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The surface characteristics of spores from thermophilic bacilli isolated from a milk powder production line and their influence on adhesion to surfaces.

A thesis submitted for the degree of Doctor of Philosophy in Food Science at the University of Otago
Dunedin
New Zealand

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Abstract

Spores of thermophilic bacilli are a common concern during the manufacture of milk powder. Spores are believed to occur in high numbers in milk powder due to their ability to survive pasteurisation, attach to stainless steel surfaces, germinate, grow as biofilms and subsequently enter the product stream and thereby contaminate the final product.

In this study, thirty one thermophilic bacilli isolates were obtained from a New Zealand milk powder production line and identified as either *Anoxybacillus flavithermus* or *Geobacillus* spp. using random amplified polymorphic DNA (RAPD) and species-specific PCR. Sporulation media and a polyethylene glycol two-phase separation system were modified to produce high yields of spores free from debris.

The spores of four *Geobacillus* spp. isolates (CGT-8, D4, E7 and E11) were characterised in terms of structure (electron microscopy), surface charge (zeta potential), hydrophobicity (contact angle and microbial adhesion to hexadecane) and attenuated total reflectance infrared spectroscopy (ATR-IR). Spores from three of the four isolates possessed an exosporium while the fourth did not. However the integrity of the exosporium varied over time. The spores were negatively charged (-10 to -20 mV) at neutral pH and high ionic strength (0.1 M KCl). Both hydrophobicity assays revealed that the spores of the four isolates were relatively hydrophilic while ATR-IR revealed the spores’ surfaces consisted of protein and polysaccharides.

The influence of these spore characteristics on adhesion to a variety of substrata under high flow rates was examined using the extended Derjaguin, Landau, Verwey and Overbeek (XDLVO) theory. Spores generally attached in higher numbers to hydrophobic surfaces compared to hydrophilic surfaces, however this observation was more prevalent for isolate D4. This result indicated that a single mechanism could not describe the adhesion of spores from different strains.

A series of glass surfaces with modified characteristics were produced in order to test the antifouling properties on the adhesion of D4 spores. Spores suspended in a high ionic strength medium (0.1 M KCl) attached in greater numbers (1 Log_{10} CFU cm^{-2}) to positively charged and hydrophobic surfaces compared with negatively charged and hydrophilic surfaces. A clean in place (CIP) procedure, reduced spore numbers on hydrophobic and hydrophilic surfaces by 1.5 and by 2.0 Log_{10} CFU cm^{-2}, respectively.
When spores were suspended in milk, there was little difference in the number of spores attaching to the different surfaces (ie. 3.5 to 3.8 $\log_{10}$ CFU cm$^{-2}$), and spore removal from surfaces via a CIP regime was unchanged (1.5 to 2.0 $\log_{10}$ CFU cm$^{-2}$ reduction) compared with spores that attached in simple 1:1 electrolyte media.

The effects of a caustic wash on spore surface characteristics and adhesion was determined. There was a significant reduction in spore viability ($2 \log_{10}$ CFU mL$^{-1}$) after a 30 min caustic wash at 65 °C in the current study, however surviving spores displayed a greater propensity to attach to stainless steel. Surface characterisation results revealed an increase in hydrophobicity and a greater negative charge on the spores’ surface after treatment with NaOH. Surviving spores could potentially recontaminate sections of the plant which are cleaned with this recycled caustic wash solution, thereby seeding surfaces with spores at the beginning of the next processing run.

In conclusion, while surfaces that reduce spore adhesion and enhance removal can be produced, exposure to complex solutions such as milk can reduce the anti-fouling effectiveness of such surfaces to spore adhesion.
List of Publications


List of Presentations

Poster Presentation: Surface characterization of thermophilic bacterial spores through zeta potential and infrared spectroscopy. New Zealand Microbiological Society Conference, Christchurch, NZ. 2008

Oral Presentation: Spore adhesion to stainless steel surfaces during the manufacture of milk powder. Industrial Research Limited (IRL) multiscale project meeting. Wellington, NZ. 2008


Poster Presentation: Determining the surface characteristics of thermophilic spores isolated from dairy plants and how this relates to their attachment to surfaces. American Society Microbiology: Biofilms Conference, Quebec, Canada. 2007

Oral Presentation: Control of spore attachment to surfaces. Fonterra PhD Day. Hamilton, NZ. 2006


Oral Presentation: Surface characteristics of spores isolated from a milk powder processing line. Industrial Research Limited multiscale project meeting. Wellington, NZ. 2006

Oral Presentation: Spore adhesion during the manufacture of milk powder. Fonterra PhD Day. Hamilton, NZ. 2005

Poster presentation: Techniques to optimise the recovery of spores from dairy plant isolates of thermophilic bacilli. New Zealand Microbiological Society Conference. Dunedin, NZ. 2005
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A multitude of techniques and materials were required for different parts of this project. A very special thanks to Jingtian Han who not only provided me with all the modified surfaces, but is also a great friend. I would like to thank Logan Cahill and other operators of the Fonterra Plant in Edendale New Zealand for their support in learning the milk powder production process and for keeping a close eye while I was taking samples. Next I would like to thank David Crump of the Pharmacy Department for use of the zetasizer machine; Brian Niven at the CASM unit for helping me with statistics; and Dr. Alexander McLellen for use of the fluorescent microscope. I would especially like to thank Liz Girvan and Richard Easingwood of the OCEM unit for sample preparation and assistance with electron microscopy.

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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>APTES</td>
<td>(3-aminopropyl)triethoxysilane</td>
</tr>
<tr>
<td>ATR-IR</td>
<td>Attenuated total reflection infrared (spectroscopy)</td>
</tr>
<tr>
<td>CaDPA</td>
<td>Calcium dipicolinic acid</td>
</tr>
<tr>
<td>CAM</td>
<td>Contact angle measurement</td>
</tr>
<tr>
<td>CIP</td>
<td>Clean in place</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DLVO</td>
<td>Derjaguin and Landau, Verwey and Overbeek theory</td>
</tr>
<tr>
<td>DMP</td>
<td>Dairy milk plant</td>
</tr>
<tr>
<td>DPA</td>
<td>Dipicolinic acid</td>
</tr>
<tr>
<td>H</td>
<td>Hamaker constant</td>
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<tr>
<td>H (medium)</td>
<td>Houston (medium)</td>
</tr>
<tr>
<td>HIC</td>
<td>Hydrophobic interaction chromatography</td>
</tr>
<tr>
<td>IRE</td>
<td>Internal reflection element</td>
</tr>
<tr>
<td>IEP</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>KS (medium)</td>
<td>Kaul and Singh (medium)</td>
</tr>
<tr>
<td>kT</td>
<td>Brownian motion of an organism. (1 \text{kT} = 4 \times 10^{21} \text{J})</td>
</tr>
<tr>
<td>MATH</td>
<td>Microbial adhesion to hexadecane (assay)</td>
</tr>
<tr>
<td>MATS</td>
<td>Microbial adhesion to solvents (assay)</td>
</tr>
<tr>
<td>MRD</td>
<td>Modified Robbins device</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Optical Density at 600 nm</td>
</tr>
<tr>
<td>PAA</td>
<td>Poly-acrylic acid</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly-ethylene glycol</td>
</tr>
<tr>
<td>PET</td>
<td>poly(ethylene terephthalate)</td>
</tr>
<tr>
<td>PHE</td>
<td>Plate heat exchanger</td>
</tr>
<tr>
<td>PWS</td>
<td>Phosphate wash solution</td>
</tr>
<tr>
<td>RAPD</td>
<td>Randomised amplified polymorphic DNA</td>
</tr>
<tr>
<td>spp</td>
<td>species</td>
</tr>
<tr>
<td>SASP</td>
<td>Small acid soluble protein</td>
</tr>
<tr>
<td>SMP</td>
<td>Skim Milk Powder</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptic Soy Agar</td>
</tr>
</tbody>
</table>
TSB  |  Tryptic Soy Broth
---|---
UHT  |  Ultra-high temperature
UV   |  Ultra-violet
WMP  |  Whole milk powder
XDLVO|  Extended Derjaguin and Landau, Verwey and Overbeek theory
1:1 (electrolyte) A salt which disassociates into mono-valent ions

**Contact Angles**

\[ \theta_{\alpha,B} \]  |  Contact angle of \( \alpha \)-bromonaphthalene
\[ \theta_F \]  |  Contact angle of formamide
\[ \theta_W \]  |  Contact angle of water

**Surface Energies**

\[ \gamma_{lv} \]  |  Liquid-vapour energy component (mJ)
\[ \gamma_{sv} \]  |  Solid-vapour energy component (mJ)
\[ \gamma^+ \]  |  Electron donor energy component (mJ)
\[ \gamma^- \]  |  Electron acceptor energy component (mJ)
\[ \gamma_{AB} \]  |  Acid-base energy component (mJ)
\[ \gamma_{lw} \]  |  Lifshitz-vander Waals energy component (mJ)
\[ \gamma_{TOT} \]  |  Total surface energy (mJ)
\[ G_{sls} \]  |  Gibbs energy of aggregation (mJ)

**Interaction Energies (XDLVO)**

\[ G_{AB} \]  |  Acid-base interaction energy (mJ m\(^{-2}\))
\[ G_{EL} \]  |  Electrostatic interaction energy (mJ m\(^{-2}\))
\[ G_{lw} \]  |  Lifshitz-van der Waals interaction energy (mJ m\(^{-2}\))
\[ G_{sm} \]  |  Interaction energy at the secondary minimum
\[ G_{Total} \]  |  Total interaction energy (mJ m\(^{-2}\))
Chapter 1

General Introduction
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1.1 Background

The New Zealand dairy industry has a worldwide reputation as a producer of high quality dairy products for the world market. New Zealand exports 95% of its milk products to other countries, and in 2008 dairy products made up 22% of New Zealand’s merchandise exports (278). Dairy products include whole (WMP) and skim (SMP) milk powders, butter, cheese, anhydrous milk fat and protein. Whole milk powder is the number one dairy export with over 669,000 tonnes being exported overseas in 2008 (74). Milk powder is used in a wide range of products such as confectionary, ice cream, baked and recombined products and is a source of milk to countries where milk is in shortage or unavailable. Consumers demand that the milk powder is of high quality, and each year, New Zealand loses millions of dollars in export earnings due to the presence of high number of thermophilic bacterial spores in milk powder. Although these organisms are not pathogenic, their occurrence in high numbers causes the milk powder to be downgraded and sold at a lower price. This is because, if conditions become suitable, the spores in the reconstituted milk can germinate and produce enzymes which cause the production of off flavours (40, 43). Strategies therefore need to be developed to ensure that spore numbers in milk powder are reduced so that New Zealand dairy industry maintains its reputation in the world market and obtain the maximum return for its dairy products.

1.2 Milk Powder Production

Milk powder production involves the removal of water from milk solids. It results in a final product that is easily transportable, small in volume and has a longer shelf life compared with liquid milk. The removal of water is achieved via three steps; milk treatment, evaporation and spray drying.

Raw milk obtained through milking of cows is chilled to 7 °C on the farm, transported to the dairy manufacturing plant (DMP) where it is stored at 7 °C until required. In the first stage of milk treatment centrifugal separators are used to separate the milk and the cream. The milk is then pasteurised (72 °C for 15 s) using plate heat exchangers (PHE). Pasteurisation is a technique used to eliminate all of the pathogenic and non-pathogenic micro-organisms associated with the spoilage of milk and has little effect on the chemical or physical composition of the milk. It should be
noted however, that pasteurisation is unable to destroy the spores of spore forming bacilli. The milk is then standardized by re-introducing the pasteurized cream to create the desired protein and fat content in the final product.

The pasteurised milk is again stored at 7 °C in a balance tank before being transferred to the powder plant (Figure 1.1). Here the milk is heated by direct or indirect heat exchangers or a combination of both. Direct heat exchangers use direct steam injection where the steam is put into direct contact with the product. Indirect contact heaters, heat the product on one side of a stainless steel surface with the heating medium being on the other side. The temperature of pre-heating generally ranges from 75 °C to 125 °C depending on the type of powder being produced. The main purpose of heat treatment is to rapidly heat the milk prior to evaporation, and to prevent ‘flash’ heating once it enters the evaporator. The heated milk is then passed through a scraped surface heater and homogenizer before reaching the evaporator.

![Figure 1.1: Schematic of milk powder manufacture. Reproduced from Scott et al., 2007 (211).](image)

Evaporators are an energy efficient way to concentrate milk by removing water and reducing the load during spray drying. To achieve this, the milk is boiled under vacuum at lower temperatures compared with the spray drier. Skim milk is concentrated from 9 – 9.5 % total solids to 45 – 50 % while whole milk is concentrated from 12 – 13 % total solids to 50 %. The most common type of evaporator in New Zealand is the falling film evaporator. The milk is pumped to the top of the evaporator and allowed to pass through heated tubes under vacuum. Each evaporator can be divided into two to five sections; each section is called an effect and operates at different temperatures ranging from 70 °C to 45 °C. Each of these effects can contain two to three passes. Each pass is made of a section of heated tubes under vacuum. Water is driven off from the milk as steam, which is then removed by
the vacuum. Once the milk undergoes one pass, it is pumped to the top of the evaporator where it can then pass through the next set of tubes thus creating a milk concentrate.

Prior to spray drying, the milk concentrate undergoes heat treatment and homogenisation. The heat treatment reduces the heat drying load and reduces milk fat globule size which prevents clumping. When the milk concentrate first enters the spray dryer, the atomiser disperses the concentrate as fine droplets into the main chamber. Hot air is then mixed with these droplets causing primary drying. Secondary drying occurs when warm air is passed through the product on fluidised beds. Cyclones are also used to recover ‘fines’ or small particles would have been lost during the air drying process. The milk powder is then packaged resulting in the final product.

After every production run, sections that come into contact with the product undergo a cleaning regime called a clean-in-place (CIP) (42, 193). A CIP allows for the plant to be cleaned without dismantling of sections of the plant. A typical regime consists of the following steps:

1. Warm water rinse
2. 1.5% caustic wash at 75 °C for 30 min
3. Water rinse
4. 0.5 % Nitric acid wash at 70 °C for 20 min
5. Water rinse

The caustic wash is designed to remove organic matter such as proteins. This high pH causes the deprotonation of functional groups present within proteins, leaving a net negative charge. These negative charges repel one another causing the protein to swell (154). The swollen protein is much easier to remove with flow. Nitric acid is a strong oxidizer and removes inorganics such as phosphate salts. In some plants, sanitisers are also used in addition to the CIP, to kill any remaining microbial contaminants. Sections of the production line may be dismantled and manually cleaned to remove fouling that the CIP failed to remove. This may occur every 5 to 10 runs, between different types of product runs, or at the end of the season. In New Zealand, DMPs usually cease operations for two months during winter due to the low supply of milk as cows are grass fed rather than grain fed as in other countries. During this period, the DMP usually undergoes maintenance and rigorous cleaning.
1.3 Microflora of a milk powder manufacture plant

Milk is produced as a sterile secretion from healthy cows, and in theory if drawn aseptically, will remain sterile. However in reality it generally becomes contaminated prior to leaving the farm with low numbers of microorganisms \((10^2 - 10^3 \text{ CFU mL}^{-1})\). The first potential source of contamination is from microorganisms that have invaded the teat opening such as micrococci, streptococci \(spp\). and the diptheroid \(Corynebacterium bovis\) (225). The second and more common source of contamination occurs during milking due to the fact that the exterior of the teats can become contaminated with a wide range of environmental isolates including thermophiles from \(Bacillus\) \(spp\.), human pathogens such as \(Listeria monocytogenes\) or \(Campylobacter\) \(spp\.), psychrotrophic spoilage organisms such as \(Pseudomonas\) \(spp\.) and \(Bacillus\) \(spp\.) and coliforms including \(Escherichia coli\), \(Enterobacter\) \(spp\.) and \(Klebsiella\) (3). As discussed later, thermoduric spore forming bacteria are of particular concern in many large milk powder manufacturing plants and their occurrence in raw milk is often correlated with the conditions on the farm, in particular the quality of silage being fed (237).

On farm the third and principal source of microbial contamination is believed to be milk handling equipment such as teat cups, pipe work and storage tanks (7, 84, 115, 138, 259). Fouling layers formed by bacteria and milk components (protein and minerals) are believed to adversely impact on the effectiveness of on farm cleaning and sanitising regimes.

The principal mechanism to control microbial growth in milk stored on the farm is low temperature storage \((< 7^\circ \text{C})\), which limits growth to psychrotrophic organisms and increases the length of time the milk can be stored before processing. During transport to and storage at the manufacturing site refrigeration may not be required as the short times involved and the volume of the bulk milk limit temperature changes and the potential for microbial growth (69, 211).

Generally, raw milk entering a DMP has a bacterial load of around \(10^2\) to \(10^3\) CFU mL\(^{-1}\) with less than 10 thermophilic bacilli mL\(^{-1}\) being present (156). Poor hygiene or inadequate cooling can, however, result in total bacterial numbers as high as \(10^6\) CFU mL\(^{-1}\) being detected in milk entering the DMP. The dairy industry relies on pasteurisation \((72^\circ \text{C for 15 seconds})\) as the main process to destroy heat sensitive
and pathogenic bacteria present in raw milk. However, thermoduric and thermophilic bacteria including certain species of streptococci, micrococcii, lactobacilli and spores from bacillus and clostridium species are able to survive this process.

1.4 Thermophiles

Thermophiles are bacteria that grow at moderately high temperatures from 45 to 65 °C. Adaptations such as heat-stable DNA, proteins and membranes enable them to grow and persist in the pre-heating and evaporation sections within milk powder production plants and to act as a source of contamination to product being processed. The major thermophilic contaminants of milk powder throughout the world are *Anoxybacillus flavithermus* and *Geobacillus* sp. (194, 202). The other bacilli found in milk powder are facultative thermophiles such as *Bacillus licheniformis*, *Bacillus coagulans*, and *Bacillus subtilis* (194). Thermophilic bacilli are not a new problem as they have been isolated from 90 year old milk powder used in an Antarctica expedition (195). While studies initially focused on the identification and enumeration of thermophilic vegetative cells (203) their spores are currently receiving more interest (204, 205).

*Anoxybacillus flavithermus* and *Geobacillus stearothermophilus* appear to be the dominant thermophilic bacteria in milk powders worldwide and are ubiquitous in milk powder plants (202). *Bacillus licheniformis* are also commonly isolated from various milk powders (202).

1.4.1 *Anoxybacillus flavithermus*

There are very few isolates of *A. flavithermus* that have been characterised. This bacterium was first isolated from a hot spring in New Zealand (89). The type strain *Anoxybacillus pushchinoensis* was first described as a strict anaerobe, and hence the name, *Anoxybacillus*. This has since been revised to species of the genus being aerotolerant anaerobes and facultative anaerobes (181).

*Anoxybacillus flavithermus* was characterised as a Gram-positive, facultatively thermophilic, facultatively aerobic, motile, rod-shaped and a spore former (89). The G + C content is 61% with a growth temperature range between 30 to 70 °C with 60 °C
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being optimal at pH 6-9. Isolates obtained from milk powder have an optimal growth temperature of 50 to 65 °C (194).

1.4.2 Geobacillus spp.

The Geobacillus group is composed of the thermophile species that were previously classified in Group 5 of the Bacillus genus (10). They are a closely related group with a 96.5 to 99.5 % 16S rDNA sequence similarity (158). They produce sub-terminal and terminal endospores. The optimal growth temperature of this group is above 50 °C. Different strains of Geobacillus spp. have been isolated from temperate areas as well as areas such as hot springs, oilfields, deep sea sediments, sugar refineries and dairy factories (12, 158, 194, 234).

The most prominent Geobacillus species to be isolated from milk powder is G. stearothermophilus (70, 194, 227). The optimal growth temperature of Geobacillus spp. milk powder isolates is between 55 and 70 °C (194). Particular strains of Geobacillus spp. can form highly heat resistant spores that can survive ultra-high temperature treatment and retort, a process used in canning (95).

1.5 Spores

Certain bacteria are able to spend part of their lifecycle as a dormant cellular structure called a spore. As a spore, the organism is able to survive adverse environmental conditions that would otherwise be lethal. The spore’s complex structure allows it to survive extremes in heat, desiccation, pH, UV and chemicals (162, 216). The dormancy is a result of the spore having little to no metabolic activity (217). However, spores can revert to their vegetative state when conditions become favourable. The structure of spores (135) and the genes involved in structural protein and regulation of sporulation, have received extensive research and review (9, 57, 93).

Spores can survive heat treatments typically used in dairy manufacture, such as during pasteurisation, some strains of Geobacillus spp. being reported to form highly heat resistant spores that can survive ultra-high temperature (UHT) treatment or retorting, involving heating regimes of 140 °C for 3 s and 110 to 120 °C for 15 to 20 min respectively (95).

The number of spores present in raw milk from mesophilic and thermophilic bacteria, tested on the farm, over a period of a year has been reported as $10^3$ and $<10$
spores mL\(^{-1}\) milk, respectively (151). The development of biofilms of spore forming bacteria in milk powder manufacturing plants is believed to ultimately originate from spores present in the raw milk surviving pasteurisation, although this has never been proven (211).

### 1.5.1 Spore Structure

Spores are a complex structure consisting of mainly protein (Figure 1.2). The core contains the genetic material and has a pH of 6.5 (145, 214). The core also contains dipicolinic acid (DPA) and small acid soluble proteins that bind to the DNA (213). Dipicolinic acid is abundant in the core of the spore (127); making up 5 to 15% of the dry weight of a spore (186). However DPA levels differ between spores of different species and even between spores of a single species (104). The dipicolinate ion forms a 1:1 chelate with predominantly calcium ions to form calcium dipicolinate (CaDPA) (216).

![Spore Structure Diagram](image)

Figure 1.2: Diagram of the spore structure displaying the spore core surrounded by a membrane, cortex and coat. Certain species contain an additional layer known as an exosporium.
Surrounding the core is the cortex, which consists of a membrane and a peptidoglycan layer which contains different sugar residues compared with the peptidoglycan of the cell wall in vegetative cells (262). The relative impermeability of the inner membrane found in the spore cortex is important for maintenance of the conditions found in the spore’s core (232).

The spore coat is a highly cross-linked protein layer, which occupies most of the spore’s volume and contains 70 to 80% of the spore’s total protein (9, 57, 93). This coat contains large amounts of cross-linked cysteine and tyrosine creating a robust structure (9, 172). The spore coat is made up of two layers containing an inner laminated layer along with an electron dense staining outer layer. The spore coat is permeable to small molecules and water and responsible for the spore’s resistance to many hydrolytic enzymes such as lysozyme and trypsin. However, if the spore coat is removed, the resultant spore is sensitive to lysozyme since the cortex containing peptidoglycan (the site for action of this enzyme) is exposed. Many of the proteins in the spore coat are glycosylated (93).

In some species the coat is the outermost structure of the spore, while spores of bacilli such as *Bacillus cereus* and *Bacillus anthracis* have an additional layer called the exosporium (81). The exosporium consists of two layers; the first is a hexagonal crystal lattice structure, while the outer layer consists of a “hair-like” nap of filaments (81). The chemical composition of the exosporium found in *B. cereus* consisted of protein, amino and neutral polysaccharides, lipids and ash (149). Specific glycoproteins and carbohydrates (76, 233) as well as proteins (188) have been characterised from the exosporium of *B. anthracis*. Currently, little is known about the function of the exosporium. However in *B. anthracis* spores, a study has shown that it protects the spore when it is ingested by a macrophage (263). Spores such as *B. cereus* also contain long appendages constructed from hydrophobic residues and carbohydrate (228). It has been shown that these appendages can influence the initial attachment of *B. cereus* spores to stainless steel (125, 236).

### 1.5.2 Spore Lifecycle

The lifecycle of sporulation, dormancy and germination of bacilli was first observed by Cohn in 1876 (45) and can be seen in Figure 1.3. There are many factors reported to initiate sporulation such as nutrient limitation, population and oxidative
stress. These all act through the phosphorylation cascade (99) ultimately activating a specific set of sigma factors (57) and a master transcription factor Spo0A (180). The first step involves the cell undergoing asymmetric division and inward folding of the cytoplasmic membrane to form a septum. This septum divides the cell into the mother cell and the forespore. The forespore is then enclosed in a membrane vesicle, and the cortex is formed, followed by the spore coat. The spore becomes denser through the uptake of CaDPA, dehydration occurs and resistance develops. The mother cell finally undergoes lysis and the spore is released. The entire process takes about 8 hours (57). Spores are the predominant contaminant in milk powder, however it still remains unclear what triggers sporulation during milk powder production since there is a constant flow of nutrients present in the milk. Perhaps the nutrients are unable to reach particular cells within a biofilm therefore triggering sporulation or sporulation within a biofilm is part of a complex lifecycle of biofilm development. Recent research has shown that sporulation of thermophilic dairy isolates requires optimal growth conditions such as nutrients and temperature (211).

Figure 1.3:

1. Vegetative cell.
2. DNA replication and formation of the septum.
3. Membrane vesicle enclosure.
4. Cortex and coat formation.
5. Exosporium formation and maturation.
6. Lysis and release of the mature endospore.
Germination occurs in three steps; activation, germination and outgrowth (59). Activation is a reversible process in which the spore is prepared for activation while retaining many of its properties such as heat resistance. If conditions are favourable, the spore will undergo the irreversible process of germination. During this time, a cascade of events occurs; H+ ions are secreted thereby raising the pH of the core from 6.5 to 7.7, CaDPA is lost and is replaced by water and the cell loses dormancy to become metabolically active. The next step involves the hydrolysis of the peptidoglycan in the spore’s cortex and further swelling of the spore core due to the ingress of water and expansion of the germ cell wall. There have been many reports of different signal substances that can induce activation and germination. Heat is commonly used for thermophilic spores (16), while amino acids such as L-alanine are used for mesophilic species (54). Low pH has also been shown to activate spores, however this does not necessarily lead to germination (111). The spore coat has also been shown to be important in the germination of \textit{B. cereus} spores since components of the coat are used as nutrients by the germinating organism (130).

Understanding how spore formation occurs may provide insight into the control of spores in the dairy industry. \textit{Bacillus subtilis} and \textit{B. cereus} have been used as model organisms for analysing sporulation (54, 63, 75). Earlier studies focused on factors important for sporulation such as nutrition, temperature and pH. These studies found that certain carbon sources increased sporulation, and that the presence of salts containing calcium and manganese was also crucial (64). The optimal temperature and pH for sporulation is similar to that required for vegetative cell growth, but the range for both parameters is smaller. Recent research has focused more on the genetic aspects of sporulation such as the activation of SpoOA and the regulation of different sigma factors and other genes important in the initiation of sporulation (99, 204).

Despite the vast amount of knowledge on the sporulation of mesophilic bacilli and on \textit{G. stearothermophilus} (119, 121, 238), little is known about the sporulation of species from other thermophilic bacilli and in particular dairy isolates. There have only been two studies that have produced spores from thermophilic bacilli isolated from the dairy industry (175, 202) with both of these protocols involving long incubation times which would be difficult if a large quantity of spores were needed quickly for surface characterisation and adhesion assays.
1.5.3 Spore resistance to adverse conditions

Spores are recognised as a major source of contamination in many food industries. This is due to their innate resistance to many of the techniques employed by food industries to limit microbial growth such as heat or the addition of acids. There are several properties that contribute to the resistance of a spore.

The features of the spore’s core are important in their resistance to adverse conditions. It is believed that the CaDPA present in the spore’s core replaces much of the water present, thereby increasing it’s resistance to wet and dry heat (169). Mineralisation has also been shown to be important in heat resistance (16, 18). However the dehydration and lower pH conditions in the core are not solely responsible for the protection of the spore’s DNA since changes in these conditions do not significantly affect the spore’s resistance. While CaDPA increases the resistance of spores to both wet and dry heat (212), it also sensitises the spore’s DNA to UV radiation (55). In recent studies, type α/β small acid soluble spore proteins (SASP) have been shown to be very important in the spore’s resistance to DNA damaging agents (215). Mutants of B. subtilis that lacked these SASPs were more sensitive to UV radiation, desiccation, and wet and dry heat. These SASPs bind to the GC rich regions of the DNA to form a tightly packed assembly (79, 80, 212). The inner membrane in conjunction with the spore coat is responsible for the spore’s innate resistance (162). The spore coat protects the spore against enzymes such as lysozyme, mechanical disruption, UV radiation and chemicals such as hydrogen peroxide (87, 189). It should be noted that the mechanisms of heat resistance are dependent on the strain and conditions during sporulation (171).

Spore resistance is dependent on environmental factors such as temperature, pH and media composition. Spores grown at temperatures greater than their optimal temperature tend to be more heat resistant (15). The metal ion content can also influence the resistance properties of spores (36). Spores grown on nutrient media supplemented with calcium and magnesium were more heat resistant compared with those grown in sporulation media fortified with manganese only (36). Spores attached to a stainless steel surface were also reported to be more heat resistant than planktonic spores (219). Spores produced during milk powder production are more heat resistant than those grown under laboratory conditions (96). Furthermore, spores of G.
stearothermophilus have higher heat resistance when suspended in milk rather than water (272).

Spores are able to survive all of the adverse conditions they face in the dairy industry including low water activity and high temperatures during the drying process, CIP treatment and the long term storage of the final product. Due to the difficulties faced in killing them, spores produced by thermophilic bacilli are a serious concern during the manufacture of milk powder.

1.6 Thermophile contamination of milk powder

Thermophilic bacilli are difficult to eliminate in the DMP environment due to their fast growth rate, resistant spores, wide temperature growth range and their ability to form biofilms (69, 95, 174). Furthermore, spores are able to survive in milk powder products for longer periods of time compared with vegetative cells (187). Initial contamination of the powder plant probably occurs from the low number of thermophiles, predominately in spore form, present in raw milk (151). Spores and cells subsequently attach to stainless steel surfaces and foulant present in the DMP (69, 175). If conditions are favourable, spores germinate while vegetative cells divide and secrete polymers to colonise the surface thereby initiating a biofilm. Thermophilic biofilms are typically found in the plate heat exchanger and evaporator sections of the plant (211). Contamination of the product being processed occurs from spores and cells sloughing from the biofilms. Sporulation within simulated dairy biofilms has been observed after 30 h for B. subtilis (140, 141) and 9 h for G. stearothermophilus and A. flavithermus isolates (211). Other research has observed spore formation in the basal layers in colonies of B. subtilis at the surface to air interface (257), and liquid to air biofilms of B. cereus (265). However this may not be the case for biofilms growing at the solid-liquid interface under turbulent flow in a dairy environment. At the end of a milk powder run, a majority of the cells are removed and killed by a CIP (173), however fouling layers may protect spores and vegetative cells (98) resulting in potential contamination of the next production run.
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1.7 Biofilms

Dairy manufacturing plant biofilms can be divided into two categories;

1) “Process biofilms” are biofilms that are unique to DMP and form on surfaces in direct contact with flowing product, or
2) “General biofilms” that form in the general food processing environment.

