Investigating the Ability of Thermal Cycling to Reduce Growth of Biofilms

Amanjeet Kaur

A thesis submitted to
Auckland University of Technology
in partial fulfilment of the requirements for the degree
of
Master of Applied Sciences (M.AppSc)

2014

School of Applied Sciences

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ATTESTATION OF AUTHORSHIP

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Amanjeet Kaur

ACKNOWLEDGEMENT

I would like this opportunity to extend my heartfelt gratitude to my supervisor, Dr. John D. Brooks, without whose help, guidance and constant support, this project wouldn't have been possible. Lot of thanks goes to Isabel Li and Tanvi Shinde for their friendly advice and Ms. Saeedah Sadoogh, lab technician for all her help. I can't finish this acknowledgement without thanking Dr. Chris Pook for his technical help. Chris, your software made my research possible. Also, I would like to acknowledge my friend, Annie's help with the final proofreading of the thesis. Last but not the least, I would like to thank my parents and my partner without whose emotional support and constant encouragement, I wouldn't have completed my work.

ABSTRACT

The objective of this research was to determine if thermal cycling can reduce the growth rate of thermophilic bacteria during milk powder production. The test bacterium selected for the experiment was *Geobacillus stearothermophilus*, which has been found in the milk powder production plant as a contaminant.G. *stearothermophilus* persists in the manufacturing plants as biofilms on the various stainless steel and seal surfaces available in the production line. A biofilm can be defined as an aggregation of microbial cells and their associated extracellular polymeric substances (EPS), actively attached to, growing and multiplying on a surface (Jonhstone, Ellar, & Appleton, 1980). Biofilm formation can cause film accumulation on food contact surfaces, and microbial colonization in milk storage tanks, fouling of heat exchangers and adhesion of contaminating spores on packaging material surfaces. The need to comply with permissible thermophilic spore count in the milk powder forces shut down of the plants for cleaning earlier than would be the case if non-microbial fouling of the heat exchangers were the only concern (Hinton, Trinh, Brooks, & Manderson, 2002). Consequently, the operational costs soar and profit decreases.

The species in question may not pose a threat to health but represent a continuing problem of spoilage and production of out-of-specification products.

The bacterial culture was grown in tryptic soy broth (TSB) in a water bath at 55°C for 6 hours. The treatment was manipulated on three parameters:
- Step change temperature (55°C to 30°C or 35°C)
- Step change duration
- Period between step changes

The milk for the experiments was prepared by dissolving milk powder acquired from Fonterra into warm deionized autoclaved water. The experimental set-up consisted of a sterile plastic bottle (milk reservoir), two inlet tubes passing from either of two water baths maintained either at step-change temperature or control temperature. The tubes then entered a two-way solenoid valve through which the milk passed into a reactor tube having 10 coupons of 1 cm^2 area inserted, and then to a pump tube, which finally drained the milk into a sealed collection vessel (a 5000 ml Schott-Duran glass bottle).

The culture was introduced into the experimental set-up by two methods. In one method, the culture was inoculated into the sterile milk and in the other, the culture was

inserted into the reactor tube and allowed to stand for 30 minutes before being pumped out rapidly with sterile milk.

The parameters for the variation were introduced using a Arduino-based software designed for the project, which switched the valve between the milk coming from the water bath at step-change temperature and control temperatures, according to the specific time pattern programmed into the software. To be sure of the temperature, a thermocouple measured the temperature of the milk as it exited from the valve and entered the reactor.

The bacterial growth rate was measured by sampling both the coupons and outflowing milk at certain points of time during the trial. The results and data collected during the project clearly indicated a significant reduction in the growth rate of *G. stearothermophilus* owing to thermal cycling. Based on the conditions tested in this project, for control of both biofilm growth rate and contamination of the outflowing milk, a step-change at 35°C for 35 min with 15 min interval between two step-changes can be recommended as the best trial regime for pilot-scale experimentation for evaluation of effectiveness of thermal cycling.

Chapter 1:

INTRODUCTION

Biofilms can be described as attached bacterial populations growing at interfaces or surfaces enveloped in a matrix. Biofilm also includes aggregation of microbes and floccules in the pore spaces of a porous medium (Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995).

