The growth of thermophilic bacteria in a milk powder plant and the formation of spores in biofilms of the dairy thermophile *Anoxybacillus flavithermus*

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Abstract

Dairy production makes up 20% of New Zealand's export earnings, with whole milk powder being the number one dairy export. However, contamination of milk powder by spores of thermophilic bacteria is an ongoing problem in the manufacture of milk powder. These spores survive manufacturing processes, contaminate final product and have potential to spoil foods manufactured with milk powder. The main thermophilic organisms that cause concern in the New Zealand dairy industry are *Geobacillus* spp. and *Anoxybacillus flavithermus*. The vegetative forms of these organisms are able to grow within biofilms, but there is very little information as to the origin of their spores found in the product and the conditions under which they are produced. We have now monitored the dynamics and location of spore formation in the industrial and controlled laboratory settings.

A survey was undertaken at the Pahiatua milk powder manufacturing plant to determine the origin and rate of spore formation. The predominant sites of spore formation were the plate heat exchanger and evaporator. Spores began to develop approximately 11 h into an 18 h manufacturing run. The spores were identified as *Anoxybacillus flavithermus* and *Geobacillus* species.

To examine the dependence of spore formation on the development of the *A. flavithermus* biofilm under controlled laboratory conditions, a continuous flow reactor was used. The release of spores and vegetative cells into the milk was measured using change in impedance. Impedance change confirmed the presence of both vegetative cells and spores on stainless steel sample tubes.

At the end of an 8.5 h run at 55°C, using the continuous flow reactor, the total number of thermophilic bacteria released into the milk reached up to 10^6 cells mL^{-1}. At least 10 % of cells attached to the stainless steel surface were spores. These results indicate that spores form readily in biofilms of *A. flavithermus* believed to colonise the surface of the manufacturing plant. When the temperature of the continuous flow reactor was decreased to 48°C no spores were detected within the biofilm.

The results from this study have provided key information about where thermophilic spores form in a milk powder manufacturing plant and how biofilms of one of the typical thermophilic bacteria, *Anoxybacillus flavithermus,* develop. This knowledge will help the dairy industry to design strategies to prevent spore formation.

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Abbreviations

Aw	water activity
CFU	colony forming unit
CIP	clean-in-place
DSI	direct steam injection
EOR	end-of-run
HILLS	hygenic live line sampling
n/d	not determined
PHE	plate heat exchanger
RAPD	randomly amplified polymorphic DNA
SEM	scanning electron microscopy
SFB	static fluid bed
SSHE	scraped surface heat exchanger
TEM	transmission electron microscopy
T/S	total solids
UHT	ultra high temperature
UPGMA	unweighted pair group method using arithmetic averages
VF	vibrofluidiser
w/v	weight per volume

Definitions

Clean-in-place (CIP)	Cleaning regime after a manufacturing run
Direct steam injection (DSI)	A direct method of heat treatment where steam is injected into the milk.
Effect	A section of an evaporator that has the same boiling temperature
Fouling	Build up of milk deposits on the internal surface of dairy plants
Raw milk treatment	Separation, pasteurisation, and standardisation of raw milk
Orifice pans	Located at the top of the evaporator to distribute milk into the pass tubes
Pass	A section of the effect, in the evaporator, that is made up of a set of tubes that the milk passes through
Plate heat exchanger (PHE)	An indirect method of heat treatment that consists of a series of plates where the heating or cooling medium passes on one side, and the milk on the other.
Randomly amplified polymorphic DNA (RAPD) profiles	An electrophoretic gel pattern produced by amplifying random sections of the genome of interest with a short arbitrary primer of approximately 10 nucleotides.
Reactor	A laboratory system used to study biofilm development
Specification	Customers requirement of a product

Static fluid bed	A non-vibrating fluid bed located at the base of the main drier chamber, used for secondary drying of milk powder. Air is directed into the layer of powder at a shallow angle.
Total thermophile count	A plate count of bacteria that grow aerobically in milk and milk products at temperatures of 55 °C or higher (Fonterra, 2000b). This includes both obligate and facultative thermophiles.
Vibrofluidiser (or vibrating fluid bed)	A low frequency vibrating fluid bed that is used for either secondary or tertiary drying of milk powder. Air is directed into the layer of powder at a perpendicular angle.

1 Introduction

The New Zealand dairy industry is a world leader in dairy production. 25% of the international dairy market consists of New Zealand milk products (McGowan, 1996). In comparison with other dairy producing nations New Zealand is unusual; in that 95% of milk products are exported, whereas worldwide only 5% of milk production is exported (McGowan, 1996). Whole milk powder is our number one dairy export, with over 300 000 t produced per year (Pearce and Archer, 1996). Milk powder is used as a source of milk in countries where milk is either unavailable or in shortage. It is also a common ingredient in foods such as confectionery, ice cream, recombined products such as sweetened condensed milk, and baked goods such as breads and cakes. However, each year the New Zealand dairy industry loses millions of dollars because of contamination with thermophilic bacterial spores in milk powder. These organisms are not pathogenic but if the conditions are right these spores can germinate in reconstituted milk, which may result in enzyme and acid production (Basappa, 1974; Chen *et al.*, 2004; Chopra and Mathur, 1984). This can produce an off flavour in the product. High numbers of bacteria are also unacceptable to customers.

1.1 Milk powder manufacture

Milk powder manufacture involves the removal of water from pasteurised milk through evaporation and drying (Bylund, 1995). This process is outlined in Figure 1. The aim of this procedure is to produce high quality milk powder that is of a strict chemical and microbiological standard. Raw milk is transported from the farm to the milk powder plant by tanker and is stored on site before being pasteurised. During transport and storage the raw milk is generally held at 7°C but may reach higher temperatures because no cooling is applied during transportation.

The production of milk powder initially requires raw milk treatment. This involves three steps. These are separation, pasteurisation, and standardisation. The order of these steps is different depending on the dairy plant (McCarthy, 1996). Centrifugal separators are used to separate milk into skim milk and cream. In the past the separation temperature ranged between 35°C and 55°C, but cold separation at 8°C is beginning to be used in some dairy plants (Lang, 2005). A heat exchanger, often a plate heat exchanger (PHE), is used for pasteurisation generally at 72°C for 15s. Pasteurisation is used as a preservation technique to kill pathogenic organisms and many non-pathogenic bacteria associated with spoilage, with little alteration to the physical and chemical composition of the milk (Bylund, 1995). The milk is standardised

by mixing the cream and skim milk to meet protein and fat composition specifications of the final milk powder product. Other ingredients, such as lactose and recycled fractions such as permeate and buttermilk from other dairy manufacturing processes may also be added during the milk treatment process. Permeate is mainly composed of lactose, minerals, and water, and is a by-product from the ultrafiltration process used to concentrate milk proteins. Buttermilk is a by-product from butter plants. Raw milk treatment runs generally last for 8 h.

Following raw milk treatment the milk may be stored transitionally at 7°C before it is directed into the powder plant. Before evaporation, the pasteurised milk is heated by either direct contact or indirect contact heat exchangers or a combination of both (Refstrup, 1998, 2000). In direct contact heaters such as direct steam injection (DSI) the heating medium is directly in contact with the product. In indirect contact heaters such as PHEs the heating medium is on one side of stainless surface and the product to be heated flows past on the other side. The temperature of a heat treatment generally ranges from 75°C to 125°C, depending on the type of powder that is being produced. This heat treatment is used to preheat the milk before entering the evaporator to ensure rapid heating within the evaporator. There are several other advantages of heat treatment. It utilizes waste heat created by the evaporator, kills bacteria and deactivates enzymes, increases the milk's heat stability and resistance to coagulation during more severe heat treatments such as the manufacture of recombined evaporated milk, and generates antioxidants which improve flavour and extend the shelf life of whole milk powders (Lewis, 1994; Newstead, 1996; Pearce, 1996)

Evaporators are used to concentrate the milk. Water is removed from the milk by boiling it under vacuum at low temperatures. Skim milk is concentrated from 9 - 9.5% total solids (T/S) to 45 - 50% T/S and whole milk is concentrated from 12 - 13% T/S to 50% T/S (Mackereth, 1996b). This reduces the evaporative load during drying. The most common New Zealand dairy evaporator is a falling film evaporator. The milk is introduced at the top of the evaporator and passes through heated tubes under vacuum. An evaporator can be divided up into two to five sections. Each of these sections is called an effect and usually operates at a different temperature ranging from 70°C to 45°C. Each effect can be further divided into two to three passes. Each pass is made up of a set of heated tubes. Steam is used to heat the outer surface of the tubes while the milk passes down through the tubes under vacuum. The milk is then pumped to the top where it passes through the next set of pass tubes.

After leaving the evaporator, milk concentrate undergoes a heat treatment and homogenisation followed by drying. The heat treatment (70°C to 80°C) lowers the drying heat load and homogenisation reduces milk fat globule size, preventing

clumping. The drying process can be divided into three stages. Firstly, the atomiser disperses the concentrate as fine droplets into the main chamber. Next, primary drying occurs in the main chamber, where hot air is mixed with the product. Finally the product undergoes secondary drying, where warm air is passed through the product in a fluidised bed. Cyclones, a component of the milk powder manufacture process, are used to recover powder, which would otherwise be lost, from the air used in the drying process. The final product is then packaged. The result is a product that is easily transportable, small in volume and has a long shelf life in comparison with pasteurised milk.

After every run the wet sections of the powder plant are cleaned. These cleaning regimes are called clean-in-place (CIP). A CIP generally consists of a warm water rinse, a 1.5% caustic wash at 75°C for 30 min, followed by a water rinse, then a 0.5% nitric acid wash at 70°C for 20 min, and finishes with a second water rinse (Bylund, 1995). The caustic wash is used to remove organic matter, and the nitric acid to remove inorganic matter. In some plants a sanitiser is also used to kill any remaining microbial contaminants. Manual cleaning (opening and scrubbing) of equipment may occur every 5 - 10 runs or once a month depending on the milk powder plant, when there is a change in specification (milk powder type), or at the end of a milk production season. In New Zealand milk powder plants generally close down for two months over winter (July and August). This is because very little milk is produced over this time because New Zealand milk production is based on grass. During this off-season milk powder plants undergo maintenance.

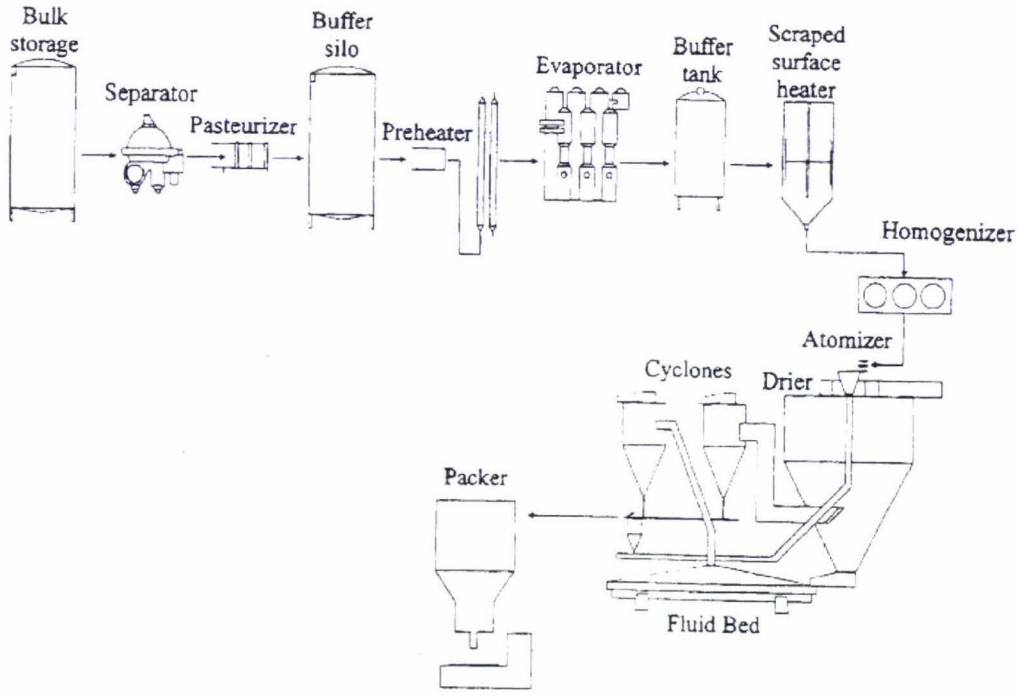

Figure 1: Schematic diagram of the milk powder manufacturing process (Mackereth, 1996a)

1.2 The microflora of a milk powder manufacturing plant

The raw milk that enters a dairy manufacturing plant generally has a bacterial load of 10^2 to 10^3 CFU/mL but may reach levels over 10^6 CFU/mL (Heeschen, 1996). The majority of raw milk contaminants originate from contamination during the milking process, the sources being the interior of the udder, the teats and milking and storage equipment (Slaghuis *et al.*, 1991). Contamination can also occur during transportation.

These contaminants range from Gram-negative bacteria such as *Pseudomonas* spp. and the Enterobacteriaceae family to Gram-positive bacteria such as the micrococci, streptococci, and bacilli (Heeschen, 1996; Slaghuis, 1996). Potential pathogenic organisms such as *Bacillus cereus, Clostridium perfringens*, *Listeria monocytogenes,* and *Staphylococcus aureus* can also be present (Bertilsson *et al.*, 1996; Loncarevic *et al.*, 2005; Sanaa *et al.*, 1993; Slaghuis *et al.*, 1997). These usually originate from a diseased udder of an infected cow (Slaghuis, 1996).

Pasteurisation is usually sufficient to kill most contaminants. Those that do survive, termed thermoduric microorganisms, include some streptococci, micrococci, and sporeforming bacteria of the genera *Bacillus* and *Clostridium* (Aaku *et al.*, 2004;

Chopra and Mathur, 1984; Crielly *et al.*, 1994; Knight *et al.*, 2004). The numbers of thermoduric bacteria in pasteurised milk are generally low and do not cause concern.

Dairy thermophiles (defined as those bacteria able to grow at elevated temperatures of 40 – 65°C), predominantly of the genus *Bacillus*, are able to grow within the preheating and evaporation sections of the milk powder plant (Murphy *et al.*, 1999; Stadhouders *et al.*, 1982; Warnecke, 2001). This results in high numbers of bacteria being released into the final product. The vegetative growth of dairy thermophiles has been well studied but there is little information as to how and where their spores form in milk powder manufacturing plants. Whole milk powder thermophilic spore counts can range from <10 CFU/g to approximately 1 x 10^5 CFU/g, whereas contamination of skim milk powder is usually less than 1 x 10^2 CFU/g (Hill and Smythe, 1994). No explanation was given for this difference in thermophilic spore counts between the two types of powders but it may be because of an increase in fouling that occurs in whole milk powder plants as will be explained in section 1.5.2.

The major thermophilic contaminants in New Zealand milk powders have been identified as *Anoxybacillus flavithermus* or members of the *Geobacillus* group (Flint *et al.*, 2001b; Hill and Smythe, 2004; Ronimus *et al.*, 2003). These organisms were formerly classified in the *Bacillus* genus. Isolates from these two groups have also been found in international powders (Rueckert *et al.*, 2004). The bacterial thermophile *Bacillus sporothermodurans* is also commonly found in European UHT (ultra high temperature) treated milk (Klijn *et al.*, 1997). Other bacilli found in milk powder are the facultative thermophiles such as *Bacillus licheniformis*, *Bacillus coagulans*, and *Bacillus subtilis* (Crielly *et al.*, 1994; Ronimus *et al.*, 2003). The identification of thermophilic isolates has mainly focused on the vegetative form not spores; therefore spore identification needs further investigation.

1.3 The phylogeny of the genus *Bacillus*

The genus *Bacillus* can be defined as Gram-positive aerobic or facultative anaerobic rods that produce endospores (Claus and Berkeley, 1986). Before the use of ribosomal RNA as a tool for studying phylogenetic relationships, the genus *Bacillus* was divided into three groups based on spore morphology (Gordon *et al.*, 1973). Fox *et al.* (1977) confirmed these groups using 16S rRNA oligonucleotide analyses.

With the advent of 16S rRNA sequencing using a reverse transcriptase in the mid 1980s taxonomic studies became more comprehensive (Lane *et al.*, 1985; Qu *et al.*, 1983). In 1991 the genus *Bacillus* was divided into five groups based on the data generated using this method (Ash *et al.*, 1991). Dairy contaminants tended to belong to

Group 1 or Group 5. Group 1 was termed the *B. subtilis* group that included the mesophilic dairy contaiminants *B. coagulans*, *B. cereus*, *Bacillus circulans* and *B. licheniformis*. Group 5 represented the thermophiles that included *Bacillus flavothermus* and *Bacillus stearothermophilus*.

Taxonomic studies provided evidence that the genus *Bacillus* is very diverse (Ash *et al.*, 1991; Rainey *et al.*, 1994; Rossler *et al.*, 1991). Throughout the 1980s there was a lot of debate as to whether the genus *Bacillus* was truly one genus or many (Rossler *et al.*, 1991; Sneath, 1986). DNA-DNA hybridisation became a key technique in defining new species (Stackebrandt and Goebel, 1994). Work throughout the 1990s resulted in the reclassification of species within the *Bacillus* genus into new genera (Goto *et al.*, 2004; Heyndrickx *et al.*, 1996a; Heyndrickx *et al.*, 1996b; Heyndrickx *et al.*, 1997; Heyndrickx *et al.*, 1998; Heyrman *et al.*, 2003; Jay, 2003; Ren and Zhou, 2005; Shida *et al.*, 1996; Waino *et al.*, 1999; Wisotzkey *et al.*, 1992; Yoon *et al.*, 2002; Yoon *et al.*, 2004). A taxonomic study reclassified *B. stearothermophilus* into the new genus *Geobacillus* (Nazina *et al.*, 2001). The isolation of a new species named *Anoxybacillus pushchinoensis* lead to *B. flavothermus* being reclassified as *Anoxybacillus flavithermus* (Pikuta et al., 2000).

1.3.1 *Anoxybacillus flavithermus*

Prior to its reclassification, *A. flavithermus* was not considered to be a valid species. There are few isolates of *A. flavithermus* that have been characterised. It was first isolated from hot springs in New Zealand (Heine *et al.*, 1982), and has since been isolated from hot springs in the USA (Yellow Stone National Park), and Turkey (Belduz *et al.*, 2000; Nold *et al.*, 1996), from gelatin extracts (De Clerck *et al.*, 2004), and milk powder (Flint *et al.*, 2001b). The type strain *A. pushchinoensis* was first described as a strict anaerobe, hence the name *Anoxybacillus* (Pikuta *et al.*, 2000). This description has since been revised to species of this genus being aerotolerant anaerobes and facultative anaerobes (Pikuta *et al.*, 2003).

A. flavithermus is a facultative aerobic thermophile (Heinen *et al.*, 1982). It is motile and has terminal endospores. The G + C content is 61%. Characterisation of the New Zealand hot spring isolate showed that the growth range is between 30 and 70°C with an optimum growth temperature of 60°C and pH of 6-9. However, isolates obtained from milk powder tend to have an optimum growth temperature ranging between 50 to 65°C (Cucksey, 2002; Hill and Smythe unpublished data, 2004; Ronimus *et al.*, 2003). Therefore, the temperature range of *A. flavithermus* growth appears to be strain dependent.

1.3.2 The *Geobacillus* genus

The *Geobacillus* group is composed of the thermophile species that were previously classified in Group 5 of the *Bacillus* genus (Ash *et al.*, 1991). They are very closely related amongst themselves, with a 96.5 – 99.5% 16S rDNA sequence similarity (Nazina *et al.*, 2001). The G+C content ranges between 45 - 69% (Priest, 1993). They produce subterminal or terminal spores. The optimum growth temperature of this group is above 50°C. *Geobacillus* strains have been isolated from temperate areas as well as hot environments such as hotsprings, oilfields, deep sea sediments, sugar refineries, and dairy factories (Banat *et al.*, 2004; Marchant *et al.*, 2002; Nazina *et al.*, 2001; Ronimus *et al.*, 2003; Tai *et al.*, 2004; Takami *et al.*, 2004; Zarilla and Perry, 1987).

To date the predominant *Geobacillus* species reported to be isolated from milk powder is *G. stearothermophilus* (Flint *et al.*, 2001b; Lane, 1982; Ronimus *et al.*, 2003; Stadhouders *et al.*, 1982). The optimum growth temperature of milk powder isolates is between 55 – 70°C (Ronimus *et al.*, 2003). Strains of *Geobacillus* spp. can produce highly heat resistant spores that survive UHT and retort (canning process) heat treatments of 140°C for approximately 3s and 110°C - 120°C for 15 – 20 min respectively (Hill and Smythe, 2004). There is debate as to whether *G. stearothermophilus* is the predominant *Geobacillus* species found in New Zealand milk powders due to the genotypic and phenotypic similarity amongst the *Geobacillus* group. There is a possibility that both *G. stearothermophilus* and *G. thermoleovorans* could be present in New Zealand milk powders (Flint *et al.*, 2001b). To date, the isolates have been identified using only Randomly Amplified Polymorphic DNA (RAPD) profiling, and partial 16S sequencing. To resolve this issue about the identification of *Geobacillus* species in New Zealand milk powder, more accurate methods, such as multiple locus sequencing or DNA-DNA hybridisation will have to be used.

1.4 The identification of milk powder contaminants

Traditionally, biochemical tests such as the API® system (bioMérieux) have been used in the dairy industry to identify contaminants. However, these tests are time consuming, can be difficult to interpret and have been developed for the identification of medical bacterial isolates and are therefore not always suitable for identifying dairy contaminants. Recently PCR (Polymerase Chain Reaction) based identification techniques such as species-specific PCR, RAPD profiling and partial 16S rDNA sequencing, have been used (Flint *et al.*, 2001b; Ronimus *et al.*, 1997; Ronimus *et al.*, 2003). These techniques provided evidence that in the past *A. flavithermus* has been mistakenly identified as *G. stearothermophilus* (Flint *et al.*, 2001b).

1.4.1 16S ribosomal RNA

During the mid 1970s Carl Woese discovered the use of 16S rRNA for studying phylogenetic relationships and with the advent of sequencing it became an important tool for identification (Woese *et al.*, 1975). More recently, many identification techniques are based on PCR amplification and sequence analysis of the 16S ribosomal RNA genes.

The 16S rRNA is an ideal molecule for taxonomic studies as it is found in all prokaryotes, and has the same function. There are three ribosomal RNA molecules in prokaryotes, namely 5S, 16S, and 23S. Woese used 16S rRNA for his studies as it is larger than 5S, hence it provides more sequence information, allowing for a more accurate classification and it is shorter than 23S and therefore more manageable (Woese *et al.*, 1975). The 16S RNA contains interspersed regions that are highly conserved and more variable regions, which are used to study respectively, distant and close relationships. Comparison of partial sequences of the DNA encoding for 16S RNA (16S rDNA) from isolates is used as a method of identification. One disadvantage of this method is that it can not differentiate closely related species (Stackebrandt and Goebel, 1994), for example, those species belonging to the *Bacillus* genus and other groups of genera that are closely related, such as *Anoxybacillus* and *Geobacillus*.

1.4.2 Species-specific PCR

(Flint *et al.*, 2001b) used a combination of partial 16S rDNA sequencing, RAPDs and Interspacer Region (ISR) amplification profiles to determine the identity of a variety of milk powder thermophilic bacterial contaminants. Partial 16S rDNA sequencing classified the majority of contaminants as either *A. flavithermus* or *G. thermoleovorans*. These results led to an attempt to develop species-specific primers for these two species, called flavo and levo respectively, which were used in combination with the universal primer Y1 (Young *et al.*, 1991). Flavo and levo were developed by comparing the 16S rDNA sequences of *A. flavithermus* and *G. thermoleovorans*. These primers were tested against a variety of bacterial species within the *Geobacillus* and *Bacillus* genera. The *A. flavithermus* primer flavo was species-specific. However, the *G. thermoleovorans* primer levo was not. The levo and Y1 primers generated a PCR product with a variety of species belonging to the *Geobacillus* genus including *G. stearothermophilus* and *G. kaustophilus*. This is probably because of the 16S rDNA sequence similarity between species belonging to the *Geobacillus* group. Therefore, it is debatable whether the true identities of the isolates used in this study were *G. thermoleovorans*.

In Belgium, subtractive DNA hybridization was used to develop species-specific primers for the detection of *Bacillus sporothermodurans* in raw milk, (Herman *et al.*,

1997). This would be a useful method for developing specific primers for species within a genus that have very similar 16S rDNA sequences such as the *Geobacillus* group. However, pure DNA must be isolated from the organism of interest to perform this method. To date attempts to isolate pure DNA from *A. flavithermus* and *Geobacillus* spp. from milk powder isolates have not been successful. This is believed to be due to DNA degradation following cell lysis (personal communication Steve Flint, 2003). In New Zealand, at present whole cell TSB cultures or crude DNA extracts are used for PCR of bacterial thermophilic isolates (Flint *et al.*, 2001b; Ronimus *et al.*, 2003).

1.4.3 Randomly amplified polymorphic DNA (RAPD) profiles

RAPD profile analysis involves the use of a short arbitrary primer of about 10 nucleotides that binds to random sections of the genome (Williams *et al.*, 1990). The amplification of these sequences results in different band patterns between strains. This method is useful in tracing a contaminant through an industrial process, and evaluating genetic variation (Ronimus *et al.*, 1997; Svensson *et al.*, 2000). The advantages of RAPD profiling are that it requires no sequence information, it is quick and easy in comparison to other identification methods such as denaturing gel gradient gel electrophoresis (DGGE) and restriction enzyme digests, and it can detect differences between closely related species. The disadvantages of this method are that up to twenty different bands can be produced making them difficult to interpret. Furthermore, these profiles are not always reproducible particularly between laboratories, and mismatches may occur between the primer and the DNA template producing weak bands. Ronimus *et al* (2003) identified seven thermophilic bacterial strains in milk powder using RAPD profiling.

1.4.4 Other *Bacillus* identification techniques

Other methods that have been used to identify *Bacillus* spp. and have a potential for use within the dairy industry include 16S-23S rDNA internal transcribed spacer-PCR, real time PCR assays, ribosomal RNA gene restriction patterns, mass spectrometry, and fourier transform infrared spectroscopy (FTIR) (Hathout *et al.*, 2003; Joung and Cote, 2002; Lin *et al.*, 1998; Nagpal *et al.*, 1998; Rueckert *et al.*, 2005).

1.4.5 Identification of spores

Previous publications have focused predominantly on the identification of vegetative thermophilic bacteria in milk powder and not the spore form, probably because of spore resistance to thermal and chemical lysis, which are used to release DNA in most procedures (Flint *et al.*, 2001b; Ronimus *et al.*, 2003). Currently, identification of thermophilic spores present in dairy products requires a heat treatment step to kill vegetative cells followed by a germination step so that DNA can easily be extracted (Hill, 2004). This process is time consuming. The use of sonication and microwave

radiation are two potential methods that could be used to release DNA from spores. A recent publication by Rueckert *et al* (2005) described the development of a method that did not require a germination step to detect and quantify both thermophilic vegetative cells and spores in milk powder. Sonication was used to release DNA from spores. However, at least 80% of spores appeared to have survived this treatment suggesting DNA release was incomplete. Microwave radiation has been shown to be useful in the release of DNA from a variety of bacterial spore suspensions, including *G. stearothermophilus* (Vaid and Bishop, 1998).

1.5 Thermophile contamination of milk powder manufacturing plants

The thermophilic bacilli (or those genera that formerly belonged to the *Bacillus* genus) are difficult to eliminate because of their fast growth rate (approximately 15 - 20 min generation time), heat and chemical resistant spores, wide temperature growth range, and their ability to form biofilms (Etoa and Michiels, 1988; Flint *et al.*, 2001a; Hill and Smythe, 1994; Parkar *et al.*, 2003). It is particularly the development of spores that is of concern to the dairy industry. Spores are able to survive in milk powder products for an extended period of time, whereas the vegetative cells are likely to die off (personal communication Bruce Hill, 2004; Reddy *et al.*, 1975).

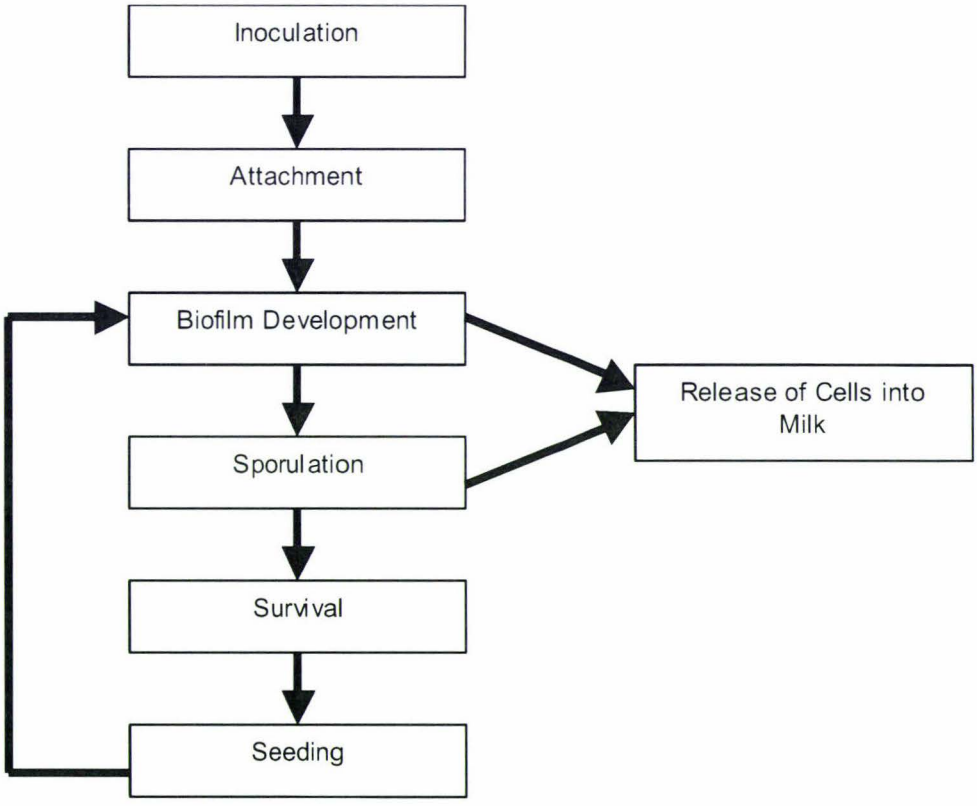

Figure 2: Flow diagram of the proposed thermophile contamination cycle within a milk powder manufacturing plant

The process of thermophile contamination is still not clearly understood. Figure 2 outlines the thermophile contamination process proposed by the New Zealand dairy industry (Personal communication Steve Flint, 2005). Initial contamination of the milk powder plant is believed to originate from low numbers of thermophiles (probably predominantly in their spore form) present in the raw milk that survive pasteurisation. Poor manufacturing practice, for example inadequate cleaning and recycle loops, can be a major factor causing thermophile growth (Hill and Smythe, 1994).

Studies indicate that in a dairy factory both spores and vegetative cells attach to the stainless steel and the foulant (Flint *et al.*, 2001a; Langeveld *et al.*, 1995; Parkar *et al.*, 2001). Once attached to the surface, the spores probably germinate, and the vegetative cells would then reproduce forming a biofilm. The next stage is unclear but it is believed sporulation occurs within the biofilm.

Contamination of the milk is likely to occur from the biofilm, as a consequence of sloughing or shedding of individual cells. At the end of a milk powder run most of the bacterial thermophilic cells would be removed or killed by CIP and a sanitiser if used (Parkar *et al.*, 2004). However, fouling or biofilms may protect spores and or vegetative cells (Hinton *et al.*, 2003). This would result in thermophilic cells remaining in the plant after CIP and would seed the next manufacturing run. There is evidence that viable bacterial cells can remain attached to dairy manufacturing surfaces following a CIP (Austin and Bergeron, 1995; Flint *et al.*, 1999).

1.5.1 Source of thermophiles

The overall raw milk quality has been shown to be unrelated to final powder quality but there is a correlation between the count of thermophilic bacteria found in pasteurised milk and the powder produced from it (Muir *et al.*, 1986). The greater the numbers of thermophilic bacteria present in the pasteurised milk the greater the final count in the final milk powder product. Long raw milk treatment runs can result in high thermophile counts in pasteurised milk. This is caused by thermophile growth in the separators and pasteuriser PHEs. In New Zealand, shortening milk treatment runs to 6 – 8 h solved this problem. However, it is not economically feasible to shorten milk powder runs, particularly since the final product is not always contaminated with high numbers of thermophiles.

Thermophile contamination can also arise from the reuse of by-products such as buttermilk and permeate (from milk ultrafiltration), ingredients such as lactose, and recycle loops in manufacturing plants (Hill and Smythe, 1994). Despite these potential sources of thermophile contamination, this does not explain all incidents where contamination of milk powder reaches spore levels of up to 10^5 CFU/g. The residence time of the milk within the powder plant is 30 minutes or approximately 1.5 – 2

generation times of thermophilic bacilli. This is much too short to account for growth in milk to achieve these observed high levels of thermophiles in the final product. Therefore contamination must arise from biofilms or growth within fouling layers. As well as providing a source of contamination, there are other adverse conditions associated with both biofilms and fouling, such as reduced flow through blocked tubes, reduced heat transfer, and corrosion of equipment (Beech and Sunner, 2004; Delsing and Hiddink, 1983; Truong, 2001a).