While there is concern in the dairy industry about the role of biofilms associated with the general food processing environment and the presence of pathogens such as *Listeria monocytogenes* (78), this chapter predominantly focuses on process biofilms. Process biofilms differ from general biofilms in two key ways (67). Firstly, in a process biofilm a single species often predominates because heat treatment operations reduce the competition from heat sensitive bacteria. Secondly, process biofilms can also be characterised by their rapid development, with numbers of up to $10^6$ CFU cm$^{-2}$ being recorded in the regeneration section of a pasteuriser after 12 hours of operation (26). In contrast biofilms in most other environments take several days or weeks to develop (67, 282).

In DMP many factors influence the initial bacterial or spore attachment and subsequent biofilm formation, initially ions and soluble macromolecules (such as proteins) will adsorb at a solid-liquid interface (17). These molecules affect the adhesion of bacteria and spores, and can lead to profound alterations in the properties of the material fluid interface (28). The bacteria that enter the interface at this stage are influenced by hydrophobic interactions, van der Waals forces, and electrostatic attraction and repulsion (102). Eventually favourable conditions are reached in the microenvironment of the interface, which allow the cells to attach and grow.

In the dairy industry, surface conditioning is often referred to as fouling and was suggested by Rosmaninho *et al.* (201) to take place as soon as a dairy product is brought into contact with stainless steel. Hinton *et al.* (97) concluded that fouled stainless steel allowed faster attachment and growth of dairy thermophiles. Conflicting opinions exist on the importance of a conditioning film in initial bacterial attachment, with Fletcher (66) reporting that the presence of proteins such as albumin, gelatin and fibrinogen inhibited attachment of a marine *Pseudomonas* to polystyrene. Parkar *et al.* (175) demonstrated that the presence of skim milk on a surface of
stainless steel, even at concentrations as low as 1%, reduced the initial attachment of spores and vegetative cells of thermophilic bacilli. Skim milk was also found to reduce the attachment of *S. aureus*, *Listeria monocytogenes* and *Serratia marcescens* to stainless steel (13). Even individual milk components such as casein and β-lactoglobulin were reported by Helke *et al.* (91) to reduce attachment of *Listeria monocytogenes* and *Salmonella typhimurium* to stainless steel. One reason for the reduced attachment may be that the proteins in the bulk fluid phase compete for binding sites on the surface of the stainless steel, reducing the number of sites for bacterial attachment. However, Speer & Gilmour (226) reported that stainless steel and rubber surfaces treated with either whey proteins or lactose demonstrated an *increase* in attachment of milk-associated micro-organisms. Flint *et al.* (69) also reported higher attachment of *Bacillus stearothermophilus* to stainless coupons coated with skim milk foulant created by denaturing skim milk on the surface by autoclaving. Milk proteins are a good substrate for bacterial growth with the presence of milk aggregates creating a suitable supporting substrate (273). Stainless steel covered in a milk foulant layer has been shown to attract up to 100 times more bacteria than clean stainless steel (69) however skim milk proteins adsorbed to stainless steel have also been shown to reduce the number of bacteria attaching to stainless steel (13, 91, 175). The effect of milk proteins on bacterial attachment to a substrate is likely to be dependent on whether they are in a denatured or native state with the denatured state presumably providing a large surface area for attachment.

In sections of the milk powder plant where nutrient and temperature conditions are ideal for thermophilic growth, vegetative cells or spores may attach to stainless steel surfaces or milk fouled surfaces (97) and develop into a biofilm. Product contamination is most likely to occur when individual cells are released from sloughing off of a surface or when breaking up of the biofilm occurs during processing, thereby releasing large numbers of bacteria directly into the product stream. This release of bacteria into the passing product is sometimes referred to as the biotransfer potential (267).

Biofilm development involves the initial adhesion of an organism to a surface, colonisation of that surface, followed by the growth of a biofilm. The rest of this review will focus on the initial stages of biofilm development and the factors that influence it after a short discussion of the materials of interest.
1.8 Materials

Bacterial colonisation can occur on all surfaces used in the dairy industry (35, 122, 226). Stainless steel is the most common food contact material used in DMPs, as it is easy to fabricate, durable, chemically and physiologically inert at a variety of food processing temperatures and pressures, generally corrosion resistant, easy to fabricate and usually easy to clean (100, 241). However, biofilm development occurs readily on stainless steel surfaces (132). Teflon, buna-N, plastic and electropolished stainless steel surfaces that are often regarded as “easy clean”, have also been shown to support biofilm development (34, 206). Materials vary in terms of their propensity for bacterial adhesion with the level of contamination. For example the adhesion of *B. cereus* spores have been reported to be the highest on high density polyethylene (1.1 x 10^7 cells cm^{-2}) and lowest on 304L stainless steel with a 2B surface finish (2.6 x 10^6 cells cm^{-2}) (123).

1.9 Initial attachment

The first phase of biofilm development involves the initial attachment of a micro-organism to a surface (170). There is a large array of factors involved in this initial attachment step. These factors include surface conditioning, mass transport and physico-chemical interactions involving surface charge and hydrophobicity (62, 170).

Conditioning films form on a surface when organic and inorganic molecules found in the bulk solution are transported to the solid-liquid interface by diffusion or flow. Conditioning films can effect initial bacterial attachment either by altering the surface charge and hydrophobicity of a surface (270) or by occupying the limited number of “binding sites” (91). Conditioning films may also provide a concentrated source of nutrients for adhered organisms (115). Milk is a colloid suspension comprising several proteins, salts and fat. Milk proteins have been shown to effect the attachment of thermophilic bacilli and spores to the surface of stainless (13, 175) and whey proteins and lactose have been reported to increase attachment of milk-associated organisms to stainless steel and rubber surfaces (226). The effect of milk proteins on bacterial attachment was discussed in greater detail in section 1.7.

Before the initial adhesion can take place, the micro-organism must be transported to the surface. Mechanisms for transport include Brownian motion, sedimentation and convective currents. Brownian motion is the random movement of
a particle in a fluid due to collisions with molecules in the surrounding environment. Sedimentation occurs when the specific gravity of the micro-organism is greater than that of the surrounding medium. Convective transport is a major contributor to the movement of micro-organisms in an environment with high flow rates, like in the dairy industry. Previous research has shown that convective mass transport contributes to the attachment of bacteria to a substratum surface (269). This type of transport resulted in higher rates of attachment in a T-junction flow cell compared with when the flow was parallel to the substratum surface. Turbulent flow may create eddies which can increase the interaction of micro-organisms with the surface, and provide sufficient energy to overcome the Gibbs energy barrier, which will be discussed later in this review.

Another form of active transport is movement by a flagellum and chemotaxis. In a study which interfered with the individual operons for flagellum and chemotaxis, it was shown that the flagellum was important in attachment while chemotaxis was not (126). However other studies have disputed this by reporting that flagella were not important in the initial adhesion of _Pseudomonas aeruginosa_ to an abiotic surface but were important in biofilm development (124). It is important to note that in contrast to vegetative bacterial cells, spores are non-motile (163) and are devoid of any metabolic activity (215) and are therefore believed not to respond to chemotactic gradients present in a solution.

The attachment of a micro-organism to a surface is governed by non-specific interactions outlined by the Derjaguin, Landau, Verwey and Overbeek (DLVO) theory (248). These include Lifshitz-van der Waals and electrostatic interactions. However acid-base interactions, or hydrogen bonding, are a third force involved in the interaction between surfaces as outlined by the extended DLVO (XDLVO) theory (254). These forces decay over different separation distances and are discussed in greater detail below and in Chapter 5 of this thesis.

The initial attachment of a micro-organism to a surface can be seen in Figure 1.4. The first step involves the transport of a bacterium close enough to the surface to interact with it. The van der Waals force acts over a range of several hundred nanometers from a surface and is usually an attractive force in an aqueous environment, thus causing the micro-organism to move closer to the surface (Figure 1.5). Over a closer range, 2 to 20 nm depending on the ionic strength of the suspending media, electrostatic interactions are present. This initial attachment is
refered to as the secondary minimum and is considered to be reversible. At this distance from the surface, the organism still displays Brownian motion and can be easily removed by fluid shear forces such as rinsing. The next step of attachment involves overcoming the energy barrier created by strong electrostatic repulsion between the two surfaces, which in most cases, share the same charge. Once this is overcome, the intimate attractive interaction between the bacteria and the surface gives rise to the primary energy minimum, which is typically < 2 nm away from the surface. However a solvation layer created by interfacial water molecules can still exist near the immediate surface of a hydrophilic substrate thus creating a strong repulsive force. This force created by the solvation layer can be overcome by the hydrophobic groups present on the micro-organism’s surface which disrupt the water molecules on the surface. Once closer to the surface, the cell surface molecules such as surface polysaccharide (44), pili or fimbriae can form specific linkages with functional groups present on the substratum. At this stage the micro-organism is said to be irreversibly attached to the surface and stronger mechanical or chemical forces are required to remove it.

Figure 1.4: The forces involved in the initial attachment of a micro-organisms to a substratum and the approximate distances at which they act. Objects are not to scale. Reproduced from Fletcher 1996 (65).
1.9.1 Surface charge

The bacterial cell surface carries a net negative charge under most physiological conditions since the bacterial surface consists of more anionic compared with cationic groups (190). Since most surfaces occurring in nature exhibit a net negative charge, micro-organisms generally experience an electrical double layer repulsion when approaching a negatively charged surface. The surface of bacterial cells and spores are complex generally consisting of surface appendages such as polymers, pili, fimbriae and flagella. These surface polymers generally consist of a heterogeneous array of charged functional groups such as carboxylates, amines and phosphates distributed throughout the surface polymers. Changes in the environment, such as pH (108) and ionic strength (49) can interact with the surface causing dramatic changes to the surface electrostatics. Decreasing the pH below the $pK_a$ of the anionic functional groups will result in their protonation, which results in only the cationic groups contributing to the overall charge on a cell’s surface. Under
these conditions the surface of the bacterium may be positively charged. Additionally culturing conditions have been shown to effect the surface characteristics of bacteria (31).

The calculation of zeta potentials is commonly used for the determination of the relative surface charge of a colloid particle in a solution. The zeta potential is the potential across a slip plane between the hydrodynamic stagnant layer surrounding a particle in solution and the bulk solution. This potential cannot be measured directly since particles in a solution are not connected to an external circuit (50). The zeta potential is therefore calculated from the measurable electrophoretic mobility, which is the magnitude of the velocity of a charged colloidal particle immersed in a liquid moving under the influence of an external field divided by the magnitude of the electric field strength. Electrophoretic light scattering is the most commonly used technique for determining electrophoretic mobility with the movement of a suspended particle in the electric field being measured by analysis of the Doppler shift of scattered light (239).

The location of the shear plane, and therefore zeta potential, can be affected by the composition of the microbial surface (ie. polymers) and by the physical properties of the surrounding medium (85, 144). In early studies, polystyrene particles were often been used as models to analyse electric double layers and bacterial adhesion. However it has been shown that the surface of synthetic polystyrene particles lacks polymeric appendages with heterogeneous functional groups and is therefore not the same as a bacterial surface and is not an appropriate representation of a bacterial cell (269). Polymers present on the surface of a micro-organism create a “soft” surface, which is penetrable by a solvent and solutes, and can influence the electric double layer (184). Currently, there are several models employed to calculate the zeta potential from the electrophoretic mobility measurements, however there is no general consensus amongst the literature as to which theory is applicable for the zeta potential of bacterial surfaces.

The zeta potential has often been used to describe bacterial adhesion to surfaces. Several groups have found relationships between the zeta potential of bacteria and their adhesion (246). However other studies have failed to observe a trend between zeta potential and bacterial (73) or spore (175) adhesion. The surface charge of a substratum is also important and is usually calculated from streaming potential measurements. The surface of a metal consists of a passive metal oxide film
resulting in a negative charge under physiological conditions (25). Stainless steel for example has a relatively low negative charge at pH 7, with an isoelectric point between pH 4 and 5 (25). While the surface charge of both the bacterium and the substratum are important factors in adhesion, the failure of electrostatic data to solely predict bacterial adhesion indicates that other forces are involved.

1.9.2 Hydrophobicity

The effects of surface hydrophobicity on the adhesion of bacteria to surfaces has been the focus of numerous studies (101, 247). Surface hydrophobicity is an interfacial property, but it has proven difficult to assign a suitable definition. Hydrophobicity or hydrophilicity arises from interactions between Lifshitz-van der Waals (LW) and acid-base (AB) forces (39, 250, 252). Lifshitz-van der Waal forces arise from the non-polar interactions including randomly orienting dipole-dipole (orientation) interactions, randomly orienting dipole induced-dipole (induction) interactions and fluctuating dipole induced-dipole (dispersion) interactions. Another force involved the interactions between electron donating and accepting polar groups, or AB interactions. Acid-base interactions often result in hydrogen bonding. As stated before, bacterial surfaces are heterogenous, containing numerous functional groups. Non-polar groups on the surface may include methyl groups, while polar groups include hydroxide and charged groups such as carboxylates, amines and phosphates. During bacterial attachment, Lifshitz-van der Waals forces are always attractive, while acid-base interactions can either be attractive or repulsive.

The measurement of microbial surface hydrophobicity has been the focus of a number of laboratories for many years (62, 196, 247). The three most commonly used methods to measure cell surface hydrophobicity will be discussed below. The microbial adhesion to hydrocarbons (MATH) method was first described by Rosenberg et al. (199) in 1980. This method involves measuring the removal of bacteria from an aqueous phase by their adherence to hydrocarbon droplets such as hexadecane or xylene. Data are presented as a percentage of bacteria bound to hydrocarbon droplets, which is then assumed to be the relative hydrophobicity of the bacterial surface. Previous studies have shown electrostatic effects to also play a role in this assay (4). Anions accumulate around the surface of the hydrocarbon droplets resulting in a negative zeta potential of -80 mV in phosphate buffer at pH 7 (32). To
overcome this, the MATH assay should be run at the isoelectric point of the hydrocarbon to eliminate the effects of electrostatic repulsion (245). Over the years, there have been many modified versions of this assay (198). The partitioning kinetics method has been suggested as a means of making the MATH assay more robust. This addition to the assay involved the measurement of the rate of removal of bacteria from the aqueous phase based on hexadecane volume and vortexing time (21).

Another method for measuring bacterial surface hydrophobicity is through hydrophobic interaction chromatography (HIC) and involves the retention and elution of bacteria bound to hydrophobic resins, usually Phenyl-Sepharose. Like the MATH assay, the HIC is influenced by electrostatic interactions. Increasing the ionic strength can overcome the electrostatic repulsion between bacteria and the beads.

The third method for measuring hydrophobicity is through contact angle measurements (CAM) and the calculation of interfacial energies. The CAM is performed by measuring the contact angle of a solvent drop on a ‘lawn’ of bacteria deposited on a membrane by negative pressure (252). From these contact angles, the total interfacial energy can be calculated. This will be described in greater detail in the later chapters of this thesis.

As stated before, the effects of bacterial surface hydrophobicity on adhesion has received plenty of attention. Several studies have found a positive correlation between hydrophobicity and attachment (14, 83, 178, 247, 255). However other studies have found little or no relationship between surface hydrophobicity and bacterial adhesion (73, 175, 222). Husmark and Ronner found that bacterial spores of *B. cereus* attach in higher numbers to surfaces when compared with vegetative cells (56, 109). This was confirmed by Faille et al, and attributed to the fact that *B. cereus* spores were more hydrophobic (62). Parkar *et al* also concluded that this could be the reason spores attached in higher numbers to stainless steel compared to their vegetative cells (175).
1.10 Summary

Bacterial attachment to surfaces is moderated by a variety of chemical and physical interactions between the bacterial and substrata surfaces. The states of these interactions outlined by the XDLVO theory are influenced by the environment under which the adhesion process takes place. The complex nature of interfacial energies outlined in the XDLVO theory can be further complicated by conditioning layers formed by complex media such as milk and mass transport caused by flow conditions in a dairy plant. A better understanding is required on the effects of these factors on the attachment of spores from thermophilic bacilli to stainless steel during the production of milk powder. This may enable the development of strategies to either reduce the attachment of spores to a surface, or reduce the strength of adhesion so spores can be easily removed from a CIP regime.

1.11 Objectives

In order to better understand the factors which contribute to the adhesion of spores from thermophilic bacilli, this thesis will analyse this problem via a series of four objectives.

1. Produce and purify spores of thermophilic bacilli isolated from a milk powder manufacturing plant
2. Characterise the surface of thermophilic spores in terms of their structure, surface charge, hydrophobicity and functional groups.
3. Examine how these different characteristics affect the initial attachment of spores to different surfaces
4. Develop surfaces, which would reduce the initial number of spores attaching, or make the surface more readily cleanable with a CIP.
Chapter 2

Spore Isolation, Production and Purification
2.1 Introduction

The growth of thermophilic bacilli and the spores they produce is a common problem during the manufacture of milk powder (157). The thermophiles of concern are from the organisms formally classified under the genus *Bacillus*. Generally, thermophiles isolated from milk powder have belonged to the genera *Geobacillus* spp. and *Anoxybacillus flavithermus* (69, 194). These organisms are not pathogenic, however they are used as indicator organisms for plant hygiene. If the milk powder is reconstituted and the conditions are favourable, spores of these organisms will germinate and generate enzymes and acids, which can result in off-flavours in the product (40, 43).

It is believed that spores enter the DMP via the raw milk stream in which they are generally found in very low numbers (<10 CFU mL\(^{-1}\)) (151). These organisms are able to survive pasteurization, and attach to stainless steel surfaces within the milk powder manufacturing plant (69, 175). Subsequently, in locations where the temperature and water activity are suitable for growth, germination of the spores occurs. These locations include the regenerative sections of the heat exchangers and the first effects of the evaporators. The vegetative cells divide, grow and secrete polymers to form a biofilm (69, 174). As these biofilms mature, large numbers (1 \times 10^6 CFU g\(^{-1}\)) of cells and spores can slough off causing contamination both further down the production line and of the final product being produced (211). Spores of thermophilic bacilli are of particular concern in a DMP due to their resistance to cleaning chemicals associated with a CIP procedure, and the high heat and low water activity associated with the drying process and long-term storage of final product. Vegetative cells are less likely to survive these adverse conditions.

Biochemical tests such as the API® system (bioMérieux, Marcy l'Etoile, France) were traditionally used to identify isolates in the dairy industry. However with the development of polymerase chain reaction (PCR)-based techniques such as species specific PCR, randomised amplified polymorphic DNA (RAPD) and 16S rDNA sequencing have shown that *A. flavithermus* has often been mistakenly identified as *Geobacillus* spp. using the API system (70).

Ronimus *et al* (194) used RAPD profiling to classify thermophilic bacilli isolated from milk powder. These isolates could be fitted into seven groups, which included *G. stearothermophilus*, three strains of *A. flavithermus*, two strains of *B.
licheniformis and B. subtilis. However, using partial 16S rDNA sequencing, Flint et al. (70) classified a majority of New Zealand thermophilic milk powder isolates as either Geobacillus thermoleovorans or A. flavithermus. These results led to the development of PCR primers specific for A. flavithermus and Geobacillus spp., and have allowed for the identification of these two major thermophilic contaminants using diagnostic PCR.

The initial attachment of micro-organisms, including spores, to surfaces is due to the physicochemical interactions that occur between their surface and the substratum (243). Such forces include Lifshitz-van der Waal’s, acid/base and electrostatic interactions. An improved understanding of the interactions that impact on spore attachment may help in the development of strategies to reduce their adherence to surfaces. However there have only been a few studies on the surface characterisation of spores and these have been confined to those from mesophilic species (62, 178). Surface characterisation techniques have involved measuring surface charge through zeta potential, relative hydrophobicity through the microbial adhesion to solvents (MATS) assay and CAM (247).

Before the surface of a spore can be characterised using these techniques, large quantities of free endospores must be obtained. Therefore a reliable method is required to produce large numbers of spores from thermophilic bacilli isolated from a milk powder production line. Traditional methods have focused on harvesting spores from mesophilic bacilli such as B. subtilis and B. cereus (54, 63, 75) with only a few papers published on spore production from the thermophile G. stearothermophilus (119, 121, 272) and even fewer on recovering spores from dairy micro-organisms (175, 202). For characterisation purposes it is important that the spore suspensions do not contain debris or vegetative cells, which may influence the surface characterisation results. Researchers have used detergents, enzymes or ultrasound to purify spores and disrupt them in order to recover proteins or DNA (207) however, these techniques can alter the spore surface (56). To overcome this problem, density gradient centrifugation using either sodium bromide (131, 161), urografin (235) or a two-phase separation technique with polyethylene glycol have been used (208).

The aims of this section were to isolate and identify thermophilic bacilli spores isolated from a milk powder production line, and develop a method to produce large quantities of endospores from isolates of interest. The next step was to develop a
purification technique to remove vegetative cells and debris that could interfere with the surface characterisation techniques used later in this study.
Chapter 2 – Spore Isolation, Production and Purification

2.2 Materials and Methods

2.2.1 Sampling

Vegetative cells and spore isolates were obtained from liquid samples and stainless steel coupons from a milk powder production line in a DMP located in Edendale, New Zealand. Liquid samples were obtained from rubber septum sampling ports in 9 mL vacuum tubes (Vacut® Greiner Labortechnik, Biolab, Auckland, New Zealand) using sterile vacutainer needles. The locations of rubber septum sampling points used in the evaporation and drying process are outlined in Figure 2.1 and the conditions at each of these points are outlined in Table 2.1. Samples were taken after 9, 15 hour and at the end of an 18 hour production run and kept at 4 °C until the isolation method described in Section 2.2.3.

2.2.2 Modified Robbins Device

In order to obtain samples of bacteria attaching to stainless steel surfaces within a milk powder production line under operating conditions a sampling device was created. Vegetative cells and spore isolates adhering to stainless steel in contact with the milk concentrate were obtained using a type 304 stainless steel modified Robbins device (MRD). Eight sampling ports were fitted into a 75 mm diameter stainless steel outlet pipe at the end of the second pass of the first effect of the evaporator (Figure 2.2). The design of the MRD was based on the original concept developed by Jim Robbins at the University of Calgary (150) (Figure 2.3). Each of the sampling ports were fitted with a stainless steel 316 2B polish stainless steel coupon (10 mm diameter). The coupons were fitted flush with the internal surface of the pipe. Stainless steel coupons were polished using 800 and 1200 grit sandpaper, washed in acetone at 20 °C, passivated by immersion in 50% nitric acid at 80 °C for 30 min, rinsed in sterile distilled water and autoclaved. Sampling in the middle of a run was achieved with the use of a bypass pipe fitted with butterfly valves upstream and downstream of the MRD. This allowed the flow of product to be diverted so that the coupons could be sampled during a production run without the product being extensively exposed to external conditions.
Figure 2.1: Diagram representing sampling points (1-6) during the milk powder production process at Powder Plant 2 in Edendale, New Zealand. Figure reproduced and modified from Scott et al., 2007 (211).

Figure 2.2: A closeup of the evaporator along with the MRD placed after the second pass of the first effect. Sampling points 3 and 4 correspond to the same points in Figure 2.1. Figure reproduced and modified from Scott et al., 2007 (211). Components are not to scale.
Table 2.1: Sampling point designations and the temperature and percentage of total solids of the product at each point.

<table>
<thead>
<tr>
<th>Sample Point</th>
<th>Name</th>
<th>Temperature (°C)</th>
<th>Total Solids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Balance tank feed</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>Preheaters</td>
<td>88</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>MRD Coupon (solid)</td>
<td>66</td>
<td>22</td>
</tr>
<tr>
<td>4</td>
<td>Post MRD (liquid)</td>
<td>66</td>
<td>22</td>
</tr>
<tr>
<td>5</td>
<td>Post Evaporator</td>
<td>53</td>
<td>48</td>
</tr>
<tr>
<td>6</td>
<td>Final Product</td>
<td>25</td>
<td>95</td>
</tr>
</tbody>
</table>
Figure 2.3: Design drawings for the MRD for sampling the surface within the milk powder production line.
2.2.3 Isolation

In order to isolate thermophilic bacilli from a milk powder production line, liquid and stainless steel surface samples were obtained at different points (outlined in Table 2.1) along the milk powder production line of Edendale Powder 2 plant in Edendale, New Zealand. Samples were also collected during two separate production runs involving skim milk and buttermilk powders. Fifteen isolates were obtained from the skim milk powder production and were designated the D-series isolates (D1 through to D15). A further fifteen isolates were obtained from the buttermilk powder production and were designated the E-series isolates (E1 through to E15). A further isolate CGT-8 was obtained from the milk powder production line of a DMP located in Pahiatua, New Zealand. Of these thirty one isolates, eight were chosen due to their reproducible growth on tryptic soy agar (TSA). The bacterial isolates used in this study are listed in Table 2.2.

2.2.4 Heat treatment

Heat treatments were used to kill vegetative cells in both milk product and on the surface of substrates (coupons) so that only spores remained for viable enumeration techniques described below (Section 2.2.5). Liquid samples were boiled for 30 min and a 10-fold dilution was carried out in 20 mL screw-capped “Universal” bottles containing 0.1% peptone (9 mL) (BBL®-Polypeptone-peptone, Becton, Dickson and Company). Coupons with attached spores were rinsed by passing the coupon through the liquid-air interface of sterile 18 MΩ cm resistivity water (Barnstead) (henceforth known as water) three times to remove loosely adherent spores. The number of spores attached to the coupons was estimated by placing the coupons in a 20 mL screw top glass Universal containing 0.1% peptone (10 mL) containing three glass beads (5 mm diameter). The surface of the coupon was swabbed with a sterile swab (LPI sterile swab). The coupon, swab and media were placed on a vortex mixer for 15 seconds and then boiled for 30 min to activate the spores and to kill any remaining vegetative cells. After heat treatment, sample tubes were places in a 20 °C water bath for 10 min. The number of viable spores was determined using the method below in section 2.2.5.
2.2.5 Enumeration of viable spore numbers

To determine the number of thermophilic bacteria in a culture containing a single strain of organism, the drop plate method was used (94). 10-fold serial dilutions were carried out in 0.1% peptone and five individual 10 μL droplets from each dilution were dispensed onto the surface of TSA agar. The droplets were allowed to air-dry in a laminar flow hood before the plates were incubated at 55 °C for 15 hours and colonies were counted.

To determine the number of bacteria in a mixed culture or when bacterial numbers were low (< 100 CFU mL⁻¹), the standard plate count method was used. 10-fold serial dilutions were carried out in 0.1% peptone and 100 μL of each dilution was pipetted onto the surface of triplicate TSA plates and spread using a disposable sterile spreader. Plates were incubated at 55 °C overnight and colonies were counted.

2.2.6 Storage of bacterial strains

All bacterial cell isolates were stored at -80 °C in 15% glycerol. Spore suspensions were stored in water at 4 °C.

2.2.7 Bacterial identification:

2.2.7.1 Species specific primers:

Isolates were identified using specific primers designed by Flint et al., (70). A section of the variable region of the 16S ribosomal RNA gene was amplified by PCR using *A. flavithermus* and *Geobacillus* spp. specific primers. The *A. flavithermus* primer FLAVO (5'TAACGCCAGTTACTACGCTACTTG-3') and *Geobacillus* spp. primer LEVO (5'CGCCGCCCTCTCTCGAACCAGCTGGCTGGCCCCG-3') were used in conjunction with the universal bacterial primer Y1 (5'TGGCTCAGAACCAGCTGGCCC-3') (275) to produce a PCR product of approximately 450 bp long. The following reaction mix was made using a MasterAmp™ PCR kit: 264 μL water, 25 μL 20 × *Tfl* buffer [400 mM (NH₄)₂SO₄, 1 M Tris HCl (pH 9.0)], 50 μL 25 mM MgCl₂, 100 μL dNTP mix containing 1.25 mM of each dNTP, 12.5 μL of 10 μM LEVO or FLAVO primer, 12.5 μL Y1 primer and 6 μL *Tfl* DNA polymerase (1 std. U μL⁻¹). Each PCR reaction was performed in a total
volume of 50 µL containing 48 µL of PCR mix and 2 µL of template (mid exponential phase cells). A water control was also prepared containing 2 µL of water instead of DNA template. The PCR reactions were performed in a Techne PHC-3 thermal cycler using the following protocol: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 45 s, followed by 1 cycle of 72 °C for 7 min. PCR reagents and products were stored at -20 °C.

2.2.7.2 Randomly amplified polymorphic DNA (RAPD) PCR:

RAPD profiles were used to differentiate strains and were generated using the primer OPR13 (5'-GGACGACAAG-3') (194). The following reaction mix was made up using MasterAmp™ Tfl PCR kit: 460 µL water, 40 µL 20 x Tfl buffer [400 mM (NH₄)₂SO₄, 1 M Tris HCl (pH 9.0)], 80 µL 25 mM MgCl₂, 160 µL dNTP mix containing 1.25 mM of each dNTP, 5 µL of 47.5 µM OPR13 primer and 10 µL Tfl DNA polymerase (1 std. U µL⁻¹). Each PCR reaction was performed in a total volume of 80 µL containing 48 µL PCR mix and 2 µL of template (mid exponential culture). A water control was also prepared containing 5 µL of water instead of DNA template. The PCR reactions were performed in a Techne PHC3 thermal cycler using the following protocol: 94 °C for 3 min and 45 s, followed by 35 cycles of 94 °C for 15 s, 36 °C for 15s and 72 °C for 2 min, followed by 1 cycle of 72 °C for 4 min.

2.2.8 Preliminary sporulation media for thermophilic bacilli

Sporulation of *A. flavithermus* isolates D3 and E1; and *Geobacillus* spp. isolates D4 and E11 was tested using several different media in microtitre trays (3 mL wells) and 72 hour culture periods. Kaul and Singh medium (KS) was based on a formulation outlined by Kaul and Singh (119), and consisted of (per litre): 9 g Oxoid No. 2 nutrient broth, 3 mg MnCl₂, 6 mg Ca(NO₃)₂ in 0.1 M phosphate buffer with the pH adjusted to 7.5 with 0.1 N NaOH. Sporulation Houston medium (H) was based on Houston *et al.* (103), and consisted of (per litre) 15 g Oxoid No. 2 nutrient broth, 20 mg MgSO₄.7H₂O. 80 mg CaCl₂.H₂O and 5 mg MnSO₄.4H₂O. The third medium designated TSB, consisted of (per litre): 15 g TSB. For all media, 10 µL of an overnight culture grown in TSB was used to inoculate each of the wells. Cultures
were incubated for 72 hours at 55 °C. The number of spores were estimated as outlined in Section 2.2.5.

