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

A thesis presented in partial fulfilment of the requirements for the
degree of

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Benjamin Thomas Somerton

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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 Ca²⁺ or Mg²⁺ 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

Abstract	iii
Acknowledgements	v
Table of contents	vi
List of publications	xv
Published manuscripts	xv
List of conference presentations.....	xvi
Oral presentations.....	xvi
Poster presentations.....	xvi
List of abbreviations.....	xvii
List of figures	xxi
List of tables.....	xxx
Chapter 1	
Literature review	1
1.1) Introduction	2
1.2) Nutritional requirement for calcium, magnesium, sodium and potassium ions in bacterial cellular functioning and homeostasis	3
1.2.1) Calcium.....	3
1.2.2) Magnesium.....	6
1.2.3) Sodium	8
1.2.4) Potassium	9
1.3) Biofilms – a definition	10

1.4)	The effect of electrostatic interactions of sodium, potassium, calcium and magnesium ions on bacterial biofilm formation	11
1.4.1)	Composition and electrostatic properties of the cell wall of Gram-positive bacteria	12
1.4.2)	The extended DLVO theory and its application to bacterial attachment and biofilm formation	14
1.4.3)	The diffuse double electric layer surrounding bacteria and stainless steel.....	15
1.4.4)	Effect of ionic strength on the diffuse double electric layer.....	16
1.4.5)	Contradictions in the application of the extended DLVO theory to predict bacterial attachment.....	18
1.4.6)	Assimilation of cations into the cell wall of Gram-positive bacteria.....	19
1.4.7)	Cation binding affinities to Gram-positive bacterial cell walls.....	20
1.4.8)	Cation binding capacity of Gram-positive bacterial cell walls.....	23
1.4.9)	Divalent cation bridges	26
1.4.10)	Monovalent to divalent cation ratio.....	28
1.4.11)	Overall conclusions for the effect of cations on electrostatic interactions in biofilms	30
1.5)	Physiological responses of bacteria in a biofilm to sodium, potassium, calcium and magnesium ions.....	31
1.5.1)	The influence of calcium ions on the conformation and function of bacterial polymers.....	31

1.5.2)	Calcium ion mediated regulation of bacterial signal transduction pathways.....	34
1.5.3)	Influences of cations on cell wall and extracellular matrix amounts and composition and its effect on biofilm formation	36
1.5.4)	Comparison of the effect of monovalent and divalent cations on physiological responses of bacteria in biofilms.....	41
1.6)	Properties of milk	42
1.6.1)	Milk composition	42
1.6.2)	Milk minerals	46
1.6.3)	Mineral interactions in milk.....	50
1.6.4)	Effects of processing on milk mineral interactions.....	51
	a. Temperature	51
	b. Concentration.....	53
	c. pH	53
	d. Addition of ions.....	54
1.7)	Biofilms in milk powder manufacturing plants	56
1.8)	Motivation	60
1.9)	Objective	63
1.10)	Aims	64
1.11)	Research questions	66
1.12)	Research hypotheses	67

Chapter 2

Influence of cations on growth of Thermophilic *Geobacillus* species

and <i>Anoxybacillus flavithermus</i> in planktonic culture	69
2.1) Abstract.....	71
2.2) Introduction	72
2.3) Methods.....	74
2.3.1) Isolation of thermophilic sporeforming bacteria from a milk powder manufacturing plant	74
2.3.2) PCR identification of bacterial isolates	75
2.3.3) Bacterial isolates and culture preparation	75
2.3.4) Evaluation of the effect of Na ⁺ , K ⁺ , Ca ²⁺ , and Mg ²⁺ on <i>Geobacillus</i> spp. and <i>A. flavithermus</i> in planktonic culture.....	76
2.3.5) Total viable cell and spore counts	78
2.3.6) Quantification of bacterial surface protein and polysaccharide.....	79
2.4) Results	80
2.4.1) Differences among the bacterial isolates	84
2.4.2) Effect of cation type	93
2.4.3) Effect of cation concentration	94
2.4.4) Effect of cation ratio	94
2.4.5) Mg ²⁺ protection from Na ⁺ , K ⁺ or Ca ²⁺ inhibition of <i>Geobacillus</i> spp. planktonic growth	94
2.4.6) Total viable cell counts	106
2.4.7) Spore counts.....	108
2.4.8) Quantification of bacterial surface protein and polysaccharide in <i>A. flavithermus</i> E16 culture	108
2.5) Discussion	110

2.5.1)	The relationship between cation composition and optical density of the cultures was unique for each isolate	110
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+}	111
	a. Effect of cation type	111
	b. Effect of cation concentration	113
	c. Effect of cation ratio.....	114
2.5.3)	Mg^{2+} protection from Na^+ , K^+ or Ca^{2+} inhibition of <i>Geobacillus</i> spp. planktonic growth	115
2.5.4)	<i>A. flavithermus</i> E16 responded to increasing cation concentrations, with equal proportions of Na^+ , K^+ , Ca^{2+} , and Mg^{2+} , by producing increased amounts of surface protein.....	117
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	118
2.6)	Conclusions.....	121

