Effect of cations on biofilm formation by *Geobacillus* species and *Anoxybacillus flavithermus* dairy isolates

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Abstract

The concentration of free cations is one factor that may influence biofilm formation and consequent contamination of milk formulations by *Geobacillus* spp. and *Anoxybacillus flavithermus* during the manufacture of milk powders.

Culture optical densities were measured to show that Ca^{2+} and Mg^{2+} predominantly increased the planktonic growth of *Geobacillus* spp. and *A. flavithermus* cultures. Culture cell numbers were enumerated, and a protein quantification assay was used to indicate that increases in optical density elicited by Ca^{2+} and Mg^{2+} supplementation was due to increased production of bacterial surface protein rather than an increase in cell numbers.

High individual concentrations of Na^{+}, K^{+} or Ca^{2+} (63 – 250 mM) inhibited the planktonic growth of *Geobacillus* spp., and Mg^{2+} protected *Geobacillus* spp. from high, inhibitory concentrations of Na^{+}, K^{+} or Ca^{2+}.

The number of viable cells attached to stainless steel coupons was enumerated to show that cation concentrations or the monovalent to divalent cation ratio (2:1 compared to 10:1) did not influence the transition of bacteria from a planktonic to surface-attached form, or the subsequent formation of an established biofilm. However, preconditioning of the bacteria with cations increased their subsequent attachment. It was proposed that the transition of bacteria from a planktonic to surface-attached form is primarily mediated by the expression of bacterial surface proteins, as induced by cation preconditioning.

The number of attached *Geobacillus* spp. was up to 4 log CFU cm^{-2} lower, for up to 18 h of biofilm formation, in a milk formulation that had a high monovalent to divalent cation ratio (greater than 10:1) relative to a milk formulation that had a monovalent to divalent cation ratio that resembled that found in unprocessed milk. Supplementation of a milk formulation that had a high monovalent to divalent cation ratio with Ca^{2+} or Mg^{2+} fully alleviated the inhibitory effect of the milk formulation on biofilm formation by *Geobacillus* spp.

It was concluded that there is potential for the total thermophile count in milk powders that have high monovalent to divalent cation ratios to be markedly reduced. This would increase the quality and selling price of the milk powders.

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Table of contents

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of publications

Published manuscripts

Somerton, B., Palmer, J., Brooks, J., Smolinski, E., Lindsay D. & Flint, S. (2012). Influence of cations on growth of thermophilic *Geobacillus* spp. and *Anoxybacillus flavithermus* in planktonic culture. *Appl Environ Microbiol* **78**, 2477-2481.

Somerton, B., Palmer, J., Brooks, J., Flint, S. & Lindsay, D. (2013). Preconditioning with cations increases the attachment of *Anoxybacillus flavithermus* and *Geobacillus* species to stainless steel. *Appl Environ Microbiol* **79**, 4186-4190.

List of conference presentations

Oral presentations

Somerton, B., Palmer, J., Brooks, J., Smolinski, E., Lindsay, D. & Flint, S. (2011). Influence of cations on growth of thermophilic *Geobacillus* spp. and *Anoxybacillus flavithermus* in planktonic culture. 57th Annual Scientific Meeting of the New Zealand Microbiological Society Conference 2011 (Palmerston North, New Zealand).

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Poster presentations

Somerton, B., Palmer, J., Brooks, J., Smolinski, E., Flint, S. & Lindsay, D. (2011). Physico-chemical and physiological role that cations play on attachment and early biofilm formation by *Anoxybacillus flavithermus*. 57th Annual Scientific Meeting of the New Zealand Microbiological Society Conference 2011 (Palmerston North, New Zealand).

Somerton, B., Palmer, J., Brooks, J., Smolinski, E., Flint, S. & Lindsay, D. (2012). Preconditioning of *Geobacillus* sp. and *Anoxybacillus flavithermus* with cations increases their attachment to stainless steel. 6th American Society for Microbiology Conference on Biofilms 2012 (Miami, USA).

g	acceleration due to gravity
ATP	adenosine triphosphate
α	alpha subunit
NH_3^+	amine ion
*	asterisk
bp	base pair(s)
β	beta subunit
Bap	biofilm-associated protein
Cd^{2+}	cadmium ion
$CaCl_2$	calcium chloride
Ca^{2+}	calcium ion
CM 1:5	calcium ion to magnesium ion ratio of 1:5
$Ca_3(PO_4)_2$	calcium phosphate
CWM	cell wall material
PS-CWM	cell wall material stripped of phosphate groups
CM-CWM	cell wall material with masked carboxylate groups
cm	centimetre
$Citr^{3-}$	citrate ion
Co^{2+}	cobalt ion
CFU	colony forming unit(s)
C	cytosine
Da	daltons
°C	degrees celcius
DNA	deoxyribonucleic acid

rDNA	deoxyribonucleic acid that encodes for a ribosomal gene
DLVO	Derjaguin, Landau, Verway, Overbeek
H_2O	dihydrogen oxide
H_2PO	dihydrogen phosphate ion
D	D orientation of an isomer
DSM	DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) bacteria collection reference number
EF	E and F helixes of a protein joined by a loop
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid
EPS	extracellular polymeric substances
Fe^{2+}	iron (II) ion
Gy	Grays
G	guanosine
His	histidine
h	hour(s)
$HCitr^{2-}$	hydrogen citrate ion
H^+	hydrogen ion
HPO_4^{2-}	hydrogen phosphate ion
κ	kappa
kg	kilogram
Pb^{2+}	lead ion
Log	logarithm
L	L orientation of an isomer
$MgCl_2$	magnesium chloride
Mg^{2+}	magnesium ion
Mn^{2+}	manganese ion

MS	mass spectroscopy
m/z	mass to charge ratio
MALDI-TOF	matrix assisted laser desorption/ionization-time of flight
µl	microliter
µmol	micromole
µM	micromole per liter
MF	milk formulation(s)
MPCA	milk plate count agar
mg	milligram
mm	millimeter
mmol	millimole
mM	millimole per liter
mV	millivolts
min	minute
mins	minutes
MDCR	monovalent to divalent cation ratio
X	multiplication factor
nm	nanometer
nM	nanomole per liter
Ni^{2+}	nickel ion
N/A	not applicable
n	number of replicates
σ	omega (symbol for the population standard deviation)
PCR	polymerase chain reaction
KCl	potassium chloride
K^{+}	potassium ion

pH	power of hydrogen
P	probability of detection
Pty Ltd	Proprietary Limited
rpm	revolutions per minute
16S	ribosomal subunit number 16
s	second(s)
NaCl	sodium chloride
Na^+	sodium ion
NKC	sodium ion to potassium ion to calcium ion ratio of 1:1:2
S-layer	Slime layer
spp.	species (plural)
sp.	species (single)
SD	standard deviation
SAS	statistical analysis software
s	subunit
TVC	total viable cells
Zn^{2+}	zinc ion

List of tables

CHAPTER 1

Literature review

1.1) Introduction

There are three proposed mechnisms for the influence of cations on bacterial attachment and the proliferation and viability of bacteria in a biofilm. Firstly, relatively high or low external cation concentrations (such as high Na^+, low Ca^{2+} or low Mg^{2+} concentrations) may interfere with the cellular functioning and homeostasis of bacteria in a biofilm. Secondly, cations may electrostatically interact with polymers in the cell envelope and extracellular matrix of a biofilm and the attachment substrate, and influence their surface charge, and the structural integrity and extent of cohesion of a biofilm. Thirdly, bacteria in a biofilm may 'sense' and respond to external cations, or cations may alter the conformation of bacterial polymers such that the physiology of bacteria may be influenced subsequently affecting the bacterial attachment process and the physiology of the biofilm.

Specialty, commercial milk formulations have a range of cation concentrations and ratios. The concentration and ratio of free Na^+, K^+, Ca^{2+} and Mg^{2+} ions is one factor which may influence the proliferation and biofilm formation of the thermophilic bacilli *Geobacillus* spp. and *A. flavithermus* in milk formulations during the manfacture of milk powder. The number of thermophilic bacilli vegetative cells and spores in final milk powder determines its grade and selling price. It is perceived that *Geobacillus* spp. and *A. flavithermus* form biofilms on product-contact surfaces of milk powder manufacturing plants acting as the main reservoir of thermophilic bacilli cells and spores. These cells and spores slough-off biofilms and foulants into milk during processing, contaminating milk powder.

This literature review will detail research which has investigated the effect of cations on bacterial attachment and biofilm formation. Insights gained from this literature review will be used to derive the research questions and research hypotheses

of this study on the effect of cations on biofilm formation by *Geobacillus* spp. and *A. flavithermus* in specialty, commercial milk formulations.

1.2) Nutritional requirement for calcium, magnesium, sodium and potassium ions in bacterial cellular functioning and homeostasis

Free Na^+, K^+, Ca^{2+} and Mg^{2+} ions are essential nutrients required by bacteria for growth and survival. Many bacterial enzymes require Na^+, K^+, Ca^{2+} and Mg^{2+} ions as co-factors for optimal functioning (Epstein, 2003; Michiels *et al*., 2002; Novakova & Smigan, 2008; Smith & Maguire, 1998). Also, Na^+, K^+, Ca^{2+} and Mg^{2+} associate with and stabilise structural polymers, such as teichoic acid and peptidoglycan (Beveridge & Murray, 1980; Neuhaus & Baddiley, 2003). Intracellular free Na^+, K^+, Ca^{2+} and Mg^{2+} ion concentrations are tightly regulated in bacteria to maintain cation homeostasis which ensures optimal cation concentrations for cellular functioning (Epstein, 2003; Michiels *et al*., 2002; Novakova & Smigan, 2008; Smith & Maguire, 1998). As Na^+, K^+, Ca^{2+} and Mg^{2+} ions are the most abundant free ions in both unprocessed milk and milk formulations available to spoilage bacteria for growth (Fox, 2003), this section will detail the importance of these cations in bacterial nutrition, cellular functioning and homeostasis.

1.2.1) Calcium

Calcium ions have an important role in enhancing the stability and cohesion of the cell envelope and extracellular matrix of bacteria, as they associate with negatively charged functional groups and may form divalent cation bridges (Neuhaus & Baddiley, 2003; Rose *et al*., 1994). In addition, Ca^{2+} associates with many structural, enzymatic and regulatory intracellular proteins in bacteria where it affects, and often optimises, their stability and function (Michiels *et al*., 2002; Norris *et al*., 1996). Most intracellular

calcium is bound to Ca^{2+}-binding proteins such that cytosolic free Ca^{2+} is maintained at concentrations that are approximately 1000 fold lower relative to the outside of the cytoplasmic membrane (Michiels *et al*., 2002). For instance, the cytosolic free Ca^{2+} concentration in *Eschericia (E.) coli* was estimated to be approximately 90 nM (Michiels *et al*., 2002), and in contrast, external free Ca^{2+} concentrations range between 0.1 and 10 mM in typical bacterial habitats. For example, free Ca^{2+} concentrations in freshwater, unprocessed bovine milk and seawater are approximately 0.3 (Garrison-Schilling *et al*., 2011), 3 (Fox, 2003) and 10 mM (Garrison-Schilling *et al*., 2011), respectively. Intracellular free Ca^{2+} concentrations are highly regulated in bacteria in a similar fashion to eukaryotes, and it is believed that the maintenance of relatively low cytosolic free Ca^{2+} concentrations is ubiquitous in all cellular organisms (Michiels *et al*., 2002; Norris *et al*., 1996). Cytosolic free Ca^{2+} concentrations are kept low in bacteria due to the low permeability of Ca^{2+} across the cytoplasmic membrane, tightly controlled Ca^{2+} influx channels, the high free Ca^{2+} buffering capacity of the cytosol, and effective Ca^{2+} efflux integral membrane proteins (Michiels *et al*., 2002; Norris *et al*., 1996). *E. coli* has a non-proteinaceous Ca^{2+} influx channel that spans the cytoplasmic membrane consisting of the lipidic polymer, poly-3-hydroxybutyrate and negatively charged inorganic polyphosphate (Michiels *et al*., 2002; Norris *et al*., 1996). This ion channel is voltage gated and may store extracellular Ca^{2+}, as calcium phosphate, ready for translocation into the cytoplasm as required (Michiels *et al*., 2002; Norris *et al*., 1996). Bacteria have a primary ATP dependant Ca^{2+} pump which transports Ca^{2+} against its concentration gradient to outside of the cytoplasmic membrane (Michiels *et al*., 2002; Norris *et al*., 1996). Additionally, bacteria utilise secondary pumps to efflux, and on occasions influx, Ca^{2+}, such as the Ca^{2+}/H^{+} and Na^{+}/Ca^{2+} antiporters (Michiels *et al*., 2002; Norris *et al*., 1996). Usually a proton electrochemical gradient is utilised to drive the Ca^{2+}/H^{+} anitporter to efflux Ca^{2+} in bacteria, however some bacteria, such as

alkaliphiles, thermophiles and pathogens, for example *Streptococcus (S.) pneunomiae* (Norris *et al*., 1996), utilise a Na^+ electrochemical gradient, which is generated across the cytoplasmic membrane, to drive the Na^+/Ca^{2+} antiporter to efflux Ca^{2+} (Hase *et al*., 2001). The *E. coli* Ca^{2+}/H^+ antiporter, ChaA has a sequence rich in negatively charged aspartic and glutamic acid residues which presumably acts as the Ca^{2+} binding site (Norris *et al*., 1996).

Interestingly, Naseem *et al*. (2008) showed that when external Na^+ or K^+ concentrations were increased to 30 mM, Na^+ and K^+ non-specifically out-competed Ca^{2+} (at an external concentration of 1 mM) binding to a non-selective ion influx channel, largely blocking Ca^{2+} influx and stimulating net Ca^{2+} efflux from *E. coli*. Furthermore, results indicated that the putative Ca^{2+}/H^+ antiporter, ChaA, and the putative Na^+/Ca^{2+} antiporter, YrbG, do not influence or compensate for the observed net Ca^{2+} efflux, and so it was proposed that neither of the antiporters play a role in the regulation of cytosolic Ca^{2+} in *E. coli*.

Since the free Ca^{2+} concentration in the cytosol of bacteria is maintained at relatively low concentrations, transient, localised Ca^{2+} fluxes in the cytosol are utilised to regulate a wide range of cellular processes, such as the cell cycle, cell division, chemotaxis, motility, heat shock, cell differentiation, and pathogenesis (Michiels *et al*., 2002; Norris *et al*., 1996). Jones *et al*. (1999) showed that increasing extracellular Ca^{2+} concentrations up to between 0.25 – 10 mM caused cytosolic Ca^{2+} concentrations to rise in *E. coli* from approximately 270 nM to a maximum of 0.85 – 5.4 μM after 30 – 40 mins, then slowly fall for an additional 30 mins. Furthermore, Garrison-Schilling *et al*. (2011) proposed that transient increases in cytosolic Ca^{2+} in response to increases in extracellular Ca^{2+} concentrations, such as those shown by Jones *et al*. (1999), may influence intracellular Ca^{2+} mediated regulatory pathways that determine the type of extracellular polysaccharide expressed by *Vibrio (V.) vulnificus*. It is thought that Ca^{2+}

influences cellular processes by interacting with and altering the structure of nucleoids, protein phosphorylation and transverse and lateral distributions of membrane lipids (Norris *et al*., 1996). For instance, Ca^{2+} may directly bind to G + C rich regions of DNA and regulate gene expression (Norris *et al*., 1996). Ca^{2+} may stimulate phosphorylation of proteins, such as regulatory proteins, and consequently alter their function (Norris *et al*., 1996). The *E. coli* heat shock protein, DnaK, has a segment that has 60% identity to the 21 amino acid sequence of the Ca^{2+} binding site of calmodulin (a comprehensively studied Ca^{2+} binding protein which, since its characterisation, many calmodulin-like Ca^{2+} binding proteins have been identified in bacteria) (Michiels *et al*., 2002; Norris *et al*., 1996). The phosphorylation of DnaK is stimulated 10 fold by Ca^{2+} *in vitro* (Norris *et al*., 1996). Interestingly, *E. coli* grows optimally at 32 and 37°C in the presence of 0.1 and 1 mM Ca^{2+}, respectively, which indicates that cytosolic Ca^{2+} may act partly as an intracellular thermometer (Norris *et al*., 1996). Additionally, Rampersaud *et al*. (1991) showed that in *E. coli*, Ca^{2+} concentrations as low as 60 μM stimulated the cytoplasmic region of an inner membrane transducer, Taz1, to phosphorylate and more readily donate its phosphate to the transcripton factor, OmpR, which promotes the upregulation of ompC encoding for the expression of OmpC – an outer membrane protein which assists the passive diffusion of small solutes.

1.2.2) Magnesium

Similarly to Ca^{2+}, free Mg^{2+} non-specifically binds to phosphate and carboxyl groups in the cytoplasmic membrane, cell wall and extracellular matrix, stabilising polymers by neutralising their overall negative charge (Heptinstall *et al*., 1970; Neuhaus & Baddiley, 2003). Additionally, Mg^{2+} stabilises ribosomes and nucleic acids (Smith & Maguire, 1998). Cytoplasmic membrane associated enzymes involved in the biosynthesis of teichoic acids in the cell wall of bacteria require Mg^{2+} for optimal functioning

(Heptinstall *et al*., 1970; Hughes *et al*., 1973). Hughes *et al*. (1973) showed that teichoic acid biosynthesis enzymes associated with the cytoplasmic membrane of *Bacillus (B.) licheniformis* preferentially utilise Mg^{2+} associated with teichoic acid rather than Mg^{2+} present in solution. It has been proposed that teichoic acids function to assimilate divalent cations from the environment surrounding bacteria and that cations transfer along anionic groups on the polymers and accumulate at the cell wall-cytoplasmic membrane interface, where they are available for utilisation by cation dependant membrane systems (Hughes *et al*., 1973; Neuhaus & Baddiley, 2003).

Bacteria constitutively express the CorA integral membrane transport system, which is the primary mode of Mg^{2+} influx (Smith & Maguire, 1998). Only one charged amino acid exists in the transmembrane segment of CorA, thus it is hypothesized that Mg^{2+} interacts via charge-lone pair interactions with carbonyl oxygen or hydroxyl groups in the transmembrane segment during influx (Smith & Maguire, 1998). In addition, bacteria upregulate the expression of the MgtA and MgtB Mg^{2+} influx transporters in response to low (less than 1 mM) extracellular Mg^{2+} concentrations (Smith & Maguire, 1998). Other cations, such as Co^{2+}, Mn^{2+} and Ni^{2+} compete with Mg^{2+} for transport with CorA, MgtA and MgtB (Smith & Maguire, 1998). However, Ca^{2+} does not compete with or inhibit Mg^{2+} transport through Mg^{2+} influx transporters (Smith & Maguire, 1998).

Of the cations required for growth by bacteria, often changes in Ca^{2+} and Mg^{2+} concentrations have the greatest influence on growth by planktonic bacteria (Aranha *et al*., 1986; Caldwell & Arcand, 1974; Jurado *et al*., 1987; Vincent, 1962). Furthermore, the preferential requirement for and predominant response of bacteria to Ca^{2+} or Mg^{2+} is unique for each bacterial strain (Caldwell & Arcand, 1974). Also, Ca^{2+} and Mg^{2+} may act co-operatively to enhance bacterial growth (Caldwell & Arcand, 1974), or, conversely, one cation type may act in replacement of the other to elicit maximum

growth (Vincent, 1962). Usually, the concentrations of Ca^{2+} and Mg^{2+} that are available to bacteria in their natural habitat exceed minimal concentrations required for optimal planktonic growth of bacteria (Aranha *et al*., 1986; Patrauchan *et al*., 2005). For example, Patrauchan *et al*. (2005) found that planktonic growth by a *Pseudoalteromonas* sp. isolate was similar when Ca^{2+} concentrations ranging between 0.25 and 10 mM in growth medium were compared. The minimum Ca^{2+} requirement of the *Pseudoalteromonas* sp. isolate is likely to be less than 0.25 mM, which is less than the concentration of Ca^{2+} readily available to this marine-dwelling bacterial isolate (10 mM) in seawater. Thus, inhibition of planktonic growth of bacteria as a result of deprivation of available Ca^{2+} or Mg^{2+} seldom occurs (Aranha *et al*., 1986). However, as both Ca^{2+} and Mg^{2+} are essentially required by bacteria for a range of cellular processes, deprivation of the optimal cytosolic acquisition of Ca^{2+} and Mg^{2+} would have a detrimental effect on metabolism and cell division. Jurado *et al*. (1987) found that although the addition of 2.5 mM Mg^{2+} stimulated growth by *B*. *stearothermophilus*, (since renamed *Geobacillus (G.) stearothermophilus*) (Nazina *et al*., 2001) growth was inhibited when the added Mg^{2+} concentration was 10 mM. However, the inhibitory effect of 10 mM Mg^{2+} was allieviated when 2.5 mM Ca^{2+} was simultaneously added. It was proposed that Ca^{2+} displaced Mg^{2+} from the divalent cation interaction sites in the bacteria and protected the bacteria from the inhibitory effect of the high Mg^{2+} concentration.

1.2.3) Sodium

Cytosolic free Na^{+} is involved in pH homeostasis mechanisms and the activation of some enzymes in bacteria (Novakova & Smigan, 2008; Quinn *et al*., 2012). Additionally, some bacteria generate a Na^{+} eleoctrochemical gradient across their cytoplasmic membrane to drive sodium ion co-transport systems and sodium ion

coupled energy transformation (Novakova & Smigan, 2008; Quinn *et al*., 2012). Excessive accumulation of Na^+ in the cytosol is toxic to bacteria and concentrations are maintained below 10 – 30 mM (Corratge-Faillie *et al*., 2010). Some bacteria use ATP dependant Na^+ exporters to transport Na^+ outside of the cytoplasmic membrane (Hase *et al*., 2001). This is energetically costly to bacteria and constant efflux of Na^+ in environments with high Na^+ concentrations can place a metabolic burden on bacteria and have a bacteriostatic effect (Hase *et al*., 2001).

1.2.4) Potassium

Potassium (K^+) is an essential ion in bacteria and is the most abundant ion in the cytoplasm, where cytosolic concentrations range between 300 – 500 mM (Follmann *et al*., 2009). The K^+ ion plays an important role in bacteria in the activation of various intracellular enzymes, the maintenance of osmotic pressure, regulation of pH and as a secondary messenger (Epstein, 2003). Potassium acts predominantly as a free ion, since it does not form covalent bonds in aqueous environments (Epstein, 2003).

Increasing concentrations of Na^+ and K^+ have been shown to increase the growth yield and growth rate of planktonic *Bacteroides* cells (Caldwell & Arcand, 1974). Caldwell and Arcand (1974) proposed that differences in the extent of the requirement for Na^+ could be used to differentiate between *Bacteroides* species.

In most instances, bacterial physiologically respond to the free or ionized form of a cation. In the study conducted by Aranha *et al*. (1986) it was stated that calcium is present in saliva both bound to proteins and in its free form, and that it is the free form of calcium that is biologically important. Furthermore, when cations interact with bacteria, whether it be non-specifically to a phosphate group of teichoic acid, or specifically to a Ca^{2+}-binding site of a protein, the cation interacts with a negatively charged region of a molecule. Thus, it is logical to assume that only free cations, which

have a positive charge, are able to attract to and interact with negatively charged regions of molecules and elicit a physiological effect. Also, only free cations are able to translocate through ion-specific channels.

It is hypothesised that the nutritional requirement of Na^+, K^+, Ca^{2+} and Mg^{2+}, and their effect on homeostasis is relevant to bacteria in a biofilm. Additional effects of Na^+, K^+, Ca^{2+} and Mg^{2+}, such as electrostatic and physiological effects, on bacteria in a biofilm will be discussed, to gain further insights of the effect of Na^+, K^+, Ca^{2+} and Mg^{2+} in milk formulations on the biofilm formation and proliferation of *Geobacillus* spp.and *A. flavithermus* during the processing of milk powders in a milk powder manufacturing plant.

1.3) Biofilms – a definition

Biofilms are defined as an aggregation of micro-organisms attached to a surface and are often surrounded by a substantial amount of bacterial-derived extracellular matrix, which may consist of polysaccharides, protein and nucleic acid; and also debris from dead cells and nutrients absorbed from the extracellular environment (Flemming & Wingender, 2010; Hall-Stoodley *et al*., 2004; Stewart & Franklin, 2008; Sutherland, 2001b). Biofilms are ubiquitous in nature and are the dominant mode of life for bacteria, where they develop on many substrates, for example, rocks in freshwater streams and marine environments, plant roots, industrial pipelines, and medical implants; and they cause disease in wounds and in the lungs of patients with cystic fibrosis (Flemming & Wingender, 2010; Hall-Stoodley *et al*., 2004; Stewart & Franklin, 2008; Sutherland, 2001b). Biofilms are typically stratified and consist of a heterogenic array of bacteria (Kolenbrander *et al*., 2010; Stewart & Franklin, 2008). Furthermore, it has been shown that bacteria of the same genotype can display varied phenotypes and physiologies in a biofilm (Stewart & Franklin, 2008). The size, structure and

composition of a biofilm varies greatly and depends on many factors, including the physiology of the species and composition of available nutrients (Stoodley *et al*., 2002). Some biofilms consist of a monolayer of cells, while others are multi-layered and consist of a large proportion of extracellular matrix (Stoodley *et al*., 2002).

Bacterial biofilm formation initiates when planktonic bacteria associate with a surface, usually via surface-exposed polymers which have adhesive properties (Stoodley *et al*., 2002). At this stage of attachment there is potential for the bacterium to dissociate from the surface, so it is described as being reversibly attached (Stoodley *et al*., 2002). Bacteria become irreversibly attached to a surface upon the secretion of extracellular matrix polymers which firmly bond them to the surface (Sutherland, 2001b). Irreversibly attached bacteria may multiply by cell division and continue to produce extracellular matrix to form biofilms with variable sizes and structures (Stoodley *et al*., 2002). Bacteria may passively slough -off a biofilm and become planktonic, or a bacterial biofilm may regulate the active dispersal of bacteria from the biofilm to allow their establishment of biofilms elsewhere (McDougald *et al*., 2012).

1.4) The effect of electrostatic interactions of sodium, potassium, calcium and magnesium ions on bacterial biofilm formation

Cations electrostatically interact with polymers comprising the cell wall and extracellular matrix of bacteria, where they have the potential to influence forces amongst these polymers, such as extended DLVO (Derjaguin, Landau, Verway, Overbeek) theory forces, which include Lifshitz-van der Waals forces, electrostatic forces, and acid-base forces (Busscher *et al*., 2008; Liang *et al*., 2007). Additionally, cations interact with negatively charged functional groups of bacterial polymers and influence the structural integrity and cohesion among the polymers (Neuhaus & Baddiley, 2003). In order to understand how cations electrostatically interact with the

surface of bacteria, it is important to consider the composition and electrostatic properties of surface-exposed polymers that comprise the cell wall and extracellular matrix of bacteria. Surface-exposed polymers interact electrostatically with the surrounding medium and with polymers of nearby cells and matrix. It is electrostatic forces among surface-exposed polymers that influence the bacterial attachment process and determine the stability and cohesive properties of a biofilm matrix.

1.4.1) Composition and electrostatic properties of the cell wall of Gram-positive bacteria

As *Geobacillus* spp. and *A. flavithermus* are Gram-positive bacteria and are of a major focus in this study, electrostatic interactions of cations with Gram-positive bacteria will be discussed in general. The cell wall of Gram-positive bacteria is comprised of peptidoglycan, teichoic acids, and a variety of proteins and polysaccharides bound to peptidoglycan (Kleerebezem *et al*., 2010; Siezen *et al*., 2006; Vollmer & Seligman, 2010; Weidenmaier & Peschel, 2008). Usually the majority of a bacterial cell wall is peptidoglycan, however teichoic acids can comprise up to 60% of the dry weight of a bacterial cell wall (Beveridge & Murray, 1980; Kleerebezem *et al*., 2010; Neuhaus & Baddiley, 2003; Vollmer & Seligman, 2010). Some bacteria also have either capsular polysaccharide or an S-layer, consisting of glycosylated protein, which overlies and is bound to the cell wall (Delcour *et al*., 1999; Hanson & Neely, 2012; Weidenmaier & Peschel, 2008). Additionally, bacteria secrete polymers that may consist of polysaccharide, protein or DNA which form an extracellular matrix (Li *et al*., 2008; Song *et al*., 2012; Subramanian *et al*., 2010). Many of the proteins, which are either associated with the cell wall or secreted are enzymatic (Flemming & Wingender, 2010; Siezen *et al*., 2006). It has been proposed that bacterial extracellular proteins act as an external digestive system and degrade larger macromolecules, such as long chain

polysaccharides, so the degraded fragments can be transported into the cytoplasm and utilised as an energy source (Flemming & Wingender, 2010; Siezen *et al*., 2006). Interestingly, it is estimated that 6 – 11% of proteins are destined to be expressed extracellularly by *Lactobacillus* (Kleerebezem *et al*., 2010). This indicates that bacteria place a significant emphasis on their ability to interact with and respond extracellularly to their surroundings. In addition, proteins have the potential to evolve to have more than one function, where such proteins are coined as moonlighting proteins - further enhancing the metabolic diversity of extracellular proteins (Copley, 2012).

Peptidoglycan, teichoic acids, and some other cell wall and extracellular matrix polymers, such as teichuronic acid and uronic acid, comprise an abundance of negatively charged functional groups, such as carboxyl, phosphate and hydroxyl groups, which confer an overall negative charge to the cell wall (Beveridge & Murray, 1980; Beveridge *et al*., 1982; Hanson & Neely, 2012; Neuhaus & Baddiley, 2003; Sobeck & Higgins, 2002; van den Brink *et al*., 2009). Peptidoglycan consists of glycan strands which are cross-linked by short peptides (Hanson & Neely, 2012; Kleerebezem *et al*., 2010; Vollmer & Seligman, 2010). The short peptides in peptidoglycan contain glutamate residues, which each have a negatively charged carboxyl group (Beveridge & Murray, 1980). Teichoic acids consist of 20 – 50 repeating units of either glycerol or ribitol phosphate (Rahman *et al*., 2009; Schirner *et al*., 2009; Weidenmaier & Peschel, 2008). Teichoic acids are polyanionic, as each glycerol or ribitol phosphate monomer comprises a single negatively charged phosphate group (Neuhaus & Baddiley, 2003; Weidenmaier & Peschel, 2008). Teichuronic acid mainly consists of a chain of glucoronic acid repeats and is also polyanionic, as each glucoronic acid has a single carboxyl group (Delcour *et al*., 1999).

Like most bacteria, thermophilic bacilli have a negative surface charge. For instance, Palmer *et al*. (2010) found that the zeta potentials of two *A*. *flavithermus*

isolates were between approximately –10 and –15 mV at a neutral pH. Seale *et al*. (2008) found that the zeta potential of spores of four *Geobacillus* spp. isolates were between –12 and –21 mV at the pH of milk (6.8).

1.4.2) The extended DLVO theory and its application to bacterial attachment and biofilm formation

The extent of attractive or repulsive forces among charged colloids, such as bacteria, and charged substrates, such as stainless steel, may be explained by the sum of forces outlined in the extended DLVO theory (Busscher *et al*., 2008; Liang *et al*., 2007). The extended DLVO theory consists of three components, which collectively determine the overall extent of attraction or repulsion among interacting entities. These components are Lifshitz-van der Waals forces, electrostatic interactions and acid-base interactions (Busscher *et al*., 2008). Extended DLVO forces are often referred to as physico-chemical forces (Busscher *et al*., 2008).

A Lifshitz-van der Waals force is a weak attractive force between two neutral molecules in close proximity (Mayer *et al*., 1999; Ninham & Parsegian, 1970). The attractive force occurs when an electron rich region surrounding one molecule is attracted to an electron deplete region surrounding another molecule (Mayer *et al*., 1999; Ninham & Parsegian, 1970). Electron rich and electron deplete regions are termed electric dipoles, which have a slight negative or positive charge, respectively (Mayer *et al*., 1999; Ninham & Parsegian, 1970).

An electrostatic interaction is described as an attractive or repulsive force among charged particles, colloids or substrates (Liang *et al*., 2007; Mayer *et al*., 1999). When colloids or particles are suspended in a medium that has a high dielectric constant, such as water, they are usually charged and repel each other (Liang *et al*., 2007). Furthermore, a diffuse double electric layer surrounds these colloids or particles

(Hermansson, 1999). The electrostatic forces present in the diffuse double electric layer often act over a longer range than weak Lifshitz-van der Waals forces (Liang *et al*., 2007). Thus, electrostatic forces among surfaces, which are typically repulsive, often prevail over weak attractive Lifshitz-van der Waals forces (Liang *et al*., 2007). The surfaces of bacteria and substrates they attach to are often charged and thus bacteria and substrates interact with each other electrostatically (Bos *et al*., 1999; Hermansson, 1999; Liang *et al*., 2007).

Acid-base interactions are described as hydrophobic attractive and hydrophillic repulsive forces (van Oss, 1995). These forces derive from the hydrogen-bonding free energy of cohesion of water molecules (van Oss, 1995). Hydrogen bonding is a form of electron-donor/ electron-acceptor and thus Lewis acid-base interaction (van Oss, 1995).

1.4.3) The diffuse double electric layer surrounding bacteria and stainless steel

As thermophilic bacilli and stainless steel typically have an overall negative surface charge when submerged in media, theoretically a diffuse double electric layer surrounds these entities (Hermansson, 1999; Poortinga *et al*., 2002). Positively charged ions are attracted to and associate with the negatively charged surfaces of bacteria and stainless steel and form a 'Stern layer' (Hermansson, 1999). Negatively charged ions associate with and overly the positively charged ions in the Stern layer (Hermansson, 1999). In the region outside the Stern layer, referred to as the 'atmosphere', the concentration of positively charged ions is relatively lower than that which exists in the bulk medium, as the positively charged ions have been attracted into the Stern layer (Hermansson, 1999). Together, the Stern layer and the atmosphere form a diffuse double electric layer (Hermansson, 1999). The atmosphere has an overall negative charge due to the negative charge of the surface (which may not be completely neutralised by positively

charged ions in the Stern layer), the presence of the negatively charged ions overlying the Stern layer, and the relatively low concentration of positively charged ions in the atmosphere (Hermansson, 1999). Outside of the atmosphere charge equilibrium is restored (Hermansson, 1999). Repulsive forces exist among bacteria and stainless steel when the negatively charged double electric layers surrounding them overlap, which is due to repulsive osmotic pressure (Hermansson, 1999). The greater the distance the atmosphere extends from the surface of bacteria and the substrate they attach to, the greater the extent of the repelling forces (Hermansson, 1999).

1.4.4) Effect of ionic strength on the diffuse double electric layer

The distance the atmosphere extends from a charged surface depends on the extent of the charge of the surface, and the ionic strength of the surrounding medium (Hermansson, 1999). As the charge of the surface increases, the extent of the atmosphere increases (Hermansson, 1999). Conversely, as the ionic strength of the surrounding medium increases, the extent of the atmosphere decreases, owing to the sheilding of charge by the ions (Bos *et al*., 1999). Media that have increased ionic strengths have an increased capacity to neutralise negatively charged surfaces (Bos *et al*., 1999; Hermansson, 1999).

It is hypothesized that a repulsive force exists among bacteria and stainless steel which hinders the transition of the bacteria from a planktonic to substrate-attached state and prevents the formation of an aggregation of bacteria as a biofilm (Bos *et al*., 1999; Busscher *et al*., 2008; Hermansson, 1999). The ionic strength of a solution, which is a measure of the concentration of both cations and anions, has the potential to alter diffuse double electric layer forces among entities (Hermansson, 1999). Thus, the ionic strength has the potential to influence attachment and biofilm formation of *Geobacillus* spp. and *A. flavithermus* on stainless steel.

This has been shown for other bacteria. For example, Long *et al*. (2009) and Zhu *et al*. (2009) investigated the influence of ionic strength on the deposition kinetics of four bacterial species on silica in the presence of NaCl and $CaCl_2$ concentrations ranging between 1 – 100 mM. *E. coli* BL21 (Gram negative, non-motile), *Pseudomonas* sp. QG6 (Gram negative, motile), *Rhodococcus* sp. QL2 (Gram positive, non-motile) and *B. subtilis* (Gram positive, motile) were used, both with and without the removal of extracellular polymeric substances. It was found that the deposition efficiency of the bacteria, both with and without extracellular polymeric substances, increased with increasing NaCl and $CaCl_2$ concentrations. This was deemed to be due to the effect that ions have on the compression of the diffuse electric double layer that surrounds the surface of the overall negatively charged bacteria and silica, thus decreasing the extent of electrostatic repulsion among them. These studies demonstrate how an increasing ionic strength can promote bacterial attachment.

By contrast, Van Hoogmoed *et al*. (1997) investigated the effect of the addition of either 0, 1 or 5 mM $CaCl_2$ on the deposition rate of three *S. thermophilus* strains. It was found that strain G1 had the highest initial deposition rate in 1 mM $CaCl_2$. No conclusive trends were found with respect to the influence of calcium concentration on the initial bacterial deposition rate, biofilm growth, bacterial surface zeta potential and bacterial surface hydrophobicity. The authors concluded that it may be essential to add calcium to buffers used to study the adhesion of *S. thermophilus* isolates of dairy origin to stainless steel, however this study does not provide strong evidence for the ability of $CaCl_2$ to enhance bacterial attachment and biofilm formation on stainless steel. This study is an example of where the ionic strength of the solution had a minimal influence on bacterial attachment.

1.4.5) Contradicitons in the application of the extended DLVO theory to predict bacterial attachment

The extended DLVO theory assumes that bacteria are inert colloids that have a uniform, consistent surface (Hermansson, 1999). In reality, the surface of bacteria is irregular, and many surface-exposed bacterial structures, such as proteinaceous fimbriae and polysaccharide polymers, have been shown to have adhesive properties that mediate the transition of bacteria from a planktonic to reversibly attached state via highly specific, stereo-chemical interactions between complementary components on interacting surfaces (Bos *et al*., 1999; Busscher *et al*., 2008; Lopez *et al*., 2010; Petrova & Sauer, 2012). For instance, Kolenbrander *et al*. (1993) detailed the many specific adhesin-receptor interactions that occur among bacteria and between bacteria and the acquired pellicle on a tooth surface in an oral plaque biofilm. Also, since bacteria are living cells they are capable of adapting their physiology in response to stimuli in their immediate environment (Stewart & Franklin, 2008). For example, upon reversible attachment to a surface, it has been found that bacteria increase the production of extracellular matrix, such as extracellular polysaccharides, which increase bonding forces between bacteria and attachment surfaces, and among bacteria aggregated at the surface, which irreversibly attaches the bacteria to the surface (Stoodley *et al*., 2002; Sutherland, 2001a; Vandevivere & Kirchman, 1993). Surface adhesins and extracellular matrix production have the potential to either promote or overcome any attractive or repulsive extended DLVO forces present at the surface of bacteria and substrates (Bos *et al*., 1999; Busscher *et al*., 2008; Hermansson, 1999).

Bos *et al*. (1999) reviewed 250 articles that investigated the influence of forces included in the extended DLVO theory on the process of bacterial attachment to a substrate. They concluded that often the extended DLVO theory could not be used to accurately predict the attachment characteristics of bacteria. The only general

conclusion that could be drawn was that negatively charged bacteria attach more readily to positively charged substrates compared with negatively charged substates. It was proposed that the presence of protein from the bulk-flow of biological systems interferes with extended DLVO forces by associating with the surface of bacteria, and forming conditioning layers on substrates.

Despite the major role that bacterial adhesins have in mediating bacterial attachment, Bos *et al*. (1999) claimed that all specific adhesin-substrate interactions initially derive from physico-chemical forces, such as Lifshitz-van der Waals forces, electrostatic forces, and acid-base forces between the tip of the adhesin the substrate or receptor. Also, it was proposed that physico-chemical forces act over a longer range than specific adhesin interactions, and that both specific adhesin interactions and physico-chemical forces should be taken into account when describing the attachment process of bacteria.

1.4.6) Assimilation of cations into the cell wall of Gram-positive bacteria

In addition to contributing to the ionic stength of the media that surrounds bacteria, cations interact with polymers in the cell wall and extracellular matrix of bacteria (Beveridge & Murray, 1980; Lambert *et al*., 1975a; Neuhaus & Baddiley, 2003). The bacterial cell wall, and the extracellular matrix in bacterial biofilms, have a large capacity to absorb cations from the environment (Beveridge & Murray, 1980; Beveridge *et al*., 1982). Free cations are attracted to and interact with negatively charged functional groups, such as carboxyl and phosphate groups, in a non-specific manner (Fowle & Fein, 1999; Lambert *et al*., 1975a; Neuhaus & Baddiley, 2003; Rose & Hogg, 1995). A wide variety of cation types may be absorbed by bacteria, such as Cd^{2+}, Fe^{2+}, Zn^{2+}, Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} (Beveridge & Murray, 1980; Fowle & Fein, 1999). The interaction of cations with negatively charged functional groups neutralises

the negative charge of both individual polymers and the surface of bacteria (Neuhaus & Baddiley, 2003). This absorption of cations enhances the stability, cohesion and structural integrity of the cytoplasmic membrane, cell wall and extracellular matrix of both planktonic bacteria and bacteria in a biofilm (Dunne & Burd, 1992; Heptinstall *et al*., 1970). Cations translocate along negatively charged functional groups in the cell wall of Gram-positive bacteria towards the cytoplasmic membrane (Hughes *et al*., 1973; Neuhaus & Baddiley, 2003). Cations, such as Ca^{2+} and Mg^{2+}, are stored at the cell wall-cytoplasmic membrane interface, and the absorption of cations by the cell wall is critical in the functioning of bacteria (Hughes *et al*., 1973; Neuhaus & Baddiley, 2003).

For example, Beveridge and Murray (1980) found that the concentration of Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} in the cell wall of *B*. *subtilis* was 2.7, 2.0, 0.4 and 8.2 μmol mg^{-1} of wall, respectively. In addition, Beveridge *et al*. (1982) found that the concentration of Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} in the cell wall of *B*. *licheniformis* was 0.02, 0.01, 0.1 and 0.05 μmol mg^{-1} of wall [dry weight], respectively. The cation concentration in the *B*. *licheniformis* cell wall was found to be much less than that in the *B*. *subtilis* cell wall, and the Ca^{2+} concentration in the *B*. *licheniformis* cell wall was greater than the Mg^{2+} concentration. Furthermore, when 1 mg of *B*. *licheniformis* cell wall was mixed with 2 ml of a 5 mM solution of either Na^{+}, K^{+}, Ca^{2+} or Mg^{2+}, so that the cation binding sites for each ion would be saturated, the cell wall concentrations were found to be 0.9, 0.6, 0.6 and 0.4 μmol mg^{-1} of wall [dry weight], respectively. These studies portray the large cation binding capacity of the cell wall of Gram-positive bacteria, and show that many cation types are assimilated, and that each cation type is assimilated to different extents.

1.4.7) Cation binding affinities to Gram-positive bacterial cell walls

All negatively charged functional groups bind all cation types (Fowle & Fein, 1999). However, the binding affinity of cations to negatively charged functional groups differs

for each cation type (Beveridge & Murray, 1980; Beveridge *et al*., 1982; Fowle & Fein, 1999; Kara *et al*., 2008). Differences among cation types are due to differences in their ionic charge, ion size and the radius of the hydration shell (Fowle & Fein, 1999; Kara *et al*., 2008). Ions with a large size and valency, have a small hydration shell radius, and are able to diffuse through cell wall and extracellular matrix polymers, and readily access their cation binding sites (Kara *et al*., 2008). Furthermore, the type of negatively charged functional group (i.e. carboxyl or phosphate), and the species origin of the polymer that contains the functional group, can influence the binding affinity of cations (Rose *et al*., 1997). Different cation types compete for binding with negatively charged functional groups in the cell wall, and the equilibrium concentration of each cation in the cell wall of bacteria depends on the binding affinity of each cation, and the concentration of each cation surrounding the bacteria (Fowle & Fein, 1999; Lambert *et al*., 1975a; Rose & Hogg, 1995).

The tendency of cations to form covalent bonds and complexes with wastewater sludge polymers, which contain both carboxyl and phosphate groups, follows the order: $Ca^{2+} > Mg^{2+} > K^{+} > Na^{+}$ (Rengasamy & Sumner, 1998). Conversely, data derived from the Protein Data Bank demonstrates that phosphate preferentially binds Mg^{2+} (Broncel *et al*., 2010). It has been shown that the relative energies of formation of a range of model phosphate-metal solvation complexes follow the trend of: $Mg^{2+} > Ca^{2+} > Na^{+} > K^{+}$ for all isostructural complexes (Marynick & Schaefer, 1975). Also, the relative affinities of cations for teichoic acid (which contains phosphate, but no carboxyl groups) were found to follow the order: $Mg^{2+} \geq Ca^{2+} > Na^{+}$ (Pal & Das, 1992).

The reported dissociation constants for the binding of Mg^{2+} to phosphate groups of teichoic acid extracted from *Lactobacillus (L.) buchneri*, *B. subtilis* and *S. sanguis* are 0.37 (Lambert *et al*., 1975a), 1.7 (Heckels *et al*., 1977) and 15 mM (Rose & Hogg, 1995), respectively. The dissociation constant for the binding of Mg^{2+} to carboxyl

groups of teichuronic acid extracted from *B. subtilis* was measured as 3 mM, which implies that the binding affinity of Mg^{2+} to teichuronic acid is slightly lower relative to teichoic acid (Heckels *et al*., 1977). It was concluded that teichuronic and teichoic acid have similar Mg^{2+} binding characteristics (Heckels *et al*., 1977).

Lambert *et al*. (1975a) compared the influence of the addition of 10 mM Na^+, K^+ or Ca^{2+} on the binding affinity of Mg^{2+} (at concentrations between 0.1 – 1.0 mM) to teichoic acid derived from *L. buchneri*. It was found that the binding affinity of Mg^{2+} to teichoic acid greatly decreased in the presence of 10 mM Ca^{2+}, however it remained unaffected by the presence of 10 mM Na^+ or K^+. An added Na^+ concentration of 50 mM was required to decrease the binding affinity of Mg^{2+} to teichoic acid to that observed in the presence of 10 mM Ca^{2+}. Rose *et al*. (1995) extrapolated from the study conducted by Lambert *et al*. (1975a) to conclude that the dissociation constants for the binding of Na^+, K^+, Ca^{2+} and Mg^{2+} to *L. buchneri* teichoic acid were 480, 350, 2.6 and 0.37 mM, respectively. Thus, the binding affinity of Na^+, K^+, Ca^{2+} and Mg^{2+} to teichoic acid extracted from *L. buchneri* followed the order of $Mg^{2+} > Ca^{2+} >> K^+ > Na^+$.

Rose and Hogg (1995) found that the dissociation constants for the binding of Ca^{2+} and Mg^{2+} to lipoteichoic acid extracted from *S. sanguis* were 8.4 and 15.0 mM, respectively. This indicated that Ca^{2+} has a higher binding affinty for lipoteichoic acid extracted from *S. sanguis* than Mg^{2+}, and contrasts results obtained by other researchers who found that Mg^{2+} has a higher binding affinity than Ca^{2+} to polymers that contain phosphate groups. As Mg^{2+} concentrations increased, the binding affinity of Ca^{2+} for *S. sanguis* lipoteichoic acid decreased. As Na^+ and K^+ were estimated to have much lower binding affinities to teichoic acids than Ca^{2+} and Mg^{2+}, it was estimated that at physiologically typical cation concentrations present in plaque fluid (which immerses *S. sanguis* in the oral cavity), K^+ and Na^+ would decrease Ca^{2+} binding to lipoteichoic acid by 3% and < 0.5%, respectively (Rose & Hogg, 1995). Trace metals, such as Zn^{2+},

Cu^{2+}, Pb^{2+}, Cd^{2+}, often have a higher binding affinity to the bacterial cell wall and negatively charged functional groups relative to the macro-elements, Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} (Fowle & Fein, 1999; Pal & Das, 1992; Rose & Hogg, 1995). It was proposed that the influence of trace metals on Ca^{2+} binding to lipoteichoic acid would be insignificant, since trace metals are present at much lower concentrations relative to Ca^{2+} (Rose & Hogg, 1995). It was concluded that Mg^{2+} is the only realistic competitive inhibitor of Ca^{2+} binding to lipoteichoic acid of *S*. *sanguis* in an oral plaque biofilm (Rose & Hogg, 1995).

Overall, it is concluded that the binding affinity of Ca^{2+} and Mg^{2+} to negatively charged functional groups of bacterial cell wall polymers is greater than Na^{+} and K^{+}, and that either Ca^{2+} or Mg^{2+} may have the greatest binding affinity, depending on the species origin of the polymer.

1.4.8) Cation binding capacity of Gram-positive bacterial cell walls

The cation binding capacity of bacteria depends on the number of cation interaction sites, such as carboxyl and phosphate groups, in the cell wall (Burnett *et al*., 2006). As the composition of the cell wall varies greatly and depends on the species and physiological state of bacteria, there is variation in the cation binding capacity when comparing among different bacteria (Beveridge & Murray, 1980; Beveridge & Fyfe, 1985; Beveridge *et al*., 1982; Ginn & Fein, 2008). Furthermore, the type of polymer and functional group that is responsible for assimilating the majority of cations, and the proportion of each cation type assimilated, differs for each bacterial species (Rose *et al*., 1997).

Beveridge and Murray (1980) found that both teichoic acids (which contain phosphate groups) and peptidoglycan (which contain carboxyl groups) bound Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} in the cell wall of *B*. *subtilis*. However the major site of cation

deposition was at the carboxyl groups associated with peptidoglycan. In contrast, Beveridge *et al*. (1982) found that teichoic and teichuronic acid were the prime Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} binding sites in the cell wall of *B*. *licheniformis*. *B*. *subtilis* was found to have a higher cation binding capacity than *B*. *licheniformis*. Also, *B*. *subtilis* assimilated more Mg^{2+} than Ca^{2+}, and, in contrast, *B*. *licheniformis* assimilated more Ca^{2+} than Mg^{2+}.

Dental plaque is largely comprised of oral streptococci, which produce large amounts of lipoteichoic acid and has a high calcium binding capacity (Kolenbrander, 2000; Rose *et al*., 1994). Rose *et al*. (1997) isolated and purified cell wall material (CWM) from six oral streptococci strains and an oral *Actinomyces (Ac.) naeslundii* and *L*. *casei* strain, and further treated the CWM by either masking the carboxylate groups (CM-CWM) or stripping the phosphate groups (PS-CWM). For the streptococci, the calcium binding capacity ranged between 130 and 1500, 90 and 1200, and 30 and 300 μmol Ca^{2+} g^{-1} (dry weight) for CWM, CM-CWM and PS-CWM, respectively. Thus, Ca^{2+} predominantly bound to phosphate groups in oral streptococci CWM. The calcium binding capacity of *Ac*. *naeslundii* and *L*. *casei* was between 160 and 200, 7 and 35, and 130 and 170 μmol Ca^{2+} g^{-1} (dry weight) for CWM, CM-CWM and PS-CWM, respectively. Thus, in contrast to the binding preference of Ca^{2+} in oral streptococci, Ca^{2+} predominantly bound to carboxyl groups, as opposed to phosphate groups, in *Ac*. *naeslundii* and *L*. *casei* CWM. Also, the Ca^{2+} binding capacity of streptococci was greater than that of *Ac*. *naeslundii* and *L*. *casei*. It was assumed that the carboxyl groups were derived from cell wall proteins and peptidoglycan cross-links and the phosphate groups were derived from teichoic and lipoteichoic acid. It was concluded that the Ca^{2+} binding capacity of Gram-positive bacteria is directly proportional to the concentration of phosphate (thus teichoic acids) in the cell wall.

Studies have found that, of the carboxyl, phosphate and amine functional groups present in the cell wall of bacteria, cations preferentially interact with the carboxyl group (Burnett *et al*., 2007; Hetzer *et al*., 2006). Of the three different functional groups, the carboxyl group has the lowest pKa value, and thus has the highest proportion of deprotonated individual groups at any given pH, which allows cations to interact more readily with carboxyl groups (Burnett *et al*., 2007; Hetzer *et al*., 2006). Hetzer *et al*. (2006) obtained results which indicated that the carboxyl group accounted for 66% and 80% of all titratable sites for *G*. *thermocatenulatus* and *G*. *stearothermophilus*, respectively, and was the main Cd^{2+} binding site.

It has been suggested that thermophilic bacteria have a lower cation binding capacity than mesophilic bacteria. This is because thermophiles have a greater abundance of long chain, saturated fatty acids in their membranes (Denich *et al*., 2003), which often extend into the cell wall. Fatty acid polymers have fewer cation binding sites (there is one carboxyl group per saturated fatty acid polymer) relative to other cell wall polymers (Burnett *et al*., 2006).