### 2.2.9 Time and base media on the sporulation of Geobacillus spp. isolates

The effects of medium, salt and time on spore yields were determined for Geobacillus spp. isolates D4, E7, E11 and CGT-8. Geobacillus spp. isolates were chosen due to their consistent spore yields in the preliminary sporulation assays above. Isolates were inoculated into 10 mL TSB, which was incubated for 18 hours at 55 °C and used as a 1% inoculum for spore media. Spore media for Geobacillus spp. isolates D4, E7, E11 and CGT-8 contained either (per litre): 30 g Bacto Tryptone (BD) or 30 g TSB (BD). These media were supplemented with salts (per litre): 0.125 g of CaCl₂, 0.15 g of MnSO₄, 0.155 g of FeSO₄, and 0.55 g of MgCl₂. All salts were analytical grade from BDH. Cultures were aerated by stirring for 72 h at 55 °C. At intervals spore numbers were determined as outlined in Section 2.2.5.

### 2.2.10 Purification

In order to remove vegetative cells and debris from harvested spore suspensions, two different purification methods were used. The first was a continuous gradient of NaBr as described by Ang and Nickerson (8). The second consisted of a two phase system containing polyethylene glycol as described by Sacks and Alderton (208).

With the density gradient, a continuous linear NaBr density gradient (1.0 to 1.4 g mL⁻¹) was prepared in a centrifuge tube using equal parts of sterile NaBr solution and water (1.5 g mL⁻¹, w/v). Solutions of NaBr consisting of different densities were carefully layered on top of each other to create a gradient. The spore suspension was then layered on top of the gradient and centrifuged in a bucket rotor at 2,400 × g, 25 °C for 45 min. Spores consistently formed a band at 1.3 g mL⁻¹ while vegetative cells formed a band at 1.1 g mL⁻¹. Bands were carefully extracted from each layer and washed several times in water.

The second purification method used a polyethylene glycol (PEG) two-phase system, which was formed by dissolving 5.6 g of polyethylene glycol 4000 in 17 mL
of 3 M phosphate buffer (pH 7.4). After phase separation, the crude spore suspension was carefully layered on the two-phase system creating a total volume of 50 mL. The sample was centrifuged at $1,500 \times g$ for 3 min at 20 °C. The two phases were carefully recovered and washed separately five times in water at 20 °C.

The effectiveness of the two separation procedures was determined by visual examination of the recovered fractions using a differential interference contrast (DIC) microscope (Olympus BX51, Olympus). Photographs were taken using a DP70 CCD camera and DP Controller software (Olympus).
2.3 Results

2.3.1 Isolation

Liquid and stainless steel surface samples obtained at different points along the milk powder production line were collected during the manufacture of skim and buttermilk powders. The number of vegetative cells and spores associated with the samples was estimated using standard microbiological techniques. Generally, vegetative cells and spores were barely detectable (< 100 CFU mL\(^{-1}\)) during the production of skim milk powder. The D-series isolates were obtained during this production run. Of the fifteen isolates obtained, four were chosen due to their reproducibility of growth on TSA and were designated D1, D2, D3 and D4. Higher counts were obtained for the buttermilk powder, with 3.0 Log\(_{10}\) CFU cm\(^{-2}\) for vegetative cells and 2.5 Log\(_{10}\) CFU cm\(^{-2}\) spores attached to the stainless steel surfaces in the MRD after 15 h into an 18 h production run. Liquid samples taken after the pre-heater and near the MRD contained 3 Log\(_{10}\) vegetative cells CFU mL\(^{-1}\) and less than 1 Log\(_{10}\) spores CFU mL\(^{-1}\). The E-series isolates were obtained from the buttermilk powder production run and were designated from E1 to E15. Isolates obtained from stainless steel surfaces of the MRD and liquid samples taken from different points along the product stream were all typical of thermophilic bacilli with all isolates able to grow at 55 °C. Colonies for isolates D1, D2, D3 and E1 were yellow in colour raised and ranged between 1 and 2 mm in diameter. Colonies of D4, E7, E11 and CGT-8 produced white colonies ranging from small 1 mm colonies to 3 mm mucoid colonies. Isolate CGT-8 was obtained from the manufacturing plant in Paihatua.

2.3.2 Identification of isolates

Random amplified polymorphic DNA analysis and species-specific primers were used to distinguish between the isolates in the D and E series (Figure 2.4), and the isolate CGT-8 (data not shown). Isolates D1 and D2 were similar in their profiles, while D3 and D4 each differed. Species specific primers were used to identify these isolates (Table 2.2). Isolates D1, D2, D3, E1 and E2 were \textit{A. flavithermus} while D4, E7 and E11 were \textit{Geobacillus} spp. Isolate CGT-8 had been previously identified as \textit{Geobacillus} spp. (personal communication with S. H. Flint).
Chapter 2 – Spore Isolation, Production and Purification

Figure 2.4: Examples of (A) RAPD profiles and species specific primers for (B) *A. flavithermus* and (C) *Geobacillus* spp. for D series isolates. Lane 1, 1 kb ladder; lane 2, negative control; Lanes 3 to 6 are isolates D1, D2, D3 and D4 respectively. E-series isolates are not included in this Figure.

Table 2.2: Isolates designations, identification and location of isolation from the plant.

<table>
<thead>
<tr>
<th>Strain Number</th>
<th>Identification$^A$</th>
<th>Source$^B$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td><em>A. flavithermus</em></td>
<td>2</td>
</tr>
<tr>
<td>D2</td>
<td><em>A. flavithermus</em></td>
<td>2</td>
</tr>
<tr>
<td>D3</td>
<td><em>A. flavithermus</em></td>
<td>2</td>
</tr>
<tr>
<td>D4</td>
<td><em>Geobacillus</em> spp.</td>
<td>4</td>
</tr>
<tr>
<td>E1</td>
<td><em>A. flavithermus</em></td>
<td>2</td>
</tr>
<tr>
<td>E7</td>
<td><em>Geobacillus</em> spp.</td>
<td>4</td>
</tr>
<tr>
<td>E11</td>
<td><em>Geobacillus</em> spp.</td>
<td>3</td>
</tr>
<tr>
<td>CGT-8</td>
<td><em>Geobacillus</em> spp.</td>
<td>3*</td>
</tr>
</tbody>
</table>

$A$: Identified isolates from Figure 2.4

$B$: Location of isolation at different points in the production line as seen in Figure 2.1. The * indicates that the isolate was obtained from a milk powder production line for the Paihatua plant rather than the Edendale plant.
2.3.3 Preliminary sporulation of thermophilic isolates

The results from a preliminary investigation into sporulation of *A. flavithermus* and *Geobacillus* spp. isolates can be seen in Figure 2.5. Generally the H medium provided the highest number of vegetative cells and spores for all isolates, while TSB provided the least. The *A. flavithermus* isolate D3 displayed the lowest spore yields of all the isolates tested with only $1 \times 10^3$ and $2 \times 10^5$ CFU mL$^{-1}$ for KS and H media respectively. The number of viable D3 spores was below the detectable limit using TSB. Spores of *A. flavithermus* isolate E1 had the highest spore yield for each of the media tested with H media providing the overall highest at $1 \times 10^7$ CFU mL$^{-1}$. The percentage of cells, which had sporulated was higher in H media for E1 (50 %) but not for D3 (2 %).

The effect of media conditions on the sporulation of *Geobacillus* spp. isolates D4 and E11 was similar to that seen for the *A. flavithermus* isolates. Medium H provided the highest spore yield for both isolates at $7 \times 10^6$ CFU mL$^{-1}$ for isolate D4 and $6 \times 10^6$ CFU mL$^{-1}$ for isolate E11. Spores of isolates D4 and E11 in H medium consisted of 30% and 21% respectively. For isolate D4, the number of spores obtained was nearly two log$_{10}$ higher in H than in KS media. Interesting, more viable spores of isolate D4 were detected after heat treatment of TSB compared with the un-treated culture containing both vegetative cells and spores. This could be due to the effect of heat activation of spores resulting in higher germination rates. On the other hand, spore yields of isolate E11 were poor in TSB.

In general, spore yields amongst *Geobacillus* spp. isolates were consistent across multiple cultures. For example, a culture of D4 grown on three separate occasions in the same media under the same conditions, consistently produced $\sim 5 \times 10^6$ CFU mL$^{-1}$. This is in stark comparison to the *A. flavithermus* isolate E1 where spore yields were inconsistent between multiple sporulation cultures. The number of spores harvested ranged from 500 to $1 \times 10^7$ spores mL$^{-1}$. For this reason, *Geobacillus* spp. isolates were chosen for further research to optimise spore yields.
Figure 2.5: The number vegetative cells (■) and spores (□) of *A. flavithermus* isolates (A) D3 and (B) E1 and *Geobacillus* spp. isolates (C) D4 and (D) E11 in either KS, H or TSB media. Values represent the mean of five measurements while error bars represent the standard deviation of the mean. NB: Viable spore counts were not detectable for isolates D3 and E11 when grown in TSB medium.
2.3.4 Effect of nutrients and time on the sporulation of Geobacillus spp. isolates

The effect of nutrient source, salts and incubation time on spore yields of Geobacillus spp. isolates D4, CGT-8, E7 and E11 were determined (Figure 2.6). Isolates D4, E7 and E11 produced higher numbers of spores when compared with isolate CGT-8. The number of spores produced from D4 and E11 grown in tryptone for 18 hours was four orders of magnitude higher than when it was grown in TSB. Spores of isolate E7 increased 2 orders of magnitude when grown in tryptone. In contrast, CGT-8 produced 500 times more spores when grown in TSB compared with tryptone. The presence of manganese, calcium, iron and magnesium caused a significant \((p < 0.05)\) increase in the number of spores obtained from all isolates at their optimal sporulation times. These differences were small for isolates D4 and CGT-8 compared with the larger differences for isolates E7 and E11. The optimal sporulation time for D4, CGT-8 and E11 was 12 h while for E7 it was 20 h. Interestingly the number of spores from D4 and E11 in TSB decreased dramatically after 20 h, presumably due to germination and subsequent cell lysis. However, when grown in tryptone, spores of these isolates including E7, were maintained in the culture even after 72 h. This affect was also seen for spores of CGT-8, however the number of spores obtained by this strain when tryptone was the base medium was much lower than the number obtained from TSB.
Figure 2.6: The number of spores recovered from either *Geobacillus* spp isolates (A) CGT-8, (B) D4, (C) E7 or (D) E11 over 72 h in Tryptone with added salt (■) or without (□) added salt, or TSB with added salt (●) or without (○) added salt. Values represent the mean of three replicates and standard deviations of the means. Spore concentration was not determined at the time 0 point.
Figure 2.6: The number of spores recovered from either *Geobacillus* spp isolates (A) CGT-8, (B) D4, (C) E7 or (D) E11 over 72 h in Tryptone with added salt (■) or without (□) added salt, or TSB with added salt (●) or without (○) added salt. Values represent the mean of three replicates and standard deviations of the means. Spore concentration was not determined at the time 0 point.
2.3.5 Comparison of two different purification methods.

Two different purification techniques were tested for their ability to separate the vegetative cells and debris present in the crude spore cultures of isolate D4 from the spores (Figure 2.7). Using a NaBr density gradient, spores were typically found at a band in the 1.3 g L\(^{-1}\) density layer, while vegetative cells were at 1.1 g L\(^{-1}\). Using the PEG two-phase system, spores were typically found in a thick layer above the PEG rich phase while debris migrated through the PEG rich phase and collected at the interface of the phosphate rich phase (Figure 2.7B).

Relatively low numbers of vegetative cells were found in the crude spore suspensions of the four isolates D4, E7, E11 and CGT-8 after the sporulation medium was determined (Section 2.3.4). These media produced fewer vegetative cells as shown by DIC microscopy (Figure 2.8A). However large amounts of debris, presumably from lysed vegetative cells, were found in these crude suspensions. While the NaBr density gradient was able to remove the remaining vegetative cells from the crude suspension, it failed to remove the debris, which remained with the spores, causing them to clump together (Figure 2.8A). In contrast, the PEG two-phase system separated large amounts of debris from the spores (Figure 2.8C). Since the PEG two-phase system provided sufficient removal of debris, it was chosen as the purification method for the four spore isolates.
Figure 2.7: Location of spore bands after purification involving density gradient centrifugation using NaBr (A) and a two-phase PEG system (B).
Figure 2.8: Sample DIC micrographs of the original crude spore suspension (A), after purification with a NaBr density gradient (B) or PEG two-phase system (C). Spores are the circular black objects while debris and any dead cells are any red materials.
Figure 2.8: Sample DIC micrographs of the original crude spore suspension (A), after purification with a NaBr density gradient (B) or PEG two-phase system (C). Spores are the circular black objects while debris and any dead cells are any red materials.
Chapter 2 – Spore Isolation, Production and Purification

2.4 Discussion

The goal of the research in this section was to produce spores from dairy isolates of thermophilic bacilli in sufficient quantity and purity to enable a subsequent study on the factors affecting the attachment of these spores to stainless steel surfaces. Over thirty different isolates of thermophilic spore forming bacteria were obtained from a DMP milk powder production line in New Zealand. From this initial group of isolates, eight were subsequently selected for further study based on their growth on TSA media. These isolates were identified using species-specific primers and RAPD analysis. Four of these isolates were identified as *A. flavithermus* and four as *Geobacillus* spp. These two organisms are frequently isolated from milk powder manufacturing plants in New Zealand and overseas (70, 194, 202). In this study, *Geobacillus* isolates were isolated after the second pass of the evaporator, while *A. flavithermus* isolates were obtained after the pre-heater but prior to the evaporator. While this study did not aim to analyse the different species with respect to their location in the manufacturing plant, the source of the isolates in this study does agree with previous findings (211).

In order to enhance spore production, several defined media were investigated. The medium produced by Houston (103) resulted in the highest spore yields amongst all of the isolates tested in this study. This medium was originally designed for the sporulation of the mesophilic sporeformer *Bacillus globigii*. In the present study, when isolates were grown in H medium at a higher temperature it resulted in an increase in spore yield compared with KS medium, which was developed for the sporulation of *G. stearothermophilus* (119). Cultures grown in TSB produced the lowest number of spores for the isolates tested. While the spore yield was consistent across multiple cultures of the two *Geobacillus* spp. isolates, this was not the case for *A. flavithermus* isolates. Previous studies have produced spores from *A. flavithermus* isolates (205, 211), however this result was not achievable in the current study. Therefore *Geobacillus* spp. isolates were chosen to further optimise the sporulation media.

Base media, culturing time and the presence of certain ions have previously been shown to effect spore yields in *G. stearothermophilus* (272). These factors were carefully examined to investigate increasing the spore yield from four different *Geobacillus* spp in liquid culture. In the current study, there was some variation in
spore yields from the different base media tested. For example, the spore concentration of D4 was higher in tryptone as the base medium than in TSB. However the opposite was seen for the sporulation of CGT-8 where TSB was optimal for spore production. Previous studies have shown that the presence of particular ions and incubation time had the most prominent effect on spore production by *Geobacillus* spp. isolates (238). It has been reported that the presence of calcium, manganese, and iron is crucial for sporulation and can promote higher yields (221). Manganese is required by the enzymes involved in sporulation (165), while calcium is chelated by dipicolinic acid and is important for heat resistance (18). Interestingly, in the present trial, media supplemented with these ions caused a subtle yet significant increase in spore yields of isolates CGT-8 and D4. This could be due to the fact these ions are already present in sufficient concentrations in the un-supplemented media. For example tryptone contains (per litre) 0.13 g Ca, 0.17 g Mg, 0.01 g Mn and 0.01 g Fe.

Spore numbers were also influenced by the incubation time of the sporulating culture. In this study, spore numbers often decreased at incubation times longer than 24 h as presumably spores germinated. However this finding was dependent on the strain and the medium. González-Pastor et al found that *B. subtilis* cells can commit to ‘cannibalism’ prior to sporulation to ensure survival (86). Cells that have entered the sporulation pathway produce a signal which causes sister cells to lyse. These lysed cells act as nutrients for other cells enabling them to grow instead of completing sporulation. Previous studies have used prolonged incubation times (4-5 days) to enhance sporulation in either a liquid culture (119) or on agar plates (121). The rapid production of spores in these experiments was similar to that observed during an 18 hour milk powder production run where spores were detected after only 9 h (211). Previous studies that have produced spores on agar plates can be laborious and costly due to amount of materials required (211). Producing spores in liquid batch sporulation media overnight was found to be a superior method due to its short incubation time, ease of use and ability to produce large numbers of free endospores from *Geobacillus* spp. isolated from dairy plants.

Obtaining spores free from any cells or debris was crucial for the accurate determination of spore surface characteristics. While previous studies had used heat or enzymes to remove vegetative cells, it was desirable to avoid these approaches as such harsh treatments have been reported to alter the hydrophobicity of spores from bacilli (56, 264). In this project, spore purification was initially attempted using a
simple density gradient made of NaBr (131, 161). While this gradient system removed vegetative cells it failed to separate cellular debris from lysed cells, and to differentiate spores from partially sporulated cells. In the current study, the PEG two-phase system reproducibly separated spores from other debris present in crude spore suspensions. Macromolecular separation using a aqueous polymer two phase system is influenced by the thermodynamic properties such as charge and hydrophobicity of polymers suspended in a saline solution (52). In this study, spores of Geobacillus spp. accumulated at the interface above the PEG rich region, while the more hydrophobic debris accumulated at the interface between the PEG and phosphate-rich phases. This is in strict contrast to a previous study which found that spores of B. cereus accumulated in the PEG rich phase while vegetative cells accumulated in the lower phosphate rich phase (208). The difference in affinities to the phases of the PEG system could be due to inherently different surface characteristics between the surface of the Geobacillus spp. and B. cereus spores and debris.

In summary, thirty-one isolates were obtained from two DMPs in New Zealand. Of these, eight were chosen for further study and identified as either A. flavithermus or Geobacillus spp. Preliminary results found that sporulation of the thermophilic isolates using previously defined media could produce large numbers of spores. However spore yields of Anoxybacillus flavithermus isolates were inconsistent between cultures. Therefore four Geobacillus spp. isolates were chosen to further optimise the sporulation media to increase spore yields. The composition of the base media, presence of salts and the length of the culture period were found to be important in maximising the spore yield. Purification of spore isolates using a PEG two-phase system removed much of the debris present in the crude spore suspensions. From these results, Geobacillus spp. isolates D4, E7, E11 and CGT-8 were chosen for characterisation and attachment assays in further studies.
Chapter 3

Spore Surface Characterisation
3.1 Introduction

The attachment of spores to stainless steel surfaces is a common problem during the production of milk powder. The aim of this chapter was to gain a better understanding of the factors contributing to the attachment of spores to surfaces by examining the structural and physico-chemical attributes of the spore surface using a variety of techniques.

The physical structure of a spore and the genetics regulating its development have been the focus of many studies and have received extensive review (57). Techniques such as electron microscopy and atomic force microscopy and more recently molecular biology techniques (9, 37, 57, 93) have revealed that spores of different species have differing structural characteristics. For example, the outer layer of spores from *Bacillus anthracis* and *Bacillus cereus* consists of pili and an exosporium made of lipid and glycoproteins (81, 149, 188). While the outer surface of spores of mesophilic *Bacillus subtilis* is a dense protein coat, which may (223, 224) or may not (58) have a tightly-bound exosporium.

The use of transmission electron microscopy (TEM) to view cross sections of spores can reveal much about their structure (261). The preparation of biological material for this technique requires fixation of the spore structure and the deposition of an electron-dense stain to impart contrast on the material (185). Ruthenium red [Ru₂O₂(NH₃)₁₄]⁶⁺ in combination with osmium tetroxide is used for the staining of polyanionic substances with high molecular weight. This technique has been used for the visualisation of acid polysaccharides and capsules of vegetative bacteria (88), and recently for examining the outer layer of *B. anthracis* spores (261). While a previous study has examined the structural characteristics of spores from the thermophile *G. stearothermophilus* (258), there is no published information on the structure of spores isolated from thermophilic dairy bacilli.

The physico-chemical characterisation of micro-organisms in general has received extensive interest (22, 248) with surface characterisation techniques relying on the measurement of hydrophobicity and surface charge. In contrast to the extensive research that has been carried out on vegetative cell surfaces there have been only a few studies examining the surface characteristics of spores, and these have been for mesophilic species such as *B. subtilis*, and *B. cereus* (62, 108, 109).
Hydrophobic interactions have been reported to be of great importance in the initial adhesion of micro-organisms to surfaces (247). In general spores of most bacilli have been found to be more hydrophobic than their vegetative cells (56, 264) with the degree of hydrophobicity varying amongst the different species of bacilli such as *B. cereus*, and *B. subtilis* (109, 196). There are several techniques commonly used for the measurement of cell surface hydrophobicity. Two of the most commonly used techniques are the microbial adhesion to hexadecane (MATH) assay (199) and determination of the surface Gibbs energies through contact angle measurements (CAM) (252). The detailed calculations used are discussed later in the materials and methods section of this chapter.

The calculation of zeta potentials is commonly used for the determination of the relative surface charge of a colloid particle in a solution. The zeta potential is the potential across a slip plane, which is the proposed interface between the hydrodynamically stagnant layer surrounding a charged particle in solution and the bulk solution. This potential cannot be measured directly since particles in a solution are not connected to an external circuit (50). Electrophoretic light scattering is the most commonly used technique for determining electrophoretic mobility with the movement of a suspended particle in the electric field being measured by analysis of the Doppler shift of scattered light (239).

The shear plane, and therefore zeta potential, can be affected by the composition of the microbial cell surface (ie. polymers) and by the physical properties of the surrounding medium (85, 144). There are several models employed to calculate the zeta potential from the electrophoretic mobility measurements and there is no general consensus amongst the researchers as to which theory is applicable for measurements of the zeta potential of bacterial surfaces (168). Nevertheless the zeta potential and its pH dependence provides evidence of surface charge variations with pH and can be used to infer the presence of ionisable functional groups at the surface of bacterial spores, based on assumed pKₘ values of known functional groups (112). However, the presence of specific ionisable groups can only be confirmed if zeta potential measurements are combined with other techniques.

Infrared (IR) spectroscopy can be used to identify functional groups found in bacteria which have been assigned to particular functional groups such as amide, carboxylic acid or phosphates (167). This approach relies on the detection of characteristic absorption bands in defined regions of an infrared spectrum.
convenient approach to obtain IR spectra of bacteria is the use of attenuated total reflection infrared (ATR-IR) spectroscopy, which enables samples to be examined directly in the liquid or solid state without further preparation (118). This method, involves an internal reflection element (IRE) coupled with a flow cell, this enables the in situ spectral monitoring of IR active functional groups in close proximity (< 1 µm) to the IRE surface. The sampling distance is comparable to the size of microorganisms, including spores. To identify the functional groups present, the physico-chemical conditions can be altered through changes in composition of the flowing solution. This technique has been used to determine cellular components of bacteria (92) as well as the functional groups present on their surface through pH-change difference spectroscopy (90, 116). Recent studies have used IR spectroscopy to examine the chemical changes in spores of bacilli as they germinate (41), the inactivation of spores by autoclaving (230) and the difference in IR spectra between spores that have dipicolinic acid (DPA) and those which lack it (179). Dipicolinic acid is present as the calcium salt (CaDPA) and is particularly abundant within the spore’s core making up 15% of the spore’s total dry weight (186) and is believed to be important in the spore’s resistance to adverse conditions (212). Calcium dipicolinate is released from the core during spore germination or if the spore is injured. While ATR-IR spectroscopy has been used to differentiate between spores of different mesophilic species (19) and determine the surface characteristics of thermophilic vegetative bacilli (90), to the author’s knowledge there has been no study applying this technique to identify the surface functional groups of spores produced from thermophilic bacilli.
Objectives:

The present study aimed to characterise the surfaces of spores of several thermophilic bacilli isolated from a milk powder manufacturing plant through:

- Structure
  - TEM
- Hydrophobicity
  - MATH
  - Contact angles
- Surface charge
  - Zeta potential
- Functional groups
  - Absolute IR spectra
  - pH dependence IR spectra

Information gathered from these surface characterisation techniques can suggest how to modify a surface to reduce the number of spores attaching to that surface.
Chapter 3 – Spore Surface Characterisation

3.2 Materials and Methods

3.2.1 Spore Preparation.

Spores of *Geobacillus* spp. were prepared as described in the previous chapter (Section 2.2.9). Spores of isolates D4, E7 and E11 were grown in medium containing 30 g Tryptone (BD), 0.125 g of CaCl₂, 0.15 g of MnSO₄, 0.155 g of FeSO₄, and 0.55 g of MgCl₂ (BDH Analar) per litre, while spores of isolate CGT-8 were grown in medium containing 30 g Tryptic Soy Broth (BD) per litre. All cultures were grown at 55 °C for 12 h and were aerated by a magnetic stir bar. The spores produced were collected by centrifugation 10,000 × g for 8 min at 4 °C and washed three times in water. Spores were purified using a two-phase polyethylene glycol system outlined in the previously (Section 2.2.10).

3.2.2 Transmission Electron Microscopy (TEM)

To obtain TEM images, spores of the different isolates were produced and washed in water three times as described above (Section 3.2.1) with the exception that they were not purified using the PEG two-phase system in order to determine the nature of the debris present in crude spore suspensions. Spores of all isolates were viewed two days after harvesting. To examine if any structural changes of spores occurred during long term storage after harvesting, a suspension of D4 spores was also examined after being stored for two weeks at 4 °C. The spore pellets in Eppendorf tubes were suspended in a primary fixative comprised of 1 mg mL⁻¹ ruthenium red (ProSciTech, Australia) and 10 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 hours as room temperature and a further 60 minutes at 37 °C. The spores were washed three times in 0.1 M sodium cacodylate buffer and added to a secondary fixative comprised of 1 mg mL⁻¹ ruthenium red and 2 % osmium tetroxide in 0.1 M sodium cacodylate buffer. The spore suspension was left on an inverting rotor for 2 hours at room temperature. The spores were centrifuged and the pellet resuspended in 3% w/v agarose (Sigma-Aldrich, USA) in 0.1 M sodium cacodylate buffer. When solid, the agarose was removed from the Eppendorf tube and cut into 1 mm³ pieces. Stepwise dehydration of the spores embedded in the agarose cubes with ethanol and infiltration with Quetol 651 epoxy resin (ProSciTech,
Chapter 3 – Spore Surface Characterisation

Australia) was carried out using a Lynx el tissue processor (Australian Biomedical Corporation, Ltd., Australia). Once cured the blocks were cut on a Reichert-Jung Ultracut E ultramicrotome (C. Reichert AG, Austria) to obtain 80-90 nm thick sections, which were placed onto Formvar-coated slot grids. The sections were stained with uranyl acetate for 25 min at 25 °C and lead citrate for 5 min at 25 °C using an LKB Ultrostainer (LKB-Produkter AB, Sweden) and viewed with a Philips CM100 transmission electron microscope (Philips/FEI Corporation, Holland).

3.2.3 Determination of spore surface hydrophobicity

3.2.3.1 MATH assay

The surface hydrophobicity of the four spore isolates was determined using the microbial adhesion to hexadecane (MATH) assay (199). Purified spores were suspended in 0.1 M KCl at either pH 3 or 6.8 to an O.D. at 600 nm of 0.6 to 1.0 and 2 mL volumes of the resulting suspensions were added to 1 mL of hexadecane in glass tubes and mixed on a vortex mixer for 1 min. The samples were then incubated at 37 °C for 10 min, vortexed for 2 min and incubated at 20 °C for a further 30 min to allow complete separation of the two phases. The absorbance of the aqueous phase at 600 nm (five replicate measurements) was taken using a quartz cuvette (1 cm path length). A 0.1 M KCl solution was used as a reference. The percentage hydrophobicity (%h) was determined from the absorbance of the original bacterial suspension ($A_i$) and absorbance of aqueous phase after mixing with hexadecane ($A_f$) as described in Eq. 3.1.

\[
%h = \frac{A_i - A_f}{A_i} \times 100 \quad \text{(Eq 3.1)}
\]

3.2.3.2 Spore lawn preparation and contact angle measurements of surfaces

To determine the contact angle of the spores, spore lawns were prepared as described by Busscher et al (33). Suspensions of spores were filtered onto a cellulose triacetate filter (0.45 μm; Pall) to a density of $10^8$ spores mm$^{-2}$. The filters containing the spore lawns were placed onto a gel surface containing 2% agar and 10% glycerol (w/v) in water for 10 min. The filter containing the spore lawn was then cut into strips
(1 cm width) using a sterile scalpel blade and the strips were allowed to dry for 45 min at room temperature. Contact angles of two polar (water and formamide) and one non-polar (α-bromonaphthalene) liquids were measured, within a further 30 minutes, by the sessile drop method using a FTA200 goniometer (First Ten Angstroms, Portsmouth, VA). The 45 min drying period was determined by measuring the stability of the solvent droplets on the surface of spore lawns over several different drying times to establish a plateau contact angle (data not shown). The solvent droplets were stable on these dried films for 10 seconds.

The sessile drop method involves adding a drop of each test liquid onto the surface of interest. In this study contact angles were determined for glass, modified glass, Thermanox®, stainless steel (316 grade 2B polish) surfaces and the filter strips containing the spore lawns. A series of 10 contact angle measurements were recorded for each surface, each within 2 seconds of the drop contacting the surface. Surface Gibbs energies were determined using the FTA32 v2.0 software (First Ten Angstroms). Surface energies of the materials were obtained by the Young-van Oss equation (Eq. 3.2) (252):

\[
(1 + \cos \theta) \gamma^{\text{TOT}} = 2 \left[ \sqrt{\gamma_{sv}^{\text{LW}} \gamma_{lv}^{\text{LW}}} + \sqrt{\gamma_{sv}^{+} \gamma_{lv}^{+}} + \sqrt{\gamma_{sv}^{-} \gamma_{lv}^{-}} \right] \quad \text{(Eq 3.2)}
\]

where \( \gamma^{\text{TOT}} \) is the total surface energy, \( \gamma^{\text{LW}}, \gamma^{+} \) and \( \gamma^{-} \) are the van der Waals, electron acceptor and electron donor components of the Gibbs surface energy, respectively, and \( \theta \) is the contact angle. A free energy balance exists between the solid, liquid and vapor phases and is noted by the subscripts sv (solid-vapor) and lv (liquid-vapor). Contact angle values of the three different solvents were put into the FTA32 software to obtained the different Gibbs free energy parameters: \( \gamma^{\text{TOT}}, \gamma^{\text{LW}}, \gamma^{AB}, \gamma^{+} \) and \( \gamma^{-} \)-parameters. This information was then used in the following formula to measure hydrophobicity. The hydrophobicity of microbial cell surfaces can be thermodynamically expressed as a Gibbs energy of aggregation (\( \Delta G_{\text{sls}} \)) (Eq. 3.3) (249):

\[
\Delta G_{\text{sls}} = -2 \left( \sqrt{\gamma_{sv}^{\text{LW}}} - \sqrt{\gamma_{lv}^{\text{LW}}} \right)^2 - 4 \left( \sqrt{\gamma_{sv}^{+} \gamma_{lv}^{+}} + \sqrt{\gamma_{sv}^{-} \gamma_{lv}^{-}} - \sqrt{\gamma_{sv}^{+} \gamma_{lv}^{-}} - \sqrt{\gamma_{sv}^{-} \gamma_{lv}^{+}} \right) \quad \text{(Eq 3.3)}
\]

Organisms with hydrophilic surfaces prefer the aqueous phase and display a \( \Delta G_{\text{sls}} > 0 \) while hydrophobic organisms tend to aggregate and therefore have a \( \Delta G_{\text{sls}} < 0 \).
3.2.4 Zeta Potentials

To determine the approximate surface charge of spores, purified spores were suspended in either 0.01 or 0.1 M KCl to an O.D. of 0.01 at 600 nm and the pH was adjusted using either concentrated HCl or NaOH in order to obtain a pH range of 3 to 11 while maintaining a 0.01 or 0.1 M ionic strength. The zeta potential was also calculated over an ionic strength range of 0.001 to 0.1 M using KCl at pH 6.8. The zeta potential of spores from the four isolates was determined using a Malvern Zetasizer Nano ZS90 (Malvern Instruments Ltd. UK). Three replicate measurements were obtained from each sample. The zeta potential was calculated from the electrophoretic mobility using the Smoluchowski equation (106).

