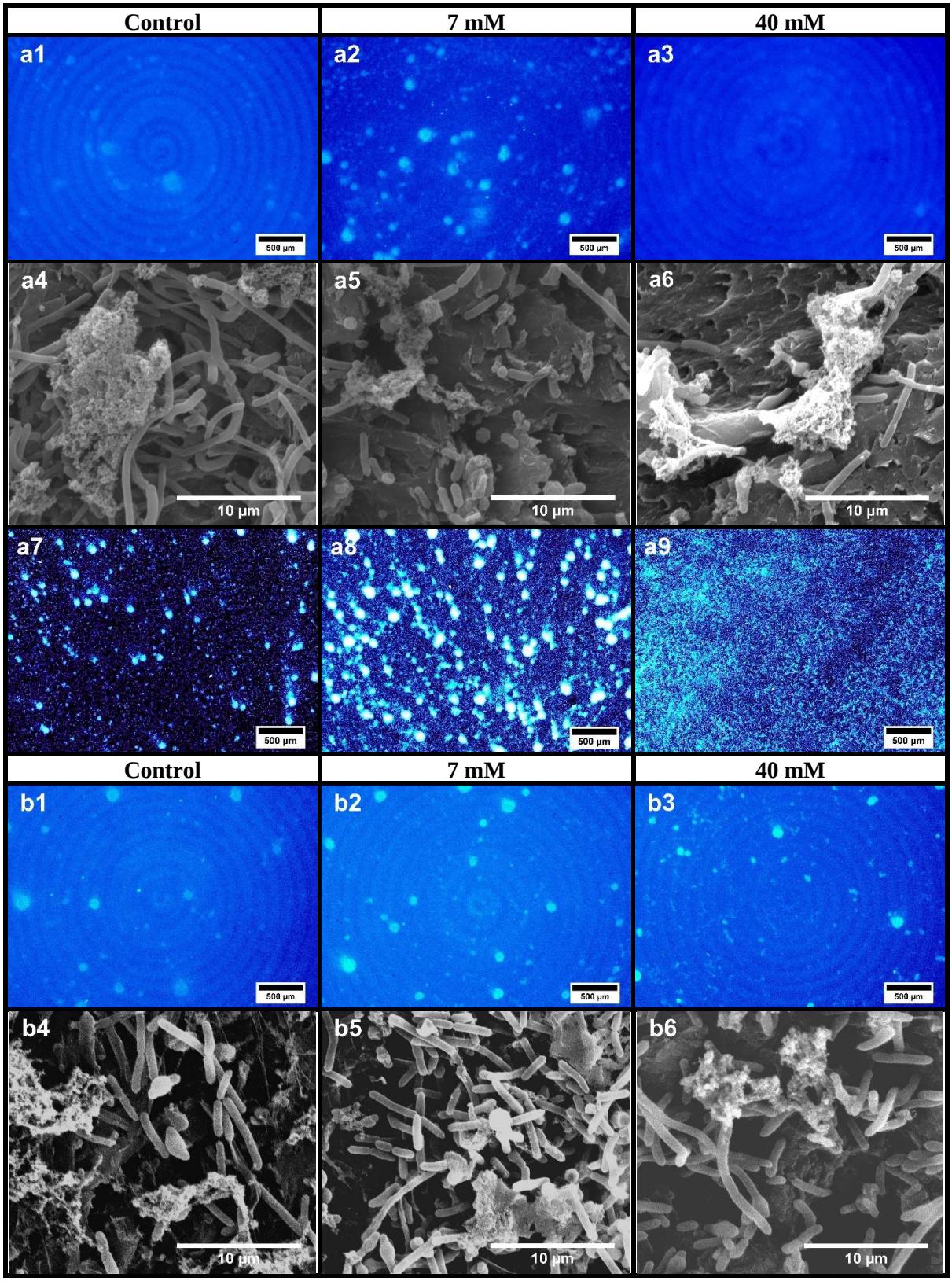
between the current study and the previous studies could be caused by differences in free calcium levels. The second explanation is that a collective effect of a calcium-protein interaction could cause inhibition of bacterial growth. As the calcium-reduced milk formulation is manufactured by an ion-exchange process, it is possible that an active bactericidal or bacteriostatic component was created during the process by stripping calcium away from native milk proteins. A similar bactericidal effect on *Streptococcus pneumoniae* was found in a folding variant of α-lactalbumin from human milk after ion-exchange, in which tightly bound calcium was removed during the preparation (Hakansson et al. 2000). The death of treated cells was accompanied by apoptosis-like morphology such as cell shrinkage, DNA condensation and degradation (Hakansson et al. 2011). Thirdly, although both current and previous studies used *G. stearothermophilus* dairy isolates as research subjects, the inconsistency in observations might have arisen from differences in the strains between the two studies.

3.3.3 Microscopic analysis

Calcofluor white and fluorescently labelled lectins (e.g. Concanavalin A-FITC) are commonly used EPS dyes in biofilm studies (Strathmann et al. 2002, Mulcahy and Lewenza 2011, Dey and Paul 2018). Initially, both dyes were used to test *G. stearothermophilus* biofilms. However, the EPS of *G. stearothermophilus* was poorly stained with Concanavalin A-FITC (ConA, supplementary materials Figure S3-11). Therefore, calcofluor white was used in the current study. Calcofluor white staining intensity of biofilms on stainless steel surfaces (Figure 3-4 a7-a9, b7-b9, c7-c9) correlated positively with the biofilm cell numbers (Figure 3-3 ABC). Specifically, A1 showed a stronger staining effect in calcium supplemented conditions (Figure 3-4 a8-a9) than the control (Figure 3-4 a7), where a similar trend was shown in biofilm cell numbers (Figure 3-3 A). This correlation was also shown in P3 and 7953. The presence of calcium also affected the distribution of biofilm polysaccharides, with larger aggregates observed at 7 mM calcium in both A1 and P3 (Figure 3-4 a8 and b8). A similar observation in was found in *P. mendocina* biofilms where clusters of cells were glued together in biofilms in the presence of divalent cations (Mangwani et al. 2014). With biofilm formation on the polystyrene surface, more polysaccharide was produced in biofilms of A1 grown under 7 mM calcium (Figure 3-4 a2) than in the control lacking calcium supplementation (Figure 3-4 a1). Polysaccharide staining in biofilms of P3 was similar under all conditions (Figure 3-4 b1-b3). Interestingly, 7953 did not show staining in any condition on the polystyrene surface. When

comparing the staining effect between stainless steel surface and polystyrene surface, only large clusters of polysaccharides were observed on the polystyrene surface (Figure 3-4 a2, b1-b3) while staining of both polysaccharide clusters and scattered polysaccharide were observed on the stainless steel surface, indicating the limitation of using polystyrene surfaces in fluorescence staining.

SEM images provided more details of EPS production. Both EPS and biofilm cells were observed in all conditions for strains A1 and P3 (Figure 3-4 a4-a6, b4-b6). However, very Little EPS production was observed in 7953 (Figure 3-4 c4, c5 and c6).


Control
7 mM
40 mM
a1
a2
a3
a4
a5
a6
a7
a8
a9
b1
b2
b3
b4
b5
b6
500 μm
10 μm

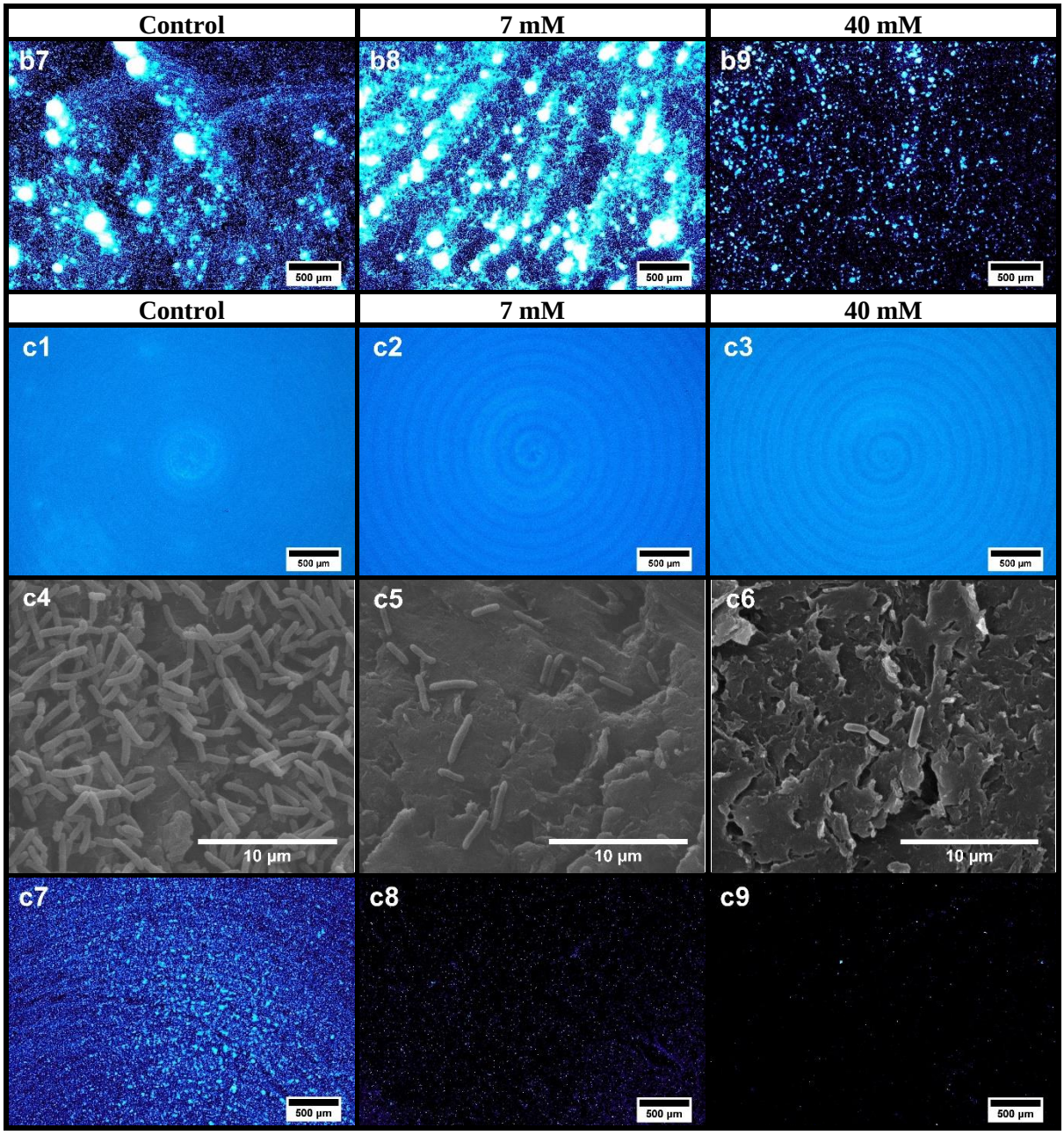


Figure 3-4 Fluorescence microscopy and SEM images of biofilms of strain A1, P3 and 7953 at 10 h under control (mTSB without calcium supplementation), 7 mM and 40 mM calcium conditions. Calcofluor white was used to stain extracellular polysaccharides in biofilms. The small letters a, b and c represent A1, P3 and 7953 respectively. For each strain, 1, 4 and 7 represent the control condition; 2, 5 and 8 represent 7 mM calcium condition; 3, 6 and 9 represent 40 mM calcium condition. Images numbered with 1-6 and 7-9 represent polystyrene and stainless steel respectively

3.3.4 EPS extraction and quantification

The EPS of A1, P3 and 7953 biofilms was extracted and quantified. As all three strains showed a considerable amount of biofilm on polystyrene coupons, biofilms on polystyrene were further examined to gain more insights into the effect of calcium on EPS production. Carbohydrates and proteins were quantified as they are usually identified to be the major components of EPS (Sheng et al. 2010). The EPS quantity varied among different strains. The presence of 7 mM calcium significantly increased the amount of extracellular protein of A1 from 0.035 mg/10 coupons to 0.14 mg/10 coupons, and extracellular polysaccharide from 4.67 μg/10 coupons to 10.48 μg/10 coupons ($P<0.05$) (Figure 3-5 AB). In contrast, calcium decreased the amount of extracellular protein of 7953 to an undetectable level at 7 mM and 40mM calcium, and no significant difference was observed in extracellular polysaccharide (Figure 3-5 AB). When comparing the EPS among three strains, A1 showed 4 times more extracellular protein than P3 and 7953 in the control and more in 7 mM and 40 mM calcium (Figure 3-5 A). However, due to the limitation of the protein detection method used, P3 with 40 mM calcium and 7953 with 7 mM and 40 mM calcium were under the detection limit. The amount of extracellular polysaccharide between the three strains was similar in different conditions (Figure 3-5 B). In all culture conditions, extracellular polysaccharide was detected in the extracted EPS of 7953 in similar amounts (Figure 3-5 B). Although due to the limitation of using polystyrene in fluorescence staining, it is not surprising that polysaccharide staining was not discernible for 7953 using fluorescence microscopy (Figure 3-4 c1-c3), but detectable in the EPS extracts. However, the amount of extracellular polysaccharide was expected to be less in calcium supplemented conditions as biofilms on both polystyrene and stainless steel showed a similar decreasing trend in the presence of calcium (Figure 3-3 C), and weaker staining was observed on stainless steel (Figure 3-4 c7-c9). As calcofluor white stains the beta-1,4 linkages of polysaccharides, it is possible that the beta-1,4 glycosidic bond between the disaccharide units of cell wall peptidoglycan were stained. Therefore, both cell wall polysaccharides and extracellular polysaccharides could be stained by calcofluor white. As it is shown on SEM images (Figure 3-4 c4-c6), limited amount of EPS was present in biofilms of 7953. Thus, the change of staining intensity of 7953 biofilms in different culture conditions on stainless steel (Figure 3-4 c7-c9) should be correlated with the change of biofilm cell numbers (Figure 3-3 C). Additionally, the current extraction method might only target the loosely bound EPS (LB-EPS, EPS that is a loose and dispersible slime layer without an obvious edge), while calcofluor white stains both tightly bound and loosely bound polysaccharides. As the content of LB-EPS is

always less than that of the tightly bound EPS (EPS that has a certain shape and is bound tightly and stably with the cell surface) (Sheng et al. 2010), it is unlikely that the amount of extracted polysaccharide can reflect well on calcofluor white staining. In addition, the standard deviations show the limitation of current method in accurate quantification of extracellular polysaccharide when only a limited amount was present. To quantify the effect of calcium on EPS synthesis, extracellular protein production per 10^8 culturable biofilm cells was calculated. Compared to the control, the extracellular protein synthesis of culturable biofilm cells of P3 and A1 was higher in 7 mM calcium and 40 mM calcium, respectively (P<0.05) (Figure 3-5 C). As the extracellular protein changes in the presence of calcium, it is possible that calcium may act as an environmental stimulus in changing EPS production of biofilm cells in a dose dependent manner. Various other studies, although not addressing strain differences, have shown the importance of divalent cations in regulating EPS gene expression of bacterial biofilms (Wang et al. 2019). Currently, the underlying mechanism in the discrepancy in strain response to calcium, especially when comparing 7953 to A1 and P3, is unclear. However, the genome comparison of A1, P3 and 7953 reported by Burgess et al. (2017) showed that A1 and P3 are more closely related to each other than 7953 is to either A1 or P3.

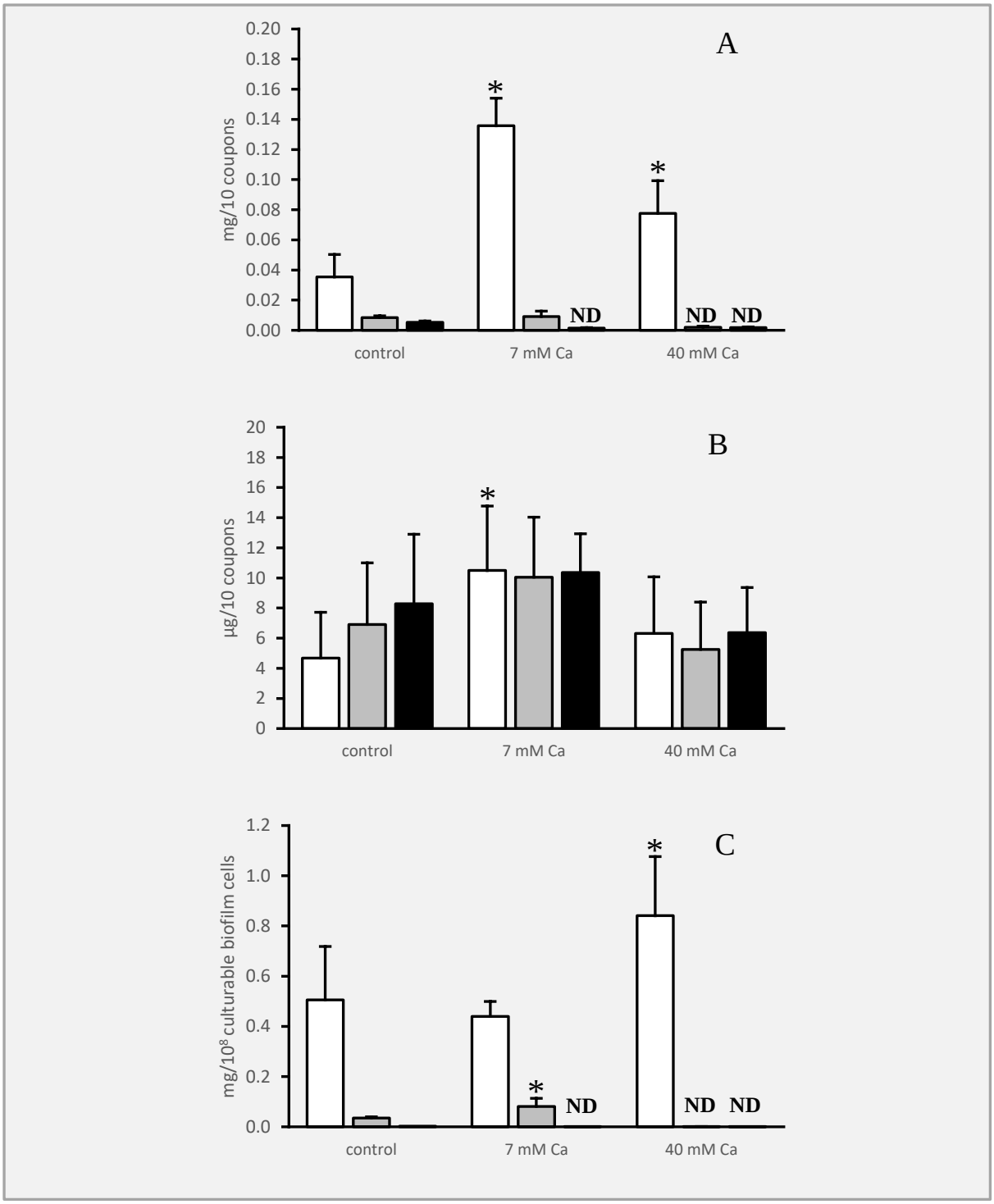


Figure 3-5 EPS quantification of A1, P3 and 7953 biofilms after incubation at 55°C, 10h on polystyrene. Extracellular protein (A); extracellular polysaccharides (B); amount of extracellular protein production per 10^8 culturable biofilm cells (C). White bars, grey bars and black bars represent A1, P3 and 7953, respectively. An asterisk indicates significant difference (P<0.05) between calcium supplemented group(s) to the respective control group. ND=not determinable (the extracted amount was under the detection limit (0.0014 mg/10 coupon) of the current method)

3.3.5 Planktonic growth

To study the difference in the effect of calcium on planktonic cells, the optical density of 8 h planktonic cultures was measured, and planktonic cells were enumerated. An eight-hour time point was chosen as this is when all the cultures reached early stationary phase and the effect of calcium on total biomass and cell numbers could be analysed (Supplementary materials Figure S3-10). Figure 3-6 A illustrates that in the presence of 40 mM calcium, the optical density of P3 and 7953 were significantly (P<0.05) higher than the respective controls. Seven mM calcium significantly (P<0.05) increased the optical density of P3 but not 7953 (Figure 3-6 A). However, the optical densities of A1 in control and calcium-supplemented conditions were similar (Figure 3-6 A).

The optical density can reflect the culture biomass or cell number of a bacterial culture (Griffiths et al. 2011), and is affected by the size, shape, and refractive index of particles within the culture (Griffiths et al. 2011, Somerton 2013). Therefore, the culturable cells were measured to check the correlation between cell numbers and optical densities. For A1, cell numbers of control and calcium-supplemented conditions were similar (Figure 3-6 B), and it reflected on the comparable optical densities (Figure 3-6 A). P3 had significantly (P<0.05) higher optical densities in 7 and 40 mM calcium-supplemented conditions than the control and the total cell numbers followed the same trend (Figure 3-6 A and B). 7953 had a significantly (P<0.05) higher optical density in the 40 mM calcium-supplemented condition and a similar optical density in 7 mM calcium-supplemented condition compared to control (Figure 3-6 A), but the cell numbers showed a opposite trend in that 7 and 40 mM calcium-supplemented conditions were significantly (P<0.05) lower than control (Figure 3-6 B). Therefore, 7953 was further studied to investigate the contradictory observation between optical densities and total culturable cell numbers.

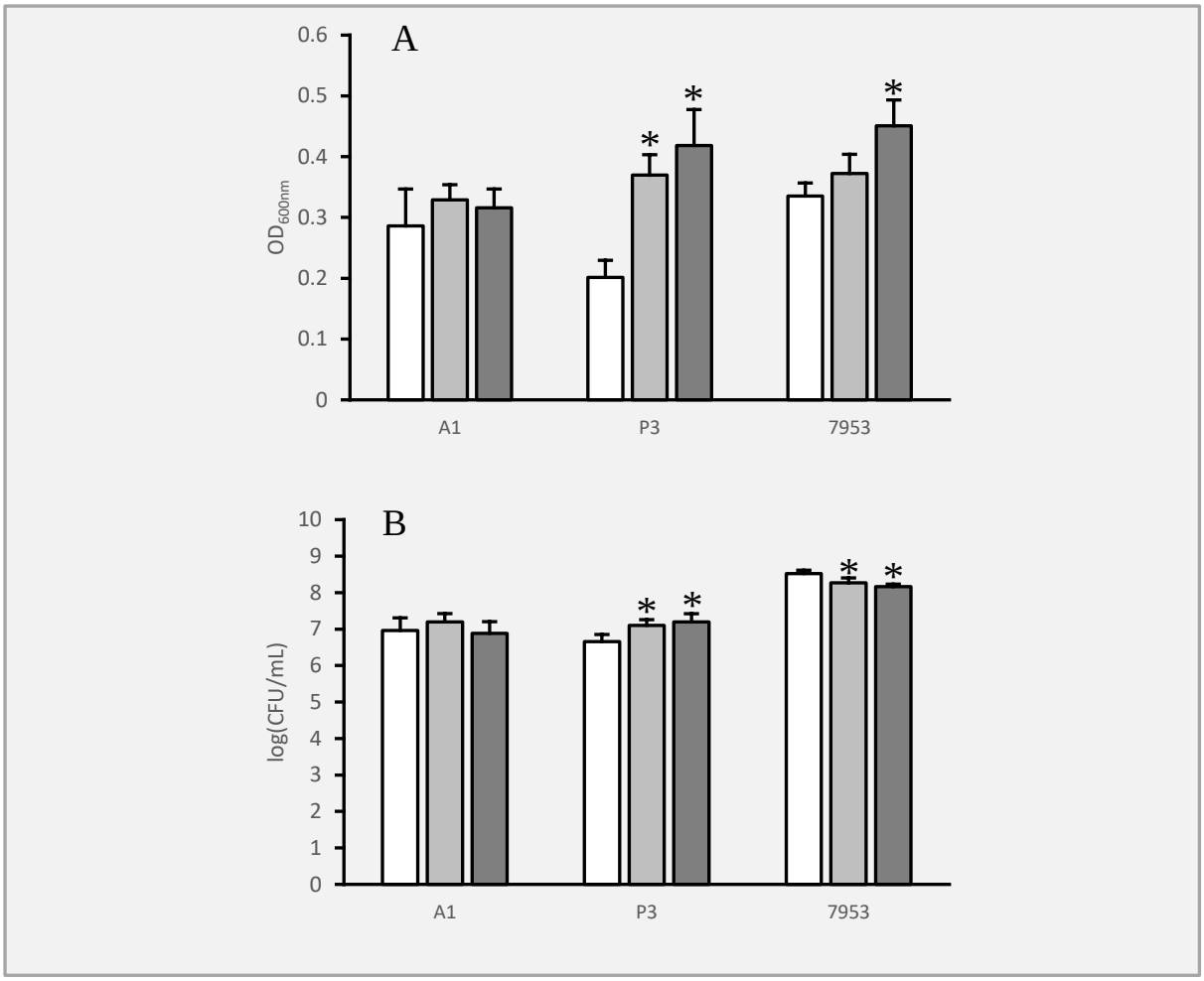


Figure 3-6 (A) Optical density of mTSB and calcium-supplemented mTSB cultures of A1, P3 and 7953 at 8 h, (B) total culturable cell numbers of mTSB and calcium-supplemented mTSB cultures of A1, P3 and 7953 at 8h. White bars, light grey bars and dark grey bars represent control (mTSB without calcium supplementation), 7 mM calcium supplemented mTSB and 40 mM calcium supplemented mTSB respectively. An asterisk indicates significant difference (P<0.05) to the control

Cell size and shape were consistent between the control and calcium-supplemented cultures of 7953 observed by light microscopy, and there was no cell aggregation in cultures (Figure 3-7). Thus, cell size, shape and cell aggregation were not the reasons for the contradictory observation. Due to the refractory properties of spores, the presence of spores can influence the optical density of a culture differently than vegetative cells. Porębska et al. (2015) reported that the germination of spores in a spore suspension of *Alicyclobacillus acidoterrestris* decreases the optical density of the suspension and indicated the influence of spores on optical density. The spore number in 7 mM calcium-supplemented culture was significantly (P<0.05) higher than the control (Figure 3-8 A) and suggests that the higher spore number could contribute to the optical density of the culture and led to a comparable OD value but lower total cell number to control. However, the spore numbers in 40 mM calcium-supplemented culture were similar to that of control. Consequently, spore numbers can be excluded as the main contributing factor to the contradictory trend between optical densities and cell numbers. Planktonic cultures of mTSB and calcium-supplemented mTSB were then centrifuged at 10000×g and then resuspended in the same volume of BPW. The optical density of supernatant of control and calcium-supplemented cultures were like that of the fresh medium (data not shown). However, the optical densities of the resuspended pellets showed a different trend to that of the original cultures. The previously observed significant (P<0.05) higher optical density in 40 mM calcium-supplemented mTSB culture than control was no longer present, and a comparable optical density of BPW resuspension appeared between the two conditions (Figure 3-8 B). Therefore, it is proposed that when 7953 was previously grown in high calcium medium (40 mM calcium-supplemented mTSB) and then exposed to low calcium medium (BPW), as the situation during BPW resuspension and serial dilution, a small proportion of bacterial cells was damaged and potentially lysed due to the sudden change of calcium concentration and became unculturable on TSA plate. Therefore, dead cells were stained with propidium iodide and observed using florescence microscopy to compare the number of dead cells in BPW resuspended cultures. Figure 3-7 shows that under calcium-supplemented conditions, relatively more dead cells were observed than in the control and this could explain the lower total culturable cells in 7 and 40 mM calcium-supplemented cultures. Overall, although 7 and 40 mM calcium-supplemented cultures had comparable or significantly higher optical densities to the control, the cell numbers could still be lower than the control potentially due to higher spore numbers in 7 mM calcium-supplemented culture, as well as cell damage and lysis in both 7 and 40 mM calcium-supplemented cultures.