1.5.2 Fouling

Fouling is the build up of milk proteins, in particular whey proteins, and calcium phosphate salts on the internal surface of a dairy processing plant (Jeurnink *et al.*, 1996). There is evidence that the degree of fouling is proportional to the amount of fat present in the milk, probably due to the migration of fat to the stainless steel surface (Ma and Trinh, 1999). Therefore, fouling tends to be a greater problem in the manufacture of whole milk powder compared with skim milk powder. Anecdotal evidence from the New Zealand milk powder industry suggests thermophilic spore contamination is worse during whole compared to skim milk powder manufacture. This correlation may indicate that fouling is a source of thermophilic spores. Studies demonstrating that thermophile attachment and growth on foulant occurs faster than on un-fouled stainless steel supports this theory (Flint *et al.*, 2001a; Hinton *et al.*, 2003).

Fouling is particularly a problem in areas of the plant where the milk comes into contact with stainless steel surfaces at temperatures greater than 70°C, if there is a large change in temperature, and in recirculation or dead end zones (Jeurnink *et al.*, 1996). Sites in a milk powder plant where fouling occurs can include heat treatment equipment such as heat exchangers and DSI units, holding tubes and evaporator orifice plates (Bennett, 2000; Truong, 2001b). Foulant may also remain in sections of the plant after a CIP, thus providing a possible source of thermophilic spores to seed the subsequent run. Parkar *et al* (2004) demonstrated the importance of following the correct CIP regime. A decrease in temperature or wash time can result in residual foulant.

1.5.3 Biofilms

Bacterial adhesion to surfaces and subsequent biofilm development is very common in many environments such as medical, marine and fresh water, water distribution units, and food processing.

Biofilms are defined as microcolonies of bacterial cells enclosed in an extracellular polysaccharide matrix actively growing on a surface (Costerton *et al.*, 1994; Flint *et al.*, 1997a).

Biofilm development occurs through a number of stages (Johnston, 2004; Kumar and Anand, 1998). First, cells attach to the substratum. Then, the initial reversible attachment is followed by irreversible attachment. Once the cells have attached, micro colony formation and production of extracellular polysaccharide (EPS) commences. These micro colonies expand, forming multilayers of bacterial cells embedded in the EPS matrix. Water channels may develop within the EPS matrix (Lewandowski *et al.*, 2002). This matrix provides a source of nutrients for bacterial cells and renders them far more resistant to harsh conditions than are the planktonic cells.

Contamination of a food product can occur via individual cell release or sloughing (Holah and Gibson, 2000; Stoodley *et al.*, 2001b; Willcock *et al.*, 1997). Individual cell release occurs during cell division, when daughter cells that have a different hydrophobicity to the biofilm are released (Allison *et al.*, 1990a; Allison *et al.*, 1990b). Sloughing is the release of biofilm segments. This process occurs when the biofilm is weakened by either mechanical process such as the shear effect from the fluid flow or during biofilm maturation when EPS lyases, which degrade the EPS, are released (Boyd and Chakrabarty, 1994; Chang *et al.*, 1991).

In the dairy industry the most common biofilm forming isolates are the thermophilic streptococci and the bacilli. Biofilms developed from these organisms are referred to as process biofilms (Flint *et al.*, 1997a). The streptococci form biofilms in PHE pasteurisers (Flint *et al.*, 1997b; Flint *et al.*, 1999; Knight *et al.*, 2004). However, because of their inability to form spores the streptococci do not pose a problem in milk powder manufacturing plants.

There are two key features of process biofilms differentiating them from the classic biofilm (Flint *et al.*, 1997a). Firstly process biofilms are generally dominated by one species due to selective pressures from the surrounding environment. Selective pressures in a milk powder manufacturing plant may include heat, product composition, pH, and water activity. Secondly process biofilms have a low cell density of approximately $10^6 - 10^7$ cells cm^{-2} (Flint *et al.*, 2001a; Flint *et al.*, 1999; Hood and Zottola, 1997; Parkar *et al.*, 2003). The low cell density appears to be a result of regular cleaning and the high shear rate at the surface. Conversely classic biofilms contain a mix of species and can be several millimetres thick (Characklis *et al.*, 1990).

Real time monitoring of biofilm development in an industrial setting is not well established. Currently biofilm detection is usually achieved through swabbing or the placement of coupons using a Robbins or Hygenic Live Line Sampling (HILLS) device. A Robbins device is a unit developed by McCoy *et al.* (1981), containing sampling ports for glass cover slips or stainless steel coupons, for the study of biofilm development. Two types exist, stainless steel units that can be used in an industrial setting under turbulent flow and perspex or acrylic units that are used in laboratory

scale reactors under laminar flow (Flint *et al.*, 1999; Millar *et al.*, 2001). Sampling can occur only when the manufacturing plant or reactor is shut down or if the Robbins device is installed in a section of the plant that can be by-passed. The HILLS device also contains sampling ports for stainless steel coupons and was constructed so that sampling can occur during the milk powder manufacturing process without breaking the vacuum (Warnecke, 2001). Biofilms have been described in preheaters, evaporators, and seals and gaskets located within milk manufacturing plants (Austin and Bergeron, 1995; Langeveld *et al.*, 1995; Warnecke, 2001).

Laboratory trials have provided information as to how thermophilic biofilms probably develop within a milk powder plant (Flint *et al.*, 2001a; Parkar *et al.*, 2001; Parkar *et al.*, 2003). It is generally accepted that both vegetative cells and spores are involved in the initial attachment stage to stainless steel though Flint *et al* (2001a) demonstrated that a greater percentage of *G. stearothermophilus* spores than vegetative cells bind to stainless steel.

Spores are more hydrophobic than vegetative cells, which probably enables them to bind more effectively to stainless steel (Wiencek *et al.*, 1990). The ability of spores to adhere to a surface varies between species (Faille *et al.*, 2002). Ronner *et al.* (1990), measured the hydrophobicity of a variety of *Bacillus* spores and tested their ability to bind to a variety of surfaces. They found that the degree to which a spore could adhere to a hydrophobic surface such as stainless steel is related to its hydrophobicity. It has been proposed that the hydrophobicity of spores is a result of more protein on their outer coats and exosporium compared with the cell surfaces of vegetative Gram-positive cells that have peptidoglycan (Wiencek *et al.*, 1990). However, there is no correlation between the removal of spore coat proteins and the ability of a spore to bind to a surface (Parkar *et al.*, 2001).

G. stearothermophilus was able to form biofilms after approximately a 6 h incubation period using a laboratory continuous flow reactor (Flint *et al.*, 2001a). After 12 h the biofilm contained up to 10^6 cells cm^{-2}. Both bacterial vegetative cells and spores were released into the milk but the authors did not look for spore formation of *G. stearothermophilus* on the surface of the stainless steel.

In the model species, *B. subtilis,* there is evidence of sporulation in both a liquid/air interface biofilm in a standing broth culture and a solid/liquid interface biofilm on glass wool in a standing culture (Branda *et al.*, 2001; Lindsay *et al.*, 2005). The liquid/air interface biofilm of *B. subtilis* took approximately 60 h to sporulate (Branda *et al.*, 2001). Lindsay *et al.* (2005) compared sporulation of *B. subtilis* in a planktonic culture with a biofilm on glass wool. Sporulation was detected after 5 h in the planktonic culture compared with 30 h in the biofilm. The author suggested that there was a window of time for a decision between biofilm formation and sporulation. Although

sporulation and biofilm formation are different pathways some of the same genes are expressed in both; 60% of genes expressed in a *B. subtilis* liquid/air biofilms are related to sporulation (Ren *et al.*, 2004).

These biofilms of *B. subtilis* differ from those expected to form in milk powder manufacturing plants because dairy biofilms tend to form at solid/liquid interfaces in areas of turbulent flow compared with no flow present during the development of the *B. subtilis* biofilms.

1.5.4 Spores

Sporulation is a natural process in the growth cycle of certain groups of bacteria such as *Bacillus* species. It is a survival mechanism, generally regarded as a process that occurs when the organism is under stress. In the dairy environment, factors other than stress may also be responsible for thermophilic bacilli spore formation as this process occurs so readily.

Structure and spore formation

Spores consist of a core otherwise known as the protoplast, which contains the nuclear material, surrounded by the cortex that is in turn enclosed in the spore coat as illustrated in Figure 3 (Russell, 1982; Tipper and Gauthier, 1972). The major difference between species is the number and structure of layers in the spore coat, whereas the cortex and core are very similar (Aronson and Fitzjames, 1976; Atrih and Foster, 2001; Tipper and Gauthier, 1972). The spore coat occupies the most of the cell volume. In some species, such as *B. cereus*, there is a layer over the spore coat called the exosporium (Ellar, 1966). Two unique components of spores are dipicolinic acid (DPA) and peptidoglycan located respectively in the core and cortex (Ellar, 1978; Warth and Stroming, 1972).

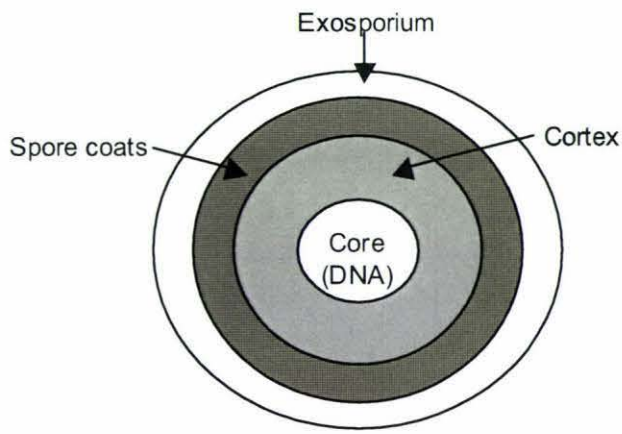

Figure 3: The 'typical' bacterial spore structure.

The spore layers are not drawn to scale. Note that some bacteria may not have an exosporium, and that the number of spore coats is dependent on the species. This figure has been adapted from (Setlow, 1995).

Sporulation is a complicated process, divided into a series of steps that is regarded as being very similar amongst spore-forming bacteria (Ellar, 1966; Palop *et al.*, 1999) (Figure 4). The first step involves the cell undergoing an asymmetric division, by the inward folding of the cytoplasmic membrane to form a septum, which divides the cell into the mother cell and the forespore. The forespore becomes enclosed in a membrane vesicle. The cortex and exosporium then form followed by the spore coat. As the spore matures it becomes denser, dehydration occurs and resistance develops. The spore is released by lysis of the mother cell. Spores may remain dormant for years if the conditions are not right for germination (Kennedy *et al.*, 1994).

Three stages are involved in the process of changing from a spore to vegetative cell. These are activation, germination, and outgrowth (Russell, 1982). Activation is a reversible process that results in a spore that is ready for germination but still keeps most spore properties. If the conditions are right an activated spore will then undergo the irreversible process of germination. This occurs when the spore changes from being dormant to a metabolically active cell. Outgrowth is the step by which a germinated spore develops into a vegetative cell that then enters the vegetative growth cycle.

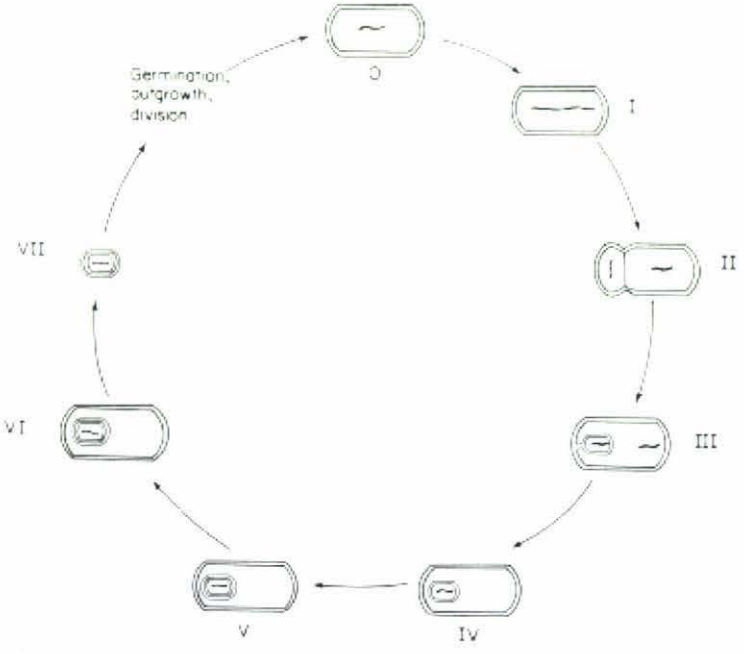

Figure 4: Stages in the development of bacterial spores (Russell, 1982)

O	Vegetative cell
I	Pre-septation
II	Formation of septum
III	Membrane vesicle enclosure
IV	Cortex formation
V	Coat formation
VI	Maturation
VII	Release of spore

Understanding how spore formation occurs is important as this knowledge may help control spores. Sporulation can be triggered by starvation, high cell density, or DNA damage (Cooney and Freese, 1976; Russell, 1982). The early stages of sporulation can be reversed if the culture is transferred into a nutrient rich environment (Cooney and Freese, 1976; Cooney *et al.*, 1977; Greene and Slepecky, 1972). Generally the optimum temperature and pH for spore formation are similar to those for vegetative cell growth, but the range of both parameters is narrower (Russell, 1982). The conditions that trigger spore formation of thermophilic bacteria within a dairy environment are poorly defined.

The presence of minerals such as manganese, magnesium, calcium, potassium and carbon compounds are known to stimulate spore formation and are commonly used in sporulation media (Cooney and Freese, 1976; Kaul and Singh, 1982; Warriner and Waites, 1999). During spore formation, minerals in particular calcium accumulate in the forespore, resulting in the core containing most of the minerals (Jonhstone *et al.*, 1980; Palop *et al.*, 1999). Despite the presence of these minerals in sporulation media, spore production by the thermophilic bacilli in the laboratory generally takes at least one week.

Resistance

Spores are resistant to heat, a wide variety of chemicals and UV radiation thus making them very hard to destroy. This creates problems for the food industry, because spore contamination of the product is not desirable. Combinations of several properties contribute to the overall resistance of spores, the two main ones being their low water content and impermeability to chemicals (Setlow, 1995). Other factors involved in resistance are the spore coat, the peptidoglycan cortex, and small acid soluble proteins (SAPs) are involved in resistance. The spore coat has been shown to act as a protection against enzymes such as lysozyme, mechanical disruption, chemicals such as hydrogen peroxide and UV-A and UV-B radiation (Gould and Hitchins, 1963; Riesenman, 2000). The peptidoglycan cortex is involved in protection against organic solvents and heat (Atrih and Foster, 2001; Imae and Strominger, 1976). Small acid

soluble proteins (SAP) that are unique to spores are present in the spore core and bind to the DNA protecting it from UV radiation (Popham *et al.*, 1995).

The mechanisms of spore heat resistance appear to be dependent on the strain as well as the conditions under which the spore is formed (Palop *et al.*, 1999). The major characteristics of spores associated with heat resistance are low water activity and mineralisation (Beaman *et al.*, 1982). The peptidoglycan cortex and SAP proteins have also been associated with heat resistance (Atrih and Foster, 2001; Popham *et al.*, 1995).

Environmental factors such as temperature, pH, and media composition may contribute to spore resistance. Spores formed at temperatures greater than the optimum growth temperature tend to be more heat resistant (Beaman and Gerhardt, 1986; Palop *et al.*, 1999). Maximum heat resistance is at neutral pH and tends to decrease with acidity (Mazas *et al.*, 1997; Periago *et al.*, 1998). There are indications that sublethal heat shock may increase the heat resistance of spores (Etoa and Michiels, 1988; Heredia *et al.*, 1997). The metal ion content of media has been shown to influence the heat resistance of spores (Cazemier *et al.*, 2001). For example, *Bacillus* spores produced on agar containing a variety of metal ions such as calcium and magnesium were found to be more heat resistant compared with those produced on nutrient agar supplemented with manganese only (Cazemier *et al.*, 2001). It has been demonstrated that spores attached to stainless steel are more heat resistant compared with those in a planktonic culture (Simmonds *et al.*, 2003). Spores produced in a milk powder factory are more heat resistant compared with those produced on laboratory media (Hill, 2004).

Resistance of spores is a problem in the dairy industry because spores that are produced during the early stages of the manufacture of milk powder are able to survive the low water activity and high temperature of the drying process, the cleaning in place system, and the long-term storage of the final milk powder product.

Activation

It is generally accepted that spores must be activated before germination can occur. Heat, chemicals, and a decrease in pH to 2 - 3 can activate spores (Kim and Foegeding, 1990).

In the dairy industry heat is the most likely mechanism of spore activation, because of the extensive use of heat as a preservation technique. The temperature of activation is strain and species-specific. For example spores of *G. stearothermophilus* can be activated at temperatures as high as 110°C, whereas spores of *B. subtilis* have a lower activation temperature of 65°C - 70°C (Finley and Fields, 1961; Leuschner and Lillford, 1999).

Investigations of chemical activation of spores showed that certain chemicals could activate the spores of certain strains. For example, ethanol treatment can activate *B. megaterium* and *B. subtilis* but not *B. cereus* (Kim and Foegending, 1990). Ito, (1981) reviewed the activation of *B. stearothermophilus* spores establishing that there is no chemical known to activate all *B. stereothermophilus* strains. It has also been suggested that spore activation may be related to hydrophobicity. Chemical treatments that disrupt hydrophobic interactions, such as an increase in pH, improve spore activation (Cravens, 1988).

To the author's knowledge, no studies have been undertaken to determine what triggers spore formation and germination within a milk powder manufacturing environment.

1.6 Conclusion

Contamination of milk powder with bacterial thermophilic spores continues to be a major problem for the New Zealand dairy industry. The two major thermophilic contaminants in New Zealand milk powder have been identified as *Geobacillus* sp. and *A. flavithermus* using molecular techniques such as organism specific PCR, partial 16S rDNA sequencing and RAPD profiling.

These organisms are particularly difficult to eliminate in milk powder manufacturing plants because of their fast growth rate, the heat resistance of their spores and their ability to form biofilms. One of the major sources of thermophilic contamination of milk powder appears to be from the release of cells from biofilms that form on equipment surfaces during milk powder manufacture.

Most previous studies have focused on the identification and growth of these organisms in their vegetative form. However, there is little information about where and when sporulation of these organisms occurs and whether it occurs within a biofilm.

1.7 Objectives of this study

The purpose of this study was to determine the origin of thermophilic bacterial spore contamination in a milk powder manufacturing plant and to investigate the role of biofilms in spore formation. This was achieved by monitoring spore formation in both a milk powder factory and in a continious flow laboratory reactor.

The specific objectives of this study were as follows:

(1) Determine the source of thermophilic spores in a milk powder manufacturing process.

(2) Isolate thermophilic spores from milk process samples and identify using specific primers.

(3) Develop techniques for the formation of biofilms in a model laboratory system from both vegetative cells and spores of the dairy isolate *A. flavithermus* CM.

(4) Investigate some of the conditions (temperature and time) required for spore formation in biofilms of *A. flavithermus* CM.

2 Materials and methods

2.1 Source of bacterial isolates

The bacterial isolates used in this study are listed in Table 1 and were cultured as detailed in section 2.2.2. These isolates originate from either reference culture collections or from milk powder. Up to 240 isolates were also obtained from the Pahiatua Powder 2 plant.

Table 1: Bacterial isolates used in this study

Species	Strain No.	Source
Anoxybacillus flavithermus	Reference DSM 2641	German Culture Collection (DSM)
Geobacillus thermoleovorans	Reference DSM 5366	German Culture Collection (DSM)
Anoxybacillus flavithermus	CM	Waikato University, Milk Powder manufacture (Te Awamutu)
Geobacillus sp.	AM	Waikato University, Milk Powder manufacture (Te Awamutu)

2.2 Bacteriological methods

2.2.1 Media preparation and storage

All media were made up with Milli-Q water (deionised water using the Mill-Q® water purification system, Millipore®) and sterilised by autoclaving at 121°C for 15 min. Solid media were cooled to 47°C before pouring. Liquid media were cooled to ambient temperature before use. Sterilised solid media were stored at 4°C and sterilised uninoculated liquid media were stored at room temperature.

2.2.2 Culture preparation

For the routine preparation of cultures all isolates were grown aerobically in trypticase soy broth (TSB; BBL, Becton, Dickinson and Company) at 55°C. Spore derived isolates (before freezing and storage) from the Pahiatua milk powder plant and *A. flavithermus* CM cultures that were used to seed the reactor, were grown for 5 – 8 h to mid exponential phase (absorbance 0.2 - 0.3 at 560nm). For the identification of isolates by PCR, cultures were incubated for 18 h (overnight).

2.2.3 Spore preparation

A. flavithermus CM was grown to mid exponential phase (outlined in section 2.2.2) and 100μL was spread plated onto sporulation agar the composition of which was: 8g nutrient broth (Difco), 0.25g $MgSO_4$, 0.97g KCl, 0.15g $CaCl_2$, 2 x 10^{-3}g $MnCl_2$, 0.3 x 10^{-3}g $FeSO_4$, 30g agar, and 1L Mili-Q water (Husmark, 1993). The plates were incubated for 7 - 10 days at 55°C. The spores were scraped from the sporulation plates, suspended in sterile Milli-Q water, then heat treated at 100°C for 30 min to kill any vegetative cells (section 2.2.6), and stored at 4°C.

2.2.4 Storage of bacterial strains

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

2.2.5 Standard plate counts

To determine the number of thermophilic bacteria, in a mixed culture or product, standard plate count methods were used as described below. These methods were based on those described in the Fonterra NZTM 2 microbiological methods manual. Milk powder and fouling were reconstituted in 0.1% peptone (BBL®-Polypeptone-peptone, Becton, Dickinson and company) by agitation for 3 min in a peristaltic mixer. Effect 1 separator foulant was blended in a Waring blender prior to reconstitution. The plates were prepared as outlined below and incubated at 55°C for 48 h. Plates of the Pahiatua undiluted liquid samples were not counted, as it was too difficult to distinguish the colonies from milk product.

Total bacterial thermophilic count

This method was used to count bacteria that grow aerobically (both obligate and facultative) in milk and milk products at temperatures of 55°C or higher and will be referred to as the total count in this study.

Serial 10-fold dilutions were carried out in 0.1% peptone. 1mL of each dilution was pour plated, in triplicate, with Milk Plate Count Agar (MPCA, Oxoid Ltd.). Composition: 19.5g MPCA/ 1 L Milli-Q water, pH=7.0.

Thermophilic aerobic spore count

This method was used to count thermophilic aerobic spores only and will be referred to as the spore count in this study.

The samples were heat-treated (refer to section 2.2.6) and serial 10-fold dilutions were carried out in 0.1% peptone. 1mL of each dilution was pour plated, in triplicate, with Milk Plate Count Agar + 2% Starch (MPCA + S). Composition: 19.5g MPCA, 2.0g soluble starch (Difco), 1L Milli-Q water, pH =7.0.

2.2.6 Heat treatment

Heat-treatments were used to kill vegetative cells in both milk product and biofilms so that only spores remained in the sample or attached to the stainless steel sample tube. The stainless steel sample tubes were transferred to a screw-capped tube containing 14mL of sterile 0.1% peptone. Solid samples were reconstituted as previously described (section 2.2.5). Milk samples from the Pahiatua evaporator pass 3, 4, and 5 of the evaporator (refer to section 2.4.1) were diluted to 10^{-1} in 0.1% peptone before heat treatment to prevent coagulation. A defined volume of the milk sample was transferred to a screw-capped test tube. A pilot tube was prepared that contained the same volume of 0.1% peptone as the milk sample or the 14mL of 0.1% peptone containing the stainless steel sample tube. The probe of a digital thermometer (Digitron, 2000T) was then inserted through a small hole in the lid. The sample tubes and pilot tube were placed in a water bath pre-heated to 100°C. When the temperature of the pilot tube reached 98°C (this must occur within 5 min), the sample tubes were heat-treated for 30 min. After the heat treatment the tubes were immediately placed in a 15 - 18°C water bath for 10 min.

2.2.7 Isolation of spores

The milk product was heat-treated (outlined in section 2.2.6), and serial 10-fold dilutions carried out in 0.1% peptone. 100µl of the appropriate dilution was spread onto MPCA + S and incubated for 48 h at 55°C. A single colony was transferred to TSB and cultured (as outlined in section 2.2.2) to mid exponential phase, then stored at -80°C in 15% glycerol.

2.2.8 Temperature growth profile of *A. flavithermus*

The temperature gradient incubator model TN-3 (Toyo Kagaku Sangyo Co. Ltd, Japan) was set to a temperature range of approximately 30 – 70°C. The incubation tubes contained 20mL of TSB and 200µL of fresh mid-exponential TSB culture. The growth was assessed by measuring the optical density of the cultures at the wavelength 560nm using the Hitachi U-2000 Spectrophotometer (Biolab Scientific). This was carried out every 2 – 2.5 h or every half hour when growth had become apparent by visual inspection. At the end of the incubation time the temperature was measured in each incubation tube using a digital thermometer.

2.3 Microscopy

2.3.1 Transmission Electron Microscopy (TEM) procedure

TEM was used to examine bacterial cells embedded in fouling sampled from the Pahiatua Milk Powder Plant 2 (section 2.4.6). Robyn Hirst at Fonterra, Palmerston North, carried out the preparation of fouling samples for TEM and examination as described below.

Fixation

The sample was cut into approximately $1mm^3$ cubes and put into a bijoux bottle containing 6.25% gluteraldehyde (Sigma) in 0.2M imidazole buffer (Sigma). This was stored at 5°C for two days. The gluteraldehyde solution was rinsed twice with 0.2M imidazole buffer over two hours. The buffer was removed and the sample placed in 1% osmium tetroxide (Sigma) in 0.2M sodium cacodylate (Sigma) overnight. The sample was rinsed twice with distilled water, placed in 1% uranyl acetate (Merc)for 30 min and then again rinsed twice with distilled water.

Dehydration

The dehydration process was carried out at 5°C in 25% acetone (15 min) then in 50%, 70% and 90% acetone for (30 min each) followed by 100% acetone (three changes over 90 min).

Embedding

The acetone was then replaced with Procure 812 embedding resin (Proscitech), and put on rollers for 24 h. A cube of the sample was placed into a BEEM embedding capsule (Proscitech) and cured at 60°C for 48 h.

Sectioning

The embedded samples were sectioned to a thickness of 90nm using the Leica Ultracut R microtome (Leica, Germany). These sections were mounted on 3mm copper/rhodium grids and stained using lead citrate before examination in a Philips Transmission Electron Microscope (TEM) (Philips, 201C, The Netherlands) at an accelerating voltage of 60 kV.

2.3.2 Scanning Electron Microscopy (SEM) procedure

SEM was used to visualise the biofilm present on stainless steel coupons from reactor run 4 (section 2.7.3). The stainless steel coupons were rinsed three times in 0.1% peptone then fixed in 1% formaldehyde solution (BDH). Doug Hopcroft at Hort Research, Palmerston North carried out the final preparation and examination of the coupons detailed as follows. The coupons were glued to aluminium SEM specimen support studs, sputter coated with gold and examined using a Cambridge 250 Mk 3 scanning electron microscope.

2.3.3 DAPI staining procedure

To determine if spores were attached to stainless steel coupons from reactor run 4 (section 2.7.3), epifluorescence microscopy was used. The stainless steel coupons were rinsed three times in 0.1% peptone then immersed in a 1µg/mL DAPI solution for 10 min at room temperature. Following staining the coupons were rinsed in distilled water three times, air-dried, and then mounted on glass slides using epoxy resin (Araldite, Sellys). The coupons were examined using a Zeiss axioskop 2 plus microscope with a G365 excitation filter and a LP420 emission filter and photographed using Axio Vision AC version 4.2 software (Carl Zeiss).

2.4 Pahiatua milk powder plant study

2.4.1 Operation of the Pahiatua milk powder plant

The milk powder manufacturing process at Pahiatua comprised of a raw milk treatment section, where pasteurisation occurred, and two powder plants, named Powder 1 and Powder 2.

The raw milk treatment section had two pasteurisation series, called train A and train B. These runs operated independently from the powder plants and lasted for 6 - 8 h. This process is outlined in Figure 5. The raw milk entered the plant at approximately 7°C. It was preheated to 50°C by a PHE and then separated into skim milk and cream. The skim and cream were then pasteurised separately. At this point permeate or lactose could be added to the skim. The skim was pasteurised at 73°C for 15s and the cream at 80°C for 15s. PHEs were used for pasteurisation. Following pasteurisation the milk was standardised to the required fat concentration by adding cream to the skim, then the standardised milk was cooled to 4°C before storage. The milk could be stored for up to 5 h before it was directed into either Powder 1 or Powder 2, where evaporation and drying occurred. It was not possible to trace a particular evaporation and drying run to a particular milk treatment run.

The focus of the present study was on Powder 2. The Powder 2 evaporation and drying process is outlined in Figure 6. This plant operated at a flow rate of

approximately 40 000 L/h. The runs lasted for 18 – 19 h. Before the milk underwent evaporation it was heat-treated. In Powder 2, this heat treatment consisted of a PHE that raised the temperature of the milk from approximately 7°C to between 50 and 65°C and a direct steam injector that raised the milk temperature to approximately 95°C (Figure 7). Following heat treatment, the milk passed through a two-effect five-pass mechanical vapour recompression (MVR) falling film evaporator (Figure 8). The evaporator was used to concentrate the milk to approximately 48% total solids before it underwent drying. The evaporator operated at a temperature of 65°C in both effects. Effect 1 had two passes whereas effect 2 had three.

Following evaporation, the concentrated milk passed through a scraped surface preheater and was then homogenised before it was dried and packed. The drying process was composed of four stages: atomisation, followed by primary, secondary, and tertiary drying. In the main chamber of the drier primary drying occurred where hot air (ranging between 185°C to 205°C) was mixed with the atomised milk. The powder fell to the base of the main chamber onto a static fluid bed (SFB). Warm air ranging between 50°C and 85°C passed up through the layer of powder. The final step of drying occurred in a vibrofluidiser (VF). This bed vibrated at a low frequency and either ambient or hot air was blown through the powder. The final product was then packaged.

After every run a CIP took place in the preheat and the evaporation sections of the plant. This took about three hours and involved a 1.6% caustic wash followed by a water rinse then a 0.9 % nitric acid wash and finished with a water rinse. To remove fouling build-up, sections of the plant, in particular the DSI and evaporator were opened and manually cleaned. This occurred every five runs, for whole milk powder production, or when there was a change of specification after the caustic wash and water rinse.

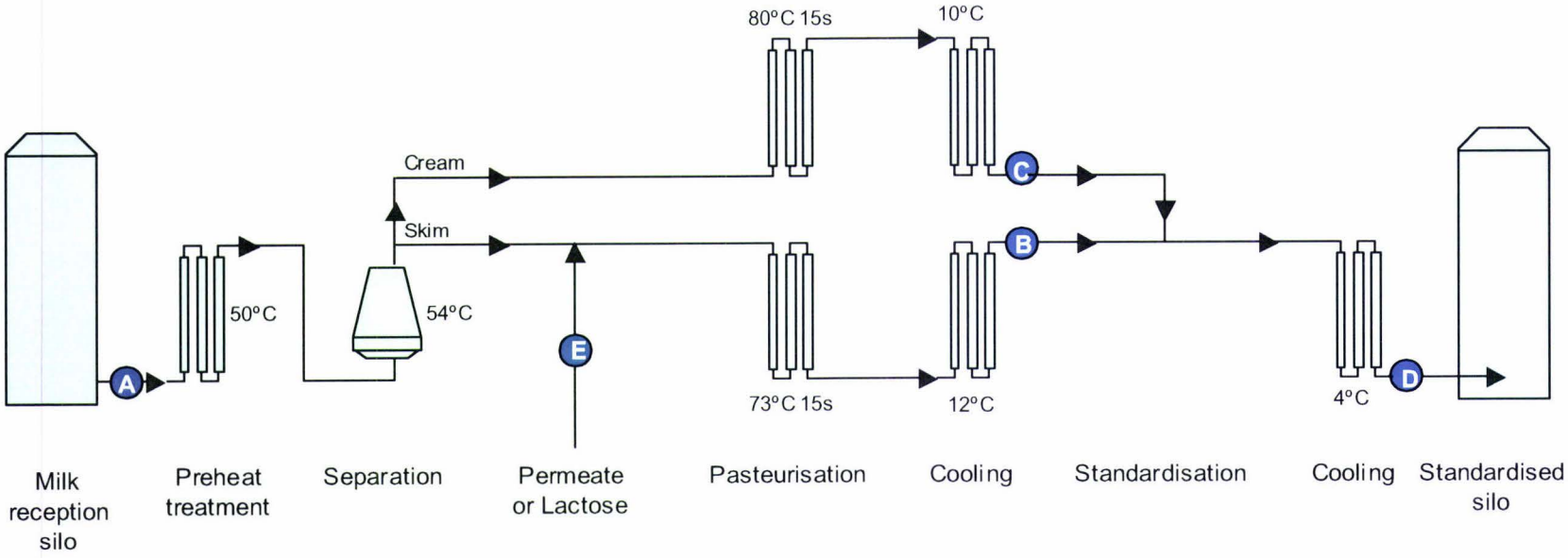

Figure 5: Schematic diagram of the raw milk treatment process at the Pahiatua milk powder factory

A, B, C, D, and E indicate the points at which samples were taken (refer to Table 3 for a detailed description of sample points).