3.2.5 Infrared Spectroscopy

ATR-IR analysis of the spores was conducted using a DigiLab FTS 4000 spectrophotometer (DigiLab, Randolph, MA) at 20 °C. The optical path was purged with dry air for 2 h prior to the experiments to reduce interference from water vapour. Samples were measured on a single 45° reflection accessory ZnSe prism (Harrick, FastIR). The prism surface was polished prior to each experiment using 0.015 μm Al₂O₃ powder supported on a polishing microcloth (Buehler) and then rinsed with water. A teflon flow cell coupled to the ZnSe prism surface with a nitrile rubber O-ring was used for flow experiments, creating a flow cell volume of 1 mL. All spectra were constructed from 64 scans at 4 cm⁻¹ resolution using DigiLab Win-IR Pro software. In this section, the spore absolute spectra are relative to a wet prism background and contain all absorptions for the functional groups in the spore. In addition pH difference spectra were obtained by subtracting the absolute spectra from lower pH conditions from the absolute spectra taken at pH 6.8 to examine changes in absorbance of peaks associated with different functional groups.

For pH titration experiments, 1 mL of spores (~1 x 10⁸ spores) was pumped into the flow cell and allowed to settle without flow for 90 min creating a ‘lawn’ of spores on the prism. The spore lawn was then rinsed with 0.1 M KCl (pH 6.8) at a flow rate of 1 mL min⁻¹ for 30 min to remove loosely adhering spores. A ‘titration’ was then carried out at the same flow rate with 0.1 M KCl solutions ranging in pH
from 6.8 to 2.5. Each solution was flowed over the lawn for 30 min while spectra were recorded every 5 min.

3.2.6 DPA Determination

The amount of DPA released from spores under acidic conditions was determined using a colorimetric assay (114). Spores suspended in 0.1 M KCl were acidified using HCl to pH 2.5 and incubated at room temperature for 30 min. To determine the total amount of CaDPA in the spores, a 10 mL suspension of spores (1 x 10^8 spores mL^-1) was autoclaved for 15 min, cooled, then treated with 0.1 mL of 1 N acetic acid for 30 min to release all of the CaDPA. Spore samples were then centrifuged at 8,000 x g for 10 min and the supernatant was carefully removed. A 800 μL aliquot of the supernatant was added to 200 μL a solution containing 1 % Fe(NH₄)₂(SO₄)₂.6H₂O and 1 % ascorbic acid in 0.5 M acetate buffer at pH 5.5. The absorbance of the resulting solution (of three replicates) was measured at 440 nm using a quartz cuvette (1 cm path length) in an Ultrospec 3300 pro spectrophotometer (Biochom, Cambridge, England). A 0.1 M KCl solution was used as a reference.
3.3 Results

3.3.1 Transmission Electron Microscopy

Cross-section TEM images of the spores from the *Geobacillus* spp. isolates CGT-8, D4, E7 and E11 stained with ruthenium red can be seen in Figures 3.1 to 3.4. By comparing these images to those previously reported, structures associated with the spores were identified. Each spore consists of a core surrounded by a darkly stained membrane and a lightly stained cortex. The core is surrounded by a spore coat, which is comprised of the stratified inner and an electron dense outer layer (57).

An exosporium was present on spores from isolates CGT-8, D4 and E7 two days after harvesting. The spores of isolate E11 did not appear to possess an exosporium at the same period of time. The exosporium surrounding spores of isolate D4 (Figure 3.2) contained two layers. The outermost layer of the exosporium was darkly stained and amorphous in appearance. However the exosporium was not clearly visible around D4 spores two weeks after harvesting. At which stage, an additional thin and darkly-stained layer is visible surrounding the outer layer of the spore coat. However it is not clear if this additional layer is the exosporium or the outer layer of the spore coat ‘peeling’ off. Spore coat debris were also visible in the background surrounding the spores.

Granular objects were visible in the space between the outer spore coat and the exosporium in spores of isolate E7 (Figure 3.3). Spores of isolates CGT-8, D4 and E7 contained ‘string-like’ structures attached to the surface of the spores while detached ones were also visible in the background. These structures ranged in length from 100 to 500 nm. Spores of isolate E11 (Figure 3.4) also contained string-like structures but it was more difficult to discern these from other debris associated with the un-purified spore samples.
Figure 3.1: TEM images of ruthenium red stained thin sections of CGT-8 spores (A and B). A darkly stained exosporium (e), spore coat (sc), core (c) and a lightly stained cortex (cx) are visible. A section of the spore coat appears to be peeling off in (B).
Figure 3.2: Figure caption on following page.
Figure 3.2: TEM images of ruthenium red stained thin sections of D4 spores two days (A and B) and two weeks (C) after harvesting. The lamellar structure of the inner spore coat (sc) is clearly seen in (B). The exopsorium (e) appears to have two layers. Structures, which could potentially be pili, are visibly attached and detached in the background. The exosporium is difficult to distinguish from the outer spore coat in the sample taken from a suspension two weeks after harvesting.
Figure 3.3: TEM images of ruthenium red stained thin sections of E7 spores (A and B).
Figure 3.4: TEM images of ruthenium red stained thin sections of E11 spores. These spores lacked an exopsorium but did contain a very thin darkly stained ring around the spore coat (sc). Notice the peeling of the spore coat layers.
3.3.2 Hydrophobicity

Purified spores of the different *Geobacillus* spp. isolates displayed a range of hydrophobicities when partitioned with hexadecane at pH 6.8 and 3 as shown in Figure 3.5. Spores of isolate D4 were the most hydrophilic (18% relative hydrophobicity) and CGT-8 the most hydrophobic (48% relative hydrophobicity) at pH 6.8. Spores of isolates D4, E7, and CGT-8 were more hydrophobic when suspended at pH 3 than when suspended at pH 7, while E11 was more hydrophilic at the lower pH.

Contact angles (in degrees) and the calculated surface tension components (mJ m$^{-2}$) for purified spores and the substratum surfaces are given in Table 3.1. Spores from isolate D4 had the lowest water contact angle (10°) and spores from isolate E11 had the highest (25°). Spores from the different isolates had similar $\gamma_{\text{TOT}}$ values, ranging from 49.4 to 58.3 mJ m$^{-2}$. Purification of the spores using the two-phase system reduced the apolar ($\gamma_{\text{LW}}$) component while increasing the total polar ($\gamma_{\text{AB}}$) component. The most prominent feature for all of the spore isolates was the electron-donating ($\gamma_{\text{-}}$) component. All spore isolates had similar $G_{\text{sbs}}$ values, and all were $> 0$, indicating that they were all hydrophilic. In contrast to the results shown for the MATH assay, CGT-8 spores appeared to be the most hydrophilic, with a $G_{\text{sbs}}$ of 31.4 mJ m$^{-2}$ and E7 spores the most hydrophobic at 20.1 mJ m$^{-2}$.
Figure 3.5: Relative hydrophobicities of spores from *Geobacillus* sp. Isolates at pH 3 (■) and 6.8 (■) in 0.1 M KCl, as determined by the MATH assay. Tabulated hydrophobicities represent the means of five replicates and the error bars represent the standard deviation of the means.
# Chapter 3 – Spore Surface Characterisation

<table>
<thead>
<tr>
<th>Surfaces</th>
<th>Contact angle (°) ± S.D.</th>
<th>Surface tension components (mJ m⁻²)</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>$\theta_W$</td>
<td>$\theta_F$</td>
</tr>
<tr>
<td>Crude D4</td>
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<td>14 ± 2</td>
</tr>
<tr>
<td>Pure D4</td>
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<td>11 ± 2</td>
</tr>
<tr>
<td>Crude E7</td>
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<td>19 ± 1</td>
</tr>
<tr>
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</tr>
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<td>Crude E11</td>
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<td>19 ± 1</td>
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<td>25 ± 2</td>
</tr>
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<td>23 ± 3</td>
</tr>
<tr>
<td>Pure CGT-8</td>
<td>20 ± 3</td>
<td>24 ± 4</td>
</tr>
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</table>

Table 3.1: Mean contact angle measurements, surface Gibbs energies, and energy of interaction ($\Delta G_{\text{sis}}$) for crude and purified suspensions of spores from *Geobacillus* sp. isolates. Contact angle of water ($\theta_W$), formamide ($\theta_F$) and $\alpha$-bromonaphthalene ($\theta_{\alpha-B}$).
3.3.3 Zeta Potential

The zeta potential measurements of spores CGT-8, D4, E7 and E11 over a pH range of pH 3 to 12 in either 0.01 M or 0.1 M KCl are shown in Figure 3.6. In general, the spores of the four different isolates have qualitatively similar pH dependence. Spores of all isolates possessed a positive zeta potential below pH 3, with the net zeta potential becoming increasingly negative as the pH increased from 3 to 6. The zeta potential then remained similar between pH 6 to 9 with the exception of spores of isolate E7, which continued to become more negatively charged. The zeta potential of spores from isolates, CGT-8, D4 and E11 increased below pH 10. Comparing the zeta potential data of the different isolates suspended in 0.1 M KCl under neutral conditions, spores of E7 and CGT-8 had greater negative zeta potential at -20 mV, while D4 and E11 had a smaller negative zeta potential at -10 and -12 mV respectively. The isoelectric point (IEP) of each of the spores occurred between pH 3 and 4. There was a sizeable net positive charge at a pH below the IEP for spores of the isolates D4, E7 and E11. Spores of all isolates suspended in the lower ionic strength medium were found to have a greater negative zeta potential at neutral pH than spores suspended at a higher ionic strength medium. This difference in the zeta potential between these different ionic strengths ranged from 3 mV for isolate D4 to 15 mV for isolate E7. The effect of ionic strength at pH 6.8 on spore zeta potential can also be seen in Figure 3.7. As the ionic strength of the medium increased from 0.001 M KCL to 0.1 M KCl the zeta potential of the spores became more positive. The largest difference in zeta potential (20 mV) with respect to change in ionic strength occurred for spores of isolate CGT-8. Spores of isolate D4 displayed the smallest difference (5 mV).
Figure 3.6: Zeta potential measurements over a pH range for four different Geobacillus spp. isolates CGT-8 (×), D4 (▲), E7 (●) and E11 (■) suspended in either 0.01 M KCl (A) or 0.1 M KCl (B). Values represent the means of three replicates and errors bars represent the standard deviations of the means.
Figure 3.7: Zeta potential measurements of four different *Geobacillus* spp. isolates CGT-8(×), D4 (▲), E7 (○) and E11 (■) suspended in a range of ionic strength solutions at pH 6.8. Values represent the means of three replicates and errors bars represent the standard deviations of the means.
3.3.4 IR Spectra

3.3.4.1 Absolute Spectra

The IR spectra of the four different spore isolates D4, E7, E11 and CGT-8 are shown in Figure 3.8. These spectra are composed of the composite bands created by the various IR absorbing groups. The most prominent of these are the amide I at 1640 cm\(^{-1}\) (mainly C=O stretching), the amide II at 1540 cm\(^{-1}\) (C-N stretching and N-H bending vibrations), and the associated amide A (N-H stretching) at 3300 cm\(^{-1}\). These peaks are generally representative of proteins and are found in the spectra obtained from all micro-organisms. Bands at 1620, 1570, 1442 and 1372 cm\(^{-1}\) are all characteristic of calcium dipicolinate (CaDPA). Different forms of phosphates (ie. phosphomonoesters and phosphodiesters) and polysaccharides are represented by a series of bands between 1150 and 950 cm\(^{-1}\). Spores of isolate E7 displayed a broad composite of overlapping peaks between 1250 and 950 cm\(^{-1}\), while other isolates had specific peaks with the most prominent at 1240, 1130, and 1080 cm\(^{-1}\). The peak at 1240 cm\(^{-1}\) has been assigned to phosphodiester groups, carboxylic acid/ester moieties, along with an amide III absorption (1280 cm\(^{-1}\)). Bands observed between 2800 to 3000 cm\(^{-1}\) and at 1450 cm\(^{-1}\) have been assigned to the methyl and methylene C-H stretch and deformation modes respectively.
Figure 3.8: Absolute spectra of the four *Geobacillus* sp. isolates in the spectral region of 3700 to 900 cm\(^{-1}\) (A) and a close-up of the region 1800 to 900 cm\(^{-1}\) (B).
3.3.4.2 IR spectra of PEG purified spores

To ensure that residual PEG was not adhering to the surface of the spores after the purification process and influencing the spore surface characterisation results, the IR spectra of a 0.1 M PEG 4000 solution was related to un-purified and purified spores of isolate D4 (Figure 3.9). Spores of isolate D4 were chosen since spore yields from isolate D4 produced the least debris during sporulation compared to other isolates. The spectrum of PEG revealed a broad peak comprised of composite peaks at 1130 and 1080 cm\(^{-1}\) due to C-O stretching. The negative peak seen at 1620 cm\(^{-1}\) is the result of the PEG solution having less water than the pure water background. Smaller peaks are also present at 1340, 1290, 1250 and 940 cm\(^{-1}\). There was very little difference in the spectra obtained from the spores, before and after purification. The broad band at 1080 cm\(^{-1}\) does overlap with the corresponding band in the spectra of the purified spores, however minor bands such as 1340 and 940 cm\(^{-1}\) do not. Therefore it appears that no residual PEG was adhering to the surface of the spores after purification.

Figure 3.9: Infrared spectra in the region of 1800 to 800 cm\(^{-1}\) of *Geobacillus* spp. isolate D4 before and after purification with PEG 4000 and of a spectrum of PEG 4000. Background spectra were taken using water.
3.3.4.3 pH dependance and pH difference spectra

The absolute spectra of spore ‘lawns’ of the four *Geobacillus* spp. spore isolates under differing conditions of pH are shown in Figures 3.10 to 3.13 spectra A. The absolute spectra of spores from isolate CGT-8 at pH values 6.8 to 2.5 are shown in Figure 3.10 A. The variation of bands associated with protein (amide I and II) along with the carboxylate (1400 cm\(^{-1}\)) and carboxylic acid (1720 cm\(^{-1}\)) were monitored over time during initial settlement and subsequent solution changes, and are plotted in Figure 3.10 B. This figure shows the increase in bacterial absorptions during the settlement of spores suspended under static conditions onto the ZeSe surface over a 60 min period. With the commencement of flow of 0.1 M KCl at pH 6.8, there was a slight reduction of the amide I band, corresponding to some loss of spores, followed by a slight increase, due to further settlement of the spores on the surface. A decrease from pH 5 to 4, caused no noticeable trends in peak absorbances except for a slight increase in the amide I peak. However when the electrolyte solution was changed to pH 3, a sharp increase in amide I, II and carboxylic acid bands was observed. When the pH of the surrounding medium was reduced to 2.5, a sharp decrease in the 1400 cm\(^{-1}\) band was seen, indicating protonation of carboxylates to carboxylic acid along with a sharp decrease of the amide I and II bands. The return to pH 6.8 at the end of the experiment, caused a reversion of the carboxylate bands back to their unprotonated state, and amide I and II peaks to reach similar absorbances to that at the beginning of the experiment.

Figure 3.11 displays the spectral trends in response to pH of spores from isolate D4. The amide I and II peaks display similar characteristics to the CGT-8 data during settlement and commencement of flow at pH 6.8. There was a small increase in all absorbing bands when shifting to pH 5, with a more remarkable increase at pH 4 for the amide I and II peaks. As the pH continued to decrease to 3 and then 2.5, the amide I and II peaks began to decrease. This was followed by an increase and decrease of the 1720 and 1400 cm\(^{-1}\) bands respectively. Reversal of the pH back to 6.8 caused these carboxylate bands to quickly revert back to initial values seen at the beginning of the experiment. The amide I and II bands did increase when the pH was increased from 3 to 6.8, but did not fully revert back to the same absorbance value observed at pH 6.8 at the beginning of the experiment. The band at 1540 cm\(^{-1}\) actually
increased back to the absorbance level seen at pH 4, rather than what was observed at pH 6.8.

Spores of isolate E7, as shown in Figure 3.12, displayed a very similar trend to that seen for D4 isolates with similar increases and decreases in the bands as the pH was changed. However the respective increase and decrease of 1720 and 1400 cm⁻¹ occurred at pH 5 for E7 spores, compared with pH 4 as for D4 spores. This was followed by a further increase of 1720 cm⁻¹, decrease of 1400 cm⁻¹ and overall decrease of the amide I and II bands between pH 5 and 2.5. Spores of E7 also displayed a similar response to reversion back to pH 6.8 after 2.5 with the amide II band reaching an absorbance level equivalent to that seen at pH 4.

Finally, spores of the isolate E11 displayed little to no change over a pH range from pH 6.8 to 5 (Figure 3.13). However when the pH was adjusted to 4, there was a sharp increase in all bands monitored. As the pH continued to decrease into 3 and 2.5, the amide I and II bands decreased. The band at 1720 cm⁻¹ increased, with the corresponding decrease at 1400 cm⁻¹. Unlike other isolates, the reversion back to pH 6.8 was not determined for E11.
Figure 3.10: (A) Absolute spectra from spores of isolate CGT-8 at different pH. (B) Absorbance changes due to changes in pH of selected peaks (∗) 1400, (▲) 1720, (●) 1640 and (■) 1540 over time. Spectra are offset vertically and unit values are for sense of scale.
Figure 3.11: (A) Absolute spectra from spores of isolate D4 at different pH. (B) Absorbance changes due to changes in pH of selected peaks (×) 1400, (▲) 1720, (♦) 1640 and (■) 1540 over time. Spectra are offset vertically and unit values are for sense of scale.
Figure 3.12: (A) Absolute spectra from spores of isolate E7 at different pH. (B) Absorbance changes due to changes in pH of selected peaks (∗) 1400, (△) 1720, (●) 1640 and (■) 1540 over time. Spectra are offset vertically and unit values are for sense of scale.
Figure 3.13: (A) Absolute spectra from spores of isolate E11 at different pH. (B) Absorbance changes due to changes in pH of selected peaks (×) 1400, (▲) 1720, (◆) 1640 and (◼) 1540 over time. Spectra are offset vertically and unit values are for sense of scale.
3.3.4.4 Spectral normalisation

The results in the present study indicated spore movement in relation to the ZnSe surface since the intensities of all of the bands in the spectra of D4 spores (Figures 3.10 to 3.13) were influenced by pH. This finding has been seen in a previous study examining the effects of pH and ionic strength on Pseudomonas aeruginosa (152). As the pH decreased below neutral pH, the spore’s net negative charge decreased due to the protonation of carboxylates as seen in changes in the bands at 1720 and 1400 cm\(^{-1}\). This caused electrostatic repulsion between carboxylates within the spore surface polymers to decrease and the polymers to compress resulting in an increase in the amide I and II peaks due to the spore moving closer to the ZeSe surface. At the IEP, these balancing charges result in net attractive interactions between the positive and negative functional groups within a polymer causing a more compressed conformation. As the pH was reduced below the IEP point, the carboxylate functional groups on the surface became fully protonated since the bands at 1720 and 1400 cm\(^{-1}\) remained unchanged between pH 3 and 2.5. At a pH below the IEP, the net positive charge on the polymers will result in repulsion between the dominant positively charged ammonium groups causing an extended polymer conformation. This explains the decrease in the amide I and II bands intensity as the pH drops below the IEP.

In order to correct the spectra for the effect of the movement of spores relative to surface of the prism due to polymer compression, spectral absorbances in the difference spectra were normalised to the amide II peak so only spectral changes in response to changes in the pH were observed. It was assumed in this normalisation that the amide absorptions are insensitive to pH changes in this range (110). The amide I band is not suitable for normalisation purposes due to interference from the over-lapping water bending mode absorption (90, 118).
3.3.4.5 pH difference spectra

To examine spectral changes of the spore isolates in response to pH, spectra recorded at pH 6.8 were subtracted from spectra obtained under more acidic conditions. The results were displayed as pH difference spectra for the four spore isolates displayed in Figures 3.14 to 3.17. These pH difference spectra were plotted as calculated (A in the Figures) and normalised to the amide II (1540 cm\(^{-1}\)) band (B in the Figures). These figures clearly showed that with decreasing pH there was an increase in the 1720 cm\(^{-1}\) band (COOH) along with a corresponding decrease in the 1400 cm\(^{-1}\) (COO\(^{-}\)) band. Additionally a prominent negative band was apparent at ~1580 cm\(^{-1}\) which corresponded to the anti-symmetric carboxylate stretch absorption which was unable to be discerned in Figure 3.10 to 3.13 absolute spectra.

The pH difference spectra for isolates CGT-8 can be seen in Figure 3.14. This spectrum was dominated by prominent increasing absorptions at 1650 and 1620 cm\(^{-1}\) as the pH decreased. Bands in the region ~1270 and ~900 cm\(^{-1}\) for isolate CGT-8 were broad making assignments to particular functional groups difficult. The broad peak which is evident from ~1100 to 1000 cm\(^{-1}\) underlying the bipolar 1080 cm\(^{-1}\) band must arise from polysaccharide absorption increases with pH, which are larger than expected from polymer compression discussed earlier. One possibility resulting from the polymer compression may be a disproportionate amount of neutralised polysaccharide being pressed onto the ZnSe prism surface.

Isolate D4 (Figure 3.15) displays similar increases in the carboxylic acid (1720 cm\(^{-1}\)) peak and a decrease in the carboxylate (1580 and 1400 cm\(^{-1}\)) peaks as was observed for CGT-8. In the 1300 to 900 cm\(^{-1}\) region of the D4 difference spectra, the broad peak at 1250 cm\(^{-1}\) scaled with pH closely with that of the 1720 cm\(^{-1}\) band which identified it as the C-O stretching mode of the –COOH group. Additional features in this region were a noticeable bipolar derivative-like band at ~1080 cm\(^{-1}\) and a small peak emerging at ~1280 cm\(^{-1}\).

There were some similarities between the pH difference spectra for spore isolates E7, CGT-8 and D4. For example, the changes in the 1400 and 1720 cm\(^{-1}\) bands were clearly evident. The negative peak at 1400 cm\(^{-1}\) was a prominent feature in the E7 difference spectrum compared to the relatively weak bands in CGT-8. On the other hand for spores of E7, the increase in absorption in the 1700 to 1600 cm\(^{-1}\) region with decreasing pH was much less pronounced compared with absorbance
changes in the carboxylate bands. Like CGT-8 the bands in the region of 1270 to 900 cm\(^{-1}\) were qualitatively similar although their broadness makes assignments to particular function groups difficult.

The pH difference spectra between E11 (Figure 3.17) and D4 were similar in many aspects. A similar absorbance increase in 1720 cm\(^{-1}\) band and a decrease in the 1580 and 1400 cm\(^{-1}\) bands were observed, indicating the protonation of carboxylates. Spores of isolate E11 displayed a sharp absorption increase at 1650 cm\(^{-1}\) together with shoulder at 1620 cm\(^{-1}\). The increase of these bands, along with the increase in absorbance at 1380 cm\(^{-1}\) under acidic conditions, was characteristic of the release of CaDPA from the core of the spore.
Figure 3.14: pH difference spectra from CGT-8 spores before (A) and after (B) normalisation to the amide II ($1540 \text{ cm}^{-1}$) band. Spectra were recorded at either pH 2.5, 3, 4, 5 and 6.8 in the region of 1800 to 800 cm$^{-1}$. The darkness of the lines is representative of the difference of the pH. Line contrast dependent upon difference in pH between two spectra, i.e. the darkest line (---) is pH 2.5 subtracted from 6.8. While the lightest line (-----) is pH 5 subtracted from 6.8. Spectra are overlayed.
Figure 3.15: pH difference spectra from D4 spores before (A) and after (B) normalisation to the amide II (1540 cm\(^{-1}\)) band. Spectra were recorded at either pH 2.5, 3, 4, 5 and 6.8 in the region of 1800 to 800 cm\(^{-1}\). The darkness of the lines is representative of the difference of the pH. Line contrast dependent upon difference in pH between two spectra, i.e. the darkest line (—) is pH 2.5 subtracted from 6.8. While the lightest line ( ) is pH 5 subtracted from 6.8. Spectra are overlayed.
Figure 3.16: pH difference spectra from E7 spores before (A) and after (B) normalisation to the amide II (1540 cm$^{-1}$) band. Spectra were recorded at either pH 2.5, 3, 4, 5 and 6.8 in the region of 1800 to 800 cm$^{-1}$. The darkness of the lines is representative of the difference of the pH. Line contrast dependent upon difference in pH between two spectra, i.e. the darkest line (—) is pH 2.5 subtracted from 6.8. While the lightest line ( ) is pH 5 subtracted from 6.8. Spectra are overlayed.
Figure 3.17: pH difference spectra from E11 spores before (A) and after (B) normalisation to the amide II (1540 cm\(^{-1}\)) band. Spectra were recorded at either pH 2.5, 3, 4, 5 and 6.8 in the region of 1800 to 800 cm\(^{-1}\). The darkness of the lines is representative of the difference of the pH. Line contrast dependent upon difference in pH between two spectra, i.e. the darkest line (—) is pH 2.5 subtracted from 6.8. While the lightest line ( ) is pH 5 subtracted from 6.8. Spectra are overlayed.
3.3.4.6 Role of CaDPA in spectra

In order to determine if DPA was being released from the spores as a result of low pH during the IR experiments, a colorimetric assay was used to measure DPA release under acidic conditions. At pH 3 and 2.5 a sharp absorption band at 1620 cm\(^{-1}\) appeared together with an increase in the absorbance of the band at 1380 cm\(^{-1}\) in pH difference spectra obtained from spores of isolate D4 (Figure 3.15), which may correspond to an increase in CaDPA absorption (179). In the 1700 to 1600 cm\(^{-1}\) region there was also a notable increase in the band at 1650 cm\(^{-1}\) as the pH was reduced. This band appears to correspond to increased water bending mode absorption. During germination water uptake occurs and CaDPA is released from the spore’s core (59). The increase of water into the spore core may be seen by the increase in the water bending mode absorption at 1650 cm\(^{-1}\). The release of CaDPA would also be expected to give a reduced absorption at 1620 cm\(^{-1}\). Thus the increase in the 1620 cm\(^{-1}\) band absorption was unexpected. However, the fate of the released CaDPA was uncertain since Ca\(^{2+}\) and DPA are released in equimolar amounts during early germination with Ca\(^{2+}\) predominating during the later stages of germination (210). Additionally, ingress of water to the spore’s core and hydration of the CaDPA would result in several changes to the water bending mode signal that overlap with the observed 1620 cm\(^{-1}\) band and complicate the analysis of this spectral region.

The release of a significant proportion of spore CaDPA under these acidic conditions was confirmed with a colorimetric assay of the D4 spore supernatant solution (Table 3.2). Spores treated under the same conditions observed during the course of the IR experiment (pH 2.5 at room temperature) released 10 μg mL\(^{-1}\) DPA compared with spores at pH 6.8 which released < 5 μg mL\(^{-1}\). Lowering the pH to 1, increased DPA release to 20 μg mL\(^{-1}\). Under these acidic conditions, spores did not release as much DPA when compared with spores that were autoclaved and treated with 1 N acetic acid. Therefore IR spectral observations (Figures 3.14 to 3.17) indicate spore activation at pH ≤ 3. Previous research has found ‘activation’ of spores due to acidic conditions, however after this low pH treatment, these spores failed to fully germinate into vegetative cells (111).
Table 3.2: Release of DPA from a D4 spore suspension (1 x 10^8 CFU mL^-1) under different conditions.

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<thead>
<tr>
<th>Conditions</th>
<th>Concentration of DPA released (µg mL^-1)</th>
</tr>
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<tbody>
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<td>Negative Control</td>
<td>&lt;5</td>
</tr>
<tr>
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</tr>
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3.3.5 Comparison of spore surface characterisation data.

In order to accurately assess the surface characteristics of the spore surface, the data obtained from the four spore isolates, CGT-8, D4, E7 and E11 were compared and summarised in Table 3.3. To understand the characterisation results, the zeta potential, MATH assay, water contact angle data and relative carboxylate concentrations obtained from the IR data were compared for all of the spore isolates. The relative concentrations of carboxylic acid groups are defined as the ratio of the difference in absorbance between the 1720 cm\(^{-1}\) (carboxylic acid) band at pH 2.5 and 6.8 relative to the protein content as indicated by the absorbance of the amide II peak (1540 cm\(^{-1}\)) at pH 6.8. These values were obtained from the pH difference infrared spectra (Figures 3.14 to 3.17).

The zeta potential data reflect the net spore surface charge which at a pH of 6.8 is assumed to correspond to the presence of functional groups such as the carboxylate, amine and phosphate with the carboxylate groups being dominant on the spore surface. The zeta potential data for three of the four spore isolates correlated fairly well with the IR spectroscopic analysis of relative carboxylic acid/carboxylate concentrations. The exception was spores from isolate CGT-8, which had a lower carboxylate concentration from the IR data than would be expected from the zeta potential data. Spores of isolate CGT-8 had the highest hydrophobicity of the four spores tested using the MATH assay, suggesting that the spore had fewer carboxylate groups. This may be due slight differences in the harvesting process between different cultures of the same isolate, and thus resulting in different carboxylic acid surface concentrations due to loss of surface polymer containing polar groups.

The water contact angle and hydrophobicity data from the microbial adhesion to hexadecane (MATH) assay are more complex to interpret in terms of the contributions of surface functional groups. In the current study, spore hydrophobicity was influenced by pH (Figure 3.5). The presence of polar functional groups such as hydroxyl, carboxyl, phosphate and amine within spore surface polymers are expected to contribute to spore surface hydrophilicity. Protonation/deprotonation reactions involving such groups in the accessible pH range are also expected to modulate hydrophilicity. The protonation of carboxylate groups when the medium pH is reduced below the pKa of the functional groups is expected to reduce hydrophilicity.
This data supports the assertion that carboxylate functional groups are major contributors to the charge and hydrophilicity of spore surfaces.