Biofilms form an area of interest for the dairy industry because of the increased difficulty in removing the comprising bacterial population as compared to the same concentration of planktonic cells. The bacteria present in the biofilms can detach and contaminate the resulting milk and milk products (Flint, Bremer, & Brooks, 1997). This sloughing can adversely influence the commercial quality, value and hygiene of the end-product. The development of biofilms can also result in a waste of energy, blocked production lines, increased rate of corrosion and other physical deterioration of detectors, equipment and sensors (Flint, Palmer, Bloemen, Brooks, & Crawford, 2001; Wirtanen, Husmark, & Mattila-Sandholm, 1996).

Dairy manufacturing plants have temperature conditions in some pieces of equipment that are selective for thermophiles. Thermophiles are defined as bacterial species having an optimum growth temperature of over 45°C (Burgess, Lindsay, & Flint, 2010). Aerobic plate countwith an incubation temperatureof 55°C is commonly used in the dairy industry to calculate, estimate, count, list, detail or itemize the thermophilic bacilli. The thermophilic bacilli that have been isolated at this temperature of incubation can be divided into two categories *viz* the obligate thermophiles and the facultative thermophiles (also called thermo-tolerant micro-organisms). The obligate thermophiles, which include *G. stearothermophilus* and *A. flavithermus* (Burgess et al., 2010), have an optimum growth temperature in the range of 40-68°C. The facultative thermophiles have an optimum growth temperature of 35-70°C. Thermophiles have the ability to form endospores (Burgess et al., 2010).

Even though these thermophilic spore forming bacteria are non-pathogenic, they are considered to be potential contaminants in the dairy industry (Murphy, Lynch, & Kelly, 1999). Owing to the formation of acids or enzymes, their presence in end products can also lead to flavour defects. Furthermore, their presence in dairy products can indicate poor hygiene. High numbers are unacceptable to customers. In production lines, these bacteria are believed to grow by attaching to and forming biofilms on the many available stainless steel surfaces (Burgess, Brooks, Rakonjac, Walker, & Flint, 2009).

As the milk powder is manufactured, thermophiles can find favourable growth conditions within the cream separation set-up and/or the regenerative sections of the heat exchangers. The use of both heat and vacuum to remove water from milk in the evaporator section of the milk powder production line can also facilitate thermophilic growth. The milk evaporators usually go up to the temperature of 70°C in the first effect and 45°C in the last effect. This temperature range provides more than sufficient opportunity for the already germinated thermophilic spores to grow and colonise on the stainless steel surfaces of the evaporator. With evaporator runs as long as 20 hours, this growth and colonisation poses a serious threat to the hygiene of the entire plant and could result in spore numbers higher than acceptable in the dried concentrate during the last hours of the manufacturing process (Murphy et al., 1999).

1.1. *Geobacillus stearothermophilus*:

The test organism selected for the current work was *Geobacillus stearothermophilus*. Stadhouders et al., in 1982 have reported finding *G. stearothermophilus* on the surface of a milk powder manufacturing plant (Stadhouders, Hup, & Hassing, 1982). *G. stearothermophilus*represents about 33% of the thermophilic isolates in foods (Deak & Timár, 1988) and 64.7% of the isolates in some milk powders (Chopra & Mathur, 1984). *G. stearothermophilus*is a thermophilicbacillus that causes flat sour spoilage in low-acid foods (Ito, 1981). The spores of this organism are ubiquitous in nature; the organism is difficult to characterise in its growth, germination and sporulation requirements and also difficult to destroy with heat and chemicals.

Conventional cleaning regimes, including cleaning in place (CIP), have provedineffectiveagainst these bacteriabecause they form resistant biofilms and can localise in cracked seals, etc (Burgess et al., 2010). Pasteurisation is the major means to ensure the required quality and safety standards of a product are met in the dairy industry. Unfortunately, thermophiles remain unaffected by this treatment. Moreover, as aerobic thermophiles are capable of forming spores under unfavourable conditions and germinate upon entering favourable growth conditions, these spore-forming thermophiles are resistant to both thermization (65°C for 15 seconds)andpasteurisation (72°C for 15 seconds). Furthermore, they have the ability to grow over a wide range of temperatures, exhibit a short generation time (approximately 15-20 min) and readily form biofilms(Burgess et al., 2010). All these factors have made development of new cleaning and sterilizing techniques of utmost importance. Some new strategies that have been developed to remove, prevent and/or

delay   the ability of thermophilic bacilli to form biofilms have met with little success. Insufficient knowledge about the composition and structure of these bacterial biofilms in the milk processing environment, or in general, is proving a hindrance in the development of new and effective cleaning routines and processes. Consequently, any new cleaning regime that has been developed has failed to give optimum results when tested(Burgess et al., 2010).