It is concluded that the cation binding capacity and preferential binding of different types of cations is highly variable among different bacterial species. Cations may be assimilated predominantly by peptidoglycan or teichoic acid, and by carboxyl or phosphate groups, depending on the bacterial species. Also, the preferential assimilation of Ca^{2+} or Mg^{2+} by bacteria depends on the bacterial species. This makes it difficult to predict the cation assimilation characteristics of bacteria. The contribution that any type of cation has in the neutralisation of the negative charge and enhancement of the structural integrity and cohesion of the bacterial cell wall and extracellular matrix of a biofilm is highly dynamic, and depends on the relative binding affinities of each cation type, and the concentration of each cation in the medium which surrounds bacteria.

1.4.9) Divalent cation bridges

Divalent cations have the potential for simultaneous interaction with two different negatively charged functional groups which are in a close proximity in the cell wall or extracellular matrix of a biofilm (Lattner *et al*., 2003; Wickham *et al*., 2009). When a divalent cation simultaneously interacts with two negatively charged functional groups it forms a divalent cation bridge (Sobeck & Higgins, 2002). A divalent cation bridge may form in one polymer, between adjacent polymers, between adjacent bacterial cells or between a bacterial cell and the conditioning film or surface substrate to which it is attached (Sobeck & Higgins, 2002; Wickham *et al*., 2009; Zhu *et al*., 2009). Divalent cations which form divalent cation bridges enhance the stability and cohesion of a biofilm (Sobeck & Higgins, 2002).

The formation of divalent cation bridges by Ca^{2+} in alginate is a classic example of the formation of divalent cation bridges in bacterial polymers (Lattner *et al*., 2003). Alginate is an extracellular polysaccharide produced by *Pseudomonas (P.) aeruginosa* (Lattner *et al*., 2003). Calcium ions specifically bind to and forms divalent cation bridges between the mannuronate-guluronate pairs in alginate forming a gel-like matrix (Lattner *et al*., 2003). In contrast to Ca^{2+}, Mg^{2+} binds relatively weakly and non-specifically to alginate and does not cause the formation of a gel-like matrix (Lattner *et al*., 2003). Thus, Ca^{2+} and Mg^{2+} may influence the extent of cohesion of a matrix of bacterial polymers differentially, depending on the different extents of divalent cation bridge formation of each cation.

Other researchers have indirectly shown that divalent cations form divalent cation bridges in the cell wall of bacteria. Magnesium ions bind to phosphate groups of *B. subtilis* and *L. buchneri* teichoic acid, and carboxyl groups of *B. subtilis* teichuronic acid with at ratio of 0.5:1 (Heckels *et al*., 1977; Lambert *et al*., 1975a). Thus, it was proposed that Mg^{2+} forms divalent cation bridges amongst teichoic acid and teichuronic

acid (Heckels *et al*., 1977; Lambert *et al*., 1975a). In addition, it was predicted that Ca^{2+} forms ionic cross-bridges between anionic groups in cell walls of *S*. *downei* and *S*. *sanguis* (Rose *et al*., 1994). This is because the dissociation constant for bidentate chelation (which indicates the potential to form divalent cross bridges) by dicarboxylic acids is below 2 mM, and it was observed that the dissociation constant for binding between Ca^{2+} and *S*. *downei* and *S*. *sanguis* whole cells was also less than 2 mM (Rose *et al*., 1994).

Often researchers conclude that the reason for greater enhancement of bacterial attachment or biofilm formation by a divalent cation relative to an equivalent concentration of a monovalent cation is due to the fact that the divalent cation forms divalent cation bridges. For example, Zhu *et al*. (2009) concluded that a reason for increased deposition efficiency of bacterial extracellular polymeric substances to a silica substrate in the presence of Ca^{2+} relative to an equimolar concentration of Na^{+}, was due to the formation of divalent cation bridges between the extracellular polymeric substances and silica by Ca^{2+}, but not Na^{+}. Similarly, Dunne and Burd (1992) proposed that reasons for the enhanced adhesion of *Staphylococcus (St.) epidermidis* to polystyrene in the presence of Ca^{2+} and Mg^{2+} was due to the stimulation of cell-to-cell aggregation through the shared binding of Mg^{2+} by cell wall teichoic acids, as shown by Lambert *et al*. (1975a, b), and that both Ca^{2+} and Mg^{2+} are known to polymerise bacterial exopolysaccharides into a gel-like matrix, thus enhancing cell-to-cell and cell-to-surface adhesion. However, in these instances, the proposition that divalent cations had formed divalent cation bridges is anecdotal, where evidence is referred to other studies, rather than proving their occurrence in the current study. There may be alternative reasons for observed increases in the promotion of attachment and biofilm formation of bacteria by divalent, relative to monovalent cations. For instance, divalent cations may have a greater capacity to compress the diffuse double electric layer. Or

divalent cations may have a greater capacity to neutralise the negative charge of cell wall and extracellular matrix polymers when they interact with functional groups in the polymers. Although it has been shown that Ca^{2+} specifically forms divalent cation bridges with mannuronate-guluronate pairs in alginate (Lattner *et al*., 2003), most bacteria do not produce alginate, or polymers which contain mannuronate-guluronate pairs. Thus, it is unreasonable to conclude that divalent cations form divalent cation bridges in bacterial polymers, unless the molecular mechanism has been proven.

Wickham *et al*. (2009) contradicted Lambert and co-workers, since they found, with the combination of phosphorus-31 solid state NMR spectroscopy and theoretical calculations using density functional theory, that the bond between magnesium and phosphate groups in teichoic acid involves bidentate coordination. Thus, it was proposed that Mg^{2+} is not able to bind to other anions or form ionic cross-bridges with other polymers when it is bound to phosphate groups in teichoic acid. Perhaps the observation that Mg^{2+} bind to phosphate groups of teichoic acids at a ratio of 0.5:1 (Lambert *et al*., 1975b) is due to a factor other than that the fact that divalent cations have formed divalent cation bridges in the polymers.

1.4.10) Monovalent to divalent cation ratio

Bioflocculation of a wastewater sludge is critical for the effective separation of wastewater solid and liquid phases and wastewater treatment (Higgins *et al*., 2004). It has been shown that the extent of bioflocculation increases with increasing Ca^{2+} or Mg^{2+} concentrations. However, increasing concentrations of Na^{+}, and to a lesser degree K^{+}, cause increasing disintegration of sludges (Higgins *et al*., 2004; Kara *et al*., 2008; Sobeck & Higgins, 2002). However Na^{+} and K^{+} are required for optimal bioflocculation, as it has been shown that bioflocculation optimally occurs at monovalent to divalent ratios of approximately 2:1 (Higgins *et al*., 2004; Sobeck &

Higgins, 2002). Monovalent to divalent cation ratios of 10:1 or above greatly compromise bioflocculation (Higgins *et al*., 2004; Sobeck & Higgins, 2002). It was proposed that Ca^{2+} and Mg^{2+} form divalent cation bridges in sludges, and since Na^{+} and K^{+} are not capable of forming divalent cation bridges, divalent cations are more efficacious than monovalent cations at promoting sludge bioflocculation (Higgins *et al*., 2004; Sobeck & Higgins, 2002). As the concentration of monovalent cations increases, the monovalent cations increasingly displace divalent cations from cation interaction sites in the sludge, which is increasingly detrimental to the performance of the sludge (Higgins *et al*., 2004; Sobeck & Higgins, 2002).

Sobeck and Higgins (2002) considered three theories: the alginate theory; DLVO theory; and divalent cation bridging theory, to evaluate the underlying reason for cation-induced bioflocculation. The alginate theory states that Ca^{2+}, but not Mg^{2+}, enhances biofilm cohesion through the formation of Ca^{2+} divalent cation bridges. Since Ca^{2+} and Mg^{2+} similarly enhance sludge bioflocculation, this contradicts the alginate theory. The DLVO theory states that an increasing ionic strength (and thus an increasing concentration of all ionic types) compresses the diffuse double electric layer surrounding charged particles, which subsequently decreases the extent of electrostatic repulsive forces among particles of the same (positive or negative) charge. The observation that increasing concentrations of the divalent cations (Ca^{2+} and Mg^{2+}) enhances bioflocculation and increasing concentrations of the monovalent cation (Na^{+} and K^{+}) causes flocs to disintegrate, contradicts the DLVO theory. It was concluded that the divalent cation bridging theory best explains the role of cations in bioflocculation. However it was not proven that the divalent cations form divalent cation bridges in wastewater sludges, only that a reduction in the monovalent to divalent cation ratio to closer to 2:1, improves floc properties and wastewater treatment performance. The observed increase in bioflocculation by divalent cations relative to

monovalent cations may be due to differences in charge and binding affinity, where the divalent cations have a greater capacity to remain bound to and neutralise the negative charge of bacterial cell wall and extracellular matrix polymers in sludges.

1.4.11) Overall conclusions for the effect of cations on electrostatic interactions in biofilms

- Cations have the potential to influence the electrostatic properties of bacteria and biofilms during biofilm formation either by contributing to the ionic strength of media surrounding biofilms, or by interacting with negatively charged functional groups in the cell wall and extracellular matrix.
- The ionic strength of media surrounding bacteria and biofilms may influence the extent of the electric double layer surrounding bacteria and stainless steel and thereby influence the extent of repulsive forces among them.
- Cations may interact with and neutralise negatively charged functional groups in a bacterial biofilm, thereby enhancing the structural integrity and cohesion of a biofilm.
- Divalent cations, such as Ca^{2+} and Mg^{2+}, have a greater capacity to enhance the cohesion of a biofilm relative to monovalent cations, such as Na^{+} and K^{+}, owing to their higher charge, higher binding affinity and potential to form divalent cation bridges.
- Cations compete for occupancy in the cell wall and extracellular matrix of bacteria, and relatively high concentrations of monovalent cations can out-compete and replace divalent cations at negatively charged functional groups, and consequently compromise the structural integrity and cohesion of a biofilm.

1.5) Physiological responses of bacteria in a biofilm to sodium, potassium, calcium and magnesium ions

Na^+, K^+, Ca^{2+} and Mg^{2+} have the potential to influence the physiology of bacteria in a biofilm. Bacteria, like all living cells, have evolved to adapt their physiology according to changing conditions in their environment, which is vital for their survival. Bacteria have molecular mechanisms that recognise, monitor and respond to a wide range of molecules acting as environmental stimuli, enabling them to adapt to changing conditions in their environment. Cations act as stimuli and influence bacterial physiological factors including the tertiary structure of polymers, signal transduction pathways and gene regulation, intracellular and extracellular protein expression, and the composition and amounts of structures and polymers that comprise the cell wall and extracellular matrix. Often physiological adaptations of bacteria in response to cations, particularly those associated with the cell wall and extracellular matrix, result in changes in physico-chemical forces among polymers in the cell wall and extracellular matrix. Thus, in addition to the direct influence of Na^+, K^+, Ca^{2+} and Mg^{2+} on physico-chemical forces in a biofilm, as described in the electrostatic interactions section above, cations also influence the physiology of bacteria in a biofilm, which consequently influences physico-chemical forces in an indirect manner (Cruz *et al*., 2012; Kara *et al*., 2008; Song & Leff, 2006). Like the direct electrostatic effect of cations on physico-chemical forces in a biofilm, indirect physiological effects also influence the structural integrity, cohesion and prosperity of a biofilm.

1.5.1) The influence of calcium ions on the conformation and function of bacterial polymers

It has been proposed that many bacterial proteins are calmodulin-like proteins which contain ‘EF’ hand motifs which specifically bind calcium ions, and upon binding

undergo folding and a conformational change, consequently affecting the stability, enzymatic function or the regulatory or buffering role of the protein (Michiels *et al*., 2002). Calmodulin-like proteins typically have multiple 'EF' hand motifs that may exist as pairs linked together by a flexible central tether (Michiels *et al*., 2002). 'EF' motifs comprise Ca^{2+}-binding loops consisting of a consensus sequence of amino acids which bind to Ca^{2+} via negatively charged regions of carboxyl groups, main-chain oxygen atoms or indirectly via water molecules (Michiels *et al*., 2002). The globular structure of 'EF' hand motifs allows for co-operative binding of Ca^{2+} with a high binding affinity (Michiels *et al*., 2002).

The first bacterial protein identified as having an 'EF' hand motif was the *Saccharopolyspora (Sa.) erythrea* protein, calerythrin (Michiels *et al*., 2002). Recently, many other 'EF' hand motifs have been identified in bacteria. For example, Yonekawa *et al*. (2005) identified that the *cadB* gene in *Streptomyces (Str.) coelicolor* A3(2) encodes for a calmodulin-like protein that contains two 'EF' hand motifs, and upon Ca^{2+} binding, the α-helix content of CadB increases. It was suggested that CadB has a role in calcium homeostasis, where it acts as a calcium buffer or transporter and is common in actinomycetes. Additionally, Dobson and O'Shea (2008) showed how Ca^{2+} increased the hydrophobicity of *Str. hygroscopius* var. *geldanus* and caused cell concentration-dependant aggregation, while in response to Mg^{2+} the bacteria were hydrophilic and grew as freely dispersed filaments. It was proposed that Ca^{2+} bound to an extracellular calmodulin-like protein that contained 'EF' hand motifs, which, upon Ca^{2+}-binding, underwent a conformational change, consequently exposing hydrophobic regions and eliciting hydrophobic attractive forces among cells. Although Mg^{2+} may weakly bind to calcium 'EF' binding motifs, calmodulin-like proteins will not elicit the same conformational change upon Mg^{2+} binding. This example demonstrates how calcium ion-calmodulin-like protein interactions can influence the morphology, and physiology

of the surface of bacterial cells which may consequently influence the cohesion and structural integrity of a biofilm.

Bacterial proteins may also have calcium binding motifs that are different from 'EF' hand motifs, but also undergo functional changes upon Ca^{2+} binding and consequently alter the physiology of bacteria. Economou *et al*. (1990) proposed that the mechanism involved in Ca^{2+} dependant attachment of *Rhizobium (R.) leguminosarum* cells to plant roots is via the binding of Ca^{2+} to the extracellular NodO protein. NodO contains multiple tandem repeats of 9 amino acids rich in the negatively charged, carboxyl group containing, glycine and aspartic acid residues, which do not resemble 'EF' hand motifs (Martinez-Gil *et al*., 2012). Similarly, Martinez-Gil *et al*. (2012) found that Ca^{2+} promoted biofilm formation by *P*. *putida*. It was found that Ca^{2+} binds to the extracellular protein LapF, which contains putative Ca^{2+} binding sites in its C terminal region and which resemble the Ca^{2+} binding sites of the *R*. *leguminosarum* NodO protein. It was proposed that the Ca^{2+} induced promotion of biofilm formation was partly due to the binding of Ca^{2+} to LapF, which causes LapF proteins to aggregate and polymerise the biofilm matrix, enhancing its structural integrity.

Some extracellular Ca^{2+}-binding proteins are enzymes that require bound Ca^{2+} in order to function optimally. For example, Razak *et al*. (1994) found that the enzyme activity of an extracellular *B*. *stearothermophilus* protease was enhanced in the presence of $CaCl_2$ at a concentration of 2 mM. Nardini *et al*. (2000) found that the His 251 loop of a *P*. *aeruginosa* lipase is stablized by an octahedrally coordinated calcium ion. Snijder and Dijkstra (2000) found that the *E*. *coli* outer membrane protein, Phospholipase A, uniquely binds Ca^{2+} with the carboxyl group of two aspartate amino acids, and after dimerisation, a second Ca^{2+} binding site is formed at the dimer interface in the active site of the protein. Eijsink *et al*. (2011) hypothesized that the binding of calcium ions to a *B*. *stearothermophilus* thermolysin-like protease functions to both

stabilize the protein and also modulate the switch between an unstable and stable state, thereby regulating its activity. Morales and Dehority (2009) found that Ca^{2+} stimulated rumen cellulolytic bacteria to degrade cellulose. It was proposed that the Ca^{2+} acted by binding to bacterial cellulosome structures, which have amino acid sequences that bind Ca^{2+}, improving the stability of the structures and enzyme activity of the cellulases.

Influences of cations on the conformation of bacterial polymers is not restricted to proteins, as it has been proposed that calcium ions also have important roles in maintaining the tertiary structure of bacterial polysaccharides, such as the formation of a gel-like matrix by alginate upon binding with Ca^{2+}, as already explained in this review. Turakhia *et al*. (1983) found that upon the addition of the Ca^{2+} specific chelant, ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid (EGTA) to wastewater overlying a wastewater sludge, the free Ca^{2+} concentration of the wastewaster decreased from 0.6 mM to 0.1 μM, and a major portion of the sludge had dispersed after 5 min. It was proposed that calcium ions acted by maintaining the tertiary structure of carbohydrate polymers in the sludge, and promoted interactions between adjacent polymers thereby enhancing the cohesion of the sludge. In this study cross-linking (or the formation of divalent cation bridges) and charge screening of negatively charged functional groups in the sludge by Ca^{2+} was ruled out. Thus, this is an example where the physiological influence of calcium ions on bacterial polymers is more important than the electrostatic effect when determining the structural integrity and cohesion of a biofilm.

1.5.2) Calcium ion mediated regulation of bacterial signal transduction pathways

Bacteria have adapted to regulate gene expression according to concentrations of external stimuli using signal transduction pathways (Stewart & Franklin, 2008; Stoodley *et al*., 2002). Signal transduction pathways typically comprise a response regulator

protein that spans the cytoplasmic membrane and regulates the pathway depending on the presence of a bound stimulus acting as a ligand (He *et al*., 2008). It has been hypothesized that many bacterial response regulators 'sense' and are influenced by external calcium ion concentrations, as has been shown in eukaryotic cells (Dominguez, 2004; Michiels *et al*., 2002; Norris *et al*., 1996). Some bacterial response regulators have been shown to have Ca^{2+} binding motifs that are regulated by extracellular Ca^{2+} concentrations. For example, He *et al*. (2008) discovered a response regulator named CiaX that contains a serine and aspartate domain which, upon Ca^{2+} binding, activates the ciaXRH operon. This operon promotes biofilm formation by *S*. *mutans*, thus this is an example of calcium mediated regulation of biofilm formation in bacteria via signal transduction.

Calcium ions have been shown to influence bacterial gene transcription. For example, Oomes *et al*. (2009) conducted a micro-array study to analyse the influence of increasing the external Ca^{2+} concentration from 0.14 to 1.38 mM on the transcriptome of sporulating *B*. *subtilis*. It was found that 305 genes were differentially expressed, and that 10 spore coat polysaccharide biosynthesis genes were induced, while one was repressed, and genes involved in biofilm formation were affected.

Calcium ions have also been shown to influence intracellular protein expression. Patrauchan *et al*. (2005) used two-dimensional gel electrophoresis to compare the influence of extracellular Ca^{2+} concentrations of 0.25 with 10 mM on intracellular proteome expression by a *Pseudoalteromonas* sp. grown as a biofilm. Patrauchan *et al*. (2005) reported that, out of a total of approximately 800 proteins, protein spot intensities of 159 proteins were at least 2 fold greater when the bacteria were grown in the presence of 10 mM Ca^{2+} relative to 0.25 mM Ca^{2+}. Of the 159 upregulated proteins, 88 were expressed only in the presence of 10 mM Ca^{2+}. This study demonstrated how elevated external Ca^{2+} concentrations have the potential to globally influence

intracellular protein expression of bacteria. It would be interesting to determine the molecular mechanisms used to sense external Ca^{2+} concentrations, and the molecular mechanisms which elicit global differences in both transcriptome expression, as demonstrated in *B. subtilis* by Oomes *et al*. (2009), and proteome expression, as demonstrated in *Pseudoalteromonas* sp. by Patrauchan *et al*. (2005), in bacteria.

1.5.3) Influences of cations on cell wall and extracellular matrix amounts and composition and its effect on biofilm formation

Early studies reported how Ca^{2+} stimulated *Chromobacterium violaceum* (Corpe, 1964) and Mg^{2+} stimulated *Aerobacter aerogenes* (Tempest *et al*., 1965) to produce exopolysaccharide. Later, Akpolat *et al*. (2003) found that increasing Ca^{2+} or Mg^{2+} concentrations increased the production of a mucoid, extracellular substance, designated as slime, by *St. epidermidis*, while it was attached to plastic The extent of slime production increased with increasing Ca^{2+} or Mg^{2+} concentrations (8 – 256 μM), particularly by the slime-positive strains tested. Slime production is a clinically important virulence factor for coagulase negative staphylococci (Akpolat *et al*., 2003), and the extracellular matrix of bacteria in a biofilm has several functions, which include aiding initial attachment, enhancing the stability of biofilms and providing a protective barrier against anti-bacterial agents (Flemming & Wingender, 2010). In addition, Patrauchan *et al*. (2005) found that as the concentration of supplemented Ca^{2+} varied from 0.25, 1.0, 5.0 and 10 mM, the amount of exopolysaccharide produced by a *Pseudoalteromonas* sp. incrementally increased with increasing Ca^{2+} concentrations.

Liu and Sun (2011) showed how extracellular protein production by bacteria in a wastewater sludge incrementally increased as the supplemented Ca^{2+} concentration was increased from 0, 50 and 100 mg l^{-1} (which corresponds to 0, 1.2 and 2.5 mM, respectively). The amount of extracellular polysaccharide in the sludge remained

unchanged as the Ca^{2+} concentration was increased. Similarly, Goode and Allen (2011) found that wastewater sludge biofilms became increasingly proteinacious as the Ca^{2+} concentration was increased up to 200 mg l^{-1} (5 mM). The amounts and ratios of polysaccharide and protein can vary in the extracellular matrix of a biofilm and mainly depend on the composition of bacterial species that comprise the biofilm, and the compositon of the available nutrients (Durmaz & Sanin, 2001; Subramanian *et al*., 2010). For example, the ratio of carbon to nitrogen in molecules, utilized by bacteria as an energy source and for the biosynthesis of polymers, can influence the proportion of polysaccharides to proteins that comprise the extracellular matrix of bacteria (Corpe, 1964; Durmaz & Sanin, 2001; Kumar *et al*., 2007). An increase in the available carbon elicits an increase in extracellular polysaccharide, and an increase in available nitrogen elicits an increase in extracellular protein (Corpe, 1964; Durmaz & Sanin, 2001; Kumar *et al*., 2007). Although extracellular polysaccharides mainly have a structural role in the biofilm matrix, extracellular proteins can have either a structural role or act as enzymes (Flemming & Wingender, 2010). An increase in the amount of protein in a biofilm matrix may indicate an increase in the metabolic diversity and capabilities of the bacteria that dwell in the biofilm (Karunakaran *et al*., 2011), and it is interesting how fluctuations in Ca^{2+} and Mg^{2+} concentrations, at extents that typically occur in environments where biofilms exist, have the potential to greatly influence the amount of extracellular protein produced by bacteria.

In addition to influencing the amount of extracellular matrix produced by bacteria, cations have been shown to influence the expression of specific surface-exposed polysaccharides and proteins, and alter the composition and physiology of the cell wall and extracellular matrix. Patrauchan *et al*. (2005) compared the influence of 0.25 and 10 mM Ca^{2+} on the expression of extracellular proteins of a *Pseudoalteromonas* sp. grown as biofilm. It was found that increasing the Ca^{2+} concentration decreased the

expression of flagellin and increased the expression of at least four unidentified extracellular proteins. As flagella are typically only required by planktonic bacteria, these findings indicated that the *Pseudoaltermonas* sp. responded to the higher Ca^{2+} concentration by adapting its physiology for a biofilm mode of living.

Garrison-Schilling *et al*. (2011) showed how the addition of 1 mM Ca^{2+} to media significantly increased the phase-shifting of *V*. *vulnificus* from the unencapsulated colony morphology to the encapsulated, with capsular polysaccharide or rugose extracellular polysaccharide, morphologies. Capsular polysaccharide increases the virulence, and rugose extracellular polysaccharide enhances the biofilm-forming capability of *V*. *vulnificus*. In addition to influencing extracellular polysaccharide production, Ca^{2+} was also shown to increase biofilm formation by all three morphologies, where it was proposed to act as a structural component of the biofilms, presumably through the formation of divalent cation bridges. *V*. *vulnificus* inhabits coastal marine and estuarine regions where seawater and freshwater often mix, both of which have contrasting Ca^{2+} concentrations. The Ca^{2+} concentrations in seawater and river water are approximately 10 and 0.3 mM, respectively. It was proposed that phase-shifting in response to external Ca^{2+} concentrations is a mechanism that *V*. *vulnificus* uses to quickly adapt to changeable conditions in estuaries. It may also be postulated that *V*. *vulnificus* expresses the rugose extracellular polysaccharide only when sufficient Ca^{2+} is present to structurally reinforce the biofilm mode of living that rugose promotes.

Cruz *et al*. (2012) found that the addition of 2 mM Ca^{2+} enhanced the force of adhesion to the substrate, biofilm thickness, cell-to-cell aggregation and twitching motility of *Xylella fastidiosa*. It was proposed that Ca^{2+} acted both by forming extracellular divalent cation bridges and by upregulating protein expression. Also, it was shown that a type I pilus had an important role in attachment and cell-to-cell aggregation, and that attachment via the pilus was mediated by Ca^{2+}. Results indicated

that Ca^{2+} mediated pilus attachment both by enhancing physico-chemical forces between pili and the substrate, and by upregulating the expression of the pili.

Although in most instances Ca^{2+} tends to play a role in stimulating attachment and biofilm formation by bacteria, there are a few examples where Ca^{2+} has been implicated in the disruption of biofilm formation. Arrizubieta *et al*. (2004) found that Ca^{2+} inhibited intercellular adhesion and biofilm formation by a Bap (biofilm-associated protein) positive *St*. *aureus* strain. It was shown that the binding of Ca^{2+} to the 'EF' hand motif of the Bap was responsible for biofilm formation by the bacteria. It was proposed that the observed response of *St*. *aureus* to Ca^{2+} at concentrations that typically exist in milk is relevant to the pathogenesis and epidemiology of the bacteria in the mastitis process. In addition, Boyd *et al*. (2012) showed that LapG is a Ca^{2+}-dependant periplasmic cysteine protease of *P*. *fluorescens* which, upon Ca^{2+}-binding, cleaves the adhesin, LapA, resulting in the loss of the ability of the bacteria to form a biofilm. It was proposed that in this instance, Ca^{2+} promotes biofilm dispersal of *P*. *fluorescens*.

Song and Leff (2006) found that as Mg^{2+} concentrations increased (0, 0.1 and 1.0 mM) attachment and biofilm formation of *P*. *fluorescens* was promoted. Both the number of attached cells and thickness of the biofilm increased with increasing Mg^{2+} concentration. It was proposed that Mg^{2+} may have acted both by decreasing the repulsive force between the negatively charged bacterial and substratum surfaces, and by stimulating the production of surface-exposed structures such as exopolysaccharide, flagella and fimbriae.

In response to elevated Na^{+} concentrations, Gram-positive bacteria have the ability to increase the quantity of teichoic acid in the cell wall and decrease the extent of incorporation of D-alanine in teichoic acid, thereby increasing surface negativity and assimilation of divalent cations (Neuhaus & Baddiley, 2003). It was shown that the

response of *B. subtilis* to an increase in the extracellular NaCl concentration was to increase the amount of teichoic acid in the cell wall and thereby maintain an adequate supply of Mg^{2+} (Ellwood, 1971; Meers & Tempest, 1970). Also, it has been noted that bacteria that are able to tolerate moderate conditions of NaCl and other salts are relatively rich in teichoic acid (Archibald *et al*., 1961). Furthermore, it has been proposed that the positively charged amine group (NH_3^+) in D-alanine, which is incorporated in teichoic acid, can form intramolecular contact ion pairs with phosphate groups, which partially neutralises the teichoic acid negative charge, and has a profound inhibitory effect on the binding capacity of Mg^{2+} ions by teichoic acid (Archibald *et al*., 1973). For example, it was shown that the D-alanine content decreased from 0.6 to 0.2 moles per mole of phosphate in the cell wall of *St. aureus*, when it was grown in a 7.5% NaCl solution, and the amount of Mg^{2+} in the cell wall increased from 0.28 to 0.51 equivalents of Mg^{2+} per mole of phosphate, respectively (Heptinstall *et al*., 1970). Lambert *et al*. (1975b), showed that at low concentrations of Mg^{2+} (0.1 – 1.0 mM) the binding capacity of Mg^{2+} to teichoic acid phosphate groups markedly increased upon the removal of D-alanine in teichoic acid, however, the binding affinity as expressed by the dissociation constant was unaffected. It was suggested that D-alanine prevents the binding of Mg^{2+} to teichoic acid phosphate groups (Lambert *et al*., 1975b). These studies indicate that high external Na^+ concentrations inhibit the assimilation of Mg^{2+} into the cell wall of bacteria, and that bacteria counteract the high Na^+ concentrations by increasing the amount of teichoic acid or decreasing the incorporation of D-alanine in the teichoic acid. It could be hypothesized that bacteria have molecular mechanisms that sense the high Na^+ concentrations in order to elicit the physiological adaptations. Alternatively, the bacteria may elicit the physiological responses by sensing low Ca^{2+} or Mg^{2+} concentrations in the cell wall and cell wall-cytoplasmic membrane interface, which occur in high Na^+ concentrations, as high Na^+ concentrations have been shown to

inhibit the assimilation of Ca^{2+} and Mg^{2+} into the cell wall of bacteria (Lambert *et al*., 1975a).

Kara *et al*. (2008) showed how the physiological response of bacteria to elevated Na^{+}, and to a lesser extent, elevated K^{+}, was to increase the amount of negatively charged, hydrophillic polymers in the extracellular matrix of a wastewater sludge, compromising the structural integrity and cohesion of the sludge. Results from this study indicate that the detrimental effect of a high monovalent to divalent cation ratio on the cohesion of a wastewater sludge is predominantly due to the physiological response of the bacteria to the cations, rather than a decrease in the number of divalent cation bridges among the polymers of the sludge, as proposed by Higgins and co-workers (Higgins & Novak, 1997; Higgins *et al*., 2004; Sobeck & Higgins, 2002).

1.5.4) Comparison of the effect of monovalent and divalent cations on physiological responses of bacteria in biofilms

Physiological responses of bacteria to divalent cations tend to enhance physico-chemical forces in extracellular polymers consequently enhancing biofilm formation. In contrast, bacterial responses to monovalent cations have the opposite effect. Given that divalent cations have a greater capacity to increase the structural integrity of a biofilm relative to monovalent cations, perhaps bacteria have evolved to respond physiologically to divalent cations in such a way as to promote biofilm formation. Conversely, their response to monovalent cations would promote biofilm dissemination, so that biofilm formation is induced in favourable conditions and delayed in unfavourable conditions. Studies conducted by Garrisson-Schilling *et al*. (2011), Cruz *et al*. (2012) and Song and Leff (2006) indicate that Ca^{2+} and Mg^{2+} act to enhance biofilm formation of bacteria, both by directly interacting with surface-exposed polymers and promoting attachment and biofilm formation, and by stimulating

physiological responses that indirectly promote biofilm formation. Conversely, studies conducted by Higgins *et al*. (1997) and Kara *et al*. (2008) indicate that relatively high Na^+ and K^+ concentrations decrease the cohesion of a wastewater sludge biofilm by displacing Ca^{2+} and Mg^{2+} from the matrix. The wastewater sludge bacteria increased the amount of negatively charged, hydrophillic polymers in the extracellular matrix, indirectly decreasing the cohesion of the biofilm. These studies are examples where the indirect physiological response of the bacteria to cations complements the direct influence of the ion on electrostatic properties of the surface of bacteria in a biofilm.

Now that the three proposed mechanisms of the effects of Na^+, K^+, Ca^{2+} and Mg^{2+} on bacteria during the biofilm formation process have been discussed, the composition of bovine milk and specialty milk formulations will be detailed. When considering the effect that cations in milk formulations have on biofilm formation by *Geobacillus* spp. and *A*. *flavithermus* during the manufacture of milk powder, it is important to consider both the quantity and form of the minerals and ions in bovine milk and specialty milk formulations. Other components of milk formulations, such as proteins, fats and lactose, may influence the biofilm formation of bacteria, thus it is also important to understand the composition of these constituents in milk formulations.

1.6) Properties of milk

1.6.1) Milk composition

Bovine milk comprises fats, protein, lactose and minerals (Table 1.1) (Bylund, 1995). An average of 39 g l^{-1} of milk is fat, of which, ~98% is composed of triglycerides, ~1% is composed of phospholipids and there are small amounts of diglycerides, monoglycerides, cholesterol, cholesterol esters and traces of fat-soluble vitamins (Banks & Dalgleish, 1990; Bylund, 1995; Fox, 2003). Milk contains an average of 34 g l^{-1}

protein which consists of ~80% casein proteins, namely: α_{s2}, α_{s1}, β and κ casein; and 20% whey proteins, such as β-lactoglobulin, α-lactalbumin and immunoglobulins (Banks & Dalgleish, 1990; Bylund, 1995; Fox, 2003). Milk contains ~ 48 g l^{-1} lactose (Banks & Dalgleish, 1990; Bylund, 1995; Fox, 2003).

The fat, protein and lactose amounts in the commercial, specialty milk formulations used in this study are detailed in Table 1.2. Relative to unprocessed (raw) milk, the fat and lactose concentrations in milk formulations 1 – 4 (reconstituted at 10 g 90 ml^{-1}) are markedly lower, and the protein concentrations are approximately 2 fold higher (Tables 1.1 and 1.2).

TABLE 1.1 Quantitative composition of bovine milk (Bylund, 1995)

Main Constituent	Limits of variation	Mean value
Water (ml l^{-1})	855 – 895	875
Total solids (g l^{-1})	105 – 145	130
Fat	25 – 60	39
Proteins	29 – 50	34
Lactose	36 – 50	48
Minerals	6 – 9	8

TABLE 1.2 Fat, protein, and lactose concentrations (g l^{-1}) in milk formulations (MF) 1–4 (reconstituted at 10 g 90 ml^{-1})

	MF 1	MF 2	MF 3	MF 4
Fat	1.6	1.5	3.5	1.7
Proteins	81.3	81.7	81.5	81.7
Lactose	4.6	3.9	2.2	3.9

1.6.2) Milk minerals

Inorganic salts make up 7 g l^{-1} of bovine milk (Banks & Dalgleish, 1990; Bylund, 1995; Fox, 2003). Mineral amounts in milk can fluctuate depending on the breed of cow, genetic factors, mastitis, diet, season and stage of lactation (Banks & Dalgleish, 1990; Bylund, 1995; Fox, 2003; Holt, 1985). Tables 1.3 and 1.4 detail the mean amounts and forms of minerals in unprocessed bovine milk. Table 1.5 details the mean total sodium, potassium, calcium and magnesium amounts in the specialty milk formulations used in this study, including those supplemented with either sodium, calcium or magnesium chloride.

TABLE 1.3 Macro- and micro-element mean and range amounts (per liter) in bovine raw milk (Fox, 2003)

Constituent	Mean	Range
Sodium (mg)	500	350-900
Potassium (mg)	1500	1100-1700
Chloride (mg)	950	900-1100
Calcium (mg)	1200	1100-1300
Magnesium (mg)	120	90-140
Phosphorus (mg)	950	900-1000
Iron (μg)	500	300-600
Zinc (μg)	3500	2000-6000
Copper (μg)	200	100-600
Manganese(μg)	30	20-50
Iodine (μg)	260	-
Fluoride (μg)	-	30-220
Selenium (μg)	-	5-67
Cobalt (μg)	1	0.5-1.3
Chromium (μg)	10	8-13
Molybdenum (μg)	73	18-120
Nickel (μg)	25	0-50
Silicon (μg)	2600	750-7000

TABLE 1.4 Macro-element amounts and forms in bovine raw milk (Fox, 2003)

Constituent	Concentration	Soluble		Colloidal
	(mg l^{-1})	%	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	67	Probably similar to calcium	33

TABLE 1.5 Total (sum of bound and free) cation concentrations of milk formulations (MF) 1 – 4 (reconstituted at 10 g 90 ml^{-1}), including those supplemented with cation chlorides

Milk formulation and cation supplementation	Na^+ (mM)	K^+ (mM)	Ca^{2+} (mM)	Mg^{2+} (mM)
MF 1	6	10	53	4
MF 2	45	6	35	3
MF 3	69	2	20	2
MF 4	101	2	7	1
MF 2 + 50 mM NaCl	95	6	35	3
MF 2 + 100 mM NaCl	145	6	35	3
MF 4 + 2 mM $CaCl_2$	101	2	9	1
MF 4 + 2 mM $MgCl_2$	101	2	7	3

1.6.3) Mineral interactions in milk

Milk minerals form an array of complexes in milk (Holt, 1985). Cations, such as sodium and potassium, but predominantly calcium and magnesium, associate with anions, such as phosphate and citrate (Fox, 2003; Gaucheron, 2005; Holt, 1985). Also, calcium and magnesium have a relatively high binding affinity with milk proteins, and play an important role in the structural integrity of the casein micelle (Fox, 2003; Gaucheron, 2005; Holt, 1985). The partitioning of minerals or ion equilibria between the bound and free (ionized) form in milk is both dynamic and complex (Fox, 2003; Gaucheron, 2005; Holt, 1985). Processing factors, such as temperature, solute concentration and the use of additives, influence the partitioning of salts and ion equilibria in milk (Fox, 2003; Gaucheron, 2005; Holt, 1985). It is proposed that it is the free Na^+, K^+, Ca^{2+} and Mg^{2+} fraction of milk formulations that have the greatest potential to influence *Geobacillus* sp. and *A. flavithermus* biofilm formation. Thus, the concentration of free Na^+, K^+, Ca^{2+} and Mg^{2+} in milk formulations 1 – 4 are of particular interest in this study.

As outlined in Table 1.4, the majority of calcium and magnesium in bovine milk is bound to caseins, citrate and phosphate, whereas the majority, being 92%, of sodium and potassium exists as soluble, free ions (Fox, 2003). The mean concentration of total calcium and magnesium in milk is 1200 mg l^{-1} and 120 mg l^{-1} (Fox, 2003), which corresponds to approximately 30 mM and 5 mM. The concentration of free Ca^{2+} and Mg^{2+} in milk is approximately 1-4 mM and 0.4-1.3 mM, respectively (Fox, 2003; Tessier & Rose, 1958). Magnesium salts in milk probably have a similar solubility to the corresponding calcium salt. Thus, as free Ca^{2+} concentrations rise and fall in milk, free Mg^{2+} concentrations proportionally rise and fall (Holt, 1985).

The majority of bound calcium and magnesium is associated with the casein micelle and is referred to as colloidal calcium phosphate (Table 1.4). Colloidal calcium

phosphate consists mainly of casein, calcium, phosphorus, magnesium, citrate and traces of other micro-elements (Banks & Dalgleish, 1990; Bylund, 1995; Fox, 2003; Holt, 1985). Casein micelles consist of an aggregation of ~400 sub-micelles (Bylund, 1995). Sub-micelles homogeneously contain α_s, β and κ caseins which bind calcium, magnesium and some mineral complexes which enhance the stability of the structure (Bylund, 1995). The amounts of calcium and magnesium that associate with fats and lactose in milk are negligable (Gaucheron, 2005).

1.6.4) Effects of processing on milk mineral interactions

During the manufacture of milk to milk powder, processing factors have the potential to change the extent of partitioning of salts between bound and free forms (Holt, 1985). Such processing factors include fluctuations in temperature, the concentration of solutes (usually affected by evaporation), the use of additives and prolonged storage.

a. Temperature

An increase in milk temperature decreases the solubility of calcium phosphate and consequently soluble calcium phosphate is transferred to the colloidal phase, with the subsequent release of H^+ ions and a pH decrease (Fox, 2003). Thus, an increase in temperature of milk decreases the free Ca^{2+} concentration, whereas a decrease in temperature increases the free Ca^{2+} concentration (Fox, 2003). Fluctuations of free Ca^{2+} ion concentrations in response to changes of temperature are usually reversible, except for when milk is exposed to extreme temperature/time processes which can occur in dairy manufacture (Fox, 2003).

Aoki *et al*. (1974) subjected skim milk to 120°C for 15 mins and then stored the milk for 15 months. It was found that over time the free Ca^{2+} concentration increased after heating. Similarly, Geerts *et al*. (1983) measured the physiological free Ca^{2+}

concentration of skim milk as 2.1 mM with a Ca^{2+} activity of 0.85±0.02 mM. In a typical experiment it was found that when the skim milk was heated at 115°C and allowed to cool for either 1 min, 30 min, 24 h or 50 h, the Ca^{2+} activity was measured as being 58%, 70%, 88%, or 89% of the initial value, respectively. The Ca^{2+} activity of reconstituted skim milk powders, which were made after heating at 115°C for various times, increased over time. The research presented by Aoki *et al*. (1974), and Geerts *et al*. (1983), shows how temperature can influence free Ca^{2+} concentrations in milk.

Bouman *et al*. (1982) found that a uniform, rough, white, calcium phosphate layer precipitated in the pre-pasteurisation region of the regenerative section of a plate heat exchanger in a dairy processing plant. The temperature range in the pre-pasteurisation region of the plate heat exchanger was between 12 and 72°C, and it was observed that the extent of calcium phosphate precipitation increased with increasing temperature. This observation was explained by the decreasing solubility of calcium phosphate in milk with increasing temperature. Furthermore, the molar ratio of calcium to phosphorus in the fouling layer was found to be about 1.5, which indicates calcium phosphate precipitation. The layer included primarily whey (as opposed to caseinate) proteins, where the weight ratio of $Ca_3(PO_4)_2$ to protein was approximately 2.73:1, which is a factor 30 times greater than the 1:14 ratio of $Ca_3(PO_4)_2$ to casein in colloidal calcium phosphate, and is evidence to suggest that the white layer formation is not due to deposition of colloidal calcium phosphate, but in fact, due to the conversion of soluble to insoluble calcium phosphate and its subsequent deposition. It was also observed in this study, that after a skim milk processing time of 12 hours, thermoresistant streptococci had grown to 10^4 CFU cm^{-2} and 10^6 CFU cm^{-2}, and occupied 0.016% and 2%, of the area of the calcium phosphate covered pre-pasteurisation region and the visibly clean post-pasteurisation region, respectively. This

study showed that calcium phosphate precipitation on stainless steel prevents biofilm formation during dairy manufacture.

b. Concentration

Similar to temperature, as the concentration of milk increases, the solubility of calcium phosphate decreases. This causes soluble calcium phosphate to form colloidal calcium phosphate and $Ca_3(PO_4)_2$, with the subsequent release of H^+ ions and a decrease in pH, and a decrease in the concentration of free Ca^{2+} and Mg^{2+} (Holt, 1985). Milk is commonly concentrated during the manufacture of milk formulations to milk powder by removing the water by evaporation (Burgess *et al*., 2010; Hill & Smythe, 2012). Thus, as chilled, unprocessed milk is heated and the water is removed by evaporation, the concentrations of free Ca^{2+} and Mg^{2+} in the processed milk decrease (Holt, 1985). Conversely, in some instances the amount of free Ca^{2+} and Mg^{2+} will increase in milk that has been concentrated by evaporation (Holt, 1985). Concentration increases the ionic strength of milk, which reduces the ion activity coefficients, enabling higher proportions of soluble calcium phosphate to exist, despite the decrease in calcium phosphate solubility (Holt, 1985).

c. pH

As the pH of milk decreases, colloidal calcium phosphate progressively dissolves which increases the concentrations of free Ca^{2+} and Mg^{2+} (Fox, 2003). If milk pH is less than approximately 4.9, calcium phosphate and colloidal calcium phosphate is completely dissolved (Fox, 2003; Lyster, 1979).

An example of the effect of pH on the concentration of Ca^{2+} in milk was shown by Geerts *et al*. (1983). The physiological free Ca^{2+} ion concentration of skim milk was measured as 2.1 mM with a Ca^{2+} activity of 0.85±0.02 mM. It was found that compared

to the Ca^{2+} activity in milk at the natural pH, the Ca^{2+} activity doubled when the milk pH was reduced to 6.0, and halved when the pH was increased to 7.5.

The free Ca^{2+} and Mg^{2+} concentrations in milk formulations can increase in circumstances with either subsequent increases or decreases in pH. Heating and concentrating milk decreases the concentrations of free Ca^{2+} and Mg^{2+} and decreases the pH of milk (Holt, 1985). In contrast, when the temperature and concentration of milk is kept constant, a decrease in pH causes an increase in the concentration of free Ca^{2+} and Mg^{2+} (Dalgleish & Parker, 1980; Holt, 1985). This is a conundrum in the salt partitioning and ion equilibria of milk during processing.

d. Addition of ions

The addition of ions to milk formulations can influence the concentration of the free form of ions by directly increasing the concentration of the free form of the ion that is added, and by altering the pH and the partitioning of salts and ion equilibria (Gaucheron, 2005; Holt, 1985; Le Ray *et al*., 1998; Philippe *et al*., 2003). This section will describe the effect of the addition of NaCl, $CaCl_2$ and $MgCl_2$ on the concentrations of free Na^+, Ca^{2+} and Mg^{2+}, and the equilibria of ions between the bound and free forms in milk formulations.

Adding NaCl, $CaCl_2$ and $MgCl_2$ to milk will increase the concentration of free Na^+, Ca^{2+} or Mg^{2+}, respectively (Gaucheron, 2005; Holt, 1985; Le Ray *et al*., 1998). Also, the addition of either NaCl, $CaCl_2$ and $MgCl_2$ increases the ionic strength of milk formulations, which decreases the pH, owing to the release of bound H^+ (Gaucheron, 2005; Holt, 1985). This decreases the activity coefficients of ions and increases the concentration of the free form of all ions in milk (Holt, 1985; Le Ray *et al*., 1998). Additionally, the added cation will displace other cations from complexes and consequently increase the concentration of the free form of cations that were not added

to the milk (Gaucheron, 2005; Holt, 1985; Le Ray *et al*., 1998; Philippe *et al*., 2003). The ion equilibria of milk are influenced differently when the effects of the addition of either NaCl, $CaCl_2$ and $MgCl_2$ are compared (Gaucheron, 2005; Holt, 1985; Le Ray *et al*., 1998). The addition of $CaCl_2$ to milk increases the concentration of free Mg^{2+} more than the increase in the concentration of free Ca^{2+} upon the addition of an equivalent amount of $MgCl_2$ (Holt, 1985; Philippe *et al*., 2003). Relatively large amounts of NaCl are required to be added to milk in order to displace calcium and magnesium from colloidal calcium phosphate and increase the concentration of free Ca^{2+} and Mg^{2+} (Philippe *et al*., 2003).

For example, Le Ray *et al*. (1998) found that the addition of 178, 19 and 19 mmol kg^{-1} of NaCl, $CaCl_2$ and $MgCl_2$ to reconstituted casein micelle dispersions decreased the pH by 0.1, 0.5 and 0.4, and increased the concentration of diffusible Ca^{2+} from ~ 0.9 mmol kg^{-1} to ~ 4.5, 18 and 4 mmol kg^{-1}, respectively. The addition of 19 mmol kg^{-1} of $MgCl_2$ to reconstituted casein micelle dispersions increased the concentration of diffusible Mg^{2+} from ~ 0.1 mmol kg^{-1} to ~ 15 mmol kg^{-1}. Although the effect of the addition of $MgCl_2$ on the concentration of free Ca^{2+} was not measured, it can be predicted that the concentration of free Ca^{2+} would have increased, owing to the decrease of pH caused by the addition of $MgCl_2$.

Similarly, Philippe *et al*. (2003) compared the influence of the addition of 4.5 mM $CaCl_2$ to skim milk on free $Ca^{2+}$and Mg^{2+} concentrations, both with and without pH adjustment (to control for the decreasing effect of $CaCl_2$ addition on the pH of skim milk). The pH decreased by ~ 0.15. Without pH adjustment the free Ca^{2+} and Mg^{2+} concentrations increased by 1.30 and 0.39 mM, respectively. With pH adjustment the free Ca^{2+} and Mg^{2+} concentrations increased by 1.11 and 0.19 mM, respectively. Thus, the effect of a decrease in pH (due to $CaCl_2$ addition), increased the concentration of both free Ca^{2+} and Mg^{2+}. Although $CaCl_2$ was added at a concentration of 4.5 mM, the

free Ca^{2+} concentration increased by less than 4.5 mM, as most of the added calcium exists bound to anions and proteins in the milk. The contribution that the decrease in pH had on the increase in the concentration of free Ca^{2+} was less than the contribution of the added calcium. Thus, the concentration of the free form of the ion that is added to milk usually increases more than the concentration of the free form of the other milk ions.

Although most research on the partitioning of minerals between the bound and free form has been performed using skim and whole milk, relationships and trends observed in skim and whole milk can be used as a guide to estimate the partitioning of ions of other milk systems, including milk formulations (1 – 4) (Table 2.5) as used in this study.

1.7) Biofilms in milk powder manufacturing plants

Biofilms on product-contact surfaces in milk powder manufacturing plants mainly comprise thermophilic bacilli that belong to the *Geobacillus* spp. and *A. flavithermus* groups (Burgess *et al*., 2010; Hill & Smythe, 2012). *Geobacillus* spp. and *A. flavithermus* are highly adapted for growth and persistence in milk powder manufacturing plants (Burgess *et al*., 2010; Hill & Smythe, 2012). Many regions of a milk powder manufacturing line are heated to between 50 and 70ºC which aligns with the growth temperature range of *Geobacillus* spp. and *A. flavithermus* (30 – 72ºC) (Burgess *et al*., 2010). Thermophillic bacilli have fast growth rates. For example, *B. stearothermophilus* was shown to have doubling times of between 19 -32 min (Flint *et al*., 2001a). Milk powder manufacture is limited to 18 – 24 h of continuous processing at which point the manufacturing plant needs to be cleaned to remove foulants and biofilms which contain aggregates of milk constituents and bacteria (Burgess *et al*., 2010; Hill & Smythe, 2012). A variety of chemicals are used during clean-in-place

regimes, including sodium hydroxide, nitric acid and sanitizers (eg. Oxonia Active®) (Bremer *et al*., 2006). Endospore forms of *Geobacillus* spp. and *A. flavithermus* are adept at resisting chemicals used during clean-in-place regimes, which is attributed to their dormant state and the presence of a protective outer proteinaceous coat (Burgess *et al*., 2010; Etoa & Michiels, 1988). Furthermore, biofilms of *Geobacillus* spp. and *A. flavithermus* are less susceptible to chemicals than their planktonic form, owing to the presence of a protective extracellular matrix (Burgess *et al*., 2010). Viable vegetative cells or spores that remain on processing surfaces after cleaning are thought to initiate biofilm formation in future processes (Flint *et al*., 1997a; Hinton *et al*., 2002). These bacteria are often referred to as 'persister' strains which can potentially seed dairy manufacturing plants (Brooks & Flint, 2008; Flint *et al*., 1997a; Marshall, 1994). Biofilms in milk powder manufacturing plants have been referred to as 'process biofilms', which, interestingly, are predominantly composed of a single bacterial species (Burgess *et al*., 2010; Flint *et al*., 1997a; Flint *et al*., 1997c). Also, biofilms formed by thermophilic bacilli often consist of a monolayer of cells (Burgess *et al*., 2010).

The majority of *Geobacillus* spp. and *A. flavithermus* which result in milk powders originate from bacteria that have sloughed from biofilms into milk as the milk transits through the manufacturing line (Burgess *et al*., 2010; Hill & Smythe, 2012). Flint *et al*. (2001a) found that a *B. stearothermophilus* strain developed a biofilm in a continuous flow laboratory reactor with a density of up to 10^6 CFU cm^{-2} releasing 10^6 CFU ml^{-1} after 6 hours of incubation. It was also found that biofilms of *B. stearothermophilus* release both vegetative cells and spores. In addition, Burgess *et al*. (2009) found that a strain of *A. flavithermus*, isolated from a milk powder plant, developed a biofilm in a continuous flow laboratory reactor, consisting of both vegetative cell and spore morphologies, at a density of 10^6 CFU cm^{-2} producing 10^5 CFU ml^{-1} in the skim milk

after 8 h of incubation. These studies portrayed the quick development of thermophilic bacilli biofilms and the consequence of milk powder contamination due to bacterial sloughing.

The total thermophile count in milk powder is of major importance, as this indicates the hygiene of the process used to maunfacture the product, is used to measure the quality of the milk powder, and determines the price that milk powders are sold for in the market (Burgess *et al*., 2010; Hill & Smythe, 2012). In addition, thermophilic bacilli have the potential to spoil milk powder after it is rehydrated, as they produce acid, and proteolytic and lipolytic enzymes which produce off-flavours (Burgess *et al*., 2010; Hill & Smythe, 2012).