Table 3.3: Spore surface characterisation data.

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<tr>
<th>Spore isolate</th>
<th>Relative concentration of COO⁻ A</th>
<th>Zeta Potential at pH 6.8 (mV) B</th>
<th>Water Contact angle (°) C</th>
<th>Relative Hydrophobicity (%) D</th>
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</thead>
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<td></td>
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<td>-20</td>
<td>20</td>
<td>84</td>
</tr>
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<td>13</td>
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<tr>
<td>E11</td>
<td>0.11</td>
<td>-13</td>
<td>10</td>
<td>30</td>
</tr>
</tbody>
</table>

(A) The difference in absorbance at 1720 cm⁻¹ between pH 2.5 and 6.8 and relative to amide II (1540 cm⁻¹) absorbance at pH 6.8. Carboxylate concentrations assume complete protonation at pH 2.5. (B) In 0.1 M KCl. (C) Water contact angles on spore lawns. (D) Relative hydrophobicity of spores determined from the microbial adherence to hexadecane (MATH) assay at pH 3 and 6.8 in 0.1 M KCl.
3.4 Discussion

Spores of the four *Geobacillus* spp. isolates CGT-8, D4, E7 and E11 were found to be morphologically different. Spores of isolates CGT-8, D4 and E7 possessed an exosporium while E11 spores did not appear to have one. The exosporium consists of a basal membrane surrounded by dark amorphous structures on the outer surface. Generally, the high contrast seen on the outer surface of the exosporium is associated with a high number of anionic binding sites for the cationic stain ruthenium red coupled with osmium tetroxide. Currently there are no stains specific for a particular anionic group therefore these images needed to be interpreted with reference to previous studies. For example it has been reported that the outer surface of the exosporium surrounding *B. anthracis* and *B. subtilis* spores contains glycoproteins (76, 77). Recent studies have also used ruthenium red to visualise the glycoprotein nap of the exosporium surrounding *B. anthracis* (261) and *B. cereus* spores (236). Therefore it seems reasonable to assume that the surface of CGT-8, D4 and E7 spores consists largely of glycoproteins. However other anionic groups associated with individual proteins, sugars and phosphates may also exist on the surface. Another notable feature of the TEM images was the appendage-like structures found attached and detached due to mechanical stress during harvesting and washing of spores. Spores of certain strains of *B. cereus* are known to have pili which have been shown to be important in the attachment of this organism to surfaces under certain conditions (236).

Another important observation from the TEM images was the changes in the physical structure of the spores after they were harvested from the sporulation medium. The exosporium surrounding D4 spores was clearly evident two days after harvesting while it was absent from spores that had been harvested two weeks prior to examination. A small darkly stained layer can be seen surrounding the spore coat. This could either be due to the peeling off of the spore coat or due to the exosporium being compressed resulting in it being in closer contact with the spore coat. Previous studies noted that incubation of harvested spores in water at 4 °C resulted in the release of spores from the sporangia, or what remained of the mother cell (54, 121). This may also be the case for spores of isolate E11 in the current study. Spores of isolate E11 may have possessed an exosporium, however it may have quickly ruptured during or after harvesting.
A potential drawback of the PEG separation method was that any residual PEG polymers remaining on the surface of the spores could alter their surface properties. To reduce this possibility, the PEG was removed by washing the spores in water at room temperature with resting periods in between each centrifugation step to allow the polymers to be released from the surface. The contact water angle measurements ($\theta_w$) of the spore lawns decreased after purification, suggesting the two-phase method removed much of the hydrophobic material such as membranes and denatured protein from the crude spore extract. Infrared spectroscopic analysis of washed spores was unable to detect any residual PEG (Figure 3.9).

The MATH assay and water contact angle results indicated that the spores of the four Geobacillus spp. isolates were all hydrophilic. Isolate D4 was the most hydrophilic with the lowest affinity for hexadecane and the smallest $\theta_w$. Spores of CGT-8 and E11 had the greatest affinity for hexadecane and the greatest $\theta_w$. However when the contact angles of the other solvents were taken into account and the $\Delta G_{sli}$ was calculated, there was no clear relationship between the MATH assay and the $\Delta G_{sli}$ values. From the $\Delta G_{sli}$ results, purified spores of CGT-8 were the most hydrophilic and E7 spores were the most hydrophobic. Other studies have found that spores of G. stearothermophilus are relatively hydrophilic through results obtained from the Hydrophobic Interaction Chromatography (HIC) (196) and MATH assays (175).

The determination of microbial surface hydrophobicity has been a focus of laboratories around the world for many years (62, 196, 246). However a common concern is that it is difficult to directly compare results obtained from different laboratories using different or even similar tests (53). Without a standardized method it becomes difficult to compare cell surface hydrophobicity of different species between numerous laboratories. Obtaining reproducible data is further complicated by the fact that surfaces of spores are heterogenous and contain a combination of functional groups such as carboxylate, amine and phosphate groups, which may account for some of the variation seen between the different tests. The MATH assay can be done in a kinetic mode with different vortexing times for more reproducible and quantitative results (137). While the hydrophobicity of D4 spores was determined using this method, this method was not performed for the other three
isolates. For spore isolates D4, a stationary endpoint after a 30 second vortexing time indicating spores of this strain were hydrophobic.

Electrostatic interactions can also affect results from the MATH and HIC assays but not contact angle (4). Previous studies have found negative ions, particularly phosphate, adsorb to the surfaces of hydrocarbon droplets creating a negatively charged surface (32). Therefore the MATH assay is not solely a hydrophobic assay but measures an interplay of long-range van der Waals and electrostatic forces and various short-range interactions (245). However, the MATH remains extremely useful as a simple assay for studying adhesion of microorganisms to a hydrophobic surface, which is clearly essentially different from being a hydrophobicity assay. It has been suggested that contact angles should be used as the universal measurement of hydrophobicity since they provide results in standard units rather than the relative results obtained from MATH or HIC (243). However contact angles are macroscopic measurements of the surface hydrophobicity of cells aggregated together in a lawn rather than of suspended single particles as measured in the MATH assay. As all methods for measuring hydrophobicity have their advantages and disadvantages, it is now recognised that more than one method of measuring hydrophobicity should be used to obtain results. In this study, the results from both MATH and contact angles show that spores from the different Geobacillus spp. were predominately hydrophilic.

Since the surface of the spores can have a net positive or a negative zeta potential depending on pH, it is assumed that the surfaces of these spores possess a heterogenous array of anionic/cationic and acid/base functional groups. Rijnaarts et al. proposed that zeta potential measurements of micro-organisms can reveal the nature of ionisable chemical species present on that surface (191). Carboxylic acid groups (-COOH/-COO⁻) can be found in proteins and peptidoglycan which have a pKₐ in the range of 4 to 5, or in polysaccharide with a pKₐ around 3. Protein and peptidoglycan associated ammonium groups (-NH₃⁺/-NH₂) have a pKₐ in the range of 9 to 10. Phosphate can be present as either phosphodiester bridges (-O-HPO₂⁻/-O-PO₂⁻-O⁻) as in phospholipids or as phosphomonoesters (-H₂PO₄⁻/-HPO₄²⁻/-PO₄³⁻) with pKₐ values typically around 2.1 and 7.2. The pronounced increase in zeta potentials below a pH of ~5 generally indicates the presence of carboxylate groups which protonate in this pH range, although deprotonation of phosphodiester groups could also contribute to this change towards the low end of the pH range. The relatively
constant negative zeta potential values in the 5-8 pH range are also consistent with carboxylate groups being present in greater concentrations compared to phosphomonoesters, as the presence of significant concentrations of phosphomonoesters would be expected to increase the negative zeta potential around a pH value of 7. The existence of net positive zeta potentials at low pH can only be accounted for if ammonium groups are present. The presence of ammonium groups is further supported by the general decrease in zeta potentials at pH values above 8 corresponding to their deprotonation. Thus the zeta potential data appears to indicate that carboxylate and ammonium groups are the predominant ionisable groups on the spore surface.

The surfaces of spores are believed to consist largely of protein and polysaccharide (149), while the spore cortex (mid section of the spore) consists of peptidoglycan. Previous reports, have shown proton diffusion through the different surface layers of the spore (120). Therefore the spore may be considered as a "soft particle." Current methodologies for calculating zeta potentials from electrophoretic mobilities call for the use of the Oshima theory instead of the Helmholtz-Smoluchowski formula since it takes into account the particle having a soft outer layer (168). However in this study, the Helmholtz-Smoluchowski formula was used so the results could be comparable to previous studies on spores. However using this formula may overestimate the zeta potential and therefore the effect of electrostatic repulsion in later results (23). Protons were able to bind to ionisable functional groups within the spore coat and cortex, but unable to penetrate the membrane that protects the core. The diffusion of protons into deeper compartments can affect zeta potentials of individual spores depending on the electro-osmotic fluid flow through these layers (182). The cortex of all four spore isolates possessed a light contrast after staining with ruthenium red compared with the darker contrast of the exosporium, indicating fewer anionic functional groups within the cortex. For this reason, the functional groups located on the polymers present on the exosporium and spore coat would have a greater effect on the negative zeta potential compared to the functional groups located in the cortex. However, further titration experiments would need to be undertaken to confirm this.

From the zeta potential experiments, it was found that spores of the isolate CGT-8 had the lowest IEP at pH 3. This may be due to larger amounts of carboxylated polysaccharides on the surface since the pK_a of this type of carboxylate
is about 3. In contrast, spores of isolate E7 had the greatest IEP at pH 4. This indicated that there is more protein and less carbohydrate on the surface since the pKa of carboxylates found in protein is between 4 and 5. Isolates D4 and E11 both had IEP at pH 3.5, which suggested moderate amounts of both protein and polysaccharide. To further elucidate the functional groups present on the surface of spores, ATR-IR spectroscopy was employed.

Infrared spectroscopy of the spores revealed a surface comprising of proteins, carbohydrates and to a lesser extent, phosphodiesters. Carboxylate was found to be the predominant functional group responsible for the net negative charge on the spore surface. However the relationship between relative carboxylate concentration and the degree of negative charge on the spore surface could not be determined. For example, spores of CGT-8 had a low relative carboxylate concentration and the second greatest negative zeta potential. Previous research have quantitatively assessed the molecular groups present on the surface of different Streptococci spp. through ratios of the CH stretch region against the amide and phosphate bands (244). However no further conclusions could be made when this method was applied in the current study.

Spore movement relative to the surface of the ZnSe prism was also detected due to the neutralisation of the net charge within surface polymers resulting in compression of these polymers. These spore movements observed using IR spectroscopy corresponded well with the IEPs determined from the zeta potential data. The spore would be at its closest point relative to the prism at its IEP. For example, spores of isolate CGT-8 were closest to the prism at pH 3, while their IEP was at 2.8. Spores of isolate E7 were closest to the prism at pH 4, which correlated well with their IEP of 4.

In this chapter, the surface characteristics of previously uncharacterised spores were determined. Electron microscopy images revealed that the surface of spores from isolates D4, E7 and CGT-8 contained a dense polysaccharide nap surrounding an exosporium. This was not apparent for spores from isolate E11 indicating that spores isolated from the same dairy environment can have dissimilar ultrastructures. The spore structure can also change over time, with D4 spores losing their exosporium two weeks after harvesting. The surfaces of the spores were negatively charged and hydrophilic under neutral pH conditions. However, the degree of these surface attributes varied amongst the four spore isolates. The surface charge of the spores varied with pH indicating ionisable functional groups such as carboxylic acids.
and ammonium groups. The pH difference IR spectroscopy confirmed the presence of carboxylates assumed to be associated with protein, carbohydrates and to a lesser extent phosphodiesters, which contribute to the spore’s charge. Acidic conditions caused polymers on the spore surface to compress through the protonation and neutralisation of carboxylates. These movements correlated well to the zeta potential data for all four spore isolates. Interestingly, these same acidic conditions also triggered the loss of CaDPA from the spore’s core indicating the initial stages of germination. While there were similarities in the pH difference spectra of the four different spore isolates, there were still inherent differences in the carboxylate content of the spores which ultimately have an affect on the surface charge and hydrophobicity of the spore. These results indicate that the surface of thermophilic spores consists of varied amounts of carboxylated glycoproteins and polysaccharides. The next step of this thesis will focus on understanding how the varied surface chemistry and structure of the different spore isolates impacts on their attachment to surfaces in the dairy industry.
Chapter 4

Effects of Environmental Conditions on Spore Adhesion
4.1 Introduction

The adhesion process of a micro-organism relies upon the mass transport, and surface characteristics of both the micro-organism and substrata which can be influenced by the suspension composition. However before these parameters can be assessed a reliable method for measuring spore adhesion needs to be established.

In order to accurately assess the initial adhesion of micro-organisms to surfaces, numerous techniques have been developed between different laboratories (22). Previous studies have carried out adhesion assays under either static or flow conditions using microscope slides or ‘coupons’ of different materials (62, 136). Under static conditions, the microbial suspension remains relatively stationary with respect to the substratum. In some cases the fluid is agitated under poorly controlled conditions. However, substrata are typically placed into, and taken out of bacterial suspensions resulting in the surfaces with adhered bacteria being exposed to a liquid-air interface where removal forces are equal to, or even several orders of magnitude higher than the adhesion force (164). Therefore these experiments are considered to measure the retention of micro-organisms on a surface rather than just the initial adhesion. With flow systems, microbial attachment can be assessed under controlled hydrodynamic and mass transport conditions. These techniques can include stagnation point flow collectors (269), rotating disc (27) and parallel plate flow chambers (153, 269). Traditionally, these methods are used to examine laminar and low velocity turbulent flow. Flow rates in the dairy industry are rapid at 1.5 m s\(^{-1}\) and are turbulent with large Reynold’s numbers and wall shear rates. The choice of which method to use comes down to modelling the conditions of the adhesion process under study. However, the choice of a particular method is dictated by cost aspects, ease of operation and familiarity amongst researchers. To model the effect of flow conditions on bacterial adhesion, biofilm development and cell removal via CIP procedures in a dairy environment, flow reactors are commonly used (29, 69, 173-175).

A major challenge in studying bacterial attachment to surfaces is ensuring the accurate assessment of the number of micro-organisms bound to a surface (101). There are several different techniques that can be used for measuring the number of micro-organisms adhered to a surface. The plate count method gives an estimate of viable cells and is commonly used after the bound organisms are removed by the use of detergents, swabbing, vortexing or sonication (30, 62). The cells in the resulting
Chapter 4 – Effects of Environmental Conditions on Spore Adhesion

Suspension undergo a serial dilution and are then plated on nutrient-supplemented agar. Direct counts on the other hand record both viable and non-viable cells (total count) adhering to translucent materials using a phase contrast microscope or on opaque materials by staining the micro-organisms with fluorescent dyes and observation under a fluorescent microscope (5). Other methods include impedance (71) and bioluminescence of metabolites such as ATP (255).

The initial stages of microbial adhesion are mostly determined by the mass transport of a micro-organism toward a substratum (153). Mass transport of organisms to a surface can also influence the reversibility of attachment. Reversibility implies a continuous exchange between free and adhering organisms. In the past, adsorption isotherms have been used to define the relationship between the adsorption capacity of an absorbent and the concentration of bacterial cells at equilibrium (147). Although initial microbial adhesion is generally said to be reversible, this is only during the first minutes of contact between an organism and a surface, after which time a virtually irreversible stage occurs in flow displacement systems (153). This is generally caused by a slow removal of interfacial water facilitating a closer approach or re-orientation of interacting sites. The Langmuir model is commonly applied and is based on the following assumptions; (1) a finite number of identical sites are available for attachment per unit area of adsorbent, (2) bacteria approach the adsorbent surface without steric hinderance and (3) adsorption is reversible up to and at the equilibrium state (82). Langmuir kinetics implies a first order rate dependence on micro-organism concentration and a \((1-e^{-kt})\) time dependence of irreversible adsorption (274).

The surfaces of spores possess structural and chemical heterogeneities, as shown in the previous chapter where ionic strength and pH influence both the zeta potential and the conformation of the polymers present on the their surface. The ionic strength and pH of the suspension has been also been shown to affect spore (108) and bacterial (190) adhesion to substrata. Increasing the ionic strength reduces the double layer thickness, which enables the micro-organism to come into closer contact to the surface. The pH of the suspending medium has also been shown to influence adhesion to substrata (264).

In the dairy industry, spores and vegetative cells attached to a surface will be exposed to a CIP regime. Spores exposed to a CIP are exposed to acidic (\(< 4\)) and alkaline (\(> 9\)) conditions (6), which can influence their surface properties. Previous studies have isolated viable *Bacillus* species from alkaline wash solutions involved in
a CIP from South African dairy factories (142, 231). In the dairy industry, it is common practice to reuse alkaline cleaning solutions to clean particular sections of the plant. While this may save money in processing costs, it can lead to re-contamination of product contact surfaces, which could ultimately cause the post-pasteurization spoilage of milk and milk products.

The aims of this chapter are to:

- Analyse the kinetics of spore adhesion to stainless steel.
  - Develop protocols to enumerate spores on the surface of stainless steel.
  - Determine if there are any differences in the number of spore adhering to stainless steel between static and flow conditions.
- Determine how ionic strength, pH and temperature of the suspension medium affect spore adhesion.
4.2 Materials and Methods

4.2.1 Spore and stainless steel preparation

The spores from isolates of thermophilic bacilli D4 were produced and washed as described in Section 2.2.9. Spores used in the adhesion experiments were not purified using the PEG two-phase system since it was only efficient at purifying small volumes (< 30 mL) of spore suspensions rather than larger volumes (> 200 mL) required for adhesion experiments. Stainless steel (316 grade 2B polish) was washed and sterilised as previously mentioned in section 2.2.2.

4.2.2 Enumeration of spores attached to surfaces.

In order to compare the viable and direct counting methods for assessing the number of spores bound to a substrate surface, three stainless steel coupons were placed into each of three separate static suspensions of D4 spores at two different concentrations (ie. $1 \times 10^7$ and $1 \times 10^8$ CFU mL$^{-1}$), in 0.1 M KCl for 20 min at room temperature. Coupons were removed and processed using the viable and direct counting methods described below in sections 4.2.2.1 and 4.2.2.2.

4.2.2.1 Viable Counts

After exposure to D4 spores at different suspension concentrations under static conditions, coupons were removed using sterile forceps and rinsed by passing the coupon through the liquid-air interface of water to remove loosely adherent spores. The number of spores attached to the coupons was estimated by placing the coupons in a 20 mL screw top glass Universal tube containing 0.1% peptone (10 mL) containing three glass beads (5 mm diameter). The surface of the coupon was swabbed with a sterile swab (LPI sterile swab). The coupon, swab and media were mixed on a vortex mixer for 15 seconds and then boiled for 30 min to activate the spores and to kill any vegetative cells. The number of viable spores was determined using the drop plate method (94). 10-fold serial dilutions were carried out in 0.1% peptone and five individual 10 μL droplets from each dilution were pipetted onto the
surface of tryptic soy agar (TSA). The droplets were allowed to air-dry in a laminar flow hood before the plates were incubated at 55 °C for 15 hours and colonies were counted.

### 4.2.2.2 Direct Counts

The number of spores of isolate D4 adhered to stainless steel was counted directly using the fluorescent stain 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI). Stainless steel coupons were removed and fixed in 9 mL of per 100 mL: 0.4 g NaH₂PO₄, 1.23 g Na₂HPO₄ and 10 mL of glutaraldehyde. Coupons were rinsed in phosphate wash solution (PWS) containing (per L): 1.36 g KH₂PO₄, 8.5 g NaCl, and 1.01 g MgCl₂.₆H₂O, then placed into 1.8 mL of PWS and 200 μL of 100 μg mL⁻¹ DAPI solution containing per 100 mL: 10 mg DAPI, 10 mL glutaraldehyde (25 %) and 90 mL water, for 20 min at room temperature. Coupons were removed, rinsed in PWS for 2 min and then air dried, before being mounted on glass microscope slides using double sided tape and viewed under 1000× magnification using a Olympus BX-51 microscope at excitation wavelength of 358 nm and emission wavelength of 461 nm. Digital micrographs were obtained using a DP70 CCD camera and DP Controller software (Olympus). The number of spores in ten random fields of view on each coupon were counted and an average number of spores per field for each coupon was calculated. A total of three coupons were used for an average for any given substrate.

### 4.2.3 Adhesion Kinetics

In order to determine the adhesion rate of spores to stainless steel, two different flow reactors were used. A small volume reactor was used to determine the adhesion rate over a period of 10 min, while a large volume flow reactor was used to determine the adhesion rate over a period of 6 hours.
4.2.4 Small Volume Reactor

In order to determine the adhesion rate of D4 spores to stainless steel over a period of seconds (0 to 600 sec), a small volume reactor (25 mL) was used (Figure 4.1). Stainless steel coupons were placed in a reactor containing 0.1 M KCl. At the start of an experiment, concentrated spore suspension in 0.1 M KCl was injected into the reactor to achieve a final spore concentration of $1.2 \times 10^7$ CFU mL$^{-1}$. After the coupons were exposed to the spore suspension for the desired time, sterile 0.1 M KCl was pumped into the reservoir at 15 mL s$^{-1}$ while the effluent was removed at a rate of 17 mL s$^{-1}$ and was turned off after three seconds. This removed all of the spores from the suspension as determined by viable plate counts. Coupons were removed at different times and the number of adhering spores was enumerated as described in section 4.2.2.1.

![Figure 4.1: Schematic of the small volume reactor used to expose stainless steel coupons to a spore suspension for shorter periods of time.](image-url)
4.2.5 Large Volume Reactor

A flow loop reactor system was used to experimentally determine the initial attachment of thermophilic spores under different conditions to a variety of substrates under different environmental conditions (29). The system consisted of a reservoir and loop (total volume was 200 mL) containing coupons of different materials (Figure 4.2). Coupons in triplicate were inserted into sections of silicon tubing (Cole Parmer). These sections of tubing were connected using barbed fittings (Cole Parmer). Spores of isolate D4 (~1 x 10⁷ spores mL⁻¹) were suspended in different media depending on the experiment and circulated at a speed of 1 m s⁻¹ (Reynolds number 1.1 x 10⁴) through the recirculating loop to model the velocity in a dairy plant. The entire system was held in a water bath to control the temperature. Coupons were removed and the number of adhered spores was enumerated as described in section 4.2.2.1.

Figure 4.2: Schematic of the large volume reactor used to expose different substrata to spores (A). Cross section of a coupon in silicon tubing (B); fluid flow occurs in unshaded areas on both sides of the coupon.
4.2.6 Effect of suspension pH, ionic strength, temperature and spore concentration on adhesion

In order to examine the effect of D4 spore concentration on adhesion to stainless steel the large volume reactor described in section 4.2.5 was used. The concentration of spores present in the reservoir between \(~8 \times 10^3\) and \(5 \times 10^8\) CFU mL\(^{-1}\) was achieved by serial dilution using 0.1 KCl. The effect of temperature on spore attachment was determined by adjusting the temperature of the water bath between 10 and 70 °C. The effect of ionic strength and pH on spore adhesion was determined by adjusting the respective parameters of the spore suspension in the reservoir. For each experiment, stainless steel coupons were exposed to each set of conditions for 30 min. Coupons were removed and the number of adhered spores was enumerated as described in section 4.2.2.1.

4.2.7 Effect of a model caustic wash on spore surface properties and adhesion

In order to examine the effects of a model CIP on spore surface characteristics, spores of isolate D4 were suspended in either water or 1% NaOH at 65 °C for 10 min in to model what occurs during a CIP. These spore suspensions will be refered to as “treated spores” throughout the rest of this thesis. Treated spores were then washed in water three times. Spore adhesion to stainless steel was determined using a large volume reactor (section 4.2.5) and viable spores adhering to stainless steel were enumerated using the technique described in section 4.2.2.1. Treatment with NaOH reduced viable spore counts, therefore spore suspensions of treated spores and controls in 0.1 M KCl were adjusted to \(1 \times 10^5\) CFU mL\(^{-1}\) prior to adhesion assays to standardise spore concentration.

Treated spores were purified using the two-phase PEG system as described in Section 2.2.10. The hydrophobicity of the treated spores was determined using the MATH assay as described in section 3.2.3.1. The zeta potential over a pH range of treated spores was determined by suspending the spores in 0.1 M KCl as described in section 3.2.4. Absolute spectra at pH 6.8 in 0.1 M KCl were obtained as described in section 3.2.5. To observe any structural changes due to the treatments, thin sections of
spores stained with ruthenium red were observed under TEM using the same methods that were described in section 3.2.2. Untreated spores were from a suspension stored at 4 °C for two weeks after harvesting.

4.2.8 Statistics

Statistical analysis was performed using Statistical Package for the Social Sciences software (SPSS, Inc., Chicago, IL). The influence of pH on attachment of isolate D4 to stainless steel was investigated by analysis of variance followed by multiple-means comparison using Tukey grouping. Correlations between the different surface parameters and spore adhesion were obtained using a Pearson’s correlation. All tests were performed with a confidence level of 95%.
4.3 Results

4.3.1 Comparison of the viable and direct counting methods

In order to accurately enumerate the number of spores bound to a surface during an adhesion experiment, two different enumeration methods, viable and direct counts, were compared in Table 4.1. An example photograph of a stainless steel surface after exposure to suspension 3 can be seen in Figure 4.3. The viable plate count method consistently detected a density of $0.6 \log_{10} \text{CFU cm}^{-2}$ fewer spores than the direct count method for all three spore suspensions ($p = 0.002$).
Table 4.1: Comparison of the viable count method with the direct count method.

<table>
<thead>
<tr>
<th></th>
<th>Viable counts in suspension (Log$_{10}$ CFU mL$^{-1}$)</th>
<th>Viable Counts Attached (Log$_{10}$ CFU cm$^{-2}$) $n = 3 \pm$ S.D.</th>
<th>Direct Counts Attached (Log$_{10}$ CFU cm$^{-2}$) $n = 3 \pm$ S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspension 1</td>
<td>7.1 ± 0.1</td>
<td>3.9 ± 0.3</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>Suspension 2</td>
<td>7.3 ± 0.1</td>
<td>4.0 ± 0.2</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>Suspension 3</td>
<td>8.0 ± 0.2</td>
<td>5.0 ± 0.1</td>
<td>5.6 ± 0.2</td>
</tr>
</tbody>
</table>

Figure 4.3: Fluorescent microscope image of DAPI stained D4 spores attached to the surface of stainless steel.
4.3.2 Attachment over time

The attachment of spores from isolate D4 suspended in 0.1 M KCl at 55 °C to stainless steel was monitored over time using two different flow systems (Figure 4.4). To analyse the adhesion of spores over a period of ten minutes, a small volume reactor was developed to rapidly expose stainless steel to a spore suspension for predetermined periods of time without passing through a liquid-air interface of a concentrated spore suspension. Attachment occurred rapidly with \(3.7 \text{ Log}_{10} \text{ CFU cm}^{-2}\) recovered from coupons that were exposed to the spore solution for 10 sec. The number of spores adhered to the surface significantly increased by \(0.5 \text{ Log}_{10} \text{ CFU cm}^{-2}\) over the next 120 seconds, and reached a stationary endpoint. There was a slight decrease in the number of viable spores recovered from the surface of the stainless steel, however this was not significant according to Tukey’s grouping.

The number of spores bound over a 6 h period was determined using a flow loop reactor. Spore surface density reached \(4.8 \text{ Log}_{10} \text{ CFU cm}^{-2}\) within the first 10 min of the stainless steel surfaces coming into contact with the spore suspension. The density of spores on the surface was higher (~ 0.3 \(\text{ Log}_{10} \text{ CFU cm}^{-2}\)) than what was achieved at the end of the previous experiment using the small volume reactor. This could be due to the differences in flow mechanics of the two systems resulting in a different mass transport mechanism of spores to the stainless steel surface. The number of spores recovered from the stainless steel surfaces did not change over two hours confirming that the stationary endpoint had already been achieved. However there was a significant decrease in the number of viable spores recovered from the surface of the stainless steel. This decrease is believed to be due to the germination of spores both on the surface and in solution. Separate experiments revealed loss of the spore’s heat resistance when held at 55 °C in the absence of nutrient media.
Figure 4.4: The adhesion of D4 spores suspended in 0.1 M KCl at 55 °C using either the small flow cell for rapid attachment (A) or flow loop reactor for the longer attachment assay (B). Values represent the means of three replicates while error bars represent standard deviation of the means. Points exhibiting no common letter are considered to be significantly different according Tukey’s grouping.
4.3.3 **Effect of spore concentration on adhesion**

The effect of spore concentration on adhesion of D4 spores to stainless steel can be seen in Figure 4.5. A linear relationship was found between the number of spores in suspension and the number recovered from the surface of the stainless steel ($R^2 = 0.98$, $p < 0.001$).

![Figure 4.5: The effect of suspension concentration on the attachment of D4 spores to stainless steel under flow (1 m s$^{-1}$) for 30 min. Values represent the means of three replicates while error bars represent standard deviation of the means.](image-url)
4.3.4 The effect of temperature of the adhesion of D4 spores to stainless steel

The effect of temperature on the attachment of D4 spores to stainless steel can be seen in Figure 4.6. There was very little difference in the number of spores recoverable from stainless steel coupons, which had been exposed to spores suspensions between 10 °C and 72 °C for 30 min. Note that while Tukey’s groupings revealed a significant difference between the attachment of spores at 40 °C and 48 °C, the difference was < 0.2 Log10 CFU cm⁻² and is unlikely to be of practical relevance.

![Figure 4.6: Effect of temperature on the attachment of D4 spores to stainless steel under flow (1 m s⁻¹) for 30 min. Values represent the means of three replicates while error bars represent standard deviation of the means. Points exhibiting no common letter are considered to be significantly different according Tukey’s grouping.](image-url)
4.3.5 Effect of ionic strength and pH on adhesion

The effect of the ionic strength of the suspending medium on spore attachment to stainless steel can be seen in Figure 4.7A. Increasing the ionic strength from 0.01 to 0.1 M resulted in a 2.5 fold increase in the number of spores adhering ($p = 0.001$).

The effect of pH on the attachment of D4 spores to stainless steel was examined under flow conditions with spores in 0.1 M KCl. Figure 4.7B shows there was a 3 fold significant increase in the number of spores adhering to stainless steel at pH 3.1 compared with pH 7.2 ($p = 0.001$). There was also a 2 fold increase at pH 10 compared with pH 7.2 however this difference was not significant ($p = 0.07$).
Figure 4.7: The effect of ionic strength (A) and pH (B) on the attachment of D4 spores to stainless steel under flow (1 m s\(^{-1}\)) for 30 min. Values represent the means of three replicates while error bars represent standard deviation of the means. Points exhibiting no common letter are considered to be significantly different according to Tukey’s grouping.
4.3.6 Effect of NaOH on the surface characteristics of D4

The effect of the NaOH step in a dairy plant CIP on spores of isolate D4 was assessed by exposing the cells to either 1% NaOH or water at 65 °C for 10 min (Table 4.2). Spores treated with water showed no decrease in their viability, while spores treated with a 1% NaOH solution had a 2 log reduction in their viability. To compensate for this decrease in spore numbers in subsequent attachment assays, the concentration of spores was standardized to $1 \times 10^5$ CFU mL$^{-1}$ to ensure that differences in numbers of attaching spores was not due to the effect of suspension concentration.