One of the new techniques that is being developed and tested involves a non-chemical approach which involves disrupting the temperature profile of the milk production lines. As an example, the application of cold processing in ultra-filtration (UF) plants and centrifugal separators to separate the cream from the milk has been shown to decrease the levels of thermophilic contamination and to extend the length of production hours (Burgess et al., 2010).

In a study by Knight et al, in 2004, temperature spiking was used to control the formation of biofilms by *Streptococcus thermophilus* in plate heat exchangers (PHE) in the pasteurisers of milk used in cheese production (Knight, Nicol, & McMeekin, 2004). This approach has also been shown to be helpful in controlling the development of thermophilic biofilms as demonstrated by Burgess et al. (Burgess et al., 2009). They were successful in preventing the formation of *Anoxybacillus flavithermus* spores in the biofilms by decreasing the temperature of the growth environment from 55°C to 48°C. A temperature of 55°C is usually presentduring production cyclesinthe dairy industry (Burgess et al., 2010).

In the research by Knight et al., a new strategy was employed to control the growth of *S. thermophilus* biofilm in a pilot plant scale cheese-milk pasteuriser with the industrial goal of achieving a 20-hour production cycle without any detectable growth of *S. thermophilus*. The process included changing the temperature regime of the regenerator stage to temperatures outside the optimum growth range periodically. An important consideration in the design of the step changes necessary to achieve this goal was to minimise any reduction in energy efficiency that resulted from the implementation of step changes to the pilot plant. The implementation of the step-change in the temperature profile at regular intervals was successful in controlling the formation of these specific bacterial biofilms. The application of a step-change cycle also prevented bacterial growth on the heating side of the pilot plant. The optimum step change conditions required to achieve a 20-hour production run without detectable growth of *S. thermophilus* comprised a step change to 55°C from a standard

temperature of 35°C, applied for 10 min, with a 60-min interval between step changes (Knight et al., 2004).

The current work is based upon the study by Knight et al. Instead of *S. thermophilus* (thermoduric bacterial species), this study was focused on investigating the effect of a step-change cycle when it is introduced within the optimum growth range temperature for *Geobacillus stearothermophilus*.

1.2. Current work:

The project was started with the hypothesis that thermal cycling does have the ability to affect the growth of thermophilic bacteria i.e. *Geobacillus stearothermophilus* in this case.

The experiment was designed to check the growth rate fluctuations (major or minor) that can be achieved by spiking the optimum growth temperature (55°C) for *G. stearothermophilus*. After some initial experimentation (as explained in the methods section), two temperatures i.e., 30°C and 35°C were selected to be used individually and in conjunction with the optimum temperature to test the hypothesis.

Chapter 2:

LITERATURE REVIEW

2.1. Biofilms in Dairy Industry:

The dairy industry is involved in the production of many perishable and semi-perishable foods. Product flavour, stability and functionality are maintained in part by strict adherence to established microbiological guidelines. Other parameters include the temperature of processing and raw material specifications. There are various points at which milk contamination can take place, although it is obtained as an essentially sterile secretion from a healthy cow (Flint et al., 1997). Contamination can occur during various stages of milk transportation, processing and storage. The microbial flora present on the udder or in the milking machine can readily cause contamination during milking. After collection, the milk can be stored at the dairy farm for up to 48 hours at 7°C which restricts the microbial growth to psychotropic micro-organisms (Flint et al., 1997). During the subsequent transportation and storage at the production site, there is a serious risk of contamination from transfer lines and storage containers. In New Zealand, most milk silos are unrefrigerated, so the processor relies on the temperature of the bulk milk just received to control growth of any unwanted micro-organisms before the production begins (Flint et al., 1997).

The manufacturing process for many products includes pasteurisation at an early stage to eliminate pathogens or heat sensitive spoilage organisms that may be present in the milk. Other steps such as decreasing pH after addition of starter, drying, cooling and addition of salt are employed to limit the growth of thermoduric bacteria that are able to resist pasteurisation. Needless to say, during the entire manufacturing process, the hygiene of the main plant is fundamental to prevent any cross contamination of the product stream from the production plant and environment (Flint et al., 1997).