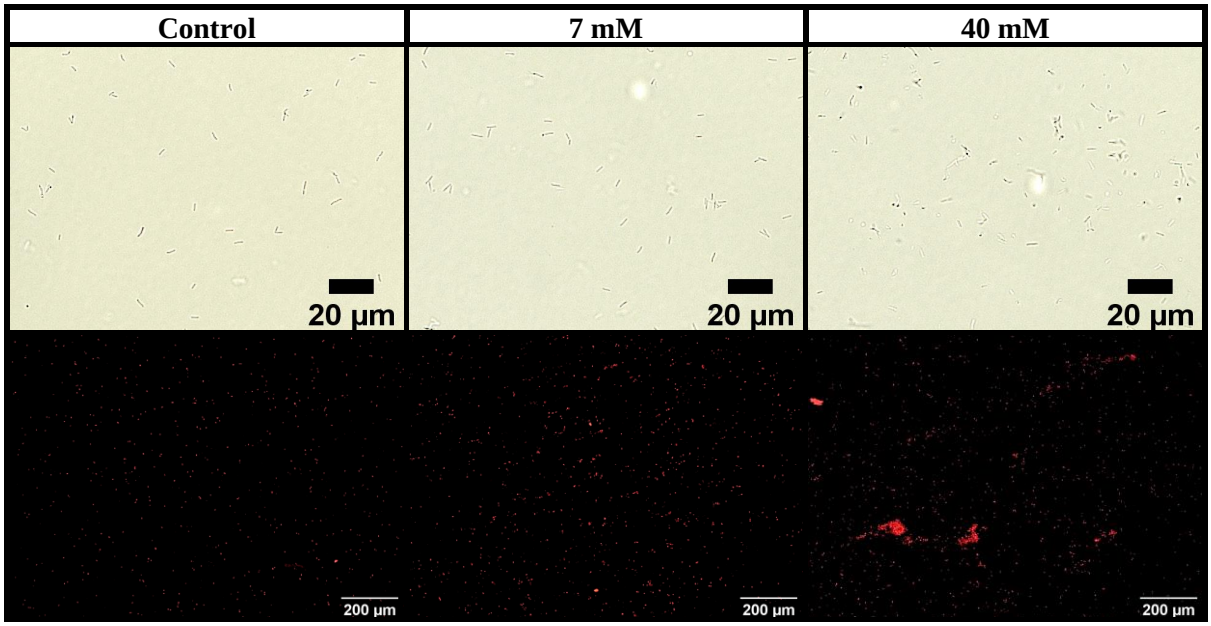


Figure 3-7 Light microscopic images (first row) of wet-mounted cells of 7953 and propidium iodide staining (second row) of BPW resuspended cells of 7953 after 8 h culture in control (mTSB), 7 mM calcium supplemented mTSB and 40 mM calcium supplemented mTSB

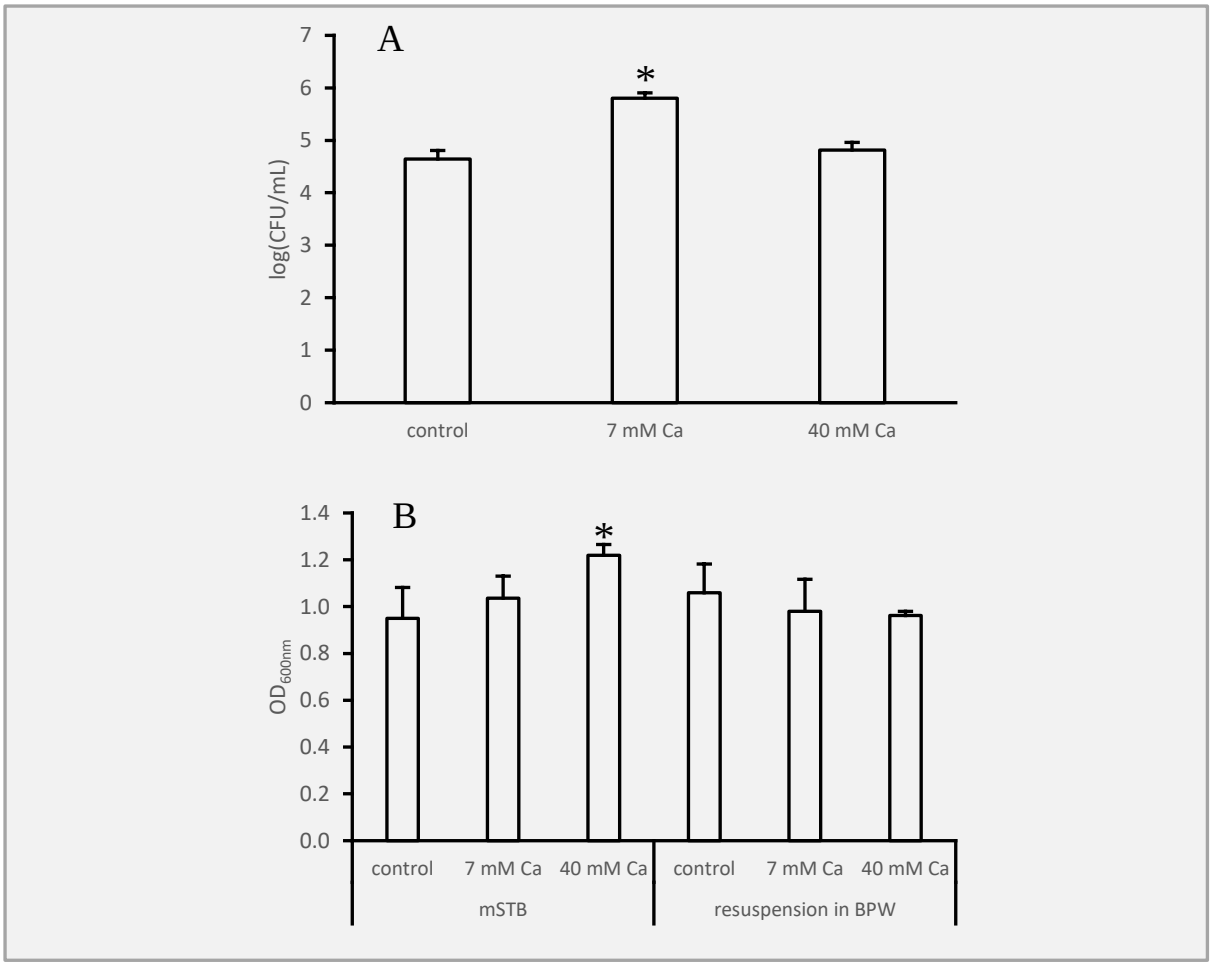


Figure 3-8 (A) total spore numbers of control (mTSB) and calcium-supplemented (7 mM calcium or 40 mM calcium) mTSB cultures of 7953 at 8h, (B) optical density of control (mTSB) and calcium-supplemented (7 mM calcium or 40 mM calcium) mTSB cultures of 7953 at 8h (three bars from the left), optical density of mTSB (control) and calcium-supplemented (7 mM calcium or 40 mM calcium) mTSB cultures of 7953 at 8h resuspended in BPW (three bars from the right). An asterisk indicates significant difference (P<0.05) to the control

The planktonic cells of A1 and P3 showed a different response to calcium compared to biofilm cells. A1 showed increased biofilm cells in the presence of calcium (Figure 3-2, Figure 3-3 A) while the planktonic cells remained constant (Figure 3-6 B). Although an increase in planktonic cells of P3 was observed in the presence of calcium (Figure 3-6 B), the trend was not observed in biofilm cells (Figure 3-2, Figure 3-3 B). Both planktonic and biofilm cells of 7953 showed a decrease in cell numbers in the presence of calcium (Figure 3-2, Figure 3-3 C and Figure 3-6 B), but the mechanism may be different. As CV staining, florescence microscopy and SEM images showed a consistency in changes of biofilm cell numbers in the presence of calcium of 7953, it was unlikely the decrease in biofilm cells in the presence of calcium was due to cell lysis or damage as was observed for planktonic cells.

3.4 Conclusions

The effect of calcium on biofilm formation was investigated for *G. stearothermophilus* strains. A strain-specific response was observed in biofilm cell number and biofilm EPS production. The heterogeneous response of biofilms to calcium in this study did not agree with the general promotive effect of calcium in milk formulations found in previous research. Although the current study fails to reproduce the effect of calcium previously observed in milk, the discrepancy suggests a diverse effect of calcium on *G. stearothermophilus* biofilms in different culture environments and the observations from previous research were possibly only limited to milk. Therefore, the mechanism of calcium and possibly other cations involved in promoting biofilm formation of *G. stearothermophilus* in milk needs further investigation.

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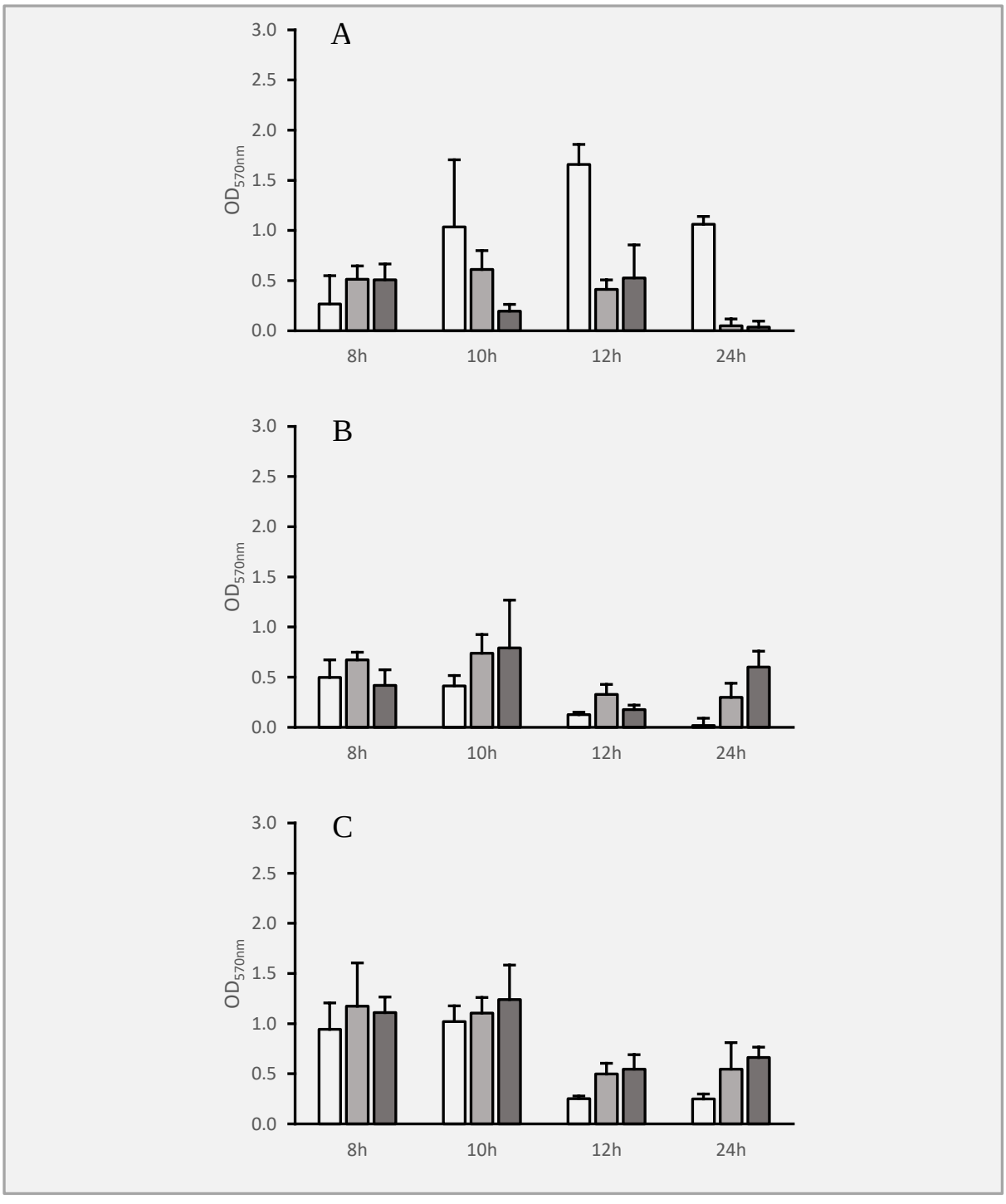
3.6 Supplementary materials



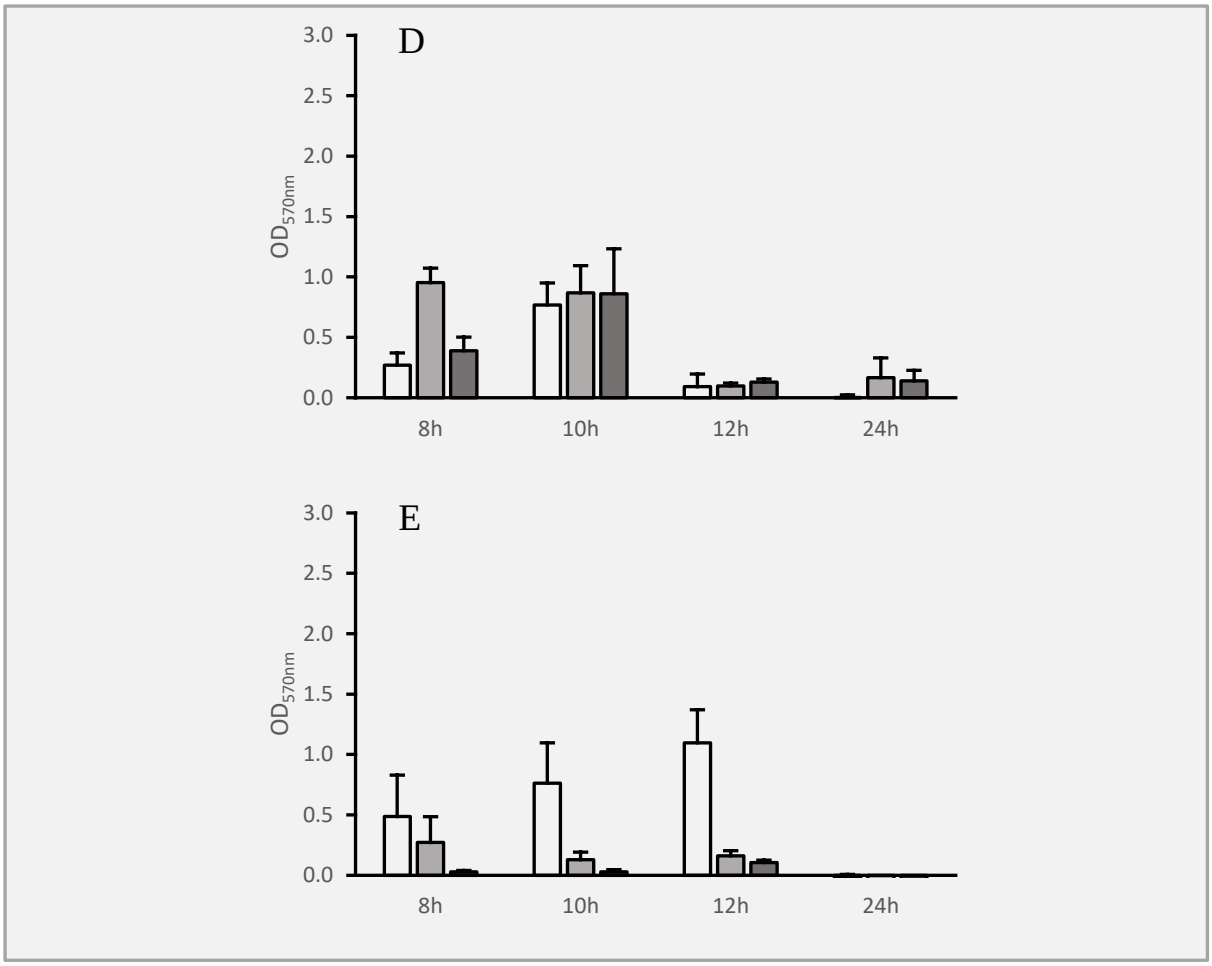


Figure S3-9 CV staining of biofilms of *G. stearothermophilus* 183 (A), A1 (B), P3 (C), F75 (D) and 7953 (F) in 96-well plates at 8, 10, 12 and 24 h. white bars indicate controls (mTSB without calcium supplementation), light grey bars indicate 7 mM calcium supplemented mTSB, dark grey bars indicate 40 mM calcium supplemented mTSB. Data are expressed as means of four replicates with standard deviations

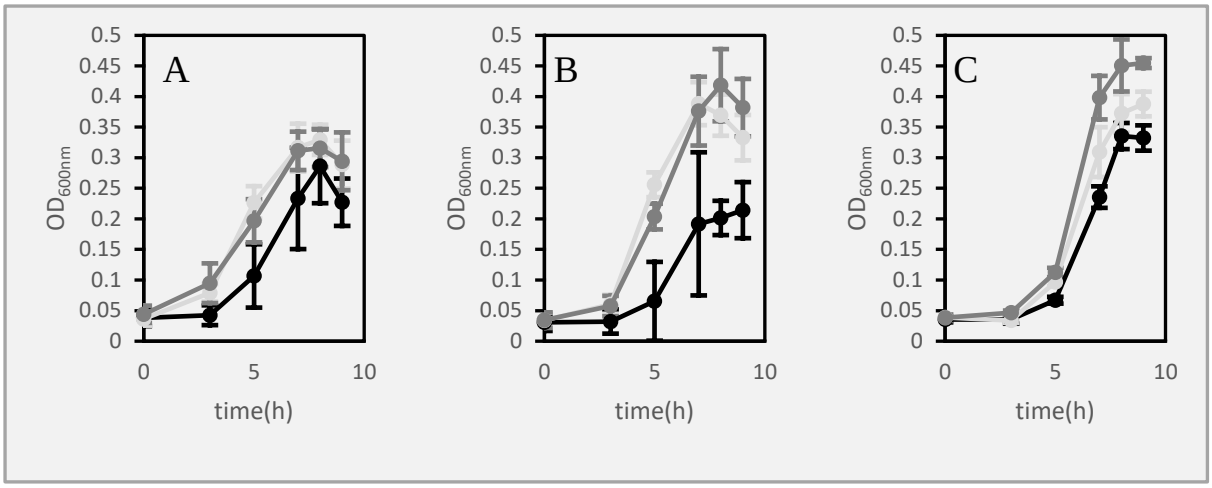


Figure S3-10 Growth curves of *G. stearothermophilus* A1 (A), P3 (B) and 7953 (C) in control (black lines, mTSB without calcium supplementation), 7 mM calcium supplemented mTSB (light grey lines) and 40 mM calcium supplemented mTSB (dark grey lines) at 55 °C

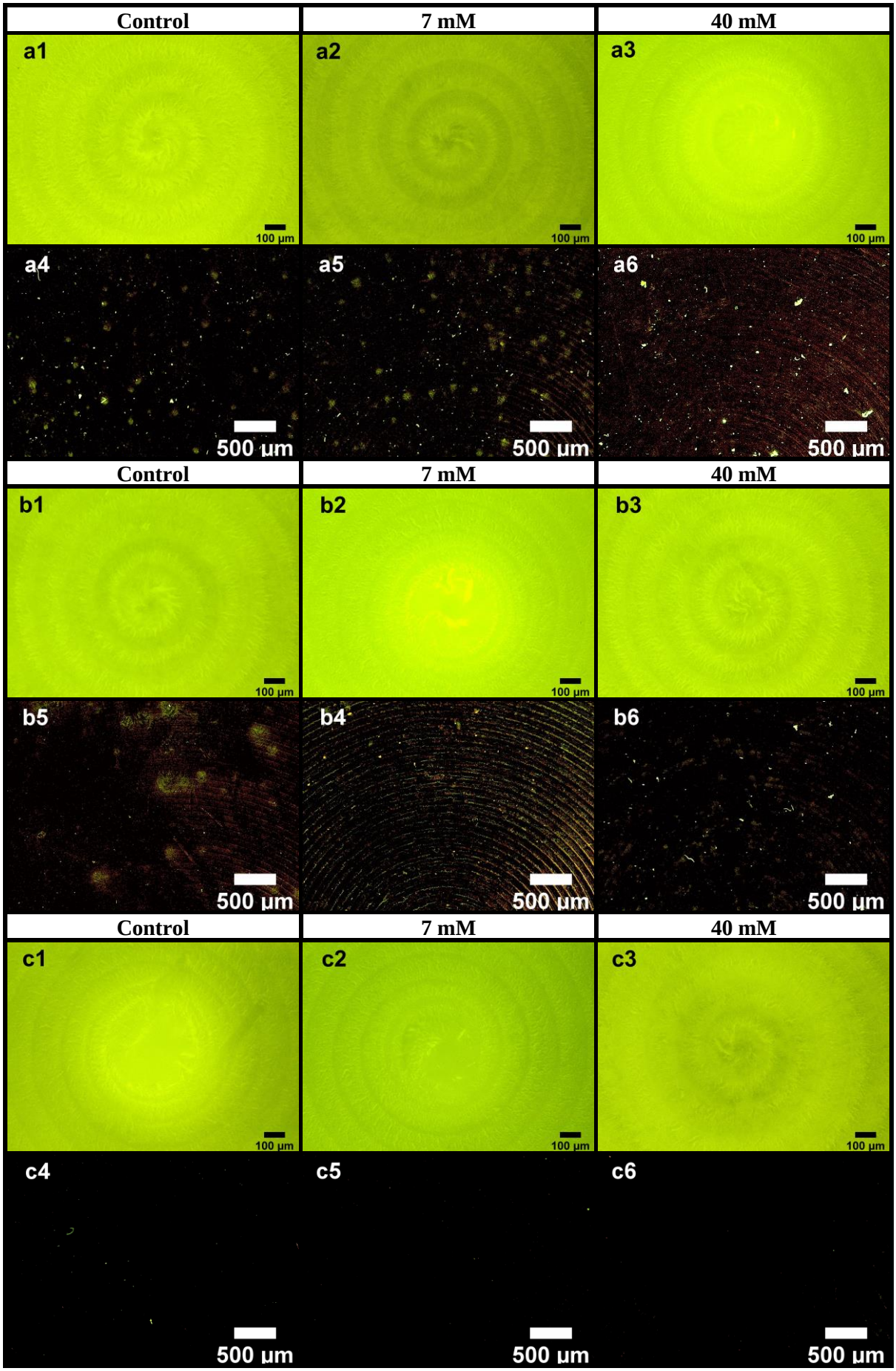

Control
7 mM
40 mM
a1
a2
a3
100 µm
a4
a5
a6
500 µm
Control
7 mM
40 mM
b1
b2
b3
100 µm
b5
b4
b6
500 µm
Control
7 mM
40 mM
c1
c2
c3
100 µm
c4
c5
c6
500 µm

Figure S3-11 Fluorescence microscopy images of biofilms of strain A1, P3 and 7953 at 10 h under control (mTSB without calcium supplementation), 7 mM and 40 mM calcium supplementated mTSB conditions. ConA was used to stain extracellular polysaccharides in biofilms. The small letters a, b and c represent A1, P3 and 7953 respectively. For each strain, 1, 4 represent the control condition; 2, 5 represent 7 mM calcium condition; 3, 6 represent 40 mM calcium condition. Images numbered with 1-3 and 4-6 represent polystyrene and stainless steel respectively