Product-contact surfaces used in milk powder manufacturing plants are made predominantly of stainless steel, as it is malleable, durable, usually easy to clean, resists corrosion, thermo-conductive and chemically and physically inert (Palmer *et al*., 2007). The metallic composition of stainless steel can vary, as can its polish, which determines its finish or surface topography (Flint *et al*., 2000). Product-contact surfaces in New Zealand milk powder manufacturing plants are commonly a 316 grade with a 2B finish. The surface properties of stainless steel, and the attachment of bacteria and milk constituents to stainless steel has been extensively studied (Barnes *et al*., 1999; Brooks & Flint, 2008; Flint *et al*., 2000; Parkar *et al*., 2001; Seale *et al*., 2008). Stainless steel has a negative surface charge and is relatively hydrophobic (Palmer *et al*., 2007; Seale *et al*., 2008). Some comparative studies on the attachment of dairy thermophilic bacterial vegetative cells and spores to stainless steel surfaces with differing surface topographies, and surfaces made of other materials, such as plastic, have found a significant difference, while others have found no significant difference, in the attachment of vegetative cells and spores to surfaces with differing physiochemical properties (Brooks & Flint, 2008; Flint *et al*., 1997b, 2000; Medilanski *et al*., 2002;

Parkar *et al*., 2001; Seale *et al*., 2008). Attachment studies can be made increasingly complex when the influence of the deposition of milk constituents on bacterial attachment is also considered in the experimental procedure (Bos *et al*., 1999; Palmer *et al*., 2007). It is important that experiments to investigate the attachment and biofilm formation of dairy thermophilic bacteria are performed using stainless steel surfaces that resemble those used in milk powder manufacturing plants, in order to simulate biofilm formation that occurs during production (Palmer *et al*., 2007).

The concentration and monovalent to divalent cation ratio of free Na^+, K^+, Ca^{2+} and Mg^{2+} in milk formulations is one aspect thought to influence biofilm development on stainless steel surfaces. Different specialty milk formulations have a range of free Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations and contrasting monovalent to divalent cation ratios (Table 1.5). Since *Geobacillus* spp. and *A. flavithermus* are the major problematic bacteria that form biofilms and contaminate milk powders, the effect of Na^+, K^+, Ca^{2+} and Mg^{2+} on the proliferation and biofilm formation of these bacteria is investigated in this study.

1.8) Motivation

Geobacillus spp. and *A. flavithermus* are the predominant bacteria that may contaminate milk as it is processed to milk powder (Burgess *et al*., 2010; Hill & Smythe, 2012). *Geobacillus* spp. and *A. flavithermus* attach to and proliferate as biofilms on product-contact surfaces in milk powder manufacturing plants (Burgess *et al*., 2010; Hill & Smythe, 2012). The biofilms harbour vegetative cells and spores which slough into milk as it transits through a milk power manufacturing line (Burgess *et al*., 2010; Hill & Smythe, 2012). The resulting number of thermophilic vegetative cells and spores in milk powder is of major importance, as the total thermophile count indicates the hygiene of the process, and determines the grade and selling price of the powders (Burgess *et al*., 2010; Hill & Smythe, 2012).

Practical observations from New Zealand milk powder manufacturing plants have indicated that the rate and extent of proliferation of dairy thermophilic bacteria during milk powder manufacture is influenced by the composition of minerals and ions in specialty milk formulations. For example, final milk powder products derived from specialty milk formulations with high monovalent to divalent cation ratios (high Na^{+}, low Ca^{2+} and low Mg^{2+} concentrations) have been found to have low (< 10 CFU g^{-1}) total thermophile counts in standard laboratory tests. These observations triggered the desire to investigate how the composition of minerals and ions in specialty milk formulations influence biofilm formation and proliferation of thermophilic bacilli.

There are three potential mechanisms for the influence of minerals and ions in milk on biofilm formation by thermophilic bacilli during the manufacture of milk powders. Firstly, relatively high or low external cation concentrations may imbalance cation homeostasis of bacteria in a biofilm. Cation homeostasis has an important role in bacterial physiology, such as in enzyme function, and the regulation of both osmotic pressure and cellular processes. An imbalance of cation homeostasis can have a toxic or

bacteriostatic effect and inhibit the growth of bacteria in a biofilm. Secondly, ions may influence the structural integrity of the biofilms. Polymers in the cell wall and extracellular matrix of bacteria have an abundance of negatively charged functional groups which electrostatically repel each other (Neuhaus & Baddiley, 2003). Positively charged ions (cations) may neutralise electrostatic repulsive forces and increase the cohesion and persistence of a biofilm (Dunne & Burd, 1992; Sobeck & Higgins, 2002). Thirdly, the physiology of bacteria in biofilms may be influenced by changes in extracellular cation concentrations. Cations may bind to cation-binding motifs in bacterial proteins and subsequently alter the conformation and function of the protein and thereby influence the physiology of bacteria. For example, cations may bind to response regulators and influence the signal transduction pathways and regulation of genes that the response regulators control (Dominguez, 2004; He *et al*., 2008). A change in the physiology of bacteria may have implications for the prosperity of the biofilms that they form.

The free (ionized) form of cations has the potential to interact with cation association sites in bacteria and elicit effects as proposed in the mechanisms listed above. Na^+, K^+, Ca^{2+} and Mg^{2+} are the most abundant free cations in unprocessed milk and specialty milk formulations (Fox, 2003). Thus, Na^+, K^+, Ca^{2+} and Mg^{2+} have the greatest potential to interact with and influence biofilm formation by *Geobacillus* spp. and *A. flavithermus* during milk powder manufacture. Research to date has investigated the influence of one or a few cation types on one aspect of biofilm formation, for example, cell surface attachment or cell wall ion assimilation by bacteria (Craven & Williams, 1998; Heptinstall *et al*., 1970; Long *et al*., 2009; Patrauchan *et al*., 2005; van Hoogmoed *et al*., 1997). In addition, most studies have been performed with bacteria of a different genus from the *Geobacillus* spp. and *A. flavithermus* groups.

This study aims to investigate the effect that a collective range of Na^+, K^+, Ca^{2+} and Mg^{2+} ion concentrations have on *Geobacillus* spp. and *A. flavithermus* throughout biofilm formation. This includes planktonic forms of the bacteria (Chapter 2), their transition from a planktonic to irreversibly attached state (Chapter 3), and bacteria that exist in an estabilised biofilm (Chapters 3-5). The research will focus on elucidating the influence that varied mineral and ion compositions in commercial, specialty milk formulations have on the proliferation and biofilm formation of *Geobacillus* spp. and *A. flavithermus* during the manufacture of milk powder.

1.9) Objective

To determine how Na^+, K^+, Ca^{2+} and Mg^{2+} ions, both individually and collectively, affect bacterial attachment, biofilm formation and the proliferation of *Geobacillus* spp. and *A. flavithermus* in conditions resembling both the bulk flow and fouling layers during the manufacture of commercial, specialty milk formulations.

1.10) Aims

This study aims to:

- Obtain bacterial isolates from product-contact surfaces and assess the milk powder manufacturing process by visiting a milk powder manufacturing plant and sampling the plant using swabs and observing the process, respectively.
- Identify and characterise suitable dairy thermophilic bacteria, such as *Geobacillus* spp. and *A. flavithermus*, isolated both from the product-contact surfaces of a milk powder manufacturing plant and from rehydrated milk formulations manufactured in the plant, by 16S rDNA PCR using *Geobacillus* spp. and *A. flavithermus* specific primers.
- Develop a base medium which can be used to detect differences in the growth characteristics, physiology and biofilm formation by *Geobacillus* spp. and *A. flavithermus* in response to the supplementation of the base medium with different Na^+, K^+, Ca^{2+} and Mg^{2+} ion ratios and concentrations, by determining the Na^+, K^+, Ca^{2+} and Mg^{2+} ion concentrations of a variety of growth media and investigating the growth capabilities of *Geobacillus* spp. and *A. flavithermus* in the media.
- Develop and perform an assay which can be used to investigate the effect of Na^+, K^+, Ca^{2+} and Mg^{2+} ions on the planktonic growth of *Geobacillus* spp. and *A. flavithermus*. Variables which could be analysed include vegetative cell density, spore density, optical density, and amounts of extracellular matrix produced.
- Develop and perform an assay which can be used to investigate the effect of Na^+, K^+, Ca^{2+} and Mg^{2+} ions on attachment of *Geobacillus* spp. and *A. flavithermus* to 316 stainless steel, by comparing the number of attached viable CFU cm^{-2}.

- Develop and perform an assay which can be used to investigate the effect of Na^+, K^+, Ca^{2+} and Mg^{2+} ions on the proliferation of *Geobacillus* spp. and *A. flavithermus* in an established biofilm on stainless steel, by comparing the number of attached viable CFU cm^{-2}. This assay would have incubation times and temperatures comparable to processing 'run' times and temperatures used in milk powder manufacturing plants of up to 18 h.
- Identify ratios and concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} ions that will inhibit the proliferation and biofilm formation of dairy thermophilic bacteria by comparing experiments which use a range of Na^+, K^+, Ca^{2+} and Mg^{2+} ions ratios and concentrations.

1.11) Research questions

How do Na^+, K^+, Ca^{2+} and Mg^{2+} ions affect biofilm formation of *Geobacillus* spp. and *A. flavithermus* during the manufacture of commercial, specialty milk formulations?

How can the ratios and concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} ions in commercial, specialty milk formulations be adjusted so that the proliferation and biofilm formation of *Geobacillus* spp. and *A. flavithermus* is inhibited during manufacture?

1.12) Research hypotheses

- The ionic strength of media will influence attachment and biofilm formation of *Geobacillus* spp. and *A. flavithermus*. As the ionic strength of media increases, the number of attached viable cells (CFU cm^{-2}) will increase during attachment (defined as the transition of bacteria from a planktonic to irrversibly attached to stainless steel form) and biofilm formation. As the ionic strength of media increases, the diffuse electric double layer will compress and the extent of repulsive forces among bacteria and stainless steel will decrease.
- Ca^{2+} and Mg^{2+} will have a greater increasing effect on attachment and biofilm formation of *Geobacillus* spp. and *A. flavithermus* on stainless steel relative to Na^{+} and K^{+}. Divalent cations have a higher binding affinity and higher charge density relative to monovalent cations. Also divalent cations have the potential to form divalent cation bridges. Thus, divalent cations have a greater capacity to increase the cohesion and structural integrity of a biofilm.
- Ca^{2+} and Mg^{2+} will stimulate the production of surface-exposed polymers and extracellular matrix, which will increase attachment and biofilm formation. In contrast, Na^{+} and K^{+} will stimulate bacteria to express surface-exposed polymers which will compromise the cohesion and structural integrity of a biofilm, such as negatively charged and hydrophillic polymers.
- Ca^{2+} and Mg^{2+} will similarly influence attachment and biofilm formation of *Geobacillus* spp. and *A. flavithermus* on stainless steel, however one may be more influential than the other, depending on the bacterial strain.
- Attachment and biofilm formation of *Geobacillus* spp. and *A. flavithermus* on stainless steel will be optimal at low monovalent to divalent cation ratios (i.e. close to 2:1). In contrast, when monovalent to divalent cation ratios are high

(i.e. 10:1 or greater), attachment and biofilm formation of *Geobacillus* spp. and *A. flavithermus* on stainless steel will be compromised.

- Relatively high individual cation concentrations will inhibit the proliferation of *Geobacillus* spp. and *A. flavithermus*, both in a planktonic and biofilm mode. At relatively high individual cation concentrations, the cation will accumulate intracellularly in bacteria at toxic amounts which will inhibit cellular metabolism and cell division.
- Relatively low individual cation concentrations will inhibit the proliferation of *Geobacillus* spp. and *A. flavithermus*, both in a planktonic and biofilm mode. At relatively low individual cation concentrations, the bacteria will not be able to assimilate an adequate amount of the cation into the cell envelope to maintain their structural integrity, and the bacteria will not be able to acquire an adequate supply of the cation intracellularly, to assist cellular processes and metabolic pathways.

CHAPTER 2

Influence of cations on growth of Thermophilic *Geobacillus* species and *Anoxybacillus flavithermus* in planktonic culture

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Ben Somerton

Name/Title of Principal Supervisor: Steve Flint

Name of Published Research Output and full reference:

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In which Chapter is the Published Work: Chapter 2

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Principal Supervisor's signature — 19 November 2013 — Date

2.1) Abstract

The influence that varied concentrations and ratios of free Na^+, K^+, Ca^{2+}, and Mg^{2+} had on the planktonic growth of cultures of thermophilic bacilli in a minimal medium was investigated. Two isolates derived from a milk powder manufacturing plant, i.e., *Anoxybacillus flavithermus* E16 and *Geobacillus* sp. F75, and two type isolates, i.e., *A. flavithermus* DSM 2641 and *G. thermoleovorans* DSM 5366, were studied. The relationship between the cation composition of the culture and optical density was unique for each isolate. Generally, free Ca^{2+} and Mg^{2+} were predominantly associated with increases in optical density, and free Na^+ and K^+ acted co-operatively with Ca^{2+} and Mg^{2+} to increase optical density. Also, supplementation with high individual concentrations (63 – 250 mM) of either Na^+, K^+ or Ca^{2+}, significantly decreased ($P \leq 0.05$) *Geobacillus* spp. culture optical densities. Mg^{2+} protected *Geobacillus* spp. from inhibitory concentrations of Na^+, K^+ or Ca^{2+} (63 – 250 mM). *A. flavithermus* E16 was selected for further study; in response to increasing cation concentrations (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}) it produced increased amounts of surface protein. It was concluded that the optical densities of the cultures, in response to different external cations, were predominantly determined by differences in the amount of bacterial surface protein, rather than differences in total viable cell count, spore count, cell size and shape, or the amount of surface polysaccharide produced. This indicated that free Ca^{2+} and/or Mg^{2+} was required for the expression of surface protein by the thermophilic bacilli.

2.2) Introduction

The thermophilic bacilli, *Geobacillus* spp. and *Anoxybacillus flavithermus*, are the predominant bacteria in foulants of heated regions (50–70°C) of milk powder manufacturing plants (Burgess *et al*., 2010; Flint *et al*., 2001a). These bacteria form biofilms and sporulate, which further enhances their resistance to high temperatures and cleaning regimes (Burgess *et al*., 2010). Enzymes and spores that originate from thermophilic bacilli may decrease the shelf life, functionality, and value of milk powder (Burgess *et al*., 2010; Chen *et al*., 2004).

The most abundant cations in milk, including the sum of both free and bound forms of each element, are, in descending order, K^+, Ca^{2+}, Na^+, and Mg^{2+} (Fox, 2003). The total free cation concentration in unprocessed milk is approximately 60 mM, consisting of free Na^+, K^+, Ca^{2+}, and Mg^{2+} ions at concentrations of approximately 20, 34, 3.6, and 1.3 mM, respectively (Fox, 2003). These concentrations are lower than the total amount of each element (free and bound) in milk, because a substantial proportion of the elements are bound or partitioned with the anions or proteins that comprise milk (Fox, 2003). The extent of the partitioning of ions within milk fluctuates as milk is processed. For example, as the temperature of chilled, unprocessed milk increases to between 50 and 70°C during the production of milk powder, the soluble fraction of milk becomes increasingly insoluble and the concentration of free cations decrease (Anema, 2009). However, as the concentration of milk increases due to evaporation during the manufacture of milk powder, the concentration of free cations increases (Anema, 2009). Furthermore, minerals and ions in milk have the potential to migrate from the bulk flow and to concentrate within surface-conditioning layers, foulants, and biofilms in milk powder manufacturing plants (Palmer *et al*., 2007). Thus, the external free cation composition that thermophilic bacilli in biofilms encounter in milk powder

manufacturing plants varies compared with the composition that exists in unprocessed milk.

Ca^{2+} has a wide range of critical physiological and structural roles in bacteria. It has been shown to regulate cellular processes (Naseem *et al*., 2007) and enzyme functionality (Onek & Smith, 1992), stimulate extracellular matrix production (Corpe, 1964; Liu & Sun, 2011), and stimulate biofilm formation by selected bacterial species (Garrison-Schilling *et al*., 2011; Oomes *et al*., 2009; Patrauchan *et al*., 2005). Mg^{2+} is a co-factor in bacterial enzymes that is involved in cell wall biosynthesis (Heptinstall *et al*., 1970; Hughes *et al*., 1973), and, similarly to Ca^{2+}, has been shown to stimulate the production of extracellular polysaccharides and biofilm formation by bacteria (Song & Leff, 2006; Tempest *et al*., 1965). Na^{+} is involved in the sodium ion X cotransport system, sodium-ion-coupled energy transformation, pH homeostasis mechanisms, and the activation of some enzymes in bacteria (Novakova & Smigan, 2008). K^{+} plays an important role in bacteria in the activation of various intracellular enzymes, the regulation of osmotic pressure, and the regulation of pH, and acts as a secondary messenger (Epstein, 2003).

Structural roles of both Ca^{2+} and Mg^{2+} in bacteria include: the stabilization of cell wall and extracellular matrix polymers and the enhancement of biofilm cohesion by neutralizing electrostatic repulsion among biopolymers, which are predominantly negatively charged, and forming Ca^{2+} and Mg^{2+} ionic bridges between the polymers (Heptinstall *et al*., 1970; Higgins & Novak, 1997; Sobeck & Higgins, 2002; Subramanian *et al*., 2010); enhancement of the thermostability of bacteria at high temperatures (i.e., above 50°C), by stabilizing the cell membrane (Mosley *et al*., 1976) and enzymes (Ward & Mooyoung, 1988); and aiding bacterial surface attachment by electrostatic neutralization (Long *et al*., 2009) and binding to bacterial surface structures involved in attachment (Thomas *et al*., 1993).

Intracellular Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations are tightly regulated by bacteria, as they have a critical role in bacterial homeostasis (Epstein, 2003; Hase *et al*., 2001; Michiels *et al*., 2002; Smith & Maguire, 1998). Ion influx and efflux translocators mediate the flux of cations through the cytoplasmic membrane and are driven by ATP and electrochemical gradients (Epstein, 2003; Hase *et al*., 2001; Michiels *et al*., 2002; Smith & Maguire, 1998). The maintenance of cation homeostasis in the presence of high external individual cation concentrations can become energetically expensive and slow the metabolism and cell divison of bacteria (Hase *et al*., 2001).

A number of bacterial species respond uniquely to different external cation concentrations, both in a planktonic form and in a biofilm form (Garrison-Schilling *et al*., 2011; Morales & Dehority, 2009; Patrauchan *et al*., 2005; Vincent, 1962). In our study, optical density was used to analyze indirectly the requirement for and the influence of a range of Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations and proportions on thermophilic bacilli in planktonic culture.

2.3) Methods

2.3.1) Isolation of thermophilic sporeforming bacteria from a milk powder manufacturing plant

Copan FLOQSwabs™ (Copan Diagnostics Inc., Murrieta, CA) were used to swab two regions in a large-scale, commercial milk powder manufacturing plant (five swabs per region, and approximately 100 cm^2 per swab). Both regions were typically heated to between 50 and 70°C during milk powder manufacture. Sampling was carried out after a clean-in-place regime. The swab tips were placed into tubes containing 10 ml of sterile reconstituted skim milk powder (110 g l^{-1}), vortex mixed, and heated at 100°C

for 35 min (Burgess *et al*., 2009). The tubes were incubated at 55°C for 18 h and the resulting cultures were serially diluted in sterile reconstituted tryptose (1 g l^{-1}) (Oxoid, Basingstoke, England). Each dilution series was spread plated on to milk plate count agar (MPCA) (Oxoid) supplemented with starch (2 g l^{-1}) (Merck, Darmstadt, Germany) to stimulate the growth of spores (Mallidis & Scholefield, 1986). The plates were incubated at 55°C for 18 h. Resultant single colonies were selected, purified, and then used to inoculate sterile 100 ml aliquots of tryptic soy broth (Merck). The cultures were incubated at 55°C until they reached mid-exponential phase (approximately 6 h). Frozen glycerol stocks of the cultures were prepared by storing 1 ml aliquots of culture, with the addition of 10% (vol/vol) sterile glycerol, at −80°C.

2.3.2) PCR identification of bacterial isolates

A. flavithermus (flavo) and *Geobacillus* spp. (levo) specific primers were used in combination with the Y1 universal primer to amplify a fragment of approximately 450 bp from the 16S rDNA region of the bacterial isolates, as previously described by Flint *et al*. (2001b). The PCR protocol used was as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 45 s; and finally 72°C for 7 min.

2.3.3) Bacterial isolates and culture preparation

A. flavithermus E16 was isolated from the product contact surface of a spiral tube heater and *Geobacillus* sp. F75 was isolated from the surface of a rubber seal, which was adjacent to the product contact surface in a milk powder manufacturing plant. These isolates were selected for further work, because of their consistent growth at 55°C, and to represent thermophilic bacilli that typically persist in a milk powder manufacturing plant. In addition, two type cultures of these thermophiles were also included: *A. flavithermus* DSM 2641, which was originally isolated from a hot spring (Heinen *et al*.,

1982), and *G. thermoleovorans* DSM 5366, which was isolated from soil near hot water effluent (Zarilla & Perry, 1987).

Frozen glycerol stocks of the bacterial isolates were prepared as described above and used in further experiments. Prior to each experiment, bacteria were grown from these frozen stocks (1 ml) to late-exponential phase by inoculating 100 ml of casein digest medium (1 g l^{-1}) (Difco™, BD Biosciences, Sparks, MD) and incubating the culture at 55°C for 9 h. A total viable cell count of approximately 5 x 10^6 CFU ml^{-1} was typically achieved by *A. flavithermus* E16, *A. flavithermus* DSM 2641, and *G. thermoleovorans* DSM 5366, whereas a total viable cell count of approximately 1 x 10^7 CFU ml^{-1} was typically achieved by *Geobacillus* sp. F75. These 9 h cultures were then diluted using casein digest medium (1 g l^{-1}) to approximately 1 x 10^4 CFU ml^{-1} for use as inocula in subsequent experiments.

2.3.4) Evaluation of the effect of Na^+, K^+, Ca^{2+}, and Mg^{2+} on *Geobacillus* spp. and *A. flavithermus* in planktonic culture

Casein digest medium (1 g l^{-1}) was used to reconstitute NaCl, KCl, $CaCl_2.2H_2O$, and $MgCl_2.6H_2O$ (Merck) powders. Into each well of Falcon™ Microtest™ 96-well, flat bottom with low evaporation lid, tissue culture plates (BD, Franklin Lakes, NJ) was dispensed 50 µl of casein digest medium (1 g l^{-1}), with various cation supplementation concentrations and ratios, and 50 µl of bacterial inoculum, which was grown as described previously. Total cation concentrations of between 2 and 250 mM and various proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+} were used, as outlined in Table 2.1. The background concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+} in casein digest medium (1 g l^{-1}) unsupplemented with cations are approximately 1.0, 0.03, 0.004, and 0.002 mM, respectively, as estimated by BD Biosciences (Biosciences, 2006). The microtiter plates were incubated at 55°C for up to 53 h.

TABLE 2.1 Cation proportions used to supplement a casein digest medium (1 g l^{-1})[a]

Cation supplementation type	Free-cation proportion				
	Na^+	K^+	Ca^{2+}	Mg^{2+}	M/D ratio
Na	1	0	0	0	–
K	0	1	0	0	–
Ca	0	0	1	0	–
Mg	0	0	0	1	–
Na/K	0.5	0.5	0	0	–
Na/Ca	0.5	0	0.5	0	1:1
Na/Mg	0.5	0	0	0.5	1:1
Ca/Mg	0	0	0.5	0.5	–
K/Ca	0	0.5	0.5	0	1:1
K/Mg	0	0.5	0	0.5	1:1
Ca/Mg (1:5)	0	0	0.17	0.83	–
Na/K/Ca (1:1:2)	0.25	0.25	0.5	0	1:1
Na/K/Ca/Mg (1:1:2:2)	0.17	0.17	0.33	0.33	0.5:1
Na/K/Ca/Mg (1:1:1:1)	0.25	0.25	0.25	0.25	1:1
Na/K/Ca/Mg (2:2:1:1)	0.33	0.33	0.17	0.17	2:1
Na/K/Ca/Mg (3:3:1:1)	0.375	0.375	0.125	0.125	3:1
Na/K/Ca/Mg (4:4:1:1)	0.4	0.4	0.1	0.1	4:1
Na/K/Ca/Mg (10:10:1:1)	0.45	0.45	0.045	0.045	10:1
Na/K/Ca/Mg (30:30:1:1)	0.484	0.484	0.016	0.016	30:1

[a] Na, K, Ca, and Mg designate free Na^+, K^+, Ca^{2+}, and Mg^{2+}, respectively. M/D represents the ratio of monovalent to divalent cations in the cation supplementation mixture.

The optical densities of the cultures were periodically measured for triplicate wells, using a wavelength of 600 nm, in an OptiMax™ tunable microplate reader (Molecular Devices, Sunnyvale, CA).

The experiment was carried out on two separate occasions, and each replicate consisted of an average of triplicate cultures. The optical density dataset from the 10 h time point was further analyzed using SAS software. The 10 h time point was chosen as this was when the bacteria reached late-exponential phase and sufficient growth had occurred such that apparent differences in optical density among the cultures could be analyzed. The resultant data and statistics were represented graphically; the *x* axes denoted the optical density of the respective bacterial isolates grown in casein digest medium (1 g l^{-1}) unsupplemented with cations (baseline control), and the optical densities of cultures that were supplemented with cations were reported relative to the baseline control. Optical density results reported with a negative value indicate a lower optical density relative to the baseline control. A one sample t-test was used to compare the optical densities of the cultures supplemented with cations to the baseline control. Population standard errors were generated and 95% confidence intervals ($P \leq 0.05$) were calculated to determine significant differences between optical density values.

2.3.5) Total viable cell and spore counts

A 1 ml aliquot of bacterial inoculum, grown as previously described, was used to inoculate 100 ml of casein digest medium (1 g 1^{-1}), supplemented with cation concentrations of 0, 2, or 125 mM (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}). The cultures were incubated at 55°C for 10 h and total viable cell counts were determined using standard microbiological plating techniques, on MPCA at 55°C for 48 h (Burgess *et al*., 2009).

To determine the spore count in the cultures, 12 ml of each culture was sampled and heated at 100°C for 35 min (Burgess *et al*., 2009). Standard microbiological plating techniques were used to determine the spore count in the heat-treated cultures, on MPCA supplemented with starch (2 g l^{-1}) (Mallidis & Scholefield, 1986). To obtain a

A 10 ml aliquot of bacterial inoculum, grown as previously described, was used to inoculate 1000 ml of casein digest medium (1 g l^{-1}), supplemented with a cation concentration of 0, 2, or 125 mM (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}). The cultures were incubated at 55°C for 10 h. Approximately 900 ml of the culture was centrifuged at 11,800 X *g* for 10 min. The supernatant of the culture was discarded, and the pellet was washed once in 450 ml of distilled water and then re-suspended in 5 ml of distilled water. The total viable cell count in both the original 1000 ml cultures and the 5 ml culture concentrates (900 ml concentrated to 5 ml) were determined using standard microbiological plating techniques, as described previously.

To quantify the amount of surface protein produced by *A. flavithermus* E16 culture, the following protocol was used: 800 µl of a 1:10 dilution of the 5 ml culture concentrate was mixed with 200 µl of Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories, Inc., Hercules, CA), and the absorbance of the mixture was read using a spectrophotometer (595 nm), in which the reference was set against a solution containing 800 µl of distilled water mixed with 200 µl of Bio-Rad protein assay dye reagent concentrate. Bovine serum albumin (Sigma-Aldrich, St. Louis, MO) was used to generate a standard curve (1–100 µg ml^{-1}).

To quantify the amount of extracellular polysaccharide produced by *A. flavithermus* E16 culture, the following protocol was used, as modified from the protocol detailed by Dall and Herndon (1989): 1 ml of the 5 ml culture concentrate was added dropwise to 8 ml of approximately 100% ethanol, incubated at 4°C for 18 h, and then centrifuged at 10,000 X *g* for 20 min. The supernatant was discarded and the pellet was re-suspended by vortex mixing in 1 ml of distilled water. To the re-suspended pellet suspension was added 7 ml of sulfuric acid (77% vol/vol) and then 1 ml of L-tryptophan (10 g l^{-1}) (BDH, Poole, England). Each suspension was thoroughly vortex mixed, dispensed into a glass test tube and then heated for 20 min at 100°C. Each suspension was vortex mixed and the absorbance was read using a spectrophotometer (500 nm), in which the reference was set against a solution containing 1 ml of distilled water mixed with 7 ml of sulfuric acid (77% vol/vol) and 1 ml of L-tryptophan (10 g l^{-1}), which had also been subjected to the heat treatment. Dextran (Sigma-Aldrich) was used to generate a standard curve (10–200 μg ml^{-1}).

The surface protein and surface polysaccharide assays and their associated standard curves were carried out on three separate occasions and the results were quoted as averages ± 1 standard deviation (σ_{n-1}).

The amounts of surface protein and surface polysaccharide, measured per CFU, in the original cultures were determined by dividing the concentration of protein or polysaccharide measured in the 5 ml culture concentrate by the total viable cell count per milliliter determined in the 5 ml culture concentrate.

2.4) Results

The magnitude and the relationship of the optical densities among the cultures supplemented with various cation concentrations and proportions were unique for each isolate studied. After 10 h of incubation, the optical densities of cultures supplemented with many different cation compositions were statistically different from those of cultures not supplemented with cations (baseline control).

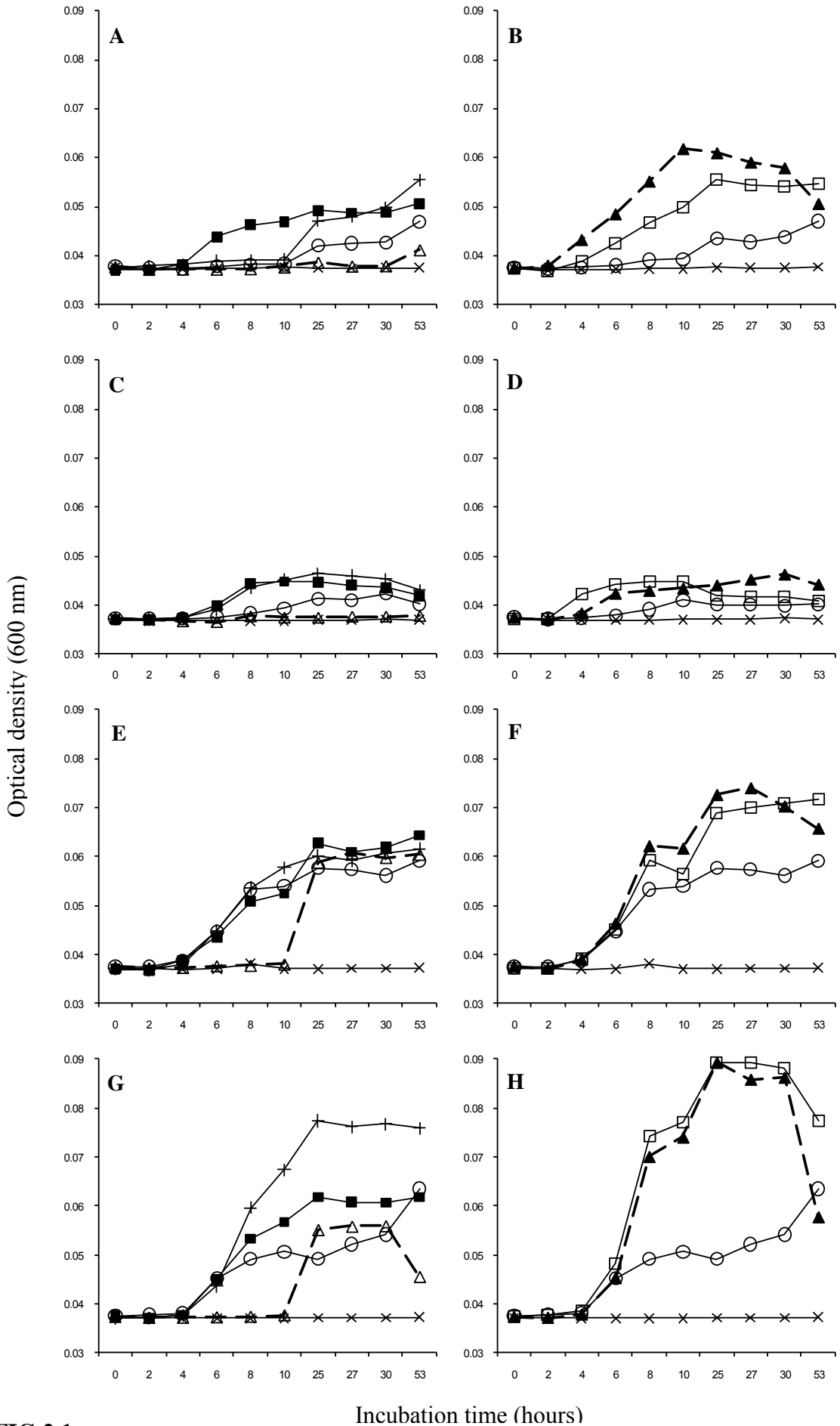
A
B
C
D
E
F
G
H
Optical density (600 nm)
Incubation time (hours)
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0
2
4
6
8
10
25
27
30
53

FIG 2.1

FIG. 2.1 Optical density of *A. flavithermus* E16 (A and B), *A. flavithermus* DSM 2641 (C and D), *Geobacillus* sp. F75 (E and F), and *G. thermoleovorans* DSM 5366 (G and H) grown in casein digest medium (1 g l^{-1}) supplemented with 2 mM Mg^{2+} (plus-hair), 2 mM Ca^{2+} (closed square), 125 mM Ca^{2+} (open triangle), a total cation concentration of either 2 mM (open square) or 125 mM (closed triangle) (consisting of equal proportions of Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}), culture unsupplemented with cations (baseline control) (open circle), and unsupplemented and uninoculated casein digest medium (1 g l^{-1}) (cross-hair). The cultures were incubated at 55°C for up to 53 h. Two replicates were done, and each replicate consisted of an average of triplicate cultures.

2.4.1) Differences among the bacterial isolates

Few conclusive trends were observed when the isolates were compared based on source, i.e., the two type isolates (DSM collection) versus the two milk powder manufacturing plant isolates (Fig. 2.1–2.9). The two *Geobacillus* spp. isolates generally showed higher absolute optical densities than the two *A. flavithermus* isolates when the isolates were compared based on genus (Fig. 2.1). Furthermore, the optical densities of the two *Geobacillus* spp. isolates in culture medium not supplemented with cations (baseline control) were greater than those of the baseline controls of the two *A. flavithermus* isolates (Fig. 2.1). The optical densities of cultures of *A. flavithermus* E16 and *G. thermoleovorans* DSM 5366 were influenced more greatly by cation supplementation, relative to their baseline controls, than were those of cultures of *A. flavithermus* DSM 2641 and *Geobacillus* sp. F75 (Fig. 2.1–2.5).

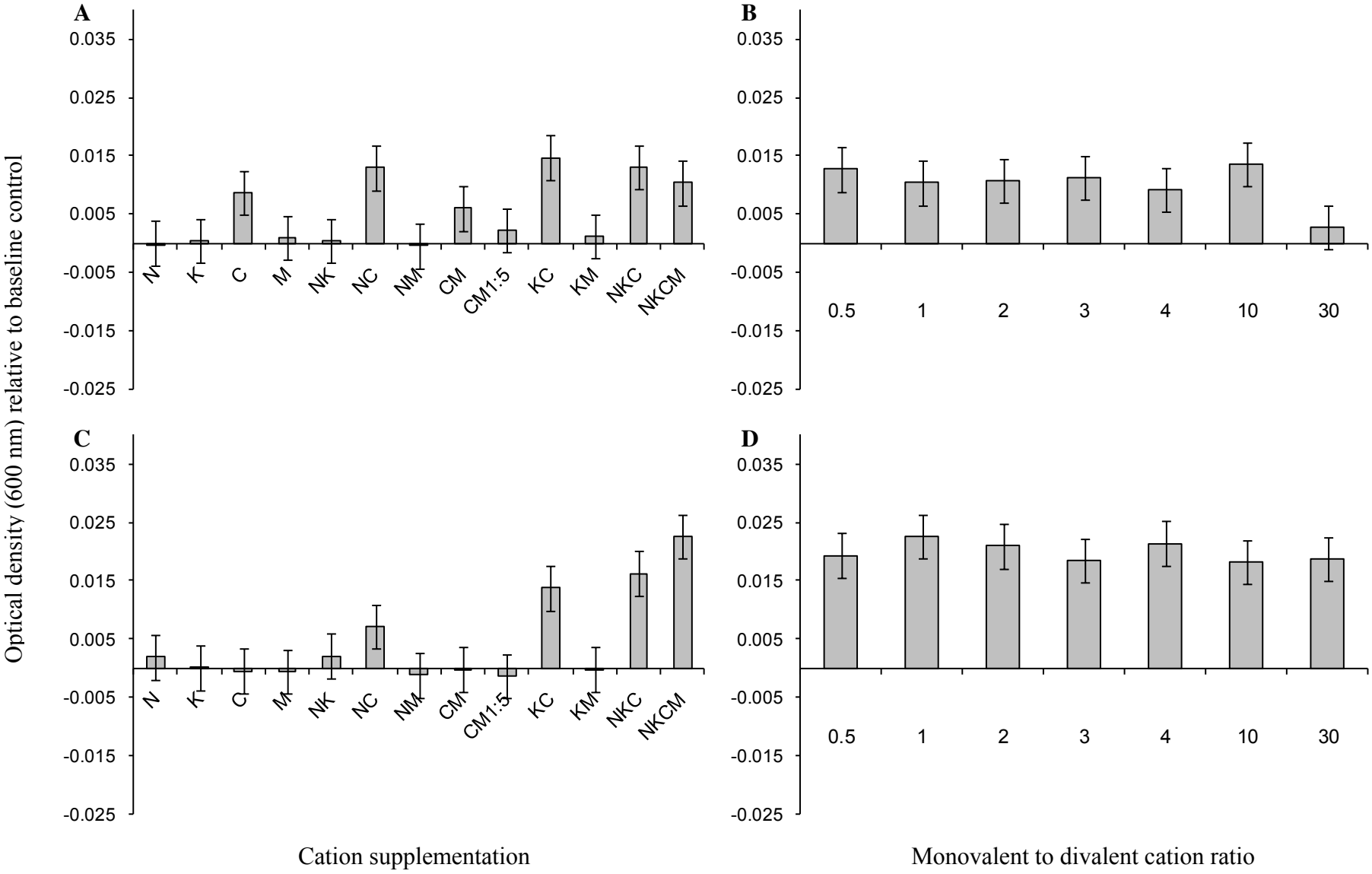
A
B
C
D
Optical density (600 nm) relative to baseline control
0.035
0.025
0.015
0.005
-0.005
-0.015
-0.025
N
K
C
M
NK
NC
NM
CM
CM1:5
KC
KM
NKC
NKCM
0.5
1
2
3
4
10
30
Cation supplementation
Monovalent to divalent cation ratio

FIG 2.2

FIG. 2.2 Optical density of *A. flavithermus* E16 grown in casein digest medium (1 g l^{-1}) supplemented with a variety of cation proportions consisting of a total cation concentration of 2 mM (A and B) or 125 mM (C and D), relative to the optical density of the bacterial isolate grown in casein digest medium (1 g l^{-1}) unsupplemented with cations (baseline control). In (A) and (C), N, K, C, and M designate free Na^+, K^+, Ca^{2+}, and Mg^{2+}, respectively, CM 1:5 refers to a Ca^{2+}:Mg^{2+} ratio of 1:5, NKC refers to a Na^+:K^+:Ca^{2+} ratio of 1:1:2, and all other treatments have equal proportions of each supplemented cation. In (B) and (D), each culture is supplemented with equal proportions of Na^+ and K^+, and equal proportions of Ca^{2+} and Mg^{2+}, at monovalent to divalent cation ratios ranging between 0.5:1 and 30:1. The cultures were incubated at 55°C for 10 h. Two replicates were done, and each replicate consisted of an average of triplicate cultures. The error bars represent 95% confidence intervals ($P \leq 0.05$), which were determined using SAS statistical analysis software.

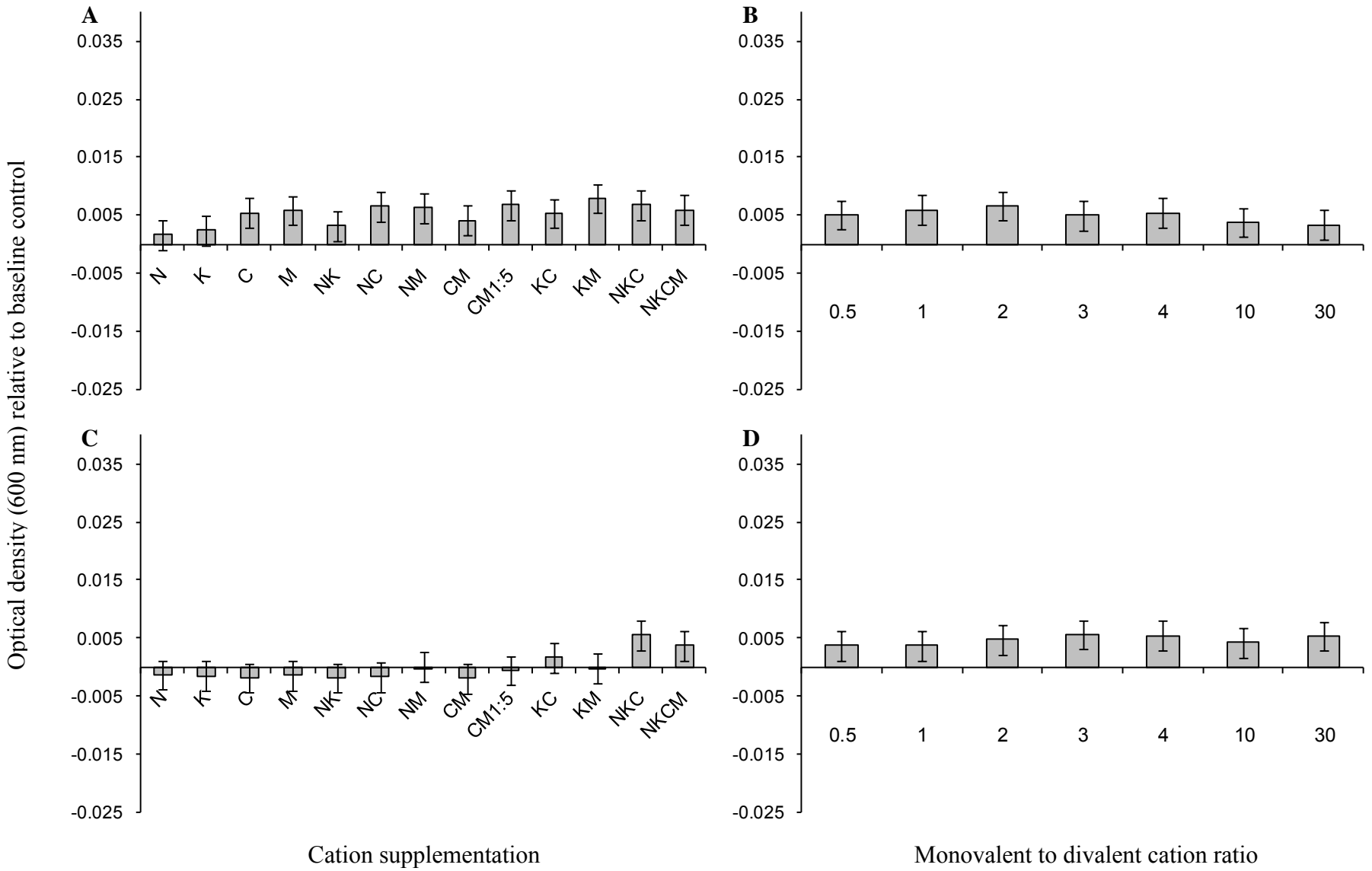
A
B
C
D
Optical density (600 nm) relative to baseline control
0.035
0.025
0.015
0.005
-0.005
-0.015
-0.025
N
K
C
M
NK
NC
NM
CM
CM1:5
KC
KM
NKC
NKCM
0.5
1
2
3
4
10
30
Cation supplementation
Monovalent to divalent cation ratio

FIG 2.3

FIG. 2.3 Optical density of *A. flavithermus* DSM 2641 grown in casein digest medium (1 g l^{-1}) supplemented with a variety of cation proportions consisting of a total cation concentration of 2 mM (A and B) or 125 mM (C and D), relative to the optical density of the bacterial isolate grown in casein digest medium (1 g l^{-1}) unsupplemented with cations (baseline control). In (A) and (C), N, K, C, and M designate free Na^+, K^+, Ca^{2+}, and Mg^{2+}, respectively, CM 1:5 refers to a Ca^{2+}:Mg^{2+} ratio of 1:5, NKC refers to a Na^+:K^+:Ca^{2+} ratio of 1:1:2, and all other treatments have equal proportions of each supplemented cation. In (B) and (D), each culture is supplemented with equal proportions of Na^+ and K^+, and equal proportions of Ca^{2+} and Mg^{2+}, at monovalent to divalent cation ratios ranging between 0.5:1 and 30:1. The cultures were incubated at 55°C for 10 h. Two replicates were done, and each replicate consisted of an average of triplicate cultures. The error bars represent 95% confidence intervals ($P \leq 0.05$), which were determined using SAS statistical analysis software.

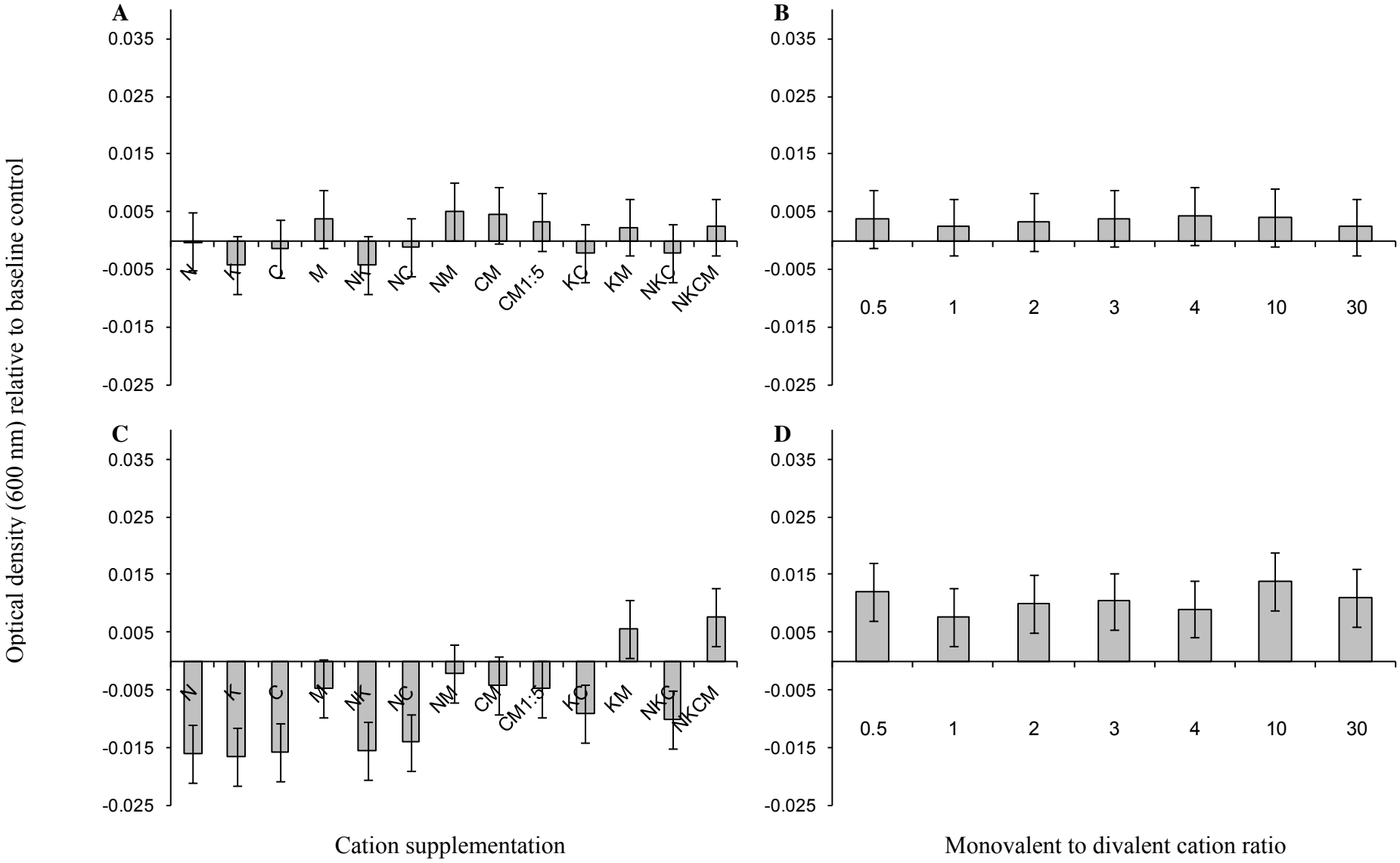
A
B
C
D
Optical density (600 nm) relative to baseline control
0.035
0.025
0.015
0.005
-0.005
-0.015
-0.025
N
K
C
M
NK
NC
NM
CM
CM1:5
KC
KM
NKC
NKCM
0.5
1
2
3
4
10
30
Cation supplementation
Monovalent to divalent cation ratio

FIG 2.4

FIG. 2.4 Optical density of *Geobacillus* sp. F75 grown in casein digest medium (1 g l^{-1}) supplemented with a variety of cation proportions consisting of a total cation concentration of 2 mM (A and B) or 125 mM (C and D), relative to the optical density of the bacterial isolate grown in casein digest medium (1 g l^{-1}) unsupplemented with cations (baseline control). In (A) and (C), N, K, C, and M designate free Na^+, K^+, Ca^{2+}, and Mg^{2+}, respectively, CM 1:5 refers to a Ca^{2+}:Mg^{2+} ratio of 1:5, NKC refers to a Na^+:K^+:Ca^{2+} ratio of 1:1:2, and all other treatments have equal proportions of each supplemented cation. In (B) and (D), each culture is supplemented with equal proportions of Na^+ and K^+, and equal proportions of Ca^{2+} and Mg^{2+}, at monovalent to divalent cation ratios ranging between 0.5:1 and 30:1. The cultures were incubated at 55°C for 10 h. Two replicates were done, and each replicate consisted of an average of triplicate cultures. The error bars represent 95% confidence intervals ($P \leq 0.05$), which were determined using SAS statistical analysis software.

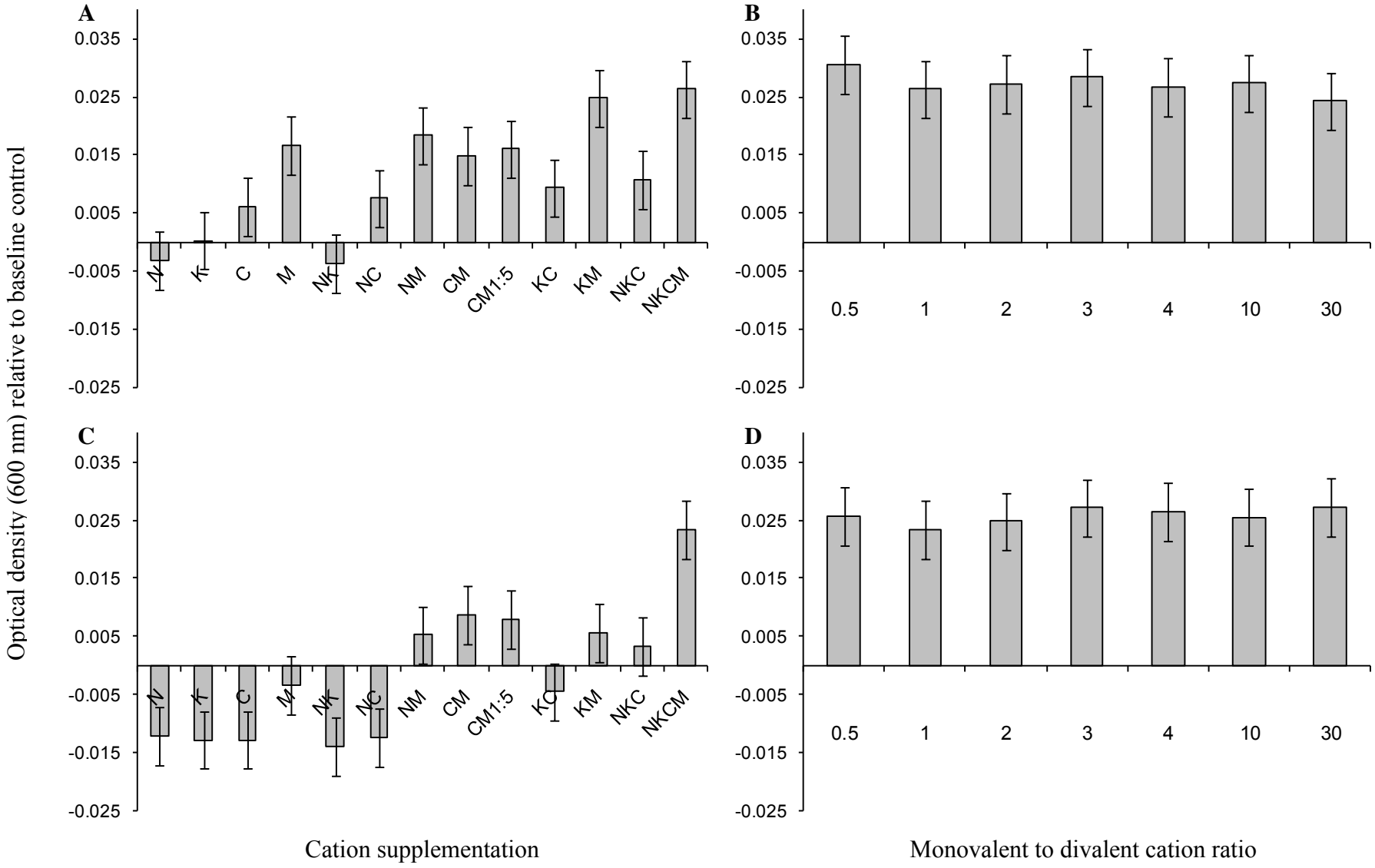
A
B
C
D
Optical density (600 nm) relative to baseline control
0.035
0.025
0.015
0.005
-0.005
-0.015
-0.025
N
K
C
M
NK
NC
NM
CM
CM1:5
KC
KM
NKC
NKCM
0.5
1
2
3
4
10
30
Cation supplementation
Monovalent to divalent cation ratio

FIG 2.5

FIG. 2.5 Optical density of *G. thermoleovorans* DSM 5366 grown in casein digest medium (1 g l^{-1}) supplemented with a variety of cation proportions consisting of a total cation concentration of 2 mM (A and B) or 125 mM (C and D), relative to the optical density of the bacterial isolate grown in casein digest medium (1 g l^{-1}) unsupplemented with cations (baseline control). In (A) and (C), N, K, C, and M designate free Na^+, K^+, Ca^{2+}, and Mg^{2+}, respectively, CM 1:5 refers to a Ca^{2+}:Mg^{2+} ratio of 1:5, NKC refers to a Na^+:K^+:Ca^{2+} ratio of 1:1:2, and all other treatments have equal proportions of each supplemented cation. In (B) and (D), each culture is supplemented with equal proportions of Na^+ and K^+, and equal proportions of Ca^{2+} and Mg^{2+}, at monovalent to divalent cation ratios ranging between 0.5:1 and 30:1. The cultures were incubated at 55°C for 10 h. Two replicates were done, and each replicate consisted of an average of triplicate cultures. The error bars represent 95% confidence intervals ($P \leq 0.05$), which were determined using SAS statistical analysis software.