When contamination does surface, there has been increasing evidence that implicates biofilms, existing on the surface of the milk processing equipment, to be the main source of contamination in the dairy products. Factors including the objective of producing products which have a longer shelf-life, requirement of fulfilling stricter hygiene guidelines, heightened awareness about health complications owing to food borne pathogens like *Listeria monocytogenes*, achieving extended production runs (Sutherland, 1995), automation of the manufacturing process and increased complexity of the production equipment (Notermans, 1994), have renewed interest in controlling contamination during the various dairy processes.

A biofilm can be defined as an aggregation of microbial cells and their associated extracellular polymeric substances (EPS), actively attached to, growing and multiplying on a

surface. The development of a biofilm usually takes place through various stages (Jonhstone et al., 1980; Stoodley, Lewandowski, Boyle, & Lappin-Scott, 1999). In the first step, the bacterial cells attach to the substratum. This attachment which is reversible is followed by irreversible attachment. After the irreversible attachment, the bacterial cells divide and form micro colonies which lead to the formation of EPS. The formation of the EPS matrix allows the micro colonies to expand and form a multilayered biofilm structure connected by water channels and embedded in the matrix (Stoodley et al., 1999). This matrix serves as a source of nutrients and other required growth supplements for the biofilm. The attachment process and development of the EPS matrix imparts increased resistance to the bacterial cells within the biofilm to harsh environments when compared to the free living cells (Burgess et al., 2010).

A biofilm can form on any surface in direct contact with an aqueous environment. The extent and rate of growth of a biofilm is determined by a number of processes including adsorption and growth (Escher & Characklis, 1990). In the attachment phase, the development of a protein conditioning film has been postulated to be an essential prerequisite for the formation of a biofilm in a dairy processing environment, with the adsorption proteins denaturing to irreversibly adsorbed species that result in further deposition and biofilm development (Kirtley & McGuire, 1989). However, some recent work by Flint et al., has demonstrated that washed cells of *S. thermophilus* and *Bacillus cereus* have the ability to attach to a clean stainless steel surface at a concentration of $>10cm^2$ within 60 seconds. This indicates that a conditioning film might not be essential for the formation of biofilms of these bacteria. Attachment of bacteria may be promoted or inhibited in dairy fluids depending on the composition of the dairy fluid and the type of bacteria (Flint et al., 1997). The inhibition of attachment has been reported in the presence of whole milk but enhanced by the presence of lactose and non-casein protein solutions (Criado, Suarez, & Ferreiros, 1994; Speers & Gilmour, 1985; Suárez, Ferreirós, & Criado, 1992). An association of the bacteria with milk fat globules or the effect of natural antibodies has been suggested as possible reasons for this inhibition of bacterial attachment (Flint et al., 1997). In another study by Maxcy et al., in 1973, a phospholipid was found to precede the attachment and growth of *Pseudomonas* spp.

on the surface of milk processing lines (Maxcy, 1973). The ability of bacteria to attach to rubber gaskets and stainless steel surfaces has also been recorded in a study by Austin and Bergeron in 1995 (Austin & Bergeron, 1995). The various factors influencing the attachment ability and rate include the growth phase of the specific bacteria, viability of the bacterial

species, time period of direct contact of bacteria with the surface, temperature of the surface and the process fluid, smoothness or roughness of the surface along with the amount of milk on the surface, flow rates, and properties of the surface cells, for example, surface charge and hydrophobicity (Bouman, Lund, Driessen, & Schmidt, 1982; Czechowski & Rapp, 1990; Hoffman, 1983; Hup & Stadhouders, 1979; Langeveld, Bolle, & Vegter, 1972; Speers & Gilmour, 1985; Van der Mei, De Vries, & Busscher, 1993).

In dairy processing plants, biofilms can be divided into two categories. Biofilms unique to a dairy environment are formed by thermophilic bacilli selectively enriched by the conditions and are called process biofilms, for example, the biofilms that are formed on surfaces which are in direct contact with the flowing product. The second category includes the biofilms that are encountered throughout the food processing industries that are not selected by the processing conditions (Flint et al., 1997).