Chapter 3

Preconditioning with cations increases the attachment of *Geobacillus*

species and <i>Anoxybacillus flavithermus</i> to stainless steel	122
3.1) Abstract.....	124
3.2) Introduction	125
3.3) Methods.....	127
3.3.1) Bacterial isolates and media.....	127
3.3.2) Culture storage	130
3.3.3) Inoculum preparation	130
3.3.4) Attachment and biofilm formation assay	130
3.3.5) Cell enumeration.....	131
3.3.6) Statistical analysis.....	132
3.4) Results	132
3.4.1) Effect of ionic strength.....	132
3.4.2) Effect of the monovalent to divalent cation ratio.....	138
3.4.3) Effect of preconditioning with cations.....	138
3.4.4) Effect of preconditioning with cations and lactose	139
3.4.5) Effect of preconditioning with milk formulations	140
3.5) Discussion	146
3.5.1) Effect of ionic strength.....	146
3.5.2) Effect of monovalent to divalent cation ratio	146
3.5.3) Effect of contrasting monovalent to divalent cation ratios in milk formulations	147
3.5.4) Effect of preconditioning with cations.....	148
3.5.5) Preconditioning of <i>A. flavithermus</i> with lactose decreased attachment.....	149

3.5.6) Effect of preconditioning with milk formulations	150
3.6) Conclusions.....	151

Chapter 4

Influence of cations on protein expression of a <i>Geobacillus</i> isolate, of dairy origin, in a biofilm as measured by MALDI-TOF MS analysis	152
4.1) Abstract.....	153
4.2) Introduction	154
4.3) Methods.....	155
4.3.1) Biofilm formation.....	155
4.3.2) Harvesting the biofilm	156
4.3.3) MALDI-TOF MS analysis	157
4.4) Results and discussion	158
4.5) Conclusions.....	169

Chapter 5

Inhibition of <i>Geobacillus</i> species biofilms by changes in sodium, calcium and magnesium ion concentrations	170
5.1) Abstract.....	172
5.2) Introduction	173
5.3) Methods.....	175
5.3.1) Bacterial isolates	175
5.3.2) Growth media.....	175
5.3.3) Culture storage	176
5.3.4) Inoculum preparation	176
5.3.5) Biofilm formation assay	176

5.3.6)	Cell enumeration.....	177
5.3.7)	Statistical analysis.....	177
5.4)	Results and discussion	178
5.4.1)	Comparison of <i>Geobacillus</i> spp. biofilm formation in milk formulations 2 and 4	178
5.4.2)	Characterization of the effect of sodium, calcium and magnesium on <i>Geobacillus</i> spp. biofilm formation.....	186
5.4.3)	Comparison of the effects of calcium and magnesium on <i>Geobacillus</i> spp. biofilm formation.....	189
5.4.4)	Effect of iron on <i>Geobacillus</i> spp. biofilm formation	190
5.4.5)	Proposed mechanisms for cation inhibition of <i>Geobacillus</i> spp. biofilm formation.....	190
5.4.6)	Comparison of <i>A. flavithermus</i> biofilm formation in milk formulations 2 and 4	194
5.5)	Conclusions.....	197

Chapter 6

	Summarising discussion and conclusion	198
6.1)	Highlights.....	199
6.2)	Summarising discussion	199
6.2.1)	Influence of cations on growth of thermophilic <i>Geobacillus</i> species and <i>Anoxybacillus flavithermus</i> in planktonic culture (Chapter 2).....	200
6.2.2)	Preconditioning with cations increases the attachment of <i>Geobacillus</i> species and <i>Anoxybacillus flavithermus</i> to stainless steel (Chapter 3).....	202

6.2.3)	Influence of cations on protein expression of a <i>Geobacillus</i> species isolate of dairy origin in a biofilm as measured by MALDI-TOF MS analysis (Chapter 4).....	203
6.2.4)	Inhibition of <i>Geobacillus</i> species biofilms by changes in sodium, calcium and magnesium ion concentrations (Chapter 5).....	204
6.3)	Conclusions.....	207
6.4)	Recommendations and future work.....	209
6.4.1)	Recommendations.....	209
6.4.2)	Future work.....	209
	References	213
	Appendix A: Influence of cations on growth of Thermophilic <i>Geobacillus</i> spp. and <i>Anoxybacillus flavithermus</i> in planktonic culture.....	234
	Appendix B: Preconditioning with cations increases the attachment of <i>Anoxybacillus flavithermus</i> and <i>Geobacillus</i> species to stainless steel.....	241
	Appendix C: Thesis embargo.....	248

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.

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List of conference presentations

Oral presentations

Somerton, B., Palmer, J., Brooks, J., Smolinski, E., Lindsay, D. & Flint, S.