Spores treated with 1% NaOH at 65 °C for 10 min, attached an order of magnitude higher than untreated spores ($p = 0.01$) (Figure 4.8). There was also a slight but not statistically significant increase in the number of water treated spores attaching to stainless steel compared with untreated spores.

Table 4.2: Viable spore adhesion counts for untreated and treated spores.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Viable Spore Adhesion Counts (Log$_{10}$ CFU mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 3 ± S.D.</td>
</tr>
<tr>
<td>Untreated</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td>Water 65 °C 10 min</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>1% NaOH 65 °C 10 min</td>
<td>5.1 ± 0.2</td>
</tr>
</tbody>
</table>
Figure 4.8: The effect of surface treatments on the adhesion of D4 spores to stainless steel. Values represent the means of three replicates while error bars represent standard deviation of the means. Points exhibiting no common letter are considered to be significantly different according Tukey’s grouping.
4.3.6.1 Effect of treatments on surface characteristics of D4 spores.

The impact of exposure to either NaOH or water at 65 °C on the hydrophobicity of spores was determined using the MATH assay at pH 3 (Figure 4.9A). Spores treated in 1% NaOH were 6 times more hydrophobic compared with untreated spores (p = 0.001). Spores that were treated in water were 3.5 times more hydrophobic compared with untreated spores (p = 0.04).

The effect of the NaOH and water at 65 °C treatments on zeta potentials can be seen in Figure 4.9B. Spores treated in water or NaOH had a similar zeta potential (-32 mV) which was significantly more negative than untreated spores at -20 mV. The IEP of the treated spores was also reduced slightly from pH 3.8 for untreated spores to 3 for treated spores. Spores which were treated 1% NaOH had a greater negative zeta potential at a pH above 10 compared with untreated spores.
Figure 4.9: Surface characteristics of spores treated with either 1% NaOH or water at 65 °C for 10 min compared to untreated spores at 25 °C. Relative hydrophobicity using the MATH assay at pH 3 in 0.1 M KCl (A) and zeta potentials over a pH range of 2.5 to 11.5 in 0.1 M KCl (B). Untreated (×), water (■) and 1% NaOH (▲) treated spores. Values represent the means of five replicates while error bars represent standard deviation of the means. Points exhibiting no common letter are considered to be significantly different according to Tukey’s grouping.
4.3.6.2 Absolute spectra of treated spores

The absolute infrared spectra of NaOH and water treated D4 spores compared to untreated spores can be seen in Figure 4.10. There are very few differences between the two spectra. The amide I and II peaks (1640 and 1540 cm\(^{-1}\)) were present in all of the spectra. For spores treated in 1% NaOH and water, there was an increase in the absorbance at 1570 cm\(^{-1}\) relative to 1540 cm\(^{-1}\) compared with untreated spores. This may be due to the anti-symmetric carboxylate stretch. There was a slight increase in a minor peak at 1310 cm\(^{-1}\) for NaOH and water treated spores, while in increase at the 1330 cm\(^{-1}\) band for NaOH treated spores. There were slight increases in the 1100 and 1030 cm\(^{-1}\) bands, which were associated with different forms of phosphates and C-O stretches of polysaccharides.
Figure 4.10: Absolute IR spectra of untreated D4 spores, and those treated with 1% NaOH and water for 10 min at 65 °C. Spectra were in the region of 1800 to 900 cm\(^{-1}\). Spectra are offset vertically.
4.3.6.3 Transmission electron microscopy of treated spores

In order to determine if there were any physical modifications to the structure of D4 spores after the NaOH or water treatments, cross-section TEM images of D4 spores stained with ruthenium red were examined (Figure 4.11). A stark difference when comparing these images of D4 spores with the ones seen in the previous chapter (Figure 3.2), was the lack of an exosporium. This was due to the 2 weeks of storage at 4 °C after harvesting. Even though the exosporium is absent, the spore coat along with the cortex and membrane around the core remain intact. Spores treated with water at 65 °C for 10 min still possessed an intact coat, cortex and core. It appears that some spore coat debris was also present with the sample. An important feature were the ‘white holes’ found within all of the spores. This could be due to the resin not infiltrating completely during the preparation for the TEM. Spores treated with 1% NaOH at 65 °C for 10 min revealed a peeling and cracking of the spore coat for nearly all the spores seen in the sample. In most cases however, the cortex along with the core remained intact. Despite the cracks in the spore coats, they still remained attached to the spore in a majority of cases. However there were some spore ‘protoplasts’ were visible lacking a spore coat.
Figure 4.11: Figure caption on following page.
Figure 4.4.11: TEM images of ruthenium red stained thin sections of D4 spores. (A) Untreated sample, (B) Water treated for 10 min at 65 °C and (C) treated with 1% NaOH for 10 min at 65 °C.
4.4 Discussion

The aim of this section was to develop accurate means of assessing the number of spores adhering to a surface and to examine the kinetics of attachment and the effects of the suspending media on the spore adhesion to surfaces.

To determine the number of spores attaching to a surface during an adhesion assay, a viable plate count method was compared with a direct count method involving staining with the fluorescent dye DAPI. The direct count method consistently detected $0.6 \text{ Log}_{10} \text{ CFU cm}^{-2}$ more spores on the surface than detected by the viable plate count method. Previous studies have examined the differences between these two approaches for vegetative cell adhesion and found similar differences (30, 277). However the direct count method has a detection limit. Under the high magnification (1000×) the method could only detect a minimum of $4 \text{ Log}_{10} \text{ CFU cm}^{-2}$. This detection limit could be decreased under lower magnification (400×) however it became difficult to distinguish between individual spores that were clustered in a group or the difference between spores and artefacts. Both of these factors could influence the final number of spores. Although the viable count method failed to enumerate the total numbers of spores present on a surface, the difference when compared with the direct count method was consistent across surfaces with different spore densities. The viable count method also had a lower detection limit (~10 CFU cm$^{-1}$), which was crucial for detecting spore densities on potential anti-fouling surfaces or the measurement of residual spores found on the surface after a CIP treatment.

There appeared to be a linear relationship between the number of spores in suspension and the number of spores adhered to a stainless steel surface (Figure 4.5). This implies that adhesion of spores may conform to the Langmuir model. However to confirm that the data conforms to this model, a detachment study would need to be performed. Despite increasing the number of spores in suspension, a stationary endpoint where spores were bound to all possible ‘binding sites’ on the surface was not obtainable. The stationary endpoint for these spores could be found if a greater concentration of spores could be produced, however this could not be achieved using present culturing techniques. Instances where adsorption kinetics does not conform to the Langmuir model have been reported. In these cases a plot of microbial suspension concentration versus adhesion density revealed a sigmoidal attachment kinetics
indicating multilayer attachment through interactions between adhered organisms and those in the suspension (143). This was not the case for the adhesion of D4 spores to stainless steel where the relationship is indicative of monolayer attachment. This was confirmed with direct microscopic observation where spores seemed isolated and there was little aggregation on the surface.

Spores adhered rapidly to the surface of stainless steel with stationary endpoint being reached within 2 min with very little change in numbers over a 3 hour period. This rapid adhesion can be attributed to the convective mass transport of spores to the surface under turbulent flow. Relating this information to the dairy industry, spore adhesion would be rapid in theory, due to convective transport. A boundary layer still exists near the surface during turbulent flow, although small, sedimentation and diffusion would still be the mechanisms of transport for spores to come into close contact with the surface. In contrast to this, previous research has found the stationary endpoint to occur over a period of hours (255, 260). However many of these studies have used vegetative cells which were metabolically active and motile. It should be noted that the flow and shear rates in the two separate reactor systems used in this study would be different. This could impact the kinetics of the experiment. To correct this, attachment and detachment studies could be carried out in a tightly controlled system with direct microscopic observation.

Temperature did not play a role in the number of spores from isolate D4 adhering to stainless steel. This contradicts a previous study of bacterial adhesion, which found that temperatures in the range of 20° to 37 °C increased the numbers of *Listeria monocytogenes* adhering to stainless steel (146). However cells were suspended in nutrient media at different temperatures and were metabolically active. This resulted in different intensities of surface polymer and flagella expression, which influence cell surface characteristics and attachment. During milk powder production, spores are suspended in nutrient rich conditions. Results from this study indicate that the adhesion of spores to stainless steel can occur anywhere within the plant regardless of temperature. Only when conditions are favourable, such as optimal growth temperature and water activity can spores germinate and grow to form biofilms. This has been seen in previous studies where biofilm growth was detected within the pre-heating and first effects of the evaporators where both temperature and water activity were optimal (211).

In the current study, spore adhesion increased as the ionic strength of the
suspending medium increased. This is a recognised phenomenon shown in previous studies (190, 246). The ionic strength of the medium can influence adhesion through two mechanisms. Firstly, as the ionic strength of the medium increases, the electric double layer decreases, thereby reducing electrostatic repulsion and allowing the organism to come into closer contact due to a decrease in the energy barrier between the primary and secondary interaction minimums. The effects of electrostatic interactions are described in greater detail in the following chapter. Secondly, the charged ions in the suspending medium can also cause electrostatic screening between functional groups within the polymers on the surface of a micro-organism, and thus influencing their conformation. This effect was seen in the previous chapter using pH dependance FTIR techniques (section 3.3.4.4).

A previous study observed similar increases in attachment under low pH conditions for *B. cereus* spore adhesion to hydrophilic and hydrophobic glass surfaces (108). This increased attachment at pH 3 relates well with the IEP of both spore and that of stainless steel at pH 4 in 0.01 M KNO₃ (25). Since the net surface charge is near zero at pH 3 for both surfaces, the electrostatic repulsion that exists between the two surfaces is also reduced thereby reducing the energy barrier for adhesion. The changes in pH also affect the conformation of polymers present on the spore surface as seen in the movement of the spore relative to the ZnSe surface in the pH difference IR data. Conditions above or below the IEP, cause the spore surface polymers to be more fully dispersed and therefore affect steric stability (191, 192, 218) resulting in a decrease in spore attachment.

The significance of the surface characterisation findings presented in this study to spore attachment in the dairy environment can be considered in two parts. Firstly, if we consider spores from a single isolate, we have shown that changes in pH can significantly affect the number of spores attaching to stainless steel (Figure 4.7). A similar result was seen from the MATH assay (Figure 3.5), where a larger number of spores were found bound to the hexadecane droplets at pH 3 compared with pH 6.8 for isolates D4, E7 and CGT-8. As previously discussed, this was attributed to both electrostatic and steric interactions of surface polymers. Secondly, it is important to consider how the substrate and spore surface characteristics impact on the number of spores attaching. This will be covered in further detail in the following chapter. Thus the spore surface pH-responsive functional groups addressed in this work play a significant but not always dominant role in the adhesion propensity of spores, which
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appears to be determined by a mixture of factors that are present to different extents in different spore isolates.

Common practice in the dairy industry involves the recycling of caustic wash solutions to clean particular portions of the plant. In the current study, the effects of a model caustic only wash on spore viability and its surface properties and how this influences their ability to attach to stainless steel surfaces was analysed. When spores of isolate D4 were suspended in 0.1 M KCl at pH 10, there was a 0.3 Log$_{10}$ CFU cm$^{-2}$ increase in attachment to stainless steel compared with adhesion at pH 6.8, however this increase was not significant. Spores of isolate D4 exposed to a 1% caustic solution at 65 °C for 10 min resulted in a 2 log reduction in spore viability, however surviving spores attached an order of magnitude higher than untreated spores. Spores that were treated with 1% caustic displayed an increase in negative charge and increase in relative hydrophobicity as determined by the MATH assay at pH 3 to reduce electrostatic interactions. This is a similar result to a previous study where Lindsay et al. found an increase in cell surface hydrophobicity of certain Bacillus spp. after treatment with 1% caustic (139). Contact angle measurements on caustic treated spores was not possible due to the inability to collect enough spores to form a spore lawn. It was difficult to distinguish any differences in the absolute spectra obtained between the caustic treated spores. However small changes in peak heights were observed within the carbohydrate and phosphate regions of the spectra. This is probably indicative of small changes in the conformational structure of polymers present on the spore surface rather than a loss of material or functional group. This is confirmed with the TEM where spore coats were ruptured but remained connected to the spore.

These small differences between the IR spectra of the untreated and treated spores relates well to what was seen in the cross-sectional TEM images. However a quantitative analysis of the differences between treated spores could not be carried out since it was unsure how much protein, represented by the amide II band, was left on the surface after NaOH treatment. Therefore spectra could not be normalized between treated samples. For water treated spores, the spore coat remained intact while for NaOH treated spores, the spore coat contained breaks but was still found surrounding the spore. The increase in hydrophobicity of NaOH treated spores could be due to the partial hydrolysis of the spore coat revealing more hydrophobic groups within the spore coat. Previous studies revealed an insoluble fraction of coat proteins after
hydrolysis of coat from *B. subtilis* spores (57). The insoluble proteins left in suspension could form clumps and adhered to the spore surface thereby increasing their relative hydrophobicity. This increased hydrophobicity could result in an increase the number of spores adhered to a substrate through attractive Lifshitz van-der Waals forces.

The practice of recycling caustic wash solutions reduces the manufacturing costs by reducing the amount of fresh caustic needed to clean the plant, however this increases the risk of re-contamination of surfaces with bacteria, and especially spores, which can still be viable. This could ultimately lead to early contamination of surfaces and eventually biofilm formation before the plant starts to process a product.
Chapter 5

Spore Adhesion to Substrata
5.1 Introduction

Biofilm formation due to bacterial adhesion to surfaces is a problem in a variety of engineered systems such as ship hulls (46), medical devices (166) and food processing (61, 67, 101). As stated earlier, the bacterial attachment process is dependent on a number of factors, including mass transport, suspending media composition and physico-chemical interactions. This chapter will investigate the physico-chemical interactions between a spore and the substratum and how modification of a substratum can influence spore adhesion.

The original DLVO theory was developed to explain the stability of colloids by two independent research teams, Derjaguin and Landau (51) and Verwey and Overbeek (256). The DLVO theory describes the interactions between colloid particles and between colloid particles and substrate surfaces due to the influence of repulsive electrostatic and attractive van der Waals forces with respect to distance. Like most naturally occurring surfaces, microbial cell surfaces are usually negatively charged and vary in their surface hydrophobicity. As they are often thought of as being colloidal particles, the DLVO model has commonly been applied to the adhesion of micro-organisms to surfaces (22, 248). However living cells are not inert particles as they contain a heterogenous array of functional groups, which respond to changes in their environment. These changes can result in the micro-organism having completely different surface characteristics under different environmental conditions.

Lifshitz-van der Waals forces arise from non-polar interactions and are typically long-ranged attractive forces for colloidal particles. These interactions include; randomly orienting dipole-dipole (orientation) interactions, randomly orienting dipole induced-dipole (induction) interactions and fluctuating dipole induced-dipole (dispersion) interactions. The most important parameter when determining the influence of Lifshitz-van der Waals forces over a given distance between interacting surfaces is the Hamaker (H) constant, which takes into account the physico-chemical aspects of the suspending medium. For simple systems there is an approximate relation between the Hamaker constant and interfacial tension (252).

As stated earlier, most bacteria and natural surfaces are usually negatively charged. This results in electrostatic repulsion between the cell and the substratum. This intensity of interaction depends on the surface potentials and electric double layer thickness of both interacting surfaces. The thickness of the electric double layer
is inversely proportional to the square root of the ionic strength. Therefore, as the ionic strength of the surrounding medium increases, the repulsion range of the force due to electrostatic interaction will reduce allowing for close range specific interactions to irreversibly bind the cell to the surface.

The original DLVO theory was modified by van Oss (254) to include Lewis acid-base interactions, thus creating the extended DLVO (XDLVO) theory. Lewis acid-base interactions are often referred to as electron acceptor and electron donor interactions respectively and for particles immersed in water they can either be repulsive or attractive. For particles having un-matched electron donor and acceptor parameters (ie. One is greater than water while the other is smaller) more water molecules are needed to balance the electron donor and acceptor potentials and the solvation layer has a greater density than that of the bulk liquid. This results in a repulsive force at a close distance (<5 nm) between the two surfaces (60). The opposite of this occurs when the electron donor and acceptor properties of a particle are near the values for water (25.5 mJ m⁻²), which results in a decrease in the density of the solvation layer resulting in hydrophobic attraction (60, 268). This phenomenon of solvation layers and hydrophobic attraction has been measured by force balance measurements (176).

Stainless steel is commonly used in the dairy industry since it is corrosion resistant and easily cleanable. However, bacteria are known to adhere to stainless steel surfaces within the dairy plant (68, 69, 175). Since microbial attachment precedes biofilm formation, surface modification to reduce the ability of bacteria to adhere, has been suggested as a possible solution to prevent biofilm formation. Several studies have examined the effects of surfaces with altered characteristics on microbial attachment (48, 136) and specifically within the dairy industry (62, 201). Modifications to reduce attachment include altering the surface characteristics of stainless steel in terms of hydrophobicity and surface charge, by impregnating the metals with different ions or applying surface coatings such as Ni-Cu-P-PTFE (209).

A number of publications have reported that micro-organisms associated with the dairy industry, particularly spores, attach in higher numbers to surfaces with a lower surface energy (hydrophobic) and attach in lower numbers to surfaces with a higher surface energy (hydrophilic) (62, 201). In contrast, it has been reported that some bacteria attach in lower numbers to hydrophobic surfaces (242, 279, 280). Surfaces that prevent non-microbial fouling are also important as the presence of
milk proteins and salts can reduce heat transfer and also influence bacterial attachment depending on the organism, surface and proteins involved (13). Hydrophobic surfaces, such as Ni-Cu-P-PTFE, have been shown to reduce calcium phosphate and protein fouling (201).

The effectiveness of surface modification in reducing bacterial attachment depends on the type of micro-organism, the nature of product being processed and conditions within the plant. If a modified surface is found to reduce bacterial and non-microbial fouling, its effect on heat conductivity and its cost also needs to be considered. For example, if a modified surface reduces heat transfer, the operation would require more energy to reach the desired temperature. Coatings also need to be durable and persist through numerous CIPs or be easy to re-apply after cleaning. Surface modification remains an attractive approach to limit biofouling within a dairy plant (129, 155). Molecular brush coatings (eg. polyethylene glycol) using steric hindrance to block bacterial attachment, have shown reduction in microbial attachment but the systems studied have largely been on glass (105).

In the dairy industry, surface conditioning is often referred to as fouling and was suggested by Rosmaninho et al. (201) to take place as soon as a dairy product is brought into contact with stainless steel. Hinton et al. (97) concluded that fouled stainless steel allowed faster attachment and growth of dairy thermophiles. Conflicting opinions exist on the importance of a conditioning film in initial bacterial attachment, with the presence of proteins such as albumin, gelatin and fibrinogen inhibiting attachment of a marine Pseudomonas to polystyrene (66). Parkar et al. (175) demonstrated that the presence of skim milk on a surface of stainless steel, even at concentrations as low as 1%, reduced the attachment of spores and vegetative cells of thermophilic bacilli. Skim milk was also found to reduce the attachment of S. aureus, Listeria monocytogenes and Serratia marcescens to stainless steel (13). Even individual milk components such as casein and β-lactoglobulin were reported by Helke et al. (91) to reduce attachment of L. monocytogenes and Salmonella typhimurium to stainless steel. One reason for the reduced attachment may be that the proteins in the bulk fluid phase compete for binding sites on the surface of the stainless steel, reducing the number of sites for bacterial attachment. However, Speer & Gilmour (226) reported that stainless steel and rubber surfaces treated with either whey proteins or lactose demonstrated an increase in attachment of milk-associated micro-organisms. Flint et al. (69) also reported higher attachment of Bacillus
**stearothermophilus** to stainless coupons coated with skim milk foulant created by denaturing skim milk on the surface by autoclaving. Milk proteins are a good substrate for bacterial growth with the presence of milk aggregates creating a suitable supporting substrate (273). Stainless steel covered in a milk foulant layer has been shown to attract up to 100 times more bacteria than clean stainless steel (69) however skim milk proteins adsorbed to stainless steel have also been shown to reduce the number of bacteria attaching to stainless steel (13, 91). The effect of milk proteins on bacterial attachment to a substrate is likely to be dependent on whether they are in a denatured or native state with the denatured state presumably providing a large surface area for attachment.

It has previously been shown that milk protein adsorbed to a stainless steel surface significantly reduced the adhesion of a number of bacterial species, with the preadsorbed β-casein fragment reducing adhesion of *Staphylococcus aureus* by around 87% compared to adhesion to untreated stainless steel (13). However, a concern with using physically adsorbed protein films is their relative instability. For example, it has been reported that covalently coupled heparin and hyaluronan modified silicone surfaces inhibited *S. epidermidis* adhesion by about 75% and were stable in saline for 30 days, while physically adsorbed protein films showed instability within hours (160).

Casein has a molecular weight ranging from 19,007 to 25,230 Daltons and makes up approximately 80% of the protein in milk (240). The major fractions in casein (~80%) are a class of four pH 4.6-insoluble phosphoproteins termed α₁s, α₂s, β- and κ-casein (78). Due to a variety of mechanisms including: steric exclusion; strong hydration effects; the presence of polar groups, such as –COOH, -NH₂, -OH; and the presence of zwitterionic structures it seems likely that the incorporation of casein into a polymer film will produce biocompatible (107) and biodegradable (11) novel antifouling biomaterials.

This chapter comprises of two parts. (1) The characteristics of substrata commonly used in cell adhesion assays such as stainless steel, Thermanox® and glass and the physico-chemical attributes affecting the adhesion of spores from four different isolates of *Geobacillus* spp. are investigated. (2) The effects of surface characteristics of modified substrate surfaces on the initial attachment of spores from isolate D4 and their removal after a model CIP procedure.
5.2 Materials and Methods

5.2.1 Spore preparation

The spores from isolates of thermophilic bacilli CGT-8, D4, E7 and E11 were produced and washed as described in section 2.2.9. Crude spore suspensions rather than spores purified using the PEG two-phase system were used in adhesion experiments in order to determine the nature of debris.

5.2.2 Substrata preparation

Stainless steel 316 2B grade cold rolled coupons (2 x 1 x 0.1 cm) were polished using 800 and 1200 grit sandpaper, washed in acetone at 20 °C and passivated by immersion in 50% nitric acid at 80 °C for 30 min, rinsed in distilled water and sterilized by autoclaving. Glass surfaces were cut from pre-cleaned and autoclaved microscope slides (Esco, Biolab, USA) into coupons (2.5 x 1 x 0.1 cm). Thermanox® is a commonly used tissue culture substrate (148) and is comprised of (PET) (276). Thermanox® (Nalge Nunc International) coupons (2 x 1 x 0.05 cm) came pre-cleaned, gamma irradiated (sterilised) and ready to use.

5.2.3 Functionalised glass substrata

Functionalised glass slides were prepared by Dr. Jingtian Han, Department of Food Science, University of Otago, Dunedin, New Zealand. Glass slides were cleaned in hot piranha solution (H₂SO₄ (98%) and H₂O₂ (30%) at a volume ratio of 7 : 3, rinsed with water and dried at 80 °C for 6 h. The glass surfaces were amine functionalized by treatment with (3-aminopropyl)triethoxysilane (APTES) in a methanol solution according to a previously reported method (47, 48). This surface was denoted as G-NH₂. The GPS-functional glass surfaces were obtained by submerging the glass slides in a (3-Glycidyloxypropyl)trimethoxysilane solution according to the method reported by Krishnan et al. (128). This surface was denoted as G-GPS. Polyacrylic acid functionalized glass was created by casting a polyacrylic acid solution (1 mg mL⁻¹) dissolved in water onto a GPS-modified glass slide. The
slide was then heated at 110 °C in vacuum oven for 1 h before being rinsed with water. This surface was denoted as G-PAA. For polystyrene functionalised glass, slides were immersed in styrene and refluxed for 3h, dip-coated in polystyrene solution dissolved in toluene (1 mg mL\(^{-1}\)) for 20 min and annealed at 110 °C for 10 min. This surface was denoted as G-polystyrene.

### 5.2.3.1 Covalent coupling casein on G-GPS surface

Casein was dissolved in water to prepare a casein solution (1.0 mg mL\(^{-1}\)), and the pH was adjusted to 10.3 with sodium hydroxide (Figure 5.1A). The GPS functionalized glass slides were immersed in the casein solution and held at 70 °C for 6 h with gentle stirring. After completion of the grafting reaction, casein modified glass slides or glass beads were thoroughly washed with water and dried under reduced pressure. This casein modified surface was denoted as G-GPS-Casein.

### 5.2.3.2 Grafting casein on G-NH\(_2\) by chemical modification

To produce casein grafted surfaces, the modification involved two steps (Figure 5.1B): (1) bonding of coupling agent glutaraldehyde to a G-NH\(_2\) surface; (2) covalent bonding of casein to the coupling agents on the modified G-NH\(_2\) surface. For glutaraldehyde modification, G-NH\(_2\) substrates were stirred in a glutaraldehyde solution (5%) in 0.1 M sodium phosphate buffer (pH=7.4) at room temperature for 12 h. The samples were washed thoroughly with buffer solution and water. The covalent attachment of casein was achieved by immersing the glutaraldehyde modified G-NH\(_2\) glass slides in a buffer solution (0.1 M, pH=7.4) containing casein (1.0 mg mL\(^{-1}\)) overnight on a shaker at room temperature (271). The samples were washed and then immersed into a sodium borohydride solution (5.0 mg mL\(^{-1}\)) dissolved in a pH 8.0 borate buffer at 4 °C for 10 min. Finally, the samples were dried as described above and denoted as G-NH\(_2\)-Glutar-Casein.
Figure 5.1: Schematic diagrams illustrating chemical processes during casein surface preparation. (A) G-GPS-Casein and (B) G-NH₂-Glutar-Casein. Notice the initial step of modification involved functionalization of glass using either G-GPS or G-NH₂ for G-GPS-Casein and G-NH₂-Glutar-Casein respectively.
Chapter 5 – Spore Adhesion to Substrata

5.2.4 **Contact angle measurements of substrata**

In order to determine the Gibbs surface energy of substrata, contact angles of two polar (water and formamide) and one non-polar (α-bromonaphthalene) solution were obtained by depositing a drop of each test liquid on the surface of glass, Termanox® or stainless steel (316 grade 2B polish). A series of 10 contact angle measurements was recorded, each within 2 seconds of the drop contacting the surface. Surface Gibbs energies were determined from contact angles using the FTA32 v2.0 software. Energetic properties of the materials were obtained by the Young-van Oss equation as previously outline in Eq. 3.2 (252) The hydrophobicity of the substratum surface can be thermodynamically expressed as a Gibbs energy of aggregation ($\Delta G_{st}$) as previously described from Eq. 3.3 (249). Substrata with hydrophilic surfaces prefer the aqueous phase and display a $\Delta G_{st} > 0$ while hydrophobic substrata tend to aggregate and therefore have a $\Delta G_{st} < 0$.

5.2.5 **Streaming potential data of several surfaces**

The streaming potential measurements for G-NH$_2$, G-PAA and G-GPS substrata were determined using a SurPASS Electrokinetic Analyzer (Anton Paar). Two samples with a planar surface were separated using a spacer creating a gap of 100 µm and placed into a clamping cell. In one of these planar samples, two holes are drilled for electrolyte transport. The streaming potential was determined in the presence of 0.01 M KCl over a pH range, which was adjusted using 0.1 M HCl and NaOH.

5.2.6 **Scanning electron microscopy of modified glass surfaces**

The surface morphology of the modified surfaces was examined using a JEOL 6700F Field Emission Scanning Electron Microscope (JEOL, Japan). Samples were sputter coated with approximately 5 nm of Cr in an Emitech K250 coating attachment (Emitech, UK), and viewed and imaged at 3 kV, using a LEI detector.
5.2.7 Adhesion Experiment

A flow loop reactor system was used to experimentally determine the initial attachment of thermophilic spores to different substrata surfaces. A large volume reactor was used as described in 4.2.5. Coupons were inserted into the silicon tubing and placed along the loop in triplicate groups. Spores of the different isolates were suspended in either 0.01 or 0.1 M KCl or reconstituted skim milk at a concentration of $\sim 1 \times 10^7$ CFU mL$^{-1}$. Spore suspensions were circulated at a speed of 1 m s$^{-1}$ (Reynolds number $1.1 \times 10^4$) through the recirculating loop to model the velocity of fluid movement in a dairy plant. The entire system was held in a water bath at 55 $^\circ$C for 30 min. Coupons were removed, processed and spores enumerated as described in the viable plate count method in section 4.2.2.1.

5.2.8 Cleaning-In-Place

A model CIP procedure using the large volume reactor described in section 4.2.5 was as follows: (i) 5 min rinse with deionized water, (ii) 10 min wash with 1% NaOH at 65 $^\circ$C, (iii) 5 min rinse with deionized water, (iv) 10 min wash with 1% nitric acid 10 min at 65 $^\circ$C, and a final (v) 5 min rinse with deionized water. Different steps of the CIP were achieved by exchanging the reservoir with pre-warmed cleaning solutions at 55 $^\circ$C. Solutions were circulated around the flow loop containing the coupons at 1 m s$^{-1}$. Coupons were removed and the number of viable spores was enumerated as in section 4.2.2.1.
5.2.9 XDLVO Calculations

The total interaction energy $\Delta G_{TOT}^{(d)}$ between a bacterium and a surface over a given distance ($d$) was calculated in the XDLVO theory using equation 5.1.

$$\Delta G_{TOT}^{(d)} = \Delta G_{LW}^{(d)} + \Delta G_{EL}^{(d)} + \Delta G_{AB}^{(d)} \quad \text{(Eq 5.1)}$$

The superscripts indicate interactions due to Lifshitz-van der Waals (LW), electrostatic (EL) and acid-base (AB) forces. Each of these parameters can be calculated using equations 5.2, 5.5 and 5.7. The $\Delta G_{LW}^{(d)}$ between a colloid particle (bacterium) and a semi-infinite flat surface can be calculated by using the following equation:

$$\Delta G_{LW}^{(d)} = -\frac{A}{6} \left[ \frac{2a(d + a)}{d(d + 2a)} \ln \left( \frac{d + 2a}{d} \right) \right] \quad \text{(Eq 5.2)}$$

where $a$ (m) is the radius of the colloid, $d$ (m) is the distance between the two surfaces and $A$ is the Hamaker constant denoted by:

$$A = -12\pi \xi_0^2 \Delta G_{LW}^{adh} \quad \text{(Eq 5.3)}$$

where $d_0$ (m) is the closest distance that can be achieved between the bacterium and a surface. This distance is commonly believed to be 0.157 nm which is regarded as the distance between outer electron shells, or van der Waals boundaries of adjoining non-covalently bound molecules (253). The $\Delta G_{LW}^{adh}$ is detailed in the following formula:

$$\Delta G_{LW}^{adh} = -2 \left( \sqrt{\gamma_{LW}^{mv}} - \sqrt{\gamma_{LW}^{lv}} \right) \left( \sqrt{\gamma_{LW}^{sv}} - \sqrt{\gamma_{LW}^{lv}} \right) \quad \text{(Eq 5.4)}$$

The subscripts stand for adhesion (adh), microbe (mv), liquid (lv) and substrata (sv).