Biofilms in the dairy processing industries possess many distinguishing characteristics in contrast to the other biofilms that are usually present in a food manufacturing environment. In such biofilms, it is usually observed that a single bacterial species predominates (Bouman et al., 1982; Hup & Stadhouders, 1979) as the dairy processing environment is believed to exert selective pressure. This selective pressure can occur in the form of heat, pH, product composition and water activity. This could also be the result of pasteurisation, which eliminates the heat sensitive Gram negative bacterial species and facilitates the growth of thermoduric bacteria such as *Streptococcus thermophilus* and thermophilic bacterial species like *G. stearothermophilus*. These biofilms are also known to show a rapid growth rate with concentrations of 10^6 bacteria per cm^2 observed in the regenerative sections of the milk pasteurisers after only 12 hours of production cycle (Bouman et al., 1982).

Various factors are considered to improve the formation and influence the nature of biofilms in dairy production industries (Flint et al., 1997). The temperature, origin and age of the raw milk is believed to modify the rate of build up of thermo-resistant streptococci in pasteurisers involved in cheese production (Hup & Stadhouders, 1979). The existence of spoilage psychrotrophic bacteria like *Pseudomonas*, *Acinetobacter* and *Flavobacteria* in pasteurised milk is postulated to occur after the pasteurisation treatment, and is believed to originate from inadequate cleaning of the production plant, with the source of contamination lying in the water used during the cleaning regimes (Koutzayiotis, 1992).

Dairy processing industries are more concerned with the biofilm problem because of the increased difficulty of eliminating bacterial cells in a biofilm as compared with the planktonic cells (Mosteller & Bishop, 1993; Wirtanen et al., 1996; Wirtanen & Mattila-Sandholm, 1992). Moreover, once the biofilms develop, they can act as a source of contamination to other products and equipment surfaces. For instance, the formation of a biofilm has been recorded to cause high concentrations($\sim 10^6$per unit area) of bacteria being released into the process stream and this in turn decreases the acceptability of the final product (Driessen & Bouman, 1979). Equipment used in the production of whey, cheese, powder and general milk processing (pasteurisation and milk transfer tubes) have encountered issues pertaining to contamination from the development of biofilms (Hartley & Flint, 1993; Hup & Stadhouders, 1979; Koutzayiotis, 1992; Somers, Johnson, & Wong, 2001)

The biofilms forming on the surface of stainless steel equipment in the dairy processing units release bacteria into the product as it is being produced, which results in decreased safety and quality of the end-product. Biofilms also affect the chemical and physical state of the equipment. Biofilms cause decreased flow, blockages in tubes, reduction in plant run times, corrosion of the stainless steel surfaces and decreased transfer of heat via the plate heat exchangers. Although disinfection and cleaning routines have been incorporated in the cleaning-in-place (CIP) regimes in food processing industries, the problem of formation of biofilms and resulting end-product spoilage has become a recurring issue (Carpentier, Wong, & Cerf, 1998; Romney, 1990; Zottola & Sasahara, 1994). This can be partly because the CIP procedures, including cleaning and disinfection, have concentrated mainly on removing food-borne pathogens (for example, *Listeria*) and neglected other non-pathogenic but still quality affecting bacterial classes like thermophilic bacilli(Parkar, Flint, & Brooks, 2004).

2.2. Thermophiles in the Dairy Industry:

To indicate the quality of the end-product in dairy production plants, thermophiles are used as a hygiene index. This is primarily due to the ability of thermophiles to form endospores. They are also considered to be potential spoilage organisms as they can impart off flavour to the end-product (Burgess et al., 2010).

These strains of thermophiles, whether obligate or facultative, have the ability to produce acids along with an array of heat-stable enzymes like proteinases and lipases that might cause a spoilage problem in dairy products (Basappa, Shroff, & Srinivasan, 1974; Chen, Coolbear, & Daniel, 2004; Chopra & Mathur, 1984; Cosentino, Mulargia, Pisano, Tuveri, & Palmas, 1997; Gundogan & Arik, 2004; Murugan & Villi, 2009). The capability of obligate thermophiles to cause spoilage in dairy products is considered to be low, owing to the storage of dairy products usually at temperatures below 37°C which does not support the growth of mostobligate thermophiles. However, *Geobacillus stearothermophilus*, the test organism selected for this study has been found to be associated with the peculiar flat-sour spoilage in various canned foods, including evaporated milk (Burgess et al., 2010; Kalogridou-Vassiliadou, 1992).