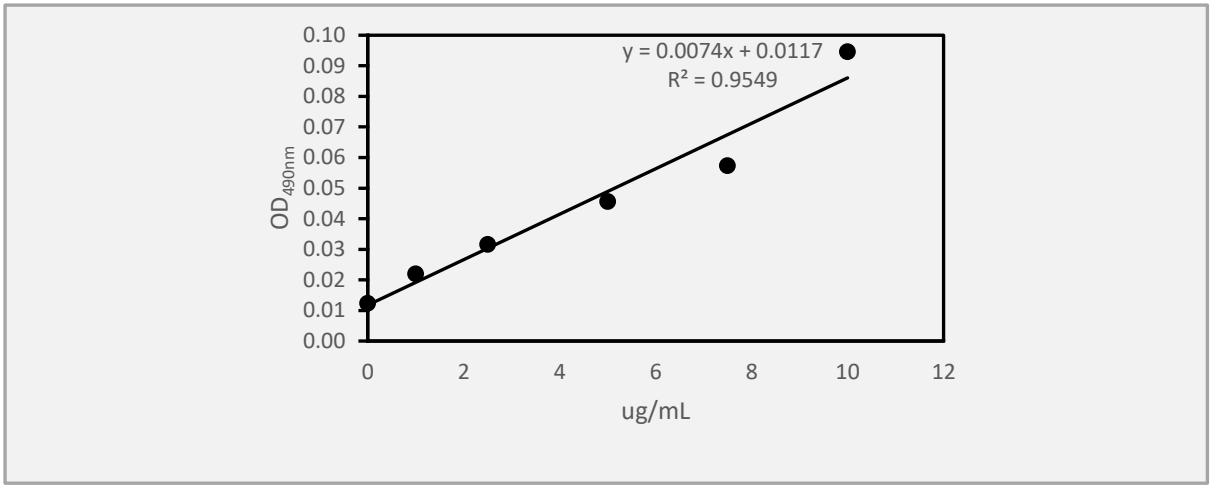


Figure S3-12 Standard curve for dextran

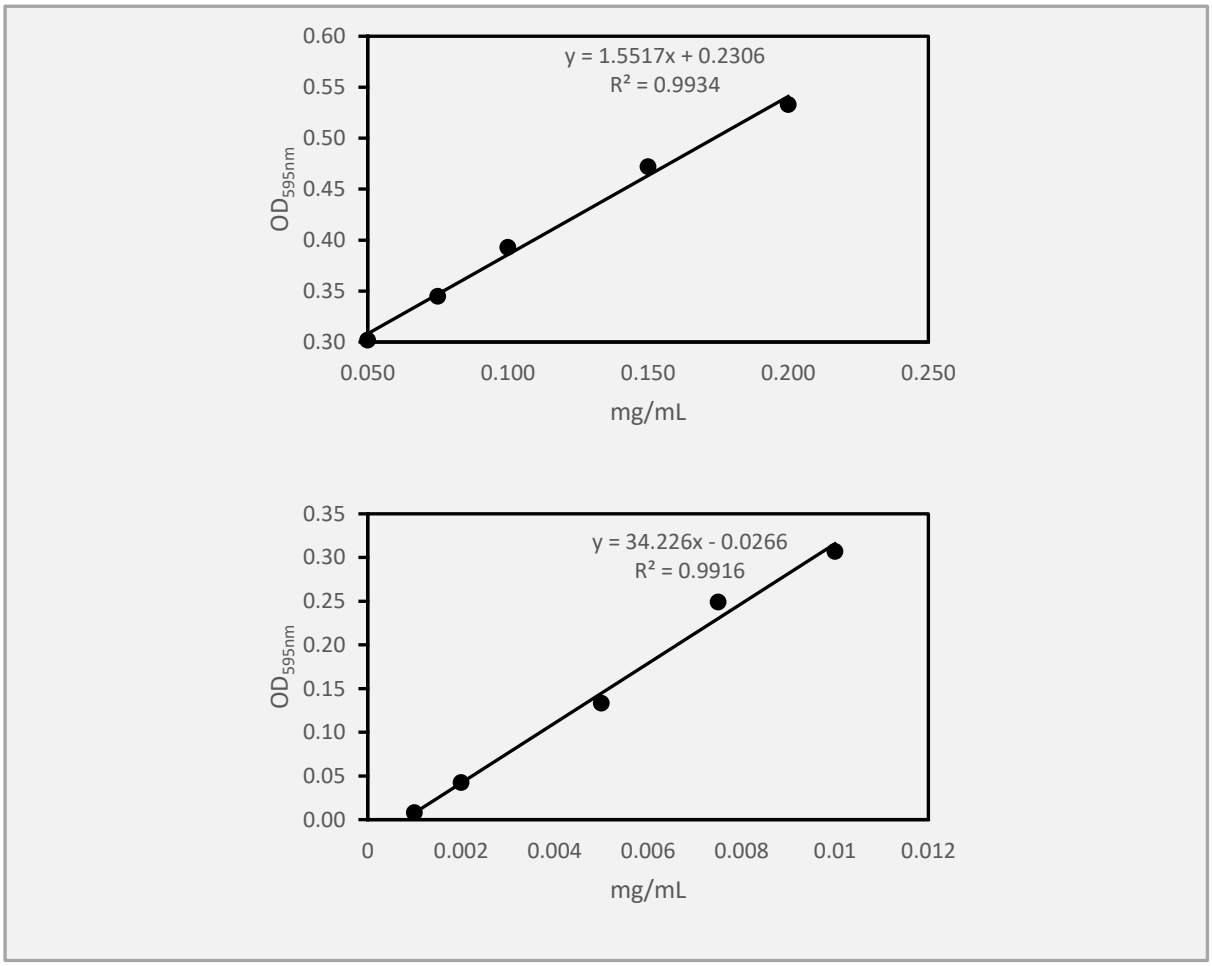


Figure S3-13 Standard curve for bovine serum albumin

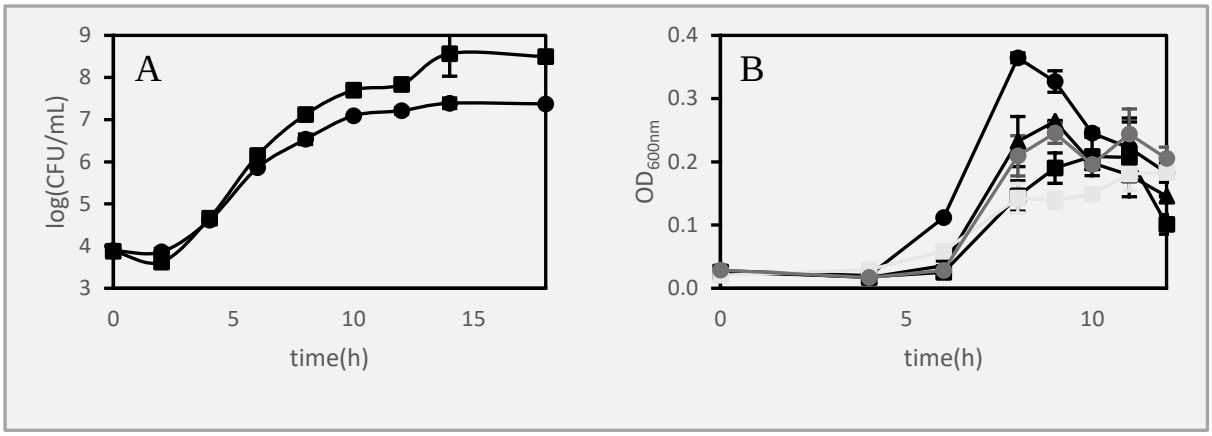


Figure S3-14 Growth curves of *G. stearothermophilus* F75 (square) and 7953 (circle) in TSB at 55 °C over 18 h culture period (A). Growth curves of *G. stearothermophilus* 183 (black closed circle), A1 (black closed square), P3 (black closed triangle), F75 (light grey closed square) and 7953 (dark grey closed square) in mTSB at 55 °C over 12 h culture period (B). Data are expressed as means of two replicates with standard deviations

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Chapter 4 The effect of calcium and milk formulations on cell attachment of *G. stearothermophilus*

4.1 Introduction

A low thermophile contamination in a calcium-reduced milk powder has been observed in industry settings and a previous study suggested that the limitation of calcium was a vital factor contributing to the reduced biofilm formation of *G. stearothermophilus* in milk formulations (Somerton et al. 2015). However, Chapter 3 in the current study showed a diversified effect of calcium on biofilm formation of *G. stearothermophilus* in modified TSB medium. Calcium was also found to affect cell attachment of *G. stearothermophilus* (Somerton et al. 2013). Therefore, cell attachment was further investigated to understand the effect of calcium on *G. stearothermophilus* in different stages of biofilm development.

Initial cell attachment is the first step in any bacterial biofilm development. Attachment commences when bacterial cells come within close proximity with substratum and overcome the electrostatic repulsion to substratum by cell structures and inherent surface properties including surface charge and hydrophobicity, after which the adhesion becomes irreversible due to production of EPS and adhesin that cling to the surface (Wang et al. 2019).

Calcium is thought to play multiple roles in the process of bacterial attachment to surfaces, ranging from assisting in the conditioning film formation, altering cell surface electrokinetic properties and hydrophobicity, modifying cell surface adhesins and bridging molecular functional groups of surfaces and/or EPS (Palmer et al. 2007, Wang et al. 2019). Calcium causes structural changes in the alginate layer on the substrata and increases the adhesion of *P. aeruginosa* cells through bridging affinity sites on bacterial surfaces and substrata for non-motile cells (De Kerchove and Elimelech 2008). In addition, it is thought that calcium shows a compression effect on the negatively charged diffuse electric double layer surrounding the cell surface, causing a decrease in electrostatic repulsion between bacteria and substrata (Somerton 2013). Increasing cell-hydroxyapatite particle aggregation in the presence of calcium for *Lactobacillus* spp. is thought to be due to the charge reversal and bridging through functional groups of hydroxyapatite particles and bacterial surfaces (Venegas et al. 2006). In addition, calcium-binding of bacterial cell surface adhesins leads to structural change that facilitates adhesion (Wang et al. 2019). Calcium is also thought to change bacterial physiology and metabolism to influence the subsequent attachment (Cruz et al. 2012, Somerton et al. 2013).

The current study investigated the effect of calcium and milk formulations on cell attachment of *G. stearothermophilus* to different surfaces. It is an extension of a previous study as more substrata and bacterial strains were used, and the electrostatic effects and physiological effects associated with calcium were investigated to understand the change of cell attachment in the presence of calcium. Additionally, cell attachment in milk formulations was checked to see whether it is associated with the varied thermophile contamination in different milk formulations observed in the dairy industry.

4.2 Materials and methods

4.2.1 Bacterial strains and growth conditions

Three *G. stearothermophilus* strains were used in this study. *G. stearothermophilus* A1, P3 are dairy isolates, ATCC 7953 was used as a reference strain. Cultures were prepared as described below unless stated otherwise. One aliquot of frozen culture was streaked onto a TSA plate (40 g/L) (DifcoTM, BD Biosciences) and incubated at 55 ℃ for 22-24 h. One colony from a TSA plate was picked and resuspended in 1 mL of 0.5 % buffered peptone water (BPW, Merck GranuCult^{TM}), each 10 µL of resuspended culture was transferred into 10 mL modified TSB medium (mTSB, Table 3-1) containing 0.2 mM total calcium and mTSB supplemented with 7 mM $CaCl_2$. The mTSB medium was used to keep consistency with the biofilm formation study in the chapter 3 of the current study. Due to calcium induced medium acidification, the pH of all media was adjusted to pH 7.2 using 1 M HCI. The calcium concentration of 7 mM was chosen based on the approximate total calcium content in milk formulation from 10% (w/w) reconstituted calcium-reduced milk protein concentrate (MF2). Cultures were incubated at 55 ℃ for 10 h.

To test cell attachment in milk formulations (Table 4-1), MF1 and MF2 were used to prepare the inocula. The milk formulations had similar fat, protein, and lactose concentrations but different calcium and sodium concentrations as outlined in Table 4-1. Milk formulations were prepared as described by Somerton et al. (2015). Different approaches were used to prepare the inocula between the dairy isolates and the reference strain. For strains A1 and P3, 5 µL thawed frozen glycerol stock was used to inoculate 5 mL MF1 and 5 mL MF2 respectively. For 7953, one aliquot of frozen stock was streaked onto a TSA plate (40 g/L) (BD Biosciences) and incubated at 55 ℃ for 22-24 h. One colony from the TSA plate was picked and resuspended in 1 mL 0.5% buffered peptone water (BPW, Maerk GranuCult) and 50 µL of the resuspended cells was transferred into 5 mL MF1 and 5 mL MF2 respectively. A different inoculum preparation was used for 7953 due to the lack of reproducibility of growth when thawed glycerol stock was used directly to inoculate MF2. The inoculated milk formulations were incubated at 55 ℃ for 24 h, which was sufficient time for bacteria to reach stationary growth phase.

Table 4-1 Total sodium, calcium, fat, protein and lactose concentrations of MF1 and MF2

Milk formulation	Sodium	Calcium	Fat	Protein	Lactose
MF1	59 mM	33 mM	1.5 g/L	81.7 g/L	3.9 g/L
MF2	108 mM	7 mM	1.7 g/L	81.7 g/L	3.9 g/L

4.2.2 Cell attachment in mTSB media

A volume of 10 mL culture was centrifuged at 10000×g for 5 min, and washed once and resuspended in BPW. The cell number of the suspension was enumerated using a bacterial counting chamber and then adjusted to approximately 7.5 log CFU/mL. The viable cell number of the adjusted suspension was confirmed by plating the suspension on TSA using a drop-plate method described in Herigstad et al. (2001). To test the effect of calcium on cell attachment, a 100 µL adjusted cell suspension from mTSB culture was used to inoculate 10 mL fresh mTSB (control) and calcium-supplemented mTSB (post-conditioning) media (Table 4-2). To test the effect of pre-treatment with calcium on cell attachment, 100 µL adjusted cell suspension from mTSB (non-conditioning) and calcium-supplemented mTSB (pre-conditioning) culture were used to inoculate 10 mL fresh calcium-supplemented mTSB respectively (Table 4-2). One millilitre well-mixed inoculated fresh medium was dispensed into 1 well of a 24-well plate containing a polystyrene coupon (RD128-PS) (Biosurface Technologies Corporation, Bozeman, MT) or a stainless steel coupon (Part #RD128-316) (Biosurface Technologies Corporation, Bozeman, MT). Prior to each experiment, polystyrene coupons were ultrasonically cleaned in 1 M NaOH solution for 30 min and washed three times with deionised water. Coupons were soaked in 70% ethanol for more than 2h, then rinsed three times in sterilized deionized water and dried at 55 ℃, after which coupons were transferred into a 24-well plate (Falcon) and placed horizontally. The plate was then incubated at 55 ℃ for 30 min. After incubation, coupons were picked out using sterilised forceps and washed three times in approximately 50 mL of 0.85 % saline by gently dipping into the saline water, a new batch of saline was used after each dipping. Washed coupons were then used for cell enumeration.

Table 4-2 Inoculum preparation and testing conditions of cell attachment in mTSB media

	Inoculum preparation condition	**Testing condition**
The effect of pre-conditioning	• mTSB (non-conditioning) • 7 mM calcium supplemented mTSB (pre-conditioning)	• 7 mM calcium supplemented mTSB
The effect of post-conditioning	• mTSB	• mTSB (non-conditioning) • 7 mM calcium supplemented mTSB (post-conditioning)

4.2.3 Quantification of Cell surface polymers

To determine the amount of cell surface protein and surface-associated exopolysaccharide of *G. stearothermophilus* strains grown in mTSB and calcium-supplemented mTSB media, the following procedure was used. Culture was prepared as follows: One aliquot of frozen culture was streaked onto a TSA plate and incubated at 55 ℃ for 22-24 h. One colony from a TSA plate was picked and resuspended in 1 mL of BPW, each 100 µL of resuspended culture was transferred into 100 mL mTSB and calcium-supplemented mTSB in a 250 mL Erlenmeyer flask. Prior to inoculation, the pH of all fresh media was adjusted to 7.2 using 1 M HCI. The culture was incubated at 55 ℃ for 10 h. The amount of surface protein was quantified based on a method used in Somerton et al. (2012) with minor modification. Briefly, 50 mL culture was centrifuged at 11800 ×g for 10 min and the supernatant was discarded. The cell pellet was washed once in 25 mL saline and resuspended in 2.5 mL saline. The cell number of 2.5-mL cell concentrate was enumerated using a bacterial counting chamber. A volume of 800 µL 100-1000 times dilution, depending on the strain and culture condition, of the 2.5-mL cell concentrate was mixed with 200 µL of Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories, Inc., Hercules, CA) and the OD_{595nm} was measured against a blank using a spectrophotometer. The blank was prepared by mixing 800 µL of deionized water with 200 µL of Bio-Rad protein assay dye reagent concentrate. To quantify the amount of cell surface-associated exopolysaccharide, a modified tryptophan assay was used based on methods described in Dall and Herndon (1989) and Somerton et al. (2012). One millilitre of 2.5-mL cell concentrate was sonicated in an ultrasonic water bath (Ultrasonic Cleaner 010T) for 1 min. The removed cells were packed by centrifugation at 10000 ×g for 5 min. 100 µL of the exopolysaccharide in the supernatant was precipitated by adding dropwise to 1 mL of absolute ethanol and then centrifuged at 10000 ×g for 20 min. The supernatant was discarded, and the

exopolysaccharide pellet was resuspended in 1 mL of deionized water and mixed by vortex. Seven millilitre of sulfuric acid (77%, vol/vol) and then 1 ml of L-tryptophan (10 g/L) were added to the 1 mL exopolysaccharide suspension, and each suspension was mixed thoroughly by vortex. One millilitre mixture was transferred to one 1.5 mL tube and incubated for 20 min at 100 ℃ in a heated block (Techne Dri-Block DB-3). After heating, the absorbance of each mixture was measured at 500 nm against a blank using a spectrophotometer. The blank was prepared by mixing 7 mL of sulfuric acid and 1 mL of L-tryptophan with 1 mL deionized water, and then subjecting to the same treatment as other samples. Concentrations of surface protein and exopolysaccharide were calculated based on the standard curves generated using either bovine serum albumin (Sigma) or dextran (Sigma-Aldrich), and results were expressed as concentrations normalized by respective cell numbers counted using a bacterial counting chamber.

4.2.4 Zeta potential analysis

The cell surface charge was determined by zeta potential analysis. Ten millilitre culture was centrifuged at 10000 × g for 5 min and washed once in saline. The cell pellet was then resuspended in saline, and the cell number of the suspension was enumerated using a bacterial counting chamber and adjusted to approximately 7.5 log CFU/mL. The cell number of the adjusted cell suspension was checked by plating the suspension on a TSA plate using the drop-plate method. To test the effect of calcium on cell surface charge, a culture was prepared by transferring each 10 µL adjusted cell suspension from mTSB culture to 1 mL saline (pH 7.2) and calcium-supplemented saline (pH 7.2). To test the effect of pre-treatment with calcium on cell surface charge, a test culture was prepared by transferring 10 µL adjusted cell suspension from mTSB and calcium-supplemented mTSB culture to 1 mL saline or calcium-supplemented saline (pH 7.2) respectively. Test cultures were mixed by vortex and zeta potential was then analysed using a Malvern ZetaSizer IV (Malvern Instruments Ltd, UK).

4.2.5 Hydrophobic interaction chromatography (HIC)

The surface hydrophobicity of cells cultured in mTSB and calcium-supplemented mTSB was measured to investigate the potential effect of calcium on the cell surface hydrophobicity. A

modified HIC method was employed as reported by Palmer et al. (2010). A chromatography column was prepared by plugging 0.03 g of glass wool to the tip of a Pasteur pipette, and the pipette was then filled with 1 mL of hydrophobic resin suspension (Sigma Phenyl-Sepharose CL-4B). The culture for testing was prepared as described below: mTSB culture and calcium-supplemented mTSB culture were centrifuged at 10000×g for 5 min and washed once in 0.05 M phosphate buffer (pH7.2) containing either 0 M or 4 M NaCl. Cell pellets were resuspended with the same buffer used in the washing step, and the cell number was adjusted to approximately 8.1 log cells/mL using a bacterial counting chamber. Prior to analysis, each column was washed with 10 mL of buffer, the NaCl concentration of buffer was the same as the culture to be used. Each column was loaded with 0.3 mL culture and eluted with 0.9 mL buffer with the corresponding NaCl concentration. The OD_{600nm} of the 4-time diluted cultures and the eluted samples were measured using a spectrophotometer. The percentage of retention was calculated as follows:

$$\%retention=[(A_0-A_1)/A_0] \times 100 \quad (1)$$

A_0= OD_{600nm} of 4-time diluted testing culture, A_1=OD_{600nm} of the eluted testing culture.

4.2.6 Cell attachment in milk formulations

The inoculum prepared using either MF1 or MF2 was diluted in fresh milk formulation MF1 or MF2 to make the initial cell number at approximately 5.5 log CFU/mL. A volume of 1.5 mL well-mixed inoculated fresh milk formulation was dispensed into 1 well of a 24-well plate containing a horizontally placed stainless-steel coupon. The plate was then incubated at 55 ℃ for 30 min. After incubation, coupons were picked out by sterilised forceps and washed three times in approximately 50 mL of 0.85 % saline by gently dipping into the saline water to rinse off planktonic and loosely attached cells. Cells attached to the coupon after washing were enumerated.

4.2.7 Cell enumeration

To enumerate biofilm cells on coupons, the beads-beating method was used as described by Wang et al. (2021). Briefly, washed coupons were transferred into glass tubes, each containing 12 g sterile glass beads with diameter of 6.35 mm and 5 mL 0.5% BPW. Glass tubes were

mixed by vortex for 1 min to detach biofilm cells into the surrounding BPW followed by serial 10-fold dilutions in 0.5% BPW. The drop-plate method was used to enumerate cell numbers on TSA plates with the overnight incubation at 55℃. The results were expressed as numbers of culturable cells per coupon.

4.2.8 Statistical analysis

All data presented are means with standard deviations from at least two biological replicates unless stated otherwise. Significant differences were determined by one-way ANOVA using Minitab Statistical Software (Minitab Version 18) at a 95% confidence (P<0.05).

4.3 Results and discussion

4.3.1 Cell attachment in mTSB media

Calcium affected cell attachment of *G. stearothermophilus* dairy isolates but not the reference strain and the effects were substratum specific. Post-conditioning with calcium significantly (P<0.05) increased the cell attachment of A1 on polystyrene compared with the control, while it did not affect P3 and 7953 (Figure 4-1 A). Pre-conditioning with calcium significantly increased cell attachment of P3 on polystyrene comparing with post-conditioning, but the same effect was not observed for A1 and 7953 (Figure 4-1 A). Similarly, an increased attachment of cells pre-conditioned with calcium was found in *Geobacillus* sp. F75 (Somerton et al. 2013). The different patterns of calcium stimulating cell attachment of *G. stearothermophilus* dairy isolates on polystyrene indicated a strain specific effect potentially associated with individual cell surface properties. Cell surface charge, hydrophobicity and surface associated polymers were characterized to understand the change of cell surface properties due to calcium.

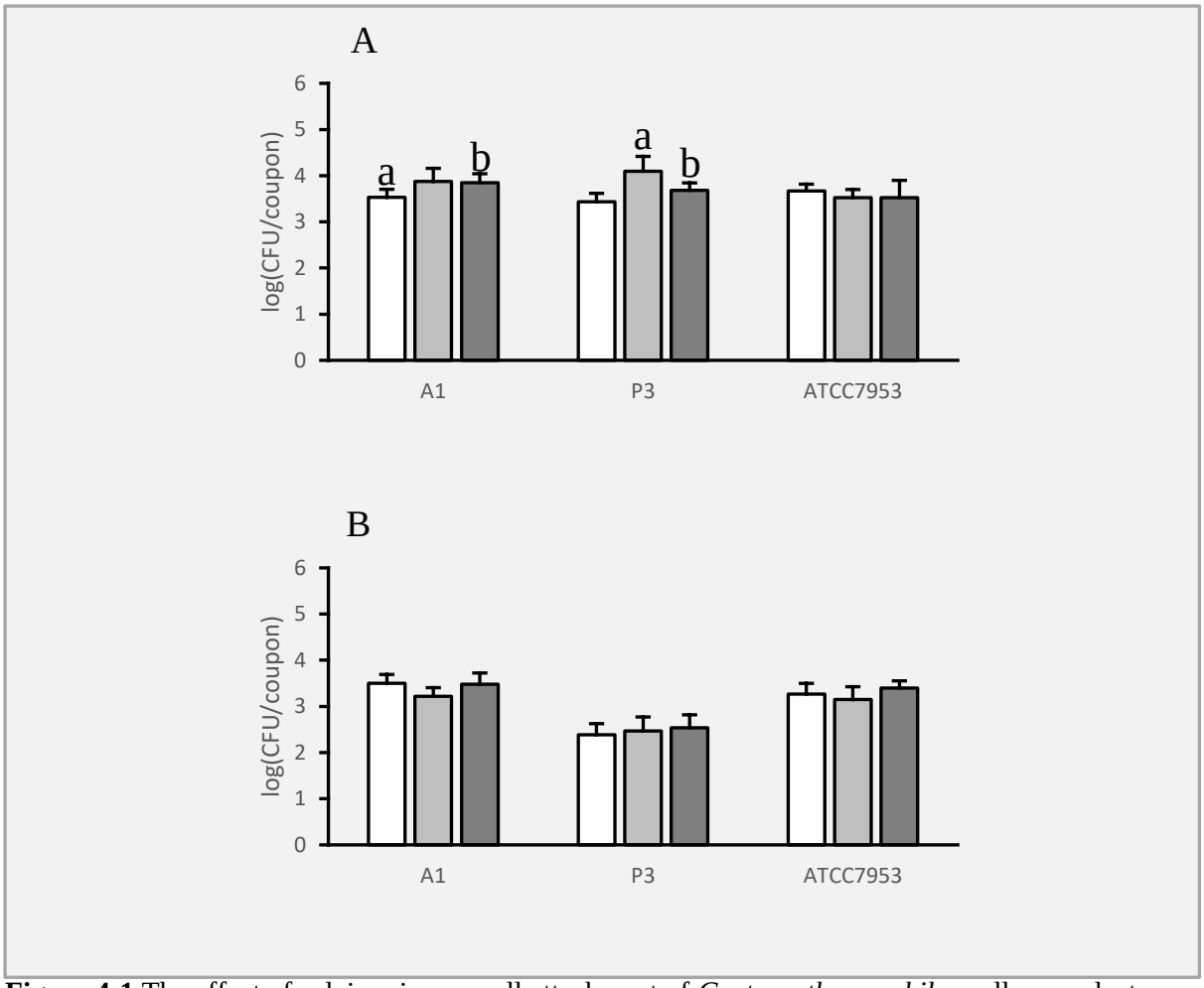

Figure 4-1 The effect of calcium ion on cell attachment of *G. stearothermophilus* cells on polystyrene (A) and stainless steel (B). White bars represent cell attachment under control (without pre-conditioning and post-conditioning), light grey bars represent attachment of cell pre-conditioned with 7 mM calcium, dark grey bars represent attachment of cell post-conditioned with 7 mM calcium. Data bars with different letters within each strain represent significant difference (P<0.05) between each other on polystyrene

Interestingly, substratum also seemed to affect the cell attachment as the promotive effect of calcium observed in cell attachment of A1 and P3 on polystyrene was absent on stainless steel (Figure 4-1 A and B). The relative lower amount of attached cells of P3 on stainless steel compared with polystyrene also indicated the effect of the substratum on cell attachment (Figure 4-1A and B). It is a consensus that the substratum characteristics are an important factor affecting bacterial cell attachment. Palmer et al. (2007) reviewed that high surface roughness and micro-topography like surface cavities assist in cell attachment. Xia et al. (2013) showed a higher maximum attachment capacity of *Acidithiobacillus ferrooxidans* to complex chalcopyrite surface than elemental sulphur or quartz surfaces. Attachment of *Salmonella*

serovars and *L. monocytogenes* to stainless steel is also lower than on plastic surfaces (Veluz et al. 2012).