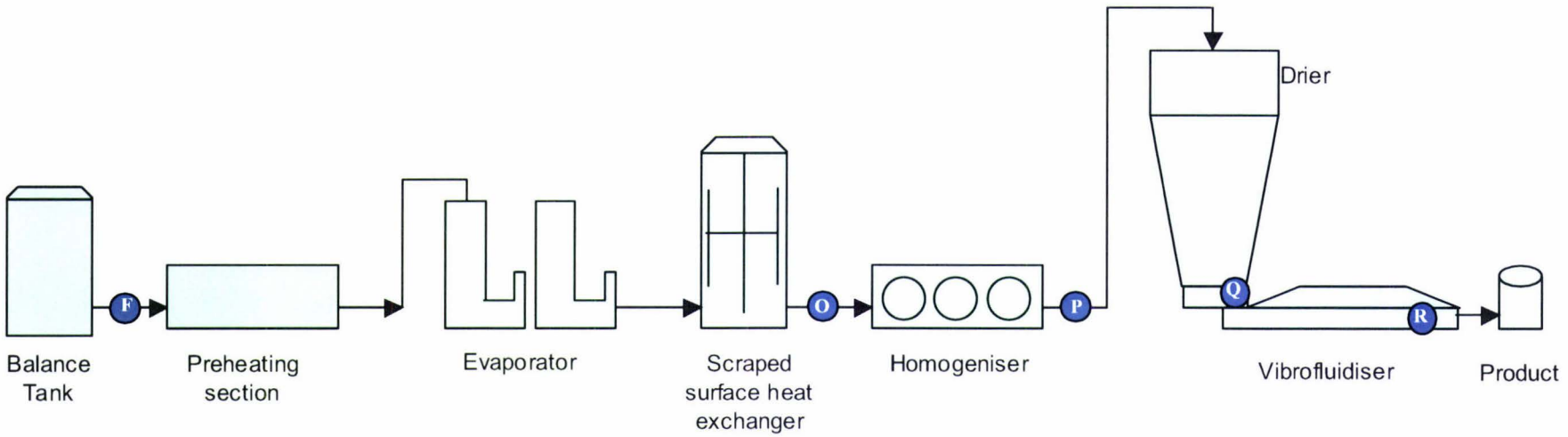

Figure 6: Schematic diagram of the Pahiatua Powder 2 evaporation and drying process

F, O, P, Q, R indicate the points at which samples were taken (refer to Table 4 for a detailed description of sample points and Figure 5 and 6 for a detailed diagram of the preheating and evaporator sections of the plant).

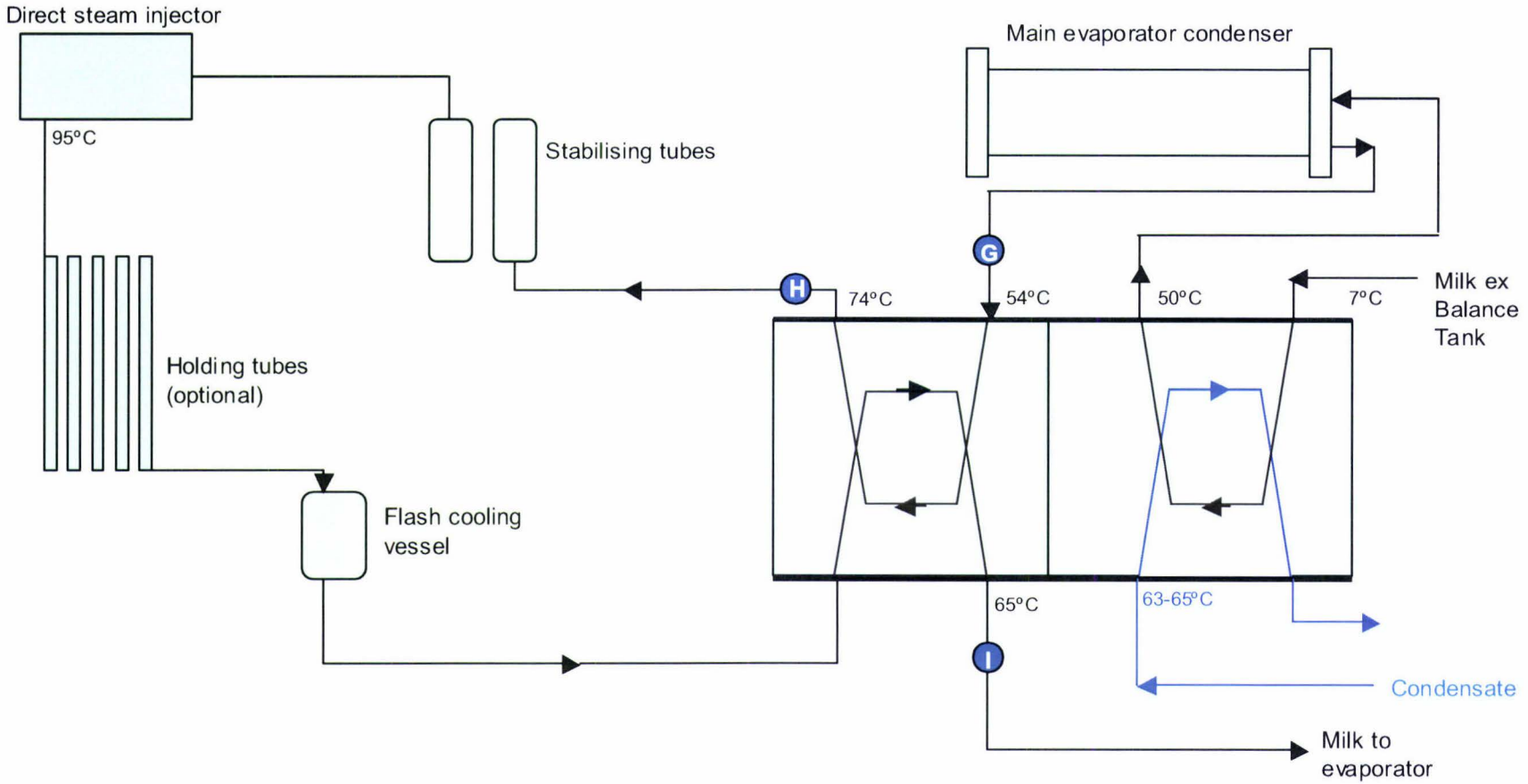

Figure 7: Schematic diagram of the preheating section of the Pahiatua Powder 2 plant

G, H, I indicate the points at which the samples were taken (refer to Table 4 for a detailed description). The temperatures noted are approximate values.

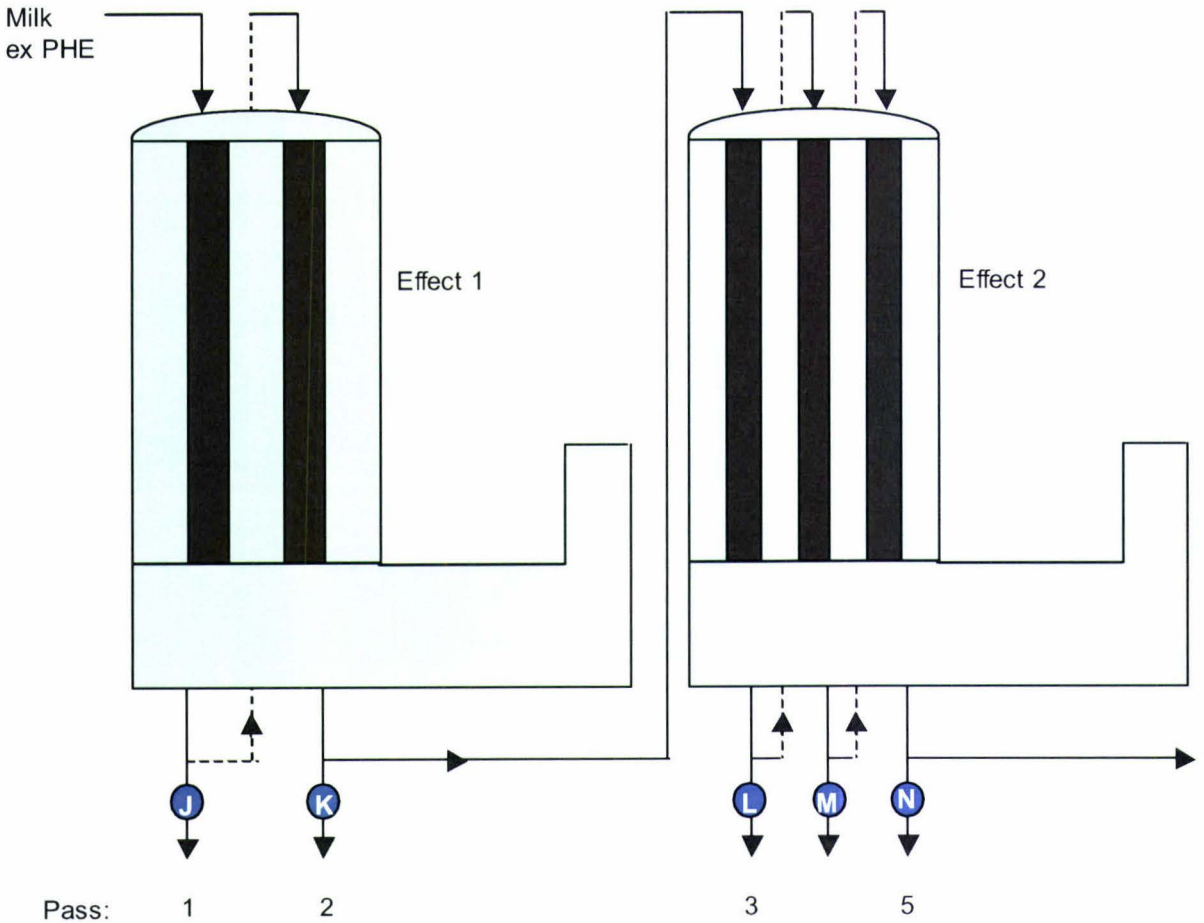

Figure 8: Schematic diagram of the evaporator of the Pahiatua Powder 2 plant

J, K, L, M, N indicate the points at which the samples were taken (refer to Table 4 for a detailed description).

2.4.2 Sampling plan

To determine the likely site of spore formation during milk powder manufacture, three sampling trials were undertaken at the Pahiatua milk powder plant. Trial 1 was undertaken in the raw milk treatment section and trials 2 and 3 were undertaken in Powder 2. Three types of whole milk powder were produced during these trials (Table 2). Total thermophile and thermophilic spore counts were performed on trial 1 and 2 samples. Thermophilic spore counts only were performed on trial 3 samples. The location of the sample points used in these trials are illustrated in Figure 5, 6, 7 and 8, and a description of the sample points is outlined in Tables 3 and 4. During these trials all liquid samples (approximately 8mL) were taken from rubber septum sample points into sterile 9mL vacuum tubes (Vacuette® grenier labortechnik, Biolab) using sterile vacutainer needles. The powder samples (from the static fluid bed and vibrofluidiser) were taken by dipping a sterile pottle into the powder.

Table 2: Types of milk powder product investigated in this study

Product Type.	Input	DSI[a] temperature	DSI[a] holding time	Vibrofluidiser temperature
Instant Whole Milk	Lecithin, Vitamin A & D	94°C	Nil	45 – 65°C
Standard Whole Milk (type one)	Nil	94°C	Nil	Ambient
Standard Whole Milk (type two)	Nil	98°C	45s	Ambient

[a] Direct Steam Injection

Table 3: Milk treatment sample points

Sample Point	Stage in Process
A	Raw milk, before separation and after heating to 50°C
B	Skim milk, after pasteurisation and cooling
C	Cream, after pasteurisation and cooling
D	Standard milk, after final cooling
E	Input, permeate or lactose

Table 4: Powder 2 sample points

Sample Point	Stage in Process
F	Balance Tank
G	Plate Heat Exchanger, Ex Preheat Condensor
H	Plate Heat Exchanger, Pre Stabilising Tubes
I	Ex Plate Heat Exchanger, Pre-evaporator
J	Evaporator Pass 1
K	Evaporator Pass 2
L	Evaporator Pass 3
M	Evaporator Pass 4
N	Evaporator Pass 5
O	Scraped surface heat exhanger
P	Homogeniser
Q	Static Fluid Bed
R	Vibrofluidiser

2.4.3 Trial 1 – Bacterial thermophilic counts of samples taken from the raw milk treatment stage

End-of-run (EOR) samples were taken from three raw milk treatment runs. The details of these runs are listed in Table 5. Five sample points were used as described in Table 3. After sampling, the samples were stored at room temperature in the dark and were plated within 8 h of sampling.

Table 5: Raw milk treatment run details

Date	Train	Input	Start time	Finish time	Run period	Sample time
6/01/04	B	Permeate	3.53	9.00	5h 7min	8.15
8-9/01/04	B	Lactose	18.52	2.52	8h	2.30
13-14/01/04	A	Permeate	22.45	6.25	7h 40 min	6.00

2.4.4 Trial 2 - Bacterial thermophilic counts from samples taken at the end of whole milk powder manufacture runs.

EOR samples were taken from four different Powder 2 runs (Table 6). Two of these runs were standard whole milk powder (type one), and the second two were instant whole milk powder. The samples were taken at different points from the balance tank through to the final product from the vibrofluidiser. The sample points used differed slightly between each run and are listed in Table 6. The balance tank sample was taken only in the third and fourth sampling trials. After collection, the samples were stored at room temperature in the dark. Run 1 and 2 samples were plated within 12 h of sampling, run 3 within 20 h and run 4 within 2 h.

Table 6: Powder 2 trial 2 runs

Run	Date	Milk powder product type	Start time	Finish time	Run period	Sample time	Sample points[a]
1	11-12/12/03	Standard Whole Milk (type one)	5:46	00:46	19 h	23:30	I, J, K, L, M, N, O, P, Q, R,
2	15/12/03	Standard Whole Milk (type one)	3:44	22:44	19 h	21:35	I, J, K, L, M, N, O, P, Q, R,
3	14-15/01/04	Instant Whole Milk	21:18	15:18	18 h	13:35	F, I, J, K, L, M, N, O, P, Q, R,
4	25-26/02/04	Instant Whole Milk	15:24	9:36	18 h 12 min	9:05	F, I, J, K, L, M

[a] Refer to Table 4, and Figures 6, 7, and 8 for sample point descriptions

2.4.5 Trial 3 - Thermophilic spore counts from samples taken throughout two 18 h whole milk powder manufacture runs

Samples were taken every two hours from two Powder 2 runs. The details of these runs are listed in Table 7. The samples were taken from the balance tank through to pass 4 of the evaporator (sample points F - M). At the last sample time samples were taken from all of the Powder 2 sample points (Table 4). The first sample of run 5 was taken at 21:25 and the last at 13:25. The first sample of run 6 was taken at 3:45 and the last at 19:45. The samples were frozen at –20°C and melted rapidly at 55°C before

plating. All samples were plated within 24 h of sampling. All remaining samples were frozen at -80°C.

Table 7: Powder 2 trial 3 runs

Run	Date	Milk powder product type.	Start time	Finish time	Run period
5	02-03/02/04	Standard Whole Milk (type two)	20:58	14:58	18 h
6	10/02/04	Standard Whole Milk (type two)	3:20	21:37	18 h 17 min

2.4.6 Foulant sampling

To determine if fouling could be a possible source of thermophilic spore contamination, foulant samples were taken during a shutdown on 6 April 2004. The five runs prior to the shutdown were standard whole milk powder (type 1). Samples were scraped off aseptically from the DSI (Figure 9), the top orifice pan of the first effect in the evaporator (Figure 10), and a structural pole within the separator body of the first effect in the evaporator (Figure 10). The samples were plated (refer to section 2.2.5) within 24 h. Any remaining sample was stored at -80°C.

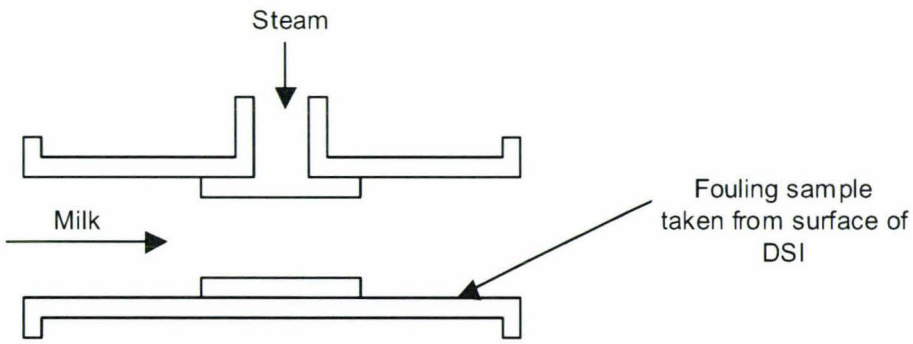

Figure 9: Schematic diagram of the direct steam injector

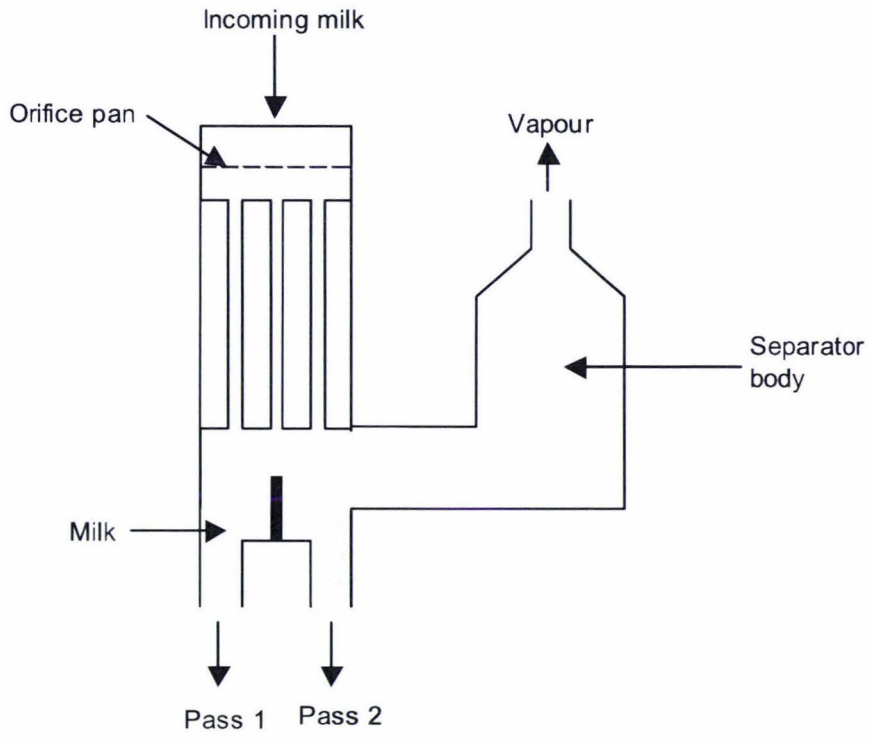

Figure 10: Schematic diagram of effect 1 of the evaporator

2.5 Identification of Pahiatua spore isolates

To determine if the Pahiatua thermophilic spore contamination was caused by *A. flavithermus* and *Geobacillus* spp., the two most common New Zealand milk powder contaminants, spore isolates were identified using specific primers designed by Flint *et al* (2001). Up to ten spore-derived colonies were isolated (refer to section 2.2.7) from each stage (sample point G through to R) at the end of run 5 and 6 (standard whole milk powder type two).

2.5.1 PCR using *A. flavithermus* and *Geobacillus* spp. specific primers

To determine the identity of the Pahiatua spore isolates, a section of the variable region of the 16S ribosomal RNA gene was amplified by PCR using *A. flavithermus* and *Geobacillus* spp specific primers (Appendix 7.1). The *A. flavithermus* primer FLAVO (5'-TAACGCCAGTTACTACGCTACTTG-3') and the *Geobacillus* spp. primer LEVO (5'-CGCCGCCCTCTTCGAACGGCGCTCC-3') were used in combination with the universal bacterial primer Y1 (5'-TGGCTCAGAACGAACGCTGGCCCG-3') (Young *et al.*, 1991) to produce a PCR product of approximately 450bp.

The following reaction mix was made up using a MasterAmp™ *Tfl* PCR kit: 264μL sterile Milli-Q water, 25μL 20 x *Tfl* PCR buffer [composition: 400mM $(NH_4)_2SO_4$. 1.0M

Tris-HCL (pH 9.0, 25°C)], 50μL 25mM $MgCl_2$, 100μL dNTP mix containing 1.25mM of each dNTP, 12.5μL of 10μM LEVO or FLAVO primer, 12.5μL of 10μM Y1 primer, and 6 μL *Tfl* DNA polymerase (1 std. U/μL). Each PCR reaction was performed in a total volume of 50μL containing 48μL of PCR mix and 2μL of template (mid exponential broth culture). A water control was also prepared containing 2μL of sterile 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 30s, 60°C for 45s, and 72°C for 45s, followed by 1 cycle of 72°C for 7 min.

All PCR reagents and products were stored at -20°C.

2.5.2 Randomly amplified polymorphic DNA (RAPD) PCR

RAPD profiles were used to differentiate strains and were generated using the primer OPR13 (5'-GGACGACAAG-3') (Ronimus *et al.*, 1997).

The following reaction mix was made up using a MasterAmp™ *Tfl* PCR kit: 460μL sterile Milli-Q water, 40μL 20 x *Tfl* PCR buffer [composition: 400mM $(NH_4)_2SO_4$. 1.0mM Tris-HCL (pH 9.0, 25°C)], 80 μL 25mM $MgCl_2$, 160μL dNTP mix containing 1.25mM of each dNTP, 5μL of 47.5μM OPR13 primer, and 10μL *Tfl* DNA polymerase (1 std. U/μL). Each PCR reaction was performed in a total volume of 80μL containing 48μL of PCR mix and 2μL of template (mid exponential broth culture). A water control was also prepared containing 5μL of sterile water instead of DNA template.

The PCR reactions were performed in a Techne PHC-3 thermal cycler using the following protocol: 94°C for 3 min 45s, followed by 35 cycles of 94°C for 15s, 36°C for 15s, and 72°C for 2 min, followed by 1 cycle of 72°C for 4 min.

2.5.3 Gel electrophoresis

Gel electrophoresis was used to visualise the PCR products. 10μL of PCR product was mixed with approximately 1μL of loading dye (composition: 0.5g Bromophenol blue, 0.25g Xylene Cyanol, 5mL Glycerol, 0.186g EDTA, made up to 10mL with 1X TBE) and loaded onto a 2% agarose (Certified™ Molecular Biology Agarose, Bio-rad) gel in 1x TBE buffer (10X TBE composition: 216g Tris, 110g Boric Acid, 14.88g EDTA, made up to 2L with Milli-Q water, pH was adjusted to 8.35 with 6M HCL or 6M NaOH). 5μL of 0.1 μg/μL 1Kb plus ladder (Invitrogen) was loaded into two lanes. Gels were run at 80V for approximately 2 h, then stained in Gel Star® Nucleic Acid Gel Stain (Cambrex BioScience Rockland, Inc., 50 μL Gel Star® in 500mL 1X TBE) for 30 min, and rinsed five times in distilled water. Gels were viewed under UV light and photographed using black and white 667 Polaroid film.

2.5.4 Randomly amplified polymorphic DNA (RAPD) analysis

The photos of the RAPD gels were scanned and analysed using Gelcompar II ™ software (Applied maths, BVBA). An unweighted pair group method using arithmetic averages (UPGMA) type dendrogram was generated using a Pearson correlation.

2.6 Enumeration of *A. flavithermus* cells using the Bactrac™ impedance analyser

Change in impedance is a standard method for measuring the number of bacteria cells in a sample (Firstenberg-Eden and Eden, 1985). The Bactrac™ (Sylab) measures the change in impedance at the electrode surface (E-value) or in the medium (M-value) as an organism grows. The change in impedance is dependent on the number of bacterial cells present in the sample and on the metabolic activity of the organism. Thus the bacterial count of a sample is inversely proportional to the impedance change. For this present study the E-values only were used to determine both vegetative cell and spore numbers of *A. flavithermus* as planktonic or adhered cells.

2.6.1 Bactrac™ calibration

The Bactrac™ was calibrated for both vegetative cells and spores of *A. flavithermus* using a similar method to that described by Flint and Brooks (2001). The vegetative cells were grown as described in section 2.2.2 and the spores were prepared as described in section 2.2.3.

The cells were washed twice by centrifugation (3000xg for the vegetative culture, and 5000xg for the spore suspension for 10 min) and resuspended in 0.1 % peptone. Serial 10-fold dilutions were performed in sterile reconstituted milk [composition: 100g skim milk powder and 1 L 0.1% peptone]. 100μL of each dilution was transferred into

quadruplet sterile bactrac tubes containing 10mL of TSB and incubated at 55°C in the Bactrac™ for 18 h and the change in impedance was recorded. The E-value threshold was set to 3%.

Standard plate counts (section 2.2.5) were performed in parallel with the change in impedance measurements. The number of colonies was recorded and a correlation graph of colony counts against the change in impedance time was produced from these data using Bactrac 4100™ software, version 4.12. The correlation graph is illustrated in Appendix 7.2 and the correlation equations are presented in Table 8. The calibration was performed over a 4 log CFU/mL range as instructed by the Bactrac™ laboratory manual and it was assumed the correlation graph was a straight line between the values of $10^1 - 10^7$ CFU/mL. The detection limit is 10 CFU/mL as described in the Bactrac™ laboratory manual (Sylab).

The different calibration curves for *A. flavithermus* in its spore form compared with the vegetative form reflects the time required for the germination of spores before vegetative growth commences.

Table 8: Bactrac™ correlation equations

CM[a] suspension	Correlation Equation	Correlation Factor
Vegetative cells	Log (CFU) = -1.3410 x t + 8.5721	R = -0.9844
Spores	Log (CFU) = -1.0803 x t + 9.3147	R = -0.9719

[a] *Anoxybacillus flavithermus*
t, time

2.6.2 Preparation of Bactrac™ tubes

Preparation of the Bactrac™ tubes consisted of washing in a laboratory dishwasher using Dishclenz (Ecolab) detergent, rinsing three times with distilled water, then autoclaving at 121°C for 15 min. Each tube was filled with 10mL sterile TSB. 100µL of milk sample or an outer surface-sterilised stainless steel sample tube was transferred to the Bactrac™ tube and incubated at 55°C in the Bactrac™, which was programmed with the appropriate calibration.

2.7 Biofilm studies

To gain an understanding of how spores form and their association with a biofilm, a reactor was set up in the laboratory. It was a modified version of the reactor designed

to study the growth of *Bacillus stearothermophilus* on stainless steel (Flint *et al.*, 2001a). The focus of this present study was on *A. flavithermus*. The reactor functions by pumping pasteurised skim milk, held at 7°C, through stainless steel tubing inoculated with the organism of interest, and held at a suitable temperature in a water bath for growth.

2.7.1 Reactor design and assembly

The reactor consisted of one 210mm and sixteen 30mm 316L stainless steel sample tubes (a total length of 690mm and volume of 9.7mL) with a grade 2B surface finish (this refers to the same type of stainless steel used in a dairy factory), connected together with red natural rubber tubing (Global) and a length of natural rubber tubing attached to either end (Figure 11). 35cm of rubber tubing was attached to the 210mm stainless steel tube and 40cm to the end of the stainless steel sample tubes. Four such setups were run in parallel

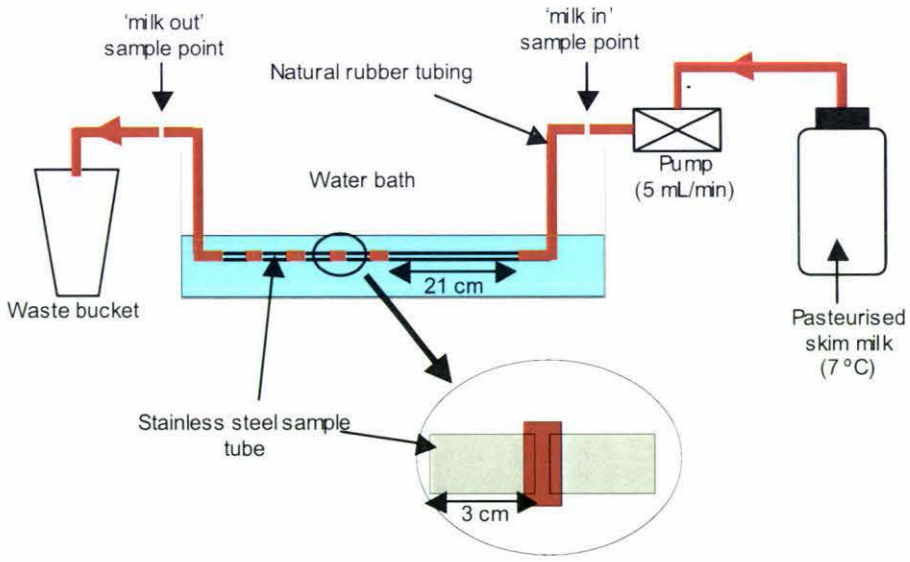

Figure 11: Schematic diagram of the laboratory reactor system

The Fonterra Palmerston North pilot plant provided fresh pasteurised skim milk the day before each reactor run. The composition of the milk was determined for each lot of milk using the milkoscan (Foss) to check for milk composition consistency. A multi-channel peristaltic pump (Buchler instruments) was used to pump the milk (5.3 ±

0.3mL/min) from a 7°C incubator through the reactor system. The flow rate was checked for each run.

The reactor was set up the day before each run, following sterilisation by autoclaving and inoculation of the reactor (refer to sections 2.7.2 and 2.7.4). A rubber tube running from the milk supply was attached to the Masterflex pump tubing (0.4m Pharmed tubing®, Cole-Parmer Instrument Co.). The pump tubing was then connected to a 5cm rubber tube that was attached to the reactor. A third rubber tube connected the reactor to the waste bucket. Sterile Masterflex plastic connectors (Cole-Parmer Instrument Co.) were used to connect rubber tubing. One exception to the set-up of the reactor system was for run 4 (section 2.7.3), where an additional 6cm of silicone tubing was attached between the last stainless steel sample tube and the 40cm natural rubber tubing at the 'milk out' end. Three stainless steel 1cm^2 coupons were placed within the silicon tubing.

The stainless steel tubing was placed in a water bath and the reactor was left overnight at room temperature. The pump and the water bath would switch on at either 1.00 am (for a 14.5 h run) or 7.00 am (for an 8.5 h run). Four reactors were run in parallel. Three of the reactors were inoculated with either a washed vegetative *A. flavithermus* CM culture or a CM spore suspension (refer to section 2.7.2 for inoculation of tubing). The fourth set was used as a control to check whether any thermophiles, present in the pasteurised milk, would attach and grow in the reactor. When the pump switched on, it would take approximately 15 min for the milk to travel from the 7°C incubator to the waste bucket, and 45 min for the water bath to reach the appropriate temperature (refer to section 2.7.3). The milk was pumped continuously through the reactor system over an 8.5 h or 14.5 h period. The reactor operated as a single pass system so once the milk had passed through the reactor it was discarded.

2.7.2 Inoculation of reactor

The reactor was inoculated using a standard protocol similar to that described by Flint *et al.* (2001a). The stainless steel tubes and the attached 35cm and 40cm rubber tubing were filled with approximately 15mL of either a mid exponential vegetative CM washed culture or a CM spore suspension, using a sterile syringe (no needle). The tubing was filled from the base of the tubes while hanging vertically. This ensured the inner surface of the tubing was exposed to the inoculum. The vegetative CM culture was grown as described in section 2.2.2 and then washed twice by centrifugation (3000xg for 10 min) and resuspended in 0.1% peptone. The spore suspension was prepared as described in section 2.2.3 and washed twice by centrifugation (5000xg for 10 min). The inoculum was incubated in the tubing for half an hour at room temperature, and then drained. Following inoculation the reactor was connected to the pump and discard bucket.

2.7.3 Sampling regime

Milk samples were taken as the milk was entering and exiting the reactor at the connecting point between the 5cm and 35cm tube, and between the waste tube and the 40cm tube. These were labelled 'milk in' and 'milk out' sample points as illustrated in Figure 11. Before and after each milk sample was taken, the connector region of the tube was swabbed with 95% ethanol. The first samples were taken half an hour after the pump was switched on, referred to as sample time 0.5 h. This was to give the milk time to pass through the tubing, and remove any cells that were loosely attached. Samples from the 'milk in' sample point and the control tube were taken at the beginning and at the end of all runs. A preliminary reactor run was performed over 8.5 h, and the 'milk out' samples were taken every 2 h. For all other runs when 'milk out' samples were taken, four stainless steel sample tubes were also removed. This was done at time 0.5 h, 4.5 h, 6.5 h, and 8.5 h for an 8.5 h run, and at time 0.5 h, 8.5 h, 11.5 h, and 14.5 h for a 14.5 h run. The 'milk out' samples were cooled in a 15 - 18°C water bath immediately after sampling. The details of all of the reactor runs are listed in Table 9.

Table 9: Reactor run conditions

Run	Inoculum (vegetative or spore suspension of CM[a])	Time period	Temperature of water bath
1	Vegetative	8.5 h	55°C
2	Vegetative	14.5 h	55°C
3	Vegetative	8.5 h	48°C
4	Vegetative	8.5 h	60°C
5	Spore	8.5 h	55°C
6	Spore	14.5 h	55°C

[a] *Anoxybacillus flavithermus*

Stainless steel sample tubes were removed to observe biofilm growth. This was achieved by stopping the pump, removing the reactor from the water bath, drying and then surface swabbing the tubes with 95% ethanol, before disconnecting four tubes. The remaining tubing was then re-connected, returned to the water bath and the pump was restarted. The outer surfaces of the four tubes were then sterilised by swabbing with 0.5% formaldehyde solution, sterile Milli-Q water, and 95% ethanol. The four tubes were then rinsed three times in 0.1% peptone, to remove loosely attached cells.