2.3. Specific Characteristics of Geobacillus stearothermophilus:

The detection of *G. stearothermophilus* is usually carried out by cultivation in agar or broth media which produces results after 24-48 hours when incubated at a temperature of 55°C (Flint & Brooks, 2001).

G. stearothermophilus was formerly known as *Bacillus stearothermophilus,* as *Geobacillus* species were previously classified within the Bacillus genus. Taxonomic investigations have shown that the genus *Bacillus* has a vast diversity (Ash, Farrow, Wallbanks, & Collins, 1991; Rainey, Fritze, & Stackebrandt, 1994;Rössler et al., 1991). Throughout the 1980's, serious debates about the classification of the genus existed. There were doubts whether some bacterial species included in the genus are in fact different genera altogether. New genetic techniques like DNA-DNA hybridisation and 16s rRNA sequence analysis were then employed to define and classify species (Rainey et al., 1994; Rössler et al., 1991;Sneath, 1986). Extensive work throughout the 1990s resulted in the reclassification of some of the bacterial species within the *Bacillus* genus into a new separate genera (Goto, Fujita, Kato, Asahara, & . 2004; Heyndrickx et al., 1998; Heyndrickx et al., 1997; Heyndrickx et al., 1996; Heyrman et al., 2003; Jay, 2003; Ren & Zhou, 2005; Shida, Takagi,

Kadowaki, & Komagata, 1996; Wainø, Tindall, Schumann, & Ingvorsen, 1999; Wisotzkey, Jurtshuk, Fox, Deinhard, & Poralla, 1992; Yoon, Kang, & Park, 2002; Yoon, Oh, & Park, 2004). A taxonomic study by Nazina et al. in 2001, reclassified *Bacillus stearothermophilus* into a new genus, *Geobacillus* (Nazina et al., 2001).Therefore, the current study will use *Geobacillus stearothermophilus* instead of *Bacillus stearothermophilus* to avoid any confusion.

Geobacillus strains have been isolated from both temperate regions and hot environments like oilfields, sugar refineries, deep sea sediments, hot water springs and dairy factories (Lin, Tai, Kuo, & Liu, 2004; Marchant, Banat, Rahman, & Berzano, 2002; Nazina et al., 2001; Ronimus et al., 2003; Scott, Brooks, Rakonjac, Walker, & Flint, 2007; Takami, Nishi, Lu, Shimamura, & Takaki, 2004; Zarilla & Perry, 1987).

2.4. Thermophilic Biofilms in the Dairy Industry:

Under most dairy processing conditions, the thermophilic bacilli are known to form a single layer on a stainless steel surfaceand hence are not able to form the last stage of a conventional biofilm comprising numbers of bacterial cells arranged in multi-layers embedded in the EPS matrix with water channels (Burgess et al., 2010). This phenomenon is likely to occur in areas of a dairy manufacturing plant that are regularly cleaned, and where there are high shear rates and no dead zones, such as on the surface of plate heat exchangers. However, in other areas of dairy manufacturing plants, where the flow is inconsistent, such as underneath the distribution plates of evaporators, conditions may allow a multi-layered structure to form or bacterial cells may become trapped within milk foulant (Burgess et al., 2010). The formation of thermophilic biofilms in dairy production plants is considered to take place in the regions at the high temperatures of 40-65°C (Flint et al., 1997; Lewis, 2003; Murphy et al., 1999; Scott et al., 2007; Stadhouders et al., 1982; Warnecke, 2001) including preheating and evaporation sections of the milk powder plants, centrifugal separators which are used to separate cream from whole milk operating at warm temperatures of 45-55°C, ultra-filtration (UF) plants running at warm temperatures (~50°C), recycle loops in butter production plants, plate heat exchangers employed in the pasteurisation process and cream heaters in anhydrous milk fat (AMF) plants (Burgess et al., 2010).

The detection of high concentrations (~10^4 CFU.g^{-1}) of thermophiles in finished dairy products including milk powder is indicative of poor hygiene during the manufacturing process. Although pending experimental verification, the source of thermophilic growth

present in the end product is believed to originate from minimal numbers (in the spore form) found in the raw milk that are able to resist pasteurisation. Laboratory scale experiments have demonstrated that both vegetative cells and spores of the thermophilic bacilli are capable of attaching to the stainless steel surfaces similar to the equipment in the dairy processing environment and milk residue foulant (Flint et al., 2001; Langeveld, van Montfort-Quasig, Waalewijn, & Wever, 1996; Parkar, Flint, Palmer, & Brooks, 2001; Seale, Flint, McQuillan, & Bremer, 2008).