2.4.2) Effect of cation type

The response of the thermophilic bacilli to Ca^{2+} and Mg^{2+} was predominantly responsible for an increase in the optical density of the culture, whereas Na^{+} and K^{+} acted cooperatively with Ca^{2+} and Mg^{2+} to increase the optical density (Fig. 2.1–2.5). The extent of the difference in optical density when Ca^{2+} or Mg^{2+} was used to supplement the growth medium, relative to the baseline control, differed depending on the bacterial isolate grown (Fig. 2.1–2.5).

Within the first 10 h of growth, the optical density readings for *A. flavithermus* E16 increased, relative to the baseline control, when cultures were supplemented with Ca^{2+}, but not Mg^{2+} (Fig. 2.1A and 2.2A). Furthermore, Na^{+} and K^{+} acted cooperatively with Ca^{2+}, but not Mg^{2+}, with K^{+} having a greater effect than Na^{+} (Fig. 2.2A and 2.2C).

Similarly, the optical density readings for *A. flavithermus* DSM 2641 increased, relative to the baseline control, when cultures were supplemented with either Ca^{2+} or Mg^{2+} (Fig. 2.1C and 2.3A). However, cooperative effects of Na^{+} or K^{+} with Ca^{2+} or Mg^{2+} were minimal (Fig. 2.3A).

In contrast, the optical density readings for *Geobacillus* sp. F75 did not increase significantly ($P > 0.05$), relative to the baseline control, when cultures were supplemented with Ca^{2+} or Mg^{2+} (Fig. 2.1E and 2.4A), and there were no cooperative effects of Na^{+} or K^{+} with Ca^{2+} or Mg^{2+} (Fig. 2.4A and 2.4C).

The optical density readings for *G. thermoleovorans* DSM 5366 increased, relative to the baseline control, when cultures were supplemented with Ca^{2+} and Mg^{2+}, with the effect of Mg^{2+} being greater than that of Ca^{2+} (Fig. 2.1G and 2.5A). Both Na^{+} and K^{+} acted cooperatively when supplemented with Ca^{2+} or Mg^{2+}, with K^{+} having a greater effect than Na^{+} (Fig. 2.5A).

2.4.3) Effect of cation concentration

When all four cation types (Na^+, K^+, Ca^{2+}, and Mg^{2+}) were used to supplement the cultures, with total cation concentrations ranging between 2 and 125 mM, the optical densities of *A. flavithermus* DSM 2641 and *G. thermoleovorans* DSM 5366 cultures did not significantly increase as the total cation concentration increased above 2 mM (Fig. 2.1D, 2.1H, 2.3A, 2.3C, 2.5A, and 2.5C). However, the optical densities of *A. flavithermus* E16 and *Geobacillus* sp. F75 cultures did increase as the total cation concentration increased above 2 mM (Fig. 2.1B, 2.1F, 2.2A, 2.2C, 2.4A, and 2.4C).

2.4.4) Effect of cation ratio

For all four isolates studied, when cultures of the same bacterial isolate and the same total cation concentration of either 2 or 125 mM were compared, there was no significant difference in the optical densities of cultures supplemented with ratios of monovalent to divalent cations ranging between 0.5:1 and 30:1 (Fig. 2.2B, 2.2D, 2.3B, 2.3D, 2.4B, 2.4D, 2.5B, and 2.5D). There was one exception. When *A. flavithermus* E16 cultures were supplemented with a cation concentration of 2 mM, there was a noticeable decrease in the optical density of the culture with a monovalent to divalent cation ratio of 30:1, relative to the cultures with monovalent to divalent cation ratios of between 0.5:1 and 10:1 (Fig. 2.2B).

2.4.5) Mg^{2+} protection from Na^+, K^+ or Ca^{2+} inhibition of *Geobacillus* spp. planktonic growth

High individual Na^+, K^+ or Ca^{2+} concentrations of at least 63 mM significantly decreased the optical density of cultures of the two *Geobacillus* spp. isolates relative to the respective baseline controls (Fig. 2.6 and 2.7). When Mg^{2+} was used as a supplement alone, concentrations of at least 250 mM significantly decreased the optical

density of cultures of the two *Geobacillus* spp. isolates relative to the respective baseline controls (Fig. 2.6D and 2.7D). When comparing cultures grown in media supplemented with Na^+, K^+ Ca^{2+} and Mg^{2+}, where the concentration of each individual cation was 63 mM, the optical densities of the cultures of the two *Geobacillus* spp. isolates were often significantly greater when Mg^{2+} was present, relative to cultures unsupplemented with Mg^{2+} (Fig. 2.8). Furthermore, cultures unsupplemented with Mg^{2+} often showed optical densities significantly lower than the baseline controls, and cultures supplemented with Mg^{2+} were equal to or significantly greater than the baseline controls (Fig. 2.8). In contrast to the two *Geobacillus* spp. isolates, the optical density of cultures of the two *A. flavithermus* isolates did not significantly decrease relative to the respective baseline controls in cultures supplemented with individual cation (Na^+, K^+, Ca^{2+} or Mg^{2+}) concentrations of up to 250 mM (Fig. 2.9 and 2.10).

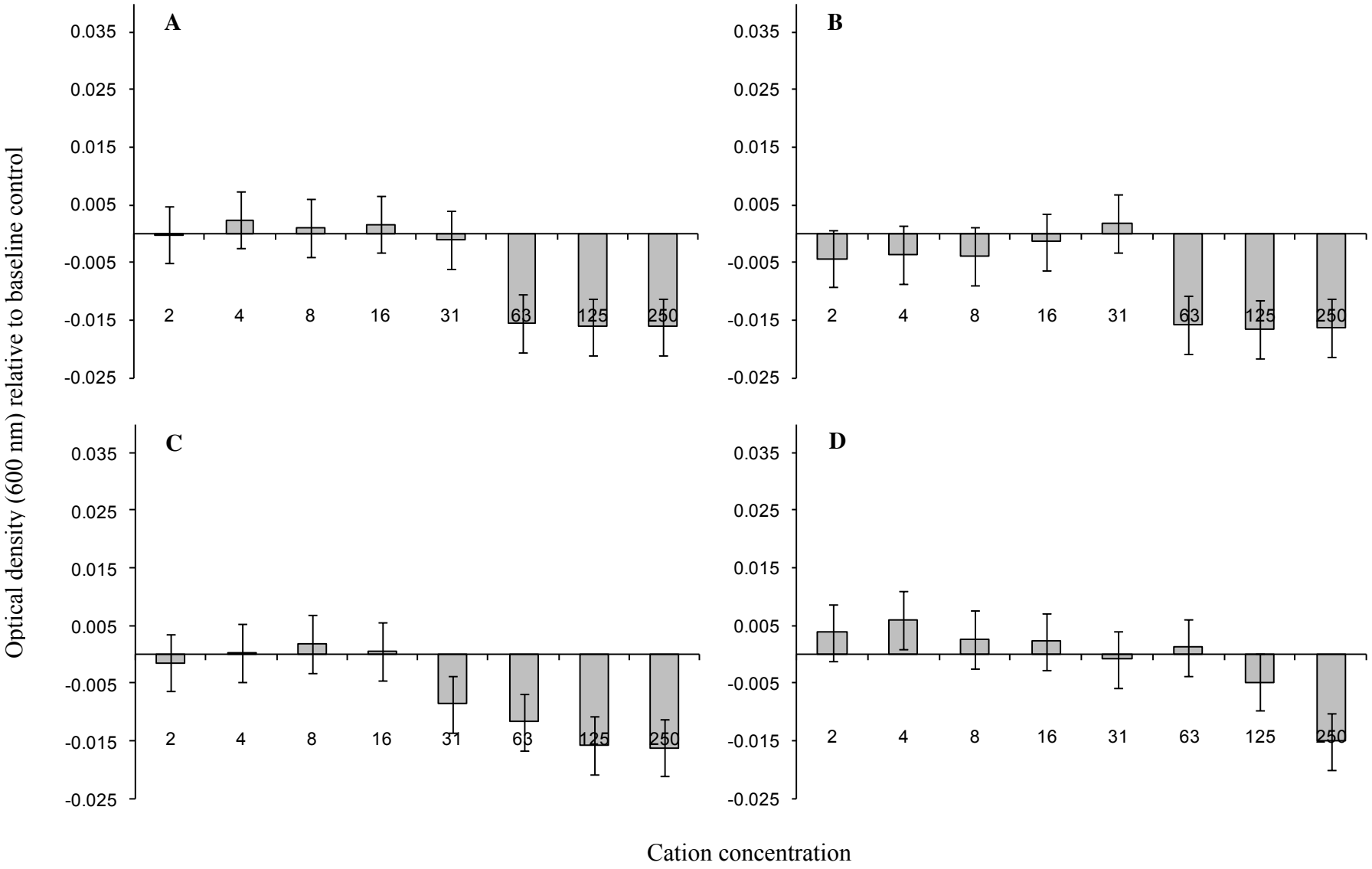
A
B
C
D
0.035
0.025
0.015
0.005
-0.005
-0.015
-0.025
2
4
8
16
31
63
125
250
Optical density (600 nm) relative to baseline control
Cation concentration

FIG 2.6

FIG. 2.6 Optical density of *Geobacillus* sp. F75 grown in casein digest medium (1 g l^{-1}) supplemented with 2 – 250 mM of either Na^{+} (A), K^{+} (B), Ca^{2+} (C) or Mg^{2+} (D), relative to the optical density of the bacterial isolate grown in casein digest medium (1 g l^{-1}) unsupplemented with cations (baseline control). The cultures were incubated at 55°C for 10 h. Two replicates were done, and each replicate consisted of an average of triplicate cultures. The error bars represent 95% confidence intervals ($P \leq 0.05$), which were determined using SAS statistical analysis software.

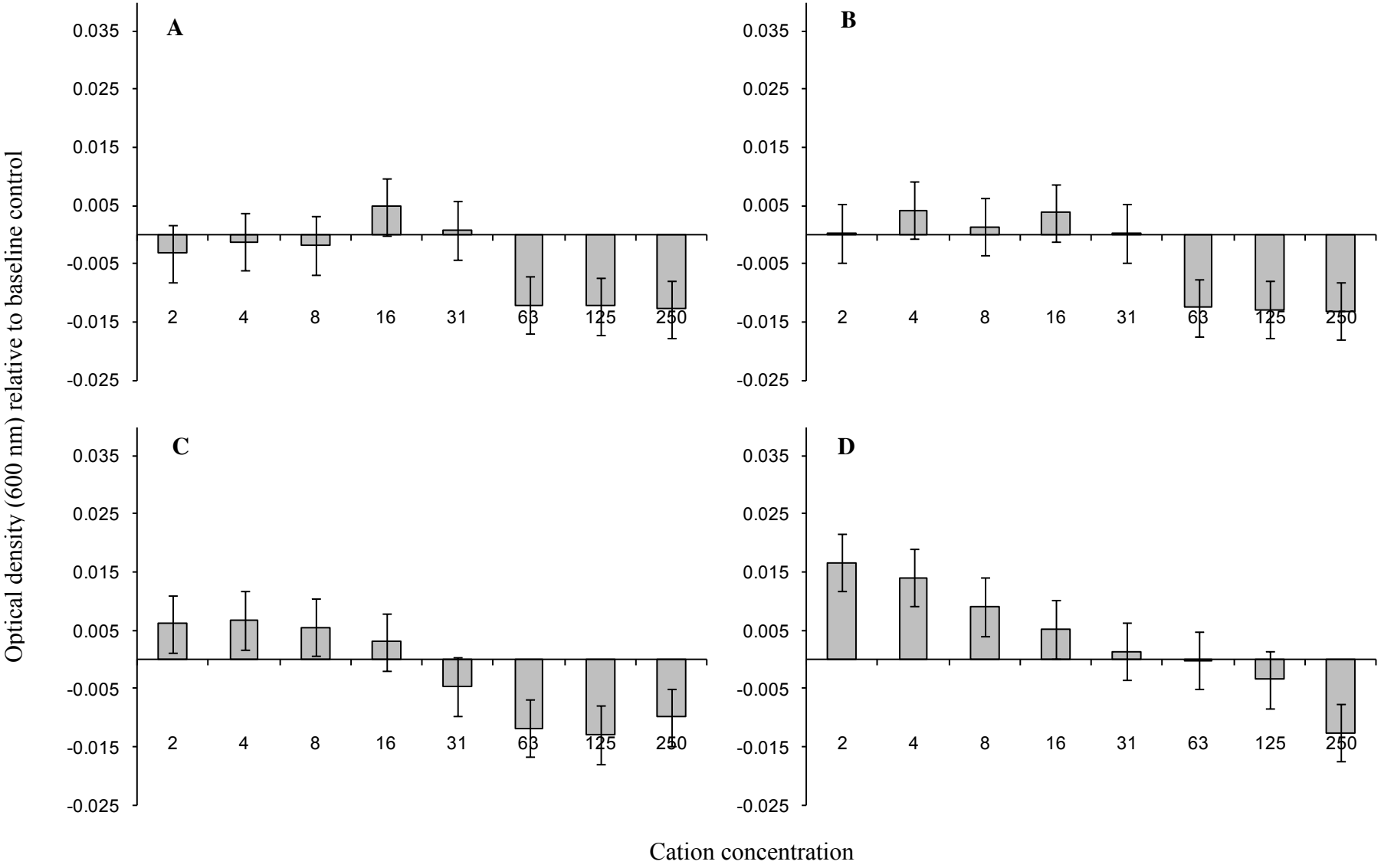
A
B
C
D
0.035
0.025
0.015
0.005
-0.005
-0.015
-0.025
2
4
8
16
31
63
125
250
Optical density (600 nm) relative to baseline control
Cation concentration

FIG 2.7

FIG. 2.7 Optical density of *G. thermoleovorans* DSM 5366 grown in casein digest medium (1 g l^{-1}) supplemented with 2 – 250 mM of either Na^+ (A), K^+ (B), Ca^{2+} (C) or Mg^{2+} (D), relative to the optical density of the bacterial isolate grown in casein digest medium (1 g l^{-1}) unsupplemented with cations (baseline control). The cultures were incubated at 55°C for 10 h. Two replicates were done, and each replicate consisted of an average of triplicate cultures. The error bars represent 95% confidence intervals ($P \leq 0.05$), which were determined using SAS statistical analysis software.

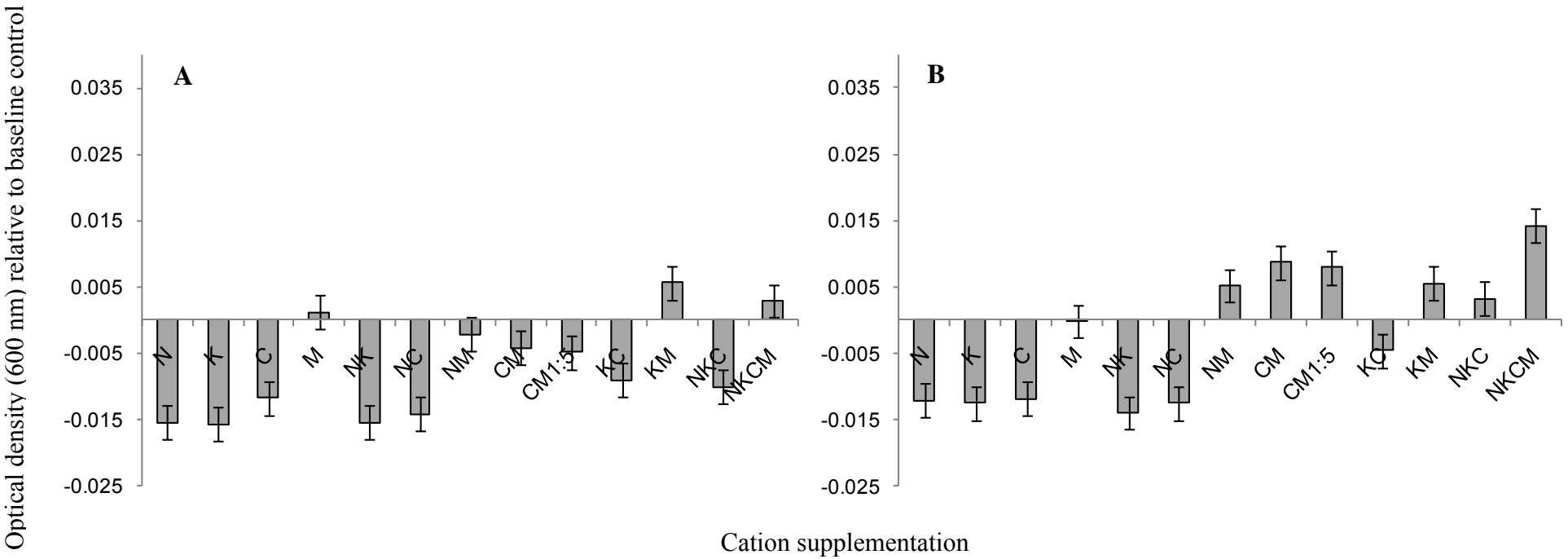
A
B
Optical density (600 nm) relative to baseline control
0.035
0.025
0.015
0.005
-0.005
-0.015
-0.025
N
K
C
M
NK
NC
NM
CM
CM1:5
KC
KM
NKC
NKCM
Cation supplementation

FIG 2.8

FIG. 2.8 Optical density of *Geobacillus* sp. F75 (A) and *Geobacillus* sp. DSM 5336 (B) grown in casein digest medium (1 g l^{-1}) supplemented with a variety of cation proportions, relative to the optical density of the bacterial isolate grown in casein digest medium (1 g l^{-1}) unsupplemented with cations (baseline control). N, K, C, and M designate free Na^+, K^+, Ca^{2+}, and Mg^{2+}, respectively, CM 1:5 refers to a Ca^{2+}:Mg^{2+} ratio of 1:5, NKC refers to a Na^+:K^+:Ca^{2+} ratio of 1:1:2, and all other treatments have equal proportions of each supplemented cation. For all treatments, whenever a cation is supplemented, it is supplemented at a concentration of 63 mM, except for the NKC treatment, where in this instance Na^+ and K^+ are each supplemented at concentrations of 31 mM. The cultures were incubated at 55°C for 10 h. Two replicates were done, and each replicate consisted of an average of triplicate cultures. The error bars represent 95% confidence intervals ($P \leq 0.05$), which were determined using SAS statistical analysis software.

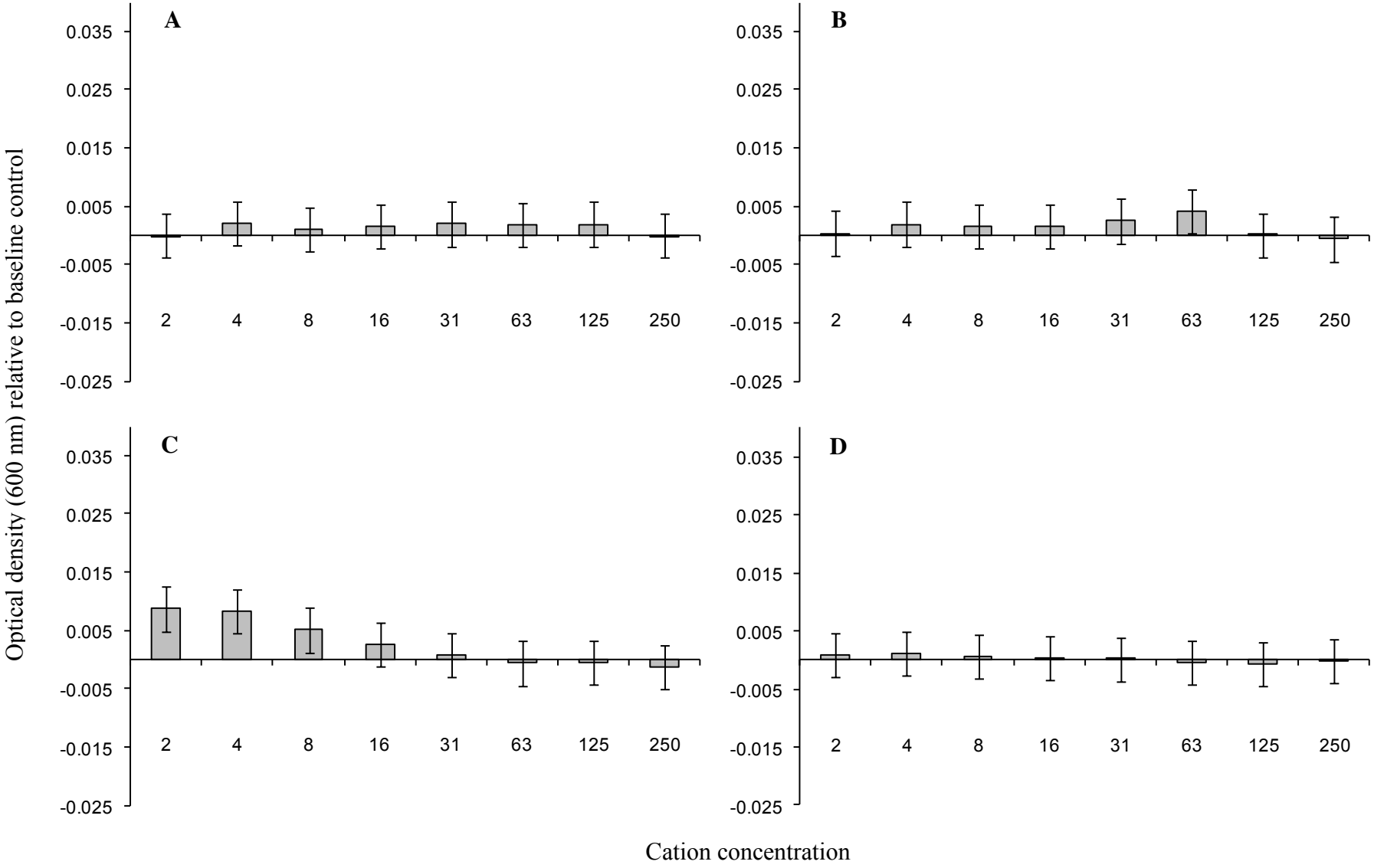
A
B
C
D
0.035
0.025
0.015
0.005
-0.005
-0.015
-0.025
2
4
8
16
31
63
125
250
Optical density (600 nm) relative to baseline control
Cation concentration

FIG 2.9

FIG. 2.9 Optical density of *A. flavithermus* E16 grown in casein digest medium (1 g l^{-1}) supplemented with 2 – 250 mM of either Na^{+} (A), K^{+} (B), Ca^{2+} (C) or Mg^{2+} (D), relative to the optical density of the bacterial isolate grown in casein digest medium (1 g l^{-1}) unsupplemented with cations (baseline control). The cultures were incubated at 55°C for 10 h. Two replicates were done, and each replicate consisted of an average of triplicate cultures. The error bars represent 95% confidence intervals ($P \leq 0.05$), which were determined using SAS statistical analysis software.

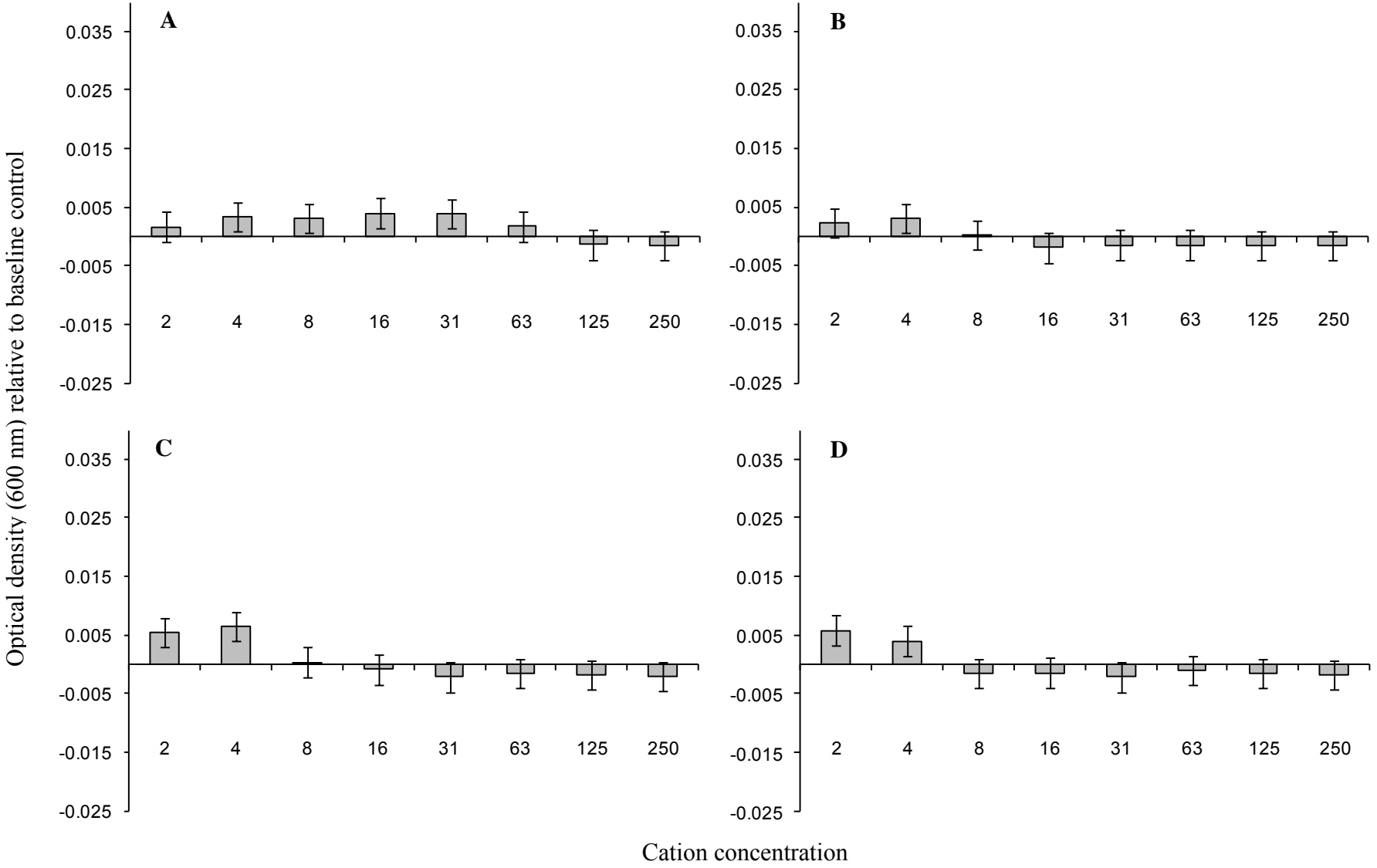
A
B
C
D
0.035
0.025
0.015
0.005
-0.005
-0.015
-0.025
2
4
8
16
31
63
125
250
Optical density (600 nm) relative to baseline control
Cation concentration

FIG 2.10

FIG. 2.10 Optical density of *A. flavithermus* DSM 2641 grown in casein digest medium (1 g l^{-1}) supplemented with 2 – 250 mM of either Na^{+} (A), K^{+} (B), Ca^{2+} (C) or Mg^{2+} (D), relative to the optical density of the bacterial isolate grown in casein digest medium (1 g l^{-1}) unsupplemented with cations (baseline control). The cultures were incubated at 55°C for 10 h. Two replicates were done, and each replicate consisted of an average of triplicate cultures. The error bars represent 95% confidence intervals ($P \leq 0.05$), which were determined using SAS statistical analysis software.

2.4.6) Total viable cell counts

After 10 h of growth, the average total viable cell counts of the cultures were similar and ranged between 5.8 and 7.3 log CFU ml^{-1} (Table 2.2). When the counts obtained from cultures of the same bacterial isolate were compared, there was a maximum difference of 0.8 log CFU ml^{-1}. When counts obtained from cultures with the same cation concentration were compared, there was a maximum difference of 1.5 log CFU ml^{-1}.

TABLE 2.2 Total viable cell (TVC) and spore (Spore) counts, as log CFU ml^{-1}, of bacterial cultures grown in casein digest medium (1 g l^{-1}) supplemented with a cation concentration of 0 mM (baseline control), 2 mM, or 125 mM (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}) after 10 h of incubation at 55°C[a]

Bacterial isolate	Cation supplementation					
	0 mM (baseline control)		2 mM $Na^+/K^+/Ca^{2+}/Mg^{2+}$		125 mM $Na^+/K^+/Ca^{2+}/Mg^{2+}$	
	TVC	Spore	TVC	Spore	TVC	Spore
A. flavithermus E16	6.6	<0.5	6.2	5.3	6.4	4.8
A. flavithermus DSM 2641	6.3	<0.5	6.0	<0.5	6.8	<0.5
Geobacillus sp. F75	7.0	<0.5	7.3	2.5	7.3	3.0
G. thermoleovorans DSM 5366	5.8	1.1	6.3	1.1	6.2	1.0

[a] All the total viable cell and spore count SDs (σ_{n-1}) were <1.0 log and ≤1.1 log, respectively (n = 3).

2.4.7) Spore counts

A. flavithermus E16 and *Geobacillus* sp. F75 produced higher spore counts in cultures supplemented with cation concentrations of 2 and 125 mM (ranging from 2.5 to 5.3 log CFU ml^{-1}), compared to unsupplemented cultures (Table 2.2). *A. flavithermus* DSM 2641 and *G. thermoleovorans* DSM 5366 produced minimal spore counts in the cultures regardless of the extent of cation supplementation. No spores were detected in any of the *A. flavithermus* DSM 2641 cultures. The spore counts for *G. thermoleovorans* DSM 5366 were approximately 1.0 log CFU ml^{-1}.

2.4.8) Quantification of bacterial surface protein and polysaccharide in *A. flavithermus* E16 culture

After high-speed centrifugation, the amount of protein, and to a lesser extent the amount of polysaccharide, associated with the pellet of *A. flavithermus* E16 cultures, per CFU, increased with increasing concentration of cation supplementation (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}) (Fig. 2.11).

In order to estimate the surface protein and polysaccharide concentrations per milliliter in the original culture, the amounts of surface protein and surface polysaccharide determined per CFU in the 5 ml concentrate were multiplied by the total viable cell count determined in the original culture. The estimated average values of surface protein and polysaccharide per milliliter of original culture were 0.42, 6.5, and 8.3 μg ml^{-1} for protein and 0.21, 1.5, and 1.7 μg ml^{-1} for polysaccharide, in cultures supplemented with total cation concentrations of 0, 2, and 125 mM (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}), respectively (n = 3).

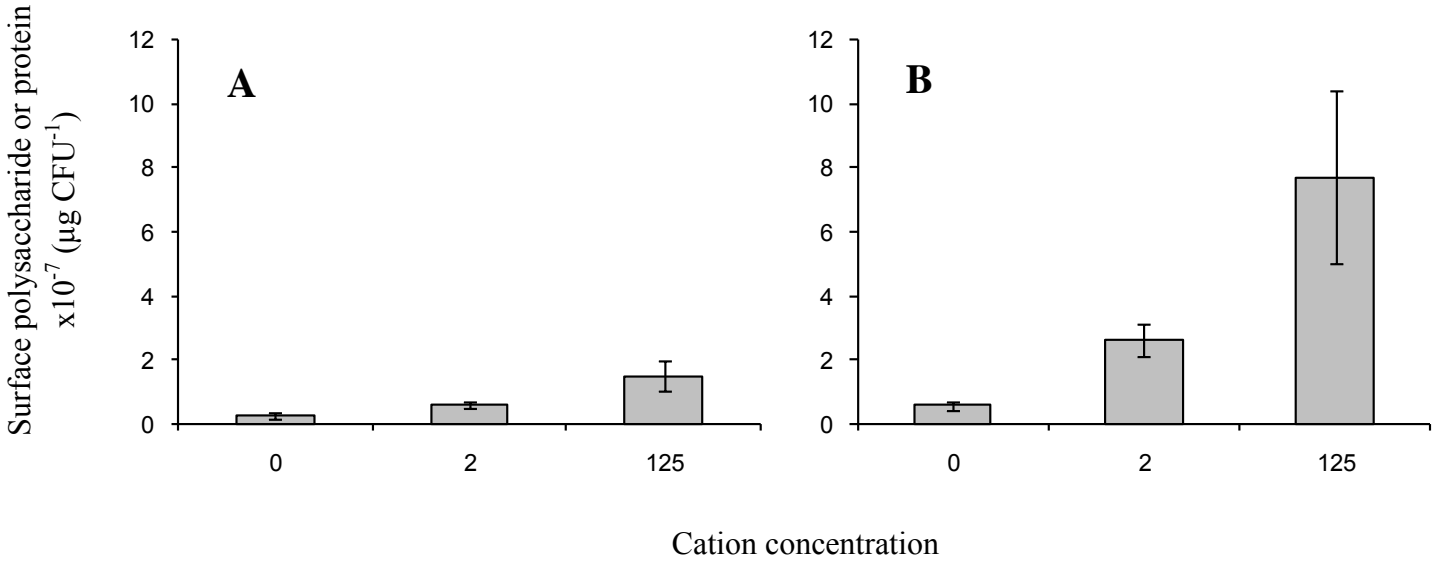

FIG. 2.11 Amount of surface polysaccharide (A) and surface protein (B), associated with the pellet after centrifugation at 11,800 X *g*, per CFU of *A. flavithermus* E16 culture after a 10 h incubation at 55°C, grown in casein digest medium (1 g l^{-1}) supplemented with a total cation concentration of, from left to right, 0, 2, and 125 mM (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}) (n = 3). Error bars represent ± 1 standard deviation (σ_{n-1}).

2.5) Discussion

2.5.1) The relationship between cation composition and optical density of the cultures was unique for each isolate

The response of the bacterial isolates to cation supplementation in planktonic culture was strain specific. Four bacterial isolates were studied; two *Geobacillus* spp. and two *A. flavithermus* isolates were intentionally selected, so that each genus pair would include an isolate derived from a milk powder manufacturing plant and a type (DSM collection) strain. This was done to derive any trends when comparing within and between the origins and genuses of the isolates. The *Geobacillus* spp. cultures had an overall greater optical density than the *A. flavithermus* cultures when grown in a minimal medium that was both unsupplemented and supplemented with cations. It was concluded that *Geobacillus* spp. are more proficient than *A. flavithermus* at increasing the optical density of planktonic cultures when grown in a minimal medium consisting of 1 g l^{-1} of casein digest.

Similarly, other studies have concluded that the optical densities of planktonic bacterial cultures differ depending on their cation composition (Aranha *et al*., 1986; Caldwell & Arcand, 1974; Jurado *et al*., 1987; Morales & Dehority, 2009; Oomes & Brul, 2004) and that, within different species and strains, there is a unique relationship between optical density and cation supplementation or composition of the growth medium (Morales & Dehority, 2009; Vincent, 1962). In contrast, Patrauchan *et al*. (2005) found that, although there were physiological differences between planktonic cultures of a *Pseudoalteromonas* sp. containing either 0.25 or 10 mM Ca^{2+}, the optical density of the cultures were identical.

Differences in the responses of bacteria to different external cation compositions potentially depend on a range of factors, including their capacity to assimilate cations

(Beveridge *et al*., 1982), the cell wall total cation-binding capacity, and the potential to translocate cations to the cell membrane (Hughes *et al*., 1973; Neuhaus & Baddiley, 2003), which depends on the compositions of the cell wall and the extracellular matrix (Vollmer & Seligman, 2010). These responses may also depend on how the cations are utilized, e.g., by enzymes involved in various metabolic pathways (Heptinstall *et al*., 1970; Hughes *et al*., 1973; Onek & Smith, 1992) and by structural components of bacteria (Heptinstall *et al*., 1970; Sobeck & Higgins, 2002; Subramanian *et al*., 2010), and the potential of changes in external cation compositions to elicit gene-regulatory responses, e.g., via signal-transduction pathways, in which the cations act as stimulatory ligands (Shemarova & Nesterov, 2005). Threshold external concentrations of cations that are influential may depend on the bacterial species and strain, and may depend on typical cationic compositions of the niche that the particular bacteria have adapted to occupy (Garrison-Schilling *et al*., 2011; Lambert *et al*., 1975a; Rose & Hogg, 1995). Any of these factors may potentially explain the unique response of different bacterial isolates, and other bacteria, to varied external cation compositions.

2.5.2) The Ca^{2+} and Mg^{2+} concentrations were the predominant factors responsible for the increase in the optical densities of the cultures, whereas the influence of Na^{+} and K^{+} was more pronounced in the presence of Ca^{2+} and Mg^{2+}

a. Effect of cation type

The response of the thermophilic bacilli to Ca^{2+} and Mg^{2+} was predominantly responsible for increases in the optical density of the cultures, whereas the influence of Na^{+} and K^{+} was more pronounced when supplemented together with Ca^{2+} and/or Mg^{2+}. Ca^{2+} has been shown to act as a regulatory ion in bacteria (Shemarova & Nesterov, 2005) and Mg^{2+} is required for the activation or optimization of bacterial enzyme

functions, especially those involved in the biosynthesis of cell wall and extracellular matrix polymers (Hughes *et al*., 1973). Additionally, Ca^{2+} and Mg^{2+} have been shown to stimulate the production of extracellular matrix by bacteria (Corpe, 1964; Liu & Sun, 2011; Tempest *et al*., 1965), and have important roles in stabilizing the cell envelope of bacteria, especially at high temperatures (Mosley *et al*., 1976; Ward & Mooyoung, 1988). Other studies have shown that Ca^{2+} and/or Mg^{2+} are required for optimal bacterial growth and cause physiological responses in bacteria (Aranha *et al*., 1986; Caldwell & Arcand, 1974; Patrauchan *et al*., 2005; Song & Leff, 2006; Vincent, 1962). Ca^{2+} and Mg^{2+} did not act cooperatively with each other to enhance the optical density of thermophilic bacilli cultures in our study. This contrasts with results found using planktonic cultures of *Bacteroides* spp.; Ca^{2+} and Mg^{2+} acted together to enhance the optical density of the cultures (Caldwell & Arcand, 1974).

Similar to our study, Caldwell *et al*. (1974) found that the monovalent cations Na^{+} and K^{+} have a crucial role in the planktonic growth of *Bacteroides* spp. K^{+} has an important role in maintaining optimal osmolarity of the cytosol (Epstein, 2003) and both Na^{+} and K^{+} have important roles in maintaining cytosolic pH, which favors optimal functioning of enzymes in bacteria (Epstein, 2003; Novakova & Smigan, 2008).

Although all four cations have important roles in bacterial physiology, Ca^{2+} and Mg^{2+} perhaps had more influence than Na^{+} and K^{+} on the optical density of planktonic thermophilic bacillus cultures because their roles in bacterial physiology are more closely linked to the responses of the bacteria that caused differences in the optical densities of the cultures. Also, the needs of thermophilic bacilli may be more easily satisfied by the background Na^{+} and K^{+} concentrations in casein digest medium (1 g l^{-1}) (1.0 and 0.03 mM, respectively) than the lower background Ca^{2+} and Mg^{2+} concentrations (0.004 and 0.002 mM, respectively), therefore nullifying the observed effect of Na^{+} and K^{+} supplementation, but allowing for the effects of Ca^{2+} and Mg^{2+}

requirement to be observed. K^+ tended to have a greater cooperative effect than Na^+, which also suggested that there is a greater requirement by thermophilic bacilli for K^+ than Na^+ to increase the optical density of the culture. However, this observed difference may have been due to the higher background concentration of Na^+ than K^+ in casein digest medium (1 g l^{-1}). Supplementing cultures with Na^+ beyond 1 mM may have had no further increasing influence on their optical density. A Na^+ concentration of 1 mM, as present in casein digest medium (1 g l^{-1}), may have been close to the minimum threshold Na^+ requirement of these bacteria.

b. Effect of cation concentration

A wide range of threshold cation concentrations have been shown to be required by different bacteria in a planktonic growth state, ranging between 0.63 µM and 10 mM for Ca^{2+} (Aranha *et al*., 1986; Caldwell & Arcand, 1974; Garrison-Schilling *et al*., 2011; Jurado *et al*., 1987; Morales & Dehority, 2009; Oomes & Brul, 2004; Patrauchan *et al*., 2005; Vincent, 1962) and between 10 µM and 0.1 mM for Mg^{2+} (Caldwell & Arcand, 1974; Jurado *et al*., 1987; Oomes & Brul, 2004; Vincent, 1962), and a sum of 16.4 mM for K^+, Ca^{2+}, and Mg^{2+}, to enable the sporulation of high-temperature-resistant *Bacillus subtilis* spores (Oomes & Brul, 2004). In this study, when a total cation concentration of 2 mM (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}) was used to supplement thermophilic bacillus cultures, maximum or close to maximum optical densities were achieved.

A. flavithermus E16 showed a significant increase in optical density as the cation supplementation of the culture increased from 2 to 125 mM (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}). In contrast, a cation concentration of 2 mM was adequate to elicit maximum optical densities of cultures of *A. flavithermus* DSM 2641 and *G. thermoleovorans* DSM 5336 isolates. Perhaps *A. flavithermus* E16

required, or was able to utilize, concentrations greater than 2 mM to increase its optical density further. The *A. flavithermus* DSM 2641 and *G. thermoleovorans* DSM 5336 isolates may have been more adept at assimilating and/or utilizing cations, so that an increase from 2 to 125 mM had no additional affect.

c. Effect of cation ratio

The influence of a range of monovalent to divalent cation ratios (0.5:1 to 30:1) on the planktonic growth of thermophilic bacilli was investigated. This was done to investigate the potential of high monovalent to divalent cation ratios to inhibit planktonic growth of thermophilic bacilli. Monovalent to divalent cation ratios of approximately greater than 10:1 compromise the structural integrity of wastewater sludge biofilms (Higgins & Novak, 1997). It was found that the monovalent to divalent cation ratio did not influence the optical density of planktonic thermophilic bacilli. Our results may contrast those observed with wastewater sludge biofilms as there may be differences in the response of thermophilic bacilli compared to bacteria which comprise a wastewater sludge, or there may be differences when comparing between the planktonic and biofilm growth states.

The optical density of a culture of *A. flavithermus* E16 supplemented with a total cation concentration of 2 mM, with a monovalent to divalent cation ratio of 30:1, was lower than those of cultures supplemented with a total cation concentration of 2 mM and monovalent to divalent cation ratios from 0.5:1 to 10:1. When the ratios of monovalent to divalent cations were 10:1 and 30:1, the Ca^{2+} concentrations were approximately 0.09 and 0.03 mM, respectively. As it was shown that Ca^{2+} was critical for *A. flavithermus* E16 to enhance the optical density of the culture, it was concluded that the minimum Ca^{2+} concentration threshold required for an increase in culture optical density lay between 0.03 and 0.09 mM. The optical densities of cultures of *A.*

flavithermus DSM 2641 and *G. thermoleovorans* DSM 5366 supplemented with a total cation concentration of 2 mM, with a monovalent to divalent cation ratio of 30:1, were similar to those of cultures supplemented with a total cation concentration of 2 mM and monovalent to divalent cation ratios of less than 30:1. This implied that, for these isolates, the minimum Ca^{2+} or Mg^{2+} concentration threshold required to enhance the optical density was less than 0.03 mM, for either cation.

2.5.3) Mg^{2+} protection from Na^{+}, K^{+} or Ca^{2+} inhibition of *Geobacillus* spp. planktonic growth

Mg^{2+} protected the two *Geobacillus* spp. isolates against relatively high, inhibitory concentrations of Na^{+}, K^{+} or Ca^{2+} during planktonic growth. At individual Na^{+}, K^{+} or Ca^{2+} concentrations of 63 mM or greater, intracellular concentrations of either cation may have increased to toxic levels and/or the *Geobacillus* spp. isolates may have had to increase energy expenditure to efflux either of the cations. This may have slowed the growth of the *Geobacillus* spp. isolates. Mg^{2+} may have acted protectively by competitively excluding the assimilation of Na^{+}, K^{+} and Ca^{2+} into the cell wall and the cell wall-cytoplasmic membrane space. *Geobacillus* spp. may be more tolerant to high external Mg^{2+} concentrations, than high external Na^{+}, K^{+} and Ca^{2+} concentration, as Mg^{2+} may have less permeability across the cytoplasmic membrane and/or increases in Mg^{2+} influx may have less impact than the other ions on imbalances in cellular homeostasis.

Planktonic growth of the two *A. flavithermus* isolates was not inhibited by high external cation concentrations. Perhaps *A. flavithermus* is more adept than *Geobacillus* spp. at tolerating high external cation concentrations, either by preventing the entry of cations into the cell cytosol, or by utilising more effective or efficient cation efflux pumps.

Results indicate that *Geobacillus* spp. which are deprived of Mg^{2+} are susceptible to growth inhibition by high Na^{+}, K^{+} or Ca^{2+} concentrations (greater than 63 mM).

2.5.4) *A. flavithermus* E16 responded to increasing cation concentrations, with equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}, by producing increased amounts of surface protein

A. flavithermus E16 responded to increasing cation concentrations by increasing its production of surface protein. There are two possible explanations for the observed increase in surface protein. Firstly, as has been described previously, cations create an environment that is favorable for optimal enzyme functionality; therefore, the scope of the metabolic diversity of the bacteria may have widened and a greater amount of enzymes may have been incorporated into the surface of the bacteria in response to the increase in external cation concentration (Flemming & Wingender, 2010). Secondly, the bacteria may have incorporated a greater amount of structural proteins into their surface (Flemming & Wingender, 2010; Van Houdt & Michiels, 2010).

Similarly to our study, Liu and Sun (2011) found that Ca^{2+} stimulated a wastewater sludge to increase its extracellular protein production, whereas the extracellular polysaccharide concentration remained unchanged and far lower than the extracellular protein concentration. Many factors influence the composition of a bacterial extracellular matrix (Bosch *et al*., 2006; Karunakaran & Biggs, 2011; Kives *et al*., 2006; Omoike & Chorover, 2004; Ras *et al*., 2011; Subramanian *et al*., 2010; Vandevivere & Kirchman, 1993). One of these factors is the carbon-to-nitrogen ratio (Corpe, 1964; Durmaz & Sanin, 2001; Kumar *et al*., 2007), which is indicative of the proportions of carbohydrate and protein available to the bacteria. In this study, the carbon-to-nitrogen ratio of the growth medium would have been relatively low, because the medium consisted of casein digest. If the bacteria had been grown in a medium that contained a significant carbohydrate source, perhaps greater amounts of surface polysaccharide would have been produced.

2.5.5) It is proposed that the underlying factor that caused significant differences in the optical density of the cultures in response to different cation compositions was a difference in the amount of surface protein produced, rather than a difference in total viable cell counts, spore counts, cell size, or the production of surface polysaccharide

The optical density of a bacterial culture can depend on a range of factors such as culture biomass, which is determined by the concentration of both bacterial cells and bacterial-derived extracellular polymers, and the size, shape, and optical properties of particles within the culture (Griffiths *et al*., 2011).

Phase contrast light microscopy showed that there was a consistent cell size, shape, and appearance of the thermophilic bacilli in the cultures when the different bacterial isolates and different cation supplementation compositions were compared (results not shown). The bacteria did not aggregate in planktonic culture (results not shown). Thus, it was concluded that cell size, shape, and co-aggregation were not factors that influenced the optical densities of the cultures.

When cultures supplemented with different cation compositions were compared, the extent of the difference in the optical density of the cultures did not consistently correlate with the extent of the difference in total viable cell counts. For example, when comparing *A. flavithermus* E16 cultures supplemented with three different cation concentrations of 0, 2 and 125 mM (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}) after 10 h of growth, the optical density of each culture was significantly different from eachother, however, the total viable cell counts of the cultures were similar. It was concluded that the total viable cell count of the cultures was not the predominant factor that influenced the optical density of the cultures.

Spores can have a greater potential than vegetative cells to influence the optical density of a suspension as they can have greater refractory properties (Rippey &

Watkins, 1992). Compared with the other isolates studied, the *G. thermoleovorans* DSM 5366 cultures had both the greatest absolute optical density and the greatest difference in optical density between cultures supplemented with cations and the baseline control; however, the cultures had similar and relatively low spore counts of approximately 1.0 log CFU ml^{-1}. It was found that substantial differences in spore counts were not necessarily required to observe differences in the optical density of the cultures. Furthermore, *Geobacillus* sp. F75 cultures had significant sporulation (2.5–3.0 log CFU ml^{-1}) only when the cultures were supplemented with cations; however, the difference in the measured optical density, when comparing between cultures supplemented with cations and the baseline control, was relatively low. This suggests that spore counts of around 3.0 log CFU ml^{-1} did not greatly increase the optical densities of the cultures. Even though there was a significant difference between the optical densities of *A. flavithermus* E16 cultures supplemented with either 2 or 125 mM cations (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}), the spore counts of the two cultures were comparable. This showed that different cultures that have comparable total viable cell and spore counts can still have significantly different optical densities. Collectively, these findings suggested that neither the total viable cell count nor the spore count of the cultures had a predominant influence on their optical densities.

After the thermophilic bacillus planktonic cultures were centrifuged at 10,000 X *g*, the optical density of the supernatant was similar to that of the uninoculated medium, and the optical density of the re-suspended pellet was similar to that of the culture prior to centrifugation (results not shown). This was observed for all thermophilic bacillus isolates tested. Thus, it was assumed that the factor that had a predominant influence on the optical density of the planktonic cultures was associated with the pellet, formed after high-speed centrifugation, rather than the culture supernatant. The amount of bacterial

surface protein and polysaccharide associated with the pellet was therefore analyzed and quantified, to investigate its potential influence on the optical density of the cultures.

A. flavithermus E16 cultures supplemented with three different cation concentrations of 0, 2 and 125 mM (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}) were chosen for surface protein and polysaccharide quantification, as the optical density of these cultures were significantly different from eachother, after 10 h of growth. The amount of surface protein in the three cultures studied correlated with their optical densities, with both the amount of surface protein and the optical density increasing with increasing cation concentration. An increase in protein on the surface of *A. flavithermus* E16 may have increased the optical density of the culture either by increasing the culture biomass or by increasing the refraction of light due to changes in the optical properties of the cell surfaces (Griffiths *et al*., 2011). It was concluded that the predominant factor that influenced the optical density of the cultures was the amount of surface protein produced, rather than differences in total viable cell counts, spore counts, cell size, or the production of surface polysaccharide.

It was also speculated that differences in the optical densities of cultures of the other three thermophilic bacilli isolates, in response to different external cation compositions, were also due to differences in the amount of surface protein produced by the bacteria. As it was found that Ca^{2+} and Mg^{2+} were the predominant influences on the optical densities of the planktonic cultures, and the optical density depended on the extent of surface protein production, it was also speculated that Ca^{2+} and Mg^{2+} stimulated surface protein production by the thermophilic bacilli.