4.3.2 Cell surface charge

Cell surface charge is often determined as the zeta potential (Palmer et al. 2007), which is affected by pH, ionic strength of the medium and concentration of the suspension (Malvern Instruments 2010). Initially, 40 mM calcium supplemented mTSB was tested. However, loss of biomass was observed during cell harvest after saline wash for all three strains when cells were cultured in 40 mM calcium supplemented mTSB, which indicated potential cell damage and lysis. The release of cytoplasmic content could interfere with the zeta potential analysis and surface polymers analysis. Therefore, only the mTSB and 7 mM calcium supplemented mTSB were included. In the post-conditioning with calcium, zeta potential of three strains decreased compared to the respective control with P3 and 7953 being significant (P<0.05, Figure 4-2). The decrease in zeta potential in the presence of cations is expected due to the compression of the diffuse double layer surrounding the bacterial surface (Somerton 2013). Zeta potentials of *Escherichia coli*, *Pseudomonas* sp., *Rhodococcus* sp. and *B. subtilis* become less negative with increasing ionic strength of both NaCl and $CaCl_2$, and it was thought to be due to the compression of the electrostatic double layer (Long et al. 2009). Similarly, less negative zeta potentials of *Streptococcus suis* and *E. coli* were shown with increasing KCl ionic strength. The increase of ionic strength is thought to mitigate the extent of electrostatic repulsion between bacteria and the substratum and facilitate bacterial attachment (Zhao et al. 2015). However, the current results indicate that lower zeta potential did not result in higher cell attachment. Post-conditioning with 7 mM calcium significantly increased the cell attachment of A1 (Figure 4-1) compared to the control with zeta potentials of the two conditions being insignificantly different (P>0.05). While post-conditioning with 7 mM calcium significantly (P<0.05) decreased the zeta potential of P3 and 7953 (Figure 4-2), it did not lead to higher cell attachment on either polystyrene or stainless steel (Figure 4-1). In addition, zeta potentials of the pre-conditioned cells of A1 or P3 in calcium-supplemented saline were significantly (P<0.05) less negative compared to the respective post-conditioned cells in calcium-supplemented saline (Figure 4-2) with only P3 showing a significant increase in cell attachment. Therefore, calcium mediated change in zeta potential did not correlate well

with the trend in cell attachment and might not be a determinant for cell attachment of the current strains.

Interestingly, when comparing zeta potentials of calcium pre-conditioned cells suspended in either saline or calcium-supplemented saline, A1 and P3 both showed significantly (P<0.05) less negative zeta potentials in calcium-supplemented saline. This could possibly be due to the change of cell surface structures during calcium pre-conditioning and the subsequent adsorption of calcium onto the cell surface.

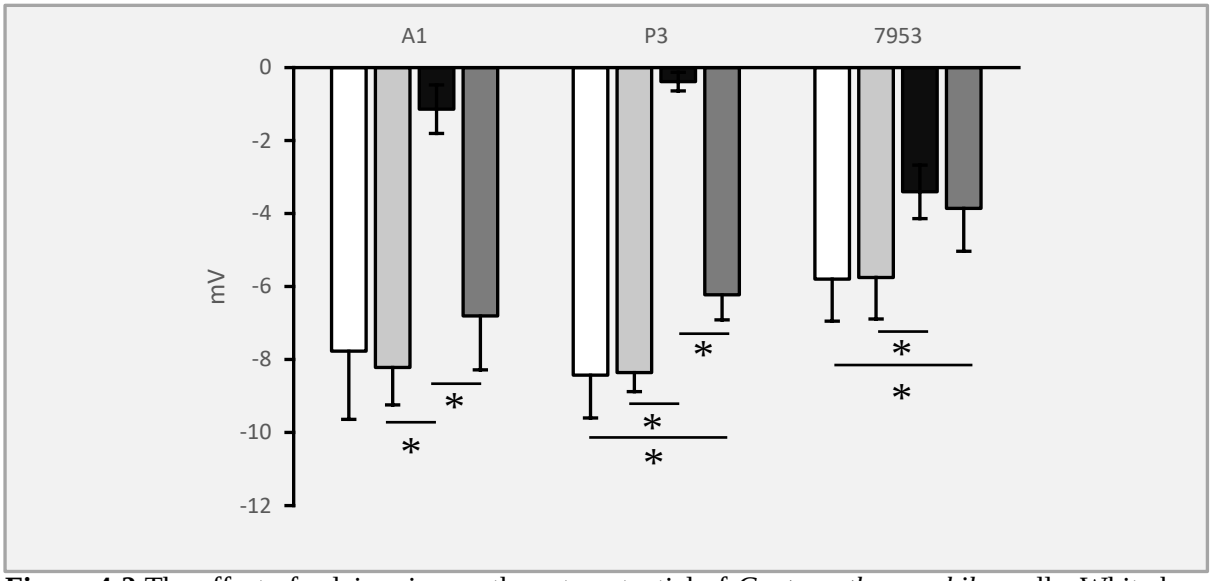

Figure 4-2 The effect of calcium ion on the zeta potential of *G. stearothermophilus* cells. White bars represent zeta potentials of non-conditioned cells in saline, light grey bars represent zeta potentials of 7mM calcium-preconditioned cells in saline, black bars represent zeta potentials of 7 mM calcium-preconditioned cells in 7 mM calcium supplemented saline, dark grey bars represent zeta potentials of non-conditioned cells in 7 mM calcium-supplemented saline. Data bars connected with brackets and asterisks are significantly different to each other (P<0.05)

The importance of zeta potential to bacterial cell attachment is still unclear due to contrasting results from different studies. During *S. suis* and *E. coli* cell attachment to soil particles, increasing cell attachment occurs with less negative zeta potentials along with the increasing ionic strength from 1 mM to 60 mM KCl (Zhao et al. 2015). Deposition efficiencies of *E. coli*, *Pseudomonas* sp., *Rhodococcus* sp. and *B. subtilis* on silica surfaces increase with increasing ionic strength of NaCl and $CaCl_2$ ranging from 1 to 100 mM and is consistent with the less negative zeta potentials (Long et al. 2009). However, Wang (2016) tested attachment of pYV+ and pYV- cells of *Yersinia enterocolitica* that varied in zeta potential in the presence or absence of calcium on polycarbonate, and found that cells with more negative zeta potentials showed

higher cell attachment on polycarbonate regardless of calcium. Palmer et al. (2010) tested two strains of *A. flavithermus* and found that the strain that has the stronger negatively charged surface shows greater ability to attach to stainless steel. In addition, the presence of calcium from 1 to 5 mM showed limited effect on zeta potentials of *Streptococcus thermophilus* dairy isolates and it was concluded that electrostatic interactions play a minimal role in bacterial adhesion (Van Hoogmoed et al. 1997). The correlation between zeta potential and bacterial attachment was also absent for thermophilic bacilli (Parkar et al. 2001). Dorobantu et al. (2009) used both the classic and extended DLVO models to describe the force between immobilized bacterial and atomic force microscopy tips and found that the extended DLVO fitted better to the experimental observation. This indicates that Lifshitz- van der Waals and electrostatic double-layer interactions described by the classic DLVO theory were not sufficient enough to describe cell-substratum interactions and acid-base interactions that account for attractive hydrophobic interactions and repulsive hydration effects should also be considered. Hitherto, the effect of calcium on cell surface charge and the role of zeta potential in cell attachment are not conclusive.

4.3.3 Cell surface hydrophobicity

Hydrophobicity is an important short-range interaction determining bacterial cell attachment as described in the extended DLVO theory (Hermansson 1999). The cell surface hydrophobicity was measured in the current study to understand its potential role in cell attachment. Hydrophobicity was tested using the HIC method at both low and high ionic strength. During hydrophobicity measurement, both electrostatic and hydrophobic interactions may occur at low ionic concentrations, while high ionic strengths of NaCl reduce the possible electrostatic interactions and indicate more accurate results (Palmer et al. 2007). However, the results at lower ionic concentration may indicate the interference of electrostatic interactions between bacterial cell surfaces and the hydrophobic resin because of pre-conditioning and show some evidence on cell surface change in response to calcium pre-conditioning. Figure 4-3 illustrates that all three strains pre-conditioned with calcium showed similar hydrophobicity to non-conditioned cells at high ionic strength. The results suggest that the increased cell attachment of P3 on polystyrene with calcium-pre-conditioning was not due to cell hydrophobicity. Interestingly, cells of both A1 and P3 became less hydrophobic at low ionic strength when pre-conditioned with calcium while 7953 remained unchanged. This observation

could be due to the change in cell surface polymers of A1 and P3 during calcium pre-conditioning and the resultant change in electrostatic interactions causing cells to be more repulsive to the resin.

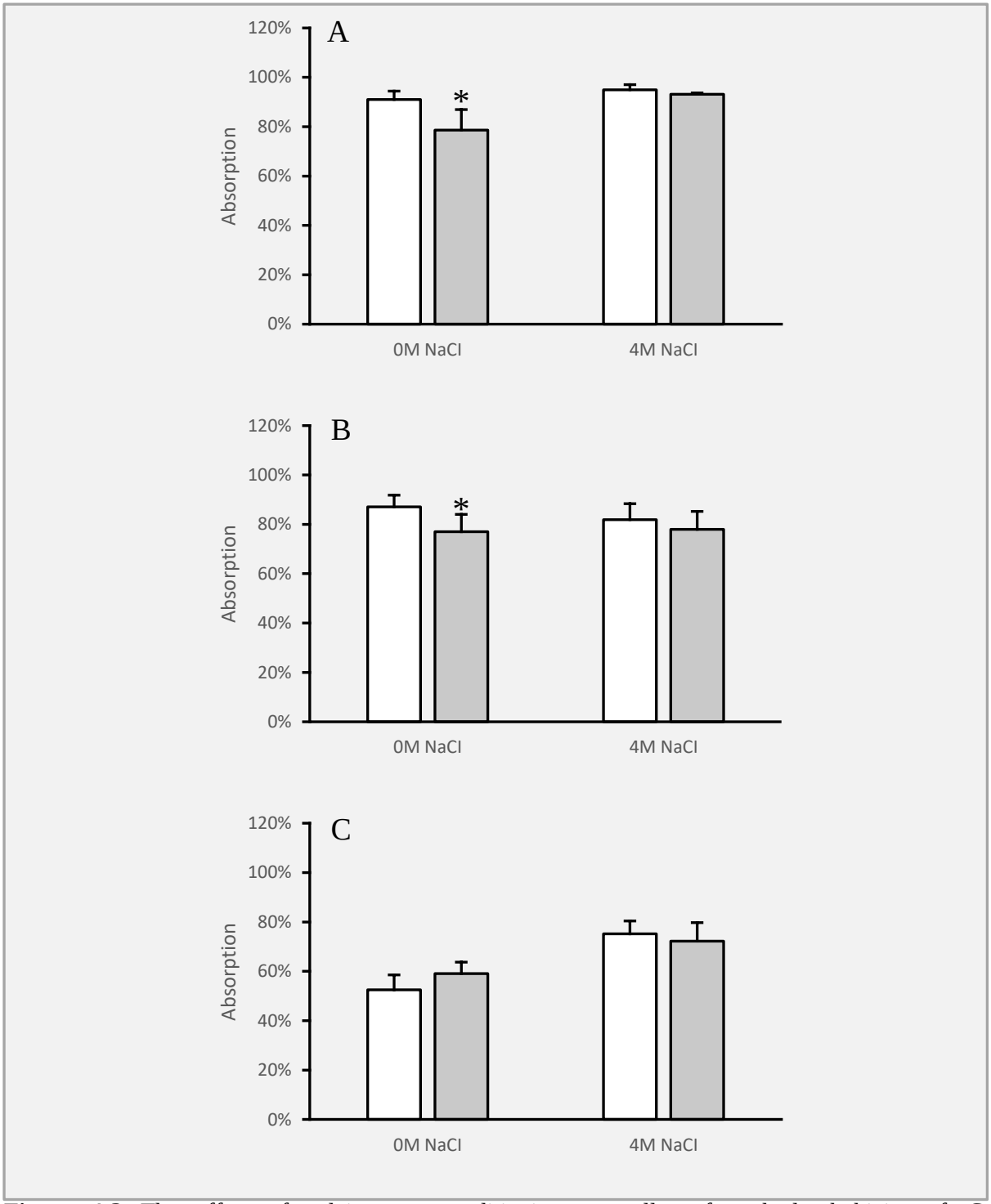

Figure 4-3 The effect of calcium pre-conditioning on cell surface hydrophobicity of *G. stearothermophilus* strain A1(A), P3(B) and 7953 (C). white bars represent non-conditioned cells (control), grey bars represent 7 mM calcium pre-conditioned cells. Data bars connected with brackets

and asterisks represent a significant difference from each other ($P<0.05$). Data are expressed as means of three replicates with standard deviations

The importance of cell surface hydrophobicity in predicting bacterial cell attachment varies between studies. Thwala et al. (2013) studied the interactions between two bacterial strains and nanofiltration membrane and found that the strain that was relatively hydrophilic and demonstrated a negatively charged surface showed the lowest level of interactive force. Peng et al. (2001) reported a positive correlation between the attachment of *B. cereus* vegetative cells on stainless steel and cell surface hydrophobicity. Liu et al. (2004) developed a mathematical model to describe the hydrophobic/hydrophilic interaction on microbial adhesion on polystyrene, bacterial adhesion to activated sludge flocs and microbial auto-aggregation of multiple bacterial species. A good agreement was found between model prediction and experimental data that increased cell surface hydrophobicity would favour cell adhesion. Van Loosdrecht et al. (1987) and Nguyen et al. (2011) also reported a good correlation between cell wall hydrophobicity of *Campylobacter* and the extent of attachment. However, Parkar et al. (2001) tested the attachment of cells and spores of thermophilic bacilli to stainless steel and found no correlation between degree of attachment and surface hydrophobicity. Flint et al. (1997) was also unable to demonstrate any relationship between hydrophobicity and cell attachment of 12 thermophilic streptococci to stainless steel. Despite a contrasting view regarding the corelation between cell surface hydrophobicity and cell attachment, it may still play an important role in cell attachment.

4.3.4 Cell surface polymers

Cell surface polymers influence cell surface charge and hydrophobicity, act as adhesins to facilitate cell attachment and are involved in steric interactions and bridging effects (Rijnaarts et al. 1999, Palmer et al. 2007). Calcium could act as an environmental stimulus in changing cell physiology and stimulate expression of adhesion related surface structures (Cruz et al. 2012), as well as interacting directly with surface adhesins in facilitating cell attachment (Wang et al. 2019). Somerton et al. (2013) concluded that pre-conditioning of *G. stearothermophilus* F75 with calcium increased the subsequent cell attachment compared to non-conditioned cells indicating a change in the metabolism and physiology induced by calcium.

In the present study, pre-conditioning with calcium significantly (P<0.05) reduced the cell associated polysaccharides of A1 but increased the amount of polysaccharide of 7953 (Figure 4-4). However, the amount of polysaccharide extracted from the surface of P3 remained unchanged (Figure 4-4). These results indicate a strain specific change of cell associated polysaccharides in response to calcium. Pre-conditioning with calcium significantly (P<0.05) decreased the amount of surface associated proteins of A1 and P3 (Figure 4-4) while the total amount of surface associated protein reduced slightly for 7953. Additionally, the total amount of surface polymers differs between different strains, with more proteins and polysaccharides found in strain A1 than P3 and 7953 of non-conditioned cells (Figure 4-4).

The presence of functional groups in cell surface polymers are associated with cell surface charge. It is thought that at physiological pH, carboxyl, phosphoryl, hydroxyl and amino functional groups present on the Gram-positive bacteria are deprotonated (Thomas and Rice 2014), rendering a negatively charged cell surface (Palmer et al. 2007). Therefore, less surface polymers could potentially lead to less negative surface charge. However, the current results suggest that the reduced amount of surface polymers did not result in the change of zeta potential. Although calcium-preconditioning reduced the total amount of cell surface proteins and polysaccharides of A1 (Figure 4-4), zeta potentials did not show a significant change (Figure 4-2). A similar observation was also observed for P3 and 7953. In addition, when comparing between strains, although A1 had relative higher amount of surface protein and polysaccharide than P3 (Figure 4-4), its zeta potential was similar to that of P3 (Figure 4-2). Two potential explanations are proposed to explain these disparate observations. Firstly, the current extraction method for polysaccharides might not be sufficient to detach all polysaccharides from cell surfaces, therefore leading to an underestimate of the polysaccharide amount and a disparate between the amount of surface polymers and zeta potentials. Secondly, the change in surface polymers could result in a different binding capacity and selectivity, as well as the numbers of binding sites towards positively charged ions in the suspension. This would lead to the similar zeta potential seen between non-conditioned and pre-conditioned cells in saline suspension despite a reduction in total amount of cell surface polymers. For example, the calcium binding capacity of whole cells and purified cell-wall material is associated with specific carboxylate and phosphate content of the cell walls and it shows differences between strains and species (Rose et al. 1997). Zhao et al. (2015) also found that decreased levels of

cell associated EPS do not confer less negative zeta potential with partial removal of EPS from *S. suis* resulting in a more negative zeta potential where an opposite trend was observed for *E. coli*. Interestingly, zeta potentials of pre-conditioned A1 and P3 cells showed significantly less negative zeta potential than unconditioned cells in calcium-supplemented saline, and this could be attributed to the biosorption of calcium to cell surface polymers following the change in cell surface structure. It was thought that the specific interaction between inorganic ions and a charged surface have a noticeable effect on the zeta potential (Malvern Instruments 2010), therefore the change in cell surface structure by calcium pre-conditioning could make calcium ions more readily adsorbed onto the cell surface. Based on the strain specific surface properties, it is assumed that the surface structure of A1 and polystyrene enable the bridging effect of calcium and facilitate the cell attachment. Similarly, Badihi Hauslich et al. (2013) found a species specific adhesion to a titanium implant surface in the presence of calcium and suggested that calcium may serve as a bridging agent in the adhesion. The bridging effect of calcium though high affinity sites on the bacterial surface and substrata was thought to play a role in the attachment of *L. salivarius*, *L. fermentum*, *S. mutans* and *P. aeruginosa* to hydroxyapatite (Wang et al. 2019). While the surface of P3 following calcium pre-conditioning promoted cell adhesion to polystyrene, the bridging effects remain unknown. It could be the interaction between the cell and the polystyrene with/without calcium bridging. Although cell surface polymers are thought to affect cell attachment in the current study, the specific role played by those polymers is still unclear. The individual function of cell surface proteins, polysaccharides and other cell surface structures needs to be further studied.

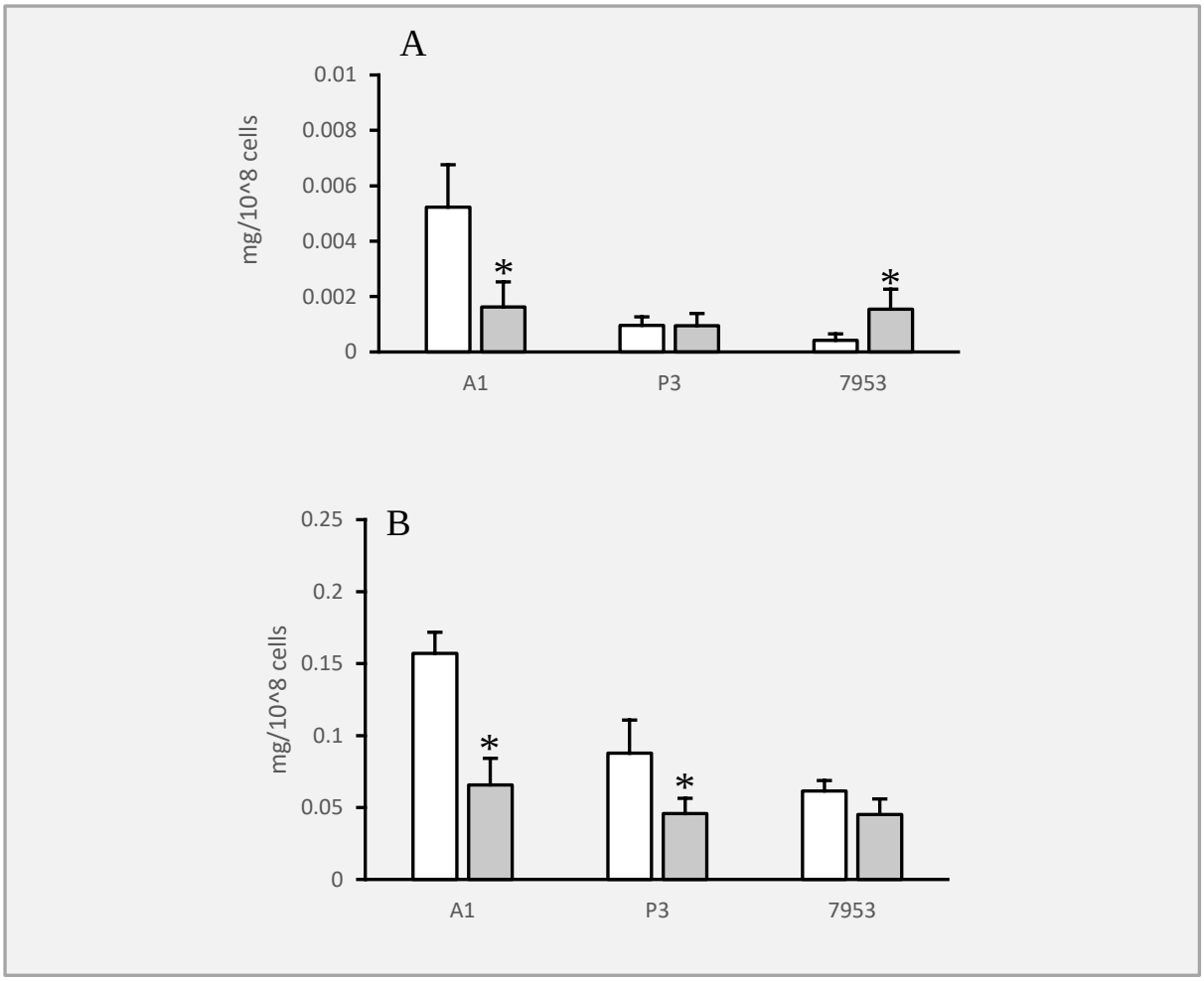

Figure 4-4 The effect of calcium ion on cell surface polysaccharide (A) and protein (B) production of *G. stearothermophilus* cells. White bars represent non-conditioned cells, grey bars represent 7 mM calcium preconditioned cells. Data bars with asterisks indicate significant difference ($P<0.05$) between control and calcium supplementation for each strain

4.3.5 Cell attachment in milk formulations

Cell attachment in milk formulations was characterized to check if attachment varied between MF1 and MF2. Both MF1 and MF2 were used to prepare the inoculum to check the effect of different pre-conditioning environments on cell attachment. The attachment assay was conducted in MF1 and MF2 to check the effect of post-conditioning with milk formulations on cell attachment. A1, P3 and 7953 had similar cell numbers attached to stainless steel in MF1 and MF2 regardless of pre-culture conditions (Figure 4-5). The present results agree with the previous study that milk formulations generally would not influence cell attachment of *G. stearothermophilus* (Somerton et al. 2013). It indicated that both pre-conditioning and post-conditioning of cells using MF1 and MF2 had little effect on the cell attachment on *G. stearothermophilus* in milk formulations.

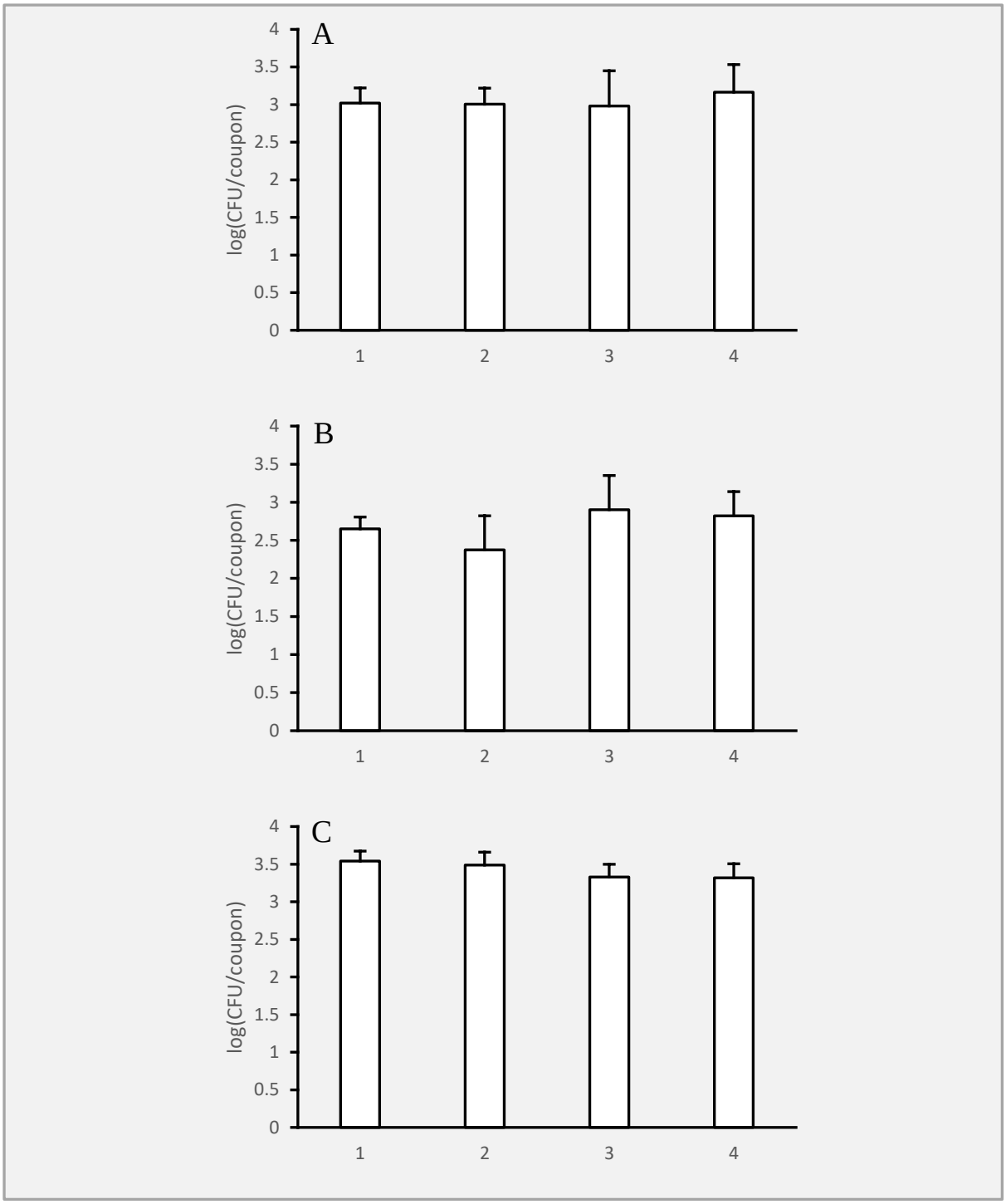

Figure 4-5 Cell attachment of *G. stearothermophilus* A1 (A), P3 (B) and 7953 (C) on stainless steel in MF1 (1 and 3) and MF2 (2 and 4) using inocula prepared in MF1 (1 and 2) and MF2 (3 and 4). Statistical comparison was performed between 1 and 2, 3 and 4 and no significant (P<0.05) differences were determined

4.4 Conclusion

Calcium increased the cell attachment of dairy isolates on polystyrene but not stainless steel. Cell surface properties such as surface charge and hydrophobicity are not correlated with the attachment. Based on the strain specificity of cell surface polymers and the change of cell surface polymers during calcium pre-conditioning, it is likely that the increased cell attachment of A1 and P3 on polystyrene was related to strain specific cell surface structures. Milk formulations with different calcium and sodium content had little effect on the attachment of *G. stearothermophilus*.