These are surface tension components (mJ m$^{-2}$) calculated from contact angles from Chapter 3 (Table 3.1) for spores, and in this chapter for substrata (Table 5.1). The electrostatic interaction over a given distance ($d$) is given by the following:

$$\Delta G_{EL}^{(d)} = \pi \varepsilon a \left( \xi_1^2 + \xi_2^2 \right) \left[ \frac{2 \xi_1^2 \xi_2^2}{\xi_1^2 + \xi_2^2} \ln \left( \frac{1 + \exp(-kd)}{1 - \exp(-kd)} \right) + \ln \left( 1 - \exp(-2kd) \right) \right] \quad \text{(Eq 5.5)}$$

where $\varepsilon$ is the permittivity of the medium of water which is $80 \times 8.854 \times 10^{-12}$ (C$^2$ J$^{-1}$ m$^{-1}$), $\xi_1$ (V) is the zeta potential of the bacterial surface (obtained from values at pH 6.8 in Figure 3.6), while $\xi_2$ (V) is the zeta potential of the substratum surface (Table 5.1). Only streaming potentials for modified substrates G-GPS, G-NH$_2$ and G-PAA were obtained in this study. While for other surfaces, values from previous research
were used. The value $\kappa$ (m$^{-1}$) is the inverse Debye length calculated for a 1:1 (ie. A salt which disassociates into mono-valent ions such as KCl) electrolyte by:

$$\kappa = 0.328 \times 10^{10} \sqrt{z_i^2 M_i} \quad \text{(Eq 5.6)}$$

where $z_i$ is the valency of the ions, and $M_i$ is the ionic strength of the medium. The interaction of acid-base $\Delta G_{AB}^{(d)}$ forces arising from hydrogen bonding between two surfaces is calculated by the following:

$$\Delta G_{AB}^{(d)} = 2\pi \lambda a \Delta G_{adh}^{AB} \exp \left[ \frac{d_0 - d}{\lambda} \right] \quad \text{(Eq 5.7)}$$

where $\lambda$ is the decay of the acid-base interaction in water which ranges between 0.2 and 1.0 nm (251) and is defined here as 0.6 nm. Below is the calculation for the interfacial acid-base interaction $\Delta G_{adh}^{AB}$ (mJ m$^{-2}$).

$$\Delta G_{adh}^{AB} = -2 \left( \sqrt{Y_{AB}^{rr}} - \sqrt{Y_{AB}^{rr}} \right) \left( \sqrt{Y_{AB}^{rr}} - \sqrt{Y_{AB}^{rr}} \right) \quad \text{(Eq 5.8)}$$

All final values will be given in the units kT, which represents the thermal or Brownian motion energy of an organism. 1 kT = 4 × 10$^{-21}$ J.

### 5.2.10 Statistics

Statistical analysis was performed using SPSS software (SPSS [Statistical Package for the Social Sciences], Inc., Chicago, IL). The influence of spore isolate and substrates was investigated by analysis of variance, followed by a multiple means comparison procedure using Tukey grouping. All tests were performed with a confidence level of 95%.
5.3 Results

5.3.1 Contact angle measurements and Gibbs surface energy of substrata

Glass was found to be extremely hydrophilic with a barely measurable water contact angle of 1° and a $\Delta G_{sls}$ of 29.2 mJ m$^{-2}$ ($\Delta G_{sls} > 0$) (Table 5.1). Thermanox and stainless steel surfaces had larger water contact angles of 63 ± 5° and 54 ± 10°, respectively and $\alpha$-bromonaphthalene contact angles of 8 ± 1° and 15 ± 3°, respectively. This resulted in greater $\gamma_{LW}$ values for Thermanox® and stainless steel of 43.9 and 42.9 mJ m$^{-2}$ respectively compared to glass (34.8 mJ m$^{-2}$). This translated into Thermanox® and stainless steel being hydrophobic with a $\Delta G_{sls} < 0$ at -40.2 and -21.7 mJ m$^{-2}$ respectively. A larger difference was seen for the $\gamma'$ component, with glass having the highest value of 54.4 mJ m$^{-2}$ and Thermanox® and stainless steel having lower values of 6.3 and 16.5 mJ m$^{-2}$ respectively.

Modified glass surfaces had higher water contact angles than unmodified glass. Polystyrene modified glass surfaces had the highest water contact angle at 96° while the G-NH$_2$ functionalised surface had the lowest water contact angle at 45°. The water contact angle for G-NH$_2$-Glutar-Casein G-GPS-Casein and were 58° and 54°. All modified glass surfaces displayed increased $\gamma_{S_{LW}}$ and decreased $\gamma_{S_{AB}}$, which resulted in the $\Delta G_{sls}$ of these surfaces being negative, and therefore hydrophobic. The G-GPS-Casein grafted and amine functionalized surfaces were the least hydrophobic with $\Delta G_{sls}$ of -10.6 and -12.3 mJ m$^{-2}$ respectively, while G-polystyrene was the most hydrophobic at -80 mJ m$^{-2}$.

5.3.2 Streaming potential of substrata

In order to determine the surface potentials of G-NH$_2$, G-PAA and G-GPS in 0.01 M KCl, streaming potential measurements were determined (Table 5.1). Unfortunately streaming potential data was not obtainable for a majority of the surfaces due to the lack of equipment. However reported values from previous research for these surfaces under similar conditions were used. Streaming potential data from reported and from measurements in this study ranged from +14 mV to -50.
mV under neutral conditions for the G-NH$_2$ and G-Polystyrene coated surfaces respectively. However the streaming potential values for several of the substrates were measured at a lower ionic strength (0.001 to 0.01 M) than the conditions used during the attachment studies here (0.1 M). Therefore it is assumed that these streaming potential values would be smaller at a higher ionic strength for these substrata. The surface of stainless steel consists of a passive oxyhydroxide film leading to an overall negative streaming potential of -40 mV in 0.01 M KNO$_3$ (25), while Thermanox® primarily consists of poly(ethylene terephthalate) (PET) (276) and contains a similar streaming potential of -42 mV in 0.001 M NaCl (281). G-GPS contains an epoxy functional group, which was negatively charged (-21 mV) in 0.01 M KCl at pH 6.8. G-polystyrene contains no charged functional groups, however polystyrene colloids suspended in 0.1 M KNO$_3$ display a negative charge (-50 mV) under neutral conditions (164). Casein contains a combination of carboxyl, amine and hydroxide groups, and therefore glass surfaces with grafted casein carry a net negative charge under neutral conditions with an IEP of 4.6.
<table>
<thead>
<tr>
<th>Surfaces</th>
<th>Streaming Potential (mV)</th>
<th>Contact angle (°) ± SDa (n = 10)</th>
<th>Surface tension components (mJ m⁻²)</th>
<th>( \gamma_s^{TOT} )</th>
<th>( \gamma_s^{LW} )</th>
<th>( \gamma_s^{AB} )</th>
<th>( \gamma_s^{C} )</th>
<th>( \gamma_s^{D} )</th>
<th>( \Delta G_{s,s} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stainless Steel</td>
<td>-40*</td>
<td>54 ± 10, 24 ± 7, 15 ± 3</td>
<td>53.8, 42.9, 10.9, 1.8, 16.5</td>
<td>-21.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermanox</td>
<td>-42*</td>
<td>63 ± 5, 17 ± 5, 8 ± 1</td>
<td>53.2, 43.9, 9.3, 3.4, 6.3</td>
<td>-40.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glass</td>
<td>-37*</td>
<td>1 ± 0, 7 ± 3, 40 ± 2</td>
<td>58.2, 34.8, 23.4, 2.5, 54.4</td>
<td>29.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-GPS</td>
<td>-21</td>
<td>47 ± 4, 13 ± 1, 16 ± 1</td>
<td>56.3, 42.6, 13.7, 2.3, 20.2</td>
<td>-14.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-NH₂</td>
<td>+14</td>
<td>45 ± 1, 9 ± 1, 19 ± 1</td>
<td>57.0, 42.0, 15.0, 0.3, 21.4</td>
<td>-12.3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>G-PAA</td>
<td>-38</td>
<td>59 ± 1, 36 ± 1, 19 ± 1</td>
<td>49.7, 42.0, 7.7, 1.0, 15.4</td>
<td>-24.9</td>
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<td></td>
</tr>
<tr>
<td>G-NH₂-Glutar-Casein</td>
<td>n/a</td>
<td>58 ± 1, 38 ± 2, 34 ± 1</td>
<td>46.7, 37.2, 9.5, 1.3, 18</td>
<td>-16.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-GPS-Casein</td>
<td>n/a</td>
<td>54 ± 2, 36 ± 1, 29 ± 1</td>
<td>48.4, 39.2, 9.2, 0.9, 22.2</td>
<td>-10.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-Polystyrene</td>
<td>-50*</td>
<td>96 ± 2, 75 ± 3, 15 ± 3</td>
<td>39.9, 42.8, -2.9, -1.1, 1.979</td>
<td>-80.1</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Table 5.1: Mean contact angle measurements, surface Gibbs energies, and energy of interaction (\( \Delta G_{s,s} \)) for crude and purified suspensions on different substrata. Contact angle of water (\( \theta_w \)), formamide (\( \theta_f \)) and \( \alpha \)-bromonaphthalene (\( \theta_{a-B} \)). * Values for streaming potentials of substrate surfaces were obtained from other studies. Some streaming potentials were not obtainable and therefore denoted as n/a.
5.3.3 **SEM images of modified glass surfaces**

The original glass, functionalised glass and casein modified surfaces were examined by SEM (Figure 5.2). Casein grafted surfaces had a distinctly different surface than the original or functionalised glass surface. The glass, G-GPS and G-NH₂ surfaces were generally featureless with little difference before and after silanization. In contrast, densely packed casein aggregates were clearly present on the G-GPS-Casein and G-NH₂- Glutar-Casein surfaces. The morphologies of the G-GPS-Casein and G-NH₂-Casein surfaces were similar with regular and spherical aggregate domains which were approximately 10 nm in diameter.
Figure 5.2: SEM images of (a) untreated glass and modified surfaces (b) G-GPS (c) G-NH$_2$ (d) G-GPS-Casein and (e) G-NH$_2$-Glutar-Casein. Scale bars represent 100 nm.
5.3.4 *Adhesion of four isolates to various substrata*

The ability of spores from either CGT-8, D4, E7 or E11 to attach to either stainless steel, Thermanox® or glass surfaces, was determined using a simple flow reactor (Figure 5.3). Generally more spores were recovered from Thermanox® and stainless steel compared with glass (5 fold increase for D4, 2 fold increase for other spore isolates). Spore attachment was greater to substrata, which had a lower $\gamma^{TOT}$ such as stainless steel and Thermanox®. According to the Tukey grouping, the substrates were separated on the number of each spore isolate adhering to the different substrates. Glass and Thermanox® were significantly different ($p < 0.05$). The test was unable to differentiate spore attachment to stainless steel from the other materials.
Figure 5.3: Viable spore adhesion counts of *Geobacillus* spp. isolates D4, E7, E11 and CGT-8 recovered from different substrata (glass, Thermanox® and stainless steel). Values represent the means of three replicate coupons and error bars are the standard deviations of the means.
5.3.5 Extended DLVO Curves

For both the spores and the various substrata studied, Lifshitz-Van der Waals and acid-base interactions were calculated using bacterial (Table 3.1) and substratum (Table 5.1) contact angles with a variety of liquids. Electrostatic interactions were determined from spore zeta potential results (Figure 3.6) and substrata streaming potential data from previous studies (Table 5.1). The various contributions of the Gibbs interaction energies according to the XDLVO theory were calculated for all combinations of bacteria and substrata involved in this study.

The XDLVO curves for the adhesion of spores from isolate D4 to glass, stainless steel and Thermanox® are shown in Figure 5.4. A classical distance dependence of the total Gibbs energy was calculated over a 25 nm range which included a predominately negative total Gibbs energy \((G^{Total})\) due largely to the long range Lifshitz-Van der Waals \((G^{LV})\) interaction energy, from 50 to 6 nm, between the spore and the substratum surface. A shallow secondary interaction minimum \((G_{min})\) was calculated at a separation distance of 6 nm for glass, 5 nm for stainless steel and 4 nm for Thermanox®. The depth of the secondary minimum varied between surfaces. Glass had the shallowest minimum at \(-6\) kT while stainless steel and Thermanox® were deeper at \(-9\) and \(-11\) kT respectively. An interaction energy barrier over 100 kT was recorded for glass and stainless steel while Thermanox® lacked an energy barrier. This was due to the acid-base interactions \((G^{AB})\), which only affect the primary minimum due to its short-range characteristics. When the interacting surfaces were hydrophilic, or had an electron donating parameter higher than water and an electron accepting parameter lower than water, the acid-base interactions resulted in a large energy barrier. However for hydrophobic substrata such as Thermanox®, there is a lack of ordering of water molecules on the surface resulting in an attractive acid-base interaction. Therefore XDLVO curves for spores for all isolates displayed a non-existent energy barrier for Thermanox®. The high ionic strength (0.1 M) conditions during the adhesion assay caused the electrostatic interactions \((G^{EL})\) to act over a similar distance to \(G^{AB}\). In all cases the \(G^{EL}\) was positive indicating a repulsive force between both the negatively charged spore and the negatively charged substratum surfaces. The electric double layer thickness \((\kappa^{-1})\) at this ionic strength (0.1 M) was calculated to be 0.96 nm.
Figure 5.4: Distance dependence of the Lifshitz-Van der Waals ($G_{(d)}^{LV}$), electrostatic ($G_{(d)}^{EL}$), acid-base ($G_{(d)}^{AB}$) and total ($G_{(d)}^{TOT}$) Gibbs energies of interaction for the adhesion of D4 spores to (A) glass, (B) stainless steel or (C) Thermanox® (in 0.1 M KCl at pH 6.8). The arrows represent the position the secondary interaction minimum ($G_{sm}$). Note the labelling on A is applicable to all graphs.
Interaction energy between spores of four isolates and stainless steel

The XDLVO curves for the adhesion of spores of isolates CGT-8, D4, E7 and E11 to stainless steel in 0.1 M KCl can be seen in Figure 5.5. Spores of all isolates with the exception of E7 displayed a secondary energy minimum ($G_{sm}$) between 5 and 6 nm away from the surface. This energy minimum varied slightly amongst isolates with CGT-8, E11 and D4 displaying a minimum of -13, -11 and -9 kT respectively. Calculations involving spores of isolate E7 did not reveal a $G_{sm}$ to any of the surfaces tested (data not shown) and therefore adhesion of E7 spores would not be thermodynamically possible. This was due to the positive values obtained for the $G^{LW}$ interaction energies. The positive $G^{LW}$ was a result of the low $\gamma_S^{LW}$ obtained from purified spores of isolate E7 (21.0 mJ m$^{-2}$), which is slightly lower than that of water (21.8 mJ m$^{-2}$). However if the interfacial energy parameters from the contact angles on unpurified spore lawns were used, a negative $G^{LW}$ value was obtained as shown in Figure 5.6. Using these values, a classical distance dependence was obtained with a $G_{sm}$ at 6 nm and a depth of –9 kT.
Chapter 5 – Spore Adhesion to Substrata

Figure 5.5: Distance dependence of the Lifshitz-Van der Waals ($G_{(d)}^{LW}$), electrostatic ($G_{(d)}^{EL}$), acid-base ($G_{(d)}^{AB}$) and total ($G_{(d)}^{TOT}$) Gibbs energies of interaction for the adhesion of spores (in 0.1 M KCl at pH 6.8) from isolates (A) CGT-8, (B) D4, (C) E7 and (D) E11 to stainless steel. The arrows represent the position the secondary interaction minimum ($G_{sm}$). Note the labelling on A is applicable to all graphs.
Figure 5.6: Distance dependence of the Lifshitz-Van der Waals ($G_{(d)}^{LW}$), electrostatic ($G_{(d)}^{EL}$), acid-base ($G_{(d)}^{AB}$) and total ($G_{(d)}^{TOT}$) Gibbs energies of interaction for the adhesion of E7 spores (in 0.1 M KCl at pH 6.8) to stainless steel. The interfacial tensions of either (A) purified or (B) crude spore suspensions were used to estimate the curves. The arrow represents the position the secondary interaction minimum ($G_{sm}$). Note the labelling on B is applicable to both graphs.
5.3.6 Relationships between interaction energy and adhesion of the four spore isolates

The impact of surface parameters such as hydrophobicity, charge and the total surface energy on the adhesion of spores from the four *Geobacillus* spp. isolates to three different substrata was examined.

Previous research had shown that attachment of micro-organisms to a substrate surface could be related to the surface charge or hydrophobicity of the organism and of the substrate (62, 109, 196). In the current study, there was no correlation between hydrophobicity of spores and the number of spores adhered to a surface ($R^2=0.44$) (data not shown). While significant differences were seen between Thermanox® and glass; stainless steel could not be differentiated from the two other substrata. However, hydrophobic substrata such stainless steel and Thermanox® displayed higher numbers of spores attaching compared with glass which is hydrophilic. A trend was seen in relation to spore zeta potential and adhesion with the least negatively charged spore D4 adhering in greater numbers compared to the most negatively charged spore E7, however this result was not significant ($R^2=0.82$) (data not shown).

Comparisons involving the hydrophobicity or zeta potential of just one surface, be it that of the spore or substrata, do not provide an accurate assessment of the adhesion process since it involves an interaction between all of these forces over separate distances. For this reason, the XDLVO has often been used to describe the process of adhesion by examining the impact of forces on the adhesion of a micro-organism as it comes into closer contact with a substratum. Previous studies found that the depth of the secondary interaction minimum governed the adsorption and desorption frequencies of several different species of bacteria to a surface (136, 153). However in the current study, we failed to find a relationship between the number of spores of all isolates, with the exception of D4, adhering to the three different substrata and secondary interaction minimums ($R^2=0.003$) (Figure 5.7). This was largely due to the fact there was not a large difference between the number of spores adhering for isolates CGT-8, E7 and E11. However spores of isolate D4 displayed a significant difference when comparing the number of spores adhering to a substrate and secondary energy minimum ($R^2=0.99$). This result is similar to what other studies
have found, where the deeper the secondary minimum the greater the number of bacteria adhering (136).

Since spores of isolate D4 displayed the greatest differences in numbers attaching to glass, stainless steel or Thermax®, D4 was used in further experiments investigating the effect of modified glass surfaces on initial spore adhesion and removal using a CIP procedure.

![Graph showing relationship between spore adhesion and Gibbs interaction energy at the secondary minimum.](image)

Figure 5.7: Relationship between the number of spores from (♦) CGT-8, E7 and E11 and (■) D4 attaching to glass, stainless steel or Thermax® and the Gibbs interaction energy at the secondary minimum ($G_{sm}$). Values represent the means of three replicate coupons and error bars are the standard deviations of the means.
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5.3.7 Attachment of D4 spores suspended in a low ionic strength medium

The number of spores from isolate D4 suspended in 0.01 M KCl attaching to glass, stainless steel, Thermanox® and modified substrata was assessed (Figure 5.8). There was a ~ 1.5 log difference in the number of spores adhering to G-PAA compared with G-polystyrene (2.78 to 4.20 Log_{10} CFU cm^{-2}). The number of spores adhered to stainless steel was 3.65 Log_{10} CFU cm^{-2}. According to the Tukey’s groupings, the nine materials were classified into different groups depending on their ability to be soiled by spores of isolate D4 (Table 5.2). Only materials not exhibiting a common letter are considered to be significantly different from each other. The G-GPS coated surface was found to be significantly different when compared with the stainless steel and other more hydrophobic surfaces. Other modified glass surfaces were not significantly different when compared with stainless steel.

The number of spores adhering to each surface after a standard caustic acid wash varied. Differences of 1 log were observed between the different surfaces while the numbers of spores remaining after the CIP was similar to the initial numbers attaching. The G-PAA modified surface had the lowest number of spores adhered after a CIP at 1.29 Log_{10} CFU cm^{-2} while G-NH_{2}-Glutar-Casein had the greatest at 2.83 Log_{10} CFU cm^{-2}. The number of spores on stainless steel after a CIP was 2.01 Log_{10} CFU cm^{-2}. According to the Tukey’s test, there was a wider variation in the number of spores left adhering after a CIP. The G-PAA surface had significantly fewer spores remaining on the surface after CIP compared with stainless steel, G-polystyrene and Thermanox® and G-NH_{2}-Glutar-Casein.
Figure 5.8: The (■) initial attachment of D4 spores suspended in 0.01 M KCl under flow for 30 min to different substrata, and the (□) number of residual spores after a CIP at 55 °C. Values represent the means of three replicate coupons and standard deviations of the means.
<table>
<thead>
<tr>
<th>Substrata</th>
<th>Initial Adhesion</th>
<th>Residual Adhered Spores after a CIP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (Log_{10}</td>
<td>Tukey’s Grouping</td>
</tr>
<tr>
<td></td>
<td>CFU cm^{-2})</td>
<td></td>
</tr>
<tr>
<td>G-PAA</td>
<td>2.78 A</td>
<td>1.29 A</td>
</tr>
<tr>
<td>G-GPS</td>
<td>2.93 A</td>
<td>1.66 AB</td>
</tr>
<tr>
<td>G-GPS-Casein</td>
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<td>1.65 AB</td>
</tr>
<tr>
<td>Stainless Steel</td>
<td>3.65 BC</td>
<td>2.01 BC</td>
</tr>
<tr>
<td>Glass</td>
<td>3.68 BC</td>
<td>1.47 AB</td>
</tr>
<tr>
<td>G-Amine-Casein</td>
<td>3.70 CD</td>
<td>2.83 D</td>
</tr>
<tr>
<td>G-NH₂</td>
<td>3.89 D</td>
<td>2.31 CD</td>
</tr>
<tr>
<td>Thermanox</td>
<td>4.12 D</td>
<td>2.53 CD</td>
</tr>
<tr>
<td>G-Polystyrene</td>
<td>4.20 D</td>
<td>2.53 CD</td>
</tr>
</tbody>
</table>

Note: Only substrata not exhibiting a common letter are considered to be significantly different.
5.3.8 Attachment of D4 spores suspended in a high ionic strength medium

The number of spores from isolate D4 suspended in 0.1 M KCl attaching to glass, stainless steel, Thermanox® and modified substrata was assessed (Figure 5.9). Spores of isolate D4 suspended in a high ionic strength medium (0.1 M) displayed a similar trend in the number of spores adhering between the nine different substrates compared with spores suspended in 0.01 M KCl (Figure 5.8). Both G-PAA and G-GPS had the lowest number of spores attaching at 3.28 and 3.50 Log_{10} CFU cm^{-2} respectively. This was 0.5 log greater than the attachment under low ionic strength (0.01 M) conditions (Figure 5.8). The substrates with the greatest number of spores attaching were G-NH₂, G-polystyrene and Thermanox® ranging from 3.98 to 4.36 Log_{10} CFU cm^{-2}. Tukey’s grouping of the nine different substrates can be seen in Table 5.3. Like spore attachment under low ionic strength conditions, G-GPS and G-PAA substrates were significantly different from stainless steel, G-NH₂, G-polystyrene and Thermanox®. The casein coated surfaces also had significantly fewer spores attached compared with hydrophobic and positively charged substrates.

The number of spores remaining on each substratum after a CIP treatment was similar to the number remaining in the low ionic strength trial. The casein coated substrates had over a 2 log reduction in spore numbers. This is in sharp contrast to the higher residual spore count at the lower ionic strength for G-NH₂-Glutar-Casein, which in this trial, had the lowest spore count after the CIP. Thermanox® only had a 1.28 log reduction in spore numbers.
Figure 5.9: The (■) initial attachment of D4 spores suspended in 0.1 M KCl under flow for 30 min to different substrata, and the (□) number of residual spores after a CIP at 55 °C. Values represent the means of three replicate coupons and standard deviations of the means.
Table 5.3: The mean number of adhering D4 spores suspended in 0.1 M KCl and the grouping of substrata according to Tukey’s test.

<table>
<thead>
<tr>
<th>Substrata</th>
<th>Initial Adhesion</th>
<th>Residual Adhered Spores after a CIP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (Log_{10} CFU cm^{-2})</td>
<td>Tukey’s Grouping</td>
</tr>
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<td>G-GPS</td>
<td>3.28 A</td>
<td>1.39 AB</td>
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<tr>
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<td>3.50 AB</td>
<td>1.72 ABC</td>
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<td>3.52 AB</td>
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<td>G-Amine-Casein</td>
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<td>Glass</td>
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<tr>
<td>Thermanox</td>
<td>4.36 C</td>
<td>3.08 D</td>
</tr>
</tbody>
</table>

Note: Only substrata not exhibiting a common letter are considered to be significantly different.
5.3.9 Attachment of D4 spores suspended in skim milk

Spores suspended in skim milk displayed slight differences in the number of spores adhering to the nine different substrata tested (Figure 5.10). In contrast to previous experiments carried out with spores suspended in 1:1 electrolyte solutions, the G-NH₂ and G-polystyrene surfaces had fewer spores attaching than the casein and G-PAA coated surfaces. However these slight differences were not statistically different, and all nine substrata were categorized under the same group according to the Tukey’s grouping (Table 5.4).

After a CIP, there was a ~2 log reduction in the number of spores on the G-GPS-casein coated surface which was statistically different from the reduction in the number of spores on the Thermanox® surface (1.5 log reduction). The number of spores remaining on all other substrata after the CIP treatment was not statistically different when compared with G-GPS-casein.
Figure 5.10: The (■) initial attachment of D4 spores suspended in skim milk under flow to different substrata, and the (□) number of residual spores after a CIP at 55 °C. Values represent the means of three replicate coupons and the error bars are the standard deviations of the means.
Table 5.4: The mean number of adhering D4 spores suspended in reconstituted skim milk and the grouping of substrata according to Tukey’s test.

<table>
<thead>
<tr>
<th>Substrata</th>
<th>Initial Adhesion Mean (Log&lt;sub&gt;10&lt;/sub&gt; CFU cm&lt;sup&gt;-2&lt;/sup&gt;)</th>
<th>Tukey’s Grouping</th>
<th>Residual Adhered Spores after a CIP Mean (Log&lt;sub&gt;10&lt;/sub&gt; CFU cm&lt;sup&gt;-2&lt;/sup&gt;)</th>
<th>Tukey’s Grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amine</td>
<td>3.53</td>
<td>A</td>
<td>1.62</td>
<td>AB</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>3.60</td>
<td>A</td>
<td>1.80</td>
<td>AB</td>
</tr>
<tr>
<td>GPS</td>
<td>3.61</td>
<td>A</td>
<td>1.72</td>
<td>AB</td>
</tr>
<tr>
<td>Thermanox</td>
<td>3.69</td>
<td>A</td>
<td>2.23</td>
<td>C</td>
</tr>
<tr>
<td>Glass</td>
<td>3.72</td>
<td>A</td>
<td>1.78</td>
<td>AB</td>
</tr>
<tr>
<td>Stainless Steel</td>
<td>3.74</td>
<td>A</td>
<td>1.96</td>
<td>AB</td>
</tr>
<tr>
<td>Amine/Casein</td>
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<td>A</td>
<td>1.68</td>
<td>AB</td>
</tr>
<tr>
<td>GPS/Casein</td>
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<td>A</td>
<td>1.32</td>
<td>A</td>
</tr>
<tr>
<td>PAA</td>
<td>3.84</td>
<td>A</td>
<td>1.56</td>
<td>AB</td>
</tr>
</tbody>
</table>

Note: Only substrata not exhibiting a common letter are considered to be significantly different.
5.3.10 Effect of modified glass surfaces on initial D4 spore adhesion and removal

Modified glass surfaces were chosen to examine the effects of different physiochemical surface properties and suspending medium on initial adhesion and removal of spores from isolate D4. Unfortunately, a comprehensive XDLVO analysis could not be carried out, as streaming potential data for several surfaces used in this study could not be obtained. In the low ionic strength solution there was nearly 1 log fewer spores adhered initially to G-PAA compared with stainless steel. The G-PAA substrate had a much larger negative charge compared with stainless steel, and therefore the force exerted by electrostatic repulsion would be much larger and at this low ionic strength would have a much larger working distance from the surface and therefore increase the depth of the $G_{sm}$. The opposite affect is seen with the spore adhesion to G-NH$_2$ where a greater number of spores were found adhered to its surface. This is due to the G-NH$_2$ surface is being positively charged due to the presence of NH$_3^+$ groups under the pH neutral and the low ionic strength conditions during the experiment. Since the spore and substratum possess opposite charge, this creates a strong attraction force between the surfaces, which would result in a deeper $G_{sm}$ and therefore a larger number of spores adhering. The effect of substratum surface charge is reduced when spores are suspended in a higher ionic strength solution. The number of spores recovered from all substrata increased as would be expected since increasing ionic strength compresses the size of the electric double layer, thereby reducing the range that electrostatic interactions (ie. repulsion) occur. Therefore greater attachment was seen for negatively charged surfaces such as G-GPS and G-PAA at a higher ionic strength. Casein modified surfaces generally had fewer spores adhering compared with stainless steel however this was not significant.

Hydrophobicity of the substrata played a large role in the number of spores attached. Spores generally attached in greater numbers to Thermanox® and G-polystyrene. Both of these substrata had much lower $\Delta G_{slb}$ values (-40.2 and -80.1 mJ m$^{-2}$ respectively). Since the $\gamma_{s}$ values of these two substrata are smaller than water, this resulted in an attractive acid-base interaction due to the disruption of the interfacial water layer at the immediate surface of the substratum. This in turn lowers the $G_{sm}$ due to the decrease in the electrostatic repulsion between the surface of the
spore and substratum resulting in increased spore adhesion, which was observed here.

When spores of isolate D4 were suspended in skim milk, there were no differences in the number of spores adhering to the different substrata. This could be due to the effects of conditioning films masking the physiochemical properties of the modified surfaces and of the spores.