2.6) Conclusions

The relationship between the cation composition and the optical density of the planktonic cultures was unique for each thermophilic bacillus isolate studied. Generally, Ca^{2+} and Mg^{2+} were predominantly responsible for increases in the optical densities of the cultures, whereas the influence of Na^{+} and K^{+} was more pronounced in the presence of Ca^{2+} and Mg^{2+}. High Na^{+}, K^{+} or Ca^{2+} concentrations (63 – 250 mM) significantly decreased the optical density of *Geobacillus* spp. cultures, whereas *A. flavithermus* cultures were unaffected by the high cation concentrations. Mg^{2+} protected *Geobacillus* spp. from inhibitory concentrations of Na^{+}, K^{+} or Ca^{2+} (63 – 250 mM). It is proposed that the underlying factor that caused significant differences in the optical density of the cultures, in response to different cation compositions, was differences in the amount of surface protein produced, rather than differences in total viable cell counts, spore counts, cell size, cell aggregation or the production of surface polysaccharide. These findings indicate that Ca^{2+} and Mg^{2+} stimulate surface protein production by thermophilic bacilli. Overall, this study indicates that cations, particularly Ca^{2+} and Mg^{2+}, are required for the metabolic processes of thermophilic bacilli to optimally proceed.

CHAPTER 3

Preconditioning with cations increases the attachment of *Geobacillus* species and *Anoxybacillus flavithermus* to stainless steel

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Ben Somerton

Name/Title of Principal Supervisor: Steve Flint

Name of Published Research Output and full reference:

Somerton, B., Palmer, J., Brooks, J., Flint, S. & Lindsay, D. (2013). Preconditioning with cations increases the attachment of Anoxybacillus flavithermus and Geobacillus species to stainless steel. Appl Environ Microbiol 79, 4186-4190.

In which Chapter is the Published Work: Chapter 3

Please indicate either:

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  and / or

- Describe the contribution that the candidate has made to the Published Work:

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Date: 2013.11.19 11:05:21 +13'00'

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19 November 2013

Date

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19 November 2013

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3.1) Abstract

The effect of cations on attachment after 30 min and biofilm formation after 6 h by *A. flavithermus* E16 and *Geobacillus* sp. F75 on 316 stainless steel coupons was investigated. Attached viable cells were enumerated after incubating coupons and bacteria in both a casein digest medium supplemented with a range of Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations (0–125 mM) and commercial milk formulations that had a range of intrinsic cation concentrations. Additionally, the effect of preconditioning planktonic *A. flavithermus* E16 and *Geobacillus* sp. F75 with cations or milk formulations, prior to attachment studies, and its subsequent effect on attachment and biofilm formation was studied. Attachment and biofilm formation by bacteria was not altered when the ionic strength of the casein digest medium ranged between 2 and 125 mM, or when monovalent to divalent cation ratios of 2:1 and 10:1 were compared. However, biofilm formation after 6 h by *Geobacillus* sp. F75 tended to decrease as the monovalent to divalent cation ratio of milk formulations increased. Preconditioning the bacteria with cations or milk formulations before attachment experiments often significantly increased ($P \leq 0.05$) the number of viable cells that attached to stainless steel after 30 min and up to 6 h (by up to 1.5 log CFU cm^{-2}) compared with unconditioned bacteria. It is proposed that the transition of *A. flavithermus* and *Geobacillus* spp. from milk formulations to stainless steel product-contact surfaces in milk powder manufacturing plants is predominantly mediated by bacterial physiological factors (e.g. surface-exposed adhesins), rather than the concentration of cations in milk formulations surrounding bacteria.

3.2) Introduction

A biofilm is described as microorganisms attached to a surface; they are often embedded within a matrix of polymers and other molecules that either originate from microorganisms in the biofilm or are absorbed from the surrounding environment (Flemming & Wingender, 2010).

Cations have two main effects on the structural integrity and proliferation of a bacterial biofilm. The first is a direct effect, such that cations interact electrostatically with surface-exposed and cell-wall-embedded polymers and the surfaces to which they attach (Hermansson, 1999; Sobeck & Higgins, 2002). The outer surfaces of bacteria generally have an overall negative charge because bacterial cell wall and extracellular matrix polymers have an abundance of negatively charged functional groups (Flemming & Wingender, 2010; Swoboda *et al*., 2010). Stainless steel also has a negative surface charge (Palmer *et al*., 2007). Thus, there is an extent of electrostatic repulsion between bacteria and the stainless steel surface to which they attach. Factors such as the ionic strength (Palmer *et al*., 2007), the ratio of the concentrations of monovalent to divalent cations in solution (Higgins & Novak, 1997), and the proportion of divalent cation bridges in a biofilm matrix (Sobeck & Higgins, 2002) have the potential to alter the extent of electrostatic repulsion in a biofilm.

The second main effect that cations have on biofilm formation is an indirect effect, such that bacteria may respond to changes in concentrations of cations in their surroundings by adjusting their metabolism and physiology (Garrison-Schilling *et al*., 2011; Kara *et al*., 2008; Patrauchan *et al*., 2005). These bacterial responses may indirectly influence their ability to transition from a planktonic form to an irreversibly attached form and prosper as a biofilm (Garrison-Schilling *et al*., 2011; Kara *et al*., 2008; Patrauchan *et al*., 2005). Ca^{2+} and Mg^{2+} have been shown to stimulate exopolysaccharide (Corpe, 1964; Patrauchan *et al*., 2005; Tempest *et al*., 1965) and

extracellular protein production by bacteria (Cruz *et al*., 2012; Goode & Allen, 2011; Liu & Sun, 2011), and often have roles in assisting the initial reversible association of bacteria with a surface or enhancing the cohesion of a biofilm (Flemming & Wingender, 2010; Sutherland, 2001a). Na^+ has been shown to stimulate bacteria to increase the proportion of negatively charged, hydrophilic polymers in a wastewater sludge and therefore to have a detrimental effect on its cohesion (Kara *et al*., 2008). The functionalities of some regulatory proteins, including response regulators, are influenced by Ca^{2+} and Mg^{2+} (He *et al*., 2008; Michiels *et al*., 2002), which may have implications in the regulation of biofilm formation.

Geobacillus spp. and *Anoxybacillus flavithermus* are thermophilic bacilli that are the predominant bacteria that contaminate milk powder (Burgess *et al*., 2010; Hill & Smythe, 2012). Thermophilic bacilli attach to and form biofilms on product-contact surfaces in milk powder manufacturing plants, which is typically comprised of stainless steel (Burgess *et al*., 2010; Hill & Smythe, 2012). Unprocessed milk typically has total (sum of bound and free) Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations of 22, 37, 30, and 5 mM, respectively (Fox, 2003); however, these concentrations can be manipulated during processing. Some milk formulations have total Na^+ concentrations as high as 100 mM, and total Ca^{2+} and Mg^{2+} concentrations as low as 7 and 1 mM, respectively. To gain insights into the extent of the influence that different cations have on biofilm formation by thermophilic bacilli, we investigated the effect of Na^+, K^+, Ca^{2+}, and Mg^{2+} on attachment to stainless steel and biofilm formation by *A. flavithermus* E16 and *Geobacillus* sp. F75 in both a casein digest medium and milk formulations. Additionally, bacteria were grown planktonically in the presence of cations prior to inoculation of stainless steel coupons. This was done to investigate if any physiological responses of the bacteria to the cations may influence subsequent attachment and biofilm formation.

Many studies have investigated the effect of cations on attachment, biofilm formation, and the physiology/phenotype of a range of bacterial species (Garrison-Schilling *et al*., 2011; Higgins & Novak, 1997; Patrauchan *et al*., 2005; Zhu *et al*., 2009). However, all studies to date have investigated the effect of a few cation types or only one aspect of the biofilm formation process. Our study is the first to investigate how various concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+} collectively influence attached viable cell numbers of single-species bacterial cultures throughout biofilm formation. In addition, our study is the first to investigate the effect of the monovalent to divalent cation ratio on attached viable cell numbers of bacteria that form biofilms in the milk processing industry.

3.3) Methods

3.3.1) Bacterial isolates and media

A. flavithermus E16 and *Geobacillus* sp. F75 were isolated from product-contact surfaces at a milk powder manufacturing plant. Casein digest medium (1 g l^{-1}) (Difco, BD Biosciences, Sparks, MD) was used because it had low background Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations of approximately 1.0, 0.03, 0.004, and 0.002 mM (Biosciences, 2006), respectively; therefore, the effect of the supplementation of cations (Table 3.1) on biofilm formation could be studied. Casein digest medium was supplemented with analytical grade NaCl, KCl, $CaCl_2.2H_2O$, or $MgCl_2.6H_2O$ powders (Merck, Darmstadt, Germany) with cation concentrations detailed in Table 3.1. Each of milk formulations 1–4 (Fonterra, New Zealand) had similar fat, protein, and lactose concentrations but different total (bound and free) Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations, and incremental differences in monovalent to divalent cation ratios (Tables 1.2 and 1.5). Each of milk formulations 1–4 were derived from the same respective batches throughout experimentation. Prior to reconstitution, the milk powders were gamma-irradiated (25,000 Gy) to inactivate any contaminating

microorganisms so that growth and analysis of the inoculated bacteria of interest was unimpeded. The milk powders were reconstituted with water that had been deionized by reverse osmosis and autoclaved (121°C, 15 mins) to sterilize.

TABLE 3.1 Cation supplementation concentrations (profiles) in casein digest medium (1 g l^{-1})

Cation profile	Na^+ (mM)	K^+ (mM)	Ca^{2+} (mM)	Mg^{2+} (mM)
0 mM	–	–	–	–
2 mM Ca^{2+}	–	–	2.00	–
2 mM Mg^{2+}	–	–	–	2.00
31 mM 2:1 [a]	10.4	10.4	5.21	5.21
31 mM 10:1 [a]	14.2	14.2	1.42	1.42
125 mM 2:1 [a]	41.7	41.7	20.8	20.8
125 mM 10:1 [a]	56.8	56.8	5.68	5.68

[a] 2:1 and 10:1 represent supplemented monovalent to divalent cation ratios of 2:1 and 10:1, respectively.

3.3.2) Culture storage

Both isolates were grown in tryptic soy broth (Merck, Darmstadt, Germany) to mid-log phase and stored with the addition of glycerol (10%, vol/vol) (Merck, Darmstadt, Germany) at –80°C.

3.3.3) Inoculum preparation

Cultures for use in the attachment and biofilm formation assays were prepared by inoculating 1 ml of a thawed bacterial culture into 100 ml of medium with one of the following compositions:

- casein digest medium unsupplemented with cations (unconditioned)
- casein digest medium supplemented with cations alone (using the cation profiles detailed in Table 3.1) (preconditioned with cations)
- casein digest medium supplemented with cations and lactose monohydrate (1 g l^{-1}) (Merck, Darmstadt, Germany) (preconditioned with cations and lactose)
- milk formulations 1–4 (preconditioned with milk formulation)

The inoculated media were incubated at 55°C for 9 h, which was sufficient time for the bacteria to reach stationary growth phase.

3.3.4) Attachment and biofilm formation assay

Stainless steel coupons were cleaned and passivated prior to use in the biofilm formation assay, as previously described by Flint *et al*. (1997b). After bacteria had been grown planktonically, for all cultures, except those preconditioned with milk formulation (1–4), 10 ml of culture was centrifuged at 10,000 X *g*, and the pellet was resuspended in 10 ml of fresh media to be used during the attachment and biofilm formation assay. Centrifugation and pellet resuspension was done to minimise cation

carry-over from the tryptic soy broth into the attachment and biofilm formation assay media. The resuspended cultures, and cultures preconditioned with milk formulation, were diluted (typically with a dilution factor of around 1:200) in medium to be used during the attachment and biofilm formation assays to achieve an inoculum of approximately 4.5 log CFU ml; 1.5 ml of this inoculum was added per well of a 24-well culture plate (BD, Franklin Lakes, NJ). Bacteria preconditioned with cations were diluted into media with the same cation profile used during preconditioning, for use in the attachment and biofilm formation assays. One 316 stainless steel coupon (Part #RD128-316) (Biosurface Technologies Corporation, Bozeman, MT), which had a surface area of approximately 4 cm^2, was placed into each inoculum using sterile forceps, so that it was fully submerged and horizontal. After the culture plate had been wrapped in a plastic bag to prevent the evaporation of water from the cultures, it was incubated at 55°C for either 30 min or 6 h.

The coupons were incubated for 30 min in the biofilm formation assay to investigate the effect of cations on the transition of bacteria from the planktonic phase to the irreversibly attached phase. Preliminary experments showed that after the coupons had been incubated for 6 h in the casein digest medium (1 g l^{-1}), a maximum number of attached viable cells was reached, therefore, this incubation time was used to investigate the effect of cations on the maximum extent of biofilm formation reached within the limits of this assay.

3.3.5) Cell enumeration

The following protocol was used to enumerate the attached viable cells per square centimeter on the coupons. The coupons were removed from the cultures using sterile forceps, dipped and rinsed three times in approximately 50 ml of deionized water to

remove any loosely attached cells, and placed into a 35 ml plastic container (Item Code LBS3722W, Thermo Fisher Scientific, New Zealand) with 5 ml of fresh casein digest medium (1 g l^{-1}) and 12 g of glass beads that had a diameter of 6.35 mm (Catalogue # 11079635, Biospec Products, Inc., Bartlesville, OK). The plastic containers were vortex mixed vigorously for 2 min to dislodge the attached cells into the surrounding medium. Standard microbiological plate counting techniques were used to enumerate the viable CFU ml^{-1} in the cell suspension using casein digest medium (1 g l^{-1}) as the diluent and milk plate count agar (MPCA; Oxoid, Basingstoke, UK). The number of attached viable cells per square centimeter was determined by multiplying the viable CFU ml^{-1} value by 1.25 (or 5/4), because the coupons had a surface area of 4 cm^2 and the attached bacteria were eluted into 5 ml.

3.3.6) Statistical analysis

The experiments were carried out on three separate occasions, and mean attached viable cell numbers (CFU cm^{-2}) + 1 standard deviation (σ_{n-1}) are reported. Minitab software was used to calculate population standard errors and 95% confidence intervals ($P \leq 0.05$) to determine significant differences among the mean values.

3.4) Results

Along with the results presented below, a summary is given in Table 3.2.

3.4.1) Effect of ionic strength

Altering the ionic strength between 0 and 125 mM did not significantly influence attachment ($P \leq 0.05$) after 30 min by unconditioned *A. flavithermus* E16 and *Geobacillus* sp. F75 (Figs. 3.1A and 3.1B). However, increasing the ionic strength from 0 to 2 mM or greater significantly increased ($P \leq 0.05$) biofilm formation after 6 h by

unconditioned *A. flavithermus* E16 and *Geobacillus* sp. F75 (Figs. 2A and 2B). There was no significant difference ($P \leq 0.05$) in biofilm formation after 6 h by unconditioned *A. flavithermus* E16 and *Geobacillus* sp. F75 when cation concentrations ranging between 2 and 125 mM were compared (Figs. 3.2A and 3.2B, ii−vii).

Generally, when cation concentrations between 2 and 125 mM were compared, there was no difference in attachment after 30 min and biofilm formation after 6 h by *A. flavithermus* E16 or *Geobacillus* sp. F75 preconditioned either with cations alone or with cations and lactose (Figs. 3.1C–3.1F and 3.2C–3.2F, ii-vii).

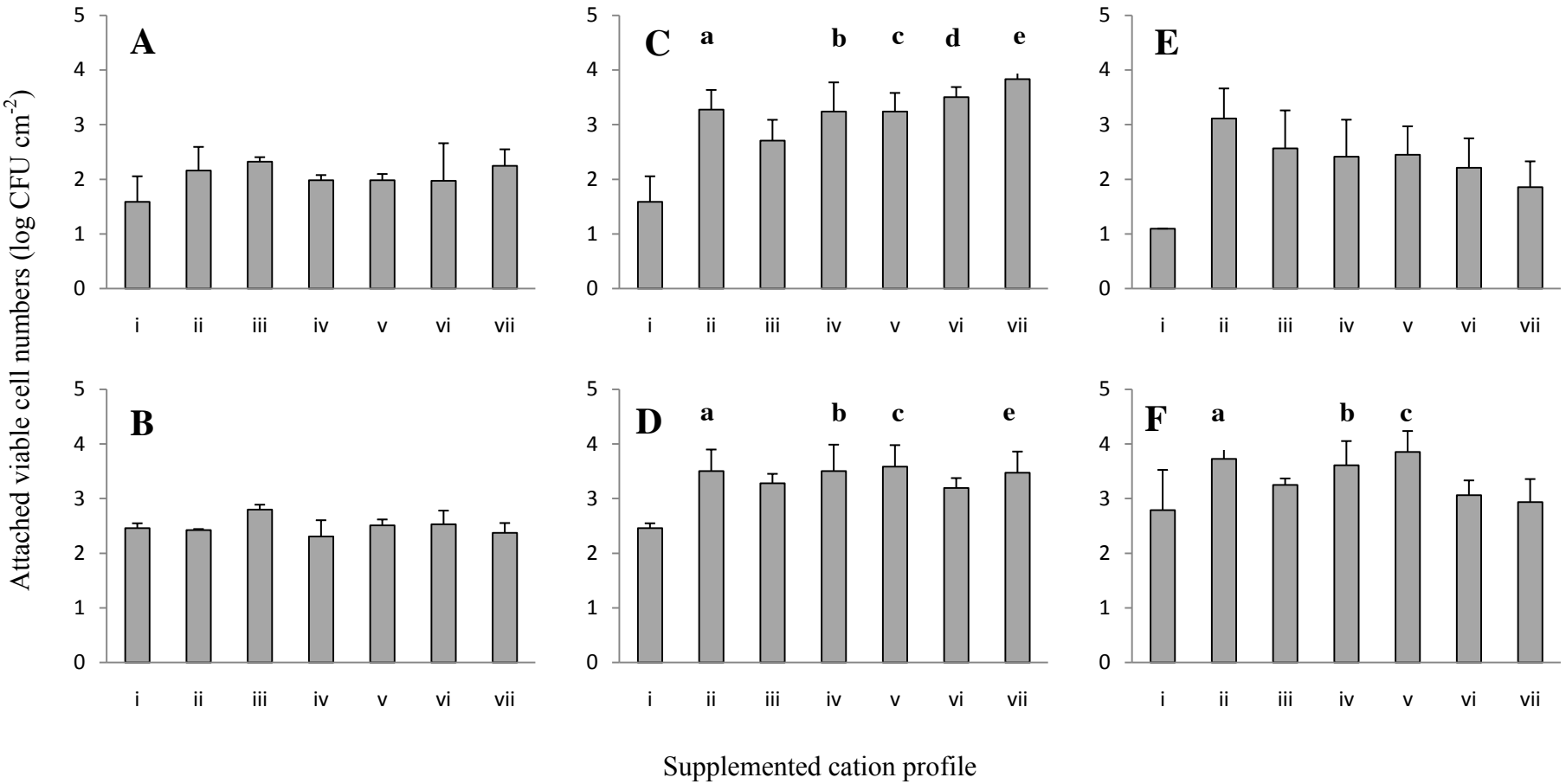
A
B
C
D
E
F
a
b
c
d
e
Attached viable cell numbers (log CFU cm-2)
Supplemented cation profile
i
ii
iii
iv
v
vi
vii
0
1
2
3
4
5

FIG 3.1

FIG 3.1 Attachment, after 30 min of incubation at 55°C, by viable *A. flavithermus* E16 (A, C, and E) and *Geobacillus* sp. F75 (B, D, and F) cells (log CFU cm^{-2}) on stainless steel coupons fully submerged in casein digest medium (1 g l^{-1}) supplemented with cation compositions of 0 mM (i), 2 mM Ca^{2+} (ii), 2 mM Mg^{2+} (iii), 31 mM 2:1 (iv), 31 mM 10:1 (v), 125 mM 2:1 (vi), and 125 mM 10:1 (vii). Total supplemented cation concentrations of 31 mM (iv and v) and 125 mM (vi and vii) had monovalent to divalent cation ratios of 2:1 (iv and vi) and 10:1 (v and vii). Each monovalent to divalent cation ratio comprised equal Na^{+} and K^{+} concentrations and equal Ca^{2+} and Mg^{2+} concentrations. Prior to the attachment assay, the bacteria were grown planktonically for 9 h at 55°C in three different media: casein digest medium (1 g l^{-1}) (unconditioned) (A and B), casein digest medium (1 g l^{-1}) supplemented with various cation compositions (preconditioned with cations) (ii–vii) (C and D), and casein digest medium (1 g l^{-1}) supplemented with lactose (1 g l^{-1}) and various cation compositions (preconditioned with cations and lactose) (ii–vii) (E and F). Experiments were repeated as triplicates and error bars represent one standard deviation (σ_{n-1}). The letters (a – e) represent significantly greater ($P \leq 0.05$) attachment by cation preconditioned cells (C, D, E and F) relative to unconditioned cells (A and B) for each respective bacterial isolate and each respective cation composition. Letter ‘a’ represents 2 mM Ca^{2+} (ii), ‘b’ represents 31 mM 2:1 (iv), ‘c’ represents 31 mM 10:1 (v), ‘d’ represents 125 mM 2:1 (vi), and ‘e’ represents 125 mM 10:1.

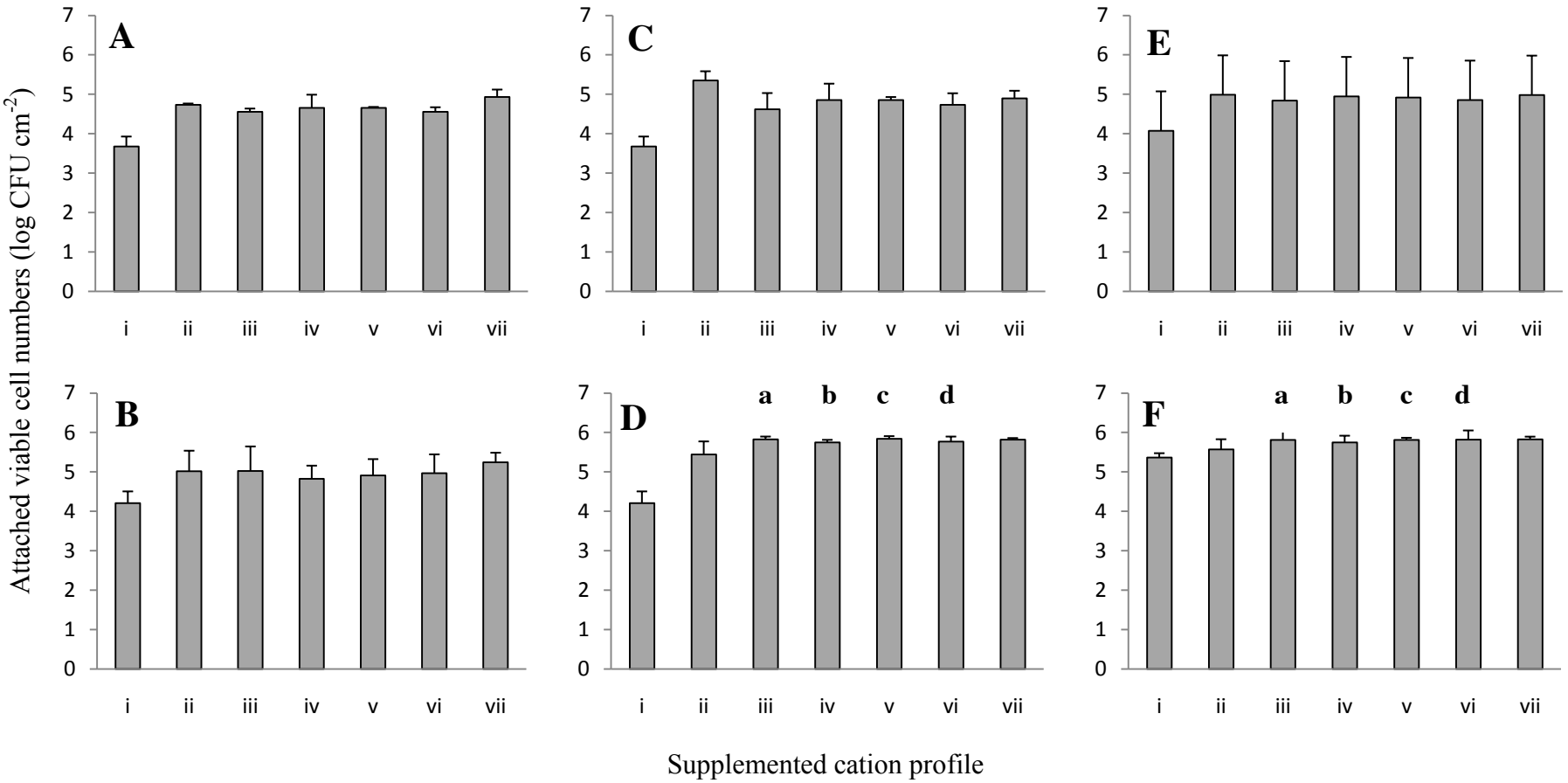
A
B
C
D
E
F
a
b
c
d
i
ii
iii
iv
v
vi
vii
0
1
2
3
4
5
6
7
Attached viable cell numbers (log CFU cm-2)
Supplemented cation profile

FIG 3.2

FIG 3.2 Biofilm formation, after 6 h of incubation at 55°C, by viable *A. flavithermus* E16 (A, C, and E) and *Geobacillus* sp. F75 (B, D, and F) cells (log CFU cm^{-2}) on stainless steel coupons fully submerged in casein digest medium (1 g l^{-1}) supplemented with cation compositions of 0 mM (i), 2 mM Ca^{2+} (ii), 2 mM Mg^{2+} (iii), 31 mM 2:1 (iv), 31 mM 10:1 (v), 125 mM 2:1 (vi), and 125 mM 10:1 (vii). Total supplemented cation concentrations of 31 mM (iv and v) and 125 mM (vi and vii) had monovalent to divalent cation ratios of 2:1 (iv and vi) and 10:1 (v and vii). Each monovalent to divalent cation ratio comprised equal Na^+ and K^+ concentrations and equal Ca^{2+} and Mg^{2+} concentrations. Prior to the biofilm formation assay, the bacteria were grown planktonically for 9 h at 55°C in three different media: casein digest medium (1 g l^{-1}) (unconditioned) (A and B), casein digest medium (1 g l^{-1}) supplemented with various cation compositions (preconditioned with cations) (ii–vii) (C and D), and casein digest medium (1 g l^{-1}) supplemented with lactose (1 g l^{-1}) and various cation compositions (preconditioned with cations and lactose) (ii–vii) (E and F). Experiments were repeated as triplicates and error bars represent one standard deviation (σ_{n-1}). The letters (a – d) represent significantly greater ($P \leq 0.05$) biofilm formation by cation preconditioned cells (D and F) relative to unconditioned cells (B) by *Geobacillus* sp. F75 for each respective cation composition. Letter ‘a’ represents 2 mM Mg^{2+} (ii), ‘b’ represents 31 mM 2:1 (iv), ‘c’ represents 31 mM 10:1 (v), and ‘d’ represents 125 mM 2:1 (vi).

3.4.2) Effect of the monovalent to divalent cation ratio

When comparing between the monovalent to divalent cation ratios of 2:1 and 10:1, at an ionic strength of either 31 or 125 mM in casein digest medium, there was no significant difference ($P \leq 0.05$) in attachment or biofilm formation by *A. flavithermus* E16 or *Geobacillus* sp. F75 (Figs. 3.1A–3.1F and 3.2A–3.2F, iv–vii). This was apparent when bacteria were both preconditioned and unconditioned with cations (Figs. 3.1A–3.1F and 3.2A–3.2F, iv–vii). Attachment after 30 min by *A. flavithermus* E16 was similar when comparing among milk formulations 2–4, however attachment in milk formulation 1 was significantly lower relative to milk formulations 2–4 (Figs. 3.3A and 3.3C); and biofilm formation after 6 h was similar in each of the milk formulations (Figs. 3.4A and 3.4C). Attachment after 30 min by *Geobacillus* sp. F75 was similar in each of the milk formulations (1–4) (Figs. 3.3B and 3.3D); however, biofilm formation after 6 h tended to decrease as the monovalent to divalent cation ratio of the milk formulations increased, particularly by the unconditioned bacteria (Figs. 3.4B and 3.4D). The number of unconditioned, attached viable *Geobacillus* sp. F75 after 6 h was approximately 2 log CFU cm^{-2} lower in milk formulation 4 relative to milk formulation 2 and the two values were significantly different ($P \leq 0.05$) (Fig. 3.4B).

3.4.3) Effect of preconditioning with cations

Preconditioning of *A. flavithermus* E16 or *Geobacillus* sp. F75 with cations often significantly increased ($P \leq 0.05$) attachment (by up to 1 log CFU cm^{-2}) after 30 min relative to unconditioned bacteria, when the same cation concentrations during attachment were compared (Figs. 3.1A, 3.1B, 3.1C, and 3.1D). For example, when *A. flavithermus* E16 attached after 30 min in the presence of 2 mM Ca^{2+}, attachment of bacteria preconditioned in 2 mM Ca^{2+} (Fig. 3.1C, ii) was significantly greater ($P \leq 0.05$)

than unconditioned bacteria (Fig. 3.1A, ii), where the number of attached viable cells were 3.3 and 2.2 log CFU cm^{-2}, respectively.

In contrast, preconditioning *A. flavithermus* E16 with cations did not significantly increase ($P \leq 0.05$) biofilm formation after 6 h relative to unconditioned bacteria, when the same cation concentrations during biofilm formation were compared (Figs. 3.2A and 3.2C). However, preconditioning had a lasting effect on *Geobacillus* sp. F75 biofilm formation after 6 h; for many cation profiles, there was a significant increase ($P \leq 0.05$) (by up to 1 log CFU cm^{-2}) in biofilm formation when bacteria were preconditioned with cations relative to unconditioned bacteria, when the same cation concentrations during biofilm formation were compared (Figs. 3.2B and 3.2D).

3.4.4) Effect of preconditioning with cations and lactose

The extent of attachment after 30 min by *Geobacillus* sp. F75 that were preconditioned with cations and lactose was similar to that when the bacteria were preconditioned with cations alone (Figs. 3.1D and 3.1F). On a few occasions, attachment by *Geobacillus* sp. F75 after 30 min was significantly greater ($P \leq 0.05$) (by up to 1 log CFU cm^{-2}) when the bacteria were preconditioned with cations and lactose compared with unconditioned bacteria, when the same cation concentration during attachment was compared (Figs. 3.1B and 3.1F). In contrast, the extent of attachment after 30 min by *A. flavithermus* E16 that were preconditioned with cations and lactose tended to be less than that observed when the bacteria were preconditioned with cations alone (Figs. 3.1C and 3.1E). Furthermore, there was no significant difference ($P \leq 0.05$) in attachment after 30 min by *A. flavithermus* E16 when bacteria preconditioned with cations and lactose were compared with unconditioned bacteria (Figs. 3.1A and 3.1E).

Preconditioning *Geobacillus* sp. F75 with cations and lactose significantly increased ($P \leq 0.05$) biofilm formation after 6 h (by up to 1 log CFU cm^{-2}) relative to

unconditioned bacteria when the same cation concentrations during attachment were compared (Figs. 2B and 2F); however, cation and lactose preconditioning did not increase biofilm formation after 6 h by *A. flavithermus* E16 (Figs. 3.2A and 3.2E).

3.4.5) Effect of preconditioning with milk formulations

Preconditioning *A. flavithermus* E16 with milk formulations 1–4 significantly increased ($P \leq 0.05$) attachment after 30 min relative to unconditioned bacteria, when each respective milk formulation (1–4) was compared (Figs. 3.3A and 3.3C); however, there was no significant difference ($P \leq 0.05$) in biofilm formation after 6 h by *A. flavithermus* E16 preconditioned with milk formulations 1–4 relative to unconditioned bacteria, when each respective milk formulation (1–4) was compared (Figs. 3.4A and 3.4C).

There was no significant difference ($P \leq 0.05$) in attachment after 30 min and biofilm formation after 6 h by *Geobacillus* sp. F75 preconditioned with milk formulations 1–4 relative to unconditioned bacteria, when each respective milk formulation (1–4) was compared (Figs. 3.4A and 3.4C), except for milk formulation 4, in which biofilm formation after 6 h by preconditioned bacteria was significantly greater ($P \leq 0.05$) than that of unconditioned bacteria (Figs. 3.4B and 3.4D).

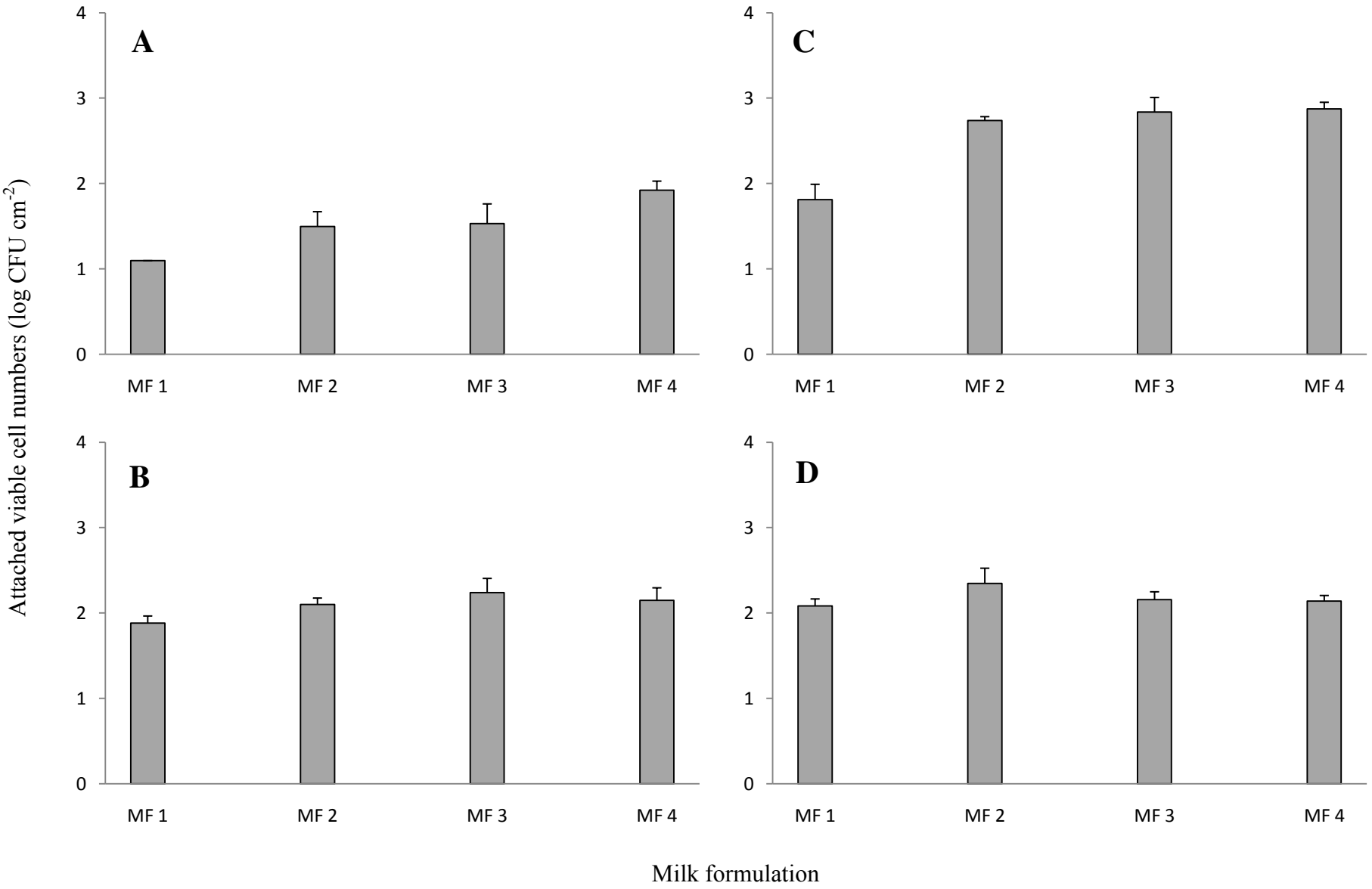
A
B
C
D
Attached viable cell numbers (log CFU cm-2)
0
1
2
3
4
MF 1
MF 2
MF 3
MF 4
Milk formulation

FIG 3.3

FIG 3.3 Attachment, after 30 min of incubation at 55°C, by viable *A. flavithermus* E16 (A and C) and *Geobacillus* sp. F75 (B and D) cells (log CFU cm^{-2}) on stainless steel coupons fully submerged in milk formulations (MF) 1–4. Prior to the attachment assay, the bacteria were grown planktonically for 9 h at 55°C in either casein digest medium (1 g l^{-1}) (unconditioned) (A and B) or milk formulations 1–4 (preconditioned with milk formulation) (C and D). Experiments were repeated as triplicates and error bars represent one standard deviation (σ_{n-1}).

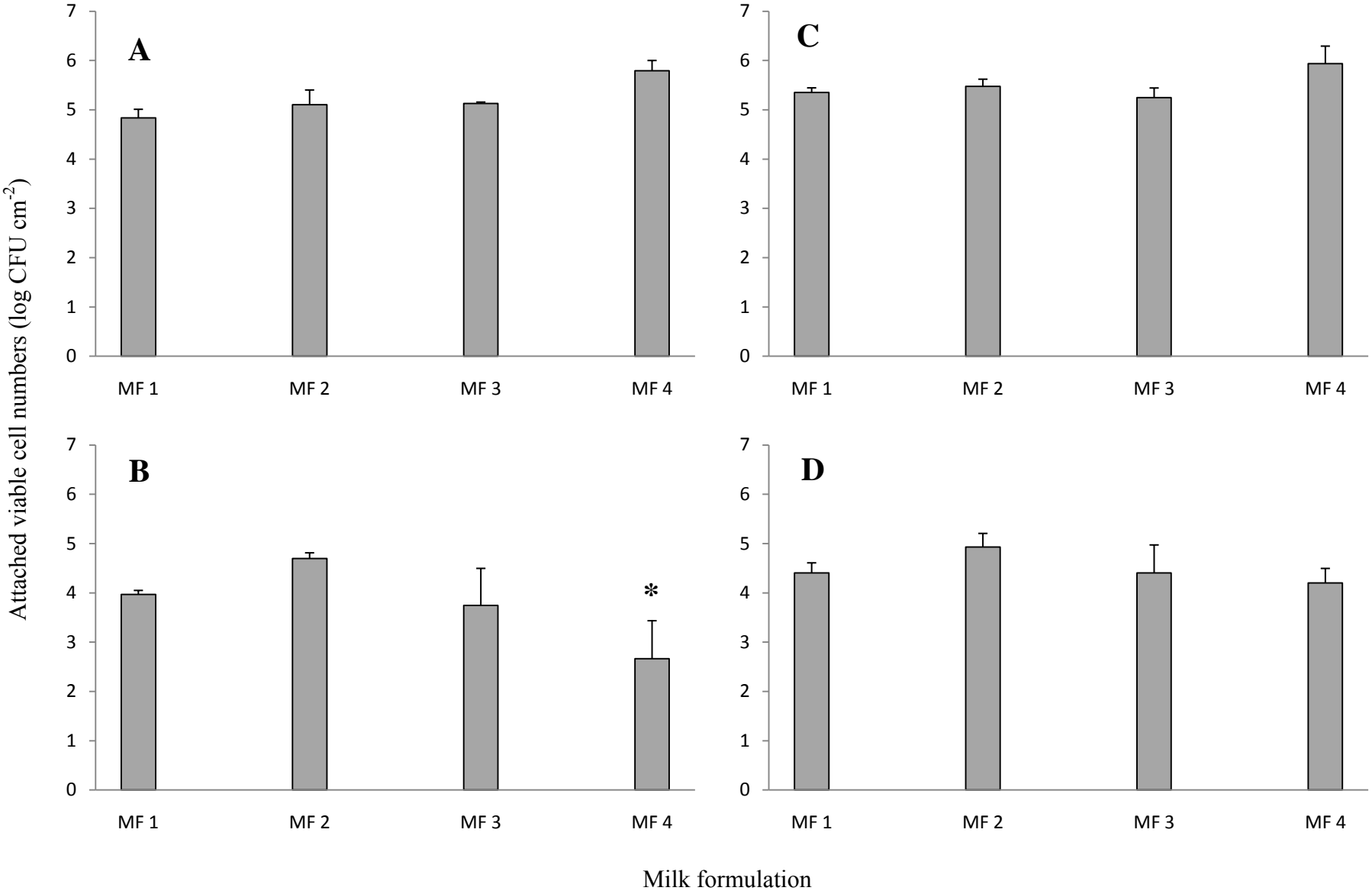
A
B
C
D
Attached viable cell numbers (log CFU cm-2)
0
1
2
3
4
5
6
7
MF 1
MF 2
MF 3
MF 4
*
Milk formulation

FIG 3.4

FIG 3.4 Biofilm formation, after 6 h of incubation at 55°C, by viable *A. flavithermus* E16 (A and C) and *Geobacillus* sp. F75 (B and D) cells (log CFU cm^{-2}) on stainless steel coupons fully submerged in milk formulations (MF) 1–4, after 6 h of incubation at 55°C. Prior to the biofilm formation assay, the bacteria were grown planktonically for 9 h at 55°C in either casein digest medium (1 g l^{-1}) (unconditioned) (A and B) or milk formulations 1–4 (preconditioned with milk formulation) (C and D). Experiments were repeated as triplicates and error bars represent one standard deviation (σ_{n-1}). The asterisk (*) depicts a significant difference ($P \leq 0.05$) between MF 2 and MF 4 in B.

TABLE 3.2 Summary of results

Incubation time	Bacterial isolate and growth medium	Treatment[a]				
		Effect of ionic strength (IS)	Effect of monovalent to divalent cation ratio (MDCR)	Effect of preconditioning with cations	Effect of preconditioning with cations and lactose	Effect of preconditioning with milk formulations (MF) 1–4
Attachment after 30 min	*A. flavithermus* E16 in casein digest medium (1 g l^{-1})	No effect observed	No effect observed	Significant increase with preconditioning	No effect observed	N/A[b]
	Geobacillus sp. F75 in casein digest medium (1 g l^{-1})	No effect observed	No effect observed	Significant increase with preconditioning	Significant increase with preconditioning	N/A
	A. flavithermus E16 in milk formulations 1–4	N/A	Attachment increased with increasing MDCR	N/A	N/A	Significant increase with preconditioning
	Geobacillus sp. F75 in milk formulations 1–4	N/A	No effect observed	N/A	N/A	No effect observed
Biofilm formation after 6 h	*A. flavithermus* E16 in casein digest medium (1 g l^{-1})	Significant increase as IS increased from 0 to 2–125 mM	No effect observed	No effect observed	No effect observed	N/A
	Geobacillus sp. F75 in casein digest medium (1 g l^{-1})	Significant increase as IS increased from 0 to 2–125 mM	No effect observed	Significant increase with preconditioning	Significant increase with preconditioning	N/A
	A. flavithermus E16 in milk formulations 1–4	N/A	No effect observed	N/A	N/A	No effect observed
	Geobacillus sp. F75 in milk formulations 1–4	N/A	Biofilm formation decreased with increasing MDCR	N/A	N/A	Significant increase with preconditioning (MF 4 only)

[a] Significant increases are $P \leq 0.05$.
[b] N/A, not applicable.

3.5) Discussion

3.5.1) Effect of ionic strength

Differences in ionic strength did not alter attachment after 30 min by *A. flavithermus* E16 or *Geobacillus* sp. F75. However, we found that increasing the concentration of supplemented ions from 0 to 2 mM increased biofilm formation after 6 h by both *A. flavithermus* E16 and *Geobacillus* sp. F75. Increases in ionic strength above 2 mM (up to 125 mM) did not further increase biofilm formation after 6 h by *A. flavithermus* E16 or *Geobacillus* sp. F75. As the addition of cations at concentrations of 2 mM may have saturated the surfaces of the bacteria and the stainless steel coupons, further increases in ionic strength may have had no further enhancing effect on biofilm formation. As the ionic strength of milk formulations and most bacterial habitats is greater than 2 mM, our results indicate that the ionic strength of milk formulations does not influence attachment and biofilm formation of *A. flavithermus* and *Geobacillus* spp. Our findings contrast other studies that have shown that bacterial attachment and biofilm cohesion increase as the ionic strength of the surrounding solution increases (Palmer *et al*., 2007; Zhu *et al*., 2009).

3.5.2) Effect of monovalent to divalent cation ratio

No significant difference was found when the numbers of attached *A. flavithermus* E16 or *Geobacillus* sp. F75 cells in casein digest medium (1 g l^{-1}) containing monovalent to divalent cation ratios of 2:1 and 10:1 were compared. These results contrast findings made in wastewater sludge research, where it has been shown that the monovalent to divalent cation ratio of wastewater is an important determinant of the extent of flocculation and cohesion of wastewater sludge biofilms (Higgins & Novak, 1997). Sludge cohesion increases as the monovalent to divalent cation ratio decreases, such

that a monovalent to divalent cation ratio of 2:1 promotes the greatest extent of cohesion, and a monovalent to divalent cation ratio of 10:1 greatly compromises the cohesion of a sludge (Higgins & Novak, 1997).

3.5.3) Effect of contrasting monovalent to divalent cation ratios in milk formulations

Similarly to observations made when biofilm formation in casein digest medium supplemented with cations was studied, attachment after 30 mins by *A. flavithermus* E16 and *Geobacillus* sp. F75 and biofilm formation after 6 h by *A. flavithermus* E16 was not influenced by different monovalent to divalent cation ratios in the milk formulations. However, in agreement with wastewater sludge research, biofilm formation after 6 h by *Geobacillus* sp. F75 decreased as the monovalent to divalent cation ratio of the milk formulations increased.

Contradictory results were obtained when the effects of the monovalent to divalent cation ratio on biofilm formation by *Geobacillus* sp. F75 in casein digest medium and milk formulations were compared. No effect was observed in casein digest medium but biofilm formation decreased as the monovalent to divalent cation ratio of the milk formulations increased. The monovalent to divalent cation ratio of milk formulation 4 is likely to be more extreme than those that exist in any of the cation profiles investigated in the casein digest medium, which may explain the apparent inhibitory influence of the high monovalent to divalent cation ratio of milk formulation 4 on *Geobacillus* sp. F75 biofilm formation. Milk formulations have higher concentrations of solutes, such as casein and anions, relative to casein digest medium, which chelate a large proportion of Ca^{2+} and Mg^{2+}, and thus lower the free Ca^{2+} and Mg^{2+} concentrations (Fox, 2003; Lewis, 2011). Furthermore, the Na^{+} concentration of milk formulation 4 was greater than that in any of the cation profiles used to supplement the

casein digest medium; the Na^+ concentration in milk formulation 4 was 101 mM (Table 1.5), whereas the highest Na^+ concentration in the casein digest medium was 56.8 mM (Table 3.1). Thus, it may have been high Na^+, low Ca^{2+}, low Mg^{2+}, or a combination of these factors that inhibited *Geobacillus* sp. F75 biofilm formation in milk formulation 4.

Our results indicate that generally the monovalent to divalent cation ratio in milk formulations does not influence attachment and biofilm formation of *A. flavithermus* and *Geobacillus* spp.

3.5.4) Effect of preconditioning with cations

During preconditioning, the metabolism and physiology of *A. flavithermus* E16 and *Geobacillus* sp. F75 may have been influenced by cations in such a way that subsequent attachment and biofilm formation by the bacteria was enhanced. For example, Ca^{2+} or Mg^{2+} may have stimulated the bacteria to increase their expression of surface-exposed proteins and polysaccharides, which promoted attachment and biofilm formation (Corpe, 1964; Cruz *et al*., 2012; Garrison-Schilling *et al*., 2011; Goode & Allen, 2011; Liu & Sun, 2011; Tempest *et al*., 1965). Surface-exposed proteins, such as pili and even flagella, have been implicated in assisting the initial reversible association of bacteria with a surface, and surface-exposed polysaccharides have been implicated in promoting the irreversible attachment of bacteria to a surface and enhancing cohesion among bacteria within a mature biofilm by acting as a cohesive component of the matrix (Flemming & Wingender, 2010). Ca^{2+} and Mg^{2+} have the potential to upregulate the expression of surface-exposed polymers by associating with response regulators and regulating signal transduction pathways (Michiels *et al*., 2002), or by acting as enzyme co-factors (Hughes *et al*., 1973; Michiels *et al*., 2002), both of which may be involved in regulation and optimization of the biosynthesis of surface-exposed polymers.

Since the electrostatic effects of ionic strength and the monovalent to divalent cation ratio generally did not influence attachment and biofilm formation of *A. flavithermus* E16 and *Geobacillus* sp. F75, and preconditioning the bacteria with cations often increased attachment, it is proposed that the transition of *A. flavithermus* and *Geobacillus* spp. from milk formulations to stainless steel product-contact surfaces in milk powder manufacturing plants is predominantly mediated by bacterial physiological factors, e.g. the expression of surface-exposed adhesins, rather than the concentrations of cations in milk formulations.

3.5.5) Preconditioning of *A. flavithermus* with lactose decreased attachment

Protein, such as casein, and carbohydrate, such as lactose, are available to bacteria as nutrient sources for biofilm formation during milk powder manufacture (Fox, 2003). Casein digest medium (1 g l^{-1}), as used in this study, has a very low carbohydrate concentration (Biosciences, 2006). To simulate biofilm formation by *A. flavithermus* and *Geobacillus* spp. in milk powder manufacturing lines, and to provide bacteria with an adequate carbohydrate source to synthesize surface-exposed polysaccharides (Dykes *et al*., 1995), lactose was added to cultures during preconditioning. Thus, when bacteria were preconditioned with cations and lactose, the effect of preconditioning bacteria with ranging cation concentrations on their subsequent attachment and biofilm formation was investigated in the presence of both a protein source and a carbohydrate source.

Preconditioning of *A. flavithermus* E16 with cations and lactose decreased its attachment after 30 min relative to when the bacteria were preconditioned with cations alone. During preconditioning, *A. flavithermus* E16 may have utilized the lactose in the medium to increase production of surface-exposed polysaccharides, which subsequently decreased attachment. This may have masked the attachment-enhancing effect of

surface-exposed proteins and consequently decreased the ability of the bacteria to transition from a planktonic state to an irreversibly attached state. Other researchers have shown that increasing the ratio of carbohydrate to nitrogen in media increases the ratio of polysaccharides to proteins in the extracellular matrix of bacteria (Corpe, 1964; Durmaz & Sanin, 2001). Furthermore, Flint *et al*. (1997b) showed that the removal of surface-exposed proteins from thermophilic streptococci decreased their attachment 100-fold; however, attachment was not altered after the removal of surface-exposed polysaccharides. Also, many studies have shown that surface-exposed polysaccharides can inhibit bacterial attachment (Abu Sayem *et al*., 2011; Guezennec *et al*., 2012). In contrast with *A. flavithermus* E16, the addition of lactose to preconditioning cultures did not affect subsequent attachment and biofilm formation by *Geobacillus* sp. F75. It is possible that either *Geobacillus* sp. F75 did not increase its production of surface-exposed polysaccharides in the presence of lactose, or its surface exposed polysaccharides did not influence subsequent attachment and biofilm formation.

3.5.6) Effect of preconditioning with milk formulations

Similar to the observed effect of preconditioning bacteria with cations in casein digest medium, on occasion, preconditioning *A. flavithermus* E16 and *Geobacillus* sp. F75 with milk formulations increased attachment and biofilm formation. Preconditioning *A. flavithermus* E16 with milk formulations 1–4 significantly increased attachment after 30 min. The bacteria may have utilized nutrients in the milk formulations, which influenced their metabolism or physiology such that it enhanced their attachment capabilities. As has been previously discussed, biofilm formation after 6 h by *Geobacillus* sp. F75 decreased as the monovalent to divalent cation ratio of the milk formulations increased. Interestingly, the difference in biofilm formation was less when the bacteria were preconditioned in the respective milk formulations relative to

unconditioned bacteria. It is proposed that, although milk formulations with relatively high monovalent to divalent cation ratios were inhibitory towards *Geobacillus* sp. F75 growth, preconditioning accustomed its growth in the milk formulations, which subsequently increased its biofilm formation.

3.6) Conclusions

The present study indicated that free cation concentrations and ratios in milk formulations generally would not influence attachment and biofilm formation by *A. flavithermus* and *Geobacillus* spp. during the processing of milk formulations in a milk powder manufacturing plant. However, as the monovalent to divalent cation ratio of milk formulations increased, biofilm formation after 6 h by *Geobacillus* sp. F75 was increasingly inhibited. This implied that either high Na^+ concentrations or low Ca^{2+} or Mg^{2+} concentrations, or a combination of these factors, have the potential to inhibit biofilm formation by some thermophilic bacilli strains during the processing of milk formulations. Interestingly, preconditioning of planktonic *A. flavithermus* E16 and *Geobacillus* sp. F75 with cations often enhanced attachment and was more influential than the electrostatic effect of cations on the attachment process. It is proposed that cations, such as Ca^{2+} and Mg^{2+}, influenced the physiology or metabolism of planktonic *A. flavithermus* and *Geobacillus* spp. such that subsequent attachment was enhanced. It is also proposed that attachment by *A. flavithermus* and *Geobacillus* spp. in milk powder manufacturing lines is predominantly mediated by bacterial physiological factors, e.g. the expression of surface-exposed adhesins, rather than the concentrations of cations in milk formulations.