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4.6 Supplementary materials

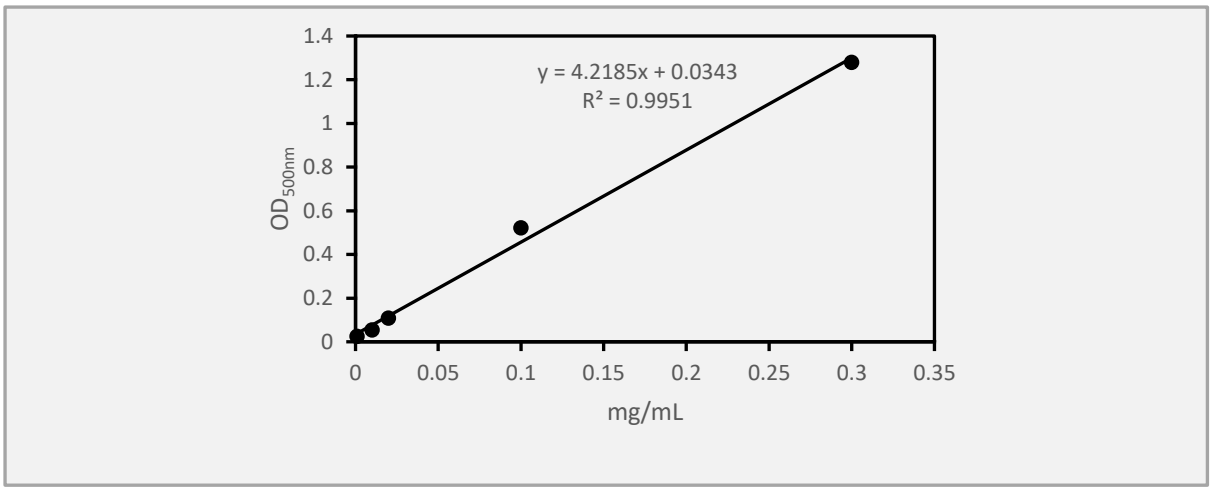

Figure S4-6 Standard curve for dextran

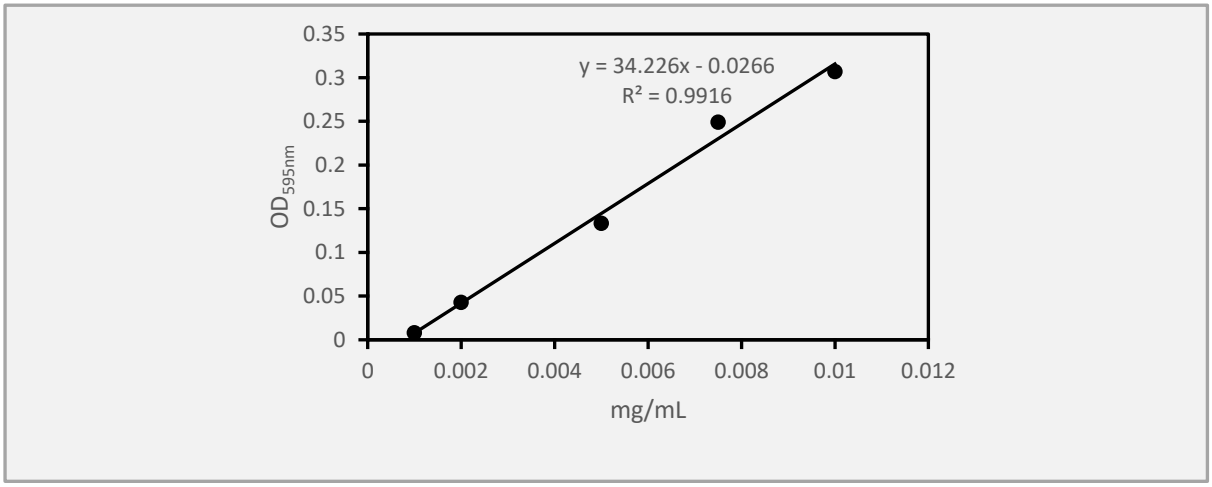

Figure S4-7 Standard curve for bovine serum albumin

Chapter 5 The effect of milk formulations on biofilm formation of *G. stearothermophilus*

Partial content was submitted to International Dairy Journal in Sep 2021

5.1 Introduction

Chapter 3 and Chapter 4 outlined the effect of calcium on cell attachment and biofilm formation of *G. stearothermophilus.* However, the effect was inconsistent between different strains and gave little indication about the role of calcium in milk formulations. Until now, we cannot conclude that the limitation of calcium results in a lowered thermophile contamination in calcium-reduced milk powder observed in the dairy industry. In addition, it is likely that the previously observed promotive effect of extraneous calcium in a calcium-reduced milk formulation was limited to that specific culture environment and chosen strains (Somerton et al. 2015). Therefore, biofilm formation was investigated in milk formulations to re-exam the potential inhibitory effect of the calcium-reduced milk formulation.

In the dairy industry, milk formulations with different cation profiles are produced via ion-exchange in order to improve the functional properties of the product (Bhaskar et al. 2004, Bhaskar et al. 2007). Specifically, calcium in the milk is partially removed in exchange for sodium, rendering a milk formulation with lower amount of calcium and excessive amount of sodium compared with original milk (Bhaskar et al. 2007). Decalcification of milk was found to affect the biofilm formation of *G. stearothermophilus* in milk formulations. Somerton et al. (2015) showed a biofilm inhibitory effect of a calcium-reduced milk formulation B (MF2 in the current study) on *Geobacillus* spp. dairy isolates F75, 183 and TRa (strain F75 and 183 were later identified as *G. stearothermophilus* by MALDI-TOF, data not shown) compared to the milk formulation A (MF1 in the current study) that has a higher amount of calcium and lower amount of sodium. Supplementation of calcium to milk formulation B counteracts the inhibitory effect and restores biofilm growth to a similar level as milk formulation A. In addition, supplementation of sodium in milk formulation A inhibits the biofilm growth of *Geobacillus* spp. (Somerton et al. 2015). It was therefore thought that low calcium and high sodium in a milk formulation are simultaneously required to inhibit *Geobacillus* spp. biofilm growth (Somerton et al. 2015). Two mechanisms were proposed for this observation including the effect of electrostatic interaction between cations and the biofilm matrix on biofilm structural integrity and the cell motility-sessility switching to different cation concentrations (Somerton et al. 2015).

However, work conducted in chapter 3 using a base medium failed to reproduce the promotive effect of calcium on biofilm formation of *G. stearothermophilus*. The discrepancy between the two studies regarding the effect of calcium was thought to be due to the different culture media used, strain specificity and bacterial growth history. To better understand the inhibitory effect of MF2 on *G. stearothermophilus* biofilm formation, three *G. stearothermophilus* strains, different from those studied by Somerton et al. (2015), were used to study the effect of strain specificity and bacterial growth history on the biofilm inhibition in MF2 using various milk formulations.

5.2 Materials and methods

5.2.1 Bacterial strains and inoculum preparation

Three *G. stearothermophilus* strains were used in this study. *G. stearothermophilus* A1 and P3 are dairy isolates and ATCC 7953 (7953) was used as a reference strain. The chosen strains represent a genotypic diversity in *G. stearothermophilus* (Burgess et al. 2017). To study the effect of the growth history, the bacterial inocula were prepared using either milk formulations (MF1 or MF2) or modified TSB media (mTSB) to simulate the conditions in milk powder manufacturing plant and typical laboratory condition respectively. MF1 and MF2 in the current study have similar cation, protein, lactose and lipid content to milk formulation A and milk formulation B respectively, as used by Somerton et al. (2015). In the preparation of inocula using milk formulations, different approaches were used between the dairy isolates and the reference strain. For strain A1 and P3, 5 µL thawed frozen glycerol stock was used to inoculate 5 mL MF1 and 5 mL MF2 respectively. For 7953, one aliquot of frozen stock was streaked onto a TSA plate (40 g/L) (BD Biosciences) and incubated at 55 ℃ for 22-24 h, one colony from TSA plate was picked and resuspended in 1 mL 0.5% buffered peptone water (BPW, Maerk GranuCult) and 50 µL of the resuspended culture was transferred into 5 mL MF1 and 5 mL MF2 respectively. A different inoculum preparation procedure was used for 7953 due to the lack of reproducibility of growth when thawed glycerol stock was used directly to inoculate MF2. The inoculated milk formulations were incubated at 55 ℃ for 24 h, which was sufficient time for bacteria to reach stationary growth phase. To prepare the inoculum using mTSB medium, one aliquot of frozen stock was streaked onto a TSA plate and incubated at 55 ℃ for22-24 h. One colony from a TSA plate was picked and resuspended in 1 mL of 0.5% BPW, 10 µL of the resuspended culture was transferred into 10 mL mTSB and incubated at 55 ℃ for 10h, which was sufficient time for bacteria to reach stationary growth phase. mTSB medium was prepared in accordance with Wang et al. (2021) and milk formulations were prepared as described by Somerton et al. (2015).

5.2.2 Biofilm formation assay

Stainless steel coupons were cleaned prior to use as described in chapter 3. Coupons were transferred into a 24-well plate (Falcon) and placed horizontally. Inoculum prepared with

mTSB media was washed once and resuspended in casein digest medium (1g/L, BD Bioscience, containing 0.004 mM total calcium), and then diluted in MF1, MF2, MF1 supplemented with 105 mM NaCl (LabServ, Thermo Fisher Scientific New Zealand Ltd.) and MF2 supplemented with 26 mM $CaCl_2$ (LabServ, Thermo Fisher Scientific New Zealand Ltd.), respectively (Table 5-1). Inocula prepared with milk formulations were diluted in the fresh milk formulation that was of the same composition as that used during the inoculum preparation. More than 200 times dilution was used to ensure the initial cell numbers after dilution were approximately 4.5 log CFU/mL. Inoculated milk formulation (1.5 mL) was added to each well of a 24-well plate. The plate was wrapped in a resealable plastic bag and incubated at 55 ℃ for 6, 10, 14 or 18 h under static conditions. At each time point, coupons were removed from the wells with sterilised forceps and washed three times in 0.85% saline by gently dipping into the saline water. Washed coupons were used for cell enumeration.

Table 5-1 Total calcium and sodium concentrations of MF 1, MF2 and cation supplemented MF1 and MF2

MF and cation-supplemented MF	**Total cation concentration (mM)**	
	sodium	calcium
MF1	59	33
MF2	108	7
MF1+105 mM NaCl	164	33
MF2+26 mM $CaCl_2$	108	33

5.2.3 Cell enumeration

To enumerate biofilm cells on coupons, the bead-beating method was used as described in chapter 3. Briefly, washed coupons were transferred into glass tubes, each containing 12 g sterile glass beads with diameter of 6.35 mm and 5 mL 0.5% BPW. Glass tubes were mixed by vortex for 1 min to detach biofilm cells into the surrounding BPW followed by serial 10-fold dilutions in 0.5% BPW. The drop-plate method was used to enumerate cell numbers on TSA plates with the overnight incubation at 55℃. The results were expressed as numbers of culturable cells per coupon.

5.2.4 Milk agar assay

The milk agar assay was done to compare with the results obtained in biofilm formation assay. The presence or absence of colonies on milk agar plates was noted at 24, 48 and 72 h. Milk agar plates were made by mixing reconstituted milk formulation MF1, MF2 and cation-supplemented MF1 and MF2 (2 g/98 mL) with equal volume of 3% (w/v) sterile agar at 50 ℃, the mixture was poured into sterile petri dishes and dried at room temperature for 2 days. The total sodium and calcium profiles were illustrated in Table 5-2. Bacterial inoculum was prepared as described in 2.1 using mTSB medium and 10 µL of 10 h culture of A1, P3 and 7953 were dropped onto the surface of milk agar plates followed by incubation at 55 ℃ for 72 h.

Table 5-2 Total calcium and sodium concentrations of milk agar and cation-supplemented milk agar

Milk agar and cation-supplemented milk agar	**Total cation concentration (mM)**	
	sodium	**calcium**
MF1	11.4	6.4
MF2	20.6	1.4
MF1+9.2 mM NaCl	20.6	6.4
MF2+5.0 mM $CaCl_2$	20.6	6.4

5.2.5 Statistical analysis

Results are expressed as means of at least two biological replicates with standard deviations. Significant differences between two different culture conditions at each time point were determined by one-way ANOVA with Tukey test (equal variances) or Games-Howell test (unequal variances) using Minitab Statistical Software (Minitab Version 18) at a 95% confidence (P<0.05). Regression analysis was conducted using Minitab Statistical Software (Minitab Version 18) at a 95% confidence (P<0.05) to determine the effects of culture time and inoculum preparation conditions on biofilm cell numbers.

5.3 Results and discussion

5.3.1 Biofilm formation of *G. stearothermophilus* in MF1 and MF2

The difference between biofilm formation of *G. stearothermophilus* in MF1 and MF2 was strain specific and affected by the growth history. When an inoculum was prepared using mTSB, MF2 inhibited biofilm formation of *G. stearothermophilus* dairy isolates A1 and P3 in comparison to MF1 (Figure 5-1 A, Figure 5-2 A). Biofilm cell numbers of A1 at 14 h and 18 h in MF2 were significantly lower ($P<0.05$) than in MF1 (Figure 5-1 A). A similar inhibition was observed for P3, where biofilm cell numbers in MF2 were significantly lower ($P<0.05$) than in MF1 at 6, 10, 14 and 18 h (Figure 5-2 A). This observation is similar to the results from Somerton et al. (2015) in that the milk formulation B with lower calcium and higher sodium content was found to inhibit biofilm formation of *G. stearothermophilus* F75 and 183 at 6, 10, 14 and 18 h. However, the reference strain 7953 showed similar biofilm cell numbers in MF2 and MF1 at all time points (Figure 5-3 A). When inocula were prepared using milk formulations, biofilm cell numbers of A1 at 10 and 18 h were significantly ($P<0.05$) higher in MF2 than in MF1 while biofilm cell numbers at 6 h and 14 h in MF2 were slightly higher than MF1 (Figure 5-1 B). In contrast, biofilm cell numbers of P3 and 7953 were generally lower in MF2 than in MF1 (Figure 5-2 B, Figure 5-3 B). Specifically, P3 showed a significant ($P<0.05$) decrease in biofilm formation in MF2 at 6, 10 and 14 h (Figure 5-2 B), while a similar decreasing trend ($P<0.05$) was shown for 7953 at all time points (Figure 5-3 B).

5.3.2 The effect of growth history on *G. stearothermophilus* biofilm formation in milk formulations

When comparing biofilm formation in MF1 and MF2, the medium used for the preparation of the inoculum influenced biofilm formation. For dairy isolates, the biofilm inhibitory effect of MF2 in comparison to MF1 observed in culture prepared from a mTSB inoculum became less or even disappeared when inocula were prepared in the milk formulation (Figure 5-1 and Figure 5-2). On the contrary, the inhibitory effect of MF2 on 7953 was greater when respective milk formulation inoculum was used (Figure 5-3). In addition, biofilm cell numbers of A1 were under the detection limit at 6 and 10 h in both MF1 and MF2 when a mTSB inoculum was used (Figure 5-1 A) while detectable cell numbers were shown in both MF1 and MF2 using milk

formulation inocula (Figure 5-1 B). P3 showed a similar trend with relatively higher biofilm cell numbers at 6 and 10 h when using milk formulation inocula (Figure 5-2 B) as compared to the mTSB inoculum (Figure 5-2 A). Regression analysis was conducted to determine the effect of culture time and growth history on biofilm cell numbers of A1, P3 and 7953 in MF1 and MF2, the results indicated that except for 7953 in MF2, both culture time and growth history significantly (P<0.05) affected the biofilm cell numbers in milk formulations. When MF1 and MF2 were used to prepare the inocula, cells were exposed to cationic variation as opposed to mTSB as the inoculum preparation medium. Additionally, mTSB has a different cationic profile to MF1 and MF2. It could be the cationic difference in MF1 and MF2 during inocula preparation that caused the following change of biofilm formation in milk formulations. The effect of growth history on biofilm formation in respect to cations, was reported by Somerton et al. 2013 in that when *G. stearothermophilus* was first cultured with additional magnesium, the subsequent biofilm formation was altered.

These results showed that growth history influences *G. stearothermophilus* biofilm formation in milk formulations. Previously, casein digest medium (1 g/L) was used to prepare the inoculum of *G. stearothermophilus* strains and the subsequent biofilm formation in milk formulation B (equivalent to MF2 in the present trial) was significantly lower than that in milk formulation A (equivalent to MF1 in the present trial) (Somerton et al. 2015). These findings also suggest that the previously shown decreased biofilm formation of *G. stearothermophilus* F75 *and* 183 in milk formulation B may be not only be due to the high sodium and low calcium concentrations, but also the bacterial growth history created by Somerton et al. (2015) using casein digest medium in the inocula preparation. Ideally, casein digest medium ought to be used in the current study to prepare inocula to maintain consistency with the previous study. However, both A1 and P3 used in the current study did not grow well in casein digest medium (data not shown), therefore mTSB medium was used to make sure there were sufficient cell numbers (at least 7 log CFU/mL) at the stationary phase. It is possible that the use of casein digest medium would mitigate the effect of growth history as both casein digest medium and milk formulations are of dairy origin. However, the preliminary study by Somerton et al. (2013) indicated that when casein digest medium was used, the biofilm formation of *G. stearothermophilus* F75 was significantly decreased in milk formulation 4 (equivalent to MF2 in the current trial) compared to milk formulation 2 (equivalent to MF1 in the current trial). But when respective milk formulations were used during inoculum preparation, the biofilm

inhibition of milk formulation 4 was reduced ((Somerton et al. 2013) supplemental material). This previous observation is consistent with the current findings and further shows that the inhibitory effect of MF2 on biofilm growth of *G. stearothermophilus* dairy isolates is associated with the growth history of the inoculum. When comparing Figure 5-1 B, Figure 5-2 B and Figure 5-3 B, A1 showed higher biofilm formation in MF2 than in MF1, P3 showed slightly lower biofilm formation in MF2, while 7953 showed a significantly ($P<0.05$) lower biofilm formation in MF2. The comparisons of biofilm formation of A1 and P3 in MF1 and MF2 are more like each other than either of them to 7953 and it is possibly due to the fact that both A1 and P3 are of dairy origin. The genome comparison of A1, P3 and 7953 reported by Burgess et al. (2017) showed that A1 and P3 are more closely related to each other than 7953 is to either A1 or P3.

The effect of bacterial growth history on biofilm formation in dairy environments does not appear to have been reported. However, bacterial growth history is known to affect the stress response of several bacterial species. This includes *Bacillus* species (den Besten et al. 2010), *L. monocytogenes* (Aryani et al. 2015), *Lactobacillus plantarum* (Aryani et al. 2016) and *Lactobacillus paracasei* (Desmond et al. 2002), where the survival after lethal treatments was observed based on pre-culture or pre-adapted conditions. For example, *L. monocytogenes* precultured in different media show a strain dependent variation in heat resistance (Aryani et al. 2015). *L. paracasei* pre-adapted by exposure to heat in reconstituted skim milk showed enhanced viability during subsequent spray-drying (Desmond et al. 2002). The present study provides new insights into the effect of growth history on biofilm formation of *G. stearothermophilus* in dairy environments.

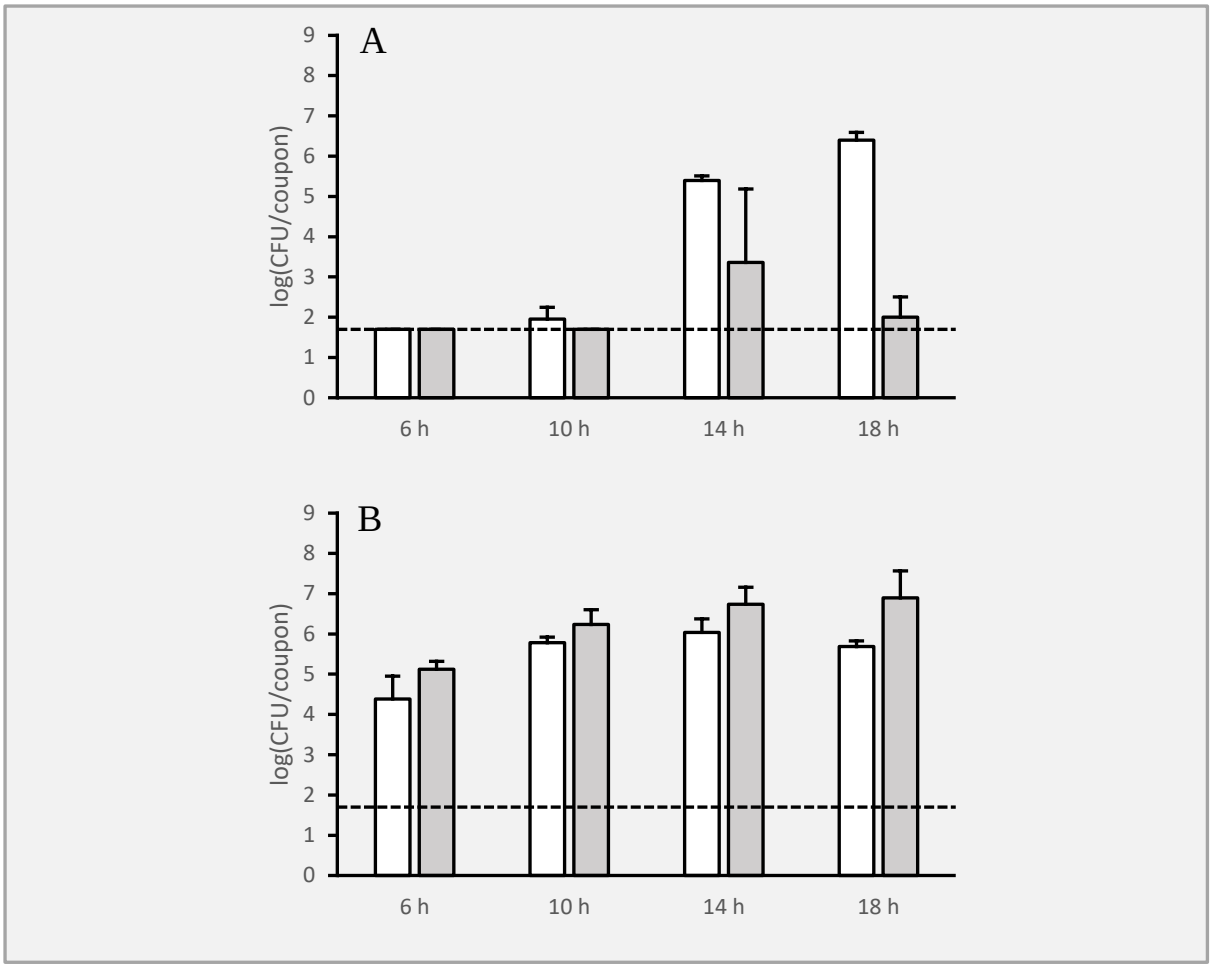

Figure 5-1 Biofilm formation of *G. stearothermophilus* A1 on stainless steel coupons from 6 to 18h at 55°C in milk formulation 1 (white bars), milk formulation 2 (grey bars). mTSB inoculum was used in A. milk formulation inocula were used in B. Dotted lines represent detection limit of the cell enumeration method. Statistical comparisons between two different conditions at respective time points are shown in **Table 5-3**

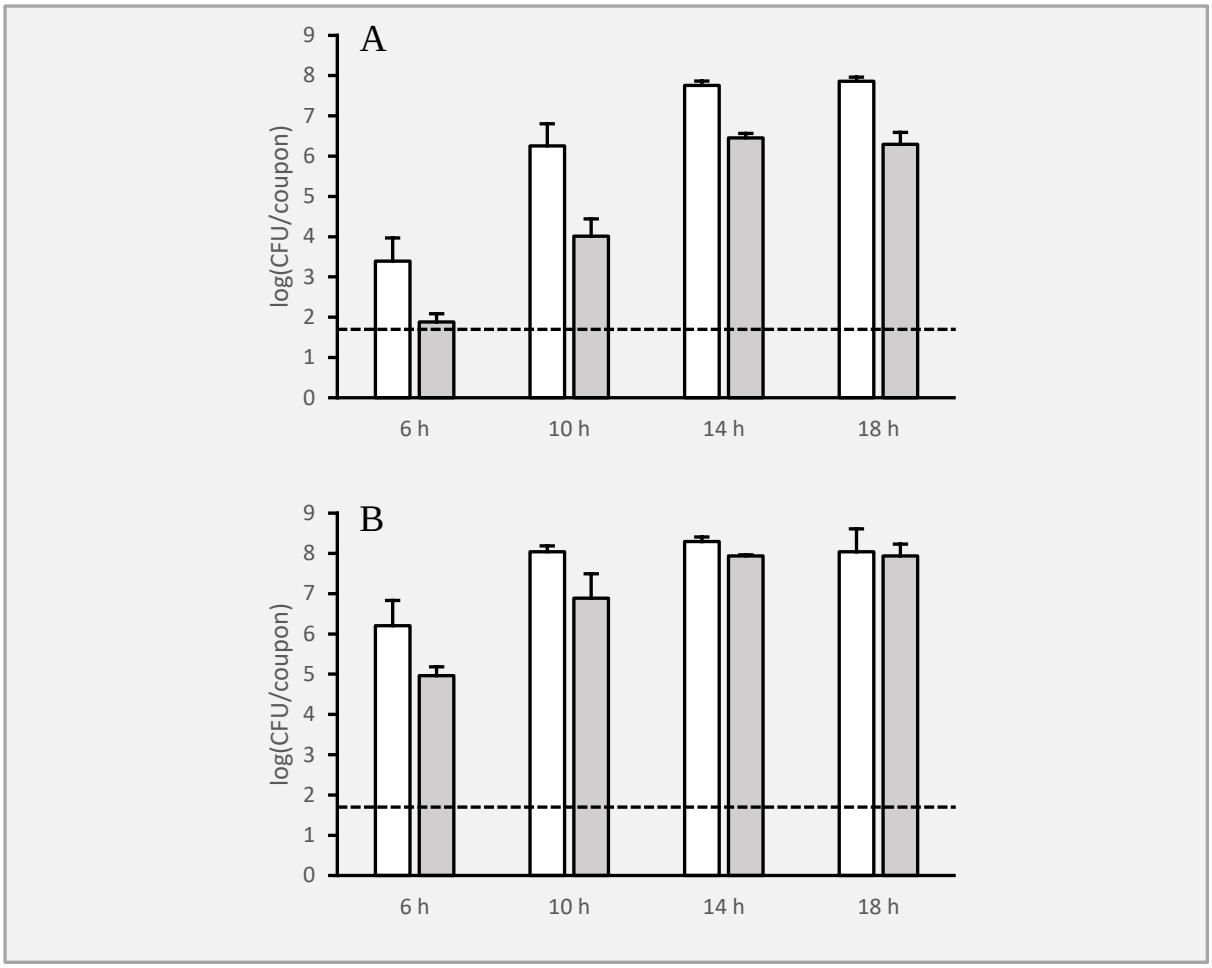

Figure 5-2 Biofilm formation of *G. stearothermophilus* P3 on stainless steel coupons from 6 to 18h at 55°C in milk formulation 1 (white bars), milk formulation 2 (grey bars). mTSB inoculum was used in A. milk formulation inocula were used in B. Dotted lines represent detection limit of the cell enumeration method. Statistical comparisons between two different conditions at respective time points are shown **Table 5-3**