The effect of a CIP on the number of spores which adhered under 0.01 and 0.1 M KCl conditions, generally followed the same trend as shown in the initial adhesion. Hydrophilic and negatively charged surfaces had fewer spores remaining on the surface. The G-GPS-Casein displayed over a 2 log reduction in the number of spores on the substrate surface after a CIP compared with a 1 log reduction for stainless steel. Substrate surfaces with a greater negative charge, which are more hydrophilic created a shallow $G_{sm}$ and a larger energy barrier between the primary interaction minimum and therefore spores would be reversibly bound to a surface and easier to remove. Thermanox® and G-Polystyrene also displayed similar 1 log reductions, however residual spore numbers were still relatively high compared with the other substrata. In the case of these surfaces, the $G_{sm}$ becomes deeper and the energy barrier between the secondary and primary interaction minimums disappears due to the attractive acid-base interactions (Figure 5.4 C), resulting in the spores becoming irreversibly bound and more difficult to remove with a CIP. While there was a two log reduction in sessile spore viability due to NaOH, the viability loss of adhered spores due to both NaOH and nitric acid is unknown, and is something that needs to be assessed in future work.

There was a small difference in the number of spores removed using a CIP after adhesion in the presence of skim milk compared with simple saline solutions. The more hydrophilic and negatively charged surfaces showed slightly higher removal compared with the hydrophobic surfaces. The G-GPS-Casein surface had a significantly greater removal of spores compared with the more hydrophobic Thermanox® after a CIP. These results just reiterate the importance of milk proteins and the influence of conditioning films on the initial adhesion and removal for isolate D4 spores.
5.4 Discussion

The goal of this study was to examine how the surface characteristics of both spore and substrata influence spore adhesion to surfaces. For this to be determined, surface characteristics such as hydrophobicity, surface charge and steric hindrance needed to be measured. The hydrophobicity, surface charge and polymers present on the surface of spores have been addressed and discussed in previous chapters. The current chapter focuses on the characteristics of the substrata and how these forces interact with each other and their influence on the adhesion of spores.

Stainless steel is commonly used in the dairy industry since it is corrosion resistant and easily cleanable. Other studies have reported stainless steel to be hydrophobic (62). Glass and Thermanox® are two other substrates that are commonly used in adhesion assays. Little information is known about the commercial product Thermanox®. Infrared spectroscopy studies revealed that it is made of poly(ethylene terephthalate) (276). Contact angle results revealed a hydrophobic surface even though product information details the surface as being “hydrophilic.” Glass was hydrophilic as shown by the barely detectable water contact angles and positive $\Delta G_{sfs}$ value. While glass is not a common substrate found in DMP, it was the chosen substrate for surface modification since a reliable surface could be created which would not be altered due to a CIP. When casein was grafted to the functionalised glass surfaces, the contact angles of the casein modified surfaces decreased. This is because polar groups, such as $-\text{COOH}$, $-\text{OH}$ or $-\text{NH}_2$, introduced to the surface by the grafting of casein, impact on surface wettability. Note that the contact angles obtained from the two casein modified surfaces were very similar despite differences in the covalent coupling procedures used. It is likely that the water contact angle of the casein modified surface is due to a complex function based on the collective numbers of the surface polar groups (113), surface roughness (observed in SEM images) and the density of the casein on the surface. Glass and surfaces functionalised with APTES or GPS remained featureless. Further modification with casein revealed small deposits around 10 nm in size. These surfaces were still considered smooth over a sub-bacterial scale ($<1\text{nm}$). Previous
studies have shown that the surface of type 304 2B stainless steel contains cracks, fissures and pits which can range in size from being smaller to larger than the size of bacteria (62, 72). While the surface roughness of substrata in this study were not quantitatively measured, several of the modified glass surfaces were qualitatively measured using SEM. These results indicated that these surfaces displayed small aggregations which were on the sub-bacterial scale (<1 μm). Various groups have observed greater cell attachment on surfaces with high surface roughness and thus concluded surface roughness is an important factor in bacterial attachment to inert surfaces (133, 177). However, other studies have reported no correlation between surface roughness and bacterial attachment to inert surfaces (72, 255).

The effects of physico-chemical interactions on the adhesion of spores from four different isolates (CGT-8, D4, E7 and E11) to three commonly used substrata (glass, stainless steel and Thermanox®) were examined. Differences in the number of spores attaching to substrates were less than one log unit between the different spore isolates while spores of isolate D4 adhered to all surfaces in the highest numbers. The number of D4 spores that adhered to stainless steel and Thermanox® was five times greater than that adhered to glass. On the other hand, the number of spores adhering from the isolates CGT-8, E7 and E11 could not be distinguished between the three different substrata. Subtle differences in the number of spores attaching to a variety of physico-chemically distinct surfaces have been reported in previous studies (62, 200). In contrast, larger differences in attachment to substrates have been observed for other species of Bacillus spp. spores (109) and vegetative cells (1, 48, 72).

There have been numerous studies examining the role physico-chemical interactions play in the adhesion of micro-organisms to surfaces (22, 136, 175, 190, 248). In the initial experiments with the four different spore isolates, there were no significant relationships between the hydrophobicity and surface charge of either spore or the substratum on adhesion. The most hydrophilic and least negatively charged isolate D4 attached in the greatest numbers to all surfaces. The surface charge of spores seemed to play an important role in adhesion, with the D4 having the least negative charge attaching in the highest numbers and E7 spores having the greatest negative charge attached the least to all the surfaces. A number of publications have reported that micro-organisms associated with the dairy industry, particularly spores, attach in higher numbers to surfaces with a lower surface energy (hydrophobic) and attach in lower numbers to surfaces with a higher surface energy (hydrophilic) (62,
201). In contrast, it has been reported that some bacteria attach in lower numbers to hydrophobic surfaces (242, 280).

The adhesion of micro-organisms involves an interplay of apolar, polar and electrostatic interactions between surfaces with respect to distance. Therefore the XDLVO theory has been used to relate the initial attachment rate and reversibility of bacterial adhesion. Previous research has found correlations between the secondary energy minimum and bacterial adsorption and desorption rates. However in the current study, no relationship was found between the $G_{sm}$ and the initial numbers of adhered spores of all four isolates. However, the $G_{sm}$ and the initial adhesion of D4 spores to glass, stainless steel and Thermanox® there was a significant correlation. Therefore a greater number of D4 spores attached to a surface as the $G_{sm}$ increased. This result is in agreement with other studies (136). However the lack of a $G_{sm}$ and positive $G_{lw}$ in the XDLVO curves for E7 spore attachment revealed that adhesion is not thermodynamically possible. This is a contradiction to the theory that $G_{lw}$ are usually negative (22). This is most likely due to errors in obtaining the correct $LW$ values for purified spores. Alternatively, other factors besides physicochemical interactions may play a role in spore adhesion.

It has been suggested that species specific exosporium and appendages among Bacillus spores play a role in adhesion (236). Appendages could promote adhesion by overcoming the energy barrier between the primary and secondary interaction minima (248). However this may not be the case in the present study since we have already shown under neutral conditions the polymers present on the spores of isolate D4 create a steric hindrance affect (see previous chapter). However the polymers on spores from different isolates or species will behave differently in different conditions.

Since the spores of isolate D4 displayed the greatest differences in the number of adhering spores between surfaces, it was chosen for further experiments to examine the effects of surfaces designed with different functional properties on initial spore adhesion and removal using a model CIP procedure. Unfortunately, a comprehensive XDLVO analysis could not be carried out since streaming potential data could not be obtained for all of the substrates used in this study. This study showed spore adhesion and removal, suspended in 1:1 electrolyte solutions, was influenced by the functional properties of substrates. Both surface charge and hydrophobicity of the substratum were shown to be important in the initial adhesion of spores. Substrata with a greater
negative charge and those that were more hydrophilic displayed fewer spores attaching and greater removal from a CIP procedure. Substrata which had a positive charge or which were more hydrophobic displayed an increase in the number of spores attaching and decreased removal rates. These results are similar to previous studies with *B. subtilis* and *B. cereus* spores (62, 108, 109). Another important finding from this experiment was the importance of milk proteins on spore adhesion. When spores were suspended in milk, substrata displayed little difference in the initial numbers of adhered spores.

In the dairy industry, surface conditioning due to milk protein adhesion is often referred to as fouling and takes place as soon as a dairy product is brought into contact with stainless steel (201). The results from the current study displayed the 'screening' effects of the foulant layer on the physico-chemical characteristics of each of the substrate surfaces. For example, spores adhered in higher number to negatively charged substrate surfaces, while numbers decreased on positively charged and hydrophobic substrate surfaces. These results agree with a majority of the reports on the importance of a conditioning film in initial bacterial attachment (66, 175). Stainless steel covered in a milk foulant layer has been shown to increase bacterial attachment (69) while other studies have shown skim milk proteins adsorbed to stainless steel reduce bacterial attachment (13, 91, 175). One reason for the reduced attachment may be that the proteins in the bulk fluid phase compete for binding sites on the surface of the stainless steel, reducing the number of sites for bacterial attachment. However these previous studies applied the conditioning film via different mechanisms, either by flow deposition or autoclaving. The effect of milk proteins on bacterial attachment to a substrate, is likely to be dependent on whether they are in a denatured or native state with the denatured state presumably providing a large surface area for attachment. In the current study, the milk proteins were assumed to be in their native conformation since they were deposited onto the substrate surfaces under turbulent flow. The effects of media suspension were also seen for spore removal, where all surfaces appeared to have similar numbers of residual spores after a CIP. This is in contrast to a previous study that found removal rates of spores from mesophilic bacilli from stainless steel increased when spores initially adhered while suspended in milk compared with an electrolyte solution (134).


**Summary**

- The initial adhesion and removal of spores from a single isolate was influenced by the physico-chemical interactions as outlined by the XDLVO theory. Negatively charged hydrophilic surfaces reduced spore attachment and enhanced spore removal. While this relationship was able to describe the adhesion of spores from one particular isolate, it may not be applicable to describe the initial adhesion of spores from all dairy isolates of *Geobacillus* spp.

- Initial adhesion and removal of spores suspended in a simple electrolyte solution does not necessarily reflect what happens when spores are suspended in milk. These results indicate that milk components such as proteins and electrolytes play an important role in the adhesion of spores to substrata through surface conditioning films.
Chapter 6

General Discussion and Future Outlook
The growth of thermophilic bacilli and the spores they produce is a common problem during the manufacture of milk powder (157). Spores found in raw milk can survive pasteurization and adhere to surfaces within the processing lines. If conditions are favourable, these spores germinate and the vegetative cells divide, grow, secrete polymers and form biofilms. Cells and spores can then slough off the biofilms and contaminate the product being processed and other surfaces downstream. These organisms are not pathogenic, however they are used as indicator organisms for plant hygiene. Also, if the milk powder is reconstituted and the conditions are favorable, spores of these organisms will germinate and generate enzymes and acids, which can result in an off-flavoured product (40, 43). Although the attachment of spores to stainless steel is known to occur in DMPs, little is known about the factors that influence the initial attachment, which is the precursor to biofilm formation and contamination of milk powder. In this study, the factors that influence the adhesion of spores from thermophilic Geobacillus spp. were investigated. Adhesion studies revealed particular characteristics were important in the adhesion of spores, and substrates with modified surfaces were produced in an attempt to reduce the number of spores adhering to a surface or reduce the strength of adhesion so a CIP was more efficient at spore removal.

Spores and vegetative cells of thermophilic bacilli *A. flavithermus* and *Geobacillus* spp. were isolated from a milk powder production line in a DMP within New Zealand. Both milk concentrate and stainless steel surfaces obtained from the MRD contained low vegetative cell and spore counts on production runs that samples were obtained from. Samples obtained from the pre-heaters and the first two effects of the evaporators contained the highest numbers of thermophiles. This observation agrees with previous studies where biofilms were established in the pre-heater and evaporator sections of the plant (98, 157, 211) and has been attributed to the optimal temperature and water activity of the milk concentrate within these sections of the production line. Thirty-one isolates were obtained from two different plants within New Zealand, and from different production runs. Of these thirty isolates, eight were chosen due to their ease and reliability of sub-culturing. Of these eight isolates, four were identified as *A. flavithermus* while the remaining four were identified as *Geobacillus* spp. The presence of these genera in milk powder products has been reported in previous studies (157, 194, 202).
The next stage of this study involved the production of large numbers of spores from thermophilic dairy isolates. It was found that base media commonly used for mesophilic bacilli produced the greatest numbers of spores for two *A. flavithermus* and two *Geobacillus* spp. isolates compared with base media used for thermophilic bacilli. The sporulation of *Geobacillus* spp. isolates was reliable across multiple cultures, however this was not the case for isolates of *A. flavithermus*. For this reason four isolates of *Geobacillus* spp. were chosen to further optimize spore yields. Spore yields of these four *Geobacillus* spp. isolates were dependent upon base media, incubation time and the presence of salts associated with sporulation. This was similar to a finding reported in a previous study on spore yields for *G. stearothermophilus* (272). Spores harvested from a liquid culture retained their heat resistance (100 °C for 30 min) despite previous studies where spores harvested from liquid culture were more heat sensitive compared with those harvested from agar plates (197). Harvesting large volumes of spores required for further characterization and adhesion assays proved easier using a liquid culture. For this reason a liquid culturing method was chosen to produce spores for subsequent experiments.

In order to remove any vegetative cells or debris from the spore suspension that could interfere with surface characterization results, two different separation techniques were examined. The PEG two-phase system was superior at removing debris present in crude spore suspensions when compared with the NaBr gradient. Interestingly, spores from the four *Geobacillus* spp. isolates collected at the interface above the PEG rich phase, which is in strict contrast to *B. cereus* spores which collected at the interface between the PEG and phosphate rich phases (208). This result could be attributed to the different surface characteristics such as hydrophobicity and charge of these two different species of spores. Using the PEG two-phase separation system the debris was removed as confirmed in later experiments by DIC microscopy and contact angle measurements. Therefore the PEG two-phase system was chosen to purify spores for further characterization experiments.

In the current study, the surface characteristics of previously un-characterised *Geobacillus* spp. spores were determined using a variety of different techniques. Thin sections viewed under TEM revealed spores of the four isolates shared similar structures with spores of mesophilic species of bacilli (37, 57, 261). Spores contained a core surrounded by a membrane, cortex and lamellar spore coat. Interestingly, of the
four isolates, only three possessed an exosporium while the fourth (E11) did not. However the integrity of the exosporium appeared to vary with spore age as the exosporium of D4 was not as apparent two weeks after harvesting. To the author's knowledge there are no reports on integrity of spore structures with age. This also brings up the question about the spore structure in a dairy environment. Spores introduced during the milking of cows may be older in age, as they may have been generated in silage (151, 156), and could potentially lack an exosporium. While spores that are generated from biofilms during milk powder processing would be much younger and could possess an exosporium. These structural differences between spores produced in silage and in biofilms could be addressed in future research.

The hydrophobicity of spores from four different Geobacillus spp. isolates was determined using two separate methods. The first method involved measuring the relative hydrophobicity through the MATH assay, while the second was through the determination of Gibbs surface energy calculated from contact angles of solvent droplets deposited on spore lawns. Both of these methods confirmed that spores of the four Geobacillus spp. isolates were relatively hydrophilic, however the degree of hydrophobicity amongst the four isolates differed between the two methods. For example, spores of isolate CGT-8 were shown to be the most hydrophobic at both pH 6.8 and 3 in the MATH assay and third most hydrophobic from water contact angles alone. However calculation of the Gibbs surface energy measurements from contact angles of three solvents revealed that spores of isolate CGT-8 had the highest ΔG_{sls} values indicating they were the most hydrophilic of the four isolates. These differences were due to the nature of the two different methods. Previous reports have shown that the MATH assay can be influenced by electrostatic interactions between the hexadecane droplets and the micro-organism (245). This was observed in the current study where spores of three isolates were more hydrophobic due to reduced electrostatic repulsion during the MATH assay at pH 3. The determination of Gibbs surface energy is considered a more reliable method for determining cell surface hydrophobicity due to providing results in standard units rather than a relative scaled as in the MATH assay. However this method determines hydrophobicity on a macroscopic scale (ie spore lawns) rather than on a microscopic scale (ie individual cells). It is also highly dependent on the amount of water bound on the surface of the cells, which could influence the conformation of appendages on the spore surface.
Nevertheless both of the methods confirmed that spores from the four different isolates were predominately hydrophilic.

The relative surface charge of four spore isolates was determined through zeta potential measurements. Spores of all four isolates were negatively charged at the pH of milk. The zeta potential of the spores could be influenced by changes in environmental conditions such as pH and ionic strength. Spores of all isolates displayed a typical profile (266) with an increasing negative zeta potential with increasing pH and an increase in positive zeta potential with increasing ionic strength. The IEP of the spores was between pH 3 and 4. These trends have been reported previously and are indicative of the surface of micro-organisms possessing a heterogeneous array of functional groups such as carboxylates, phosphates and amino groups (191). Rijnaarts et al. proposed that zeta potential measurements of micro-organisms can reveal the nature of ionisable chemical species present on that surface (191). From the zeta potential data in the current study, the surface of the spores of the four spore isolates contained carboxylate, amino and to a lesser extent, phosphate groups. Spores of the isolate CGT-8 had the lowest IEP at pH 3. This may be due to larger amounts of carboxylated polysaccharides on the surface since the pKₐ of this type of carboxylate is 3. In contrast, spores of isolate E7 had the greatest IEP at pH 4, which is indicative of carboxylate associated with protein. To further elucidate the functional groups present on the surface of spores, ATR-IR spectroscopy studies were carried out.

Infrared spectroscopy of spores from the four isolates revealed a surface comprising of carboxylate and amines associated with proteins and carbohydrates. Carboxylates were found to be the functional group mainly responsible for the net negative charge on the spore surface. However the relationship between the relative carboxylate concentration and the degree of negative charge on the spore surface could not be determined. Spore movement relative to the surface of the ZnSe prism was also detected due to the neutralisation of the net charge within surface polymers resulting in compression of these polymers. The spore movements with regards to the surface observed using IR spectroscopy corresponded well with the IEPs determined from the zeta potential data. The spore was at its closest point relative to the prism at its IEP. For example, spores of isolate CGT-8 were closest to the prism at pH 3, while their IEP was at 2.8. Spores of isolate E7 were closest to the prism at pH 4, which correlated well with the IEP of 4. From these characterization results spores of these
four isolates were relatively hydrophilic and negatively charged under neutral conditions. These characteristics were primarily attributed to the presence of carboxylates and amines associated with proteins and polysaccharides on the spore surface.

The next section of the thesis involved analysing the adhesion of spores to surfaces. A critical aspect of this study was the validation of a method to accurately assess the number of spores adhering to a surface. The viable plate count method was compared with a direct count method involving staining with the fluorescent dye DAPI. The direct count method consistently detected more spores on the surface of stainless steel compared with the viable count method. The detection limit for the viable plate count method was below $4 \log_{10} \text{CFU cm}^{-2}$. Since the number of spores attaching to most surfaces was near the detection limit of the direct count method, the viable count method was superior since potential anti-fouling surfaces would have fewer spores than the direct count method could detect.

The adhesion kinetics of D4 spores to stainless steel was also examined. A linear relationship was found between the number of spores in suspension and the number recovered from the stainless steel indicating that the adhesion of spores from this isolate occurred as a mono-layer on the surface of the stainless steel. This was confirmed using direct epifluorescence microscopy where spores were seen on the surface of the stainless steel with little aggregation.

Spores adhered rapidly to the surface of stainless steel with stationary endpoint being reached within two minutes with very little change in numbers over a further three hour period. In contrast to this, previous research has found the stationary endpoint to occur over a period of hours (255, 260). However, these studies used flow systems with low shear rates and vegetative cells that were metabolically active and motile. Previous studies have shown the importance of flow conditions on the initial adhesion of micro-organisms to substrata (20, 269). While the affect of flow conditions and shear rates were not determined in this study, the rapid adhesion observed in the current study may be attributed to the convective mass transport of spores to the surface under turbulent flow. Relating this information to the dairy industry, spore adhesion would be rapid in theory, due to convective transport. Previous studies have shown that increased fluid flow towards or parallel to a substratum surface results in faster adhesion rates of micro-organisms due to higher mass transport (220). While a small boundary layer still exists near the surface during
turbulent flow, sedimentation and diffusion would still be the mechanisms of transport for spores to come into close contact with the surface.

In the current study, spore adhesion increased as the ionic strength of the suspending medium increased and as the pH decreased. Environmental conditions such as pH and ionic strength of the suspending medium have been previously shown to influence adhesion of micro-organisms to surfaces (190, 246). The ionic strength and pH of the medium can influence adhesion through two mechanisms. Firstly, as the ionic strength of the medium increases, the thickness of the electric double layer decreases, thereby reducing electrostatic repulsion and allowing the organism to come into closer contact with the substratum surface. Secondly, the charged ions in the suspending medium can also cause electrostatic screening between functional groups within the polymers on the surface of a micro-organism, thereby influencing their conformation and reducing steric hinderance. This effect was seen using the pH dependance FTIR techniques. In the dairy industry spores are suspended in milk and not simple electrolyte solutions. However it was necessary to investigate the influence of simple environmental conditions on spore adhesion without the confounding effects of proteins and fats in milk. These assays also highlighted the importance of proteins on adhesion when spores were suspended in skim milk in later adhesion assays.

The adhesion of spores from the four spore isolates to a variety of different substrata commonly used in adhesion assays was analysed. In this experiment, spores suspended in a high ionic strength solution (0.1 M KCl) attached in greater numbers to stainless steel and Thermanox® compared with glass. Previous research had shown that the attachment of micro-organisms to a substrate surface could be related to the surface charge or hydrophobicity of the organism and of the substrate (62, 109, 196). In the current study, there was no correlation between hydrophobicity of spores and the number of spores adhered to a surface. Spores of isolate D4 which had the least negative zeta potential adhered in the greatest numbers compared with spores of E7 which had the greatest negative zeta potential, however this result was only significant for stainless steel and Thermanox®.

Comparisons involving the hydrophobicity or zeta potential of just one surface, be it that of the spore or substrata, do not provide an accurate assessment of the adhesion process since it involves an interaction between all of these forces over distance. For this reason, the XDLVO has often been used to describe the process of
adhesion by examining the impact of forces on the adhesion of a micro-organism as it comes into closer contact with a substratum. Previous studies found that the depth of the secondary interaction minimum governed the adsorption and desorption frequencies of several different species of bacteria to a surface (136, 153). However in the current study, there was no relationship between the number of spores of all isolates adhering to the three different substrata and secondary interaction minimum values and distances. The lack of a relationship was due to the fact there was not a large difference between the number of spores of isolates CGT-8, E7 and E11 to the three different substrates. However spores of isolate D4 displayed a significant difference when comparing the number of spores adhering to a substrate and secondary energy minimum. This result is similar to other studies, where it has been reported that the deeper the secondary minimum, the greater the number of bacteria adhered (136). This difference in the number of adhering spores was larger for isolate D4 than it was for the other three isolates. These findings illustrate that the physicochemical interactions that govern the adhesion of spores from a particular species of thermophilic bacilli to a substrata may not act in the same way for spores from different species. Previous research has postulated that electron transfer may exist between the spore and a conducting surface (183). There may also be image charge forces developing which are not accounted for in the DLVO theory (2). Despite this finding, spores of isolate D4 demonstrated more clearly the application of the XDLVO theory and therefore this isolate was chosen for further experiments with modified surfaces.

To investigate the effect of surface properties on spore adhesion, a series of coated glass substrata were produced which contained a range of different surface characteristics such as hydrophobicity and surface charge. When spores were suspended in a 1:1 electrolyte solution, substrata surface characteristics influenced the number of spores adhering. Spores attached in greater numbers to hydrophobic and positively charged surfaces such as polystyrene and amine functionalised substrata. Surfaces which were more hydrophilic or negatively charged such as poly-acrylic acid and epoxy functionalised surfaces had fewer spores adhering compared with stainless steel. However the differences in the number of spores adhering to these surfaces was greatly reduced when spores were suspended in a complex solution such as reconstituted skim milk powder. Under these conditions there were no significant differences in the number of spores attaching between all of the substrata tested. This
finding is due to the complex nature of the suspending medium with proteins depositing on all surfaces creating a conditioning layer and thereby causing the physico-chemical characteristics of all substrata to be similar (24, 117). This result was also observed in a previous study looking at mesophilic spores adhering to different types of surface modified stainless steels suspended in hot milk (62, 200). Individual milk proteins have also been shown to influence bacterial attachment to stainless steel (13). However these findings highlight the point that the constituents of milk play an important role in the adhesion of spores from *Geobacillus* spp.

Frequent cleaning of equipment in a DMP via a CIP is the current method to control biofilms produced by thermophilic organisms during the manufacture of milk powder. Therefore the removal of spores using a caustic/acid wash from substrata with variable surface characteristics was examined. The removal of spores from modified glass substrata, stainless steel and Therma
cox® displayed similar trends that were observed in the initial adhesion. For example, when spores were suspended in an 1:1 electrolyte solution, fewer spores remained attached to hydrophilic and negatively charged surfaces after a CIP than to the hydrophobic and positively charged surfaces. However when substrata were exposed to spores suspended in milk, there were no significant differences in the number of spores removed by a CIP from all of the different surfaces. This result was also seen for the removal of *B. subtilis* spores adhered to modified stainless steels using a CIP (200). In contrast, Faille *et al.* (2000) found larger differences in residual spores and cells remaining on a surface after a CIP (62). A number of factors such as temperature, concentration, flow and shear rates have been shown to influence the cleanability of surfaces during a CIP (38, 229). These studies used different flow systems to wash substrata with bound spores, and therefore it is difficult to compare the results from previous studies to the current one. Nevertheless, the constituents of milk are important in the initial attachment and removal of spores from any type of surface.

Spores of bacilli are generally regarded as being resilient to harsh environments like those faced during a CIP. While there was a significant reduction in spore viability after a 30 min caustic wash at 65 °C in the current study, surviving spores displayed a greater propensity to attach to stainless steel. Surface characterisation results revealed an increase in hydrophobicity and a greater negative charge on the spores’ surface after treatment with NaOH. The increase in hydrophobicity would likely be due to the ruptured spore coats revealing more hydrophobic groups. This
could have serious consequences for a DMP since the re-use of caustic to reduce costs is a common practice. Surviving spores could potentially recontaminate sections of the plant which are cleaned with this recycled caustic wash solution, thereby seeding surfaces with spores at the beginning of the next processing run.

This study has made many contributions into understanding the factors involved in the adhesion of previously unstudied spores from *Geobacillus* spp. to the types of surfaces that may be found in a DMP. This study has used a large variety of commonly used methods to characterise the previously unknown surface of spores from thermophilic bacilli. These results have shown that the thermophilic spore adhesion is not too dissimilar to spores of mesophilic species or even vegetative cells and have confirmed the general consensus that physico-chemical interactions do play a role in spore adhesion. However these forces do not solely govern adhesion. This research has set up numerous avenues for further research using the methods and building on the findings of the current study. Spores of *A. flavithermus* are just as prevalent as a source of milk powder contamination as *Geobacillus* spp. spores (202). Therefore a reliable culturing method needs to be developed to produce large spore yields of *A. flavithermus* for further study. In terms of the initial adhesion of spores, the influences of flow and shear rates were not determined in this study. This could be analysed using a flow cell with tightly controlled fluid dynamics. Furthermore, Nejadnik *et al.* showed that polymer brush coated surfaces reduced the initial adhesion of bacterial cells and increased removal rates with increased shear rates (159). The application of these brush coatings on spore adhesion and removal could be analysed since surfaces are exposed to high shear rates during the manufacturing of milk powder. An important direction into better understanding spore adhesion in the dairy industry is to closely analyse the interaction between a spore, substrate surface and the surface modifying components within milk. Previous studies have found that specific milk proteins can influence bacterial attachment (13). Therefore this should be examined in order to find specific milk proteins which could potentially inhibit or promote adhesion of spores. Furthermore, the implication of the recycling caustic wash solutions to clean dairy manufacturing plant should be investigated, in order to prevent recontamination of manufacturing surfaces with the spores of thermophiles.

In conclusion, thirty one isolates belonging to *A. flavithermus* or *Geobacillus* spp. were isolated from milk powder production lines from two separate DMP in New Zealand. Culturing medium and methods were developed to produce large spore
yields of *Geobacillus* spp. A purification method was also used to remove debris from spore crops which could interfere with surface characterisation results. Three of the four spore isolates possessed an exosporium. However this exosporium was absent from spores two weeks after harvesting, indicating that spore structure can change over time. All four spore isolates were relatively hydrophilic and negatively charged at the pH and ionic strength of milk. These characteristics were attributed to the carboxylates associated with proteins and polysaccharides present on the spore surface. There were differences between the spore isolates in the way they adhered to surfaces with different physico-chemical characteristics. While anti-fouling substrata could be produced to reduce spore attachment in a simple 1:1 electrolyte solution, these anti-fouling properties were reduced when the spores were suspended in complex media such as milk.


29. **Bremer, P., S. Fillery, and A. J. McQuillan.** 2006. Laboratory scale Clean-In-Place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. International Journal of Food Microbiology **106**:254-262.


electron donor and acceptor characteristics under different environmental


defined, synthetic media for sporulation and for germination and growth of

dipicolinic acid, a major component of spores of *Bacillus* species.
Photochemical and Photobiological Sciences 4:591-597.

characteristics of *Bacillus* spores. Current Microbiology 10:329-332.

57. Driks, A. 1999. *Bacillus subtilis* spore coat. Microbiological and Molecular


germination of spores of *Bacillus cereus*. Journal of General Microbiology
65:101-104.

60. Elimelech, M. 1990. Indirect evidence for hydration forces in the deposition
of polystyrene latex colloids on glass surfaces. Journal of the Chemical

adhering living Bacillus spores in milk product processing lines. Journal of
Applied Microbiology 90:892-900.

and T. Benezech. 2002. Adhesion of Bacillus spores and *Escherichia coli*
cells to inert surfaces: role of surface hydrophobicity. Canadian Journal of
Microbiology 48:728-38.

63. Faille, C., V. Lebret, F. Gavini, and J. F. Maingonnat. 1997. Injury and
lethality of heat treatement of *Bacillus cereus* spores suspended in buffer and

64. Fleming, H. P., and Z. J. Ordal. 1964. Responses of *Bacillus subtilis* spores
to ionic environments during sporulation and germination. Journal Of
Bacteriology 88:1529-1537.

Adhesion: Molecular and Ecological Diversity. Wiley.


77. **Fox, A., G. C. Stewart, L. N. Waller, K. F. Fox, W. M. Harley, and R. L. Price.** 2003. Carbohydrates and glycoproteins of *Bacillus anthracis* and


140. **Lindsay, D., V. S. Brözel, and A. von Holy.** 2006. Biofilm-spore response in *Bacillus cereus* and *Bacillus subtilis* during nutrient limitation. Journal of Food Protection **69**:1168-1172.


extraterrestrial environments. Microbiological and Molecular Biology Reviews 64:548-572.


221. **Slepecky, R., and J. W. Foster.** 1959. Alterations in metal content of spores of *Bacillus megaterium* and the effect on some spore properties. Journal of Bacteriology **78**:117-123.


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