CHAPTER 4

Influence of cations on protein expression of a *Geobacillus* isolate, of dairy origin, in a biofilm as measured by MALDI-TOF MS analysis

4.1) Abstract

Matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectroscopy (MS) was used to investigate the influence of Na^+, K^+, Ca^{2+} and Mg^{2+} on protein expression by a *Geobacillus* sp. isolate of dairy origin grown in a biofilm. Analysis of the resulting spectra indicated that the expression of 16 proteins were down-regulated by *Geobacillus* sp. F75 grown as a biofilm in media supplemented with 2 mM Mg^{2+}, relative to when it was either grown in media unsupplemented with cations, or media supplemented with a mixture of Na^+, K^+, Ca^{2+} and Mg^{2+} (total of 31 mM, and with a monovalent to divalent cation ratio of 10:1). The mass of the proteins were estimated from the resulting MALDI-TOF MS spectra, and TagIdent software was used to predict the identity of the proteins. Of the 16 down-regulated proteins, it was estimated that five of the proteins are involved in sporulation. We propose that Mg^{2+} stimulates vegetative cell growth over sporulation in *Geobacillus* spp. biofilms, thus promoting cell division and metabolism, as opposed to sporulation.

4.2) Introduction

External free Na^+, K^+, Ca^{2+} and Mg^{2+} ions have the potential to influence the protein expression, physiology and metabolism of bacteria in a biofilm (Cruz *et al*., 2012; Garrison-Schilling *et al*., 2011; Kara *et al*., 2008; Patrauchan *et al*., 2005; Song & Leff, 2006). For instance, in response to increasing external Ca^{2+} and Mg^{2+} concentrations, bacteria generally adjust their physiology to promote biofilm formation. For example, Patrauchan *et al*. (2005) showed that as the external Ca^{2+} concentration varied among 0.25, 1, 5 and 10 mM, exopolysaccharide production by a *Pseudoalteromonas* sp. isolate increased with increasing Ca^{2+} concentrations, promoting its biofilm formation. Cruz *et al*. (2012) showed that *Xylella fastidiosa* responded to the addition of 2 mM Ca^{2+} by upregulating its expression of a type I pilus subsequently increasing its attachment. Additionally, Song & Leff (2006) proposed that 1 mM Mg^{2+} stimulated *Pseudomonas fluorescens* to increase its production of exopolysaccharide, flagella and fimbriae thereby increasing attachment and biofilm formation. In contrast, the response of bacteria to increasing external Na^+ and K^+ concentrations is to adjust their physiology to prevent biofilm formation. For example, Kara *et al*. (2008) showed that in response to increasing concentrations of Na^+ or K^+, bacteria in a wastewater sludge increased the production of negatively charged and hydrophilic extracellular polymers, compromising the structural integrity and cohesion performance of the sludge.

Thermophilic bacilli, mainly belonging to the *Geobacillus* spp. and *Anoxybacillus flavithermus* groups, form biofilms and proliferate in regions of milk powder manufacturing plants which are heated to between 50 and 80°C, such as pasteurising plate heat exchangers and evaporators (Burgess *et al*., 2010; Hill & Smythe, 2012). These biofilms contaminate milk formulations as they are processed to milk powder, and the total thermophile viable cell count determines the grade and selling price of milk powders (Burgess *et al*., 2010; Hill & Smythe, 2012). Our previous studies

showed that biofilm formation by a *Geobacillus* sp. isolate was inhibited in a milk formulation with a relatively high monovalent to divalent cation ratio (Somerton *et al*., 2013). However, the mechanisms behind the observed decrease in biofilm formation were not fully elucidated.

MALDI-TOF MS is used to predict the mass of proteins and other polymers, and to identify the species of cultured bacteria, as a quicker and more cost-effective alternative to traditional biochemical testing and microscopy methods (Wieser *et al*., 2012). MALDI-TOF MS can also be used to analyse the protein expression profile of bacteria (Hathout *et al*., 2003; Wieser *et al*., 2012), thereby providing insights into bacterial physiology. In an attempt to better understand how cations influence biofilm development of thermophilic bacilli, MALDI-TOF MS was used to compare the influence of 0 mM, 2 mM Mg^{2+} and 31 mM of Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} (with a monovalent to divalent cation ratio of 10:1) on the protein expression profile of *Geobacillus* sp. F75 grown as a biofilm for 6 h in casein digest medium. The influence of Mg^{2+}, in both the absence and presence of Na^{+}, K^{+} and Ca^{2+}, on the physiology of a *Geobacilllus* sp. isolate in a biofilm was evaluated.

4.3) Methods

4.3.1) Biofilm formation

To propagate *Geobacillus* sp. F75 prior to biofilm formation, 1 ml of a thawed culture was used to inoculate 100 ml of casein digest medium (1 g l^{-1}) (Difco, BD Biosciences, Sparks, MD). The inoculated medium was incubated at 55°C for 9 h, which was sufficient time for the bacteria to reach mid-stationary growth phase. One milliliter of the planktonic culture was used to inoculate 100 ml of casein digest medium (1 g l^{-1}) in a glass flask containing 1 g of sterile, reagent grade glass wool (Product # 18421,

Sigma-Aldrich, New Zealand). Glass wool was chosen as it has a high surface area which allows for the growth of a biofilm with a high biomass (Oosthuizen *et al*., 2002). In order to further increase the biomass of the biofilms, three 100 ml cultures, each containing glass wool, were grown for each replicate. The casein digest medium was either unsupplemented with cations (0 mM), or supplemented with analytical grade NaCl, KCl, $CaCl_2.2H_2O$, and $MgCl_2.6H_2O$ (Merck, Darmstadt, Germany) powders to obtain final concentrations of either 2 mM $MgCl_2.6H_20$ or 1.42 mM each of $CaCl_2.2H_20$ and $MgCl_2.6H_20$, and 14.2 mM each of NaCl and KCl (a total cation concentration of 31 mM with a monovalent to divalent cation ratio of 10:1). The cultures were incubated at 55°C for 6 h, which was sufficient time for the *Geobacillus* sp. F75 to establish a biofilm on the glass wool (Somerton *et al*., 2013). Biofilms grown in each of the three cation profiles tested (0 mM, 2 mM Mg and 31 mM 10:1) were grown on three separate occasions in preparation for MALDI-TOF MS analysis.

4.3.2) Harvesting the biofilm

To remove loosely attached bacteria from the biofilm, sterile forceps were used to remove the glass wool from the culture flask and the glass wool and biofilm was dipped in approximately 800 ml of sterile, distilled water three times to rinse the biofilm. The glass wool was placed into a plastic container with 10 ml of casein digest medium (1 g l^{-1}). To dislodge the biofilm, the plastic container was alternatively subjected to 1 min of vigorous vortex-mixing, and 1 min in a sonicating waterbath, for a total of 3 mins each. Using sterile forceps, the glass wool was removed from the biofilm suspension and squeezed to minimize the loss of biofilm suspension. To collect the biofilm, the biofilm suspension was centrifuged for 10 mins at 10, 000 X *g* at 20°C (Oosthuizen *et al*., 2002). The supernatant was discarded and the pelleted biofilm (which contained a collation of the biomass of the three original, identical 100 ml cultures) was

resuspended in 370 µl of sterile, double distilled water. The biofilm suspension was centrifuged again for 5 mins at 10, 000 X *g*. The supernatant was discarded and the pelleted biofilm was resuspended in 370 µl of sterile, double distilled water. The protein concentration of each collated biofilm sample was measured using the Qubit Protein Assay Kit (Product # Q33211, Invitrogen, Carlsbad, CA) in combination with the Qubit 2.0 Fluorometer (Product # Q32866, Invitrogen). Briefly, 1 µl of collated biofilm sample was thoroughly vortex-mixed and added to 199 µl of Qubit working solution. Each collated, biofilm sample was diluted using sterile, double distilled water to achieve a final protein concentration of 195 µg ml^{-1}, and 300 µl of each diluted sample was added to 900 µl of pure ethanol, and the samples were stored at – 20°C until they were utilised for MALDI-TOF MS analysis.

4.3.3) MALDI-TOF MS analysis

Biofilm samples were thawed on ice and prepared for MALDI-TOF MS analysis as described by Altoom *et al*. (2011). The biofilm suspension was centrifuged for 2 mins at 13, 000 rpm, and the supernatant was discarded. The biofilm was again centrifuged for 2 mins at 13, 000 rpm and residual supernatant was aspirated by pipetting. The pelleted biofilm was resuspended in 20 µl of 70% (vol/vol) formic acid and mixed thoroughly by vortex-mixing and repeated pipetting. Acetonitrile (20 µl) was added to the samples which were then mixed thoroughly by vortex-mixing and repeated pipetting (Alatoom *et al*., 2011). The biofilm suspension was centrifuged for 2 mins at 13, 000 rpm. One microliter of the supernatant of each biofilm suspension was added to the polished steel, target plate (Product # 224989, Bruker Daltonic GmbH, Germany) in quadruplicate and allowed to air-dry. One microliter of α-cyano-4-hydroxycinnamic acid matrix (Part # 255344, Bruker Daltonic GmbH, Germany) was used to overlay each spot on the target plate, and allowed to air-dry. All three biofilm replicates of each cation profile were

analysed on the same MALDI-TOF MS run using the Microflex LT (Bruker Daltonic GmbH, Germany). MALDI Biotyper Version 3.1 and flexControl Version 3.3 software (Bruker Daltonic GmbH, Germany) was used to identify peaks that were absent or present when comparing among MALDI-TOF MS spectra of the three cation profiles. It was only concluded that a peak was absent or present if it was apparent when comparing among all three replicate cultures and all four target plate spots. This was based on a subjective assessment of the amplitude of the peaks in the spectra.

It was assumed that each peak in the MALDI-TOF MS spectra represented a putative protein. The mass/charge (m/z) value depicted in the MALDI-TOF MS spectra was used to estimate the mass of putative proteins (Daltons), as it was assumed that the charge of each protein is 1. TagIdent software (Swiss Institute of Bioinformatics, Lausanne, Switzerland) was used to estimate the identity of proteins represented by peaks of interest in the MALDI-TOF MS spectra. The mass of the putative *Geobacillus* sp. F75 proteins were compared to the mass of *Geobacillus* as well as *Anoxybacillus* and *Bacillus* proteins in the TagIdent database to predict the identity of the putative *Geobacillus* sp. F75 proteins, as the *Anoxybacillus* and *Bacillus* genera are closely related to the *Geobacillus* genus (Flint *et al*., 2001b). A successful protein match was considered if the protein in the TagIdent database had a mass that was within ± 0.1% of the estimated mass, in Daltons, of the putative *Geobacillus* sp. F75 protein.

4.4) Results and discussion

Spectral analysis showed that 16 peaks in the MALDI-TOF MS spectra were absent from the *Geobacillus* sp. F75 biofilm preparations derived from cultures supplemented with 2 mM Mg^{2+}, which were present in biofilm preparations derived from cultures supplemented with 0 mM and 31 mM 10:1. Figures 4.1 – 4.3 show examples of MALDI-TOF MS spectra peaks which were absent in biofilm preparations derived from cultures supplemented with 2 mM Mg^{2+}, and present in biofilm preparations derived

from cultures supplemented with 0 mM and 31 mM 10:1. Six of the spectra peaks of interest were successfully matched to a total of nine proteins in the TagIdent database (Table 4.1). The remaining ten spectra peaks of interest were not successfully matched with proteins in the TagIdent database. These proteins had an estimated mass of 3034, 3686, 4788, 5197, 5588, 6208, 6236, 7006, 7374 and 7916 Da.

The complete down-regulation of 16 proteins by *Geobacillus* sp. F75 in the presence of 2 mM Mg^{2+}, relative to cultures supplemented with the cation profiles of 0 mM and 31 mM 10:1, indicated that *Geobacillus* sp. F75 altered its physiology in response to 2 mM Mg^{2+}. Other *Geobacillus* sp. F75 proteins may have been partially up-regulated or partially down-regulated in response to 2 mM Mg^{2+}. As MALDI-TOF MS was used to predict the complete presence or absence of proteins, partially up-regulated or partially down-regulated proteins would have been undetected in our study. Patrauchan *et al*. (2005) found that, when comparing the proteome expression of a *Pseudoalteromonas* sp. in external Ca^{2+} concentrations of 0.25 with 10 mM, the majority of proteins which had altered expression were partially altered, rather than completely up- or down-regulated (Patrauchan *et al*., 2005). Thus, it could be anticipated that in our study, in addition to the 16 proteins that were completely down-regulated by *Geobacillus* sp. F75 in response to 2 mM Mg^{2+}, the expression of many other proteins may have been partially altered. Mg^{2+} is an important nutrient requirement in bacteria and has many structural and enzymatic roles (Heptinstall et al., 1970; Hughes et al., 1973; Smith & Maguire, 1998; Song & Leff, 2006). Thus, Mg^{2+} may act as an environmental cue or stimulus which Geobacillus species respond to, subsequently modifying their physiology depending on the external Mg^{2+} concentration.

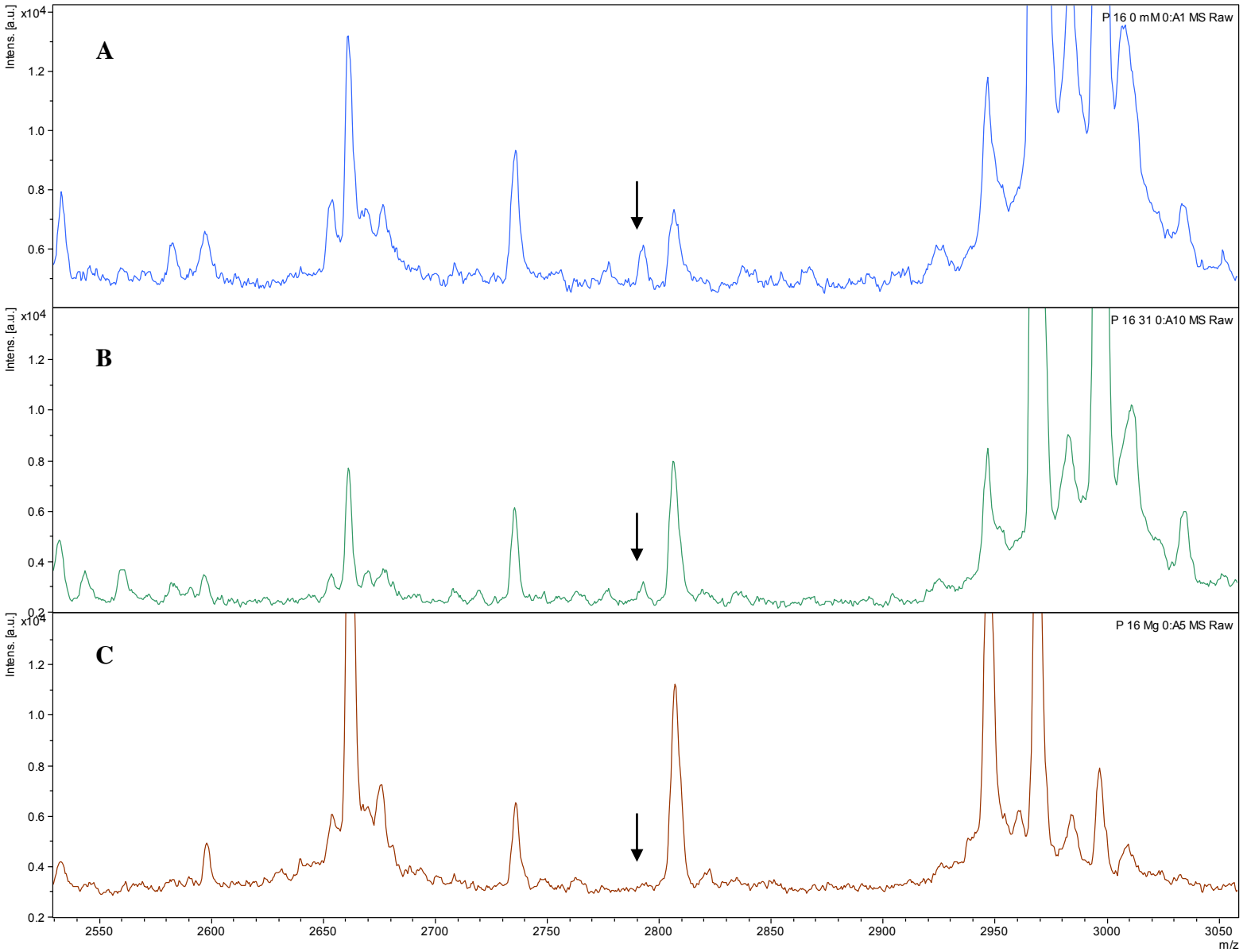
A
B
C
P 16 0 mM 0:A1 MS Raw
P 16 31 0:A10 MS Raw
P 16 Mg 0:A5 MS Raw
Intens. [a.u.]
x10^4
m/z
2550
2600
2650
2700
2750
2800
2850
2900
2950
3000
3050

FIG 4.1

FIG 4.1 MALDI-TOF MS spectra of a whole cell extract of *Geobacillus* sp. F75 grown as a biofilm in casein digest medium (1 g l^{-1}) either unsupplemented with cations (A), supplemented with a total Na^+, K^+, Ca^{2+} and Mg^{2+} concentration of 31 mM with a monovalent to divalent cation ratio of 10:1 (B), or supplemented with 2 mM Mg^{2+} (C). The arrows identify spectra peaks indicating a mass/charge (m/z) value of approximately 2792, which represents a putative protein with an estimated mass of 2792 Da. The *Geobacillus* sp. F75 biofilm cultures were grown on three separate occasions and each replicate was analysed on the MALDI-TOF Microflex LT target plate in quadruplicate. Each spectrum shows the analysis of one target plate spot of one replicate.

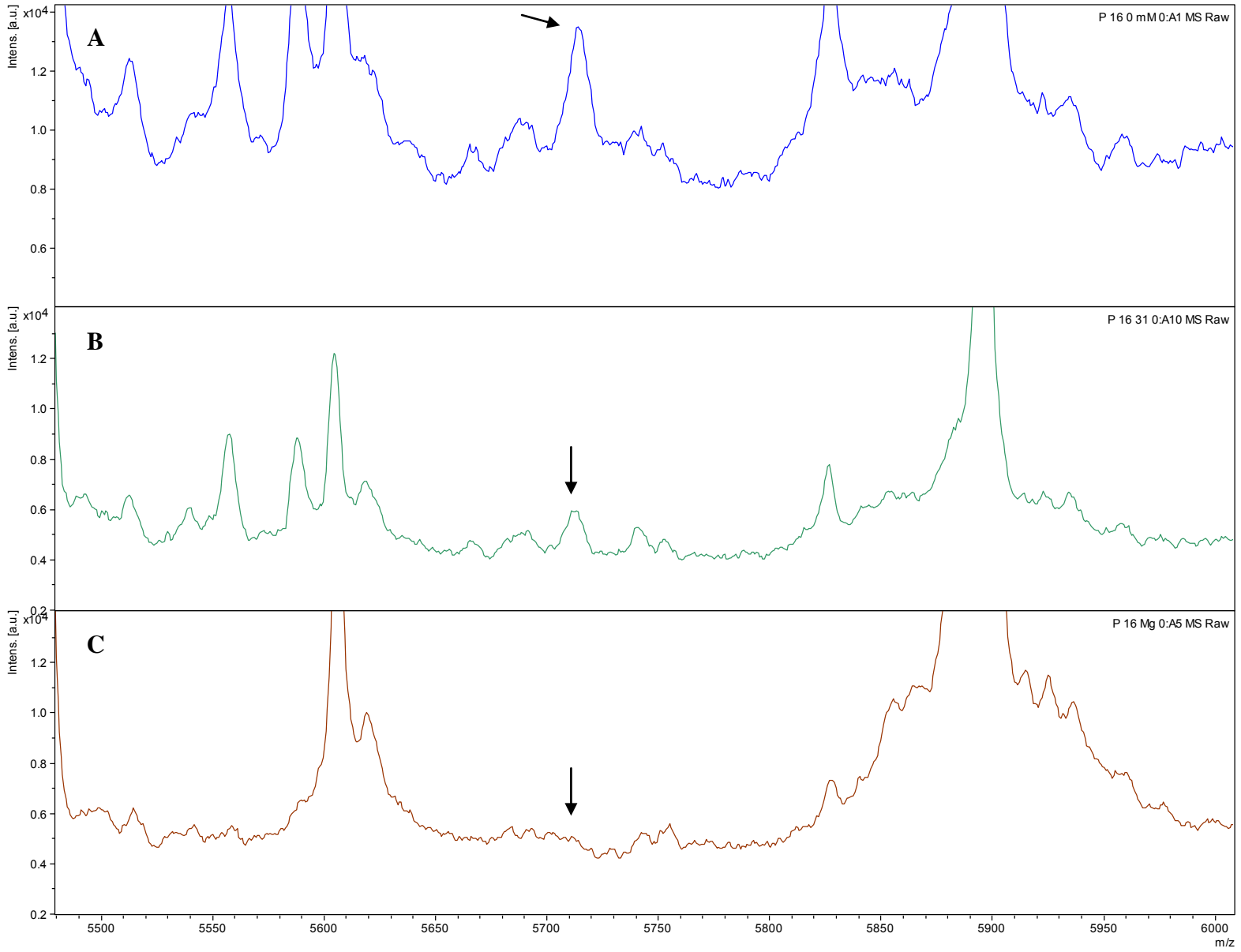
A
P 16 0 mM 0:A1 MS Raw
B
P 16 31 0:A10 MS Raw
C
P 16 Mg 0:A5 MS Raw
Intens. [a.u.]
x10^4
1.2
1.0
0.8
0.6
0.4
0.2
5500
5550
5600
5650
5700
5750
5800
5850
5900
5950
6000
m/z

FIG 4.2

FIG 4.2 MALDI-TOF MS spectra of a whole cell extract of *Geobacillus* sp. F75 grown as a biofilm in casein digest medium (1 g l^{-1}) either unsupplemented with cations (A), supplemented with a total Na^+, K^+, Ca^{2+} and Mg^{2+} concentration of 31 mM with a monovalent to divalent cation ratio of 10:1 (B), or supplemented with 2 mM Mg^{2+} (C). The arrows identify spectra peaks indicating a mass/charge (m/z) value of approximately 5714, which represents a putative protein with an estimated mass of 5714 Da. The *Geobacillus* sp. F75 biofilm cultures were grown on three separate occasions and each replicate was analysed on the MALDI-TOF Microflex LT target plate in quadruplicate. Each spectrum shows the analysis of one target plate spot of one replicate.

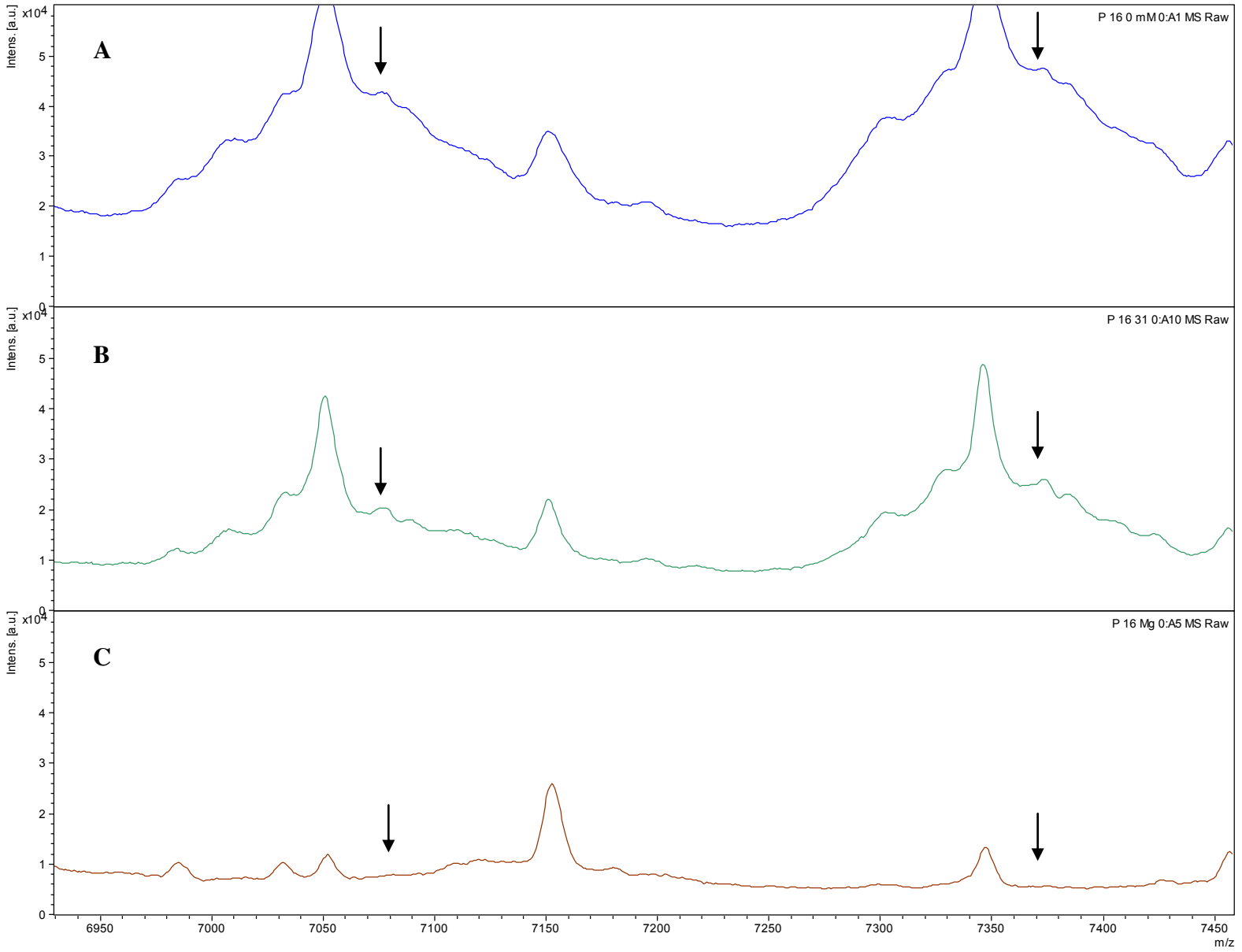
A
P 16 0 mM 0:A1 MS Raw
B
P 16 31 0:A10 MS Raw
C
P 16 Mg 0:A5 MS Raw
Intens. [a.u.]
x10^4
0
1
2
3
4
5
6950
7000
7050
7100
7150
7200
7250
7300
7350
7400
7450
m/z

FIG 4.3

FIG 4.3 MALDI-TOF MS spectra of a whole cell extract of *Geobacillus* sp. F75 grown as a biofilm in casein digest medium (1 g l^{-1}) either unsupplemented with cations (A), supplemented with a total Na^+, K^+, Ca^{2+} and Mg^{2+} concentration of 31 mM with a monovalent to divalent cation ratio of 10:1 (B), or supplemented with 2 mM Mg^{2+} (C). The arrows identify spectra peaks indicating mass/charge (m/z) values of approximately 7076 and 7374, which represent putative proteins with estimated masses of 7076 and 7374 Da, respectively. The *Geobacillus* sp. F75 biofilm cultures were grown on three separate occasions and each replicate was analysed on the MALDI-TOF Microflex LT target plate in quadruplicate. Each spectrum shows the analysis of one target plate spot of one replicate.

TABLE 4.1 Predicted identities of successfully matched putative proteins absent in *Geobacillus* sp. F75 biofilms grown in 2 mM Mg^{2+}

Molecular weight of putative *Geobacillus* sp. F75 protein absent from 2 mM Mg^{2+} biofilm culture	Strain which predicted protein belongs to	Molecular weight of predicted protein	Name of predicted protein	Function of predicted protein
2792	*B. licheniformis* DSM 13/ATCC 14580	2795	PanD, Aspartate 1-decarboxylase	Alanine biosynthesis, pantothenate biosynthesis
4687	*B. cereus* ssp. *cytotoxis* NVH 391-98	4682	SspN, small acid soluble spore protein N	Sporulation
5714	*B. amyloliquefaciens* FZB42	5718	SspK, small, acid soluble spore protein K	Sporulation
6156	*B. subtilis* 168	6155	Sda, sporulation inhibitor	Sporulation
	B. subtilis 168	6155	YxzL, Uncharacterized protein	Unknown
6918	*G. kaustophilus* HTA426	6913	RpmB, 50S ribosomal protein	Ribosomal protein
	A. flavithermus DSM 21510/WK1	6917	S-adenosylmethionine decarboxylase proenzyme	Required for the synthesis of spermine and spermidine
	B. amyloliquefaciens FZB42	6914	SspH, small acid soluble spore protein H	Sporulation
7076	*B. subtilis* 168	7071	SspA, small acid soluble spore protein A	Sporulation

Mg^{2+}, present in cultures supplemented with 31 mM of cations with a monovalent to divalent cation ratio of 10:1 (and a Mg^{2+} concentration of 1.42 mM), may not have had the same effect on *Geobacillus* sp. F75 as Mg^{2+} in cultures supplemented with 2 mM Mg^{2+}. The Na^{+}, K^{+} and Ca^{2+} present in cultures supplemented with Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} (31 mM, 10:1) may have competitively excluded Mg^{2+} from the cell envelope of *Geobacillus* sp. F75 (Lambert *et al*., 1975a; Rose & Hogg, 1995). This may have prevented the *Geobacillus* sp. F75 from responding physiologically to Mg^{2+}, via structures which may sense Mg^{2+}, such as response regulators.

Of the proteins absent from the *Geobacillus* sp. F75 biofilm preparations derived from cultures supplemented with 2 mM Mg^{2+}, an involvement in sporulation was the most common predicted function of the proteins. Studies have shown that small, acid soluble spore proteins act as reliable biomarkers for the differentiation of *Bacillus* spore species using MALDI-TOF MS, as they have consistent conformation when *Bacillus* are cultured in different growth media (Hathout *et al*., 2003). Furthermore, small acid soluble spore proteins are extremely similar in amino acid sequence, and thus molecular weight, both within and across species (Hathout *et al*., 2003). Also, small acid soluble spore proteins have no sequence similarities to other proteins or protein motifs in available databases (Hathout *et al*., 2003). Thus, it is feasible to use *Bacillus* spp. and *Anoxybacillus* spp. small acid soluble spore proteins to predict the identity of *Geobacillus* spp. small acid soluble spore proteins. It is proposed that Mg^{2+} prevents sporulation and thereby stimulates cell division and metabolism of *Geobacillus* spp. in a biofilm.

Scribner *et al*. (1974) showed that both the rate of Mg^{2+} influx and the intracellular concentration of Mg^{2+} in a *Bacillus subtilis* strain decreased during sporulation. It could be hypothesized that Mg^{2+} has a similar role during the sporulation of *Geobacillus* spp., and that low Mg^{2+} concentrations are required for sporulation to proceed. The heightened availability of Mg^{2+} to *Geobacillus* sp. F75 in the culture supplemented with 2 mM Mg^{2+} may have prevented sporulation, thus causing the apparent down-regulation of sporulation proteins.

Sporulation may be utilized by bacteria as a survival mechanism when they are subjected to conditions that are unfavourable for vegetative growth, for example, when bacteria face starvation in low nutrient conditions (Setlow, 2007). *Geobacillus* sp. F75 grown in cultures supplemented with either 0 mM, or Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} (31 mM, 10:1) may have been starved of Mg^{2+}, inducing sporulation. Conversely, *Geobacillus* sp. F75 grown in cultures supplemented with 2 mM Mg^{2+} would have had an adequate supply of Mg^{2+}, preventing sporulation.

Previously, it has been shown that biofilm formation after 6 h by *Geobacillus* sp. F75 in a milk formulation with a high monovalent to divalent cation ratio was approximately 2 log CFU cm^{-2} lower relative to a milk formulation with a monovalent to divalent cation ratio resembling unprocessed milk (Somerton *et al*., 2013). Perhaps the relatively high Na^{+} and K^{+} concentrations in the milk formulation with a high monovalent to divalent cation ratio competitively excluded Ca^{2+} and Mg^{2+} from *Geobacillus* sp. F75, preventing Ca^{2+} and Mg^{2+} from stimulating *Geobacillus* sp. F75 to grow as a biofilm (Higgins & Novak, 1997; Kara et al., 2008). In the current study, results suggest that Mg^{2+} stimulates the biofilm form of *Geobacillus* sp. F75 to refrain from sporulating. It is postulated that the low concentration and availability of Mg^{2+} to *Geobacillus* sp. F75 during biofilm formation in a milk formulation with a high

monovalent to divalent cation ratio may have stimulated the *Geobacillus* sp. F75 to sporulate, resulting in a dormant biofilm form compared to an actively metabolising and growing biofilm. This is evidence which suggests that biofilm formation by *Geobacillus* spp. during milk powder manufacture is reduced when milk formulations with a high monovalent to divalent cation ratio are processed.

4.5) Conclusions

MALDI-TOF MS analysis of whole cell extracts of *Geobacillus* sp. F75 grown in a biofilm indicated that *Geobacillus* spp. alter their protein expression profile and physiology in response to external free Mg^{2+} concentrations. Also, the presence of Mg^{2+} stimulates *Geobacillus* spp. to refrain from undergoing sporulation. It is proposed that in milk formulations that have a high monovalent to divalent cation ratio, *Geobacillus* spp. respond to the low concentration and availability of Mg^{2+} by increasing their sporulation. This would prevent cell division, metabolism and proliferation of the *Geobacillus* spp. in a growing biofilm. Thus, the proliferation of *Geobacillus* spp. may be markedly reduced during the manufacture of milk powders with high monovalent to divalent cation ratios.

CHAPTER 5

Inhibition of *Geobacillus* species biofilms by changes in sodium, calcium and magnesium ion concentrations

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Ben Somerton

Name/Title of Principal Supervisor: Steve Flint

Name of Published Research Output and full reference:

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In which Chapter is the Published Work: Chapter 5

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Candidate's Signature — 19 November 2013 — Date

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Principal Supervisor's signature — 19 November 2013 — Date

5.1) Abstract

This study investigated the effect of varied sodium, calcium and magnesium concentrations in specialty milk formulations on biofilm formation by *Geobacillus* spp. and *Anoxybacillus flavithermus*. The numbers of attached viable cells (log CFU cm^{-2}) after 6–18 h of biofilm formation by three dairy-derived strains of *Geobacillus* spp. and three dairy-derived strains of *A. flavithermus* were compared in two commercial milk formulations. Milk formulation '4' had relatively high sodium and low calcium and magnesium concentrations compared with milk formulation '2', but both had comparable fat, protein and lactose concentrations. Biofilm formation by the three *Geobacillus* spp. isolates was up to 4 log CFU cm^{-2} lower in milk formulation 4 compared with milk formulation 2 after 6–18 h and the difference was often significant ($P \leq 0.05$). However, no significant differences ($P \leq 0.05$) were found when biofilm formation by the three *A. flavithermus* isolates was compared in milk formulations 2 and 4. Supplementation of milk formulation 2 with 100 mM NaCl significantly decreased ($P \leq 0.05$) *Geobacillus* spp. biofilm formation after 6–10 h. Furthermore, supplementation of milk formulation 4 with 2 mM $CaCl_2$ or 2 mM $MgCl_2$ significantly increased ($P \leq 0.05$) *Geobacillus* spp. biofilm formation after 10–18 h. It was concluded that relatively high free Na^+ and low free Ca^{2+} and Mg^{2+} concentrations in milk formulations are collectively required to inhibit biofilm formation by *Geobacillus* spp., whereas biofilm formation by *A. flavithermus* spp. is not impacted by typical cation concentration differences of milk formulations.

5.2) Introduction

Thermophilic bacilli belonging to the *Geobacillus* spp. and *Anoxybacillus flavithermus* groups are the predominant spoilage bacteria that may contaminate milk during its manufacture into milk powder (Burgess *et al*., 2010; Hill & Smythe, 2012). The number of thermophilic bacilli in milk powder is of major importance because it is a measure of its quality and determines its market selling price (Burgess *et al*., 2010; Hill & Smythe, 2012). *Geobacillus* spp. and *A. flavithermus* grow as biofilms on product-contact surfaces in regions of milk powder manufacturing plants, such as in plate heat exchangers and evaporators, that are held at high temperatures (up to 70 °C) (Burgess *et al*., 2010; Hill & Smythe, 2012). It is perceived that these biofilms act as a reservoir of cells that slough off and disperse into milk as it transits through the plant (Burgess *et al*., 2010; Hill & Smythe, 2012). The majority of thermophilic bacilli that appear in milk powder originate from biofilms on product-contact surfaces (Hill & Smythe, 2012).

The concentrations and ratios of free cations in the aqueous phase that immerses a biofilm can influence biofilms in many ways. Free cations electrostatically interact with bacterial polymers in a biofilm matrix, which can influence the structural integrity and cohesion of a biofilm (Hermansson, 1999; Sobeck & Higgins, 2002). In addition, bacteria respond to fluctuations in free cation concentrations by adapting their physiology, which may impact the prosperity of a biofilm. For example, Kara *et al*. (2008) showed how bacteria in a wastewater sludge increase the proportion of negatively charged, hydrophilic extracellular polymers in response to increasing Na^+ concentrations, which causes a decrease in the cohesion of the sludge. Conversely, Patrauchan *et al*. (2005) showed how Ca^{2+} stimulates a *Pseudoalteromonas* sp. to increase both the amount and the composition of extracellular proteins it expresses, which primes the bacteria for biofilm formation. Additionally, Song & Leff (2006)

proposed that Mg^{2+} may enhance biofilm formation by *Pseudomonas fluorescens* by influencing the production of flagella and fimbriae or the production and structure of exopolysaccharide.

Furthermore, Na^{+}, Ca^{2+} and Mg^{2+} have important roles in bacterial homeostasis and are required as a nutrient source (Hase *et al*., 2001; Michiels *et al*., 2002; Smith & Maguire, 1998). High Na^{+} concentrations can have a toxic effect on bacteria (Hase *et al*., 2001). Ca^{2+} and Mg^{2+} are required for the optimal functioning of many bacterial proteins, including enzymes (Heptinstall *et al*., 1970; Hughes *et al*., 1973; Martinez-Gil *et al*., 2012; Michiels *et al*., 2002), and localized fluxes of Ca^{2+} have an integral role in the regulation of important bacterial cellular processes, such as the cell cycle and cell division (Michiels *et al*., 2002).

In the dairy industry, many different milk formulations are processed into milk powder. Milk formulations have a range of cation concentrations, and the cation concentrations of some milk formulations differ from those of unprocessed milk. Typical total (sum of bound and free) sodium, potassium, calcium and magnesium concentrations in unprocessed milk are 22, 37, 30 and 5 mM respectively (Fox, 2003). However, in some specialty milk formulations, total sodium concentrations can reach as high as 100 mM and total calcium concentrations can reach as low as 7 mM (Table 1.5). There is potential for different cation concentrations and ratios in milk formulations to differentially influence biofilm formation and proliferation by *Geobacillus* spp. and *A. flavithermus* during milk powder manufacture. To understand how varied cation concentrations in milk formulations differentially impact biofilm formation by *Geobacillus* spp. and *A. flavithermus*, we investigated the influence that different sodium, calcium and magnesium concentrations in milk formulations had on the number of viable cells attached per square centimetre to 316 stainless steel coupons.

5.3) Methods

5.3.1) Bacterial isolates

A. flavithermus E16 and *Geobacillus* sp. F75 were isolated from product-contact surfaces at milk powder manufacturing plant '1'. *A. flavithermus* 136 and *Geobacillus* sp. 183 were derived from milk powders manufactured at plant 1. *A. flavithermus* TRb and *Geobacillus* sp. TRa were isolated from product-contact surfaces at milk powder manufacturing plant '2'. Plant 1 is situated on the South Island and plant 2 is situated on the North Island of New Zealand.

5.3.2) Growth media

Casein digest medium (1 g l^{-1}) (Difco, BD Biosciences) and bovine, commercial, specialty milk formulations 2 and 4 (10 g 90 ml^{-1}) (Fonterra) (Table 1.5) were used as bacterial growth media. Milk powders were reconstituted with water that had been deionized by reverse osmosis and autoclaved (121 °C, 15 min) to sterilize. Milk formulations 2 and 4 were derived from the same respective batches throughout experimentation. Milk formulations 2 and 4 had similar fat, protein and lactose concentrations: 1.5, 81.7 and 3.9 g l^{-1} respectively in milk formulation 2 and 1.7, 81.7 and 3.9 g l^{-1} respectively in milk formulation 4. However, milk formulations 2 and 4 had different total (sum of bound and free) sodium, calcium and magnesium concentrations (Table 1.5). Milk formulation 2 was supplemented with analytical grade NaCl (Merck) powder and milk formulation 4 was supplemented with analytical grade $CaCl_2.2H_2O$ (Merck) or $MgCl_2.6H_2O$ (Merck) powder, which was achieved by dissolving the NaCl, $CaCl_2.2H_2O$ or $MgCl_2.6H_2O$ powder in deionized water prior to dissolving the milk powder. Prior to reconstitution, the milk powders were gamma

irradiated (25,000 Gy) to inactivate any contaminating micro-organisms present, so that growth and analysis of the inoculated bacteria of interest were unimpeded.

5.3.3) Culture storage

The bacterial isolates were grown in tryptic soy broth (Merck) to mid-log phase and were stored with the addition of glycerol (10%, vol/vol) (Merck) at –80 °C.

5.3.4) Inoculum preparation

To propagate bacteria for use in the biofilm formation assay, 1 ml of a thawed bacterial culture was used to inoculate 100 ml of casein digest medium (1 g l^{-1}) (Difco, BD Biosciences). The inoculated medium was incubated at 55 °C for 9 h, which was sufficient time for the bacteria to reach mid-stationary growth phase. Bacteria were propagated in casein digest medium (reconstituted at a low concentration of 1 g l^{-1} with deionized water) prior to the initiation of biofilm formation so that they would be in a nutrient-starved metabolic state, which simulated the growth of remaining viable bacteria on surfaces in milk powder manufacturing plants after a cleaning regime and at the commencement of a manufacturing run.

5.3.5) Biofilm formation assay

Stainless steel coupons were cleaned and passivated prior to use in the biofilm formation assay as previously described by Flint *et al*. (1997b). After bacteria were grown planktonically, they were diluted (typically with a dilution factor of around 1:200) in either milk formulation 2 or milk formulation 4 to achieve an inoculum of approximately 4.5 log CFU ml^{-1}; 1.5 ml of this inoculum was added per well of a 24-well culture plate (Becton Dickinson). One 316 stainless steel coupon (Part #RD128-316, Biosurface Technologies Corporation), which had a surface area of approximately

4 cm^2, was added, using sterile forceps, to each inoculum-containing well so that it was completely submerged and horizontal. The plate, wrapped in a plastic bag to prevent evaporation, was incubated at 55°C for 6, 10, 14 or 18 h. Biofilm formation was investigated for up to 18 h to simulate the duration of a typical milk powder manufacturing run (Burgess *et al*., 2010). Biofilm formation was investigated on coupons made from 316 stainless steel, as this is the grade of stainless steel that typically comprises product-contact surfaces in milk powder manufacturing plants (Burgess *et al*., 2010).

5.3.6) Cell enumeration

The following protocol was used to enumerate the number of attached viable cells per square centimetre on the coupons. The coupons were removed from the cultures using sterile forceps, dipped and rinsed three times in approximately 50 ml of deionized water to displace any loosely attached cells, and placed into a 35 ml plastic container (Item Code LBS3722W, Thermo Fisher Scientific) with 5 ml of fresh casein digest medium (1 g l^{-1}) and 12 g of glass beads with a diameter of 6.35 mm (Catalogue # 11079635, Biospec Products, Inc.). The plastic containers were vortex mixed vigorously for 2 min to dislodge the attached cells into the surrounding medium. Standard microbiological plate counting techniques were used to enumerate the viable CFU per millilitre in the dislodged cell suspension, using casein digest medium (1 g l^{-1}) as the diluent and milk plate count agar (Oxoid). The number of attached viable cells per square centimetre was determined.

5.3.7) Statistical analysis

The experiments were carried out on three separate occasions, and mean attached viable cell numbers (CFU cm^{-2}) ± 1 standard deviation (σ_{n-1}) are reported. Minitab software

(Minitab Pty Ltd) was used to calculate the population standard error and 95% confidence intervals ($P \leq 0.05$) were used to determine significant differences among the mean values.

5.4) Results and discussion

5.4.1) Comparison of *Geobacillus* spp. biofilm formation in milk formulations 2 and 4

Biofilm formation, as determined by the number of viable cells per square centimetre, by all three of the *Geobacillus* spp. isolates was significantly lower ($P \leq 0.05$) in milk formulation 4 compared with milk formulation 2 at all four time points (6, 10, 14 and 18 h) (Fig. 5.1A, 5.1D, 5.2A, 5.2D, 5.3A and 5.3D). The extent of the difference in biofilm formation by the three *Geobacillus* spp. isolates, in milk formulation 2 compared with milk formulation 4, ranged between 1 and 4 log CFU cm^{-2} (Fig. 5.1A, 5.1D, 5.2A, 5.2D, 5.3A and 5.3D). The one exception, when there was no significant difference ($P \leq 0.05$) in biofilm formation in milk formulations 2 and 4, was for *Geobacillus* sp. TRa after 18 h (Fig. 5.2A and 5.2D).

It is proposed that biofilm formation by a large proportion of the dairy *Geobacillus* spp. population is inhibited throughout the duration of a manufacturing run when milk formulations with high sodium and low calcium and magnesium concentrations are processed. As the *Geobacillus* spp. isolates used in this study were isolated from different geographical regions (i.e. two different manufacturing plants) and different sources (i.e. product-contact surfaces and milk powders), they were likely to represent a range of dairy *Geobacillus* spp. phenotypes. Furthermore, a substantial proportion of thermophilic bacilli that contaminate milk powder manufacturing plants belong to the *Geobacillus* genus (Burgess *et al*., 2010; Hill & Smythe, 2012). Also, *Geobacillus* spp.

are the main bacteria to survive in reconstituted milk powder products destined for ultrahigh temperature (UHT) or retort treatment when used by customers in downstream applications, as *Geobacillus* spp. spores have a greater tolerance to high temperatures than *A. flavithermus* spores (Hill & Smythe, 2012). Thus, there is potential for the thermophilic bacilli count in the final milk powder product to decrease markedly, and the quality and selling price of the final milk powder product to increase, when a specialty milk formulation with relatively high sodium and low calcium and magnesium concentrations is processed.

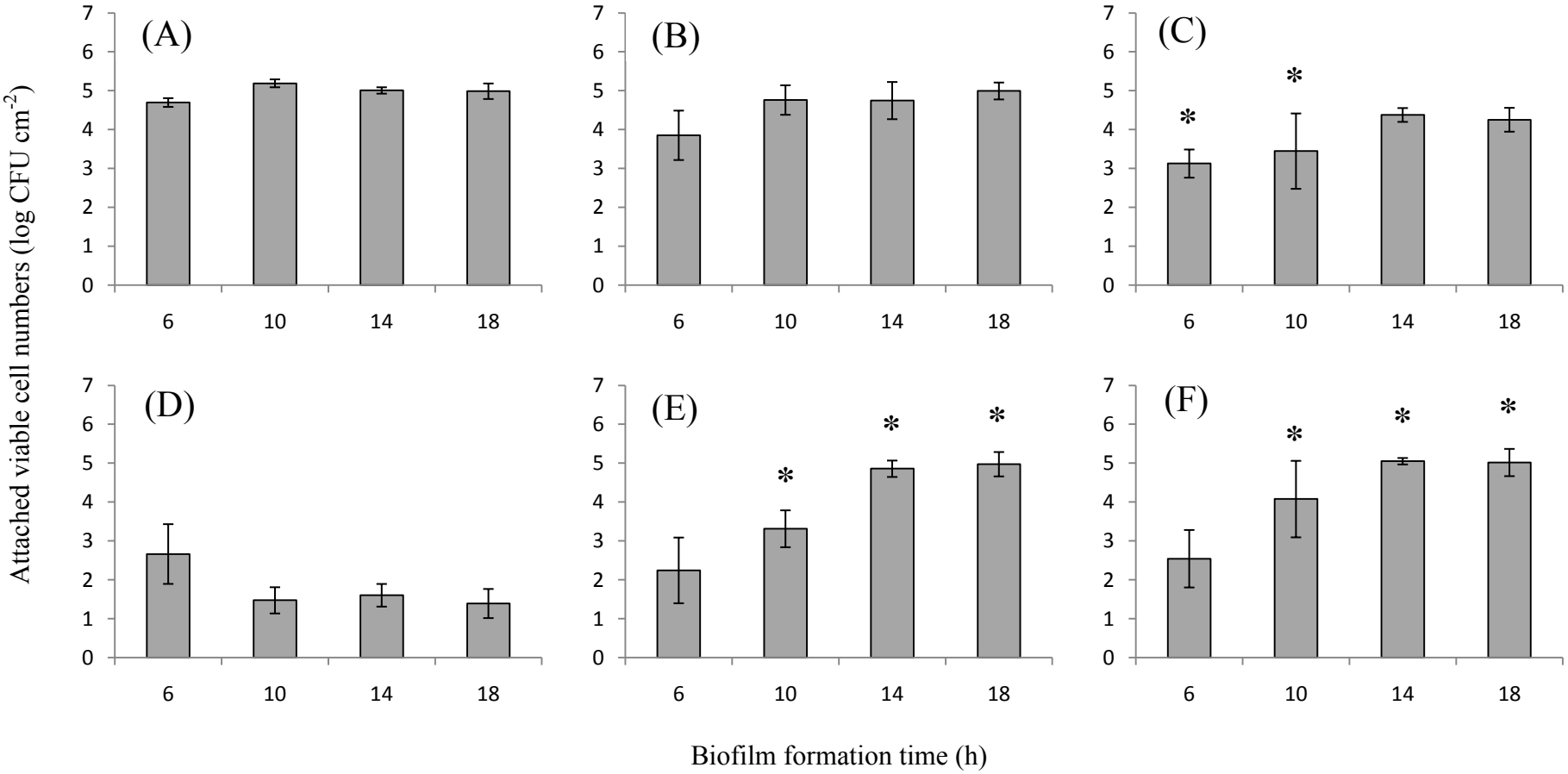
(A)
(B)
(C)
(D)
(E)
(F)
Attached viable cell numbers (log CFU cm-2)
Biofilm formation time (h)
0
1
2
3
4
5
6
7
6
10
14
18
*

FIG 5.1

FIG 5.1 Biofilm formation, after 6–18 h of incubation at 55°C, by viable *Geobacillus* sp. F75 cells (log CFU cm^{-2}) on stainless steel coupons completely submerged in milk formulation 2 (A), milk formulation 2 supplemented with 50 mM NaCl (B), milk formulation 2 supplemented with 100 mM NaCl (C), milk formulation 4 (D), milk formulation 4 supplemented with 2 mM $CaCl_2$ (E) and milk formulation 4 supplemented with 2 mM $MgCl_2$ (F). Experiments were repeated as triplicates and error bars represent ± 1 standard deviation (σ_{n-1}). An asterisk (*) depicts a significant difference ($P \leq 0.05$) between cation-supplemented and unsupplemented milk formulations for the respective milk formulation and time point.