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High sodium and low calcium and magnesium concentrations in milk collectively inhibit biofilm formation by *Geobacillus* spp. 58th Annual Scientific Meeting of the New Zealand Microbiological Society Conference 2012 (Dunedin, New Zealand).

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).

List of abbreviations

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
$\text{Ca}_3(\text{PO}_4)_2$	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
$^{\circ}\text{C}$	degrees celcius
DNA	deoxyribonucleic acid

rDNA	deoxyribonucleic acid that encodes for a ribosomal gene
DLVO	Derjaguin, Landau, Verway, Overbeek
H ₂ O	dihydrogen oxide
H ₂ PO	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
<i>n</i>	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
<i>P</i>	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

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.82

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 $\text{Ca}^{2+}:\text{Mg}^{2+}$ ratio of 1:5, NKC refers to a $\text{Na}^+:\text{K}^+:\text{Ca}^{2+}$ 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.85

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 $\text{Ca}^{2+}:\text{Mg}^{2+}$ ratio of 1:5, NKC refers to a $\text{Na}^+:\text{K}^+:\text{Ca}^{2+}$ 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.87

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 $\text{Ca}^{2+}:\text{Mg}^{2+}$ ratio of 1:5, NKC refers to a $\text{Na}^+:\text{K}^+:\text{Ca}^{2+}$ 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.89

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⁺, Ca²⁺, and Mg²⁺, respectively, CM 1:5 refers to a Ca²⁺:Mg²⁺ ratio of 1:5, NKC refers to a Na⁺:K⁺:Ca²⁺ 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 Ca²⁺ and Mg²⁺, 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.91

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), Ca²⁺ (C) or Mg²⁺ (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.96

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), Ca²⁺ (C) or Mg²⁺ (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.98

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 $\text{Ca}^{2+}:\text{Mg}^{2+}$ ratio of 1:5, NKC refers to a $\text{Na}^+:\text{K}^+:\text{Ca}^{2+}$ 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. **100**

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. **102**

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. **104**

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⁺, Ca²⁺, and Mg²⁺) (*n* = 3). Error bars represent ± 1 standard deviation (σ_{n-1}). **109**

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 Ca²⁺ (ii), 2 mM Mg²⁺ (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 Ca²⁺ and Mg²⁺ 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* ≤ 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 Ca²⁺ (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..... **134**

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 Ca²⁺ (ii), 2 mM Mg²⁺ (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 Ca²⁺ and Mg²⁺ 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 Mg²⁺ (ii), ‘b’ represents 31 mM 2:1 (iv), ‘c’ represents 31 mM 10:1 (v), and ‘d’ represents 125 mM 2:1 (vi). **136**

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}). 141

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. 143

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⁺, Ca²⁺ and Mg²⁺ concentration of 31 mM with a monovalent to divalent cation ratio of 10:1 (B), or supplemented with 2 mM Mg²⁺ (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. 160

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⁺, Ca²⁺ and Mg²⁺ concentration of 31 mM with a monovalent to divalent cation ratio of 10:1 (B), or supplemented with 2 mM Mg²⁺ (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..... **162**

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..... **164**

FIG 5.1 Biofilm formation, after 6–18 h of incubation at 55°C , by viable *Geobacillus* sp. F75 cells ($\log \text{CFU cm}^{-2}$) 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..... **180**

FIG 5.2 Biofilm formation, after 6–18 h of incubation at 55°C , by viable *Geobacillus* sp. TRa cells ($\log \text{CFU cm}^{-2}$) 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 CaCl₂ (E) and milk formulation 4 supplemented with 2 mM MgCl₂ (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. 182

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 CaCl₂ (E) and milk formulation 4 supplemented with 2 mM MgCl₂ (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. 184

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}). 195

List of tables

TABLE 1.1 Quantitative composition of bovine milk	44
TABLE 1.2 Fat, protein, and lactose concentrations (g l^{-1}) in milk formulations (MF) 1–4 (reconstituted at $10 \text{ g } 90 \text{ ml}^{-1}$)	45
TABLE 1.3 Macro- and micro-element mean and range amounts (per liter) in bovine raw milk	47
TABLE 1.4 Macro-element amounts and forms in bovine raw milk.....	48
TABLE 1.5 Total (sum of bound and free) cation concentrations of milk formulations (MF) 1 – 4 (reconstituted at $10 \text{ g } 90 \text{ ml}^{-1}$), including those supplemented with cation chlorides.....	49
TABLE 2.1 Cation proportions used to supplement a casein digest medium (1 g l^{-1}) ^a	77
TABLE 2.2 Total viable cell (TVC) and spore (Spore) counts, as $\log \text{CFU ml}^{-1}$, 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	107
TABLE 3.1 Cation supplementation concentrations (profiles) in casein digest medium (1 g l^{-1}).....	129
TABLE 3.2 Summary of results.....	145
TABLE 4.1 Predicted identities of successfully matched putative proteins absent in <i>Geobacillus</i> sp. F75 biofilms grown in 2 mM Mg^{2+}	166