The number of both vegetative cells and spores in the milk samples or attached to the stainless steel sample tubes was estimated by measuring the change in impedance

using the Bactrac 4100 ™ impedance analyser run with the appropriate calibration (refer to section 2.6). It was assumed for the vegetative cell counts that because the spores were not activated by a heat treatment that they would not germinate within the short incubation time. Before the spore only counts were carried out both the milk samples and the stainless steel tubes were heat-treated (refer to section 2.2.6 for heat treatment procedure).

The 'milk out' samples were analysed in triplicate. The remaining samples including the 'milk in', control milk samples, and the stainless steel sample tubes were analysed in duplicate. Total thermophile and thermophilic spore plate counts (refer to section 2.2.5) were also performed on all of the control tube milk samples. This was to ensure any thermophiles naturally present in the pasteurised milk were not growing. The Bactrac™ was calibrated for a pure culture of *A. flavithermus* CM, therefore any thermophilic contaminants may not be detected by change in impedance or may contribute to the change in impedance in an undefined way.

2.7.4 Cleaning and sterilisation of reactor

Following a reactor run, each reactor was disassembled and cleaned. The 210mm stainless steel tubes were soaked in 10% NaOH overnight then rinsed in distilled water. This was to remove foulant at the entrance of the tube. The 35mm stainless steel sample tubes, rubber tubing, and connectors were soaked in a 5% solution of Pyroneg (Pyrogenic Negative Cleaner, Intermed Scientific Ltd.) overnight, and then rinsed in distilled water. Following cleaning the reactor was assembled.

Prior to use the assembled reactor was autoclaved at 121°C for 15 min. The additional rubber tubing (connected to the milk supply, discard bucket, and the 5cm tubing attached to the pump tubing), was autoclaved separately. New pump tubing was used for each reactor run. The pump tubing was sterilised by pumping 50mL 0.5% formaldehyde solution through the tubing followed by a rinse of 100mL sterile Milli-Q water.

3 Pahiatua milk powder plant study – Results and discussion

3.1 Survey at Pahiatua

A survey for thermophilic spores was undertaken in a milk powder manufacturing plant to determine the likely site of spore formation. The Pahiatua milk powder plant was chosen, as at the time Powder 2 had a bacterial thermophile contamination problem. Three sampling trials were set up. The aim of trial 1 was to eliminate the raw milk treatment section (the pasteurisation process before the milk enters the powder plant) as the source of thermophilic spores. Trial 2 was set up to get a preliminary result as to where the spores may be forming. The aim of trial 3 was to confirm the location of spore formation and to examine how fast spores form within the plant.

3.1.1 Trial 1: Bacterial thermophilic counts of samples taken from the milk treatment stage

In this trial both total and spore thermophile counts were performed on milk samples taken from three milk treatment runs (Table 10). The spore counts were all below 15 CFU/mL, demonstrating that this process was unlikely to be a major source of spores.

There was no noticeable change in thermophile numbers between the raw milk and pasteurised milk. The total counts of the standardised milk (taken at the end of the milk treatment process) ranged from less than 10 CFU/mL up to 93 CFU/mL demonstrating that the pasteurised milk destined for the milk powder plant may be a possible source of low numbers of vegetative thermophilic cells. It was not possible to trace a particular milk powder run to a milk treatment run.

These results are in agreement with previous studies that have demonstrated that raw milk generally contains low numbers of thermophiles (< 100 CFU/mL) and that thermophilic spores are seldom detected (Chopra and Mathur, 1984; Hill and Smythe, 1994; Kwee *et al.*, 1986; Muir *et al.*, 1986; Reddy *et al.*, 1975)

There is evidence that raw milk treatment has very little influence on thermophile numbers in pasteurised milk destined for milk powder manufacture (Muir *et al.*, 1986; Reddy *et al.*, 1975). This is probably because of the shorter run times of pasteurisation. However, Murphy *et al.* (1999) has recorded thermophile counts in pasteurised milk as high as 3500 CFU/mL of thermophiles, perhaps due to different handling of the raw milk before pasteurisation.

Table 10: Thermophilic bacterial counts of samples from the raw milk treatment process

	Run 1 thermophile count (CFU/mL)		Run 2 thermophile count (CFU/mL)		Run 3 thermophile count (CFU/mL)	
Sample	**Total**	**Spore**	**Total**	**Spore**	**Total**	**Spore**
Raw	< 10	< 10	50	< 10	10	< 10
Skim	No sample	< 10	43	< 10	< 10	< 10
Cream	13	< 10	167	< 10	27	< 10
Standard	10	< 10	93	< 10	< 10	< 10
Input[a]	< 10	13	< 10	< 10	< 10	< 10

[a] permeate or lactose

3.1.2 Trial 2: Bacterial thermophilic counts from samples taken at the end of whole milk powder manufacture runs.

Samples were taken at the end of four whole milk powder manufacture runs from sample points throughout the manufacture process as detailed in Table 4 & 6 section 2.4.2. Thermophiles were detected throughout Powder 2 (Figure 12). Thermohiles were detected in low numbers and the spores were not detected in the incoming pasteurised milk from the balance tank. The largest increase in bacterial counts from the balance tank to the pre-evaporator indicates that the preheat section was the predominant site of both thermophile growth and spore formation. This result is in agreement with other studies that have shown the preheating section is probably the most active site of thermophile growth, and also indicates that spore formation probably occurs at the same site as the vegetative growth (Murphy *et al.*, 1999; Warnecke, 2001).

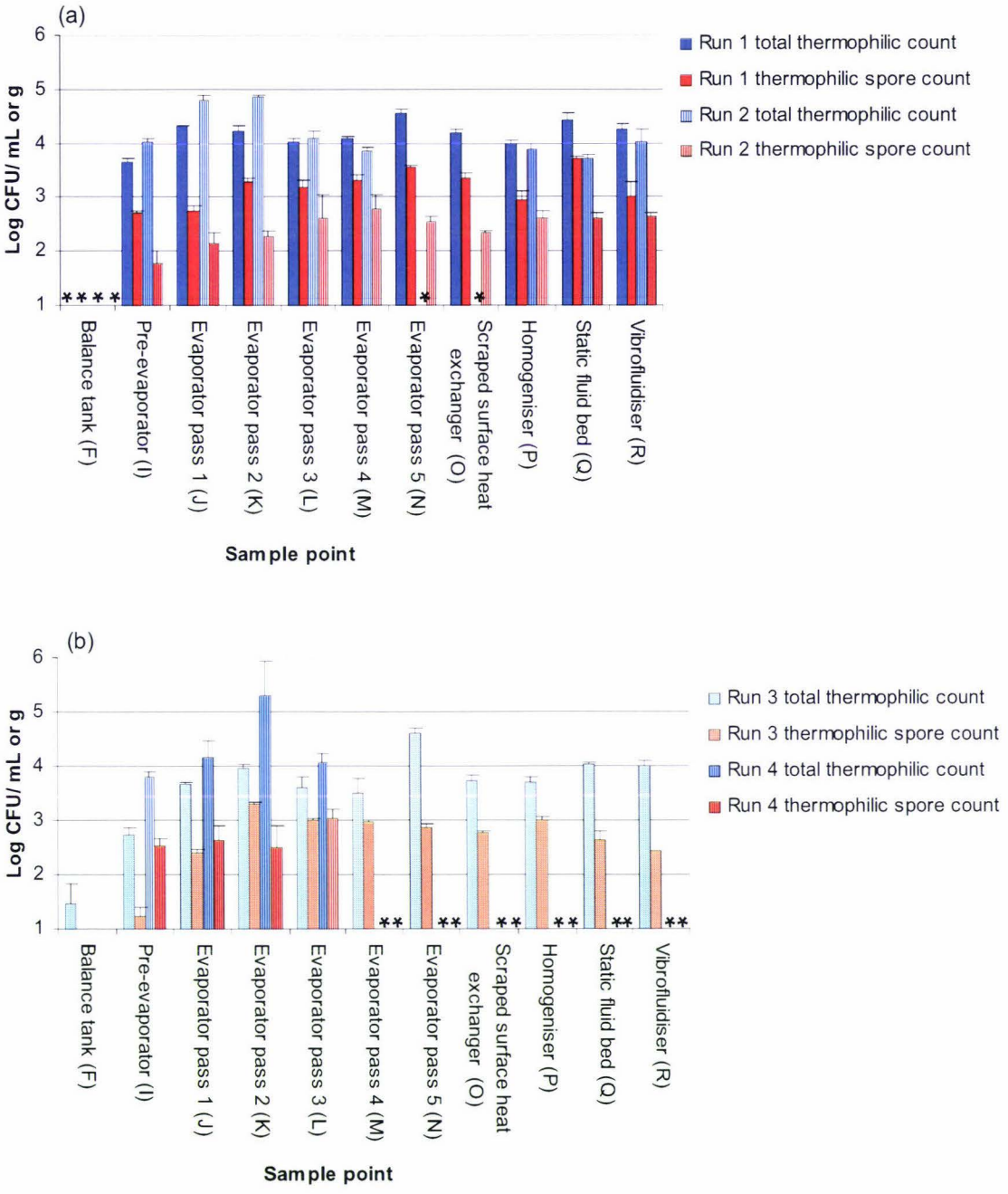

Figure 12: Thermophilic bacterial counts from samples taken at different stages at the end of various whole milk powder runs

Product samples were taken approximately 1 h prior to the end of two standard whole milk powder runs (a) and two instant whole milk powder runs (b). Thermophilic bacterial counts were determined by standard plate methods as described in section 2.2.5. Where * is marked no sample was taken. Each bar represents the mean and standard deviation of triplicate counts. The powder sample counts (Q and R) were measured in $\log_{10}$ CFU/g and the remaining samples were measured in $\log_{10}$ CFU/mL.

In Powder 2 a PHE and DSI were used for the preheat treatment. A PHE could provide a very extensive surface area for thermophile growth. To the author's knowledge there is no visual evidence in the literature that thermophilic biofilms develop on evaporator PHE surfaces or any information about the predominant sites of thermophilic growth in a PHE. This is probably because of the cost and time involved in dismantling a PHE. However, PHEs in the New Zealand dairy industry are considered to be a major site of thermophile growth based on regular manufacturing plant monitoring (Personal communication Steve Flint, 2005). Bacterial adhesion and growth on tube heat exchangers, gaskets, and spiral heating tubes have been recorded (Austin and Bergeron, 1995; Giffel *et al.*, 1997; Langeveld *et al.*, 1995; Warnecke, 2001).

There is evidence that preheat treatment before evaporation actually favours growth of thermophilic species (Murphy *et al.*, 1999). The authors showed that a typical heat treatment of 77°C for 15s did not inactivate vegetative cells. On the contrary, the more intensive heating schedule, with three heat treatments instead of one resulted in a noticeble increase in thermophile numbers but not spores in the final product. This was probably a result of a more extensive surface area for the development of a thermophilic biofilm.

Thermophile growth and sporulation can also occur in evaporators (Stadhouders *et al.*, 1982; Warnecke, 2001). This present study suggests spore formation probably also occurs in the evaporator as there is more than 1 $\log_{10}$ increase in spore counts between the pre-evaporator and the evaporator pass 1 samples from run 3 (Figure 12b).

The total thermophile counts, in all of the samples, were consistently higher than the spore counts. Generally both the total and spore counts from the pre-evaporator sample point through to the vibrofluidiser ranged from 2 $\log_{10}$ – 5 $\log_{10}$ CFU/mL. The thermophilic spore counts, of the pre-evaporator samples, were an exception, only 60 CFU/mL were present in run two (Figure 12a) and 17 CFU/mL for run three (Figure 12b).

The concentration of total solids in the milk increases as the milk passes through the process. The total solids (T/S) concentration in the milk, as it exits each pass of the evaporator, is given in Table 11. The total and spore count calculations were not corrected for this decrease in volume due to evaporation. This is because bacterial numbers are treated exponentially; therefore any correction would be less than 0.5 $\log_{10}$ for the evaporation samples and up to 1 $\log_{10}$ for the powder samples (Q and R sample points).

Thermophile growth is unlikely to occur in passes 3, 4, and 5 due to the lower water activity compared with passes 1 and 2. Lane, (1982) demonstrated that as the T/S in milk increases from 12.9% to 45% the growth of the dairy thermophile *G. stearothermophilus* decreased and a noticeble growth decrease occurred at approximately 32% T/S. This is strain dependent, and some strains are known to be more tolerant to a lower water activity (A_w). The limiting water activity of most spoilage bacteria is approximately 0.90 – 0.91 (Banwart, 1989). As T/S increases A_w decreases. The exact relationship between A_w and T/S differs due to variation in milk composition.

Table 11: Total solids concentration as the processed milk exits each pass of the evaporator

Sample point	Total solids of whole milk
Balance tank	13%
Pass 1	17%
Pass 2	22%
Pass 3	35%
Pass 4	44%
Pass 5	48%

Data obtained from the Pahiatua Powder 2 Plant Manual

Both standard whole milk and instant whole milk powders produced similar results. This is not surprising as the manufacturing processes for these two products were very similar. The two major differences were the addition of lecithin, vitamin A and D to instant whole milk, and the operation of the vibrofluidiser at 45 - 65°C during the production of instant whole milk compared with ambient temperature for standard whole milk.

3.1.3 Trial 3: Thermophilic spore counts from samples taken throughout two 18 h whole milk powder manufacture runs

Thermophilic spore counts were performed on samples taken every two hours throughout two whole milk powder runs (5 and 6) as detailed in section 2.4.2. Spores were not detected in any samples until approximately 11 h into both runs (Figure 13). By the end of the run spores reached levels of 3.5 $\log_{10}$ to 4.1 $\log_{10}$ CFU/mL during Run 5 (Figure 13a) and 2.7 $\log_{10}$ to 3 $\log_{10}$ CFU/mL during run 6 (Figure 13b). This confirms spores were forming within the Powder 2 manufacturing plant.

The rate of spore formation, the initial sites of spore formation and the spore numbers in the final product were different for the two runs. Spore formation occurred at a faster

rate during run 5 where spores were detected after 11 h in all of the samples except the pre-evaporator, and reached levels of 3 $\log_{10}$ CFU/mL within 13 h. Whereas during run 6, spores were only detected in the second and third passes of the evaporator after 11 h but they were not detected in pass 4 until 13 h into the run and after 15 h in the preheat samples.

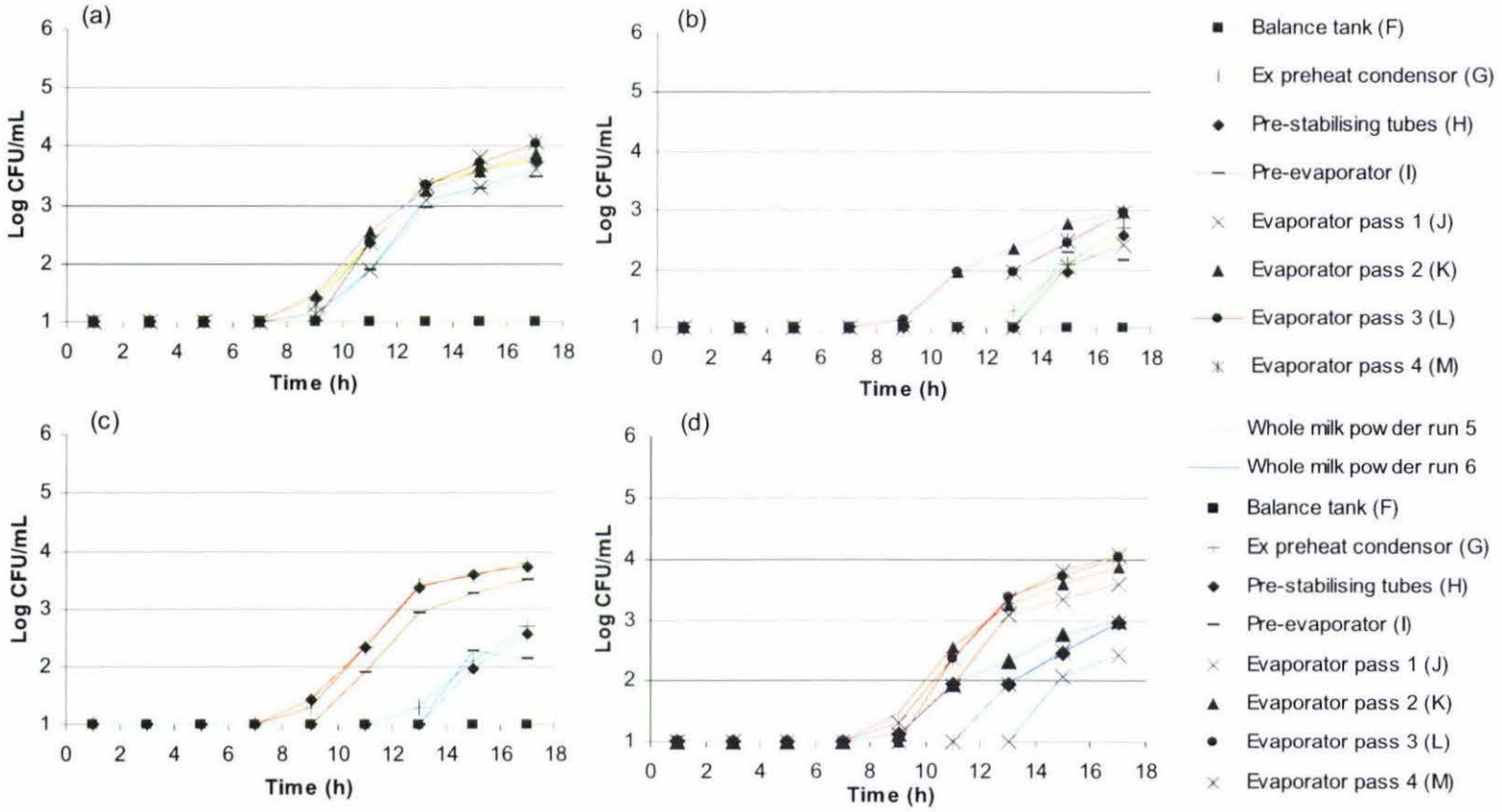

Figure 13: Thermophilic spore counts of samples taken throughout the 18 h run period of two whole milk powder runs

Product samples were taken every two hours over an 18 h run period of two standard whole milk powder runs. Thermophilic spore counts were detected using standard plate counts as described in section 2.2.5. (a) run 5 plate counts, (b) run 6 plate counts, (c) comparison of preheat plate counts between runs 5 and 6, (d) comparison of evaporator plate counts between runs 5 and 6. Each point represents the mean of triplicate counts.

The final samples were taken from all of the sample points, from the balance tank through to the vibrofluidiser. The thermophilic spore counts of these samples are presented in Figure 14. Spores were present in all of the samples. At the end of run 5 spore counts were approximately the same in all samples, ranging from 3.5 to 4.3 $\log_{10}$ CFU/mL or g. However, in run 6 the counts were lower in the pre-evaporator, at a level of approximately 2 $\log_{10}$ CFU/mL, and increased towards the downstream process stages to a level of approximately 4 $\log_{10}$ CFU/g at the end of the process.

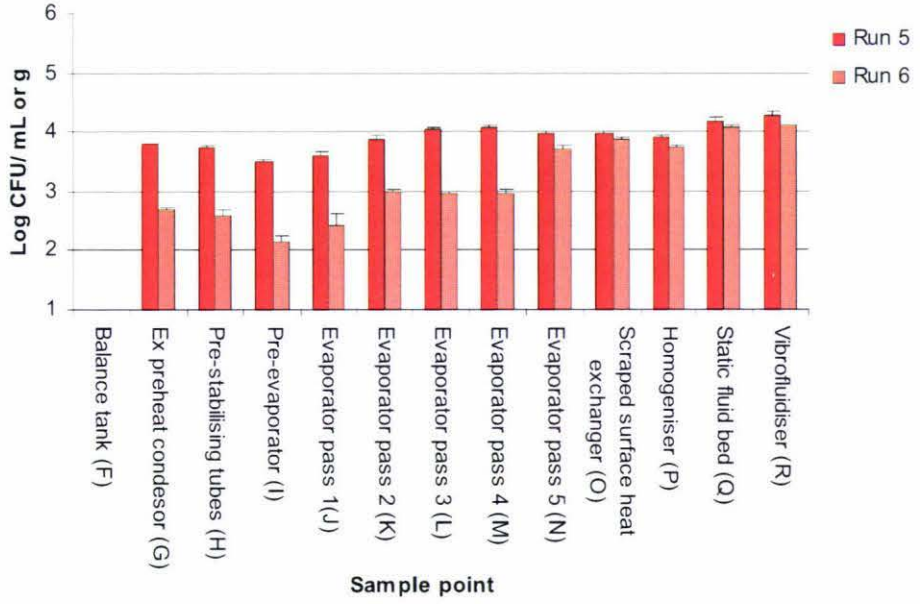

Figure 14: Thermophilic spore counts of samples taken at the end of two whole milk powder runs

Product samples were taken from all sample points 1 h prior to the end of both whole milk powder runs 5 and 6. Thermophilic spore counts were detected using standard plate counts as described in Section 2.2.5. Each bar represents the mean and standard deviation of triplicate counts. The powder sample counts (Q and R) were measured in $\log_{10}$ CFU/g, the remaining samples were measured in $\log_{10}$ CFU/mL.

There are many variables that can occur between two manufacturing runs and between manufacturing plants. For example, milk quality (age, chemical, and microbiological); temperature of stored milk; product type; equipment; and temperature regimes may be different. These factors may have an impact on sporulation. With the exception of raw milk microbial quality (Muir *et al.*, 1986), there appears to be little information on the effect of these variables on thermophilic sporulation.

It is difficult, because of run variability, to determine why the rate of sporulation and spore counts differed between runs 5 and 6. One possibility is the timing of manual cleans. A manual clean occurred immediately before run 5. It is possible that foulant was dislodged during this clean and resulted in greater contamination during the following run. During the manual clean that occurred four runs after run 5 it was noted that the DSI unit was very dirty from extensive fouling, which had also spread into the surrounding pipe work (personal communication Robin Davenport – Pahiatua milk powder plant supervisor, 2004). Based on this information it is also possible that the DSI was not adequately cleaned during the previous manual clean. A manual clean occurred 4 runs before run 6 and no extensive fouling was noted during the manual clean that occurred after run 6.

The results from this study differ from similar studies by Warnecke (2001) and Murphy *et al.* (1999). These studies monitored the growth and spore formation of thermophilic bacteria during the manufacture of whole milk powder at the Te Awamutu (New Zealand) whole milk powder plant and skim milk powder in a pilot scale manufacturing plant respectively.

Warnecke (2001) demonstrated that thermophile growth and sporulation predominated in the preheat section and to a lesser degree during the initial stages of evaporation. Spores started to be detected 7 h into the run. The main difference at the Te Awamutu site, compared with Pahiatua, is the presence of a seven-effect evaporator that ranges in temperature from 43°C to 74°C instead of a two-effect evaporator operating at 65°C. Warneke (2001) also used a different plate count method that involved using various heat treatments (mainly 10 min at 100°C) and different media. This may explain why spores were detected in 7 h compared with 10 h in this present study.

In the study by Murphy *et al.* (1999) there was no notable difference in spore numbers between the start of the run and the end of the run or between the preheater evaporator samples and powder samples. However, thermophile counts increased throughout the run and the authors concluded that vegetative cell growth was preferred over sporulation. One reason for their observations may have been because skim milk powder was produced instead of whole milk powder. Anecdotal evidence in the New Zealand dairy industry suggests spore formation is worse during whole milk powder production compared with skim.

3.2 Identification of Pahiatua spore isolates

A PCR assay (Flint *et al.*, 2001b), was used to determine whether *A. flavithermus* and the *Geobacillus* group were the predominant organisms causing spore contamination in the Pahiatua Powder 2 plant. Up to ten spore isolates that originated from samples collected at different stages during the manufacture of milk powder at the end of runs 5 and 6, were identified. The spore-derived colonies were randomly picked from MPCA + S medium spread plates. There was no difference in colony morphology between *A. flavithermus* and *Geobacillus* spp. on MPCA + S medium (Figure 15). Two primers (flavo and levo), each specific for one of the two organisms (*A. flavithermus and Geobacillus* spp.), were used in conjunction with a universal primer (Y1) to identify the spore isolates as described in section 2.5.1. The presence of a PCR product, of approximately 450bp in length, with the appropriate primer set identifies the isolate as the organism corresponding to that primer set.

Examples of these PCR products run on electrophoretic gels are shown in Figures 16 and 17. The spore isolates used for these PCR assay examples originated from run 6, pass 2 of the evaporator. Three of the ten spores isolated were *A. flavithermus* and seven were *Geobacillus* spp. Two reference strains, *A. flavithermus* DSM 2641, and *Geobacillus thermoleovorans* DSM 5366, were used as controls. Each control produced a PCR product of the correct size only with their corresponding primer set, and not with the alternative primer set.

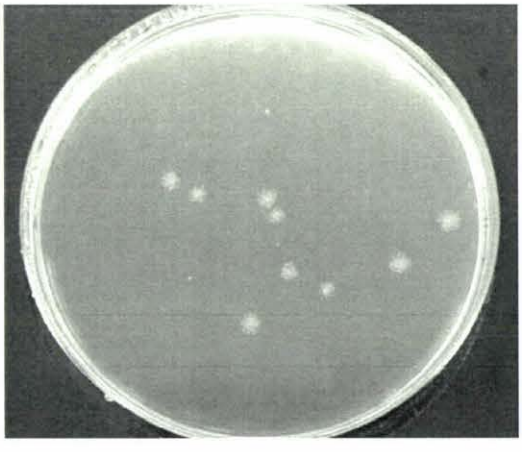

Figure 15: Spread plate of bacterial thermophilic spore-derived colonies isolated from the Pahiatua Powder 2 plant

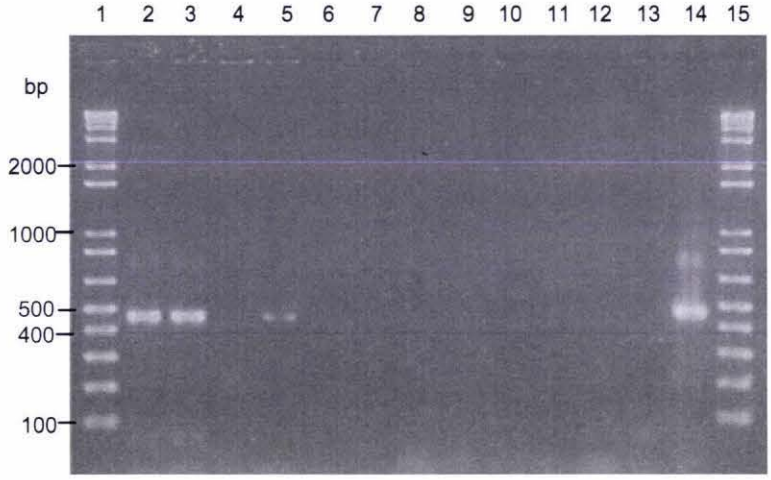

Figure 16: PCR products from *Anoxybacillus flavithermus* specific PCR assays

Specific primers for *A. flavithermus* were used to amplify a region of the 16S ribosomal gene (described in section 2.5.1) of Pahiatua spore isolates from evaporator pass 2 end-of-run (EOR) samples collected from run 6. The PCR products were separated on a 2% agarose gel and stained with Gel Star ® Nucleic Acid Gel stain. The molecular band size is specified on the left in base pairs (bp).

Lanes	1	1Kb plus ladder
	2 - 11	EOR pass 2 evaporator spore isolates
	12	Water control (no DNA template)
	13	*G. thermoleovorans* DSM 5366 (negative control)
	14	*A. flavithermus* DSM 2641 (positive control)
	15	1Kb plus ladder

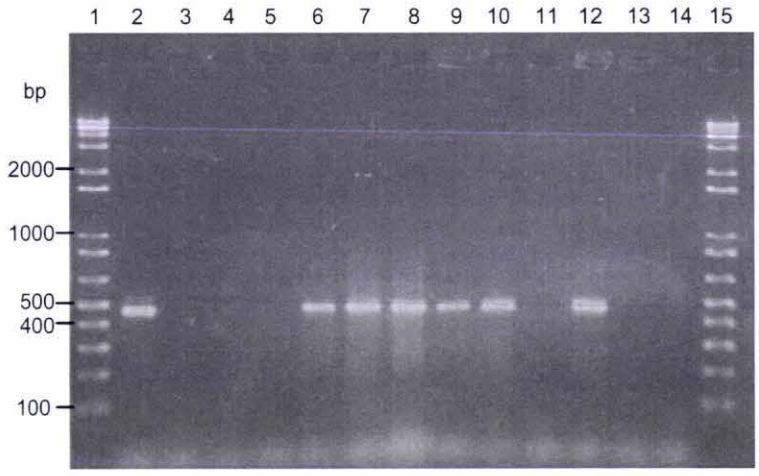

Figure 17: PCR products from *Geobacillus* spp. specific PCR assays

Specific primers for *Geobacillus* spp. were used to amplify a region of the 16S ribosomal gene (described in section 2.5.1) of Pahiatua spore isolates from evaporator pass 2 end-of-run (EOR) samples collected from run 6. The PCR products were separated on a 2% agarose gel and stained with Gel Star ® Nucleic Acid Gel stain. The molecular band size is specified on the left in base pairs (bp).

Lanes	1	1Kb plus ladder
	2	*G. thermoleovorans* DSM 5366 (positive control)
	3	*A. flavithermus* DSM 2641 (negative control)
	4	Water control (no DNA template)
	5 – 14	EOR pass 2 evaporator spore isolates
	15	1Kb plus ladder

Throughout the different stages in Powder 2 (sampling points G through to R) at the end of both run 5 and run 6 all of the spores were identified as either *A. flavithermus* or *Geobacillus* spp (Figure 18). These two organisms have been frequently isolated from New Zealand milk powders, and have also been found overseas (Flint *et al.*, 2001b; Hill, 2004; Ronimus *et al.*, 2003; Rueckert *et al.*, 2004; Warnecke, 2001).

A. flavithermus predominated in the preheating stage of the milk powder process whereas there was a mix of the two types of organisms in the evaporation and drying stages of the process. In run 5 the *Geobacillus* group did not appear until the second pass of the evaporator, whereas in run 6 it appeared earlier, before evaporation and after preheating.

This is the first time to the author's knowledge that thermophilic spores have been identified throughout the different stages of milk powder manufacture. There is evidence that certain bacterial species can predominate in different sections of milk powder manufacturing plant equipment (Hinton *et al.*, 2001; Langeveld *et al.*, 1995). Warnecke (2001) also demonstrated that the dominant thermophile in the preheat section of the Te Awamutu powder plant during the 1999/2000 season was probably *A. flavithermus*. However, it was unclear whether this study focused on thermophilic vegetative cells only or both thermophilic vegetative cells and spores.

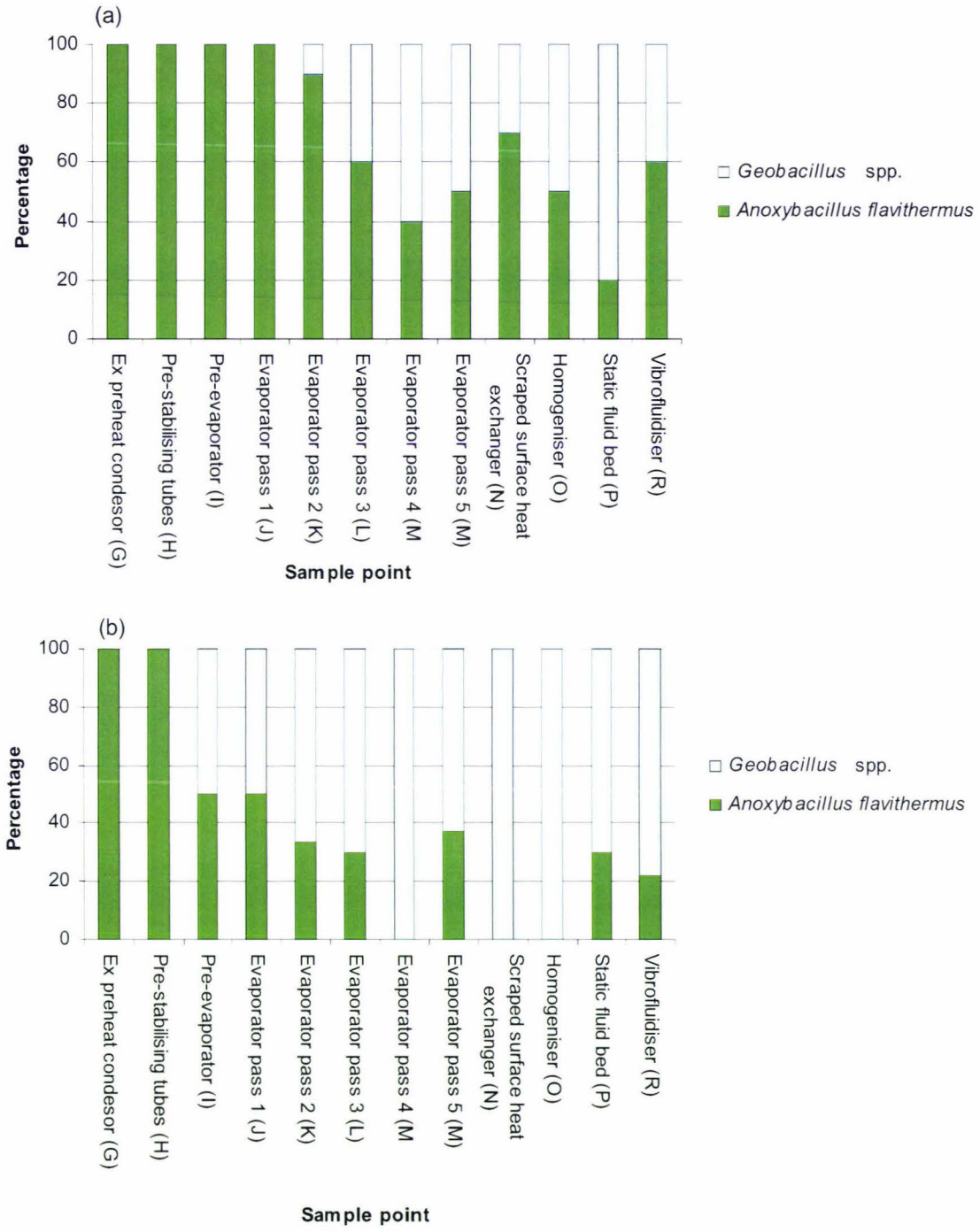

Figure 18: Identification of the predominant thermophilic bacterial spores in the Pahiatua Powder 2 plant

Pahiatua spore isolates from whole milk powder runs 5 (a) and 6 (b) were identified using *Anoxybacillus flavithermus* and *Geobacillus* spp. specific primers. Up to ten spores were isolated and identified from each sample point.