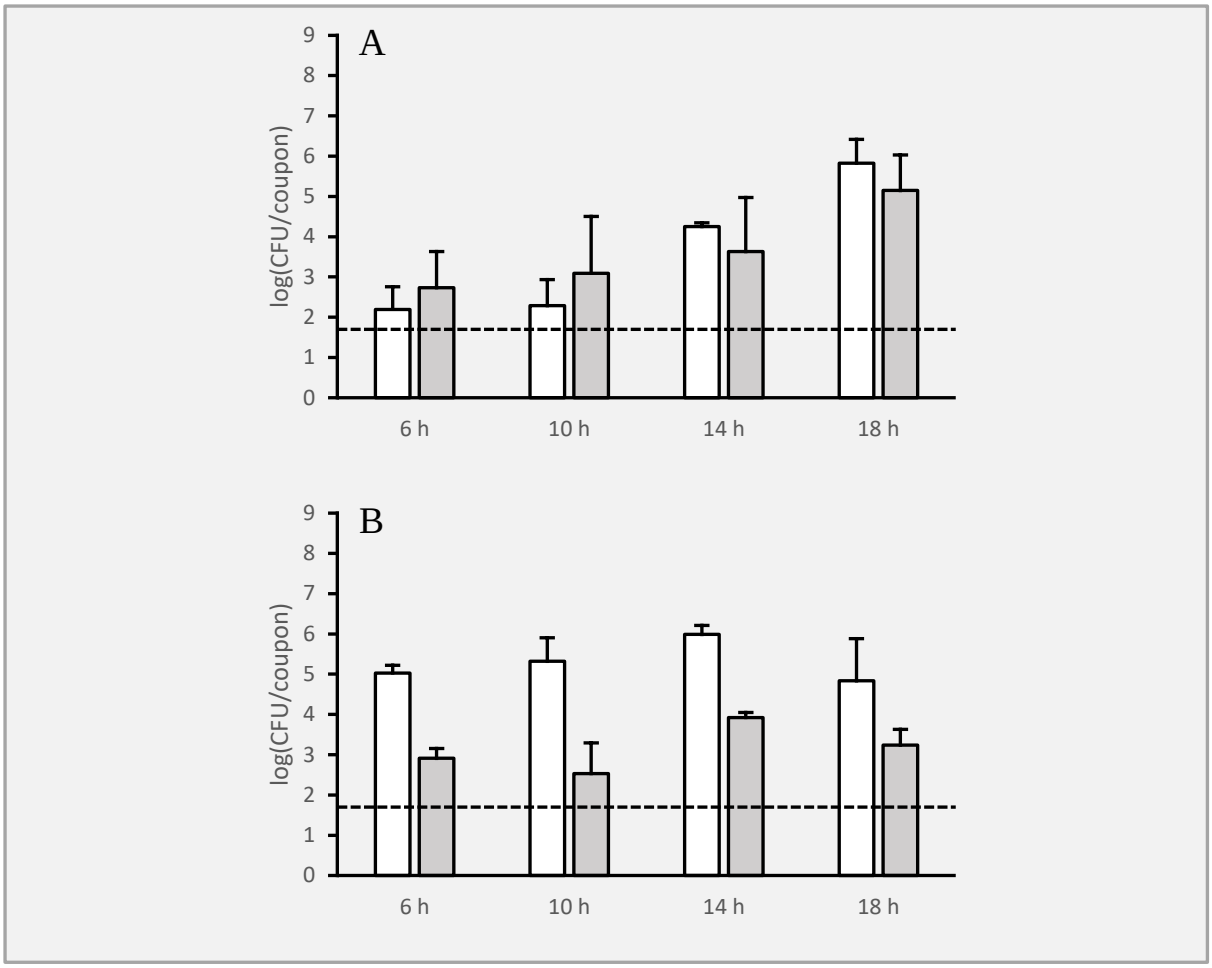

Figure 5-3 Biofilm formation of *G. stearothermophilus* 7953 on stainless steel coupons from 6 to 18h at 55℃ in milk formulation 1 (white bars), milk formulation 2 (grey bars). mTSB inoculum was used in A. milk formulation inocula were used in B. Dotted lines represent detection limit of the cell enumeration method. Statistical comparisons between two different conditions at respective time points are shown **Table 5-3**

5.3.3 The effects of sodium and calcium on *G. stearothermophilus* biofilm formation

As both dairy isolates showed reduced biofilm in MF2 compared to MF1 using an inoculum prepared in mTSB while no significant difference was observed for 7953, mTSB inocula were used to examine the effect of different cation concentrations on biofilm formation of dairy isolates A1 and P3. As a starting point, 105 mM of NaCl, similar to the concentration in the previous study (Somerton et al. 2015) that was reported to significantly inhibit biofilm formation of *G. stearothermophilus* in milk formulation A, was used to supplement MF1. Calcium was used to supplement MF2 to check if the presence of extraneous calcium can resume the biofilm growth observed in MF1. Supplementation of MF2 with 26 mM $CaCl_2$ ensured that supplemented MF2 had a similar calcium level to MF1. Adding NaCl to MF1 significantly (P<0.05) decreased the biofilm cell numbers after 18 h incubation for A1 and P3, while the biofilm formation at 6, 10 and 14 h was generally similar in MF1 and MF2 (Figure 5-1 A and Figure 5-4 A, Figure 5-2 A and Figure 5-4 C). This led to the hypothesis that the biofilms start to detach from the surface at 18 h in MF1 due to the addition of sodium. The detrimental effect of sodium has been shown to weaken and disintegrate floc structures, as well as decrease floc strength in wastewater treatment studies (Higgins and Novak 1997, Sobeck and Higgins 2002, Kara et al. 2008, Berde and Jolis 2020). Although, both the current study and the previous study by Somerton et al. (2015) showed decreased biofilm formation in sodium supplemented MF1 compared with MF1, the pattern of inhibition was different. In the previous study, the reduced biofilm formation occurred at 6 h and 10 h for all *Geobacillus* spp. with one strain showing a prolonged decreased biofilm formation up until 18 h. In the current study, the inhibition only happened at 18 h. It is possible that the inconsistency is due to the different strains in the two studies. Additionally, as growth history was shown to affect biofilm formation in milk formulations, the culture medium used during inoculum preparation between the two studies could also lead to this discrepancy.

Biofilm formation in response to supplemented calcium also showed a discrepancy between the current and the previous study by Somerton et al. (2015). In the current study, calcium was supplemented in MF2 to a total amount that was close to the level in MF1 and it significantly (P<0.05) increased biofilm formation of A1 at 18 h (Figure 5-1 A and Figure 5-4 B). Calcium significantly (P<0.05) increased the biofilm formation of P3 at 10 h but not 14 and 18 h (Figure

5-2 B and Figure 5-4 D). P3 generally showed a reduced biofilm formation in MF2 regardless of inoculum preparation conditions (Figure 5-2), it is possible that P3 was more sensitive to MF2 and calcium supplementation could not completely neutralize its inhibitory effect. In addition, the maximum biofilm cell numbers of dairy isolates in calcium-supplemented MF2 were generally lower than that in MF1 during the 18 h period (Figure 5-1 A and Figure 5-4 B, Figure 5-2 A and Figure 5-4 D). In the previous study by Somerton et al. (2015), supplementation of 2 mM calcium in MF2 significantly increased the biofilm formation at 10, 14 and 18 h for *G. stearothermophilus* dairy isolates and the maximum biofilm cells in calcium-supplemented milk formulation reached a similar level as in MF1. Apart from strain specificity, it is reasonable to assume that a different cell physiology was induced during inoculum preparation due to the choice of inoculum culture media, influencing the biofilm formation in response to cation supplementation. Additionally, the amount of calcium supplementation in the current study was different from the previous study in that only 2 mM calcium was used by Somerton et al. (2015). To exclude the potential effect of calcium concentration, 2 mM calcium was also added to MF2 in the current study for dairy isolates. Supplementation of 2 mM calcium showed a similar effect to 26 mM calcium for A1 and P3 (supplementary materials). Therefore, either strain specificity or growth history is likely to be the cause of the discrepancy in biofilm response to calcium supplementation between the two studies. The effect of cell physiology and strain variation on biofilm formation of *G. stearothermophilus* in the dairy environment needs to be further elucidated.

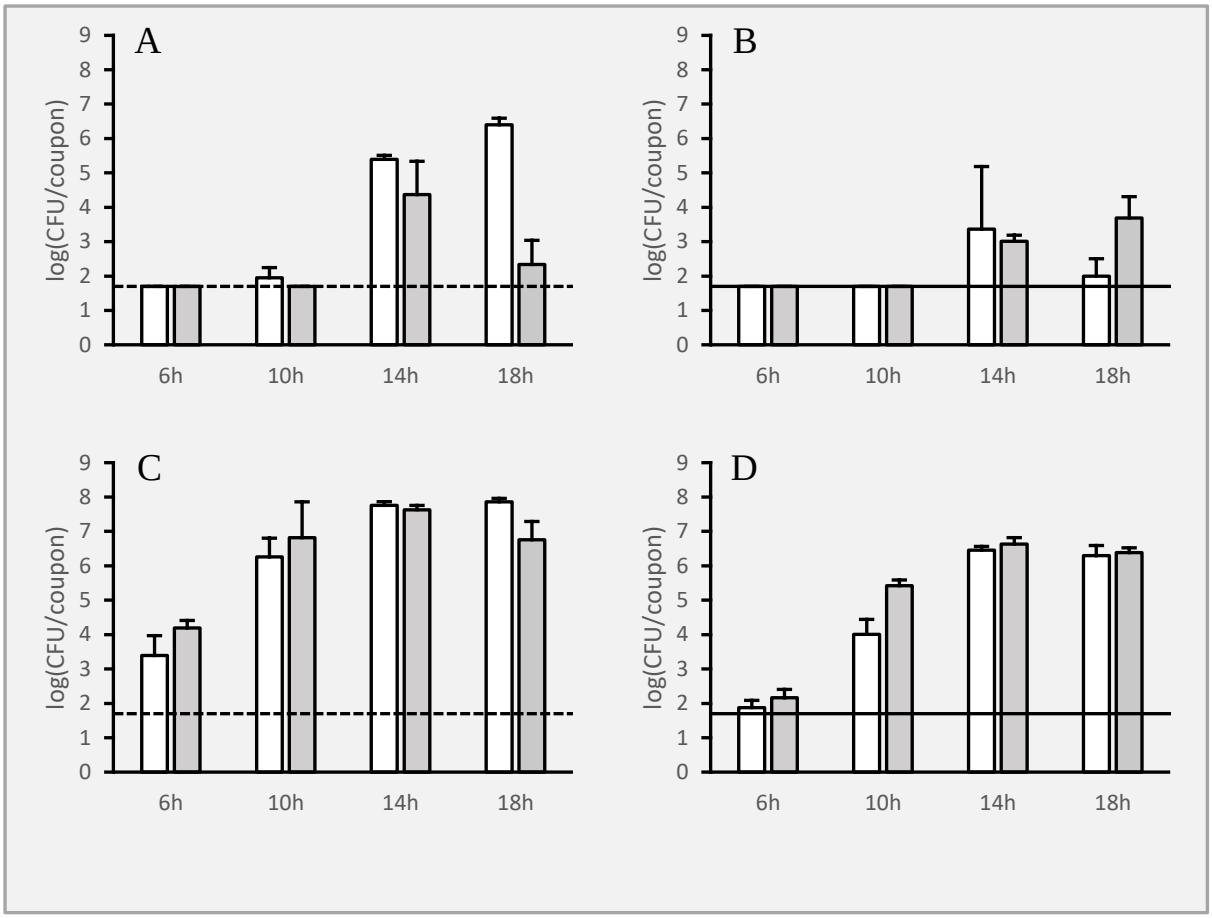

Figure 5-4 Biofilm formation of *G. stearothermophilus* A1 (A, B), P3 (C, D) on stainless steel coupons from 6 to 18 h at 55°C in milk formulation 1 (white bars in A,C) and milk formulation 1 supplemented with 105 mM NaCl (grey bars in A,C), milk formulation 2 (white bars in B,D) and milk formulation 2 supplemented with 26 mM $CaCl_2$ (grey bars in B,D). mTSB inoculum was used in all conditions. All experiments were repeated at least on two different occasions and error bars stand for standard deviations. Dotted lines represent detection limit of the cell enumeration method. Statistical comparisons between two different conditions at respective time points are shown in **Table 5-3**. data of white bars were duplicated from **Figure 5-1** A and **Figure 5-2** A.

Table 5-3 Statistical comparison of biofilm cell numbers of A1, P3 and 7953 in two milk formulations at respective time points from MF inocula or mTSB inoculum.

		mTSB inoculum		**MF inocula**
A1	**MF1-MF2**	**MF1-MF1+Na**	**MF2-MF2+26 mM Ca**	**MF1-MF2**
6 h	ND	ND	ND	NS
10 h	ND	ND	ND	S
14 h	S	NS	NS	NS
18 h	S	S	S	S
P3				
6 h	S	NS	NS	S
10 h	S	NS	S	S
14 h	S	NS	NS	S
18 h	S	S	NS	NS
7953				
6 h	NS	-	-	S
10 h	NS	-	-	S
14 h	NS	-	-	S
18 h	NS	-	-	S

ND: Not Determinable as the cell number of at least one experiment condition was under the detection limit of the current method, NS: Not a Significant difference (P>0.05), S: Significant difference (P<0.05), "-": Not determined as no significant difference was present between MF1 and MF2 when cultures were prepared using mTSB inoculum

5.3.4 Biofilm formation on milk agar plates

Bacterial colonies formed on the surface of an agar plate is considered as biofilm by some researchers (Marsden et al. 2017, Keren-Paz et al. 2018). Therefore, the colony growth on milk agar plates was investigated in the current study to provide supplementary information to biofilm formation in milk formulations. mTSB medium was used to prepare the inoculum as the milk formulation assay showed a strong inhibitory effect of MF2 to both dairy isolates using this preculture condition. As illustrated in Table 5-4, A1 and 7953 showed no growth on MF2 agar over the 72 h culture period, while growth was present on MF1, sodium-supplemented MF1 and calcium-supplemented MF2 agar. An equivocal growth of P3 was observed on MF2 agar at 24 h and 48 h as the growth was inconsistent between biological repeats. However, even when growth was present on MF2 agar, only a few colonies were observed compared to the densely grown bacterial lawn on the inoculated area on MF1 agar (data not shown). At 72 h, both biological repeats showed growth of P3 on MF2 agar. Similar

to A1 and 7953, P3 grew on MF1, sodium-supplemented MF1 and calcium-supplemented MF2 agar over the 72 h culture period. MF2 agar showed an inhibitory effect on A1, P3 and 7953 which is in line with the decreased biofilm formation of A1 and P3 in MF2 but not 7953, where the growth of 7953 was present in MF2 but absent on MF2 agar. Supplementing MF1 milk agar with sodium showed minimal effect on the presence of growth of A1, P3 and 7953. While supplementing MF2 milk agar with calcium eliminated the inhibitory effect of MF2 over the 72 h culture period. This indicated the importance of calcium in supporting *G. stearothermophilus* biofilm growth in a milk environment. Even though a direct comparison of biofilm formation results from milk agar plates and milk formulations is not possible, the current results of milk agar plates showed a consistent effect of MF2 on *G. stearothermophilus* biofilms in different culture systems and the importance of calcium over sodium to the biofilm inhibitory effect of MF2.

Table 5-4 The presence/absence of growth of *G. stearothermophilus* A1, P3 and 7953 on milk agar plates from mTSB inoculum

Culture time (h)	Milk agar	A1	P3	7953
24	MF1	+	+	+
	MF2	-	+/-	-
	MF1+Na	+	+	+
	MF2+Ca	+	+	+
48	MF1	+	+	+
	MF2	-	+/-	-
	MF1+Na	+	+	+
	MF2+Ca	+	+	+
72	MF1	+	+	+
	MF2	-	+	-
	MF1+Na	+	+	+
	MF2+Ca	+	+	+

"+": the presence of growth, "-": the absence of growth, "+/-": the presence of growth was inconsistent between biological repeats

5.3.5 Proposed mechanisms for the decreased biofilm formation in MF2

Various factors might contribute to the decreased biofilm formation in MF2, including the electrostatic interactions between cations and biofilm, effect of sodium and calcium on regulating the motile/sessile switch in bacteria and the structural change of milk proteins after decalcification.

Calcium is thought to neutralize and bridge negatively charged functional groups of bacterial cell walls and extracellular polymeric substances (Wang et al. 2019), and the presence of a high concentration of sodium can presumably displace divalent cations in flocs and weaken the floc structure (Kara et al. 2008). A relatively high concentration of sodium has been shown to compromise the structural integrity of wastewater sludge biofilms (Berde and Jolis 2020). The electrostatic interactions associated with high sodium and low calcium concentrations may not be the dominant effect in the current study as 7953 and A1 showed similar or even increased biofilm formation in MF2 compared with MF1 (Figure 5-1 B, Figure 5-3 A). In addition, MF2 agar still showed the inhibitory effect on A1, P3 and 7953 where the electrostatic effect on the colony biofilm structures was absent. Therefore, an excessive amount of sodium in MF2 may only play a minor role in inhibiting biofilm formation of *G. stearothermophilus*. In addition, one previous study showed similar biofilm formation of *G. stearothermophilus* in culture medium with 2:1 and 10:1 monovalent to divalent cation ratios indicating the ratio of monovalent to divalent cations did not strongly affect biofilm formation of *G. stearothermophilus* (Somerton et al. 2013).

Calcium ion has the potential to affect biofilm formation of *G. stearothermophilus* and several other bacterial species by influencing cell physiology (Somerton et al. 2013, Wang et al. 2019). Supplementing MF2 milk agar with calcium reduced its inhibitory effect. In addition, increasing free calcium concentration was shown to increase biofilm formation of A1 but not P3 and 7953 (Wang et al. 2021). When 26 mM or 2 mM $CaCl_2$ was added to MF2, it is thought that free calcium increased (Somerton et al. 2015).Therefore, the increased biofilm formation of A1 in calcium-supplemented MF2 compared to MF2 (Figure 5-2 A and Figure 5-4 B)could potentially be due to the higher amount of free calcium and the resultant cell physiological

response. However, this cannot explain why A1 formed similar or even stronger biofilms in MF2 than in MF1 when milk formulation inocula were used. The increase in free calcium might not play a role in promoting biofilm formation of P3 in milk formulations as it was observed in chapter 3 that biofilm formation was maintained or reduced for P3 with an increasing amount of free calcium. In addition, increasing calcium concentration of a base medium was shown to inhibit biofilm formation of 7953 compared to the control that had a total calcium content of 0.2 mM, which was lower than that in MF1 or MF2 (Wang et al. 2021). However, the inhibition of biofilm formation of 7953 occurred in MF2 where the calcium content was lower than that in MF1. Therefore, in the current study, calcium might affect cell physiology in milk formulations and the effect remain on accustoming cells to the environment of MF2 to facilitate biofilm formation, rather than regulating cells to switch between biofilm and planktonic forms.

It is not only the total amount of sodium and calcium that are different between MF1 and MF2. Decalcification of milk also results in a change in milk protein structure. Casein micelle structures are mostly dissembled when calcium is removed from the milk by ion-exchange and sodium-caseinate is thought to be generated during the process (Xu et al. 2016). The addition of sodium-caseinate to reconstituted skim milk delayed the biofilm formation of strain A1 (Kumar et al. 2021). During ion-exchange treatment, calcium is also removed from some of the cation-binding whey proteins causing a structural change in the whey proteins. For example, calcium deprived α-lactalbumin after ion-exchange treatment showed a bactericidal effect on *S. pneumoniae* (Håkansson et al. 2000). In the current study, it is possible that the structural change in milk proteins after calcium removal delayed or inhibited biofilm formation. Although the current study attempted to revert the process by supplementing MF2 with extraneous calcium, but the effect on the protein structural change is still unknown. Therefore, to investigate the effect of milk protein on biofilm formation, it is recommended to separate whey and casein fractions in MF2 and test their independent effect on biofilm formation.

5.4 Conclusion

Milk formulation with high sodium and low calcium concentrations inhibited biofilm formation of *G. stearothermophilus* but the inhibitory effect was strongly affected by the growth history and the strain. Calcium supplementation of MF2 promoted biofilm formation of A1 and P3, while sodium supplementation of MF1 was detrimental to biofilm formation. The electrostatic interactions of sodium and calcium were less likely to be the main cause for the inhibition in biofilm formation in MF2. The effect of calcium-milk protein interaction on biofilm formation of *G. stearothermophilus* and the effect of calcium on cell physiology in milk formulations needs to be further addressed to understand the inhibitory effect of MF2.

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5.6 Supplementary materials

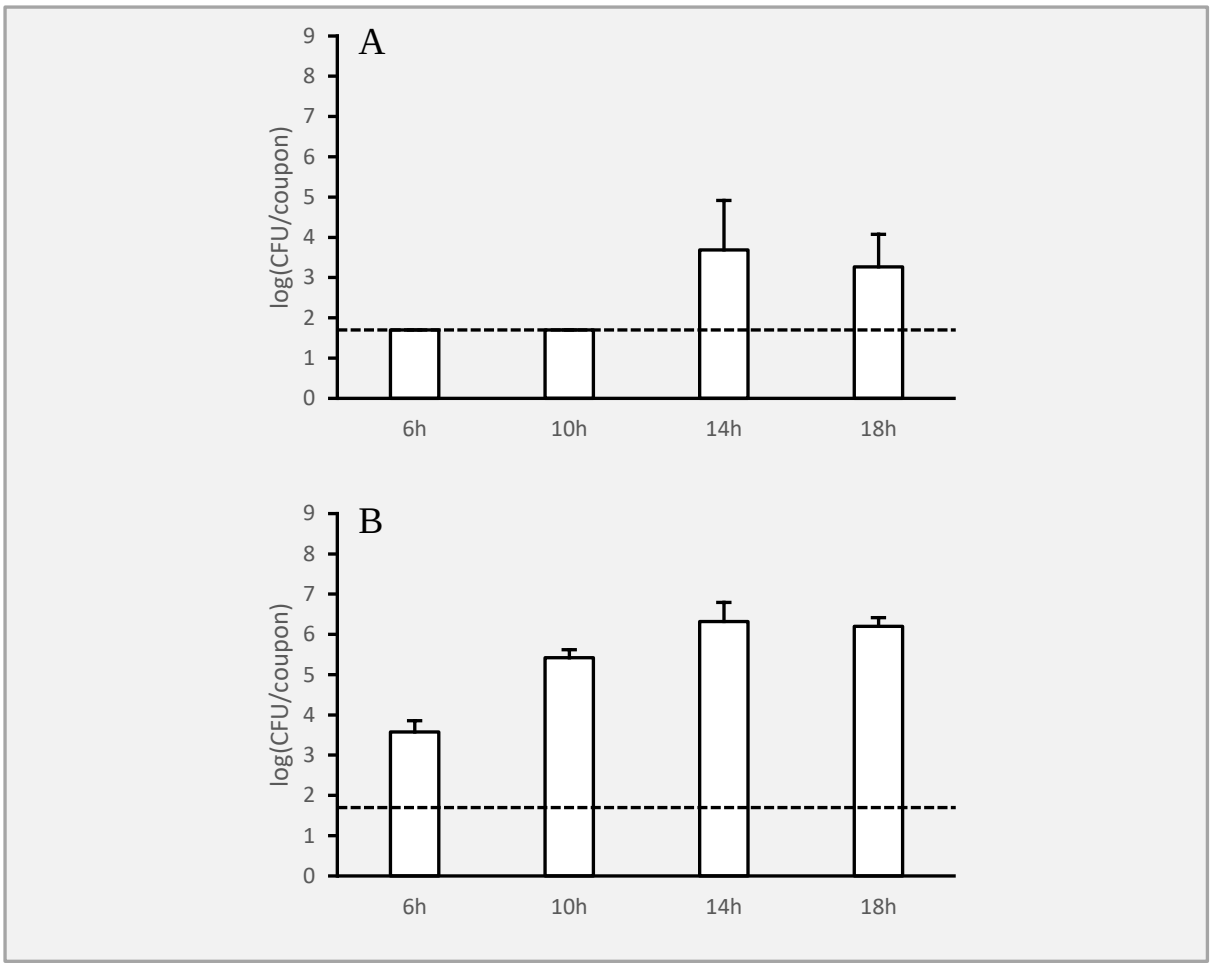

Figure S5-5 Biofilm formation of *G. stearothermophilus* A1 (A), P3 (B) on stainless steel coupons from 6 to 18 h at 55℃ in milk formulation 2 supplemented with 2 mM $CaCl_2$. mTSB inoculum was used in all conditions. Dotted lines represent detection limit of the cell enumeration method

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Name/title of Primary Supervisor:	Jon Palmer
In which chapter is the manuscript /published work:	Chapter5

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Date:	29-Aug-2021
Primary Supervisor's Signature:	Jon Palmer Digitally signed by Jon Palmer Date: 2021.08.30 14:35:11 +12'00'
Date:	30-Aug-2021

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Chapter 6 The effect of milk formulations on sporulation and spore heat resistance of *G. stearothermophilus*

6.1 Introduction

During biofilm formation of *G. stearothermophilus* in a milk environment, spores are produced within biofilms and released into the environment, which results in the contamination of milk products (Burgess et al. 2014, Seale et al. 2015). There are several process steps in milk powder manufacture where the spores of *G. stearothermophilus* are produced within the biofilms, which contributes to the final thermophile contamination level in milk powder products. Growth of *G. stearothermophilus* occurs at process steps where elevated temperatures prevail (40 to 65 ℃) (Burgess et al. 2010). For example, a field study conducted by Burgess et al. (2014) reported the prevalence of thermophilic spores in product streams and on equipment surfaces. Specifically, spores isolated from product streams out of the evaporator and pre-evaporator process steps consist of *Anoxybacillus*, while spores isolated from the fouling in the direct steam injection are predominantly *Geobacillus* spp.. The number of spores produced by biofilm during milk powder manufacture and their heat resistance influences the quality and potential use of milk powders.