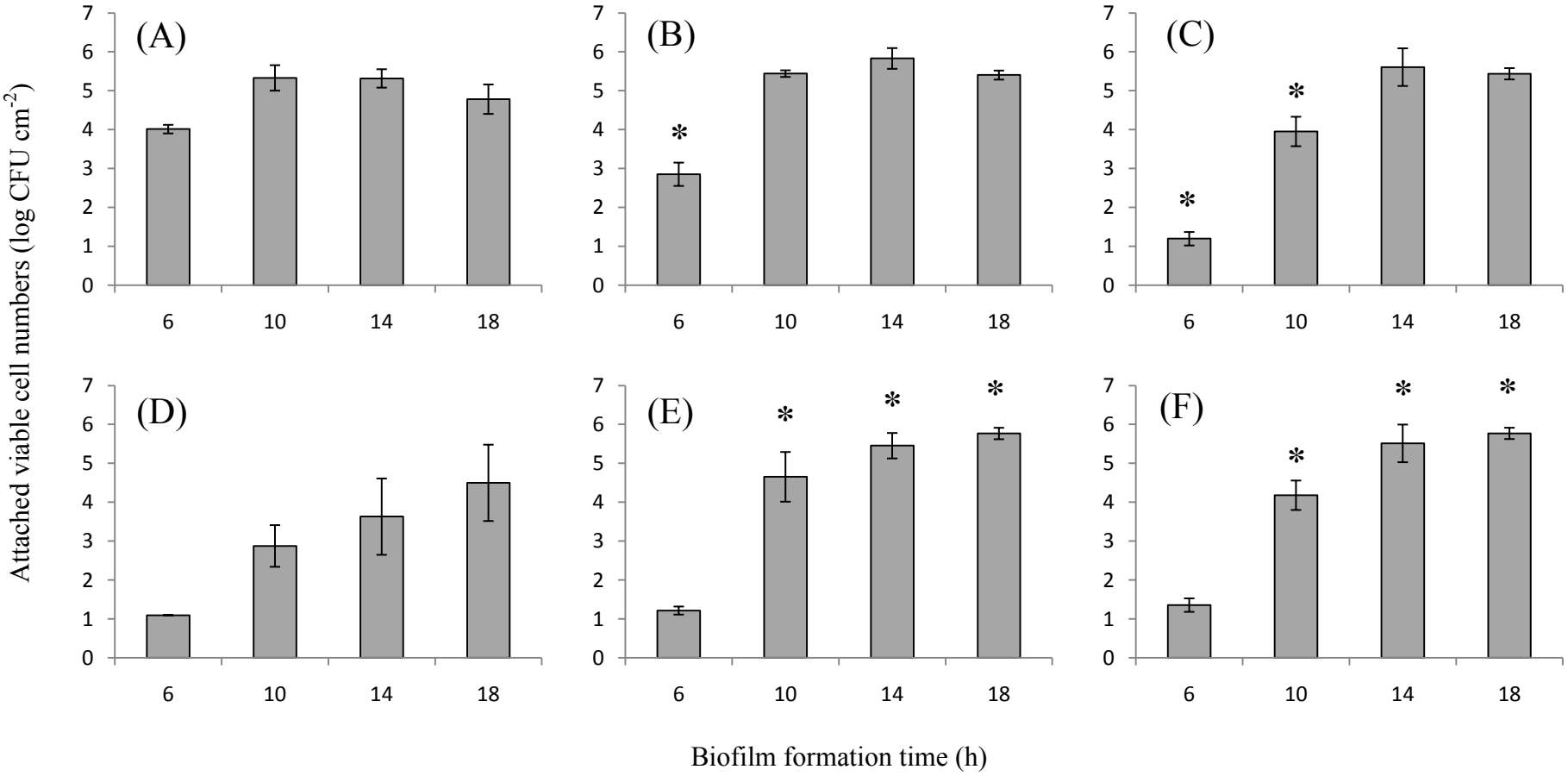
(A)
(B)
(C)
(D)
(E)
(F)
Attached viable cell numbers (log CFU cm-2)
Biofilm formation time (h)
0
1
2
3
4
5
6
7
6
10
14
18
*

FIG 5.2

FIG 5.2 Biofilm formation, after 6–18 h of incubation at 55°C, by viable *Geobacillus* sp. TRa cells (log CFU cm^{-2}) on stainless steel coupons completely submerged in milk formulation 2 (A), milk formulation 2 supplemented with 50 mM NaCl (B), milk formulation 2 supplemented with 100 mM NaCl (C), milk formulation 4 (D), milk formulation 4 supplemented with 2 mM $CaCl_2$ (E) and milk formulation 4 supplemented with 2 mM $MgCl_2$ (F). Experiments were repeated as triplicates and error bars represent ± 1 standard deviation (σ_{n-1}). An asterisk (*) depicts a significant difference ($P \leq 0.05$) between cation-supplemented and unsupplemented milk formulations for the respective milk formulation and time point.

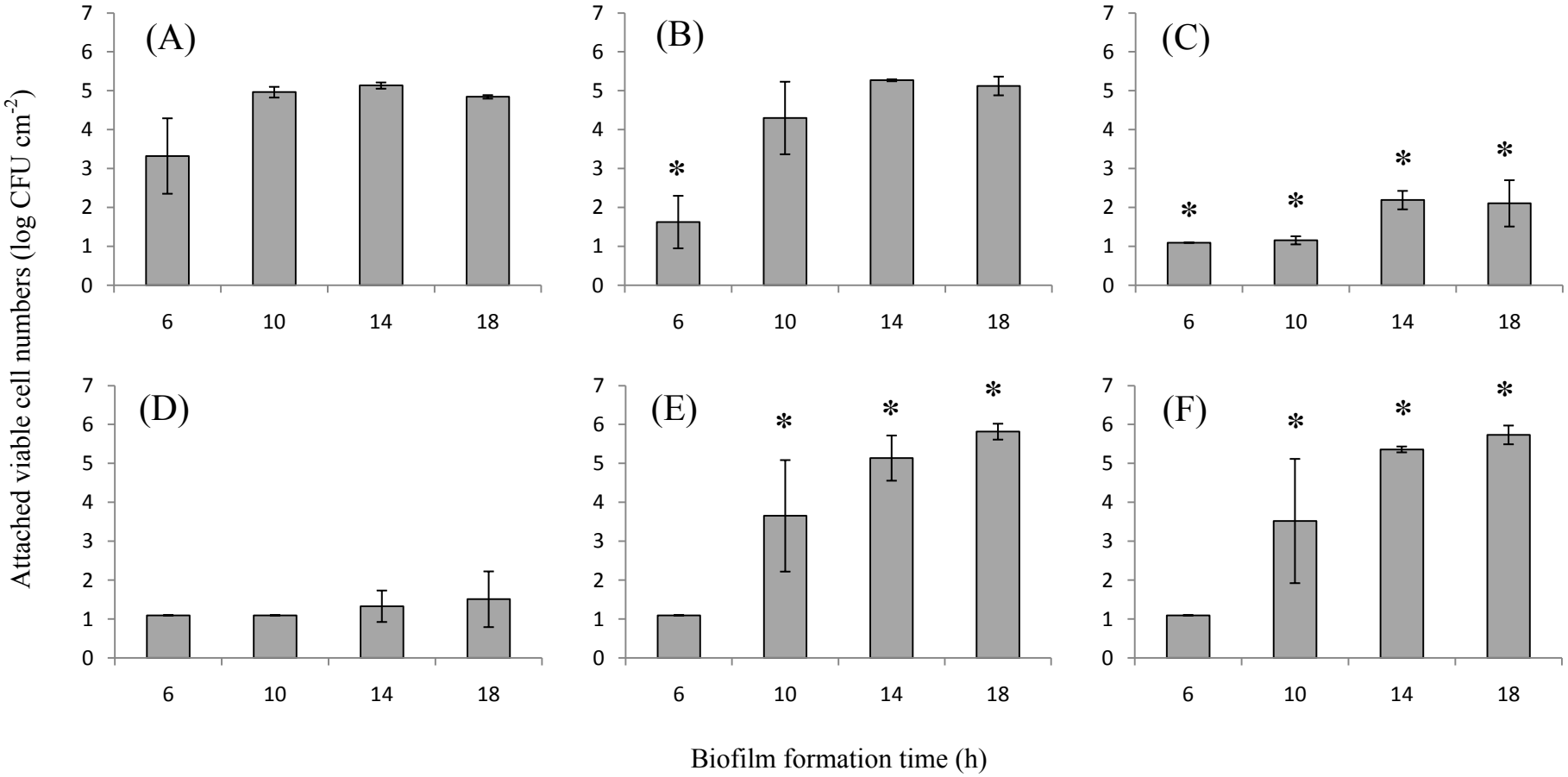
(A)
(B)
(C)
(D)
(E)
(F)
Attached viable cell numbers (log CFU cm-2)
Biofilm formation time (h)
0
1
2
3
4
5
6
7
6
10
14
18
*

FIG 5.3

FIG 5.3 Biofilm formation, after 6–18 h of incubation at 55°C, by viable *Geobacillus* sp. 183 cells (log CFU cm^{-2}) on stainless steel coupons completely submerged in milk formulation 2 (A), milk formulation 2 supplemented with 50 mM NaCl (B), milk formulation 2 supplemented with 100 mM NaCl (C), milk formulation 4 (D), milk formulation 4 supplemented with 2 mM $CaCl_2$ (E) and milk formulation 4 supplemented with 2 mM $MgCl_2$ (F). Experiments were repeated as triplicates and error bars represent ± 1 standard deviation (σ_{n-1}). An asterisk (*) depicts a significant difference ($P \leq 0.05$) between cation-supplemented and unsupplemented milk formulations for the respective milk formulation and time point.

5.4.2) Characterization of the effect of sodium, calcium and magnesium on *Geobacillus* spp. biofilm formation

To characterize the role that high sodium and low calcium and magnesium concentrations had on the inhibition of biofilm formation by *Geobacillus* spp., we investigated the influence of supplementation of milk formulation 2 with 50 or 100 mM NaCl and supplementation of milk formulation 4 with 2 mM $CaCl_2$ or 2 mM $MgCl_2$ on biofilm formation by three dairy *Geobacillus* spp. isolates.

Relative to unsupplemented milk formulation 2, supplementation of milk formulation 2 with 100 mM NaCl significantly decreased ($P \leq 0.05$) biofilm formation by *Geobacillus* spp. isolates F75 and TRa at the earlier biofilm formation times of 6 and 10 h by between 1.4 and 2.8 log CFU cm^{-2} (Fig. 5.1A, 5.1C, 5.2A and 5.2C); and significantly decreased ($P \leq 0.05$) biofilm formation by *Geobacillus* sp. 183 at all time points (6–18 h) by between 2.2 and 3.8 log CFU cm^{-2} (Fig. 5.3A and 5.3C). Supplementation of milk formulation 2 with 50 mM NaCl did not inhibit biofilm formation, by the *Geobacillus* spp. isolates studied, as greatly as supplementation with 100 mM NaCl. Relative to unsupplemented milk formulation 2, supplementation of milk formulation 2 with 50 mM NaCl did not significantly decrease ($P \leq 0.05$) biofilm formation by *Geobacillus* sp. F75 at all time points studied (Fig. 5.1A and 5.1B), and significantly decreased ($P \leq 0.05$) biofilm formation by *Geobacillus* spp. isolates TRa and 183 after 6 h by 1.2 and 1.7 log CFU cm^{-2} respectively (Fig. 5.2A, 5.2B, 5.3A and 5.3B).

Supplementation of milk formulation 4 with either 2 mM $CaCl_2$ or 2 mM $MgCl_2$ significantly increased ($P \leq 0.05$) biofilm formation by all three *Geobacillus* spp. isolates at 10, 14 and 18 h by up to 4 log CFU cm^{-2} (Fig. 5.1D, 5.1E, 5.1F, 5.2D, 5.2E, 5.2F, 5.3D, 5.3E and 5.3F).

These results indicated that high sodium, low calcium and low magnesium concentrations each act as a hurdle to inhibit *Geobacillus* spp. biofilm formation and that all three hurdles are required to maximize the inhibition of *Geobacillus* spp. biofilm formation over an 18-h period.

It is likely that the free (ionized) form of the cations influenced biofilm formation by the *Geobacillus* spp. isolates, because it is the free form of cations that is biologically active and has the potential to interact with and influence bacteria (Aranha *et al*., 1986). Milk formulations have a high concentration of solutes, such as proteins and anions, which readily form complexes with cations (Holt, 1985). Furthermore, the partitioning of ions between the bound and free forms is dynamic (Holt, 1985). In any given milk formulation, approximately 10% of the total calcium and magnesium and approximately 90% of the total sodium exist in the free form (Fox, 2003; Holt, 1985). Thus, the free Na^+, Ca^{2+} and Mg^{2+} concentrations in milk formulation 4 were estimated to be 91, 0.7 and 0.1 mM respectively. It is proposed that differences in the free Na^+, Ca^{2+} and Mg^{2+} concentrations in the milk formulations (both intrinsic to the milk formulations and from supplementation) caused the differences in biofilm formation by the *Geobacillus* spp. isolates.

A calcium-selective electrode was used to determine the free Ca^{2+} concentration in milk formulations 2 and 4. Although the total calcium concentration in milk formulations 2 and 4 differed by approximately 28 mM (Table 1.5), the free Ca^{2+} concentrations were similar (0.4 and 0.2 mM in milk formulations 2 and 4, respectively). Both milk formulations 2 and 4 had a high protein concentration of 81.7 g l^{-1}. Perhaps the high protein concentration caused a relatively high proportion of the total calcium to exist bound to protein in the milk formulations, leaving a low proportion of the total calcium to exist in the free form. In any given milk formulation, the partitioning of magnesium between the bound and free forms follows that of

calcium (Fox, 2003; Holt, 1985). Thus, the free Mg^{2+} concentrations in milk formulations 2 and 4 were also likely to be similar. These findings indicate that, although low Ca^{2+} and Mg^{2+} concentrations were required to inhibit biofilm formation by the *Geobacillus* spp. isolates, it was the higher free Na^{+} concentration of milk formulation 4, relative to milk formulation 2, that was responsible for the inhibition of biofilm formation by the *Geobacillus* spp. isolates in milk formulation 4.

Supplementation of milk formulation 2 with 50 or 100 mM NaCl did not inhibit biofilm formation by the *Geobacillus* spp. isolates as greatly as milk formulation 4 (Fig. 5.1B, 5.1C, 5.1D, 5.2B, 5.2C, 5.2D, 5.3B, 5.3C and 5.3D), even though the total sodium concentration of milk formulation 2 supplemented with 50 or 100 mM NaCl was close to or higher than that of milk formulation 4 (Table 1.5). The supplementation of any given milk system with NaCl, (or $CaCl_2$ or $MgCl_2$) causes bound divalent cations to dissociate and increases the concentrations of free Ca^{2+} and Mg^{2+} (Gaucheron, 2005; Holt, 1985). Thus, supplementing milk formulation 2 with NaCl would have increased the free Ca^{2+} and Mg^{2+} concentrations. Although supplementing milk formulation 2 with 50 or 100 mM NaCl would have increased the free Na^{+} concentration, and caused the observed decrease in biofilm formation by the *Geobacillus* spp. isolates, the slight increase in free Ca^{2+} and Mg^{2+} concentrations (due to NaCl supplementation) may have increased biofilm formation relative to that observed in milk formulation 4.

The estimated free Ca^{2+} and Mg^{2+} concentrations in milk formulation 4, of 0.4−0.7 and 0.1 mM, respectively, were low relative to those in many milk formulations. For example, the free Ca^{2+} and Mg^{2+} concentrations in unprocessed milk are 1–4 and 0.4–1.3 mM (Fox, 2003) respectively. When milk formulations are supplemented with $CaCl_2$ or $MgCl_2$, only part of the supplemented calcium or magnesium exists in the free form. For example, Philippe *et al*. (2003) found that the addition of 4.5 mM $CaCl_2$ to skim milk increased the free Ca^{2+} concentration from 1.56 to 2.86 mM. Thus, it was

predicted that, when milk formulation 4 was supplemented with 2 mM $CaCl_2$ or $MgCl_2$, the free Ca^{2+} or Mg^{2+} concentration would have increased by less than 2 mM. However, this increase was enough to elicit the observed increase in biofilm formation by the *Geobacillus* spp. isolates. This observation is feasible given that the estimated intrinsic free Ca^{2+} and Mg^{2+} concentrations in milk formulation 4 were low.

5.4.3) Comparison of the effects of calcium and magnesium on *Geobacillus* spp. biofilm formation

There was no significant difference ($P \leq 0.05$) when the effects of Ca^{2+} and Mg^{2+} on biofilm formation by the three *Geobacillus* spp. isolates studied were compared (Fig. 5.1E, 5.1F, 5.2E, 5.2F, 5.3E and 5.3F). Divalent cations, such as Ca^{2+} and Mg^{2+}, have structural, functional and regulatory interactions with many bacterial polymers, enzymes and regulatory proteins (Dobson & O'Shea, 2008; Lambert *et al*., 1975a; Rose & Hogg, 1995). Often the interaction is specific to a particular cation species, or the interaction of one cation species is more efficacious than that of other cation species (Dobson & O'Shea, 2008; Lambert *et al*., 1975a; Rose & Hogg, 1995). It could be hypothesized that Ca^{2+} and Mg^{2+} acted in a non-specific manner to enhance biofilm formation by the *Geobacillus* spp. isolates in the milk formulations.

Conversely, it is also possible that only one of the two cations, either Ca^{2+} or Mg^{2+}, increased biofilm formation by the *Geobacillus* spp. isolates. Supplementation of milk formulation 4 with either $CaCl_2$ or $MgCl_2$ would have increased the concentration of both free Ca^{2+} and free Mg^{2+}. For example, if an increase in the free Ca^{2+} concentration was responsible for increasing *Geobacillus* spp. biofilm formation, the increase in the free Ca^{2+} concentration after $MgCl_2$ supplementation may have been sufficient to increase biofilm formation.

5.4.4) Effect of iron on *Geobacillus* spp. biofilm formation

As it has also been proposed that iron may influence the growth of *Geobacillu*s spp. in sodium caseinate media (Ashton *et al*., 1968), we determined whether an intrinsically low Fe^{2+} concentration in milk formulation 4 was responsible for the inhibition of the *Geobacillus* spp. isolates. Milk formulation 4 was supplemented with 0.5, 1 or 5 mM $FeCl_2$ and its effect on *Geobacillus* sp. F75 biofilm formation was investigated. In contrast to $CaCl_2$ and $MgCl_2$ supplementation, $FeCl_2$ supplementation did not increase biofilm formation by *Geobacillus* sp. F75 (results not shown). This implied that, in our study, the low Fe^{2+} concentration did not inhibit biofilm formation by the *Geobacillus* spp. isolates. Our results contrast with those of Ashton *et al*. (1968), who showed that iron ion chelation by sodium caseinate inhibited both the outgrowth of spores of *Bacillus stearothermophilus* (since renamed *Geobacillus stearothermophilus*) (Nazina *et al*., 2001) and the subsequent development of colony forming units on agar. Ashton *et al*. (1968) focused on the effects of cation deprivation on spore germination, whereas we did not distinguish between vegetative cells and spores. The contrasting observations could have been due to differences in the requirements and responses of vegetative cells compared with spore forms of *Geobacillus* spp. to iron ions.

5.4.5) Proposed mechanisms for cation inhibition of *Geobacillus* spp. biofilm formation

Three mechanisms can be proposed to explain the observed inhibition of *Geobacillus* spp. biofilm formation by relatively high sodium and low calcium and magnesium concentrations. Firstly, this combination of cation concentrations may have compromised the structural integrity of the biofilm. Bacterial cell wall and extracellular matrix polymers are often composed of an abundance of negatively charged functional groups, such as phosphate and carboxyl groups (Neuhaus & Baddiley, 2003). Free

cations associate with negatively charged functional groups on polymers and enhance the overall stability and cohesion of the cell wall and the extracellular matrix (Dunne & Burd, 1992; Lambert *et al*., 1975a). Furthermore, Ca^{2+} and Mg^{2+} have greater cohesive properties than Na^{+}, because divalent cations have a higher charge density and a greater capacity to neutralize negatively charged functional groups, and may form divalent cation bridges (Kara *et al*., 2008; Sobeck & Higgins, 2002). Although Ca^{2+} and Mg^{2+} have greater binding affinities than Na^{+}, as the Na^{+} concentration surrounding bacteria and biofilms increases, Na^{+} can displace Ca^{2+} and Mg^{2+} within the biofilm matrix and consequently decrease the cohesion and structural integrity of the biofilm (Higgins & Novak, 1997; Lambert *et al*., 1975a). There was a stark contrast in the estimated free Na^{+} concentration relative to the free Ca^{2+} and Mg^{2+} concentrations in milk formulation 4, such that it had a very high monovalent to divalent cation ratio. This may have sufficiently decreased the electrostatic forces within the biofilms formed by the *Geobacillus* spp. isolates and inhibited biofilm formation. This hypothesis agrees with results for wastewater sludges, in which high monovalent to divalent cation ratios compromised the structural integrity of wastewater sludge biofilms (Higgins & Novak, 1997). Negatively charged functional groups are ubiquitous in bacterial cell wall polymers and cations usually associate with these groups in a non-specific manner (Neuhaus & Baddiley, 2003). Given that *A. flavithermus* biofilms were not inhibited in milk formulation 4, it is proposed that the inhibitory effect on *Geobacillus* spp. may have been related to cellular functions specific to that group of bacteria.

The second proposed mechanism is that the combination of cation concentrations may have influenced regulatory pathways that altered the metabolism or physiology of the *Geobacillus* spp. isolates and compromised the growth or structural integrity of the biofilms. Elevated extracellular Na^{+} concentrations have been shown to stimulate an increase in the proportion of negatively charged, hydrophilic bacterial cell wall

polymers in a wastewater sludge, which decreases its structural integrity (Kara *et al*., 2008). Conversely, Ca^{2+} and Mg^{2+} have the potential to enhance biofilm formation by binding to regulatory proteins, such as response regulators and secreted proteins, which have implications in biofilm formation (He *et al*., 2008; Martinez-Gil *et al*., 2012; Michiels *et al*., 2002; Song & Leff, 2006). In additon, Ca^{2+} has been shown to influence the morphology and proteome expression of bacteria, which consequently enhances their biofilm formation (Garrison-Schilling *et al*., 2011; Patrauchan *et al*., 2005).

Na^{+} competes with Ca^{2+} and Mg^{2+} for assimilation into the cell wall and accumulation at the cell wall−cytoplasmic membrane interface (Heptinstall *et al*., 1970; Lambert *et al*., 1975a; Neuhaus & Baddiley, 2003). When the *Geobacillus* spp. isolates formed biofilms in milk formulation 4, relatively high concentrations of Na^{+} and low concentrations of Ca^{2+} and Mg^{2+} may have accumulated at the cell wall−cytoplasmic membrane interface. The high Na^{+} concentrations may have directly stimulated the *Geobacillus* spp. isolates to enter a metabolic or physiological state that decreased their ability to grow as a biofilm. Alternatively, the low Ca^{2+} and Mg^{2+} concentrations may have resulted in inadequate stimulation of regulatory proteins that recognize Ca^{2+} or Mg^{2+} as a stimulus. The lack of stimulation by Ca^{2+} or Mg^{2+} may have decreased the ability of the *Geobacillus* spp. isolates to grow as a biofilm (Patrauchan *et al*., 2005; Song & Leff, 2006). Supplementation of milk formulation 4 with either $CaCl_2$ or $MgCl_2$ may have caused sufficient amounts of Ca^{2+} or Mg^{2+} to accumulate at the cell wall−cytoplasmic membrane interface of the *Geobacillus* spp. isolates and buffered the cells from the high Na^{+} concentration. This would have either prevented the high Na^{+} concentration from influencing the metabolism or physiology of the *Geobacillus* spp. isolates or provided an adequate supply of Ca^{2+} and Mg^{2+} at the cell wall−cytoplasmic membrane interface to bind to and stimulate Ca^{2+}- or Mg^{2+}-binding regulatory proteins.

The third proposed mechanism is that the combination of cation concentrations may have imbalanced cation homeostasis and limited cellular functioning and cell division of the *Geobacillus* spp. isolates. Cation homeostasis is critical, as cations have important roles in bacterial physiology (Hase *et al*., 2001; Michiels *et al*., 2002; Smith & Maguire, 1998). Na^+ is translocated outside the cell to establish an environment in the cytosol that has a low Na^+ concentration and creates an optimal pH and enzyme function (Hase *et al*., 2001). Relatively high extracellular Na^+ concentrations may force the accumulation of Na^+ to unfavourable concentrations in the cytosol of bacteria, and may have a toxic effect by impairing cell function (Hase *et al*., 2001). Although Ca^{2+} is required intracellularly, most is bound to cellular structures such as Ca^{2+}-binding proteins (Michiels *et al*., 2002). Free Ca^{2+} concentrations in the cytosol are typically 100–1000 fold lower relative to those outside the cytoplasmic membrane (Michiels *et al*., 2002). This Ca^{2+} concentration gradient is utilized by bacteria, as localized cytosolic fluxes of Ca^{2+} regulate bacterial cellular processes such as the cell cycle and cell division (Michiels *et al*., 2002). In addition, Ca^{2+} and Mg^{2+} are required as co-factors by many enzymes for optimal functioning (Heptinstall *et al*., 1970; Hughes *et al*., 1973; Martinez-Gil *et al*., 2012; Michiels *et al*., 2002). An inadequate supply of intracellular Ca^{2+} and Mg^{2+} may limit bacterial cellular function (Lambert *et al*., 1975a; Michiels *et al*., 2002).

Na^+ competes with Ca^{2+} for binding to Ca^{2+} influx channels, and high Na^+ concentrations can block Ca^{2+} influx and acquisition into the cytosol (Naseem *et al*., 2008). Naseem *et al*. (2008) showed that, when external Na^+ concentrations are increased to 30 mM, Na^+ competes with Ca^{2+} for binding to a Ca^{2+} influx ion channel and stimulates net Ca^{2+} efflux from *Escherichia coli*. Bacterial Mg^{2+} transporter proteins have been identified in *Salmonella typhimurium* (Smith & Maguire, 1998). It could be hypothesized that Na^+ also competes with Mg^{2+} for binding to Mg^{2+} influx ion

channels, and high Na^+ concentrations may reduce cytosolic acquisition of Mg^{2+}. Thus, high extracellular Na^+ concentrations may reduce Ca^{2+} and Mg^{2+} translocation and intracellular acquisition via ion transport proteins.

The high Na^+ concentration in milk formulation 4 may have reduced the acquisition or promoted the loss of Ca^{2+} and Mg^{2+} from the cytosol and had a bacteriostatic effect on the *Geobacillus* spp. isolates. The high Na^+ concentration may have acted either by reducing the assimilation of Ca^{2+} and Mg^{2+} into the cell wall and the cell wall−cytoplasmic membrane interface or by reducing the translocation of Ca^{2+} and Mg^{2+} through ion transport proteins. Alternatively, the high Na^+ concentration may have had a toxic effect on the *Geobacillus* spp. because of excessive accumulation of Na^+ in the cytosol. The supplementation of milk formulation 4 with either $CaCl_2$ or $MgCl_2$ may have either enabled the *Geobacillus* spp. isolates to acquire sufficient amounts of intracellular Ca^{2+} and Mg^{2+} or prevented excessive accumulation of Na^+ into the cytosol.

5.4.6) Comparison of *A. flavithermus* biofilm formation in milk formulations 2 and 4

There was no significant difference ($P \leq 0.05$) in biofilm formation after 6–18 h by any of the three *A. flavithermus* isolates studied when growth in milk formulations 2 and 4 at each time point was compared (Fig. 5.4). It is proposed that *A. flavithermus* is more adept than *Geobacillus* spp. at tolerating the high sodium and low calcium and magnesium concentrations that existed in milk formulation 4. For instance, *A. flavithermus* may have a greater capacity than *Geobacillus* spp. to tolerate the Na^+ concentrations present in milk formulation 4, or may have a greater capacity to acquire Ca^{2+} and Mg^{2+}.

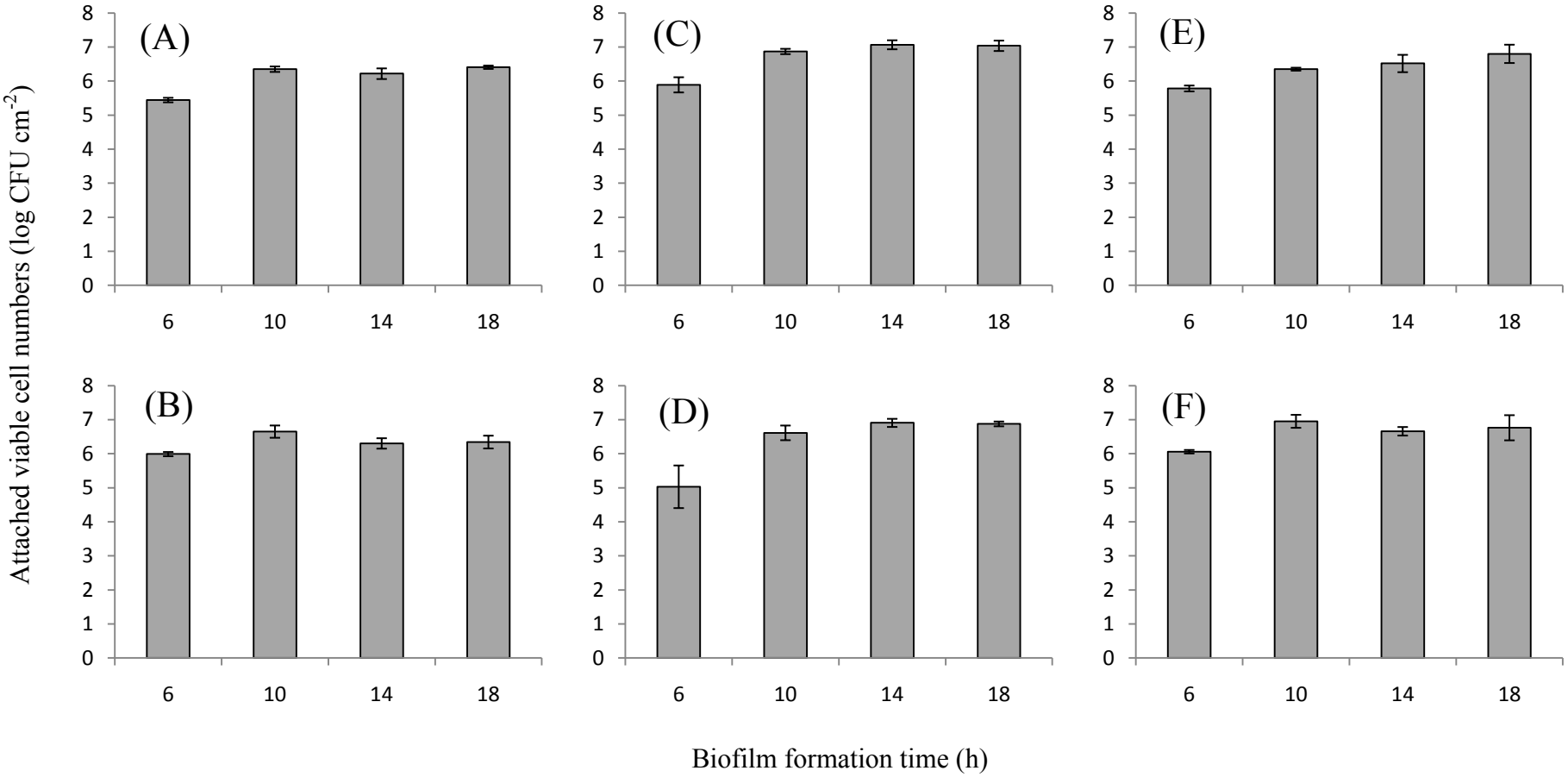
(A)
(B)
(C)
(D)
(E)
(F)
Attached viable cell numbers (log CFU cm-2)
Biofilm formation time (h)
0
1
2
3
4
5
6
7
8
6
10
14
18

FIG 5.4

FIG 5.4 Biofilm formation, after 6–18 h of incubation at 55°C, by viable *Anoxybacillus flavithermus* E16 (A) and (B), *A. flavithermus* TRb (C) and (D) and *A. flavithermus* 136 cells (E) and (F) (log CFU cm^{-2}) on stainless steel coupons completely submerged in milk formulation 2 (A), (C) and (E), and milk formulation 4 (B), (D) and (F). Experiments were repeated as triplicates and error bars represent ± 1 standard deviation (σ_{n-1}).

5.5) Conclusions

Geobacillus spp. biofilm formation was inhibited for up to 18 h in a milk formulation that had relatively high sodium and low calcium and magnesium concentrations. High sodium, low calcium and low magnesium concentrations were collectively required for *Geobacillus* spp. biofilm formation to be maximally inhibited. In contrast, biofilm formation by *A. flavithermus* was not inhibited in the milk formulation with relatively high sodium and low calcium and magnesium concentrations. High free Na^{+} and low free Ca^{2+} and Mg^{2+} concentrations may have inhibited *Geobacillus* spp. biofilm formation, either by decreasing electrostatic forces and consequently compromising the structural integrity of the biofilm − influencing the metabolism or physiology of the *Geobacillus* spp. − or by imbalancing cellular cation homeostasis. As a substantial proportion of thermophilic bacilli that may contaminate milk powder manufacturing plants and milk powders belong to the *Geobacillus* genus, and since *Geobacillus* spp. spores have a greater tolerance to high temperatures than *A. flavithermus* spores, these findings indicate that milk powders derived from milk formulations that collectively have high sodium and low calcium and magnesium concentrations may have markedly decreased thermophilic bacilli counts, may have superior quality and may fetch higher selling prices.

CHAPTER 6

Summarising discussion and conclusion

6.1) Highlights

The highlights of this study supporting the hypothesis that biofilm formation by *Geobacillus* spp. and *A. flavithermus* is compromised in milk formulations with high monovalent to divalent cation ratios are:

- *Geobacillus* spp. and *A. flavithermus* had a greater requirement for Ca^{2+} and Mg^{2+}, than Na^{+} and K^{+}, for surface protein production (Chapter 2).
- Mg^{2+} protected *Geobacillus* spp. from inhibitory concentrations of Na^{+}, K^{+} or Ca^{2+} (63 – 250 mM) (Chapter 2).
- Mg^{2+} prevents the expression of sporulation proteins, and thereby promotes cell division and metabolism of *Geobacillus* spp. in a biofilm (Chapter 4).
- High Na^{+}, low Ca^{2+} and low Mg^{2+} concentrations in milk formulations were collectively required to maximally inhibit *Geobacillus* spp. biofilm formation (Chapters 3 and 5).

6.2) Summarising discussion

Observations made in New Zealand milk powder manufacturing plants have indicated that during processing of milk formulations high in sodium and low in calcium and magnesium ions, biofilm formation and contamination by thermophilic bacilli, predominantly consisting of *Geobacillus* spp. and *A. flavithermus*, is markedly abated. As it is perceived that biofilms in the manufacturing lines of milk powder manufacturing plants act as the main reservoir of thermophilic bacilli, biofilm formation was a major focus in this study. This study investigated the influence of a range of free Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} concentrations and ratios on *Geobacillus* spp. and *A. flavithermus* throughout biofilm formation involving the transition of planktonic cells to an irreversibly attached form, and the subsequent establishment of a biofilm. This aimed to increase our understanding of the observed decrease in thermophile counts in

final milk powder products with high monovalent to divalent cation ratios, and to obtain insights of a practical significance.

Three mechanisms for the effect of Na^+, K^+, Ca^{2+} and Mg^{2+} on *Geobacillus* spp. and *A. flavithermus* biofilm formation were proposed:

1. Their effect on cation homeostasis and requirement as a nutrient source.

2. Their direct electrostatic effect on cohesive forces among bacterial cells, the stainless steel attachment substrate and extracellular matrix polymers.

3. Their effect on the physiology and metabolism of bacteria which may indirectly influence attachment and cohesive forces of a biofilm.

The influence of each proposed mechanism was compared throughout biofilm formation, in order to obtain insights of a fundamental significance on the effects of cations on *Geobacillus* spp. and *A. flavithermus* biofilm formation.

6.2.1) Influence of cations on growth of thermophilic *Geobacillus* species and *Anoxybacillus flavithermus* in planktonic culture (Chapter 2)

The effect of Na^+, K^+, Ca^{2+} and Mg^{2+} on planktonic *Geobacillus* spp. and *A. flavithermus* was investigated to gain insights into the effect of cations on the bacteria prior to their transition to a surface-attached form. It was hypothesized that if cations influence *Geobacillus* spp. and *A. flavithermus* in the planktonic form, this may subsequently influence their ability to transition from a planktonic to stainless steel-attached form.

It was found that the response of *Geobacillus* spp. and *A. flavithermus* to Ca^{2+} and Mg^{2+} was predominantly responsible for an increase in the optical density of the planktonic cultures, whereas Na^+ and K^+ acted cooperatively with Ca^{2+} and Mg^{2+} to increase the optical density. It was concluded that the optical density of the cultures

depended on surface protein production, rather than differences in total viable cell counts, spore counts, cell size, cell aggregation or the production of surface polysaccharide. This is a novel finding, as usually the optical density of planktonic bacterial cultures is proportional to cell and spore numbers (Griffiths *et al*., 2011; Rippey & Watkins, 1992). Also, it was proposed that Ca^{2+} and Mg^{2+} stimulated the production of surface protein by *Geobacillus* spp. and *A. flavithermus*, which increases the metabolic diversity of the bacteria, increases their interaction with the environment, and may enhance their ability to attach to a substrate. These findings indicated that the cations had a physiological effect on planktonic *Geobacillus* spp. and *A. flavithermus*, and conversely, electrostatic effects of the cations had no apparent influence on culture optical density. These findings are of a fundamental significance to the effect of cations on *Geobacillus* spp. and *A. flavithermus* metabolism, physiology and biofilm formation.

In addition, it was found that when a cation was supplemented alone, high Na^+, K^+ or Ca^{2+} concentrations of between 63 and 250 mM significantly decreased the optical density of *Geobacillus* spp. cultures. It was proposed that the high individual cation concentrations imbalanced cation homeostasis of the *Geobacillus* spp. which inhibited their metabolism and growth. This is an example of an effect of the cations on homeostasis of the *Geobacillus* spp. Furthermore, Mg^{2+} protected the *Geobacillus* spp. strains from inhibitory concentrations of Na^+, K^+ or Ca^{2+} (63 – 250 mM). These results have a practical significance as they indicate that growth of *Geobacillus* spp. in a milk formulation with a high monovalent to divalent cation ratio (i.e. high Na^+ and low Mg^{2+} concentrations) may be inhibited. In addition, this result has a fundamental significance as it indicates that cations, at high monovalent to divalent cation ratios, inhibit the growth and metabolism of bacteria by imbalancing cation homeostasis.

Overall, results obtained from investigations of the effect of cations on planktonic *Geobacillus* spp. and *A. flavithermus* indicate that the divalent cations, Ca^{2+} and Mg^{2+},

promote growth and physiologically prime the bacteria for biofilm formation, and that high concentrations of the monovalent cations, Na^+ and K^+, inhibit the growth of *Geobacillus* spp. These findings have a practical significance as they indicate that *Geobacillus* spp. growth and biofilm formation may be inhibited in a milk formulation with a high monovalent to divalent cation ratio.

6.2.2) Preconditioning with cations increases the attachment of *Geobacillus* species and *Anoxybacillus flavithermus* to stainless steel (Chapter 3)

The effect of different Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations and monovalent to divalent cation ratios on both the transition of planktonic *Geobacillus* spp. and *A. flavithermus* to an irreversibly attached form on stainless steel, and the subsequent establishment of a biofilm was investigated. Also, the effect of preconditioning planktonic *Geobacillus* spp. and *A. flavithermus* with different cation concentrations and monovalent to divalent cation ratios prior to attachment and biofilm formation was investigated.

It was found that attachment and biofilm formation of *Geobacillus* spp. and *A. flavithermus* was not altered when the ionic strength of the growth medium ranged between 2 and 125 mM, or when monovalent to divalent cation ratios of 2:1 and 10:1 were compared. This indicated that electrostatic effects of the cations did not influence the transition of planktonic *Geobacillus* spp. and *A. flavithermus* to a stainless steel-attached form or the proliferation of the bacteria in an established biofilm.

Preconditioning *Geobacillus* spp. and *A. flavithermus* with cations often increased subsequent attachment of the bacteria relative to unconditioned bacteria. This is a novel finding which indicated that the bacteria physiologically responded to the cations during preconditioning subsequently increasing their ability to attach to stainless steel. For

example, the *Geobacillus* spp. and *A. flavithermus* may have responded to the cations by up-regulating the expression of surface-exposed polymers which assist attachment. These findings have a fundamental significance as they indicate that the transition of *Geobacillus* spp. and *A. flavithermus* from milk formulations to stainless steel product-contact surfaces in milk powder manufacturing plants is predominantly mediated by bacterial physiological factors (e.g. surface-exposed adhesins), rather than the direct electrostatic effect of cations surrounding bacteria.

Interestingly, biofilm formation after 6 h by *Geobacillus* sp. F75 tended to decrease as the monovalent to divalent cation ratio of milk formulations increased. This demonstrated the potential for *Geobacillus* spp. biofilm formation to be inhibited in milk formulations with high monovalent to divalent cation ratios during milk powder manufacture.

6.2.3) Influence of cations on protein expression of a *Geobacillus* species isolate of dairy origin in a biofilm as measured by MALDI-TOF analysis (Chapter 4)

MALDI-TOF mass spectroscopy was used to investigate the influence of Na^+, K^+, Ca^{2+} and Mg^{2+} on protein expression by *Geobacillus* sp. F75 grown in a biofilm. Protein expression was investigated to gain insights of the influence of the cations on the physiology of *Geobacillus* spp., to test the hypothesis that there are differences in physiologies resulting from the presence of cations.

In cultures supplemented with 2 mM Mg^{2+}, 16 *Geobacillus* sp. F75 proteins were not expressed (i.e. speculated to be down-regulated), compared to cultures unsupplemented with cations or cultures supplemented with all cations (Na^+, K^+, Ca^{2+} and Mg^{2+}). This finding has a fundamental significance as it indicated that Mg^{2+} influences the physiology of Geobacillus spp. during biofilm formation.

Five of the down-regulated proteins were identified as having functions involved in sporulation, thus it was proposed that Mg^{2+} prevents sporulation, and thereby promotes the cell division and metabolism of *Geobacillus* spp. in a biofilm. This is a novel proposal, as it is the first time it has been suggested that Mg^{2+} prevents sporulation of *Geobacillus* spp. Also, this finding provides evidence to suggest that in milk formulations with high monovalent to divalent cation ratios (which have low Mg^{2+} concentrations), *Geobacillus* spp. would have a tendency to opt for sporulation as opposed to cell division and growth. Thus, this finding has a practical significance as it indicates that the proliferation of *Geobacillus* spp. biofilms during the processing of milk formulations with high monovalent to divalent cation ratios may be abated, consequently lowering the thermophillic bacilli cell counts in derived final milk powder products.

6.2.4) Inhibition of *Geobacillus* species biofilms by changes in sodium, calcium and magnesium ion concentrations (Chapter 5)

To further investigate the observation that biofilm formation after 6 h by *Geobacillu*s sp. F75 was inhibited in a milk formulation with a high monovalent to divalent cation ratio, biofilm formation for up to 18 h by three *Geobacillus* spp. isolates and three *A. flavithermus* isolates was investigated in milk formulations with varied Na^{+}, Ca^{2+} and Mg^{2+} concentrations and monovalent to divalent cation ratios. This study was conducted for three reasons. Firstly, to investigate the prevalence of the inhibition of biofilm formation by isolates from the *Geobacillus* spp. and *A. flavithermus* groups in milk formulations with high monovalent to divalent cation ratios. Secondly, to investigate the potential for the inhibition of biofilm formation by the bacterial isolates

for the entire 18 h duration of operation of a typical milk powder manufacturing plant. Thirdly, to characterise the role of each of high Na^+, low Ca^{2+} and low Mg^{2+} concentrations in the inhibition of biofilm formation of the bacterial isolates in milk formulations with high monovalent to divalent cation ratios.

Biofilm formation by all three *Geobacillus* spp. isolates was inhibited for up to 18 h in a milk formulation with a high monovalent to divalent cation ratio, whereas biofilm formation between 6 and 18 h by all three *A. flavithermus* isolates was similar in a milk formulation with a high monovalent to divalent cation ratio compared to a milk formulation with a monovalent to divalent cation ratio typically found in milk. These results demonstrated that biofilm formation by *Geobacillus* spp. in manufacturing lines of milk powder manufacturing plants is markedly compromised throughout the duration of the processing of milk formulations with high monovalent to divalent cation ratios. This has a practical significance, as given that a substantial proportion of thermophillic bacilli that may contaminate milk powders belong to the *Geobacillus* spp. group and since *Geobacillus* spp. spores have a greater tolerance to high temperatures than *A. flavithermus* spores, it is proposed that milk powders derived from milk formulations with high monovalent to divalent cation ratios have the potential to record markedly decreased thermophilic bacilli counts, and as a consequence, have a superior quality and may fetch higher selling prices.

It was concluded that high Na^+, low Ca^{2+} and low Mg^{2+} concentrations were collectively required to maximally inhibit *Geobacillus* spp. biofilm formation. When a milk formulation with a high monovalent to divalent cation ratio was supplemented with either Ca^{2+} or Mg^{2+}, the increased Ca^{2+} and Mg^{2+}concentrations protected the *Geobacillus* spp. isolates from the toxic effect of the high Na^+ concentration. This finding is similar to results observed in Chapter 2, where it was observed that Mg^{2+} protected *Geobacillus* spp. from toxic concentrations of Na^+, K^+ or Ca^{2+}. These

findings have a fundamental and practical significance, as they show that Mg^{2+} has a protective effect against toxic Na^{+} concentrations, and a high monovalent to divalent cation ratio can inhibit *Geobacillus* spp. both in a planktonic and biofilm form, respectively.

It was proposed that it was unlikely that electrostatic effects of the cations in a milk formulation with a high monovalent to divalent cation ratio were responsible for the inhibition of biofilm formation by the *Geobacillus* spp. isolates. Electrostatic effects of cations with surface-exposed polymers and the extracellular matrix of bacteria are similar for all types of bacteria (Neuhaus & Baddiley, 2003). Given that biofilm formation by the *Geobacillus* spp. isolates, but not the *A. flavithermus* isolates, was inhibited in the milk formulation with a high monovalent to divalent cation ratio, this finding has a fundamental significance as it is proposed that the predominant mechanism influencing the inhibition of the *Geobacillus* spp. was not an electrostatic effect.

It is proposed that the predominant mechanism influencing the inhibition of *Geobacillus* spp. was either an imbalance of cation homeostasis, or physiological responses of the bacteria to the high Na^{+}, low Ca^{2+} and low Mg^{2+} concentrations. These findings are of a fundamental significant as they provide insights of how cations, at high monovalent to divalent cation ratios, inhibit *Geobacillus* spp. biofilm formation. High Na^{+} concentrations may have either had a toxic effect or caused a physiological response compromising the growth of the *Geobacillus* spp. in a biofilm. Low Ca^{2+} and Mg^{2+} concentrations may have deprived the *Geobacillus* spp. of sufficient Ca^{2+} and Mg^{2+} for growth and metabolism or caused the bacteria to elicit a physiological response decreasing the growth of the *Geobacillus* spp. in a biofilm. Also, the suggestion that high monovalent to divalent cation ratios inhibit growth and biofilm formation of bacteria by imbalancing cation homeostasis of the bacteria is a novel

insight. Furthermore, this is the first study to show that high monovalent to divalent cation ratios decrease viable cell numbers in a bacterial biofilm.

6.3) Conclusions

High monovalent to divalent cation ratios in milk compromise biofilm formation by a range of *Geobacillus* spp. strains that typically form biofilms in milk powder manufacturing plants. This indicates that milk powders derived from milk formulations with high monovalent to divalent cation ratios may lower counts of thermophilic bacilli (particularly *Geobacillus* spp.) and therefore be of superior quality and may fetch higher selling prices.

Relatively high Na^+, low Ca^{2+} and low Mg^{2+} concentrations in milk formulations were collectively required to cause maximum inhibition of biofilm formation by *Geobacillus* spp. High Na^+ concentrations in milk may be toxic to *Geobacillus* spp., and low Ca^{2+} and Mg^{2+} concentrations may cause Ca^{2+} and Mg^{2+} nutrient deprivation. Also, high Na^+, low Ca^{2+} or low Mg^{2+} may elicit a physiological response by *Geobacillus* spp. decreasing their growth and metabolism in a biofilm. In addition, increasing Ca^{2+} and Mg^{2+} concentrations protect *Geobacillus* spp. from inhibitory effects of high Na^+ concentrations. These findings indicate that cations, at high monovalent to divalent cation ratios, influence *Geobacillus* spp. biofilm formation by either affecting cation homeostasis or the physiology of the bacteria.

In contrast to results found with *Geobacillus* spp., biofilm formation by *A. flavithermus* is not affected by high monovalent to divalent cation ratios present in some milk formulations. *A. flavithermus* may have a greater tolerance than *Geobacillus* spp. to the Na^+ concentrations present in the milk formulations studied with a high monovalent to divalent cation ratio, or may have a greater capacity to acquire Ca^{2+} and Mg^{2+}. This suggests that *A. flavithermus* growth and biofilm formation in

manufacturing lines during the manufacture of milk powders with high monovalent to divalent cation ratios is not inhibited. Also, this finding indicates that there are differences in physiology when comparing between *Geobacillus* spp. and *A. flavithermus*.

Cations have the potential to influence *Geobacillus* spp. and *A. flavithermus* throughout the biofilm formation process. For instance, Ca^{2+} and Mg^{2+} stimulated planktonic *Geobacillus* spp. and *A. flavithermus* to increase production of surface-exposed protein. Preconditioning planktonic *Geobacillus* spp. and *A. flavithermus* with cations enhanced their ability to attach to stainless steel. Mg^{2+} influenced protein expression and caused the down-regulation of the expression of sporulation proteins by *Geobacillus* spp. in an established biofilm. The establishment of a biofilm by *Geobacillus* spp. was inhibited for up to 18 h when grown in a milk formulation with a high monovalent to divalent cation ratio.

Cations influenced biofilm formation of *Geobacillus* spp. and *A. flavithermus* predominantly by affecting cation homeostasis and the physiology of the bacteria, rather than affecting electrostatic properties of the bacteria and their biofilms. Increasing the ionic strength of media between 2 and 125 mM did not influence the aggregation, attachment or biofilm formation of *Geobacillus* spp. and *A. flavithermus*. These findings indicate that direct electrostatic effects of cations do not influence biofilm formation of *Geobacillus* spp. and *A. flavithermus*. High Na^{+} concentrations were found to inhibit the growth of *Geobacillus* spp. both in planktonic culture and in a biofilm, when Ca^{2+} and Mg^{2+} concentrations were low. It was proposed that the high Na^{+} concentrations had a toxic effect on *Geobacillus* spp., and increased Ca^{2+} and Mg^{2+} concentrations protected the bacteria from toxic Na^{+} concentrations. This is an example of the effect of cations on *Geobacillus* spp. cation homeostasis. Ca^{2+} and Mg^{2+} increased surface protein production of planktonic *Geobacillus* spp. and *A. flavithermus*,

and enhanced the ability of the bacteria to attach to stainless steel. Also, Mg^{2+} influenced protein expression of *Geobacillus* spp. and down-regulated the expression of sporulation proteins. These findings are examples of effects of cations on the physiological responses of *Geobacillus* spp. and *A. flavithermus* during biofilm formation.

6.4) Recommendations and future work

6.4.1) Recommendations

If the Na^{+} concentration of a milk formulation is to be increased and the Ca^{2+} and Mg^{2+} concentrations of a milk formulation are to be reduced, the cation concentrations should be manipulated as early in the manufacturing process as possible. This will have the benefit of preventing biofilm formation of *Geobacillus* spp. during milk powder manufacture. This would have the potential to lower the thermophilic bacilli count in milk powder product which would increase the quality and selling price of the product.

6.4.2) Future work

- Further investigate the prevalence of *Geobacillus* spp. strains that are inhibited when grown in milk formulations that have high monovalent to divalent cation ratios. This will more accurately and conclusively determine the extent of growth inhibition of the *Geobacillus* spp. group in milk formulations with a high monovalent to divalent cation ratio. Furthermore, if the growth inhibition of *Geobacillus* spp. strains in milk formulations with high monovalent to divalent cation ratios is wide-spread, a Na^{+} toxicity test may be developed which could be used to differentiate between the *Geobacillus* spp. and *A. flavithermus* groups.

- Investigate the potential that *Geobacillus* spp. biofilm formation is inhibited for longer than 18 h (i.e. for up to 30 h) when grown in milk formulations with high monovalent to divalent cation ratios. If it is found that many *Geobacillus* spp. strains are inhibited for up to 30 h, then the manufacturing run time could be extended. This would decrease manufacturing costs associated with cleaning regimes such as the cost of cleaning chemicals and loss of production time.
- Further investigate if it is low Ca^{2+}, low Mg^{2+} or both low Ca^{2+} and Mg^{2+}, which inhibits *Geobacillus* spp. biofilm formation in milk formulation with a high monovalent to divalent cation ratio. If it is found that only one of the two cation concentrations is required to be low to inhibit *Geobacillus* spp. biofilm formation, then only one cation will be required to be targeted when developing strategies to decrease *Geobacillus* spp. biofilm formation during milk powder manufacture.
- Identify the minimum inhibitory concentration of Na^{+}, and the maximum inhibitory concentrations of Ca^{2+} and Mg^{2+} which inhibit *Geobacillus* spp. biofilm formation. This would provide target concentrations when developing strategies to decrease *Geobacillus* spp. biofilm formation during milk powder manufacture.
- Identify the molecular mechanisms used by *Geobacillus* spp. and *A. flavithermus* to detect, monitor and respond to fluctuations in external cation concentrations. For example, external cations may interact with response regulators, ion channels, or may induce conformation changes of extracellular polymers or influence the activity of extracellular enzymes. This will provide insights for the molecular mechanisms which liberate the

effects of cations on cation homeostasis and physiology of *Geobacillus* spp. and *A. flavithermus*.