To give some indication if more than one strain of *A. flavithermus* and *Geobacillus* spp. was present in Powder 2 RAPD profiles were determined for two spore isolates isolated from each EOR sample taken at different stages during milk powder manufacture. These RAPD profiles were generated using the arbitrary primer OPR13. Examples of some of the resulting profiles are illustrated in Figure 19. To ensure reproducibility of each PCR reaction and subsequent RAPD profiles generated on each electrophoresis gel two controls were included. These were two reference isolates that originate from milk powder, *A. flavithermus* CM and *Geobacillus* sp. AM.

The RAPD profiles were analysed using Gelcompar™ software. This is a relative comparison between *A. flavithermus* and *Geobacillus* spp. only. The isolates were classified into two major groups, representative of *A. flavithermus* and *Geobacillus* spp., and and many subgroups (Figure 20). The further division of the two major groups is probably because of the presence of many faint bands on the RAPD profile. By eye, all of the *A. flavithermus* profiles looked identical, as did all of the *Geobacillus* sp. profiles. RAPD profiles of the two controls, *A. flavithermus* CM and *Geobacillus* sp. AM (milk powder reference isolates), were each repeated seven times and these profiles were not grouped together. This indicates that there was one strain only present for each thermophilic group.

The primer OPR13 has been previously used to classify thermophilic milk powder isolates in the New Zealand Dairy Industry (Flint *et al.*, 2001b; Ronimus *et al.*, 1997; Ronimus *et al.*, 2003). The band patterns of *A. flavithermus* and *Geobacillus* sp. profiles from these previous studies appear to be similar to those presented in this study. RAPD profiling may also be used to trace a contaminant through an industrial process. The results from this study indicate that the same strains of both *A. flavithermus* and *Geobacillus* spp. were present throughout the manufacturing process.

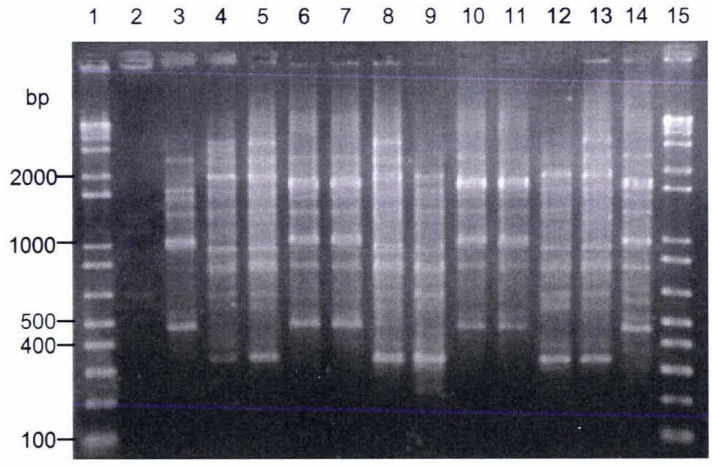

Figure 19: **RAPD profiles of a selection of Pahiatua spore isolates**

An example of RAPD profiles generated using the primer OPR 13. The PCR products were separated on a 2% agarose gel and stained with Gel Star Nucleic Acid Gel stain. The molecular band size is specified on the left in base pairs (bp).

Lanes	1	1kb plus ladder
	2	Water control (no DNA template)
	3	*Anoxybacillus flavithermus* CM
	4	*Geobacillus* sp. AM
	5	Evaporator Pass 3 *Geobacillus* sp.
	6	Evaporator Pass 3 *A. flavithermus*
	7	Evaporator Pass 4 *A. flavithermus*
	8	Evaporator Pass 4 *Geobacillus* sp.
	9	Evaporator Pass 5 *Geobacillus* sp.
	10	Evaporator Pass 5 *A. flavithermus*
	11	Scraped surface heat exhanger *A. flavithermus*
	12	Scraped surface heat exhanger *Geobacillus* sp.
	13	Homogeniser *Geobacillus* sp.
	14	Homogeniser *A. flavithermus*
	15	1 kb plus ladder

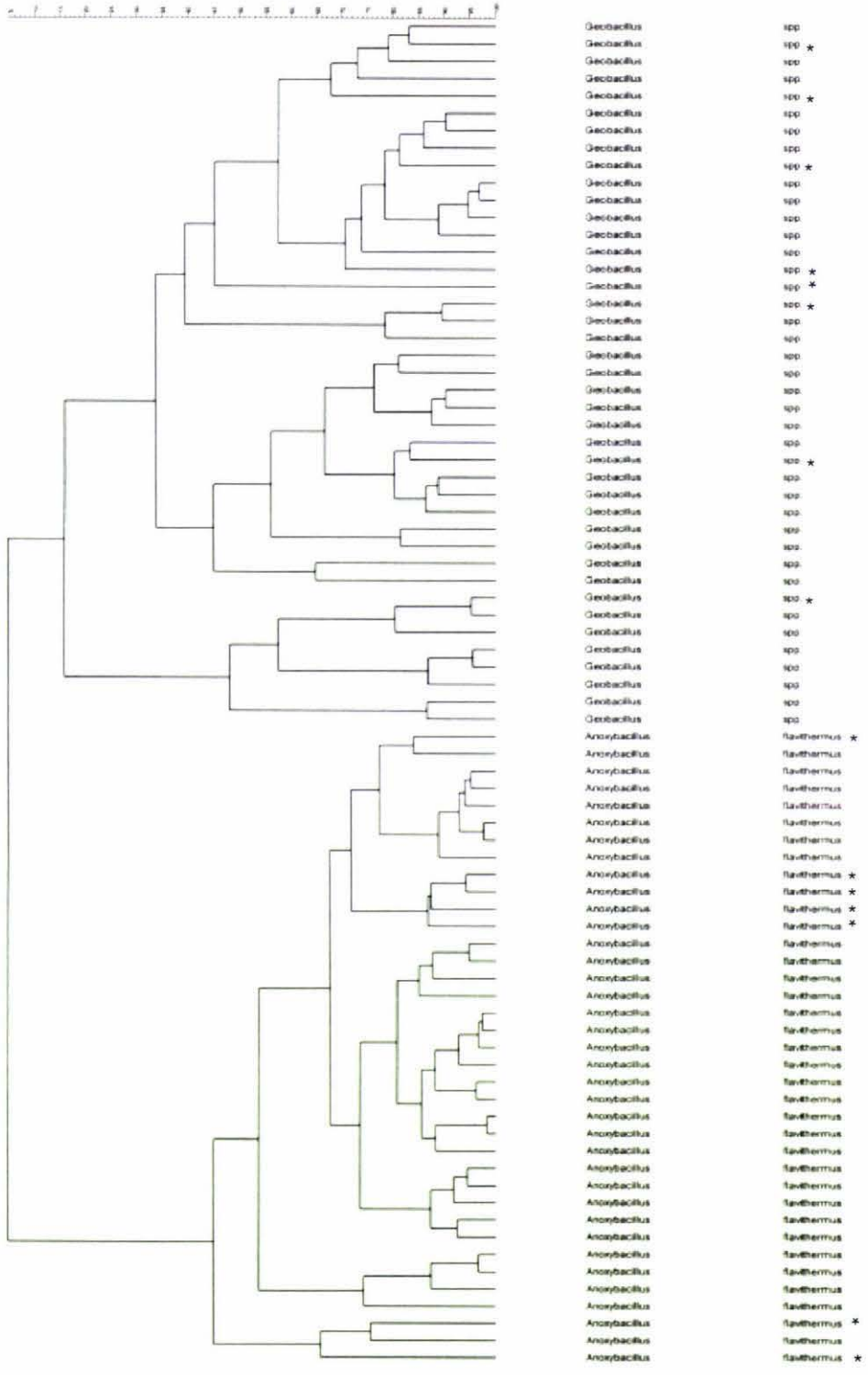

Figure 20: The classification of thermophilic spore isolates from the Pahiatua Powder 2 manufacturing plant

Gelcompar was used to generate an UPGMA type dendrogram using a Pearson correlation based on RAPD profiles of *Geobacillus* spp. and *Anoxybacillus flavithermus*. Those isolates marked with an asterisk are the controls *A. flavithermus* CM and *Geobacillus* sp. AM.

The identification of the predominant spore isolates is important. Firstly, it may help determine the origin of bacterial thermophilic spore contamination. Secondly it could enable the New Zealand dairy industry to determine the suitability of powders based on their bacterial content for different export markets. For example *Geobacillus* spores are highly heat resistant and can survive retort and UHT treatment (Hill and Smythe, 2004). Powders containing the more heat sensitive *A. flavithermus* spores would be more suitable for retort and UHT processing.

3.3 Fouling

In order for thermophiles to grow in a milk powder plant there must be an initial source of inoculum. One possible source is bacteria trapped in foulant that remains in the plant between CIPs and may only be partially removed during a manual clean (Hinton *et al.*, 2003). Fouling is the build up of milk proteins on the inner surface of dairy factory equipment. To determine if fouling was a possible source of inoculum, foulant samples were taken during a shutdown of Powder 2 and total and spore plate counts were performed.

Foulant was taken from the DSI, the orifice pans from effect 1, and a support pole in the separator of effect 1. Samples were not taken from the PHE or the second effect of the evaporator. The PHE is pulled apart and manually cleaned only if it is not functioning correctly at the end of a dairy-manufacturing season. Very little fouling occurred in the second effect of the evaporator. This is possibly because it was running at a lower volume capacity than the first effect therefore it might have had a higher flow rate to compensate for the low volume (personal communication Brian Mildenhall – Pahiatua powder plant supervisor, 2004).

Accumulation of fouling in the Pahiatua plant would have occurred for a variety of reasons. In areas where temperatures are greater than 65°C, for example in the separator and DSI, fouling will gradually build up. In the DSI unit the steam did not mix very well with the milk resulting in layers of burnt milk. Fouling can also occur in areas of flow recirculation for example under the orifice pans in the evaporator.

Photographs of foulant samples show the variation in appearance and consistency of foulant (Figure 21). The DSI sample was burnt on the side in contact with the stainless steel (Figure 21a), and milky and mushy on the other (Figure 21b). The pass 1 pan sample was similar to the DSI sample but not burnt (Figure 21c). Some parts were quite mushy and others were more rubbery with a light brown colour. The pass 2 pan sample was very fluffy and white (Figure 21d). The effect 1 separator sample was like burnt biscuit (Figure 21e).

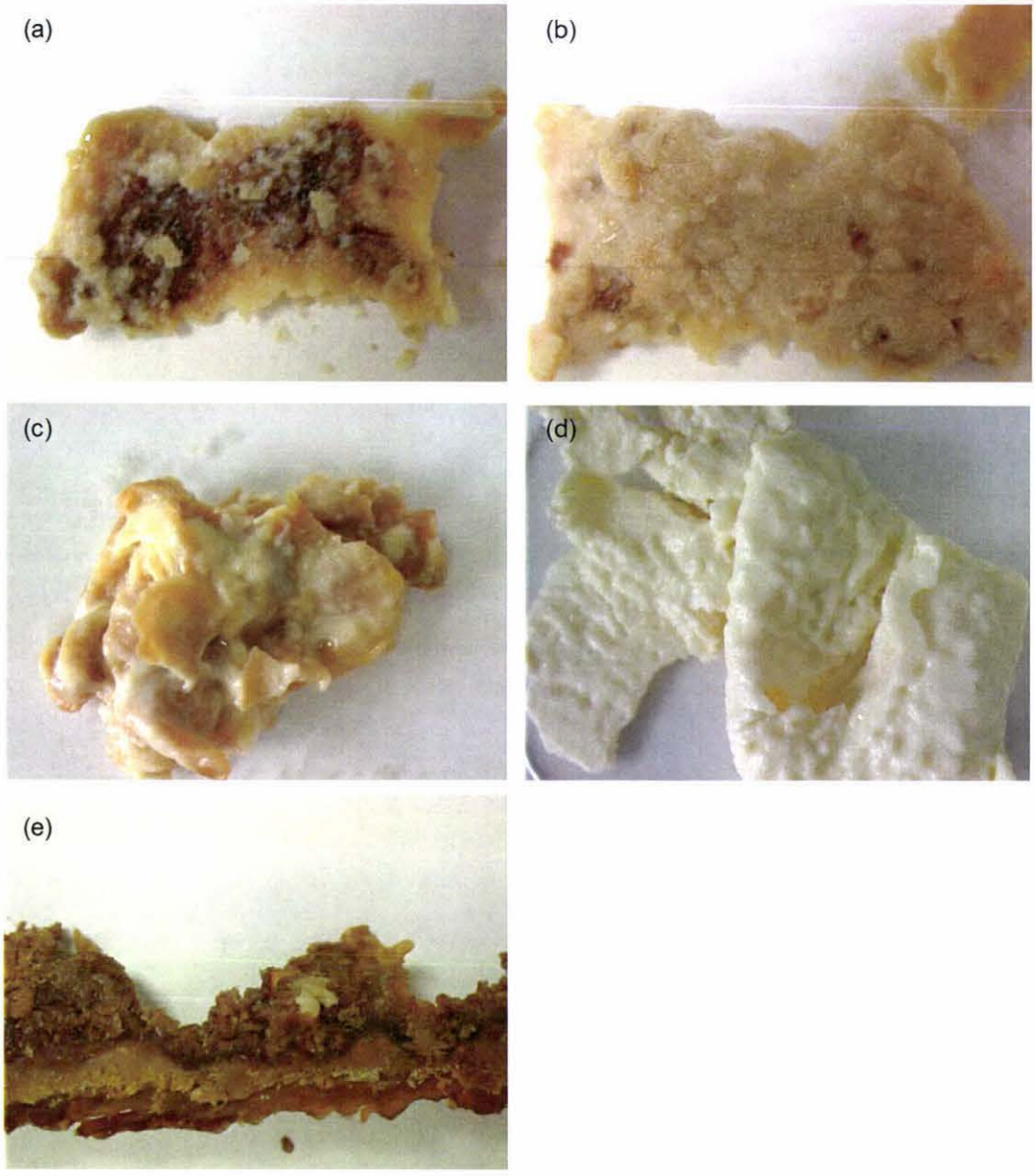

Figure 21: Foulant scraped from the surface of the Pahiatua Powder 2 plant
(a), direct steam injection (DSI) unit foulant stainless steel contact side; (b), DSI foulant, milk contact side; (c), pass 1 pan foulant; (d), pass 2 pan foulant; (e), effect 1 separator foulant.

The total and spore counts of all of the fouling samples were similar, suggesting that the bacteria present in these samples were predominantly in their spore form (Figure 22). The effect 1 separator had the highest total thermophile and spore count of approximately 7 $\log_{10}$ CFU/g. The pass 1 and pass 2 pans counts ranged between 5.3 – 6.1 $\log_{10}$ CFU/g. The DSI had the lowest count of approximately 2 $\log_{10}$ CFU/g.

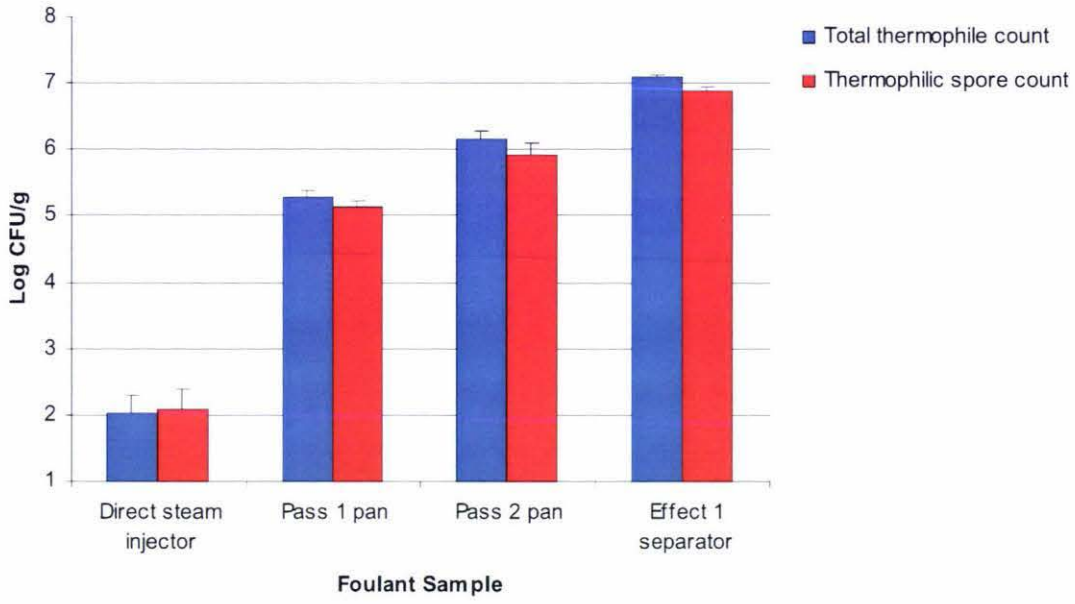

Figure 22: Bacterial total thermophile and thermophilic spore counts of Pahiatua foulant samples

Foulant was scraped aseptically from the surface of the Powder 2 manufacturing plant after a standard whole milk powder run. Bacterial counts were determined using standard plate counts as described in section 2.2.5. Each bar represents the mean and standard deviation of triplicate results.

To visualise the thermophilic bacteria within the foulant, TEM was performed on foulant from the pass 1 and 2 pans and the separator. This was not performed on the DSI sample because of the low thermophile count. Bacteria were seen in all three samples as shown in Figure 23.

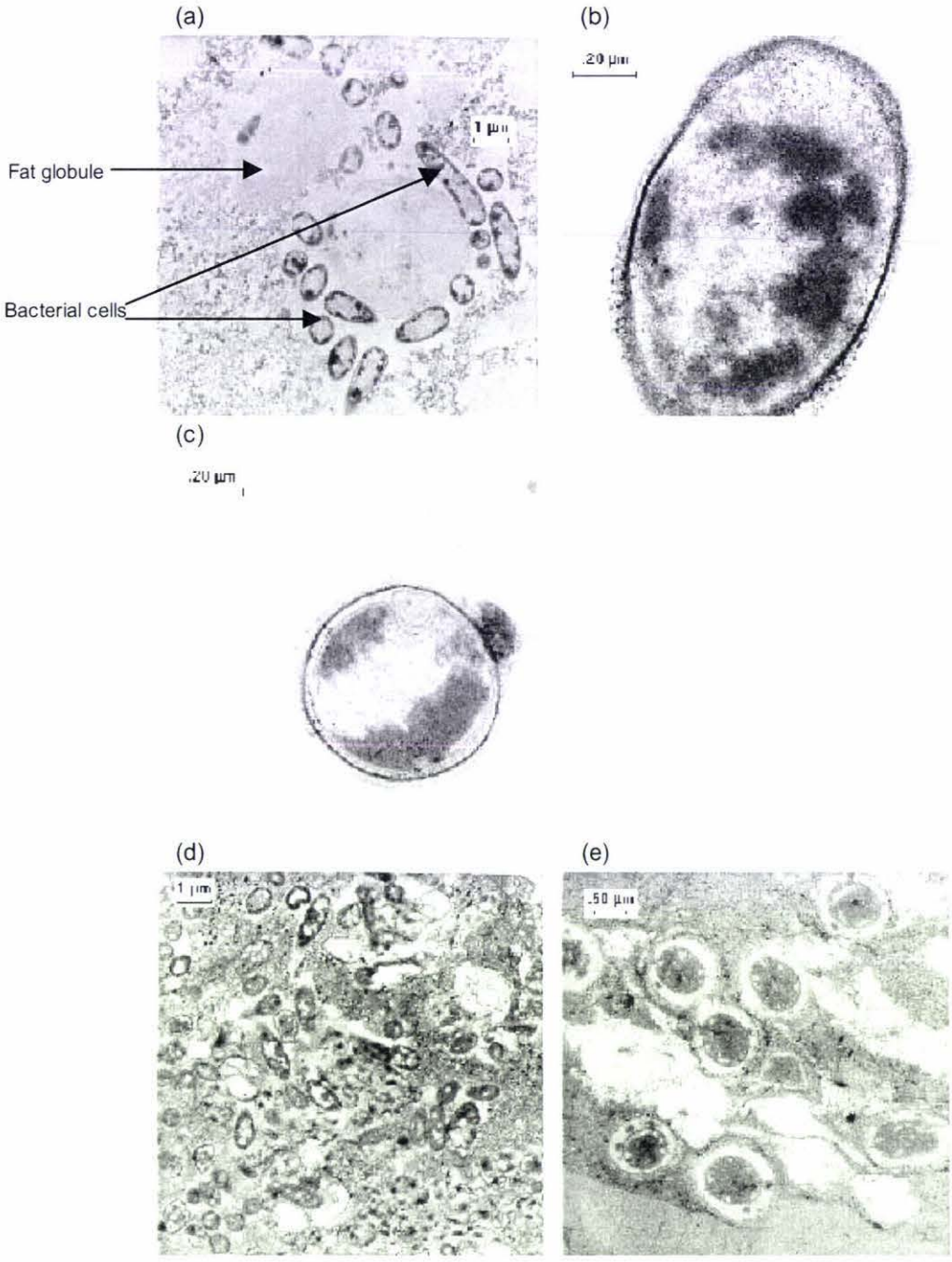

Figure 23: TEM of Pahiatua foulant samples containing thermophilic bacteria
Foulant was scraped aseptically from the surface of the Powder 2 manufacturing plant after a whole milk powder run. TEM was performed to visualise bacteria contained within foulant.from: (a), pass 1 pan; (b), pass 1 pan; (c), pass 2 pan; (d), effect 1 separator; (e); effect 1 separator.

Up to ten spore isolates were identified from each of the foulant samples, using the *A. flavithermus* and *Geobacillus* spp. specific primer sets. Examples of these PCR

products run on electrophoretic gels are shown in Figure 24. The predominant organism in all of the fouling samples was *Geobacillus* spp. No *A. flavithermus* was detected.

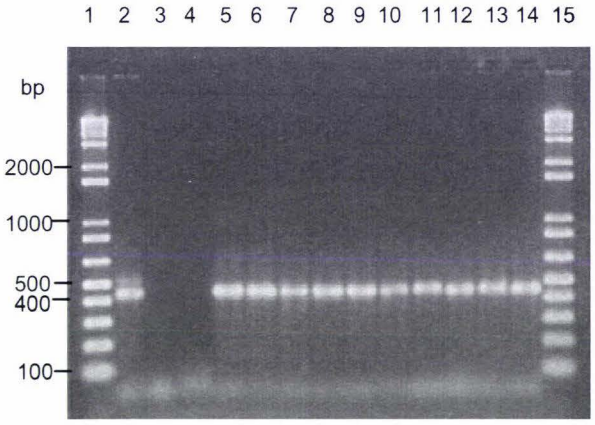

Figure 24: PCR products from *Geobacillus* spp. specific PCR assays of spore isolates from foulant

An example of a *Geobacillus* spp. specific PCR assay of spores isolated from Pahiatua pass 1 pan foulant. The primers Y1 and Levo were used to amplify a region of the 16S ribosomal gene. The PCR products were separated on a 2% agarose gel and stained with Gel Star ® Nucleic Acid Gel stain. The molecular band size is specified on the left in base pairs (bp).

Lanes	1	1Kb plus ladder
	2	*G. thermoleovorans* DSM 5366 (positive control)
	3	*A. flavithermus* DSM 2641 (negative control)
	4	Water control (no DNA template)
	5 – 14	Spore isolates taken from pass 1 pan foulant
	15	1Kb plus ladder

Identification of spore isolates isolated from the different milk powder manufacturing stages demonstrated that *Geobacillus* spp. spores were only present in the evaporator stages and onwards. These results provide further evidence that *Geobacillus* spp. predominates in the evaporator. It may also indicate that *A. flavithermus* found in the evaporator milk samples (section 3.2) could originate from cells shedding from biofilm or fouling material in the preheat section.

3.4 Summary

This study demonstrated that high thermophilic spore numbers in the final whole milk powder product were a result of sporulation occurring within the manufacturing plant. The predominant site of both vegetative growth and sporulation appeared to be the preheat section. Sporulation also occurred in the evaporator.

The major thermophilic spore contaminants were identified as *A. flavithermus* and *Geobacillus* spp. using primers specific for these organisms. *A. flavithermus* dominated in the preheat section whereas there was a mix of the two isolates later in the manufacturing process. One possible source of thermophilic contamination is foulant, which remains in the manufacturing plant after a CIP. Spore numbers, in the foulant obtained from the Powder 2 DSI and evaporator, were as high as 7 $\log_{10}$ CFU/g. The predominant thermophilic spore contaminant isolated from the foulant was identified as *Geobacillus* spp.

In summary, the results of this survey helped to understand the sporulation dynamics in an industrial setting of the thermophiles *A. flavithermus* and *Geobacillus* spp. These findings helped design the experimental parameters of a laboratory reactor to monitor the growth of dairy thermophilic biofilms and their relation to spores.

4 Spore formation in *Anoxybacillus flavithermus* biofilms – Results and discussion

To gain an understanding of how *A. flavithermus* spores form and associate with a biofilm, a laboratory reactor was used (refer to section 2.7.1 for reactor design). This reactor was a modification of that used by Flint *et al* (2001). Two major modifications were made. The reactor was extended from five to sixteen stainless steel sample tubes, to monitor biofilm development over time. Secondly the long stainless steel tube, used to bring the milk up to temperature, was straight instead of curved at one end. This enabled easier cleaning of the tube to remove fouling build-up.

A. flavithermus strain CM was chosen as the organism of study because this is a reference strain derived from milk powder, it is defined as a species, and grows faster in the laboratory than the other common thermophile group found in milk powder, the *Geobacillus* group.

Six reactor runs were performed as described in Table 9 section 2.7.3. The aims of these runs were to examine both biofilm formation and spore formation from a vegetative cell-initiated biofilm at three different temperatures (55°C, 48°C, and 60°C) and from a spore-initiated biofilm at 55°C. For each run, three reactors were used in parallel as described in section 2.7.1. The reactors were inoculated with a vegetative cell suspension containing approximately 7 $\log_{10}$ cells mL^{-1} or a spore suspension of approximately 5 $\log_{10}$ cells mL^{-1} as described in section 2.7.2. The cell count (measured using change in impedance) of the bacterial suspension used to inoculate each reactor run is detailed in Table 12.

Table 12: Bacterial counts of *Anoxybacillus flavithermus* CM cell suspensions

Run Number	*Bacterial Count*
1	7.0 ± 0.1 $\log_{10}$ cells mL^{-1}
2	7.2 ± 0.1 $\log_{10}$ cells mL^{-1}
3	6.4 ± 0.3 $\log_{10}$ cells mL^{-1}
4	7.2 ± 0.1 $\log_{10}$ cells mL^{-1}
5	5.2 ± 0.1 $\log_{10}$ spores mL^{-1}
6	5.5 ± 0.1 $\log_{10}$ spores mL^{-1}

For all of the reactor runs it was assumed that bacterial cells in the 'milk out' samples were derived from biofilm sloughing or cell release. The reactor and the attached rubber tubing held a volume of approximately 7.8mL - 10.8mL of milk that was incubated at the set water bath temperature. The milk flow, in all of the reactor runs, was 5.3 ± 0.3mL min^{-1}. As a result, any volume of milk was retained in the reactor for approximately 1.5 - 2 min. This is less than 10% of the estimated generation time. The generation time of an *A. flavithermus* planktonic culture is approximately 22 minutes (Heinen *et al.*, 1982).

4.1 Preliminary reactor run

A preliminary reactor run was set up to determine when spores begin to form in order to establish the time frame that should be used for future reactor runs. Only the release of bacteria into the flowing milk was monitored, not the biofilm. The stainless steel tubes were held at 55°C for 8.5 h and 'milk out' samples were taken every two hours at the exit point. In previous studies, thermophilic biofilms were generated at 55°C (Flint *et al.*, 2001a; Parkar *et al.*, 2003). This temperature is considered to be the optimum growth temperature of dairy thermophiles and is used for the routine cultivation of these organisms.

Both vegetative cells and spores were detected in the milk within the 8 h run period (Appendix 7.3). Spores were not detected for 4 – 6 h into the run and reached a level of 4.5 ± 0.3 log_{10} spores mL^{-1} at the end of the run. The vegetative cell counts in the milk increased from approximately 1 log_{10} cells mL^{-1} at the start of the run to 5.4 ± 0.7 log_{10} cells mL^{-1} at the end of the run. Based on these results the optimal time frame for monitoring the dynamics of *A. flavithermus* growth in this reactor system was deemed to be 8.5 h. Flint *et al.* (2001a) have examined the development of *G. stearothermophilus* biofilms over a 6 h time period with similar results.

4.2 The development of biofilms at 55°C

4.2.1 Run 1: The development of biofilms at 55°C over 8.5 h

After the preliminary run, a run under the same conditions was carried out to monitor the development of the biofilm over an 8.5 h period at 55°C (Figures 25 and 26). This was achieved by removing stainless steel sample tubes at 0.5 h, 4.5 h, 6.5 h, and 8.5 h and measuring the attachment of cells to the inner surface of the tubes by change in impedance as described in section 2.6.2.

Following inoculation, the attachment of vegetative cells to the stainless steel of the three parallel reactors ranged between 1 – 3.7 $\log_{10}$ cells cm^{-2} but by the end of the run had reached an almost constant level of approximately 6 $\log_{10}$ cells cm^{-2}. This variation in attachment of the inoculum was also seen with the growth of *G. stearothermophilus* on stainless steel (Flint *et al.*, 2001a).

Spores were detected on the surface of the stainless steel within 4.5 h and at the end of the 8.5 h run had reached levels of up to 6.3 $\log_{10}$ spores cm^{-2}. At the start of the run spores were also detected on the surface of one reactor. This is unusual, as experiments in our laboratory have repeatedly shown that spores do not to form in a TSB culture. When this experiment was repeated no spores were detected at the beginning of the run. It is possible that during the heat treatment in the spore counting procedure, the heat-transfer between the diluent and the stainless steel tube was not sufficient (for example, due to an air bubble or the stainless steel tube not being completely submerged in the diluent) or that the impedance change was measured incorrectly.

'Milk-out' samples were also taken whenever the stainless steel sample tubes were removed. There was a large variation in the vegetative counts of the milk samples at the start of the run. These ranged between 2.9 – 3.7 $\log_{10}$ cells mL^{-1}. At the end of the 8.5 h run the vegetative counts reached levels up to 6.1 ± 0.9 $\log_{10}$ cells mL^{-1} (Figure 26). The high counts in the 'milk-out' samples at the beginning of the run were probably derived from the residual inoculum washed off the surface of the stainless steel. Spores were not detected in the milk at the beginning of the run, but were present after 4 – 6 h. After 8.5 h they reached a level of 2.9 ± 0.8 $\log_{10}$ spores mL^{-1}.

The formation of *A. flavithermus* biofilms has not been well characterised as yet. Previous publications on *A. flavithermus* biofilms have focused on the physiology of mature 18 h biofilms and the effect of different cleaning regimes (Parkar *et al.*, 2003, 2004). This thesis is the first to characterise the dynamics of biofilm formation by *A. flavithermus*. The results presented here are in agreement with a similar study that focused on *G. stearothermophilus* biofilm development (Flint *et al.*, 2001a). After 6 h growth the maximum number of *G. stearothermophilus* cells released into the milk was 1×10^6 cells mL^{-1} from a biofilm that reached a maximum of 5.0×10^6 cells cm^{-2}.

It is an important observation that approximately 10 – 50% of an 8 h *A. flavithermus* biofilm developed in pasteurised milk is composed of spores. Biofilms are known to be more resistant to harsher conditions such as heat, chemicals, and radiation compared with a planktonic culture (Kumar and Anand, 1998). The presence of spores within the biofilm could greatly increase the resistance of the biofilm in a dairy environment. Spores have been shown to be more adhesive to stainless steel surfaces, heat

tolerant, and resistant to chemicals compared with vegetative cells (Husmark, 1993; Palop *et al.*, 1999; Parkar *et al.*, 2001; Popham *et al.*, 1995; Riesenman, 2000).