The sporulation and heat resistance of bacterial spores were studied in the role of cations on the spore production and the heat resistance of *G. stearothermophilus* spores (Bender and Marquis 1985, Wells-Bennik et al. 2019). Calcium increased sporulation and spore heat resistance (Slepecky and Foster 1959, Lenz and Vogel 2014), while sodium was reported to be detrimental to the heat resistance of bacterial spores (Bender and Marquis 1985). The distinct cation profiles of MF1 and MF2 in the amount of calcium and sodium may result in a different sporulation and spore heat resistance, and therefore influence thermophilic bacterial contamination. In this chapter, the sporulation of *G. stearothermophilus* in MF1 and MF2, and heat resistance of spores produced from growth in MF1 and MF2 were characterized.

6.2 Materials and methods

6.2.1 Bacterial strains and inoculum preparation

Three *G. stearothermophilus* strains were used in this study. *G. stearothermophilus* A1, P3 are dairy isolates, ATCC 7953 was used as a reference strain. The inocula were prepared in MF1 and MF2 as described in Chapter 5 2.1.

6.2.2 Biofilm sporulation in milk formulations

For sporulation in biofilm cells, the inoculum was diluted in either the fresh milk formulation that was of the same composition as that used during the inoculum preparation or the fresh milk formulation that was of the same composition and supplemented with extra cations (MF1 supplemented with 105 mM NaCl or MF2 supplemented with 26 mM $CaCl_2$). More than 200 times dilution was used to ensure the initial cell numbers after dilution were approximately 4.5 log CFU/mL. A volume of 1.5 mL inoculated milk formulation was added to each well of a 24-well plate. The plate was wrapped in a resealable plastic bag and incubated at 55 ℃ for 6, 10, 14 or 18 h under static conditions. At each time point, coupons were removed from the wells with sterilised forceps and washed three times in 0.85% saline by gently dipping into the saline water.

Washed coupons were transferred into glass tubes, each containing 12 g sterile glass beads with diameter of 6.35 mm and 5 mL 0.5% BPW. Glass tubes were mixed by vortex for 1 min to detach biofilm cells into the surrounding BPW followed by serial 10-fold dilutions in 0.5% BPW. To enumerate spore numbers, bacteria resuspended in BPW were heat-treated at 90 ℃ for 30 min to inactivate vegetative cells. The drop-plate method was used to enumerate cell and spore numbers on TSA plates with the overnight incubation at 55℃. The sporulation percentages were calculated using the spore number divided by the respective total cell number.

6.2.3 Spore production for heat resistance test from planktonic cultures in milk formulations

The inoculum prepared as described in 6.2.1 was diluted 100 times in the fresh milk formulation that was of the same composition as that used during the inoculum preparation. The inoculated milk formulations were cultured at 55 ℃, 150 rpm for 24 h. Spore numbers of MF1 and MF2 cultures were enumerated at 6, 10, 14, 18 and 24 h by heating samples of cultures (6, 10 h) or 10-fold diluted sample cultures (14, 18, 24 h) at 90 ℃ for 30 min to inactivate vegetative cells followed by drop-plate method on TSA with the overnight incubation at 55℃.

To harvest spores, 20 mL MF1 and 100 mL MF2 cultures were used to produce 2 mL spore suspension. Briefly, 24 h MF1 and MF2 cultures were both mixed with 10 % (v/v) of 10 % (w/v) SDS (Bio-Rad) and set at room temperature for 30 min. The treated cultures were then centrifuged at 9000 × g for 10 min and resuspended with 2 mL 1% (w/v) SDS to maintain a homogenous resuspension of bacterial spores, the resuspension was further subjected to two times of wash with sterile deionized water. The washed pellet was then resuspended in 2 mL water and heat-treated at 90 ℃ for 30 min to inactivate vegetative cells. The drop-plate method was used to enumerate spore numbers on TSA plates with the overnight incubation at 55℃. The spore numbers produced from MF1 and MF2 were adjusted to approximate the same on the following day based on the spore enumeration results and used for the heat resistance test.

6.2.4 Heat resistance of spores

A capillary tube method was used to estimate the heat resistance of spores as described by Kumar et al. (2019). Briefly, 50 µL of spore suspension was injected into a glass capillary tube (Globe Scientific, USA, I.D. 1.1-1.2 mm, wall 0.2±0.02 mm, length 75 mm) using a pipette. The tube was heat-sealed using a flame. Each tube was completely submerged in the oil bath for a selected time, at a temperature of 107.5 ℃ for A1 and P3 and 115 ℃ for 7953. After each time interval, tubes were immediately taken out of the oil bath and submerged into cold water. The tubes were washed with 70 % ethanol and then sterile water before being aseptically cut open. The heat-treated spore suspension was 10-fold serial diluted with BPW and plated on

TSA followed by incubation at 55 ℃ for 24 h. D-values were calculated through the negative inverse of the slope of the regression line by plotting survival numbers of spores versus time.

6.2.5 Statistical analysis

Results are expressed as means of two biological replicates with standard deviations unless stated otherwise. Significant differences were determined by one-way ANOVA using Minitab Statistical Software (Minitab Version 18) at a 95% confidence (P<0.05).

6.3 Results and discussion

6.3.1 Sporulation in milk formulations

An 18 h time frame for incubation was selected to represent the common industry manufacture run length and to maintain a consistency with the biofilm formation experiment in the previous chapter. The sporulation of A1 occurred from 14 h and onwards (Figure 6-1 A). For P3, the sporulation occurred from 6 h (Figure 6-1 B). A slight delay in sporulation was observed in MF2 for P3 and 7953 where the spores were first detected at 6 h and 14 h in MF1 but not in MF2, respectively (Figure 6-1 B and C).

For dairy isolates A1 and P3, a consistently lower spore numbers were observed in MF2 than in MF1 over an18 h culture period at respective time points (Figure 6-1 A and B). However, 7953 showed a similar total spore numbers in MF1 and MF2 at 18 h (Figure 6-1 C). A similar observation was also shown for planktonic cultures in that spore numbers in MF2 were lower than MF1 over the 24 h culture period for A1 and P3 (Figure 6-2). Therefore, A1 and P3 were used to study the effect of calcium and sodium on sporulation in milk formulations. The consistent lower spore numbers in MF2 for dairy isolates could potentially account for the low thermophile contamination level in calcium-reduced milk protein powder observed in industry.

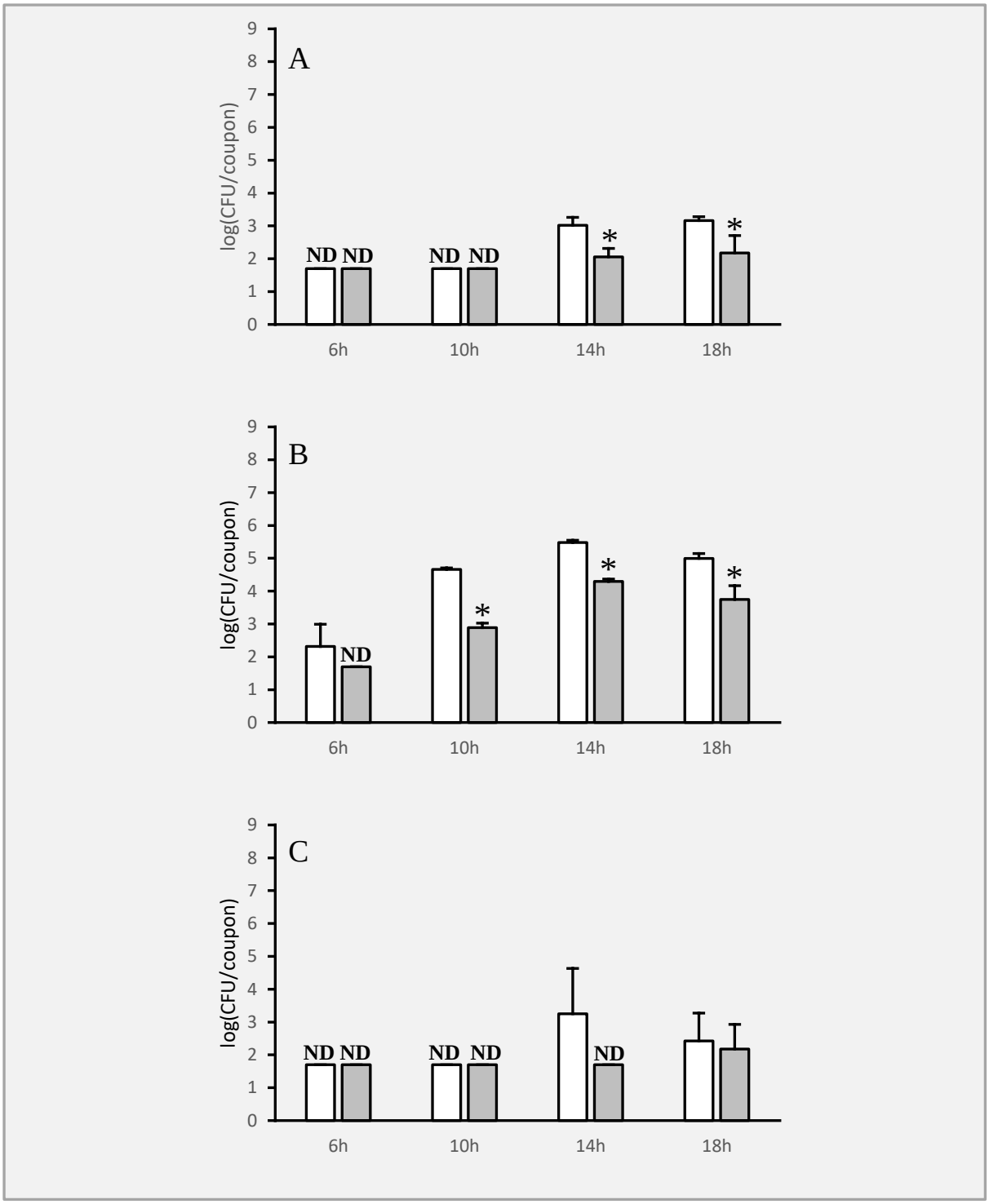

Figure 6-1 Sporulation in biofilms of *G. stearothermophilus* A1 (A), P3 (B) and 7953 (C) on stainless steel coupons from 6 to 18h at 55°C in milk formulation 1 (white bars), milk formulation 2 (grey bars). Statistical comparisons between MF1 and MF2 for each strain at respective time points are indicated with asterisks. ND=not determinable (the spore number was under the detection limit of the current enumeration method)

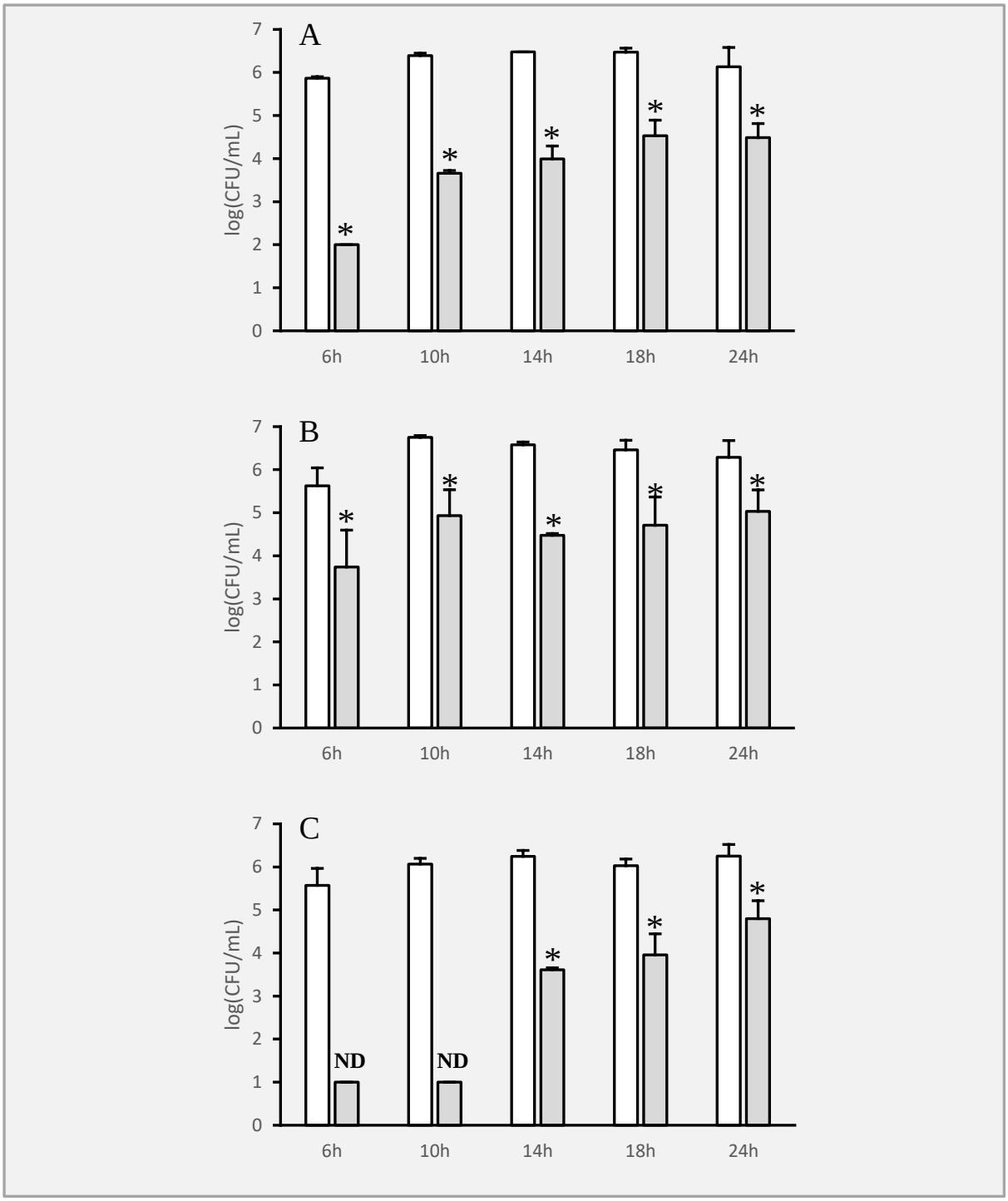

Figure 6-2 Sporulation in planktonic cultures of *G. stearothermophilus* A1 (A), P3 (B) and 7953 (C) in MF1 (white bars) and MF2 (grey bars). Data are expressed as means of three replicates with standard deviations. Statistical comparisons between MF1 and MF2 for each strain at respective time points are indicated with asterisks. ND=not determinable (the spore number was under the detection limit of the current enumeration method)

6.3.2 The effect of sodium and calcium on sporulation in milk formulations

To further investigate which cation level in the MF2 results in the reduced sporulation of A1 and P3, sodium and calcium were added to MF1 and MF2 respectively. The addition of calcium in MF2 significantly (P<0.05) increased the spore numbers of A1 at 14 and 18 h (Figure 6-3 A), with no effect on P3 observed at 14 and 18 h (Figure 6-3 B). The addition of sodium to MF1 did not significantly (P>0.05) change the spore numbers of A1 at 14 and 18 h (Figure 6-3 C), but it significantly (P<0.05) reduced the spore numbers at 10 and 14 h for P3 (Figure 6-3 D). While similar spore numbers of P3 were observed at 18 h in MF1 and MF1 with sodium added (Figure 6-3 D). Both sodium and calcium influenced the total spore numbers of dairy isolates, but the effect was strain specific. Interestingly, the sporulation of P3 was delayed in cation supplemented milk formulations until 14 h (Figure 6-3 B and D), whereas the spores were first detected at 10 h in MF1 and MF2 (Figure 6-1 B). This could be due to the change of culture conditions from the milk formulations used to prepare the inocula to the cation supplemented milk formulations for the sporulation experiment, resulting in an extended time for cells to adapt to the new environment.

It is worth noting that when cation was added into the milk formulations, the total cell numbers also changed. For example, total cell numbers of P3 decreased approximately 1 log CFU/mL at 6, 10 and 14 h with the extra amount of sodium added to MF1 (Figure 6-4 D), and this could cause the total spore numbers to decrease. Therefore, sporulation percentage was calculated to compare the effect of calcium and sodium on the sporulation efficiency of *G. stearothermophilus* dairy isolates (Table 6-1).

As illustrated in Table 6-1 the sporulation percentages were consistently higher in MF1 than MF2 at 14 and 18 h for A1. The sporulation percentages were higher in MF1 than MF2 at 10, 14 h but not 18 h for P3. Supplementing calcium significantly (P<0.05) increased the sporulation percentage for A1 at 14 and 18h while for P3, the sporulation percentage was increased at 14 h but not 18 h. It is not surprising that calcium addition did not increase the sporulation percentage of P3 at 18 h, as it was already comparable between MF1 and MF2 at 18 h. Adding sodium decreased the sporulation percentage for A1 at 18 h, while minimal effect of sodium was shown for P3. The increase in sporulation caused by calcium observed in the current study agrees with several previous studies in which the presence of calcium increases

the sporulation rate of *C. botulinum* (Lenz and Vogel 2014), or the absence of calcium reduces the sporulation of *B. megaterium* and spore yield of *B. subtilis* (Slepecky and Foster 1959, Minh et al. 2011). Limited research has reported on the effect of sodium on spore productions, although the addition of sodium was reported to decrease the spore production of *B. subtilis*, but it was thought to be associated with the osmotic pressure due to the large amount of NaCl (from 32 to 70 g/L) added to the sporulation medium (Minh et al. 2008, Minh et al. 2011).

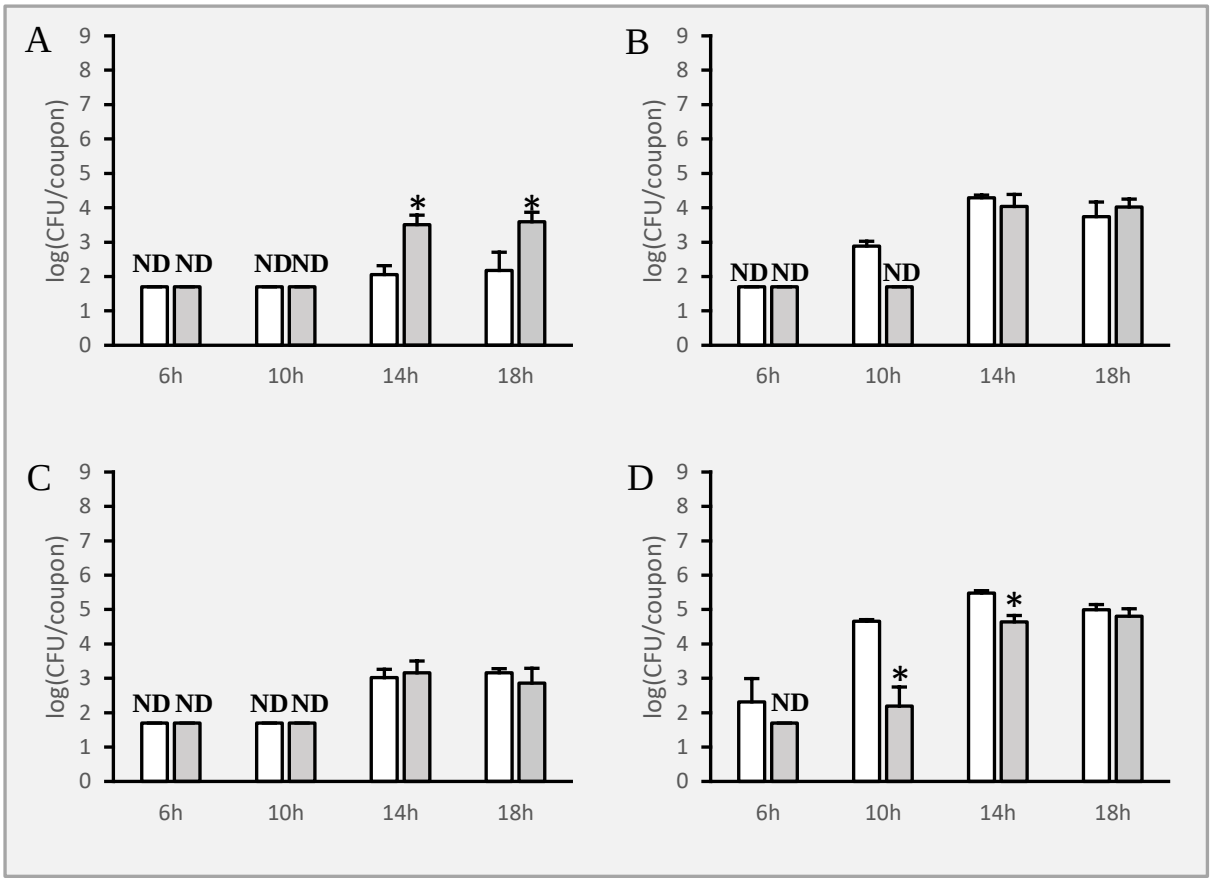

Figure 6-3 Sporulation in biofilms of *G. stearothermophilus* A1 (A,C), P3 (B,D) on stainless steel coupons from 6 to 18 h at 55°C in MF2 (white bars in A,B) and MF2 with calcium added (grey bars in A,B),MF1 (white bars in C,D) and MF1 with sodium added (grey bars in C,D). Statistical comparisons between milk formulation (**Figure 6-1**)and the respective milk formulation with added cations for each strain at each time point are indicated with asterisks. ND=not determinable (the spore number was under the detection limit of the current enumeration method). data of white bars were duplicated from **Figure 6-1**.

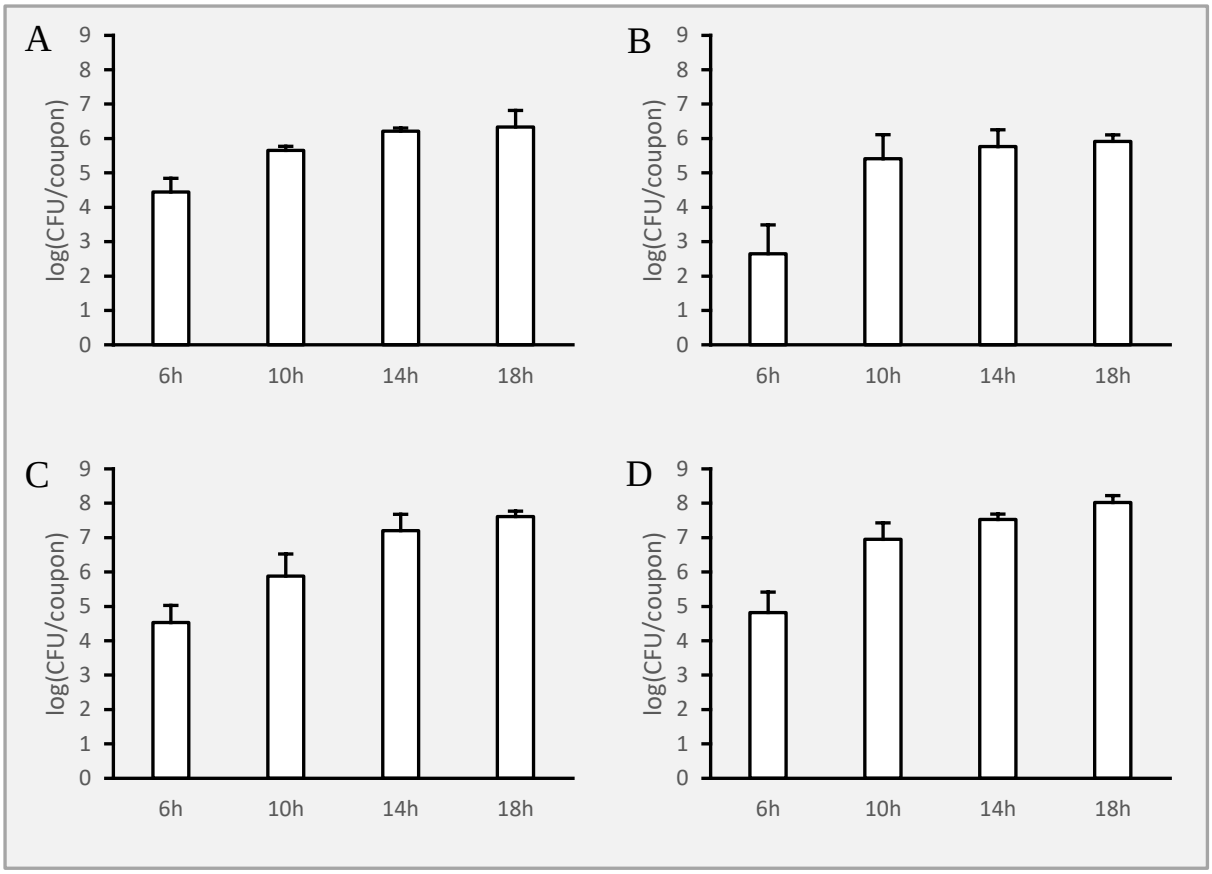

Figure 6-4 Biofilm formation of *G. stearothermophilus* A1 (A, B), P3 (C, D) on stainless steel coupons from 6 to 18 h at 55 ℃ in milk formulation 1 with the addition of 105 mM NaCl (B, D), milk formulation 2 with the addition of 26 mM $CaCl_2$ (A, C). Milk formulation inocula were used in all conditions.