- Identify the physiological factors which either make *Geobacillus* spp. susceptible to high monovalent to divalent cation ratios in milk formulations, or identify the physiological factors which enable *A. flavithermus* to tolerate high monovalent to divalent cation ratios in milk formulations.
- Investigate the potential for Na^{+} to competitively exclude Ca^{2+} and Mg^{2+} from the cell envelope. This would provide further insights for the molecular mechanisms which liberate the effects of cations on cation homeostasis and physiology of *Geobacillus* spp. and *A. flavithermus*.
- Identify and characterise the proteins up-regulated by *Geobacillus* spp. and *A. flavithermus* in response to Ca^{2+} and Mg^{2+} in planktonic culture, in order to further investigate the involvement of the surface-exposed proteins in attachment and biofilm formation.
- Identify and characterise the physiological factors, for instance, adhesin/s, which assist attachment by *Geobacillus* spp. and *A. flavithermus*. Understanding the attachment mechanism used by thermophilic bacilli will aid the development of strategies which negate their attachment to stainless steel in manufacturing lines of milk powder manufacturing plants.
- Investigate the effect of Mg^{2+} on the number of spores in *Geobacillus* sp. biofilms. This may support results obtained from protein expression experiments which indicated that Mg^{2+} induces a down-regulation of sporulation protein expression, and thus sporulation.
- Investigate the effect of a range of cation concentrations and monovalent to divalent cation ratios on *Geobacillus* spp. and *A. flavithermus* biofilm formation in a continuous flow reactor. This would create circumstances

more closely aligned to those found in a milk powder manufacturing plant. Under these circumstances the direct electrostatic effects of cations on bacterial attachment and biofilm formation may be more influential.

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Appendix A: Influence of cations on growth of Thermophilic *Geobacillus* spp. and *Anoxybacillus flavithermus* in planktonic culture

(Pages 234 - 240)

Applied and Environmental Microbiology

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Influence of Cations on Growth of Thermophilic *Geobacillus* spp. and *Anoxybacillus flavithermus* in Planktonic Culture

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Free ions of Na^+, K^+, Ca^{2+}, and Mg^{2+} influenced the optical density of planktonic cultures of thermophilic bacilli. *Anoxybacillus flavithermus* E16 and *Geobacillus* sp. strain F75 (milk powder manufacturing plant isolates) and *A. flavithermus* DSM 2641 and *G. thermoleovorans* DSM 5366 were studied. Ca^{2+} and Mg^{2+} were associated with increases in optical density more so than Na^+ and K^+. Overall, it appeared that Ca^{2+} and/or Mg^{2+} was required for the production of protein in thermophilic bacilli, as shown by results obtained with *A. flavithermus* E16, which was selected for further study.

The thermophilic bacilli *Geobacillus* spp. and *Anoxybacillus flavithermus* are the predominant bacteria within foulants of heated regions (50 to 70°C) of milk powder manufacturing plants (3). The manufacturing process used for the production of milk powder selects for the growth of these bacteria, which prosper at high temperatures (50 to 70°C) (3). Thermophilic bacilli are the predominant spoilage organisms in the final milk powder product, and their presence determines the product selling price (3). If their numbers exceed acceptable levels, this has a negative financial impact on the milk powder manufacturer (3). Furthermore, since thermophilic bacilli are biofilm and spore formers, they are adept at persistence in milk powder manufacturing plants (3).

The total free cation concentration in unprocessed milk is approximately 60 mM, consisting of free Na^+, K^+, Ca^{2+}, and Mg^{2+} ions at concentrations of approximately 20, 34, 3.6, and 1.3 mM, respectively (9). Processing factors, such as heating and evaporation, change the equilibrium between free and complexed cations within milk (1). Thus, the external free cation compositions that thermophilic bacilli encounter vary through the milk powder manufacturing process as chilled, unprocessed milk is heated and evaporated to produce milk powder and as products with different specifications are manufactured between runs (1). Furthermore, minerals and ions in milk have the potential to migrate from the bulk flow and concentrate within surface-conditioning layers, foulants, and biofilms in milk powder manufacturing plants (15).

A number of bacterial species respond uniquely to different external cation concentrations, both in a planktonic form and in a biofilm form (10, 16, 22). In our study, optical density was used to analyze indirectly the requirement for and the influence of a range of Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations and proportions on thermophilic bacilli in planktonic culture, as a preliminary screening experiment leading into a study of the influence of cations on thermophilic bacilli propagated as a biofilm. Four bacterial isolates were studied; two *Geobacillus* sp. and two *A. flavithermus* isolates were intentionally selected so that each genus pair would include an isolate derived from a milk powder manufacturing plant and a type (DSMZ collection) strain.

Evaluation of the response of *Geobacillus* spp. and *A. flavithermus* to Na^+, K^+, Ca^{2+}, and Mg^{2+} in planktonic culture. Casein digest medium (1 g/liter) (Difco, BD Biosciences, Sparks, MD) was used to reconstitute NaCl, KCl, $CaCl_2 \cdot 2H_2O$, and $MgCl_2 \cdot 6H_2O$ (Merck, Darmstadt, Germany) powders. Into each well of a Falcon Microtest 96-well culture plate (BD, Franklin Lakes, NJ) 50 μl of casein digest medium (1 g/liter) was dispensed, with various cation supplementation concentrations and proportions (Table 1), as was 50 μl of bacterial inoculum (approximately 1×10^4 CFU/ml), which was previously grown in casein digest medium (1 g/liter) to early stationary phase (9 h). The background concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+} in casein digest medium (1 g/liter) unsupplemented with cations were approximately 1.0, 0.03, 0.004, and 0.002 mM, respectively, as estimated by BD Biosciences (2). The microtiter plates were incubated at 55°C for 10 h. The optical densities of the cultures were measured for triplicate wells, using a wavelength of 600 nm, in an OptiMax tunable microplate reader (Molecular Devices, Sunnyvale, CA).

The experiment was carried out on two separate occasions. The optical density data set from the 10-h time point was further analyzed using SAS software, with a population standard error and 95% confidence intervals ($P \leq 0.05$) being calculated. The 10-h time point was chosen as this was when the bacteria reached late-exponential phase and sufficient growth had occurred such that apparent differences in optical density among the cultures could be analyzed. The resultant data and statistics were represented graphically; the zero values on the *y* axes denote the optical density of the respective bacterial isolates grown in casein digest medium (1 g/liter) unsupplemented with cations (baseline control), and the optical densities of cultures that were supplemented with cations are reported relative to the baseline control.

Effect of cation type. The response of the thermophilic bacilli to Ca^{2+} and Mg^{2+} was predominantly responsible for an increase in the optical density of the cultures, whereas Na^+ and K^+ acted cooperatively with Ca^{2+} and Mg^{2+} to increase the optical density (Fig. 1). The extent of the differences in optical densities was unique for each bacterial isolate studied (Fig. 1).

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TABLE 1 Cation proportions used to supplement a casein digest medium (1 g/liter)[a]

Cation supplementation type	Free-cation proportion			
	Na^+	K^+	Ca^{2+}	Mg^{2+}
Na	1	0	0	0
K	0	1	0	0
Ca	0	0	1	0
Mg	0	0	0	1
Na/K	0.5	0.5	0	0
Na/Ca	0.5	0	0.5	0
Na/Mg	0.5	0	0	0.5
Ca/Mg	0	0	0.5	0.5
K/Ca	0	0.5	0.5	0
K/Mg	0	0.5	0	0.5
Ca/Mg (1:5)	0	0	0.17	0.83
Na/K/Ca (1:1:2)	0.25	0.25	0.5	0
Na/K/Ca/Mg (1:1:1:1)	0.25	0.25	0.25	0.25

[a] Na, K, Ca, and Mg designate free Na^+, K^+, Ca^{2+}, and Mg^{2+}, respectively.

After 10 h of growth, optical density readings for *A. flavithermus* E16, *A. flavithermus* DSM 2641, and *Geobacillus thermoleovorans* DSM 5366 cultures increased when supplemented solely with Ca^{2+} (Fig. 1A, B, and D) and/or Mg^{2+} (Fig. 1B and D), relative to the baseline control. Na^+, and to a greater extent K^+, acted cooperatively with Ca^{2+} or Mg^{2+} to induce an increase in optical density readings in *A. flavithermus* E16 and *G. thermoleovorans* DSM 5366 cultures (Fig. 1A and D).

In contrast, there was no significant difference in the optical densities of *Geobacillus* sp. strain F75 cultures supplemented with Ca^{2+} or Mg^{2+} (Fig. 1C) relative to the baseline control, and the cooperative effect of Na^+ or K^+ in *A. flavithermus* DSM 2641 and *Geobacillus* sp. F75 cultures was either minimal or unapparent (Fig. 1B and C).

Other studies with different species have shown that Ca^{2+} and/or Mg^{2+} increases the optical density of planktonic bacterial cultures (4, 22). Furthermore, Ca^{2+} and Mg^{2+} have important physiological roles in the cell envelope of bacteria, where they have been shown to stimulate extracellular matrix production by bacteria (5, 16, 20); they are required to optimize enzyme functionality, particularly for enzymes involved in the biosynthesis of cell wall polymers (12), and have an important role in maintaining the structural integrity of the cell envelope (19). Na^+ and K^+ have largely intracellular physiological roles, such as in the optimization of enzyme functionality and in osmotic pressure and pH homeostasis (7, 14). Similarly to our study, Caldwell and Arcand (4) found that the monovalent cations Na^+ and K^+ have a crucial role in the planktonic growth of *Bacteroides* spp. The optical density of a bacterial culture can depend on a range of factors, such as culture biomass, which is determined by the concentration of both bacterial cells and bacterium-derived extracellular polymers, and the size, shape, and optical properties of particles within the culture

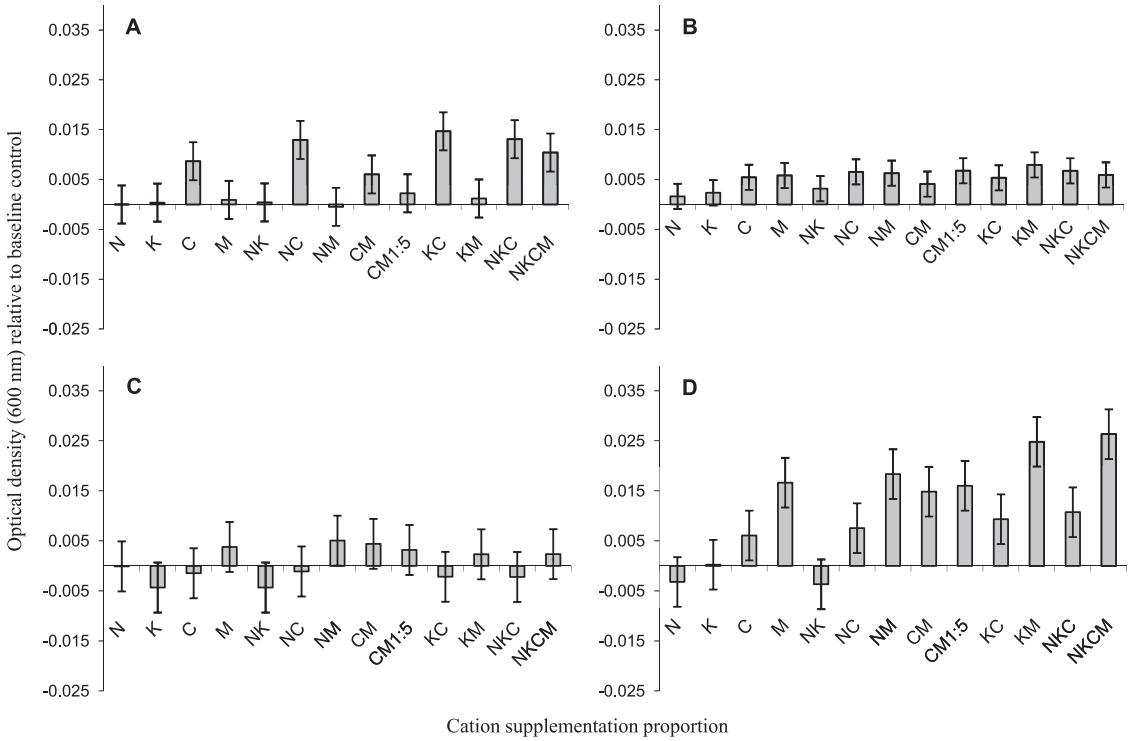

FIG 1 Optical densities of *A. flavithermus* E16 (A), *A. flavithermus* DSM 2641 (B), *Geobacillus* sp. F75 (C), and *G. thermoleovorans* DSM 5336 (D) planktonic cultures grown in casein digest medium (1 g/liter) supplemented with a total cation concentration of 2 mM with varied proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}, relative to the optical density of the respective bacterial isolate grown in casein digest medium (1 g/liter) unsupplemented with cations (baseline control). N, K, C, and M designate free Na^+, K^+, Ca^{2+}, and Mg^{2+}, respectively. CM1:5 refers to a Ca^{2+}-Mg^{2+} ratio of 1:5, NKC refers to a Na^+-K^+-Ca^{2+} ratio of 1:1:2, and all other treatments have equal proportions of each supplemented cation. The cultures were incubated at 55°C for 10 h. Two replicates were done, and each replicate consisted of an average of triplicate cultures. The error bars represent 95% confidence intervals ($P \leq 0.05$), which were determined using SAS statistical analysis software.

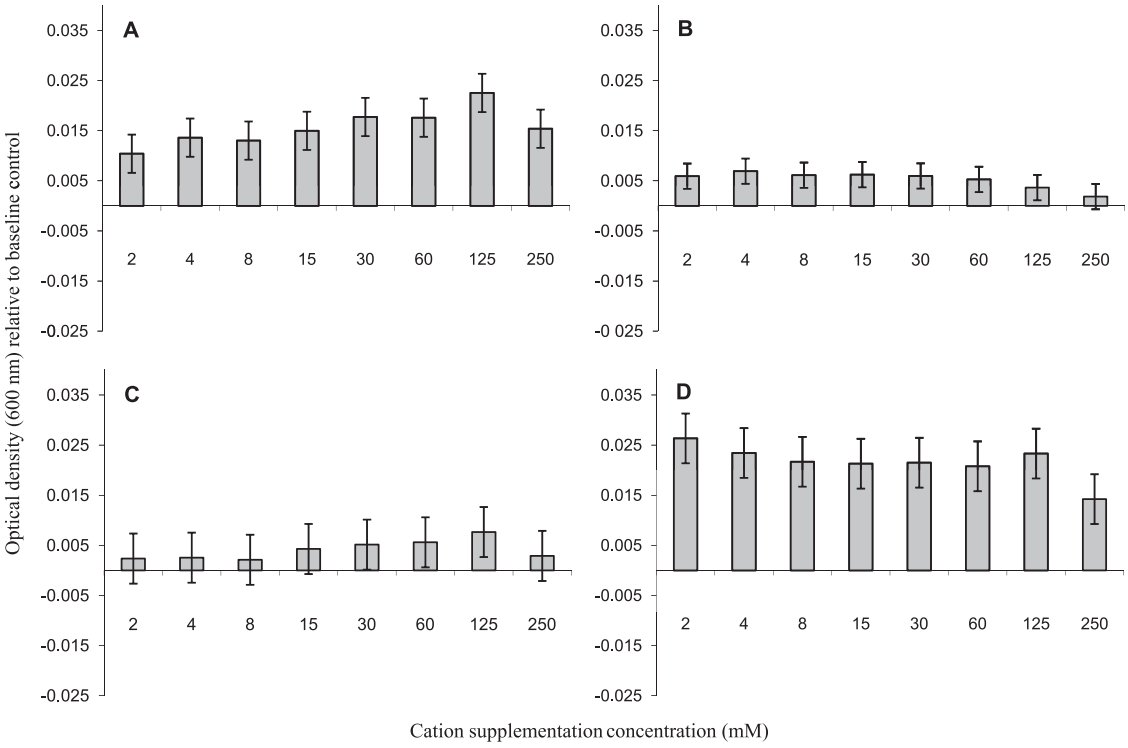

FIG 2 Optical densities of *A. flavithermus* E16 (A), *A. flavithermus* DSM 2641 (B), *Geobacillus* sp. F75 (C), and *G. thermoleovorans* DSM 5336 (D) planktonic cultures grown in casein digest medium (1 g/liter) supplemented with equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+} at total cation concentrations of between 2 and 250 mM, relative to the optical density of the respective bacterial isolate grown in casein digest medium (1 g/liter) unsupplemented with cations (baseline control). The cultures were incubated at 55°C for 10 h. Two replicates were done, and each replicate consisted of an average of triplicate cultures. The error bars represent 95% confidence intervals ($P \leq 0.05$), which were determined using SAS statistical analysis software.

(11). Since it was found that predominantly Ca^{2+} and Mg^{2+} caused an increase in thermophilic bacillus culture optical densities and other research has shown that Ca^{2+} and Mg^{2+} have important physiological roles in the cell envelope of bacteria, it is proposed that changes in the cell envelope of the thermophilic bacilli were responsible for eliciting differential culture optical densities in response to cation supplementation.

Additionally, the needs of thermophilic bacilli may be satisfied more easily by the background Na^+ and K^+ concentrations in casein digest medium (1 g/liter) (1.0 and 0.03 mM, respectively) than the lower background Ca^{2+} and Mg^{2+} concentrations (0.004 and 0.002 mM, respectively), therefore nullifying the observed effect of Na^+ and K^+ supplementation but allowing for supplementation effects of Ca^{2+} and Mg^{2+} to be observed. K^+ tended to have a greater cooperative effect than Na^+, which also suggested that there is a greater requirement by thermophilic bacilli for K^+ than Na^+ to increase the optical density of the culture. However, this observed difference may have been due to the higher background concentration of Na^+ than K^+ in casein digest medium (1 g/liter). Supplementing cultures with Na^+ beyond 1 mM may have had no further increasing influence on their optical density. A Na^+ concentration of 1 mM, as present in casein digest medium (1 g/liter), may have been close to the minimum threshold Na^+ requirement of these bacteria.

Effect of cation concentration. When all four cation types (Na^+, K^+, Ca^{2+}, and Mg^{2+}) were used to supplement cultures, the optical densities of the milk powder manufacturing plant isolates (*A. flavithermus* E16 and *Geobacillus* sp. F75 cultures) increased as the total cation concentration increased between 2 and 125 mM (Fig. 2A and C). A similar trend was not apparent for the DSM strains (Fig. 2B and D). This trend was indicative of the potential of thermophilic bacilli in milk powder manufacturing plants to be influenced as cation concentrations vary during dairy processing.

Total viable cell and spore counts of *A. flavithermus* E16 cultures. To determine the factors that influenced the optical densities of the cultures in our study, *A. flavithermus* E16 cultures supplemented with three different cation concentrations of 0, 2, and 125 mM (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}) were further analyzed, as these cultures had significantly different optical densities after 10 h of growth.

Initially, the total viable cell and spore counts were measured to investigate their potential influence on culture optical densities. One-hundred-milliliter casein digest medium (1 g/liter) aliquots, supplemented with cation concentrations of 0, 2, or 125 mM (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}), were inoculated with approximately 1×10^4 CFU/ml of *A. flavithermus* E16 which was previously grown in casein digest medium (1 g/liter) to early stationary phase (9 h). The cultures were incubated at 55°C for 10 h, and total viable cell counts were determined using standard microbiological plating techniques on milk plate count agar (MPCA; Oxoid, Basingstoke, United Kingdom) at 55°C for 48 h. Spores reportedly have a greater potential than vegetative cells to influence the optical density of a suspension, as they have refractory properties (17). To determine the spore count in the cultures, 12 ml of each culture was sampled and heated at

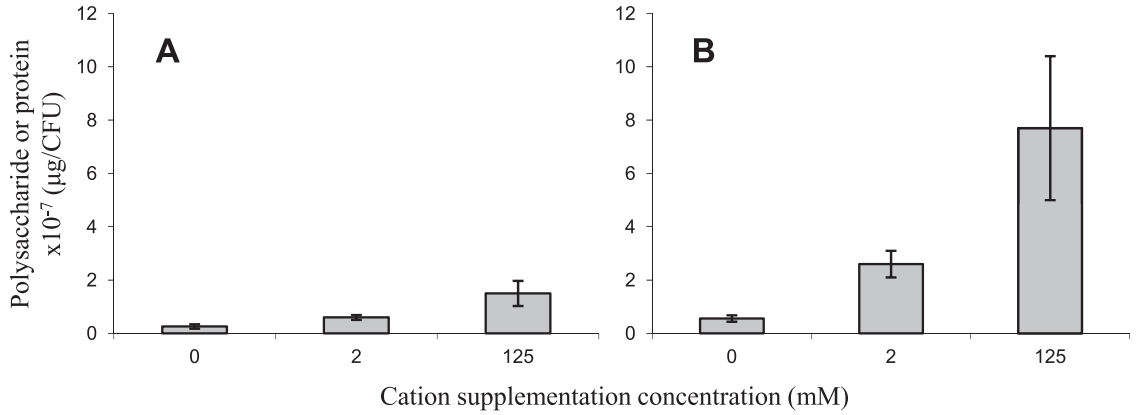

FIG 3 Amounts of polysaccharide (A) and protein (B), associated with the pellet after centrifugation at 11,800 × *g*, per CFU of *A. flavithermus* E16 culture after a 10-h incubation at 55°C, grown in casein digest medium (1 g/liter) supplemented with a total cation concentration of 0, 2, or 125 mM (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}) ($n = 3$). Error bars represent ±1 standard deviation (σ_{n-1}).

100°C for 35 min (18). Standard microbiological plating techniques were used to determine the spore count in the heat-treated cultures on MPCA supplemented with starch (2 g/liter) (13). To obtain a lower spore detection limit of <0.5 log CFU/ml, 10 1-ml aliquots of each of the heat-treated cultures were spread plated. The total viable cell and spore counts were determined on three separate occasions.

The average total viable cell counts of the cultures supplemented with 0, 2, and 125 mM cations were similar, at 6.6, 6.2, and 6.4 log CFU/ml, respectively. Higher spore counts were determined in cultures supplemented with cation concentrations of 2 and 125 mM (5.3 and 4.8 log CFU/ml, respectively) than in the unsupplemented culture (<0.5 log CFU/ml). The total viable cell and spore count standard deviations (σ_{n-1}) were <1.0 log and ≤1.1 log, respectively. Neither the total viable cell nor the spore counts correlated with the differences in optical densities seen among the three cultures. Furthermore, phase-contrast light microscopy showed that there was a consistent cell size, shape, and appearance among the cultures and that the bacteria did not aggregate. Thus, it was concluded that total viable cell or spore counts and cell size, shape, and coaggregation were not factors that influenced the optical densities of these cultures.

Quantification of bacterial surface protein and polysaccharide in *A. flavithermus* E16 culture. The amounts of bacterial protein and polysaccharide in cultures of *A. flavithermus* E16 containing 0, 2, or 125 mM cations were quantified to investigate their contribution toward the optical densities of the planktonic cultures.

A 10-ml aliquot of bacterial inoculum, grown as described above, was used to inoculate 1,000 ml of casein digest medium (1 g/liter), supplemented with a cation concentration of 0, 2, or 125 mM (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}). The cultures were incubated at 55°C for 10 h. Approximately 900 ml of the culture was centrifuged at 11,800 × *g* for 10 min. The supernatant of the culture was discarded, and the pellet was washed once in 450 ml of distilled water and then resuspended in 5 ml of distilled water. The total viable cell counts in both the original 1,000-ml cultures and the resulting 5-ml culture concentrates were determined using standard microbiological plating techniques, as described above.

To quantify the amount of protein produced by *A. flavithermus* E16 in culture, the following protocol was used. A 1:10 dilution (800 μl) of the 5-ml culture concentrate was mixed with 200 μl of Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories, Inc., Hercules, CA), and the absorbance of the mixture was read using a spectrophotometer (595 nm), in which the reference was set against a solution containing 800 μl of distilled water mixed with 200 μl of Bio-Rad protein assay dye reagent concentrate. Bovine serum albumin (Sigma-Aldrich, St. Louis, MO) was used to generate a standard curve (1 to 100 μg/ml).

To quantify the amount of extracellular polysaccharide produced by *A. flavithermus* E16 in culture, the following protocol was used, as modified from the protocol detailed by Dall and Herndon (6). One milliliter of the 5-ml culture concentrate was added dropwise to 8 ml of approximately 100% ethanol; the solution was incubated at 4°C for 18 h and then centrifuged at 10,000 × *g* for 20 min. The supernatant was discarded, and the pellet was resuspended by vortex mixing in 1 ml of distilled water. To the resuspended pellet suspension was added 7 ml of sulfuric acid (77%, vol/vol) and then 1 ml of L-tryptophan (10 g/liter) (BDH, Poole, England). Each suspension was thoroughly vortex mixed, dispensed into a glass test tube, and then heated for 20 min at 100°C. Each suspension was vortex mixed, and the absorbance was read using a spectrophotometer (500 nm), in which the reference was set against a solution containing 1 ml of distilled water mixed with 7 ml of sulfuric acid (77%, vol/vol) and 1 ml of L-tryptophan (10 g/liter), which had also been subjected to the heat treatment. Dextran (Sigma-Aldrich) was used to generate a standard curve (10 to 200 μg/ml).

The bacterial protein and polysaccharide assays and their associated standard curves were carried out on three separate occasions, and the results are quoted as averages ± 1 standard deviation (σ_{n-1}).

After high-speed centrifugation, the amount of protein and to a lesser extent the amount of polysaccharide in *A. flavithermus* E16 cultures, per CFU, increased with increasing concentration of cation supplementation (consisting of equal proportions of Na^+, K^+, Ca^{2+}, and Mg^{2+}) (Fig. 3).

Thus, it appeared that the predominant factor that influenced the optical densities of the cultures was the amount of bacterial protein produced. An increase in protein on the surface of *A. flavithermus* E16 may have increased the optical density of the

culture either by increasing the culture biomass or by increasing the refraction of light due to changes in the optical properties of the cell surfaces (11).

It is known that cations create an environment that is favorable for optimal enzyme functionality; therefore, the scope of the metabolic diversity of the bacteria in our study may have widened, and a greater amount of enzyme may have been produced in response to the increase in external cation concentration (8). The bacteria may also have produced a greater amount of structural protein (8, 21).

Overall, it can be postulated that Ca^{2+} and/or Mg^{2+} was required for or stimulated the production of protein in thermophilic bacillus planktonic cultures. Although the cellular location of the protein that increased in response to varied external cation concentrations was not determined, it could be hypothesized that the protein was located in the cell envelope, as Ca^{2+} and Mg^{2+} have physiological roles focused at the cell envelope (12, 19).

It was observed that as the collective concentration of all four cation types (Na^+, K^+, Ca^{2+}, and Mg^{2+}) fluctuated between concentrations typically present in milk products during their manufacture (2 to 125 mM), the optical densities of the milk powder manufacturing plant isolates (*A. flavithermus* E16 and *Geobacillus* sp. F75) increased as the total cation concentration increased. This indicated that there is the potential that external free cation concentrations may influence the metabolic and physiological state of thermophilic bacilli, which may influence their proliferation during the manufacture of milk powders, for example, during biofilm formation. This hypothesis is currently being studied.

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Appendix B: Preconditioning with cations increases the attachment of *Anoxybacillus flavithermus* and *Geobacillus* species to stainless steel

(Pages 241 - 247)

Applied and Environmental Microbiology

Preconditioning with Cations Increases the Attachment of Anoxybacillus flavithermus and Geobacillus Species to Stainless Steel

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Preconditioning with Cations Increases the Attachment of *Anoxybacillus flavithermus* and *Geobacillus* Species to Stainless Steel

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Preconditioning of *Anoxybacillus flavithermus* E16 and *Geobacillus* sp. strain F75 with cations prior to attachment often significantly increased ($P \leq 0.05$) the number of viable cells that attached to stainless steel (by up to 1.5 log CFU/cm^2) compared with unconditioned bacteria. It is proposed that the transition of *A. flavithermus* and *Geobacillus* spp. from milk formulations to stainless steel product contact surfaces in milk powder manufacturing plants is mediated predominantly by bacterial physiological factors (e.g., surface-exposed adhesins) rather than the concentrations of cations in milk formulations surrounding bacteria.

A biofilm is described as microorganisms attached to a surface; they are often embedded in a matrix of polymers and other molecules that either originate from microorganisms in the biofilm or are absorbed from the surrounding environment (1).

Cations have two main effects on the structural integrity and proliferation of a bacterial biofilm. The first is a direct effect, such that cations interact electrostatically with surface-exposed and cell wall-embedded polymers and the surfaces to which they attach (2, 3). The outer surfaces of bacteria generally have an overall negative charge because bacterial cell wall and extracellular matrix polymers have an abundance of negatively charged functional groups (1, 4). Stainless steel also has a negative surface charge (5). Thus, there is an extent of electrostatic repulsion between bacteria and the stainless steel surface to which they attach (3). Factors such as the ionic strength (6), the ratio of the concentrations of monovalent to divalent cations in solution (7), and the proportion of divalent cation bridges in a biofilm matrix (2) have the potential to alter the extent of electrostatic repulsion in a biofilm.

The second effect that cations have on biofilm formation is an indirect effect, such that bacteria may respond to changes in concentrations of cations in their surroundings and adjust their metabolism and physiology (8–11). These bacterial responses may indirectly influence their ability to transition from a planktonic form to an irreversibly attached form and prosper as a biofilm (8–10). Ca^{2+} and Mg^{2+} have been shown to stimulate exopolysaccharide (8, 12, 13) and extracellular protein (9, 14, 15) production by bacteria and often have physiological roles in assisting the initial reversible association of bacteria with a surface or enhancing the cohesion of a biofilm (9, 11, 16). Na^+ has been shown to stimulate bacteria to increase the proportion of negatively charged, hydrophilic polymers in a wastewater sludge and, therefore, have a detrimental effect on its cohesion (10).

Geobacillus and *A. flavithermus* attach to and form biofilms on stainless steel product contact surfaces in milk powder manufacturing plants and are the predominant bacteria that contaminate milk powder (17, 18). Unprocessed milk typically has total (sum of bound and free) Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations of 22, 37, 30, and 5 mM, respectively (19); however, these concentrations can be manipulated during processing. Some milk formulations have total Na^+ concentrations as high as 100 mM and total Ca^{2+} and Mg^{2+} concentrations as low as 7 and 1 mM, respectively. To gain insight into the extent of the influence that different cation concentrations and ratios have on biofilm formation by *Geobacillus* and *A. flavithermus*, we investigated the effect of Na^+, K^+, Ca^{2+}, and Mg^{2+} on attachment to stainless steel and biofilm formation by *A. flavithermus* E16 and *Geobacillus* sp. strain F75 in both a casein digest medium and milk formulations.

Evaluation of the effect of Na^+, K^+, Ca^{2+}, and Mg^{2+} on attachment and biofilm formation of *A. flavithermus* and *Geobacillus*. *A. flavithermus* E16 and *Geobacillus* sp. F75 were isolated from product contact surfaces at a milk powder manufacturing plant. Prior to attachment and biofilm formation, each bacterial isolate was planktonically preconditioned (to mid-stationary phase after 9 h at 55°C) in medium with one of the following compositions: casein digest medium (1 g/liter) (Difco, BD Biosciences) unsupplemented with cations, referred to as "unconditioned"; casein digest medium supplemented with cations alone (see Table S1 in the supplemental material), referred to as "preconditioned with cations"; casein digest and lactose monohydrate (1 g/liter) (Merck, Darmstadt, Germany) medium supplemented with cations, referred to as "preconditioned with cations and lactose"; milk formulations 1 to 4 (10 g milk powder rehydrated with 90 ml deionized, sterile water) (Fonterra, New Zealand) (see Tables S2 and S3 in the supplemental material), referred to as "preconditioned with milk formulation."

To remove medium used to planktonically grow and condition the bacteria, cultures, except those preconditioned with milk formulation (1 to 4), were centrifuged at 10,000 × *g* and the bacterial pellet was resuspended in fresh medium to be used during the attachment and biofilm formation assay, which consisted of casein digest medium (1 g/liter) supplemented with a range of cation concentrations and ratios (see Table S1 in the supplemental material) or milk formulations 1 to 4 (see Tables S2 and S3 in the supplemental material). The resuspended cultures and cultures

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preconditioned with milk formulation (1 to 4) were diluted in medium to be used during the attachment and biofilm formation assays to achieve an inoculum of approximately 4.5 log CFU/ml; 1.5 ml of this inoculum was added per well of a 24-well culture plate (BD, Franklin Lakes, NJ). Stainless steel coupons (part number RD128-316; Biosurface Technologies Corporation, Bozeman, MT), which had a surface area of approximately 4 cm^2, were cleaned and passivated prior to use in the biofilm formation assay, as previously described by Flint et al. (20). One coupon was placed into each inoculum using sterile forceps so that it was completely submerged and horizontal. After the culture plate had been wrapped in a plastic bag to prevent the evaporation of water from the cultures, it was incubated at 55°C for either 30 min or 6 h.

Casein digest medium (1 g/liter) (Difco, BD Biosciences, Sparks, MD) was used because it had low background Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations of approximately 1.0, 0.03, 0.004, and 0.002 mM (21), respectively; therefore, the effect of the supplementation of a range of cation concentrations and ratios on attachment and biofilm formation was able to be studied. Casein digest medium was supplemented with analytical grade NaCl, KCl, $CaCl_2 \cdot 2H_2O$, or $MgCl_2 \cdot 6H_2O$ powder (Merck, Darmstadt, Germany). Milk formulations 1 to 4 (Fonterra, New Zealand) had similar fat, protein, and lactose concentrations but different total (sum of bound and free) Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations (see Tables S2 and S3 in the supplemental material). The higher the designated milk formulation number (1 to 4), the higher the monovalent-to-divalent cation ratio of the milk formulation (see Table S3 in the supplemental material). Milk formulations 1 to 4 were used to investigate the effect of the monovalent-to-divalent cation ratio on attachment and biofilm formation in milk formulations. Milk formulations 1 to 4 were derived from the same respective batches throughout experimentation. Prior to reconstitution, the milk powders were gamma irradiated (25,000 Gy) to inactivate any contaminating microorganisms so that growth and analysis of the inoculated bacteria of interest were unimpeded.

The bacteria were planktonically preconditioned with a range of cation concentrations and ratios (both in casein digest medium and milk formulations) to allow the bacteria to physiologically adapt to the cationic conditions so that the effect of cation preconditioning on subsequent attachment and biofilm formation could be investigated. This was done to investigate if a physiological response of the bacteria to particular cation concentrations and ratios may abate their ability to attach to and form biofilms in milk powder manufacturing lines. Bacteria preconditioned with cations or milk formulations, except bacteria preconditioned with cations and lactose, for which lactose was omitted from medium in the attachment and biofilm formation assay, were subjected to medium in the attachment and biofilm formation assays that was of the same composition as that used during preconditioning.

To enumerate the attached viable cells per square centimeter on the coupons, the coupons were removed from the cultures using sterile forceps, dipped and rinsed three times in approximately 50 ml of deionized water to remove any loosely attached cells, and placed into a 35-ml plastic container (item code LBS3722W; Thermo Fisher Scientific, New Zealand) with 5 ml of fresh casein digest medium (1 g/liter) and 12 g of glass beads that had a diameter of 6.35 mm (catalogue number 11079635; Biospec Products, Inc., Bartlesville, OK). The plastic containers were vortex mixed vigorously for 2 min to dislodge the attached cells into the surrounding medium. Standard microbiological plate-counting techniques were used to enumerate the viable CFU/ml in the cell suspension using casein digest medium (1 g/liter) as the diluent and milk plate count agar (MPCA; Oxoid, Basingstoke, United Kingdom). The experiments were carried out on three separate occasions, and mean attached viable cell numbers (CFU/cm^2) and 1 standard deviation (σ_{n-1}) are reported. Minitab software was used to calculate population standard errors and 95% confidence intervals ($P \leq 0.05$) to determine significant differences among the mean values.

Effect of ionic strength. Altering the ionic strength between 2 and 125 mM did not significantly influence ($P \leq 0.05$) attachment after 30 min or biofilm formation after 6 h by *A. flavithermus* E16 or *Geobacillus* sp. F75 (Fig. 1 and 2). This was apparent for unconditioned bacteria, bacteria preconditioned with cations, and bacteria preconditioned with cations and lactose (Fig. 1 and 2). Our results contradict those of other studies, which showed that bacterial attachment and biofilm cohesion increase as the ionic strength of the surrounding solution increases (5, 6). Perhaps the addition of cations at concentrations of 2 mM saturated the surfaces of the bacteria and the stainless steel coupons such that further increases in ionic strength may have had no further enhancing effect on biofilm formation. As the ionic strength of milk formulations and most bacterial habitats is greater than 2 mM, our results indicate that the ionic strength of solution does not influence attachment and biofilm formation of *A. flavithermus* and *Geobacillus*.

Effect of the monovalent-to-divalent cation ratio. When comparing the monovalent-to-divalent cation ratios of 2:1 and 10:1 at an ionic strength of either 31 or 125 mM in casein digest medium, there was no significant difference ($P \leq 0.05$) in attachment or biofilm formation by *A. flavithermus* E16 or *Geobacillus* sp. F75 (Fig. 1 and 2, iv to vii). This was apparent when bacteria were unconditioned, preconditioned with cations, and preconditioned with cations and lactose (Fig. 1 and 2, iv to vii). Our findings contrast observations made with wastewater sludge biofilms, in which it has been shown that sludge cohesion decreases as the monovalent-to-divalent cation ratio increases, such that a monovalent-to-divalent cation ratio of 2:1 promotes the greatest extent of cohesion, and at a monovalent-to-divalent cation ratio of 10:1, the cohesion of a sludge is greatly compromised (7). Generally, attachment after 30 min and biofilm formation after 6 h by *A. flavithermus* E16 and *Geobacillus* sp. F75 were similar when comparing milk formulations 1 to 4 (see Fig. S1 and S2 in the supplemental material). However, in agreement with wastewater sludge research, biofilm formation after 6 h by *Geobacillus* sp. F75 tended to decrease as the monovalent-to-divalent cation ratio of the milk formulations increased, particularly by the unconditioned bacteria (see Fig. S2B in the supplemental material). The number of unconditioned, attached viable *Geobacillus* sp. F75 cells after 6 h was approximately 2 log CFU/cm^2 lower in milk formulation 4 than in milk formulation 2, and the two values were significantly different ($P \leq 0.05$) (see Fig. S2B in the supplemental material). The monovalent-to-divalent cation ratio of milk formulation 4 is likely to be greater than those that exist in any of the cation concentrations and ratios investigated in the casein digest medium, which may explain the apparent inhibitory influence of the high monovalent-to-divalent cation ratio of milk formulation 4 on *Geobacillus* sp. F75 biofilm formation. Milk formulations have higher concentrations of solutes, such as casein and anions, than

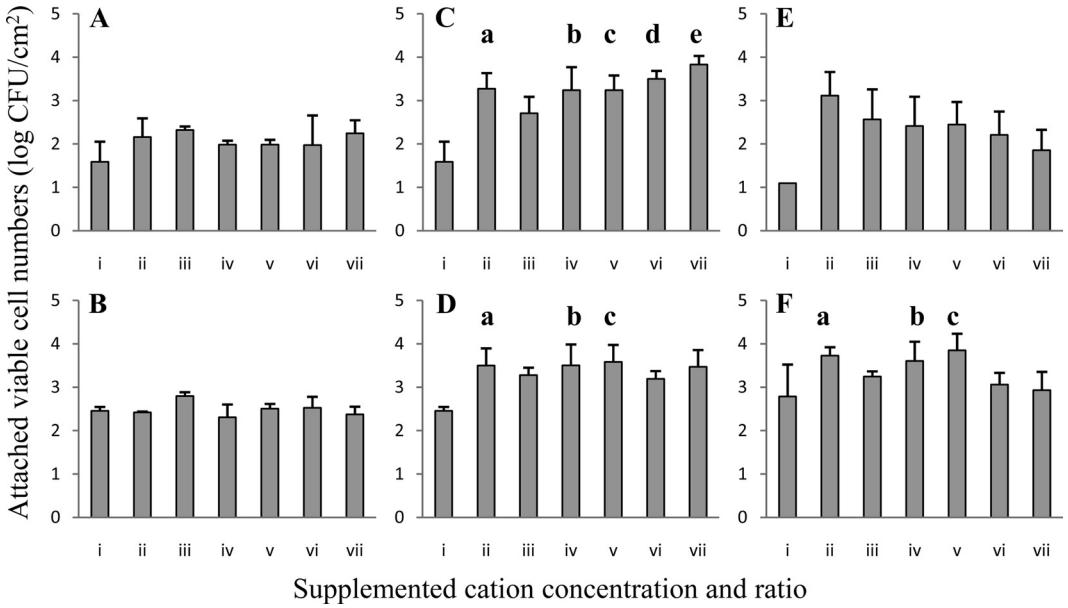

FIG 1 Attachment, after 30 min of incubation at 55°C, by viable *A. flavithermus* E16 (A, C, and E) and *Geobacillus* sp. F75 (B, D, and F) cells (log CFU/cm^2) on stainless steel coupons completely submerged in casein digest medium (1 g/liter) supplemented with cation compositions of 0 mM (i), 2 mM Ca^{2+} (ii), 2 mM Mg^{2+} (iii), 31 mM 2:1 (iv), 31 mM 10:1 (v), 125 mM 2:1 (vi), and 125 mM 10:1 (vii). Total supplemented cation concentrations of 31 mM (iv and v) and 125 mM (vi and vii) had monovalent-to-divalent cation ratios of 2:1 (iv and vi) and 10:1 (v and vii). Each monovalent-to-divalent cation ratio comprised equal Na^+ and K^+ concentrations and equal Ca^{2+} and Mg^{2+} concentrations. Prior to the attachment assay, the bacteria were grown planktonically for 9 h at 55°C in three different media: casein digest medium (1 g/liter) (unconditioned) (A and B), casein digest medium (1 g/liter) supplemented with various cation compositions (preconditioned with cations) (ii to vii) (C and D), and casein digest medium (1 g/liter) supplemented with lactose (1 g/liter) and various cation compositions (preconditioned with cations and lactose) (ii to vii) (E and F). Experiments were repeated as triplicates, and error bars represent one standard deviation (σ_{n-1}). The letters (a to e) represent significantly greater ($P \leq 0.05$) attachment by cation-preconditioned cells (C, D, E, and F) than by unconditioned cells (A and B) for each respective bacterial isolate and each respective cation composition. "a" represents 2 mM Ca (ii), "b" represents 31 mM 2:1 (iv), "c" represents 31 mM 10:1 (v), "d" represents 125 mM 2:1 (vi), and "e" represents 125 mM 10:1 (vii).

casein digest medium. Thus, in milk formulations, a large proportion of Ca^{2+} and Mg^{2+} is chelated, maintaining low free Ca^{2+} and Mg^{2+} concentrations (19, 22). Furthermore, the Na^+ concentration of milk formulation 4 was greater than the Na^+ concentration in any of the cation concentrations and ratios used to supplement the casein digest medium; the Na^+ concentration in milk formulation 4 was 101 mM (see Table S3 in the supplemental material), whereas the highest Na^+ concentration in the casein digest medium was 56.8 mM (see Table S1 in the supplemental material). Thus, it may have been high Na^+, low Ca^{2+}, low Mg^{2+}, or a combination of these factors that inhibited *Geobacillus* sp. F75 biofilm formation in milk formulation 4.

Our results indicate that the monovalent-to-divalent cation ratio generally does not influence attachment and biofilm formation of *A. flavithermus* and *Geobacillus* spp. in processed milk formulations during milk powder manufacture.

Effect of preconditioning with cations. Preconditioning of *A. flavithermus* E16 or *Geobacillus* sp. F75 with cations often significantly increased ($P \leq 0.05$) attachment (by up to 1 log CFU/cm^2) after 30 min relative to unconditioned bacteria when the same cation concentrations during attachment were compared (Fig. 1A to D). For example, when *A. flavithermus* E16 attached after 30 min in the presence of 2 mM Ca^{2+}, attachment of bacteria preconditioned in 2 mM Ca^{2+} (Fig. 1C, ii) was significantly greater ($P \leq 0.05$) than attachment of unconditioned bacteria (Fig. 1A, ii), with the numbers of attached viable cells at 3.3 and 2.2 log CFU/cm^2, respectively.

In contrast, preconditioning *A. flavithermus* E16 with cations did not significantly increase ($P \leq 0.05$) biofilm formation after 6 h relative to that of unconditioned bacteria when the same cation concentrations during biofilm formation were compared (Fig. 2A and C). However, preconditioning had a lasting effect on *Geobacillus* sp. F75 biofilm formation after 6 h; for many cation concentrations and ratios, there was a significant increase ($P \leq 0.05$) (by up to 1 log CFU/cm^2) in biofilm formation when bacteria were preconditioned with cations relative to unconditioned bacteria when the same cation concentrations during biofilm formation were compared (Fig. 2B and D).

During preconditioning, the metabolism and physiology of *A. flavithermus* E16 and *Geobacillus* sp. F75 may have been influenced by cations in such a way that subsequent attachment and biofilm formation by the bacteria was enhanced. For example, Ca^{2+} or Mg^{2+} may have stimulated the bacteria to increase their expression of surface-exposed proteins and polysaccharides, which promoted attachment and biofilm formation (9, 12–16). Surface-exposed proteins, such as pili and even flagella, have been implicated in assisting the initial reversible association of bacteria with a surface, and surface-exposed polysaccharides have been implicated in promoting the irreversible attachment of bacteria to a surface and enhancing cohesion among bacteria within a mature biofilm by acting as a cohesive component of the matrix (1).

Since the electrostatic effects of ionic strength and the monovalent-to-divalent cation ratio generally did not influence attachment and biofilm formation of *A. flavithermus* E16 and *Geobacillus* sp. F75 and preconditioning the bacteria with cations often increased attachment, it is proposed that the transition of *A. fla-*

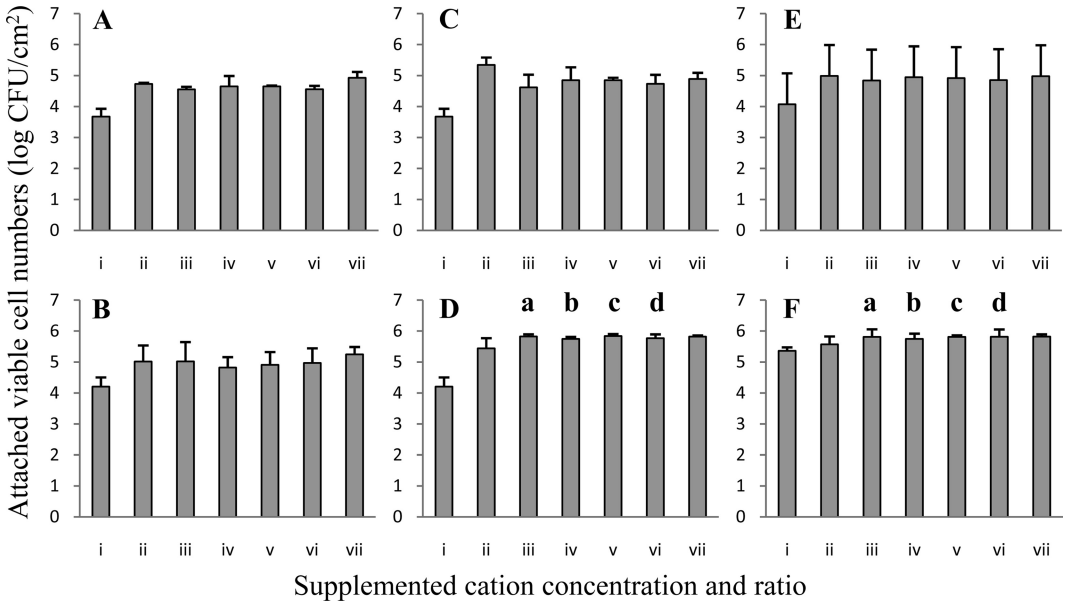

FIG 2 Biofilm formation, after 6 h of incubation at 55°C, by viable *A. flavithermus* E16 (A, C, and E) and *Geobacillus* sp. F75 (B, D, and F) cells (log CFU/cm^2) on stainless steel coupons completely submerged in casein digest medium (1 g/liter) supplemented with cation compositions of 0 mM (i), 2 mM Ca^{2+} (ii), 2 mM Mg^{2+} (iii), 31 mM 2:1 (iv), 31 mM 10:1 (v), 125 mM 2:1 (vi), and 125 mM 10:1 (vii). Total supplemented cation concentrations of 31 mM (iv and v) and 125 mM (vi and vii) had monovalent-to-divalent cation ratios of 2:1 (iv and vi) and 10:1 (v and vii). Each monovalent-to-divalent cation ratio comprised equal Na^+ and K^+ concentrations and equal Ca^{2+} and Mg^{2+} concentrations. Prior to the biofilm formation assay, the bacteria were grown planktonically for 9 h at 55°C in three different media: casein digest medium (1 g/liter) (unconditioned) (A and B), casein digest medium (1 g/liter) supplemented with various cation compositions (preconditioned with cations) (ii to vii) (C and D), and casein digest medium (1 g/liter) supplemented with lactose (1 g/liter) and various cation compositions (preconditioned with cations and lactose) (ii to vii) (E and F). Experiments were repeated as triplicates, and error bars represent one standard deviation (σ_{n-1}). The letters (a to d) represent significantly greater ($P \leq 0.05$) biofilm formation by cation-preconditioned cells (D and F) than by unconditioned cells (B) by *Geobacillus* sp. F75 for each respective cation composition. "a" represents 2 mM Mg (iii), "b" represents 31 mM 2:1 (iv), "c" represents 31 mM 10:1 (v), and "d" represents 125 mM 2:1 (vi).

vithermus and *Geobacillus* spp. from milk formulations to stainless steel product contact surfaces in milk powder manufacturing plants is mediated predominantly by bacterial physiological factors, e.g., the expression of surface-exposed adhesins, rather than the concentrations of cations in milk formulations.

Preconditioning with cations and lactose decreased attachment by *A. flavithermus* E16. Protein, such as casein, and carbohydrate, such as lactose, are available to bacteria as nutrient sources for biofilm formation during milk powder manufacture (19). To simulate biofilm formation by *A. flavithermus* and *Geobacillus* spp. in milk powder manufacturing lines and to provide bacteria with an adequate carbohydrate source to synthesize surface-exposed polysaccharides (23), lactose was added to cultures during preconditioning. Thus, when bacteria were preconditioned with cations and lactose, the effect of preconditioning bacteria with ranging cation concentrations on their subsequent attachment and biofilm formation was investigated in the presence of both a protein source and a carbohydrate source. The extent of attachment after 30 min by *A. flavithermus* E16 preconditioned with cations and lactose tended to be less than that observed when the bacteria were preconditioned with cations alone (Fig. 1C and E). During preconditioning, *A. flavithermus* E16 may have utilized the lactose in the medium to increase production of surface-exposed polysaccharides, which subsequently decreased attachment. Many studies have shown that surface-exposed polysaccharides can inhibit bacterial attachment (24, 25).

The present study indicated that cation concentrations and ratios in milk formulations generally would not influence attachment and biofilm formation by *A. flavithermus* and *Geobacillus* during the processing of milk formulations in milk powder manufacturing plants. However, as the monovalent-to-divalent cation ratio of milk formulations increased, biofilm formation after 6 h by *Geobacillus* sp. F75 was increasingly inhibited. This implied that either high Na^+ concentrations or low Ca^{2+} or Mg^{2+} concentrations, or a combination of these factors, have the potential to inhibit biofilm formation by some *A. flavithermus* and *Geobacillus* spp. strains during the processing of milk formulations. Interestingly, preconditioning of planktonic *A. flavithermus* E16 and *Geobacillus* sp. F75 with cations often enhanced attachment and was more influential than the electrostatic effect of cations on the attachment process. It is proposed that cations, such as Ca^{2+} and Mg^{2+}, influenced the physiology or metabolism of planktonic *A. flavithermus* and *Geobacillus* spp. such that subsequent attachment was enhanced. It is also proposed that attachment by *A. flavithermus* and *Geobacillus* spp. in milk powder manufacturing lines is mediated predominantly by bacterial physiological factors, e.g., the expression of surface-exposed adhesins, rather than the concentrations of cations in milk formulations.

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Appendix C: Thesis embargo

(Pages 248 - 249)

Appendix D

MASSEY UNIVERSITY
Application for Approval of Request to Embargo a Thesis
(Pursuant to AC98/168 (Revised 2), Approved by Academic Board 17/02/99)

Name of Candidate: Ben Somerton ID Number: 1101437 3

Degree: PhD Food Technology Dept/Institute/School: IFNHH

Thesis title: Effect of cations on biofilm formation by Geobacillus species and Anoxybacillus flavithermus dairy isolates

Name of Chief Supervisor: Steve Flint Telephone Ext: 81418

As author of the above named thesis, I request that my thesis be embargoed from public access until (date) 31 June 2015 for the following reasons:

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Denise Winkworth
fonterra Supervisor

Signed (Candidate): Date: 10 June 2013

Endorsed (Chief Supervisor): Date: 13/6/2013

Approved/Not Approved (Representative of VC): Date: 5/07/2013

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