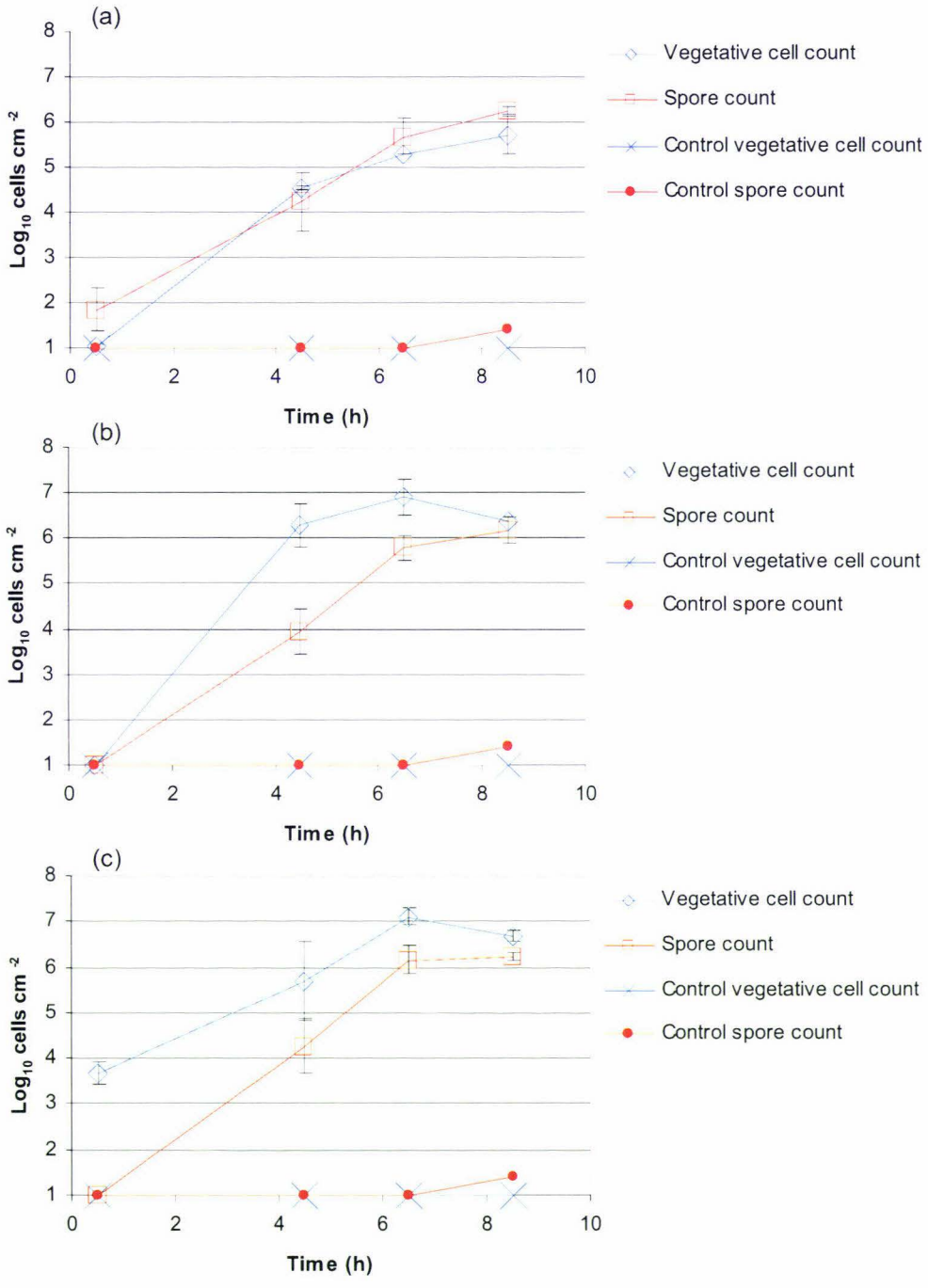

Figure 25: The development of the biofilm in the laboratory reactor at 55 °C over 8.5 h (run 1)

Bacteria, attached to the inner surface of the stainless steel tubing of three reactors run in parallel (a, b, and c), were enumerated using change in impedance at four indicated time points. Each point represents the mean and standard deviation of duplicate results.

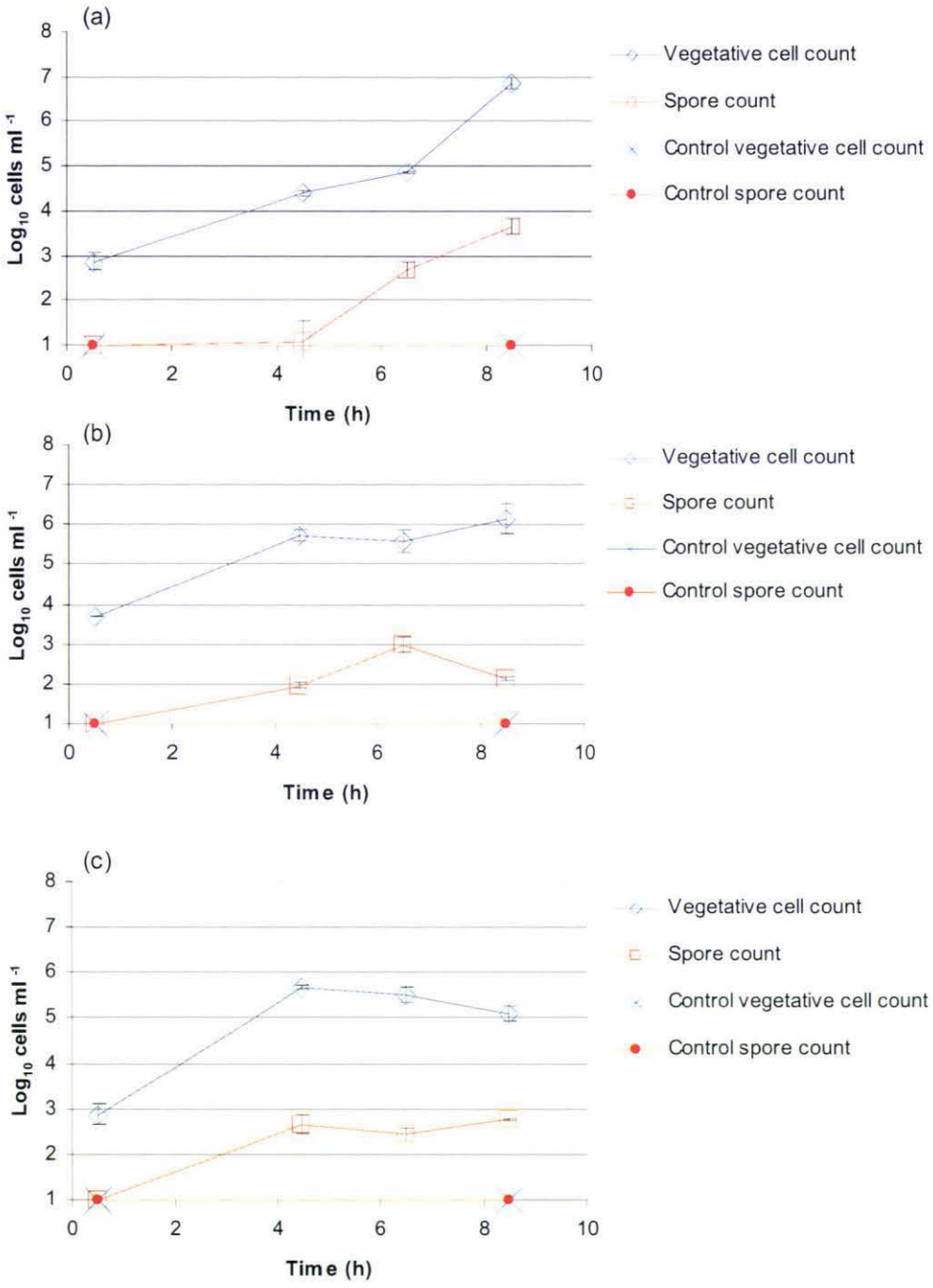

Figure 26: The release of bacteria into flowing milk from biofilms growing in the laboratory reactor at 55°C over 8 h (run 1)

The numbers of bacteria in the exiting milk from three laboratory reactors run in parallel (a, b, and c) were enumerated using change in impedance at four indicated time points. Each point represents the mean and standard deviation of triplicate results.

4.2.2 Run 2: The development of biofilms at 55°C over 14.5 h.

To determine what happens to the biofilm beyond an 8.5 h run, a similar experiment was repeated but samples were taken at 0.5 h, 8.5 h, 11.5 h, and 14.5 h (Figures 27 and 28). The growth of the biofilm reached a maximum level of 6.5 ± 0.1 log_{10} cells cm^{-2} after 8.5 h, and then maintained this level of approximately 6 log_{10}cells cm^{-2} for both vegetative cells and spores until the end of the run.

A maximum cell density of approximately 6 - 7 log_{10} cells cm^{-2} within 6 – 8 h was expected as previous studies have demonstrated this occurs with thermophilic biofilms (Flint *et al.*, 2001a; Parkar *et al.*, 2003). Process biofilms of other dairy contaminants such as *S. thermophilus* and *Pseudomonas* also reach a maximum cell density in the range of 5 – 7 log_{10} cells cm^{-2} (Flint *et al.*, 1999; Hood and Zottola, 1997). This differs from the classic biofilm, containing mixed species, which can form a thick layer of several hundred micrometres (Characklis *et al.*, 1990).

Vegetative cells and spores found in the exiting milk varied throughout the run (Figure 28). The vegetative cell and spore counts were approximately 6 log_{10} cells mL^{-1} and 4.5 log_{10} spores mL^{-1}, respectively, after 11.5 h and decreased by approximately 1 log to 4.9 ± 0.2 log_{10} cells mL^{-1} and 5.4 ± 0.2 log_{10} spores mL^{-1} after 14.5 h. This variability in the milk probably demonstrates that the release of cells did not occur at a constant rate. This is consistent with a well known fact that release of cells from biofilms in many environments often occurs in clumps, otherwise known as sloughing (Stoodley *et al.*, 2001a; Willcock *et al.*, 1997).

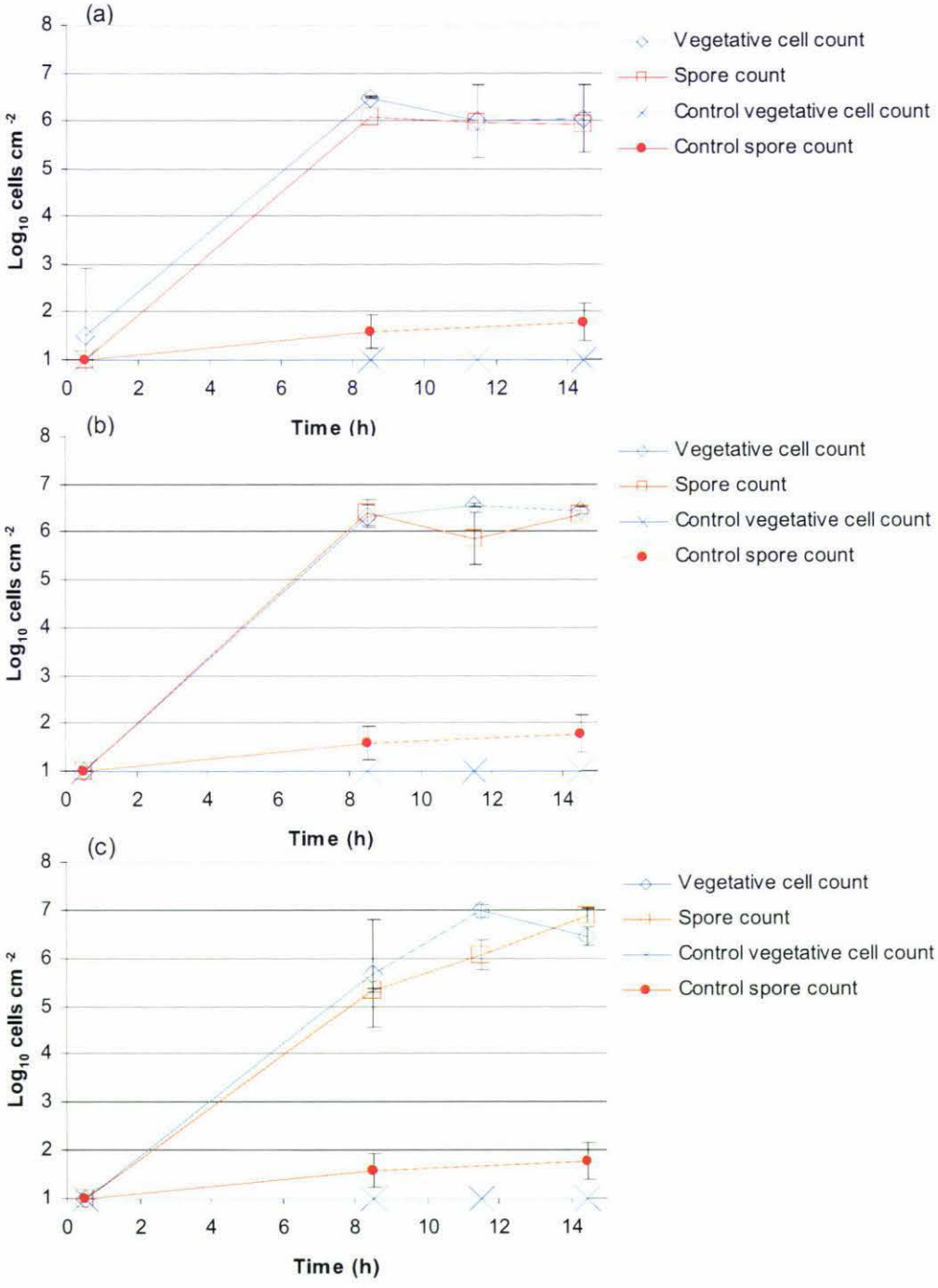

Figure 27: The development of the biofilm in the laboratory reactor at 55°C over 14.5 h (run 2).

Bacteria attached to the inner surface of the stainless steel tubing of three reactors run in parallel (a, b, and c), were enumerated using change in impedance at four indicated time points. Each point represents the mean and standard deviation of duplicate results.

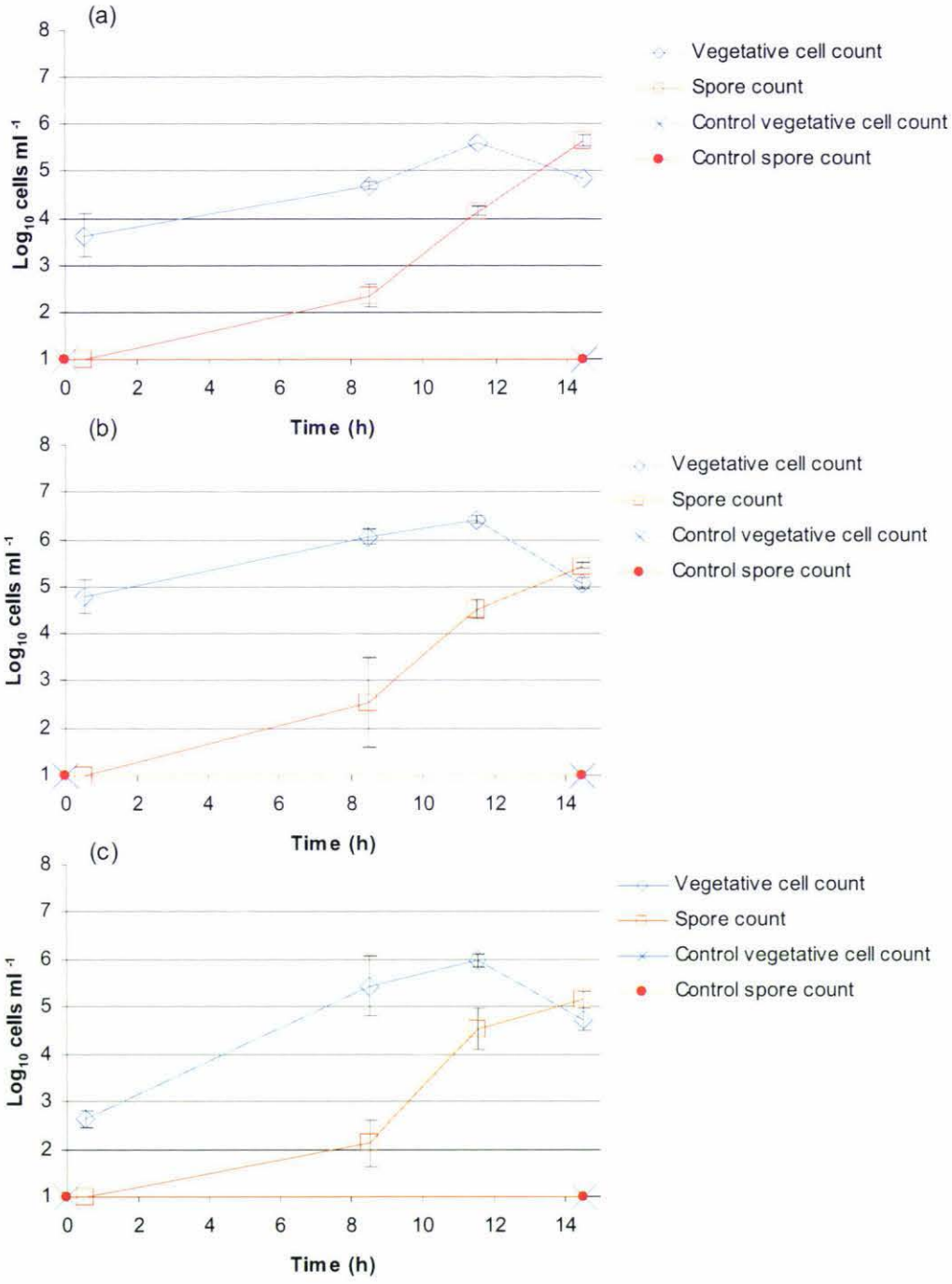

Figure 28: The release of bacteria into flowing milk from biofilms growing in the laboratory reactor at 55°C over 14.5 h (run 2)

The numbers of bacteria in the exiting milk from three laboratory reactors run in parallel (a, b, and c) were enumerated using change in impedance at four indicated time points. Each point represents the mean and standard deviation of triplicate results.

4.3 The development of biofilms at various temperatures

In the Pahiatua milk powder plant, the milk was exposed to a variety of temperatures ranging between 7°C and 95°C. The temperature used for routine cultivation of thermophilic bacteria from dairy samples is 55°C as this is considered to be their optimum growth temperature. Biofilms of *A. flavithermus* have been shown to grow readily at this temperature, but the effect of temperature variation on the growth of these biofilms is unknown. To determine the effect temperature has on biofilms and spore production, the reactor was run at two different temperatures. The aim was to choose two temperatures, one higher and one lower than 55°C, but within the temperature range of the PHE in the preheat section of the Powder 2 manufacturing plant. To select temperatures for this trial, a temperature profile for *A. flavithermus* CM was needed.

4.3.1 Temperature profile of *A. flavithermus* CM

To select two temperatures that were within the growth range of *A. flavithermus*, a temperature growth profile was determined. The culture aliquots were incubated in a temperature gradient incubator and the growth was assessed by measuring the optical density of the cultures at the wavelength of 560nm as described in section 2.2.8.

In two independent experiments, the optical density was measured over an 8 h time period (Appendix 7.4). In the laboratory, it generally takes 4 - 5 h to reach the mid-exponential growth phase of CM at 55°C. Figure 29a shows the temperature growth profile of CM after 4.5 - 5 h growth and Figure 29b after 8 h. The first growth curve had two peaks at 48°C and 54°C after 4.5 h growth, whereas the second growth curve had two peaks at 50°C and 55°C after 5 h growth. After 8 h growth the peaks of the first growth curve remained the same, but the second growth curve only had 1 peak at 48°C. The presence of two peaks could represent contamination with a mesophile or clumping. However, this result has previously been observed for an *A. flavithermus* growth curve (Cucksey, 2002).

The optimum growth temperature appeared to be between 48°C and 56°C (Appendix 7.4). This value disagrees with Heinen *et al.* (1982), which states the optimum growth temperature of *A. flavithermus* is 60°C. However, Ronimus *et al* (2003) have demonstrated that the growth range can vary between *A. flavithermus* strains. It is also possible that repeat sub culturing of this isolate at 55°C may have selected for clones of *A. flavithermus* CM with a lower optimium growth temperature than that reported by Heinen *et al* (1982). The temperature range for CM growth was also determined for a 24 h incubation period (Figure 30). At this later time, growth was also detected at temperatures ranging from 34 – 67°C, suggesting that very slow growth occurs from 34 – 43°C and 58 – 67°C.

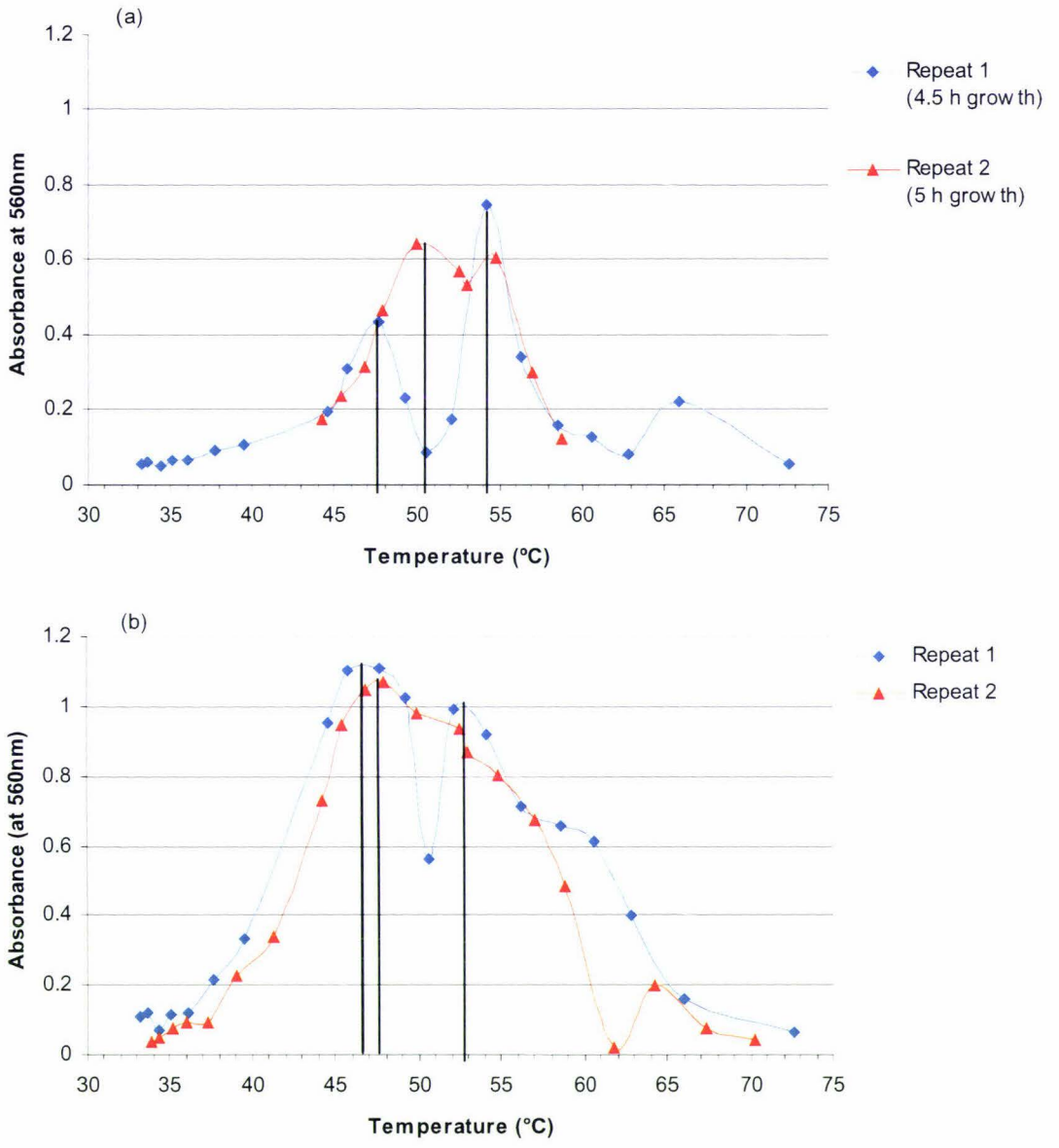

Figure 29: Temperature growth curves for *A. flavithermus* CM after 4.5 – 8 h growth
The cell density (absorbance at 560nm) was measured after (a) 4.5 - 5 h growth and (b) 8 h growth in TSB in a temperature gradient incubator. Each point represents the mean of duplicate results. The results of two experiments are shown in each graph.

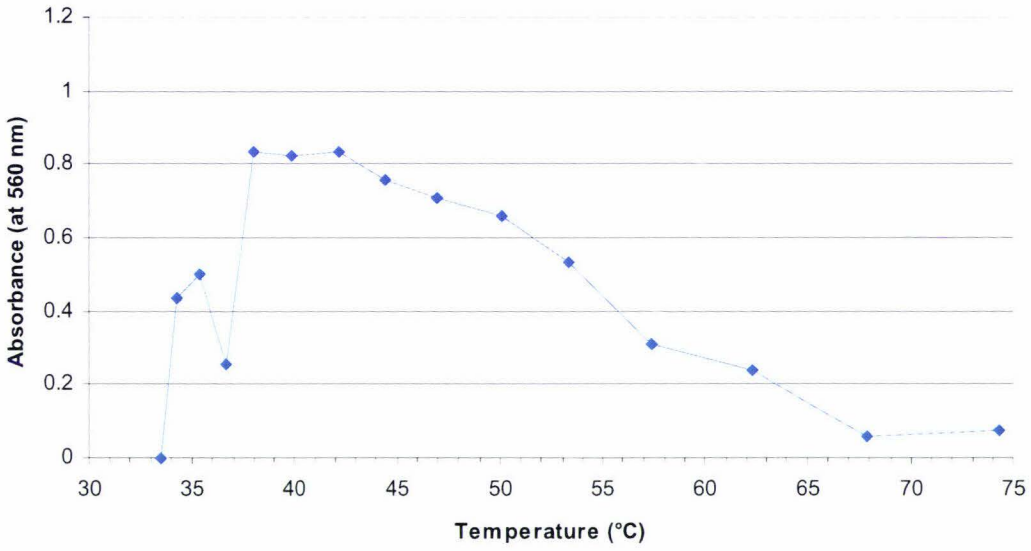

Figure 30: Temperature growth curve for *A. flavithermus* CM after 24 h growth
The cell density (absorbance at 560nm) was measured after 24 h growth in TSB using a temperature gradient incubator. Each point represents the mean of duplicate results.

Based on the temperature-dependence of *A. flavithermus* growth and the conditions in the preheat section of the Pahiatua plant, the two temperatures chosen to investigate biofilm development were 48°C and 60°C. The temperature of 48°C was chosen because it was within the optimum growth temperature range of *A. flavithermus* CM, and in the Pahiatua plant the milk is exposed to this temperature when it passes into the PHE for the first time, as it is heated from approximately 7°C to 50°C as outlined in Figure 7 section 2.4.1. The temperature of 60°C is outside the optimum temperature range but CM was still able to grow at this temperature at a slower growth rate. In the Pahiatua plant the milk reaches this temperature when it passes through the PHE for the second and third time as outlined in Figure 7 section 2.4.1.

4.3.2 Run 3: The development of biofilms at 48°C over 8.5 h

There was a 1 log reduction in biofilm growth at 48°C compared with 55°C. The maximum cell density of the biofilm was 4.9 ± 0.2 log cells cm^{-2}, after 6.5 h (Figure 31). This was an unexpected result given that the optimum temperature of CM in a planktonic culture appeared to be 48°C. Surprisingly, no spores formed on the surface of the stainless steel during the entire run. However, low numbers of spores were detected in the milk (Figure 32).

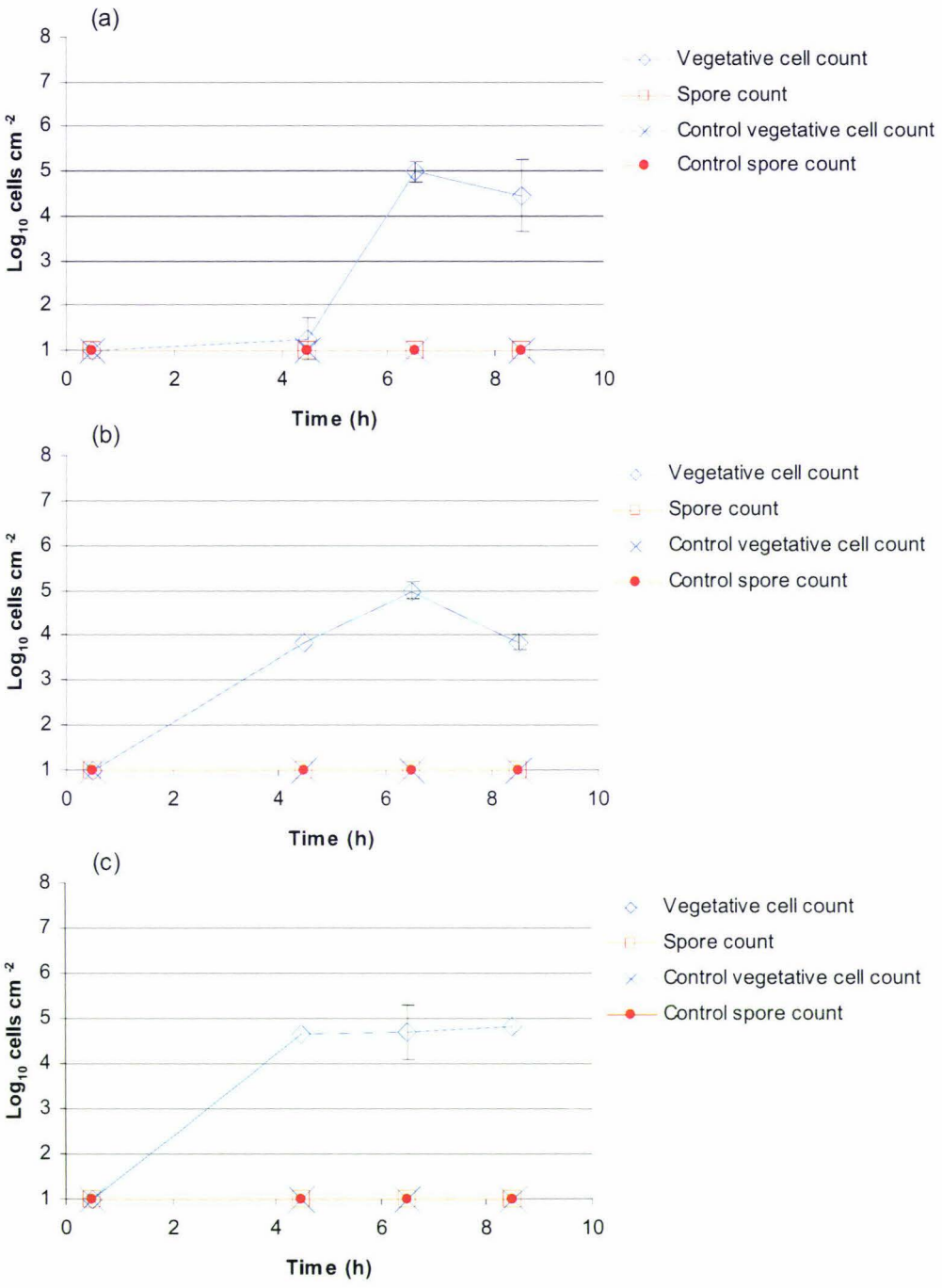

Figure 31: The development of the biofilm in the laboratory reactor at 48°C over 8.5 h (run 3)

Bacteria attached to the inner surface of the stainless steel tubing of three reactors run in parallel (a, b, and c), were enumerated using change in impedance at four indicated time points. Each point represents the mean and standard deviation of duplicate results.

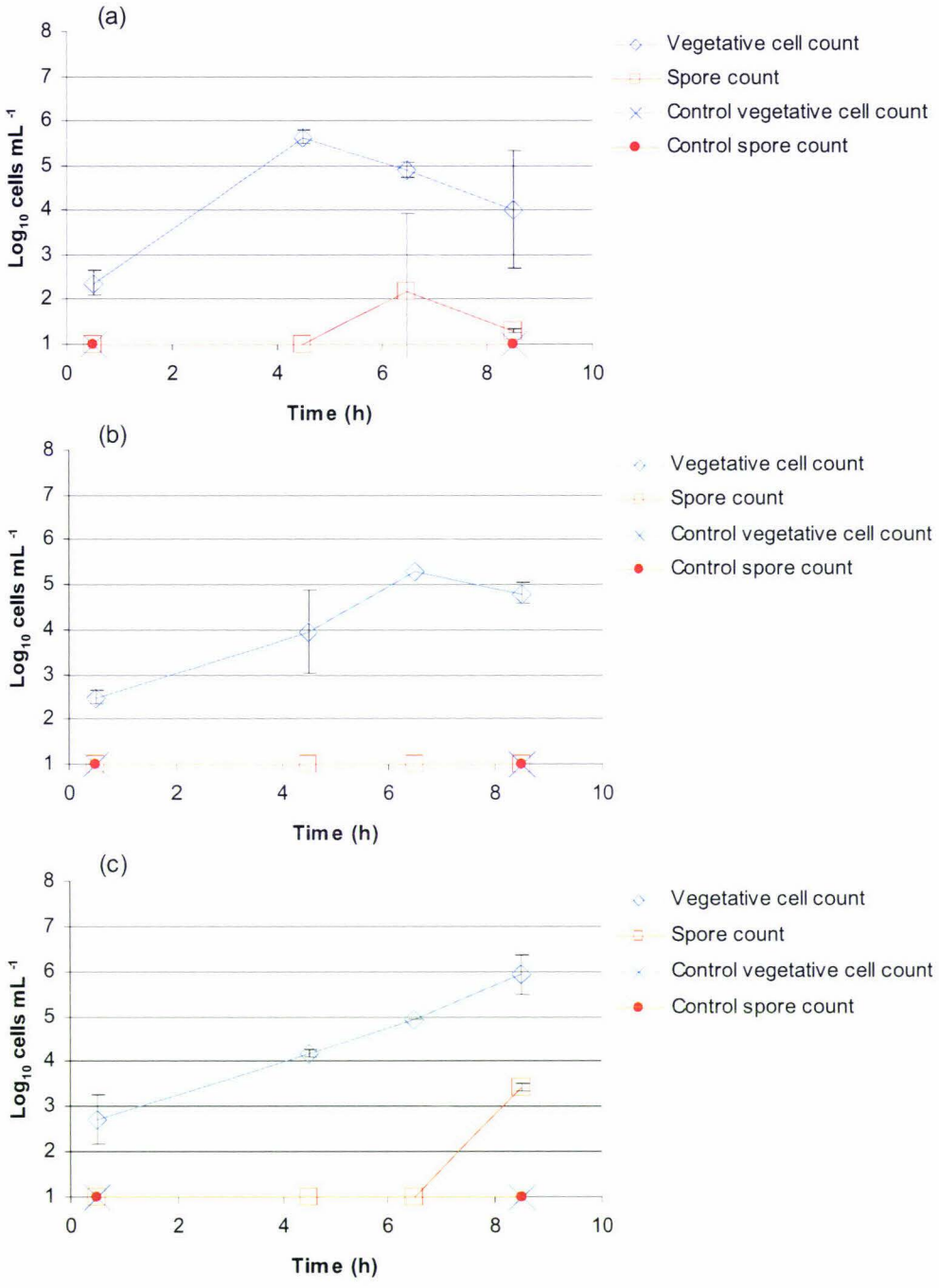

Figure 32: The release of bacteria into flowing milk from biofilms growing in the laboratory reactor at 48°C over 8.5 h (run2)

The numbers of bacteria in the exiting milk from three laboratory reactors run in parallel (a, b, and c) were enumerated using change in impedance at four indicated time points. Each point represents the mean and standard deviation of triplicate results.