Table 6-1 Sporulation percentages (±standard deviations) of A1 and P3 in milk formulations and statistical comparison of sporulation percentages of A1, P3 in two milk formulations at respective time points

A1	**MF1**	**MF2**	**MF1+Na**	**MF2+Ca**	**MF1-MF2**	**MF1-MF1+Na**	**MF2-MF2+Ca**
6 h	ND	ND	ND	ND	ND	ND	ND
10 h	ND	ND	ND	ND	ND	ND	ND
14 h	0.099±0.030	0.0028±0.0021	0.28±0.18	0.21±0.086	S	NS	S
18 h	0.32±0.12	0.0020±0.0010	0.11±0.061	0.24±0.16	S	S	S
P3							
6 h	ND	ND	ND	ND	ND	ND	ND
10 h	0.055±0.0049	0.0023±0.00087	ND	ND	S	ND	ND
14 h	0.18±0.072	0.023±0.0037	0.14±0.040	0.072±0.028	S	NS	S
18 h	0.12±0.11	0.030±0.028	0.076±0.063	0.038±0.025	NS	NS	NS

ND: Not Determined (the spore number was under the detection limit of the current enumeration method), NS: Not a Significant difference (P>0.05), S: Significant difference (P<0.05)

6.3.3 Heat resistance of spores

Planktonic cultures of MF1 and MF2 were used to produce spores for heat resistance testing as limited amount of biofilm spores were produced on stainless steel coupons (Figure 6-1).The heat resistance of spores was quantified at 107.5 ℃ for A1 and P3, and 115 ℃ for 7953. The heat resistance was initially conducted at 105 ℃ for A1, P3 and 7953. However, the decimal reductions were at approximately 1 log CFU/mL or less during a 20 min treatment time (Figure 6-5). Therefore, an incremental temperature of 2.5-5 ℃ was used to find the appropriate temperature to give more than 1 log CFU/mL reduction during the treatment time to calculate D values (Figure 6-5). A similar heat resistance was observed in spores produced from MF1 and MF2 for A1, P3 and 7953 (Figure 6-6). The current study did not agree with the previous studies as the increased calcium concentration has been repeatedly reported to be correlated with an increase in heat resistance of bacterial spores (Slepecky and Foster 1959, Levinson and Hyatt 1964, Bender and Marquis 1985, Lenz and Vogel 2014), while NaCl addition can result in reduced heat resistance (Fleming and Ordal 1964).

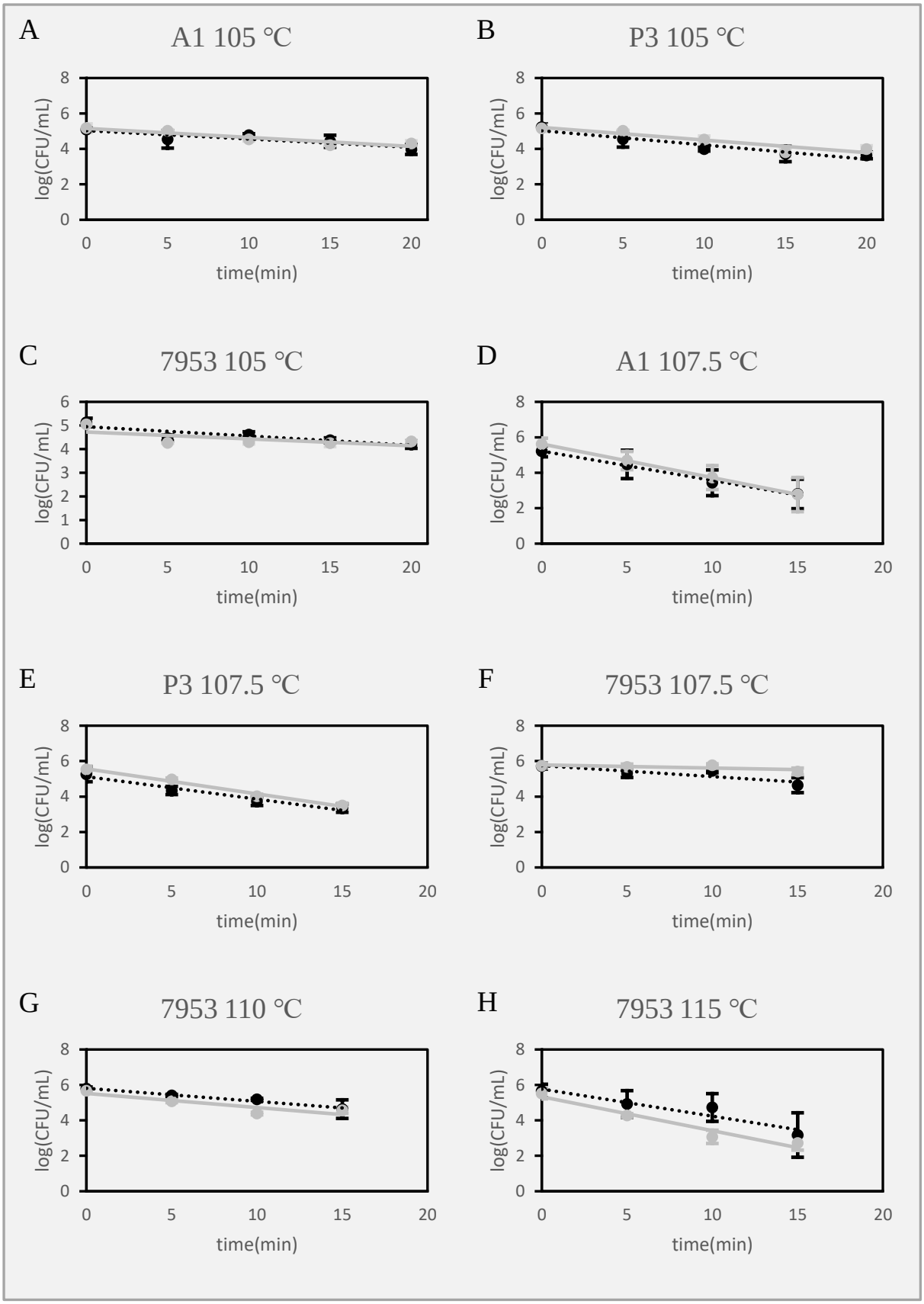

Figure 6-5 Heat inactivation curves of A1, P3 and 7953 at different temperatures. Blank lines indicate spores produced from MF1, grey lines indicate spores produced from MF2. Data are expressed as means of at least three replicates with standard deviations

Interestingly, the D values of spores produced from MF2 consistently showed slightly but not statistically lower values than those produced in MF1 for all strains (Figure 6-6). One hypothesis is that during spore harvest, vegetative cells were harvested simultaneously with the spores. Due to the culture volume used for spore harvest, the spore suspension produced from MF2 might have more vegetative cell debris than that from MF1 when similar amounts of spores were present (supplementary materials Figure S6-7). The presence of cell debris might interfere with the heat transfer within the spore suspension and affect the spore heat resistance, causing an overestimation of the D values of spores produced from MF2. However, limited research has been done to investigate the effect of vegetative cell debris on the heat resistance of spores. Wedel et al. (2019) compared the heat resistance of spores between pure spore suspension and the spore suspension with milk residue and reported that the presence of milk residues reduced the log reduction of *G. stearothermophilus* and *A. flavithermus* during heat treatment. Therefore, one limitation of the current heat resistance experiment was the use of impure spore suspension. The current study used spores produced from planktonic cultures of MF1 and MF2 as spore production within biofilms on stainless steel coupons was insufficient and would have required large number of coupons to produce sufficient spores for heat resistance experiments. Therefore, an efficient biofilm spore production method in milk formulations should be developed in the future study.

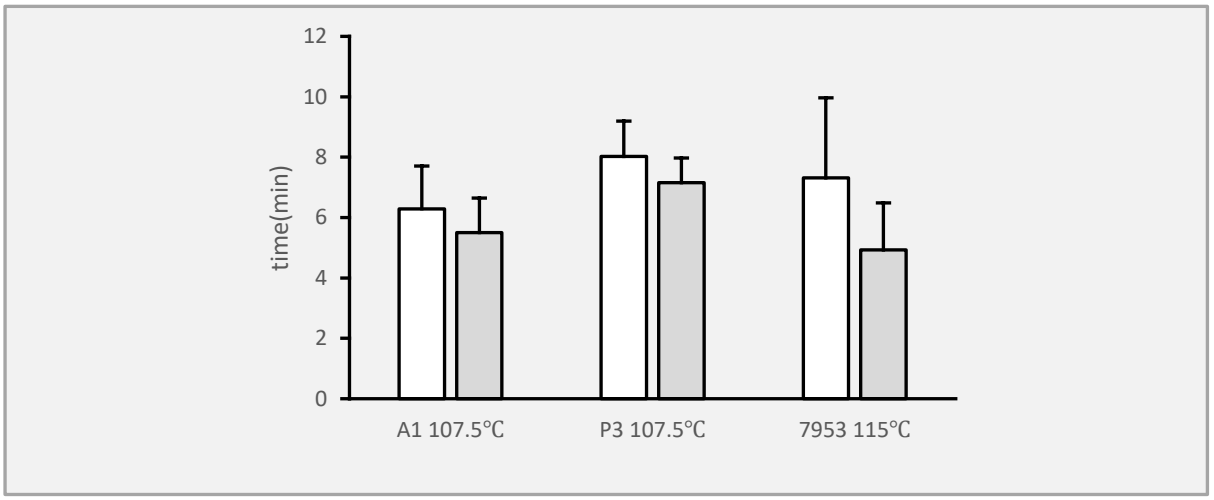

Figure 6-6 The calculated D values of A1, P3 and 7953 spores produced from MF1 (white bars) and MF2 (grey bars) at specific temperatures

6.4 Conclusion

Spore production of *G. stearothermophilus* dairy isolates A1 and P3 was reduced in MF2 compared with MF1. This observation could potentially explain the reduced thermophile contamination in MF2 powder observed in industry. The sporulation percentage of A1 and P3 was lower in MF2 than in MF1 during most of the biofilm formation time. Both sodium and calcium affected the sporulation percentage of A1 and P3 in milk formulations. Addition of calcium increased the sporulation percentage of A1 and P3, and the addition of sodium was detrimental to the sporulation percentage of A1. The heat resistance of spores of A1, P3 and 7953 produced from MF1 and MF2 were not significantly different but further investigation considering spore purity and the production of biofilm spores needs to be done.

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6.6 Supplementary materials

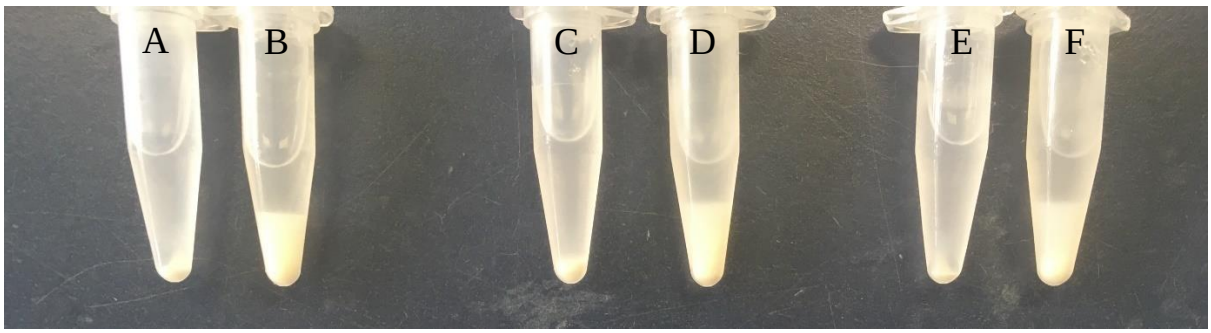

Figure S6-7 Spore suspensions of A1 (A, B), P3 (C, D) and 7953 (E, F) produced from MF1 (A, C and E) and MF2 (B, D, F)

Chapter 7 Final discussion and future work

7.1 The indication of a reduced thermophile spore contamination in calcium-reduced milk powder in industry

The dairy industry has observed the calcium-reduced milk protein powder is associated with reduced spore contamination of thermophilic bacteria during milk powder manufacture. The presence of thermophilic spores in the milk powder affects the selling price and increase the risk of sensory or functional defects in the manufactured powder (Hill and Smythe 2012, Somerton 2013). Investigating the causes of the issue, it could lead to a novel control method to reduce the thermophile contamination in milk powder manufacture.

Two thermophilic bacterial species *Geobacillus* spp. and *A. flavithermus*, are considered to be the major cause for thermophilic bacterial (thermophile) contamination in dairy products (Burgess et al. 2010). However, the biofilm formation and cell attachment of *A. flavithermus* in MF1 and MF2 were similar, while the biofilm formation of *G. stearothermophilus* was significantly reduced in MF2 and the addition of calcium into MF2 seemed to counteract the inhibitory effect of MF2 and restore biofilm formation (Somerton 2013). Therefore, *G. stearothermophilus* was chosen to further investigate the effect of calcium and milk formulations on cell attachment, biofilm formation, spore production and spore heat resistance for the potential causes of the reduced thermophile spore contamination observed in calcium-reduced milk protein powder. Previously, cell attachment and biofilm formation in milk formulation were investigated in milk formulations (Somerton 2013). In the present study, the effect of calcium ions on biofilm formation and cell attachment was studied in more detail than reported previously. Sporulation and spore heat resistance of *G. stearothermophilus* were included in the current study as these could be important factors in thermophile contamination and not previously reported.

7.2 The effect of calcium ions on biofilm formation of *G. stearothermophilus*

The current study showed different responses of *G. stearothermophilus* strains to the presence of calcium ions. The presence of calcium decreased the biofilm cell numbers of 7953 on stainless steel surfaces, while an increase in biofilm cell numbers of A1 was observed in the

presence of calcium. The addition of 40 mM calcium decreased the biofilm cell numbers of P3 on stainless steel, but little effect was observed at 7 mM. This result does not agree with the consistent enhancement of biofilm numbers in the presence of calcium observed in MF2 in the previous study (Somerton et al. 2015), and indicates calcium may act differently in mTSB media compared with milk. The biofilm CV staining generally followed the same trend as the cell enumeration results with calcium resulting in increased biofilm cell numbers and CV staining of A1, comparable biofilm cell numbers and CV staining of P3 and decreased biofilm cell numbers and CV staining of 7953. In addition, calcofluor white staining indicated that the presence of calcium resulted in a different distribution of biofilm of dairy isolates on the surface with biofilm cells clustered more closely to each other in 7 mM calcium. A similar structure arrangement was also observed in biofilms of *P. mendocina* in the presence of divalent cations (Mangwani et al. 2014). SEM images showed a matrix structure surrounding bacterial cells, particularly A1 and P3, which indicate the presence of EPS. The presence of calcium was shown to facilitate extracellular protein production of *G. stearothermophilus* A1 and P3. The study of biofilm structure and EPS composition could provide useful information to design novel cleaning methods to eradicate biofilms. Several researchers have attempted to use an enzymatic cleaning method to remove biofilms (Augustin et al. 2004, Nguyen and Burrows 2014). Proteinase agents effectively inactivate *P. aeruginosa* biofilms, but the effect was compromised with the presence of milk in the treatment suspension (Augustin et al. 2004). The addition of proteinase K to bacterial culture inhibits biofilm formation of *L. monocytogenes*, and the presence of proteinase K dispersed the pre-formed biofilms on both polystyrene and stainless steel (Nguyen and Burrows 2014). These studies showed the promising outcome of enzyme treatment in controlling bacterial biofilms. However, careful consideration needs to be taken as where to apply proteinase-based cleaning agents in dairy manufacture due to the potential effect on milk proteins if the enzymes contaminate the milk products.

Planktonic cells of A1, P3 and 7953 showed a different response to calcium ions than biofilm cells. For example, the presence of calcium increased the total planktonic cell concentrations of P3 and total biomass (determined by optical density) of planktonic cultures of 7953, but a similar effect was not observed in the biofilm. The presence of calcium did not increase the biofilm cell numbers of P3 and the total biofilm biomass (determined by CV staining) of 7953 decreased with increasing concentration of calcium. It is not surprising as planktonic and biofilm cells represent two different life forms of bacteria (Sadiq et al. 2017). The different

response between planktonic and biofilm cells to cations was also observed in *B. subtilis* in the presence of magnesium (Oknin et al. 2015). The presence of 50 mM or more magnesium inhibits pellicle and biofilm colony formation of *B. subtilis* but has minimal effect on the planktonic culture growth (Oknin et al. 2015). High concentrations of calcium ions changed viabilities of *G. stearothermophilus* planktonic cells, especially 7953, and it is hypothesised that the high concentration of calcium could render them to be more susceptible to the change of osmotic pressure with respect to calcium. The specific mechanism still needs to be further investigated.

7.3 The effect of calcium ions and milk formulations on cell attachment of *G. stearothermophilus*

Multiple factors affect bacterial cell attachment, some of these factors include the electrostatic interactions between bacteria surfaces and the substratum, hydrophobicity of bacteria surfaces and the substratum, the presence of conditioning films on the substratum and the presence of cell surface polymers (Palmer et al. 2007). The current study saw that an increased cell attachment of A1 and P3 on polystyrene in the presence of calcium but not on stainless steel. This is not surprising as the two surfaces might have different charges, hydrophobicity and topography properties. The effect of the surface on the cell attachment was reported in several other bacterial species including *Salmonella* and *L. monocytogenes* where attachment on stainless steel is less than on plastic surfaces (Veluz et al. 2012).

On the polystyrene surfaces, the presence of calcium increased the cell attachment of *G. stearothermophilus* dairy isolates. The zeta potential of the cell surface and cell surface hydrophobicity were measured, and they did not correlate with cell attachment. Therefore, the cell surface polymers were investigated in more detail. The amount of cell surface protein and cell surface polysaccharides were higher in A1 than P3 and 7953 and a bridging effect of calcium between cell surface polymers and substratum could contribute to the increased cell attachment of A1 when calcium was added to the medium for attachment. Preconditioning bacteria in media containing calcium decreased the cell surface proteins of dairy isolates A1 and P3, and it increased the cell attachment of P3 on polystyrene. Therefore, it was hypothesized that calcium changed of cell surface polymers of *G. stearothermophilus* dairy

isolates and altered the cell attachment. This observation is in partial agreement with the previous study by Somerton et al. (2013) where calcium promoted cell attachment when it was used to precondition *G. stearothermophilus* cells, but little effect was seen when calcium was just present during attachment.

However, the results of cell attachment in mTSB media gave little indication of the cell attachment in milk formulations as no differences were observed on stainless steel surfaces. Cell attachment in MF1 and MF2 using inocula prepared with either MF1 or MF2 was similar for all strains. Similarly, cell attachment of *Geobacillus* sp. F75 in MF1 and MF2 is similar on stainless steel regardless of the preculture conditions (Somerton 2013). Interestingly, other studies suggest the effect of milk on bacterial cell attachment. The attachment of *L. monocytogenes* is reduced in the presence of skim milk (Helke et al. 1993), and stainless steel pre-treated with skim milk reduces the attachment of *S. aureus*, *L. monocytogenes* and *Serratia marcescens* (Barnes et al. 1999). The difference between the current and previous findings may be due to the bacterial species and the milk composition involved.

7.4 The effect of milk formulations on biofilm formation, spore production and spore heat resistance of *G. stearothermophilus*

The current study indicated that the initial cell growth history played a vital role in the subsequent biofilm formation in milk formulations, especially in MF2. These results expand our understanding of the previously thought universal inhibitory effect of MF2 on *G. stearothermophilus* and indicated that the reduced biofilm formation in MF2 could contribute to the reduced thermophile spore contamination in calcium-reduced milk powder in industry.

The addition of calcium and sodium to milk formulations showed both cations played a role in promoting and inhibiting biofilm formation, respectively. And it was partially in line with the previous findings that addition of calcium in MF2 increased the biofilm cell numbers and addition of sodium to MF1 reduced the biofilm cell numbers of *G. stearothermophilus* (Somerton et al. 2015). As the majority of sodium in milk formulations exists as free ions (Fox 2003), the sodium might directly affect biofilms and result in reduced biofilm formation.

However, as the majority of calcium exists in the colloidal phase that is associated with the casein micelle in milk (Fox 2003), calcium could either affect cells directly or interact with milk proteins and in return change the biofilm formation.

The sporulation of biofilms in MF2 was consistently lower than that of MF1 and the reduced spore numbers in MF2 could explain the reduced thermophile spore contamination of MF2 in industry. Both calcium and sodium seemed to play a role in the sporulation of biofilms. The addition of calcium increased the sporulation percentage of A1 and P3, and the addition of sodium decreased the sporulation percentage of A1. Similarly, increasing calcium concentration of sporulation media slightly increases the spore yield of *Clostridium* spp. (Lenz and Vogel 2014), while excluding calcium from the sporulation media diminishes the spore yield of *Bacillus* sp. (Minh et al. 2011). However, as discussed earlier, the specific effect of calcium in milk formulations remains unknown.

The inactivation of vegetative cells is thought to occur during the spray drying process, where the product heats up to a maximum temperature of normally 60-80 ℃ (Bylund 2015), however spores will survive. The heat resistance of spores will determine the ability to survive industrial such as UHT processing and this can influence the market for milk powders. The heat resistance of spores grown in MF2 was consistently lower than spores grown in MF1 for all three strains, although this difference was not statistically significant. The current findings are not in line with the previous studies. Increasing calcium concentration of the spore production media increased the spore heat resistance of *C. botulinum* and *B. subtilis* (Fleming and Ordal 1964, Lenz and Vogel 2014). While the addition of sodium in the sporulation medium resulted in lower spore heat resistance of *B. subtilis* (Fleming and Ordal 1964). However, in the present study, it could be that the relative higher impurity of the MF2 spore suspension, caused by the remaining vegetative cell debris, interfered with the effect of heat treatment and thus further research needs to be done to confirm the results obtained.

A summary of results that either support or do not support the reduced the thermophile contamination in calcium-reduced milk protein powder are presented in Table 7-1. It is possible that the sporulation percentage and growth history dependent biofilm formation of *G.*

stearothermophilus dairy strains result into the varied thermophile spore contamination levels between normal and calcium-reduced milk protein powder

Table 7-1 Experimental observations that give insights into the reduced thermophile contamination levels in calcium-reduced milk protein powder

The effect of calcium on cell attachment in mTSB	**The effect of milk formulations on cell attachment**	**The effect of calcium on biofilm formation in mTSB**	**The effect of milk formulations on biofilm formation**	**The effect of milk formulations on sporulation and spore heat resistance**
G. stearothermophilus showed a similar cell attachment pattern on stainless steel in mTSB with different calcium concentrations	*G. stearothermophilus* showed a similar cell attachment pattern in milk formulations with different cation profiles	Calcium showed a strain-dependent effect on biofilm formation of *G. stearothermophilus*	Calcium-reduced milk formulation showed an inhibitory effect on biofilm formation of *G. stearothermophilus*	Calcium-reduced milk formulation reduced the sporulation percentage of *G. stearothermophilus* dairy isolates
			The inhibitory effect of calcium-reduced milk formulation on *G. stearothermophilus* biofilms was associated with the bacterial growth history	The heat resistance of spores produced from calcium-reduced milk formulation did not show a significant reduction compared to the normal milk formulation
			The inhibitory effect of calcium-reduced milk formulation on *G. stearothermophilus* biofilms was associated with the concentrations of calcium and sodium	

Note: experimental observations that could explain the reduced thermophile contamination levels in calcium-reduced milk protein powder are highlighted with grey background.

7.5 Future recommendations

It is shown in the current study that when planktonic cells were cultured in 40 mM calcium, a decrease in optical density was observed in the resuspended cells using BPW and this was thought to represent the damage of the cells due to the sudden change in calcium concentrations. The mechanisms for calcium affecting cell wall integrity should be investigated. Fluorescence staining in combination with a fluophotospectrometer or flowcytometer could assist in identifying the ratio between intact cells and damaged cells before and after cell resuspension. TEM analysis could help to visually distinguish the change of cell wall, influenced by calcium.

The effect of cell surface polymers of *G. stearothermophilus* on cell attachment needs to be studied. An efficient surface structure removal method targeting proteins, polysaccharides and eDNA could be developed to check how the absence of certain cell surface polymers affects the cell attachment in the presence or absence of calcium.

The biofilm formation, spore production and heat resistance of spores in a flow system which closely mimics the dynamic system in the industry, could be explored. The effect of growth history could also be confirmed using different inoculum preparation methods in a flowing system.

A field study in the manufacture of MF1 and MF2 is needed. Samples could be taken before spray drying, after spray drying and in the final products. Samples taken prior to spray drying from the balance tank, plate heat exchanger and evaporator passes could be useful to monitor thermophile and spore formation at different processing points during the manufacture of milk formulations. Both total cell and spore numbers should be enumerated, and bacteria species could be identified using MALDI-TOF, PCR or metagenomic analysis. If the similar total cell numbers are present in MF1 and MF2 prior to spray drying but reduced spore numbers occurred in MF2, it means MF2 reduces the sporulation efficiency during manufacture, and this is beneficial to the industry. If total cells are reduced in MF2 than MF1, this indicates MF2 inhibits biofilm formation. The results of final products could be used to correlate with the results obtained from process samples.

The effect of a calcium-milk protein interaction on the biofilm formation and sporulation needs further investigation. One approach is to separate the whey and casein fractions and test the biofilm formation in each separately. An alternative approach is to compare the inhibitory effect of MF2 on *G. stearothermophilus* biofilms in different concentrations and the effect of adding sodium-caseinate and whey to the lower concentrations of milk formulations. In addition, SEM could be used to observe the structure of biofilms when inhibited in MF2. The same approach could be used to investigate the effect of milk proteins on sporulation and spore heat resistance of *G. stearothermophilus*.

A comprehensive spore cleaning procedure could be developed that consists the use of surfactant, enzymatic digestion, ultrasonication and gradient centrifugation to produce pure spore suspensions and investigate the effect of cell and milk residues on the heat resistance of spores.

An RNA-seq analysis should be undertaken to determine the differential expression of sporulation genes of *G. stearothermophilus* planktonic cells and biofilms in MF1 and MF2 as greater spore formation was observed in MF1 than MF2.

A larger set of *G. stearothermophilus* dairy isolates could be used in the future study to understand the role of strain variation in biofilm formation, sporulation and spore heat resistance in milk formulations with different cation profiles.

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