4.3.3 Run 4: The development of biofilms at 60°C over 8.5 h.

The biofilm of CM grew well at 60°C and sporulation occurred (Figure 33). Within 4.5 h the biofilm density reached a maximum of 6.9 ± 0.4 log cells cm^{-2} for the three reactors. However, there were between 2.8 – 4.8 log cells cm^{-2} attached to the surface at the beginning of the run, more cells compared to any other run although a similar inoculum level was used. Sporulation occurred within 4.5 h and reached a level of 4.0 ± 1.0 log spores cm^{-2} by the end of the run. After 8.5 h 6.1 ± 0.4 log vegetative cells mL^{-1} and 3.6 ± 0.4 log spores mL^{-1} were being released into the milk (Figure 34).

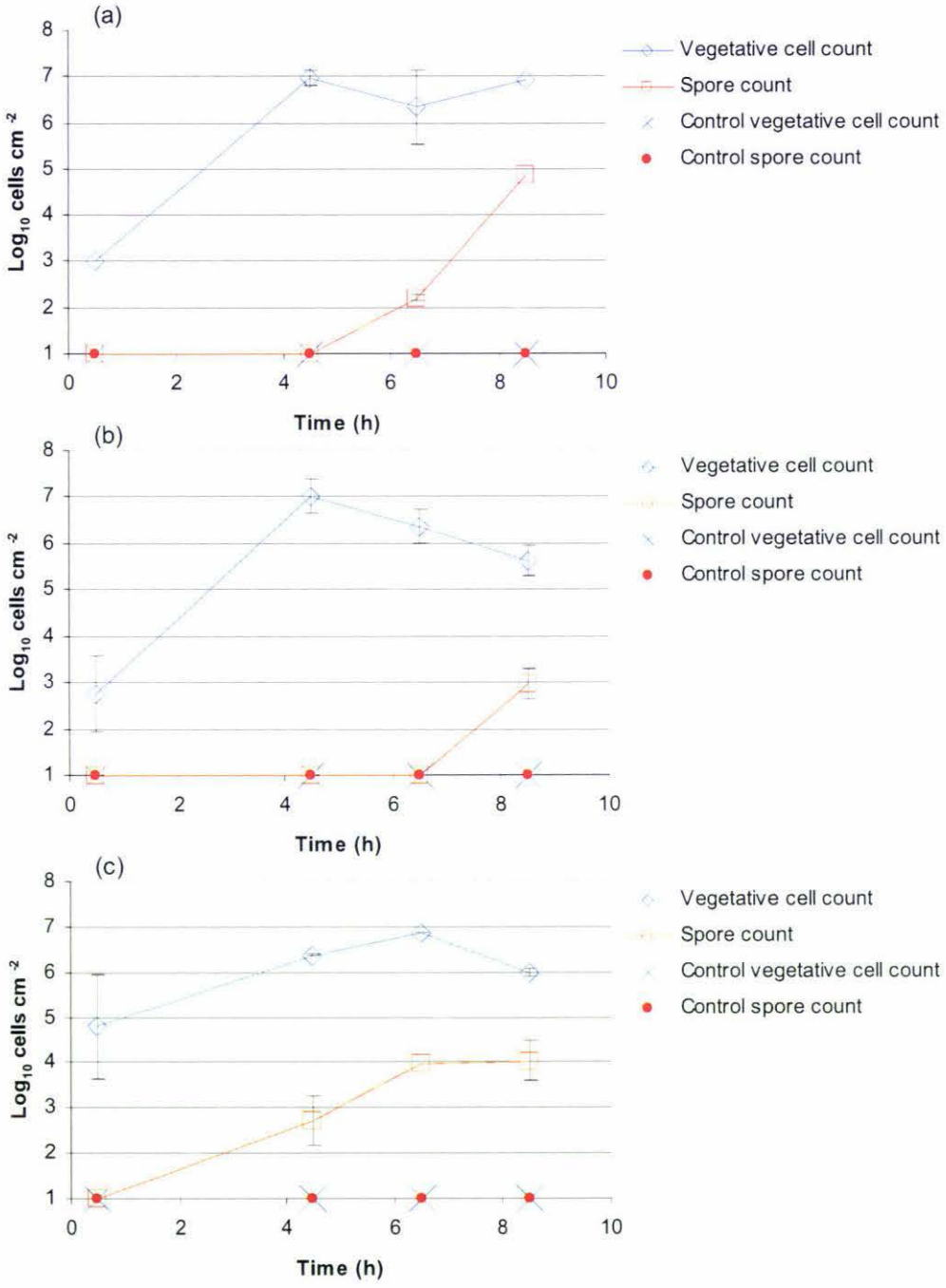

Figure 33: The development of the biofilm in the laboratory reactor at 60°C over 8.5 h (run4).

Bacteria, attached to the inner surface of the stainless steel tubing of three reactors run in parallel (a, b, and c), were enumerated using change in impedance at four indicated time points. Each point represents the mean and standard deviation of duplicate results.

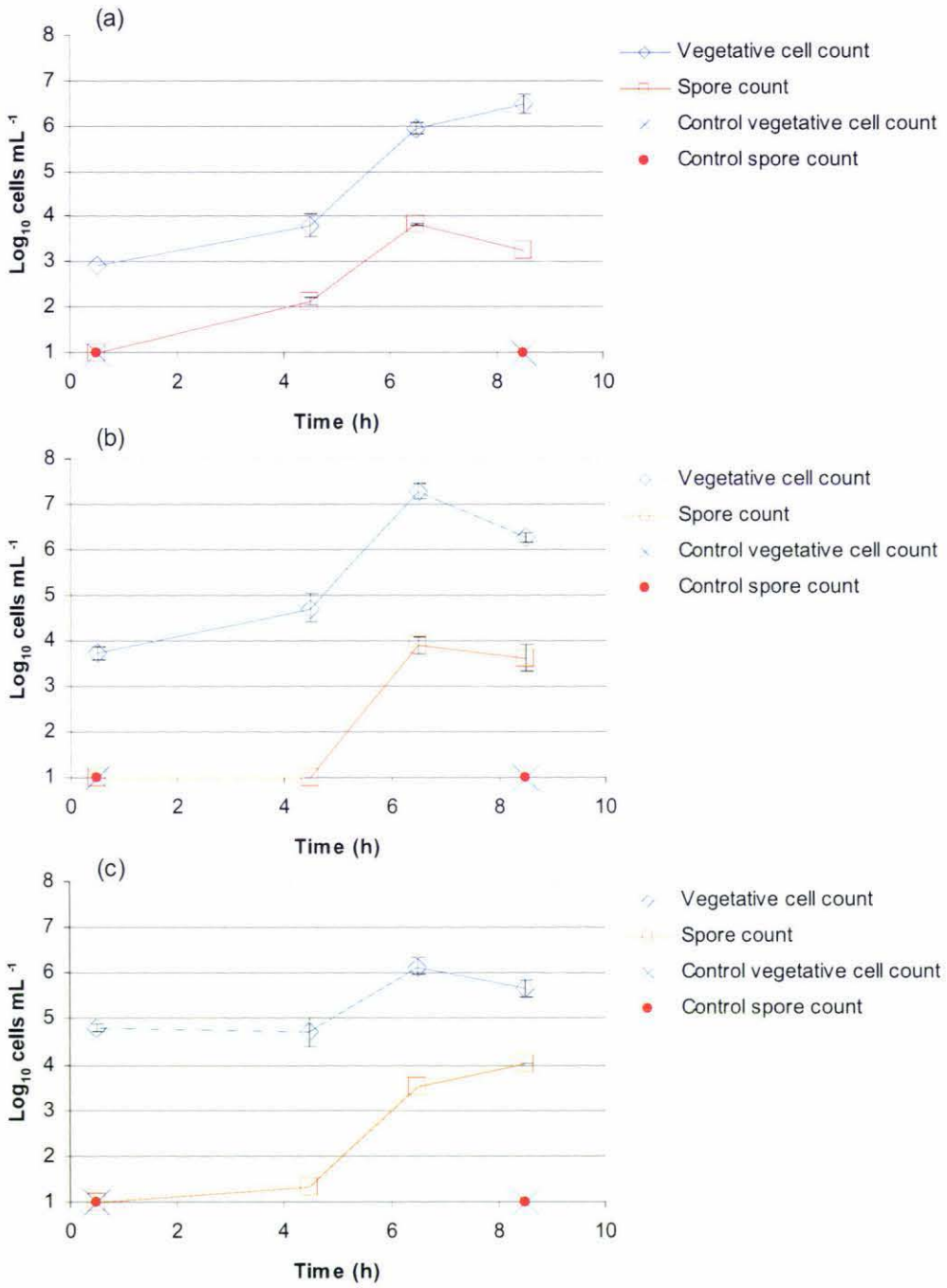

Figure 34: The release of bacteria into flowing milk from biofilms growing in the laboratory reactor at 60°C over 8.5 h (run 4)

The numbers of bacteria in the exiting milk from three laboratory reactors run in parallel (a, b, and c) were enumerated using change in impedance at four indicated time points. Each point represents the mean and standard deviation of triplicate results.

No sporulation at 48°C and rapid sporulation at 60°C are important findings. This information will enable the dairy industry to design strategies to avoid the temperature regimes, where sporulation occurs.

4.4 The development of biofilms from spores

4.4.1 Run 5: The development of biofilms from spores at 55°C over 8.5 h

Spores are more resistant to conditions during the pasteurisation process compared with vegetative cells. Therefore, it is thought that spores seed thermophile contamination in a milk powder plant. To determine if spores could germinate and produce a biofilm, three reactors were inoculated with a CM spore suspension and were run over 8 h as described previously to examine whether vegetative cells were produced.

The number of cells attached to the surface increased by approximately 1 log only for both vegetative cells and spores (Figure 35). Because of the high error between the stainless steel tube counts throughout the run it was difficult to determine if this figure was a true increase or within the error range. At the beginning of the run the number of spores attached to the stainless steel surface ranged between 1 – 1.6 $\log_{10}$ spores cm^{-2} and increased to 2.8 ± 0.2 $\log_{10}$ spores cm^{-2} after 8 h. The number of vegetative cells attached to the stainless steel sample tubes increased by less than1 $\log_{10}$ cells cm^{-2}.

The release of vegetative cells into the milk at the end of the run indicated that germination of spores had probably occurred (Figure 36). In the 'milk out' sample vegetative cells were detected after 6.5 h from one reactor and reached a level of 3 $\log_{10}$ ± 0.6 cells mL^{-1} from all three reactors. It is possible that the attached spores remained dormant for 6 – 8 h at which time vegetative growth began, and low numbers of vegetative cells were detected in the milk. There were low numbers of spores detected in the milk samples at the start of the run but none were detected for the remainder of the run. This is possibly because spores that were loosely attached after the initial inoculation, were washed into the milk.

These results indicate that in an 8 h time frame spores can germinate, resulting in the release of vegetative cells. However, sporulation of the germinated cells did not appear to occur.

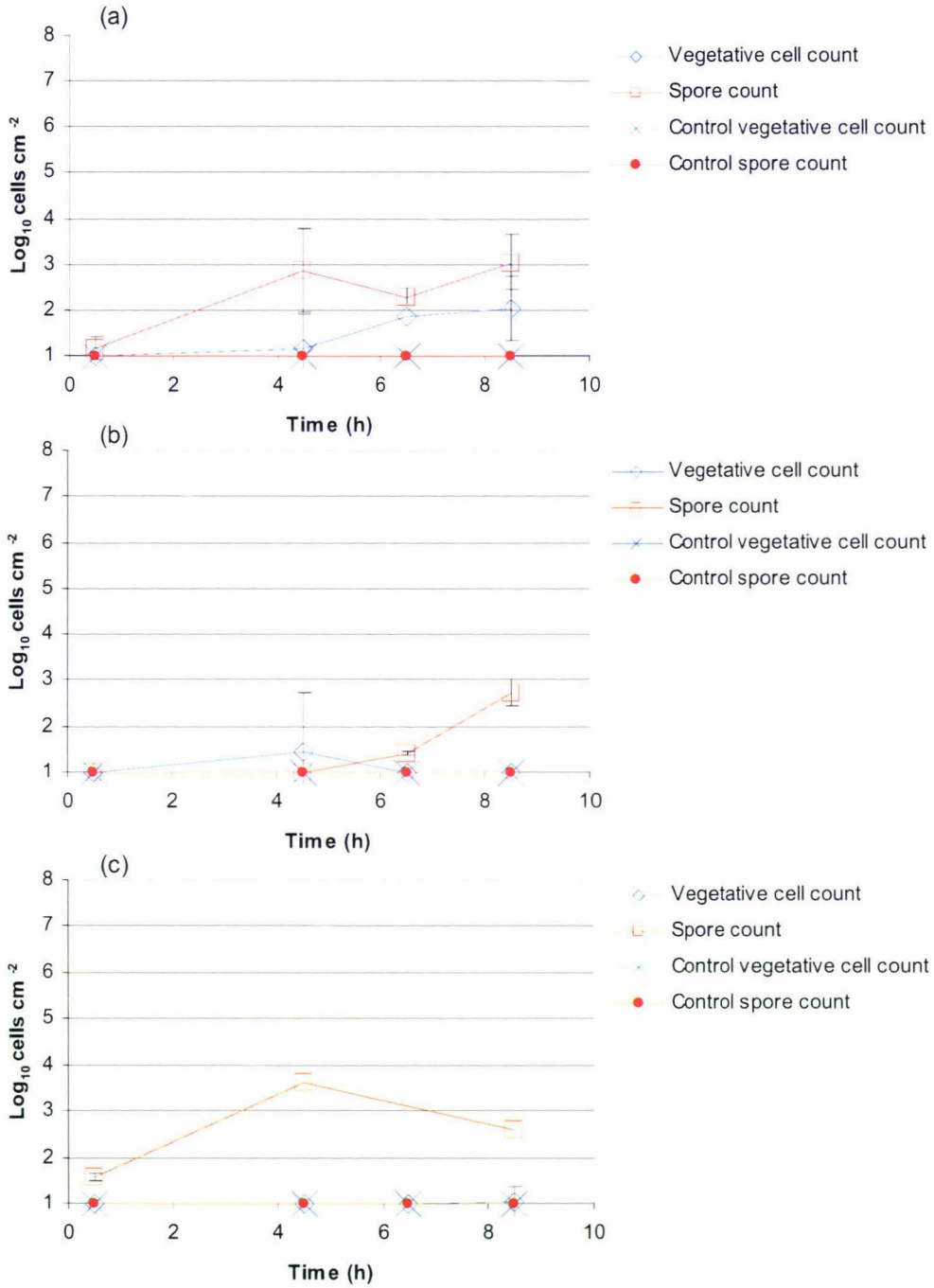

Figure 35: The development of a spore-intiated biofilm in the laboratory reactor at 55°C over 8.5 h (run 5)

Bacteria, attached to the inner surface of the stainless steel tubing of three reactors run in parallel (a, b, and c), were enumerated using change in impedance at four indicated time points. Each point represents the mean and standard deviation of duplicate results.

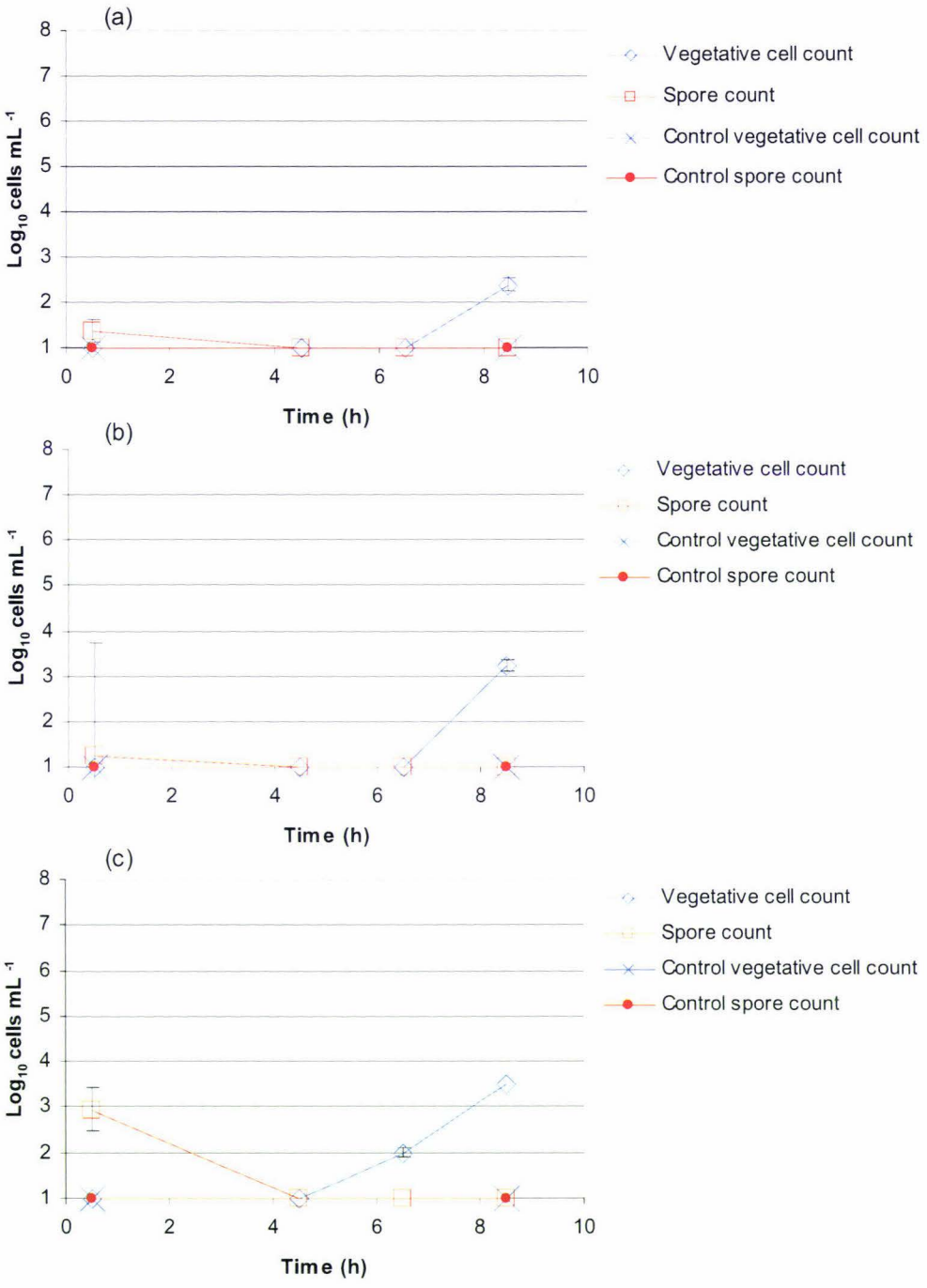

Figure 36: The release of bacteria into flowing milk from a spore-initiated biofilm growing in the laboratory reactor at 55°C over 8.5 h

The numbers of bacteria in the exiting milk from three laboratory reactors run in parallel (a, b, and c) were enumerated using change in impedance at four indicated time points. Each point represents the mean and standard deviation of triplicate results.

4.4.2 Run 6: The development of biofilms from spores at 55°C over 14.5 h

To determine if a biofilm will continue to develop from spores after 8.5 h, a similar experiment was repeated over a 14.5 h time frame. The reactors were again inoculated with an *A. flavithermus* spore suspension and both the biofilm density and the release of bacteria into the milk was measured after 0.5 h, 8.5 h, 11.5 h, and 14.5 h.

Throughout these 14.5 h reactor runs there was no noticeble increase in biofilm density (Figure 37). This was probably because a high number of spores attached to the stainless steel following inoculation. At the start of the run the number of attached spores ranged between 3.3 - 5.3 $\log_{10}$ spores cm^{-2}. For the remainder of the run the biofilm spore density was maintained at approximately 5 $\log_{10}$ cells cm^{-2}. However, the number of attached vegetative cells increased from between 1.3 – 3.4 $\log_{10}$ cells cm^{-2} at the start of the run to 5.1 ± 0.8 $\log_{10}$ cells cm^{-2} at the end of the run. Because the biofilm spore count was maintained during the run rather than decreased at the expense of the vegetative cells it is possible that only a small percentage of the spores germinated resulting in proliferation of vegetative cells and therefore an overall increase in the biofilm density.

Spores were also detected on the control stainless steel tubing. This indicates that a biofilm had also begun to develop from thermophiles that were naturally present in low numbers in the pasteurised milk. Biofilm formation from a low concentration ($<10^0$ cell/mL) of thermophilic bacteria present in pasteurised milk has been shown to occur within 18 h using a laboratory reactor (Parkar *et al.*, 2003). A similar situation would occur in milk powder manufacturing plants.

Vegetative cells were released into the milk in the laboratory reactor after 8 h at a similar level of 2.5 – 4.9 $\log_{10}$ cells mL^{-1} (Figure 38) to the previous run (run 5). At the end of the 14.5 h run, 6.3 $\log_{10}$ ± 0.3 cells mL^{-1} were being released into the milk. Spores were not detected until after 14.5 h at low levels of 2 $\log_{10}$ spores mL^{-1}. Periodic release and low numbers of spores may explain why spores were detected from the third reactor after 11.5 h but not 14.5 h.

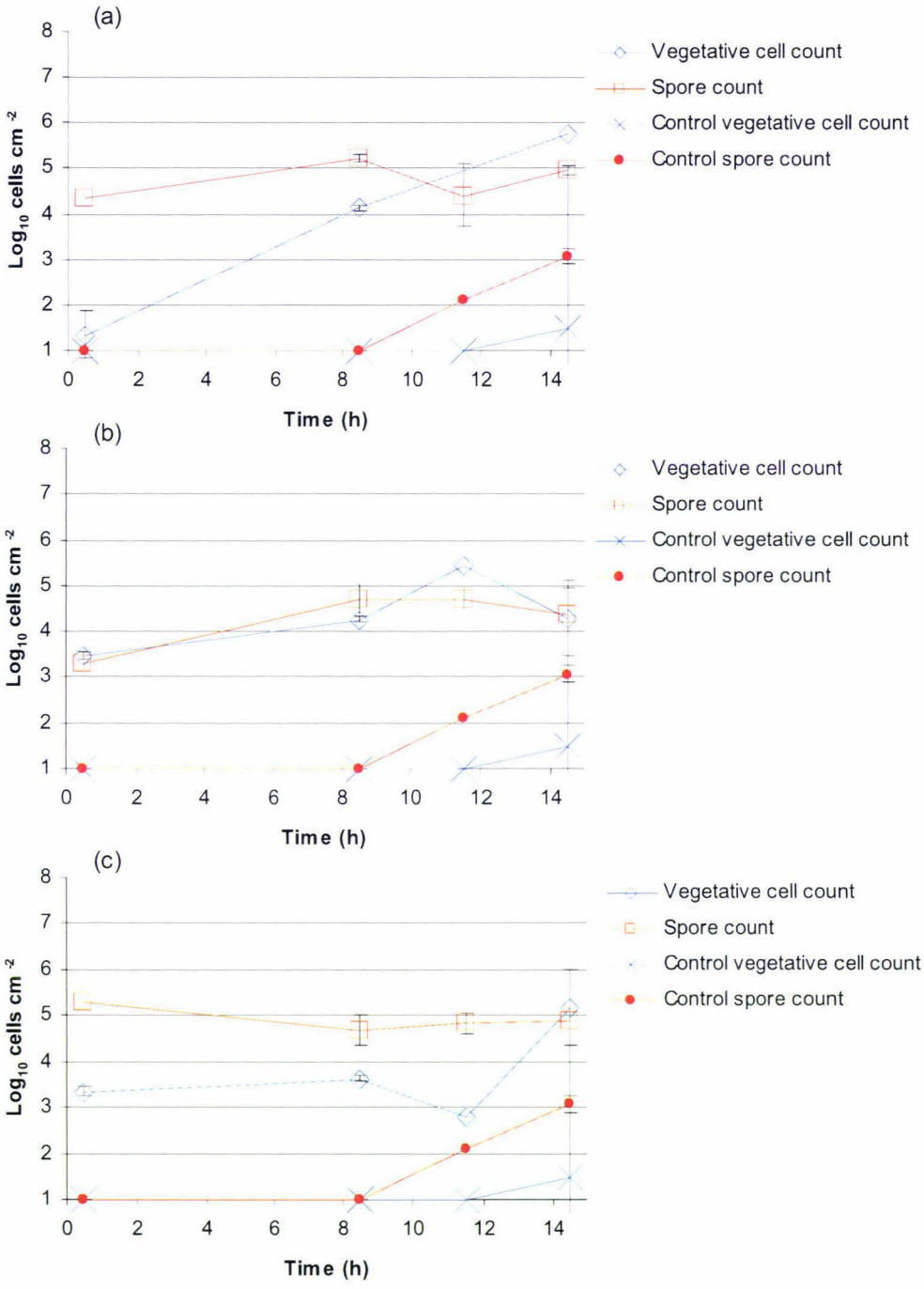

Figure 37: The development of a spore-initiated biofilm in the laboratory reactor at 55°C over 14.5 h (run 6)

Bacteria attached to the inner surface of the stainless steel tubing of three reactors run in parallel (a, b, and c), were enumerated using change in impedance at four indicated time points. Each point represents the mean and standard deviation of duplicate results.

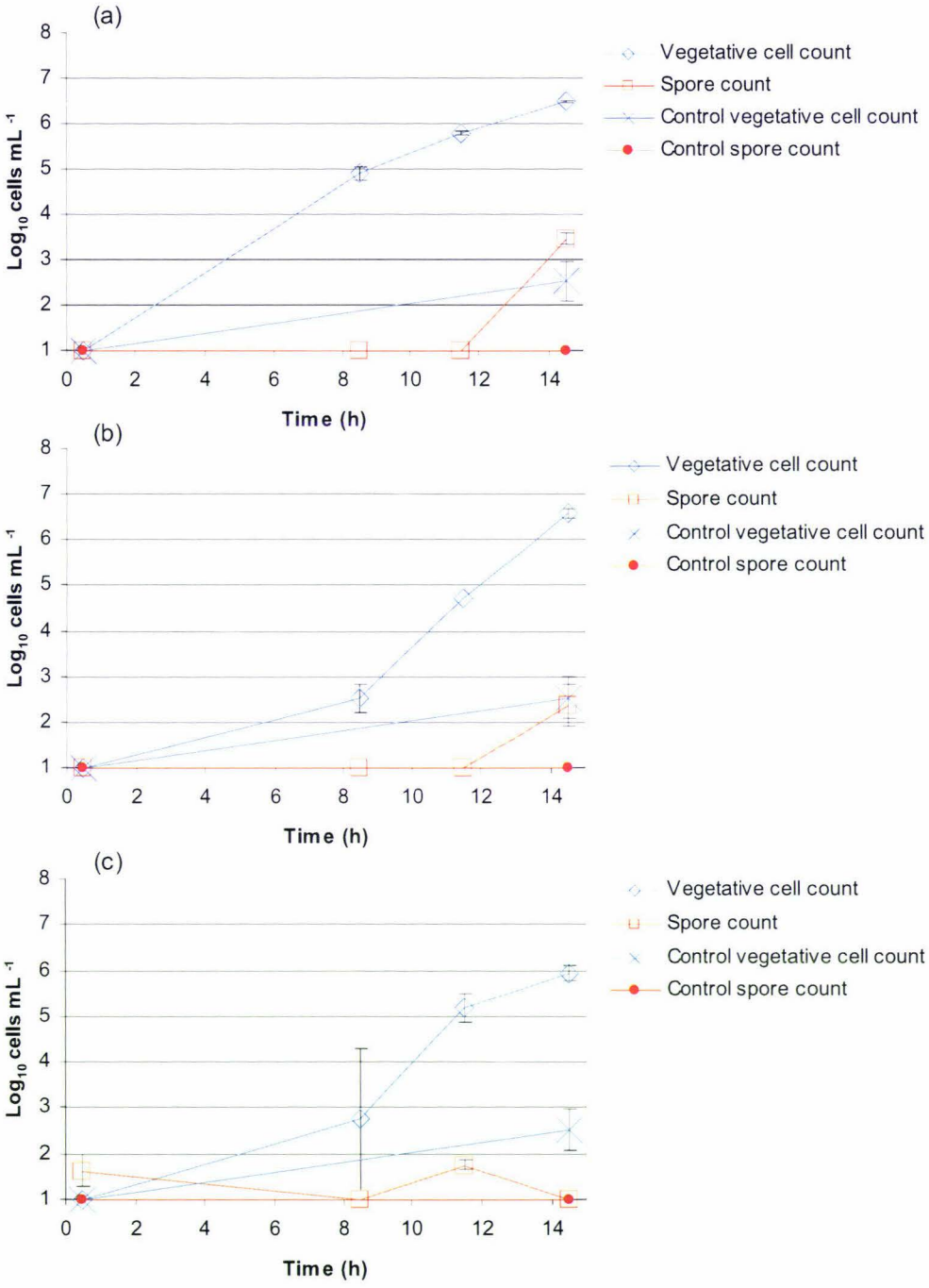

Figure 38: The release of bacteria into flowing milk from a spore-initiated biofilm growing in the laboratory reactor at 55°C over 14.5 h (run 6)

The numbers of bacteria in the exiting milk from three laboratory reactors run in parallel (a, b, and c) were enumerated using change in impedance at four indicated time points. Each point represents the mean and standard deviation of triplicate results.

4.5 Error and control reactor runs

Throughout these reactor runs, the vegetative and spore counts results in the milk did not always correlate with the biofilm counts.

At the beginning of those of reactor runs inoculated with vegetative cells, the vegetative cell counts in the exiting milk were high. Theoretically at the start of the run the cell counts in the incoming milk should have been the same as the exiting milk. At this time the biofilm would not have developed, therefore no cells should have been released. The high number of cells (up to 5 $\log_{10}$ cells mL^{-1}) detected in the milk was probably the consequence of loosely attached cells of the original inoculum still being washed off the reactor surface. The lag in washing off could be due to the slower flow rate at the surface interface (the region called the boundary layer).

During the reactor runs, both the vegetative and spore counts were sometimes quite variable in the exiting milk between the three parallel reactor sets. This was possibly because there were periodic releases of biofilm material, resulting in some milk samples containing larger numbers of cells, clumped together. This was not due to contamination from the incoming milk, because the vegetative and spore counts of milk from the control reactor at the beginning and the end of the run, were less than 10 CFU/mL.

Large differences between some of the biofilm counts were observed. This may be due to the variation in biofilm coverage between the individual tubes. Vegetative and spore counts were performed on duplicate tubes (as described in section 2.7.3), therefore this was not necessarily a representation of all of the tubes. The growth of the biofilm on the stainless steel was probably not uniform between the analysed tubes. Previous studies have demonstrated that biofilm growth initiates as microcolonies on random sections before it expands over the entire surface (Johnston, 2004). There was also a variation in the initial attachment of both the vegetative cells and spores. As previously mentioned this has been seen with the attachment of *G. stearothermophilus* vegetative cells to stainless steel (Flint *et al.*, 2001a).

Total thermophile and thermophilic spore plate counts were also performed on all of the milk samples from the control, uninoculated reactors. The thermophile counts were all less than 50 CFU/mL and spore counts were always less than 10 CFU/mL. One exception was the thermophile count of the exiting milk at the end of the 14.5 h spore inoculation run (section 4.4.2). Thermophile plate counts reached levels of up to 3 $\log_{10}$ CFU/mL but no spores were detected. This indicates that over this time frame a biofilm had developed from thermophiles naturally present in the milk. This was not seen for the other 14.5 h run (run 2, section 4.2.2), possibly because of a difference in composition or quality of the pasteurised milk used for this particular run.

4.6 Limitations of the reactor

The laboratory reactor system is a much more relevant model for the development of thermophilic biofilms in a dairy manufacturing plant compared with the use of stainless steel coupons in a standing culture. Despite that, there were three major limitations of this reactor set up: a) The number of samples that could be analysed, b), the flow was laminar and c), only skim milk could be used:

a) The reactor consisted of only sixteen stainless steel sample tubes; therefore the biofilm density could be measured at four different time points only. The number of stainless steel sample tubes could not be increased due to the lack of space in the water bath. The other limitation came from the impedance method used to measure the biofilm density and the number of bacteria present in the milk samples. The number of both milk samples and stainless steel sample tubes was limited because the Bactrac™ could analyse only 100 samples in one day. In some cases the measurement also failed resulting in no duplicate result for the biofilm count or triplicate count for the milk count.

b) In this laboratory reactor the flow was laminar, whereas in milk powder manufacturing plants the flow is turbulent. In order to study biofilm formation under turbulent flow, pilot plant scale equipment or a new laboratory reactor would need to be used. There are currently laboratory reactor systems that can be used under turbulent flow conditions but these appear to be limited to the use of glass cover slips rather than stainless steel coupons (Stoodley *et al.*, 2001a). Previous studies that have compared biofilm development under laminar and turbulent flow conditions have demonstrated that the major difference is the structure of the biofilm but there was no noticeble difference in cell density (Stoodley *et al.*, 1999). Under laminar flow the biofilm was made up of immobile circular microcolonies, whereas under turbulent flow, ripples formed that moved along the surface. With regard to the present study it is possible that under turbulent flow more cells that were loosely attached would be released into the milk.

c) This reactor could not be used to study biofilm development in the presence of pasteurised whole milk. This is because of fouling that occurs at the inlet of the stainless steel tubing, causing blockage.

This reactor system was used for preliminary investigation of the temperature and time dependence of *A. flavithermus* biofilm formation. Further research to confirm findings from the laboratory reactor need to be carried out using pilot plant scale equipment. However, the knowledge that spores can be generated from an *A. flavithermus* biofilm

grown in a laboratory reactor will provide a platform to study other variables that may have an effect on biofilm and spore formation.

4.7 Microscopy

Epifluorescence microscopy and Scanning Electron Microscopy (SEM) were used to visualise spores within the *A. flavithermus* biofilm. This was attempted only after run 4 (8.5 h run at 60°C). Before the start of the run two stainless steel coupons were placed in silicone tubing and attached to the end of the stainless steel sample tubes in each reactor. At the end of the run, one coupon was stained with the fluorescent stain DAPI and the second coupon was analysed using SEM.

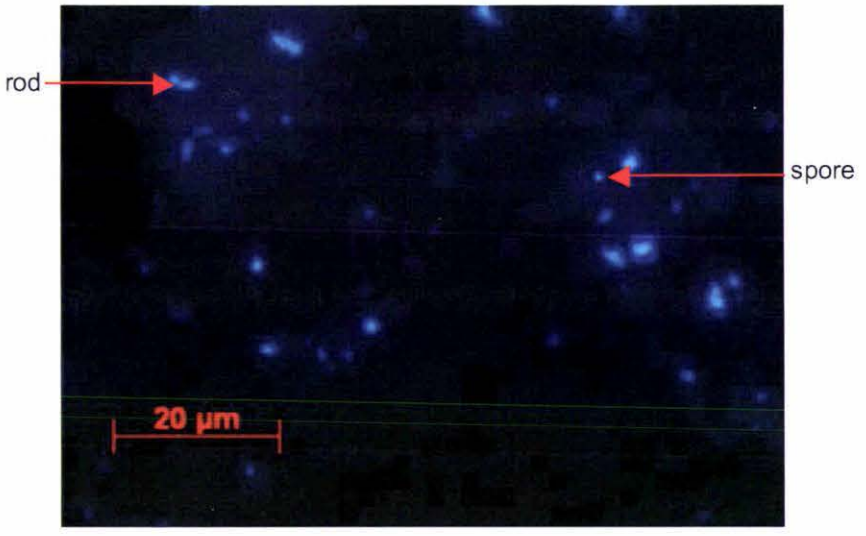

Figure 39: DAPI stain of thermophilic cells attached to a stainless steel coupon

All of the DAPI stained coupons appeared to have both bacterial rods and round spores attached to the surface. An example of one of the DAPI stained coupons is illustrated in Figure 39. On the coupons from the three inoculated reactors, bacteria were evenly spread across the entire surface, whereas on the control coupon sporadic patches of cells were detected.

DAPI is a non-specific DNA binding stain, therefore it does not differentiate between dead and live cells. It is possible that dead bacteria or low numbers of thermophilic bacteria naturally present in pasteurised milk may have attached to the stainless steel surface, hence the presence of bacteria on the control coupons. DAPI has been previously used to stain *Bacillus* spores, which can be differentiated from bacterial rods by the cell shape (Laflamme *et al.*, 2004). The LIVE/DEAD BacLight viability kit and the

fluorescent stain CTC have been used to differentiate live cells from dead cells and to detect viable endospores (Laflamme *et al.*, 2004). However, there is currently no epifluorescent staining method to differentiate spores from vegetative cells.

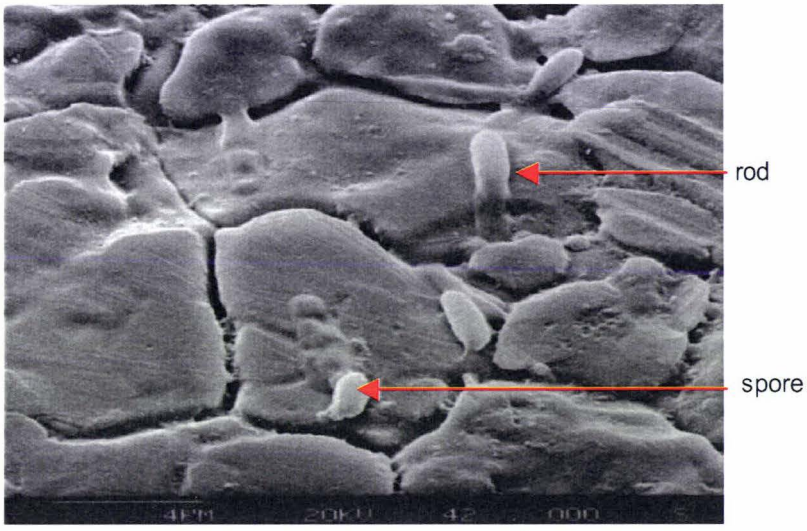

Figure 40: Scanning electron micrograph of thermophilic cells attached to a stainless steel coupon

Both bacterial spores and rods appear to have attached to the stainless steel as indicated.

SEM detected less cells attached to the stainless steel coupons than expected. An example of a scanning electron micrograph of one of the coupons is illustrated in Figure 40. One possible reason for the low number of cells observed is that there may have been a layer of milk over the surface covering a large proportion of the cells. However both spores and bacterial rods appear to be present. The spores are a similar shape to the rods but are short and stumpy in comparison. The spore shape seen here is similar to those observed in the scanning electron micrographs of *G. stearothermophilus* spores produced on media and suspended in water (Vaid and Bishop, 1998).

4.8 Summary

Results from this laboratory reactor system have demonstrated that sporulation from an *A. flavithermus* vegetative cell initiated biofilm can occur very rapidly within the temperature range of an evaporation preheat PHE. At 55°C these biofilms reached a maximum cell density of approximately 6 – 7 $\log_{10}$ cm^{-2} within 6 – 8 h and up to 6 – 7 $\log_{10}$ cells mL^{-1} were released into the milk. At the end of the 8.5 h run there was a similar level of spores and vegetative cells in the biofilm. Spore formation in the biofilm

was prevented within the 8.5 h run time when the temperature was decreased to 48°C. A biofilm was also developed from spores, releasing vegetative cells into the milk within 8.5 h and spores within 14.5 h.

5 General Discussion

5.1 Summary

The aim of this study was to determine the origin of contamination by thermophilic bacterial spores in a milk powder manufacturing plant. This was achieved by monitoring the formation of spores in both an industrial setting and a controlled laboratory reactor. At the start of this project the origin of spores in the milk powder manufacturing process was not clear. While there was some thought that biofilms played a role, this was by no means sure. A lot of work had been done on the vegetative growth of thermophilic bacteria in biofilms, but the conditions leading to spore formation were not well understood (Flint *et al.*, 2001a; Parkar *et al.*, 2001; Parkar *et al.*, 2003, 2004). This project has now confirmed two sites of spore formation in a milk powder manufacturing plant and the presence of spores within a biofilm of the typical dairy thermophile *A. flavithermus*.

The results from a survey undertaken at the Pahiatua site in the Powder 2 plant established the zones in a typical milk power manufacturing plant where thermophilic spores developed. Spore formation occurred predominantly in the preheat section and to a lesser extent during the initial stages of evaporation. Spores were detected within 11 – 15 h of the start of two manufacturing runs.

The proportions of two genera of thermophilic bacteria were determined at different stages during the manufacturing process. The predominant thermophilic spore organisms in samples taken at the end of the two manufacuring runs from the Pahiatua Powder 2 plant were identified as *A. flavithermus* and *Geobacillus* spp. using primers specific for these organisms. *A. flavithermus* predominated in the preheat section and there was a mix of both *Geobacillus* spp. and *A. flavithermus* in the evaporation section and onwards.

Given that previous studies have shown that *A. flavithermus* dairy isolates tend to have a lower optimum growth temperature compared with *Geobacillus* spp. isolates it is not surprising that *A. flavithermus* spores were isolated predominantly from the preheat section whereas *Geobacillus* spp.spores were isolated only from the evaporation section onwards (Cucksey, 2002). Previous studies have indicated that the evaporation temperature of 65°C would probably be too high for substantial growth of most *A. flavithermus* strains (Cucksey, 2002). However, one study by Ronimus *et al.* (2003) has reported the isolation of *A. flavithermus* strains from milk powder that can grow at temperatures of 65°C. This variation in results may reflect a broad growth temperature range of *A. flavithermus*, a difference in growth conditions used or a strain difference.

The presence of both *A. flavithermus* and *Geobacillus* spp. in product from the Pahiatua Powder 2 plant agrees with previous studies that have demonstrated the microflora of New Zealand milk powders is dominated by these two thermophilic organisms (Flint *et al.*, 2001b; Hill, 2004; Ronimus *et al.*, 2003; Warnecke, 2001). However, these studies have focused mainly on the identification of vegetative cells in the final product, rather than spores. This study is unique in that thermophilic spore contaminants were identified throughout the manufacturing process of milk powder.

A laboratory reactor was used in this study to grow biofilms of the typical dairy thermophile *A. flavithermus* (strain CM). The aim was to examine the rate and extent of spore formation in these biofilms under controlled conditions, and to test one key variable – temperature – and its effect on spore formation.

In the Pahiatua Powder 2 plant spore formation occurred within 11 h, whereas in the laboratory reactor system release of spores from an *A. flavithermus* biofilm into flowing milk took only 4 – 6 h. This is possibly because the biofilm was derived from vegetative cells. The initial contamination of thermophilic organisms in a milk powder plant is believed to arise from spores present in the raw milk in low numbers that survive pasteurisation and/or from biofilms or foulant that remain on equipment surfaces following CIP (Hinton *et al.*, 2003; Parkar *et al.*, 2003). Therefore the process of spore germination, biofilm formation, and subsequent sporulation would take longer compared with a biofilm initiated from vegetative cells. Further laboratory trials demonstrated that an *A. flavithermus* biofilm could develop from spores and release vegetative cells in 8 h and spores in 14 h. This is close to the behaviour observed in the manufacturing environment.

It is difficult to compare the laboratory reactor system with a milk powder plant because they are operated under different conditions. A milk powder plant is operated under turbulent flow, ingredients such as lactose or lethicin may be added during milk powder manufacture, milk varies in total solids concentration throughout the manufacturing process and is exposed to a wide variety of temperatures. On the other hand a laboratory reactor is operated under laminar flow conditions, at one temperature, and the composition of the pasteurised milk supplied by the Fonterra Palmerston North pilot plant is generally very consistent. Despite these differences between industry and laboratory scale systems, the use of a laboratory reactor is an ideal way to study the effect of different variables, such as temperature, on formation of spores by thermophiles. In this study the development of an *A. flavithermus* biofilm and spore formation was examined under three temperature regimes: 48°C, 55°C and 60°C. The biofilm developed at all three temperatures and spores formed at 55°C and 60°C but not at 48°C. Therefore a lower temperature of 48°C prevents the formation of spores.

To date, two methods have been employed in milk powder manufacture to avoid thermophile growth. These are the use of direct contact heating systems such as DSI, and dual preheating systems (Refstrup, 1998, 2000). DSI rapidly increases the temperature of the milk therefore avoiding the optimum growth temperature of thermophiles. This system is very expensive compared with indirect heating systems such as PHEs and tubular heat exchangers used in many dairy plants for preheating milk before evaporation. The use of DSI also results in dilution of the milk. Indirect heating methods are commonly used in conjunction with a DSI, where the PHE is used to heat the milk to temperatures of approximately 72°C and the DSI is used to raise the milk temperature up to about 120°C, depending on the product that is being manufactured. Dual preheating systems involve the use of two PHEs or tubular preheaters where after 8 – 10 h of production the milk is directed from one preheater to the second preheater so that the first preheater can be cleaned using a CIP.

The correct use of temperature could potentially prevent the formation of spores in thermophilic biofilms that develop in milk powder manufacturing plants. Temperature step changes have been shown to control *S. thermophilus* in cheese milk PHE pasteurisers (Knight *et al.*, 2004). In this system the cool down section of the PHE was adjusted to 67 – 55°C instead of 67 – 35°C for 10 minutes every two hours. The optimum growth temperature of *S. thermophilus* is 35 – 50°C. This temperature step change is believed to disrupt the growth and runs can be extended from 8 – 14 h to 22 h. A similar method could probably be used to control the thermophiles *A. flavithermus* and the *Geobacillus* group. In the preheat section of an evaporator, milk is heated from 7°C up to 75 – 120°C. If the preheater were run at 48°C with temperature step changes up to 70 – 85°C this may prevent sporulation and disrupt vegetative cell growth.

The results from this study will help the dairy industry design cost effective strategies to reduce the contamination of milk powder product with spores. Two key facts are important to the dairy industry. Firstly, approximately 10 - 50% of a biofilm of one of the typical thermophilic bacteria, *A. flavithermus,* may be in the form of spores and this occurs after 8 h under ideal conditions. Secondly, temperature variation has a considerable effect on the formation of spores, allowing us to manipulate conditions to avoid the temperature range where spores form. This will form part of a total control package to extend the running time of milk powder manufacturing plants while making product that meets customer specifications.

5.2 Future directions

5.2.1 Development of species-specific primers for *Geobacillus* species

Currently the primers used to identify the *Geobacillus* group do not differentiate the species. The identification of the predominant *Geobacillus* specie(s) may be important in tests of milk powder destined for the retort and UHT milk industry. In New Zealand only *Geobacillus* spp. spores appear to survive these two processes (Hill and Smythe, 2004). However, it is not known whether these spores belong to one or more species of *Geobacillus*.

In New Zealand, RAPD profiling and partial 16S rDNA sequencing have been used to identify and group *Geobacillus* isolates from milk powder. More accurate methods, such as multiple locus sequencing will need to be used to identify the species. To design primers to differentiate this group a more variable gene other than the 16S rRNA gene needs to be used.

5.2.2 Spore formation in *Geobacillus* spp. biofilms

The development of biofilms *of G. stearothermophilus* in pasteurised milk has been examined, but these studies have focused on either the development of vegetative cells in a biofilm or the mechanisms of attachment of both spores and vegetative cells (Flint *et al.*, 2001a; Parkar *et al.*, 2001). To design strategies to prevent spore formation in thermophilic biofilms using temperature in the dairy industry more information is needed about the formation of spores in *Geobacillus* spp. biofilms.

5.2.3 The effect of milk processing variables on spore formation of dairy thermophiles

There are many variables in the operation of a milk powder plant that may influence spore formation of thermophiles. Examples of these variables include pH, total solids concentration, milk composition, milk age, and raw milk quality. To give a more complete understanding of biofilms and spore formation, these variables need to be studied using a laboratory reactor based system. A miniature stainless steel Robbins device has recently been constructed for use in the laboratory. This device has a larger diameter than the reactor used in this present study and could potentially be used with both whole and skim milk to study spore formation of thermophiles.

5.2.4 Pilot plant studies

To give a more accurate representation of what occurs in a milk powder factory any strategies designed such as the use of temperature to prevent spore formation need to be repeated using a pilot plant scale system, where the flow regime can be turbulant.

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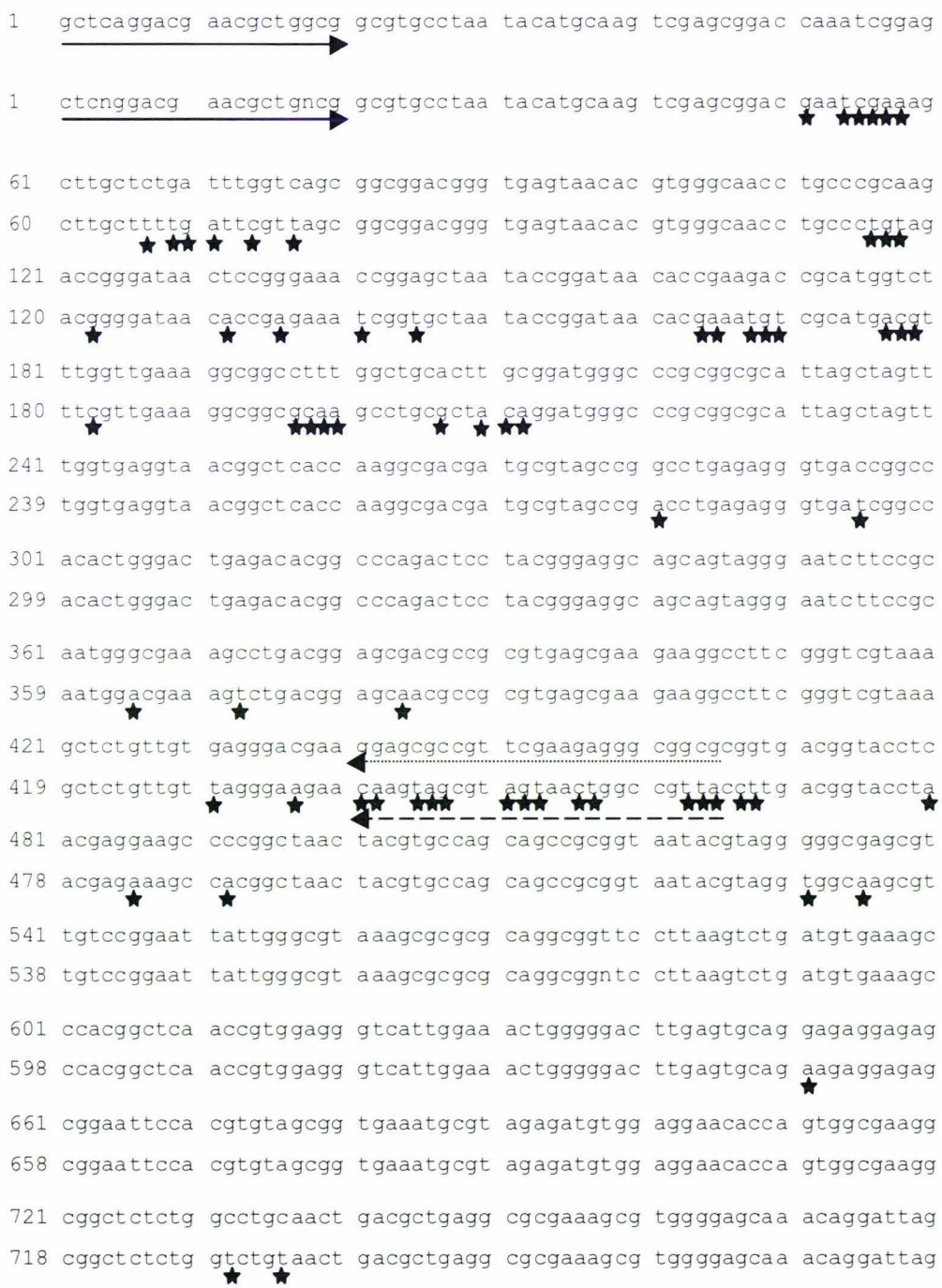
7 Appendix

7.1 Partial 16S rRNA gene alignment for *A. flavithermus* and *G. thermoleovorans*

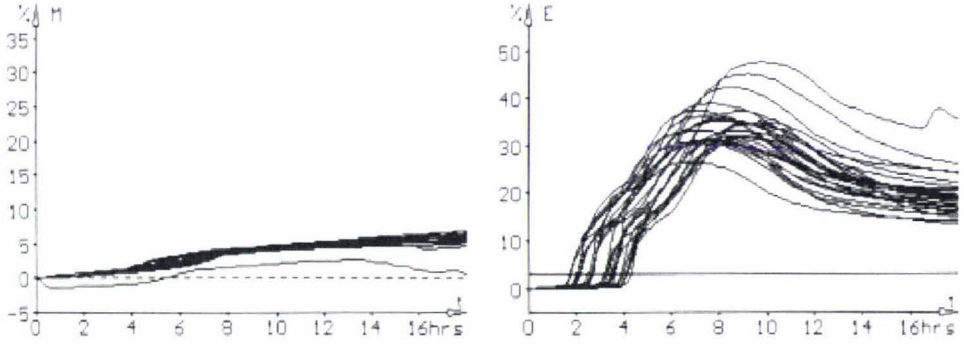


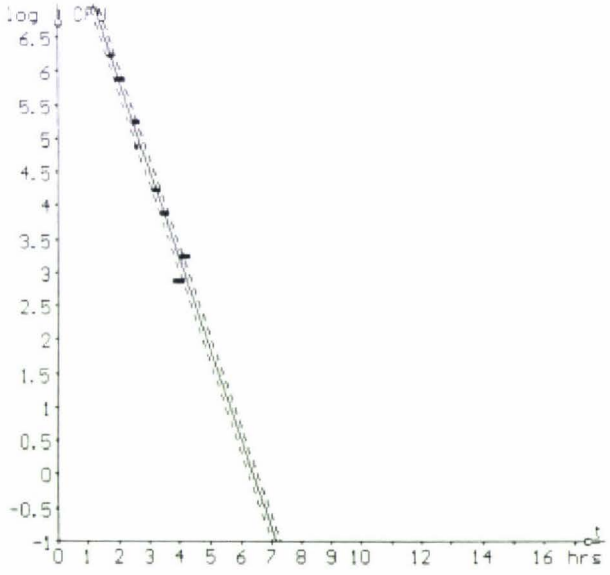
Alignment of the partial sequences of the 16S rRNA gene for *G. thermoleovorans* (ATCC 43513) and *A. flavithermus* (DSM 2641) (Flint *et al.*, 2001b). The sequences which the Y1 (——▶), LEVO (·····▶), and FLAVO (– – –▶) primers anneal to are underlined. The bases that vary between the two sequences are marked with an asterisk.

7.2 Bactrac calibration curves

7.2.1 Vegetative cells of *A. flavithermus* CM

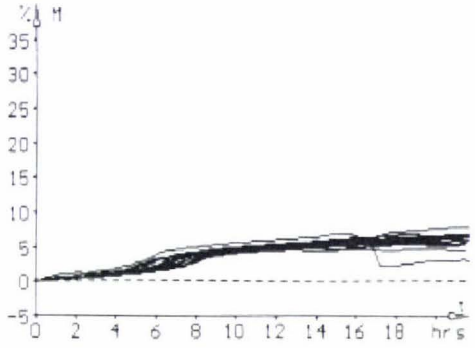
```
File:              Text:
Threshold M:    0 %    Threshold E:    3 %    In Use: E-Value
  No Limits
Correlation: log(CFU) = -1.3410 * t + 8.5721   correlationfactor: r = -0.9844
Dispersion: Syx = 0.2100
```

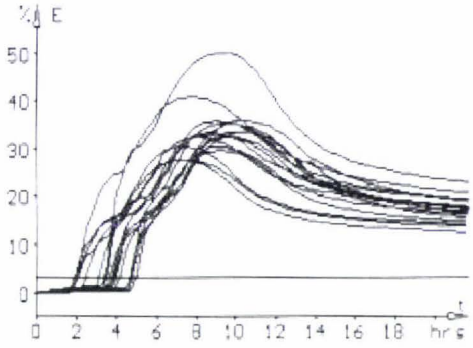


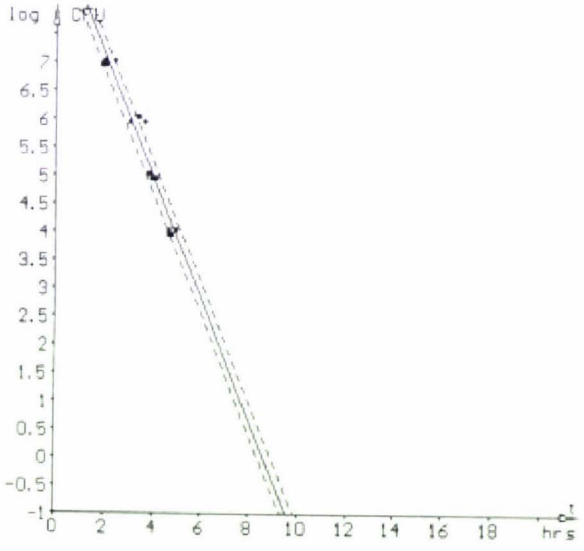


7.2.2 Spores of *A. flavithermus* CM

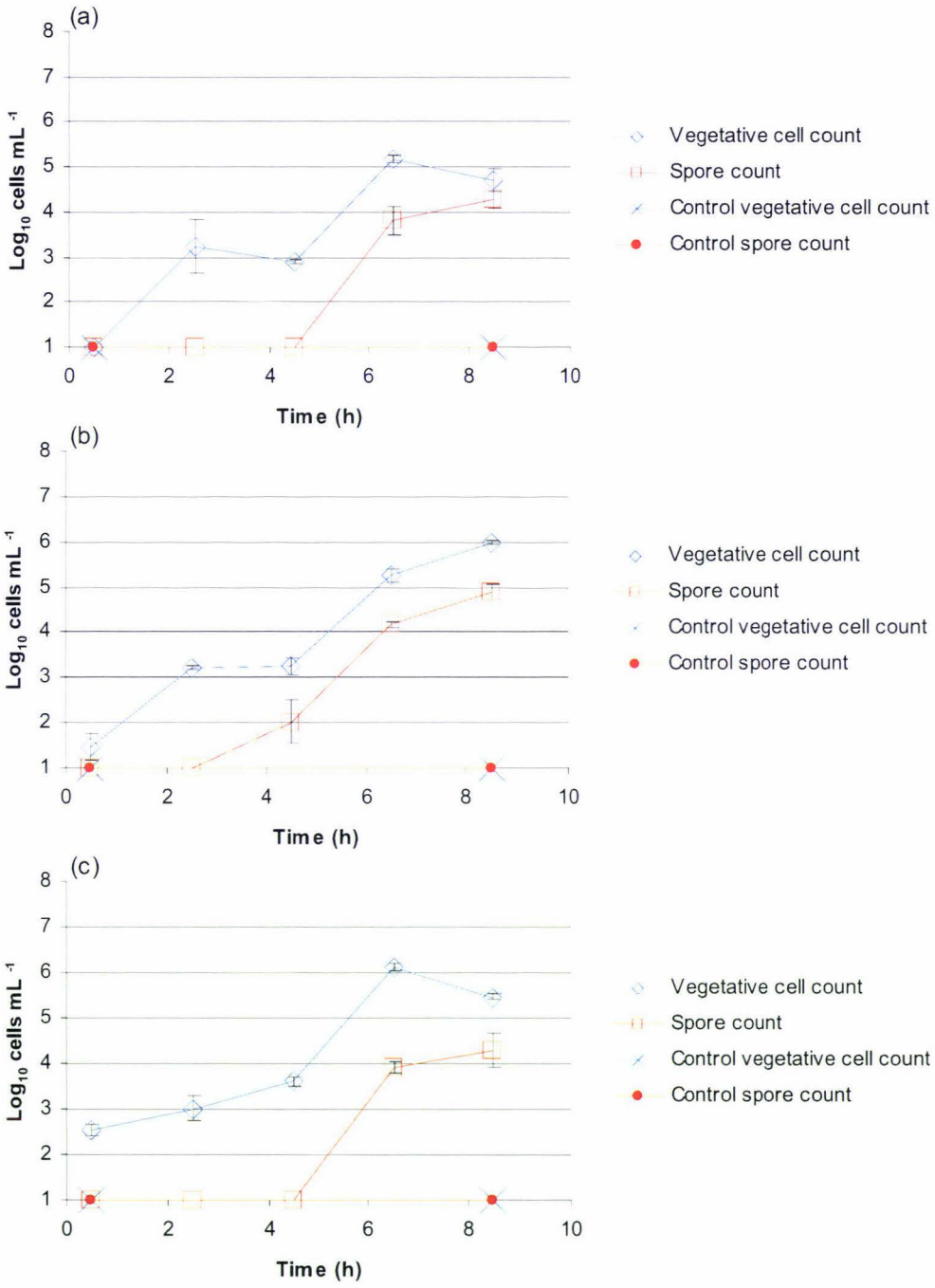
```
File:              Text:
Threshold M:    0 %    Threshold E:    3 %    In Use: E-Value
  No Limits
Correlation: log(CFU) = -1.0803 * t + 9.3147   correlationfactor: r = -0.9719
Dispersion: Syx = 0.2853
```





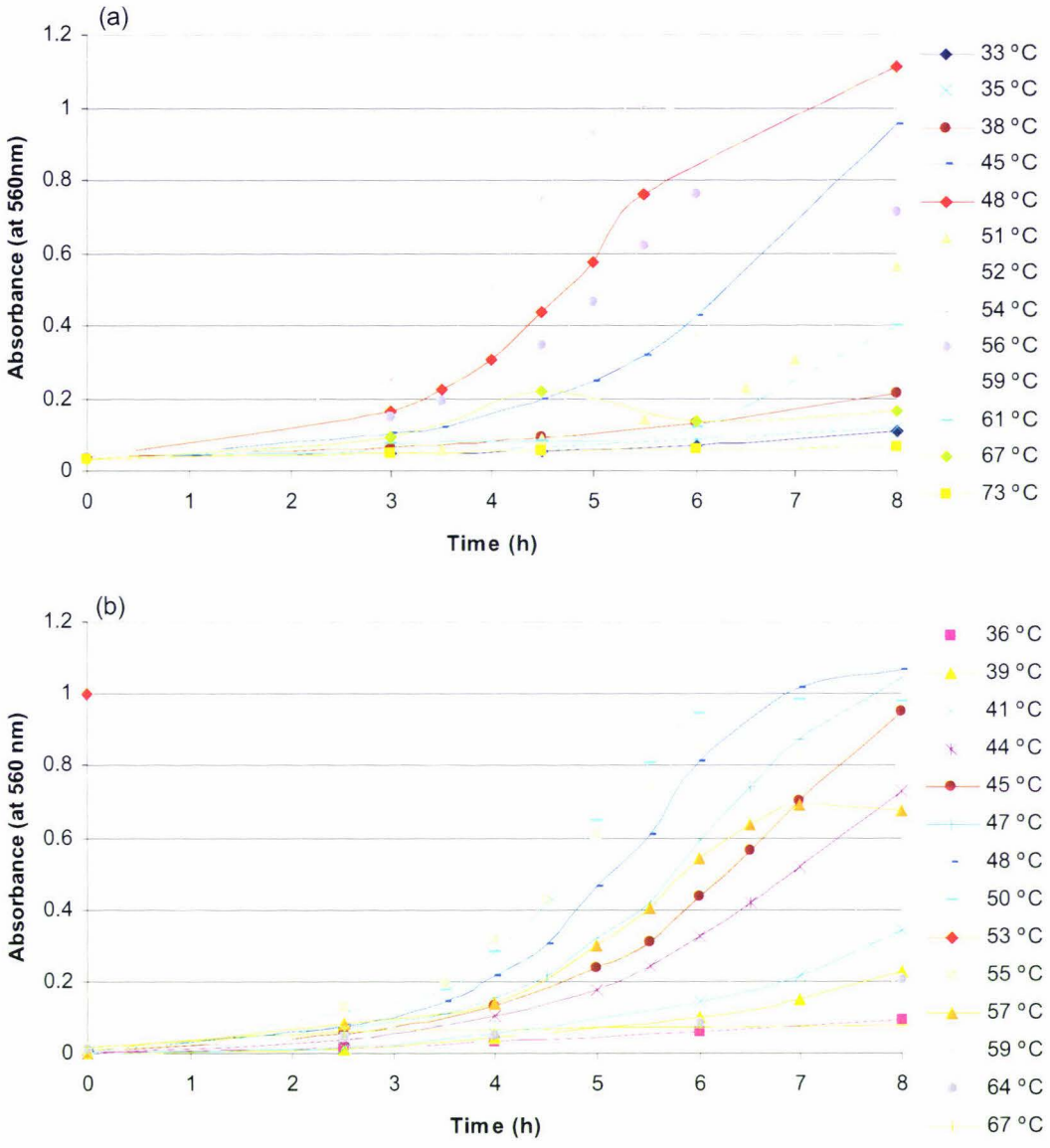


7.3 Preliminary reactor run



The release of bacteria into flowing milk from biofilms growing in three parallel laboratory reactors (a, b, and c) at 55°C over 8 h. The numbers of bacteria in the exiting milk were enumerated using change in impedance at five indicated time points. Each point represents the mean and standard deviation of triplicate results.

7.4 Temperature profile of *A. flavithermus* CM



The temperature profile of *A. flavithermus* was determined in two independent experiments (a and b) by incubating culture aliquots in a temperature gradient incubator and measuring the optical density at a wavelength of 560nm over an 8 h time period.