The formation of a biofilm is considered to take place in severalsteps. The first step is the reversible attachment of the planktonic cells or spores to the appropriate surface. The vegetative cells initiate biofilm formation while spores undergo germination and become a part of already forming biofilm. The next step though unclear is assumed to involve the sporulation within the biofilm. The sloughing of the cells from the biofilm or release of individual cells and spores by a detachment process is the likely cause of the contamination of the process liquid during production (Burgess et al., 2009). The formation of *G. stearothermophilus* biofilms on stainless steel surfaces with milk as the fluid being processed has been investigated by Flint et al., in 2001 (Flint et al., 2001).

The formation of spores is a major concern to the dairy industry (Burgess et al., 2009). Spores are able to remain viable in the milk powder products for a prolonged time period, while the vegetative cells are most likely to die off with time (Reddy, Atwal, & Srinivasan, 1975). Under favourable conditions, these spores can easily undergo germination when the milk powder is reconstituted. This can lead to formation of enzymes and acid production, which in turn results in the development of an undesirable off-flavour in the resulting product (Chen et al., 2004; Chopra & Mathur, 1984). In addition, the presence of high numbers of any kind of undesirable bacteria will tend to decrease the consumer demand as this is not acceptable to customers.However, the concentration of thermophilic strains in the raw milk is unrelated to the concentration of these bacteria present in the final milk powder product (Muir, Griffiths, Phillips, Sweetsur, & West, 1986). In fact, the numbers of thermophiles in the raw milk in New Zealand are normally very low (~10 CFU.ml^{-1}) (Hill & Smythe, 1994; McGuiggan, McCleery, Hannan, & Gilmour, 2002). On rare instances when high numbers are present (10^2 CFU.ml^{-1}), the predominant strains detected in the raw milk are usually of *B. licheniformis* and *B. coagulans* species (Hill, 2004). But the analysis of the final product from this quality raw milk indicates the predominant species to be *Anoxybacillus flavithermis* and *G. Stearothermophilus.* Consequently, the low concentration

of spores in the raw milk and a short residence time for milk in the dairy processing plant cannot account for the final high concentrations of thermophilic bacteria in the milk powder (Burgess et al., 2010).

However, this observation can be explained on the basis of growth of these bacteria in the processing plant itself. In particular, the biofilms of thermophilic bacilli have been observed on the surface of production equipment or within the foulant milk residual layers. Prolonged production runs, improper and incomplete cleaning of processing equipment between production runs, the use of recycle loops and ingredients and by-products that may have thermophilic contamination can result in an extensive biofilm growth (Burgess et al., 2010). Dairy processes like the production of milk powder, whey, pasteurised milk and buttermilk are affected by the growth of thermophilic biofilms and the subsequent contamination issues (Flint et al., 1997; Langeveld et al., 1996; Muir et al., 1986; Scott et al., 2007).

2.5. Early study of Thermal Cycling:

Geoff Knight and co-workers performed some initial experiments to test the effectiveness of thermal cycling in controlling the growth of *Streptococcus thermophilus* in pasteurisers during the production of cheese. During the production of cheese, raw milk is pasteurised to eliminate the existing pathogenic and spoilage bacteria presumed to be present. Pasteurisation involves heating the milk from its storage temperature of 10°C to 72°C. The milk is maintained at 72°C for 15seconds and then cooled down to a temperature suitable for cheese production like 35°C (Knight et al., 2004). For energy conservation purposes, a cheese-milk pasteuriser includes a regenerative section that is used to pass incoming cold milk (from storage) and the pasteurised hot milk concurrently through opposite sides of the heat exchangers. This movement enables the hot milk to heat up the incoming milk and the cold milk to cool down the outgoing pasteurised milk, thus recovering some of the input heat (Knight et al., 2004).

That study involved the design and construction of a pilot plant-scale cheese-milk pasteuriser to examine the formation of biofilms by *Streptococcus thermophilus*. Under usual production cycles and operation regimes, *S. thermophilus* was able to grow on surfaces in the regeneration sections of the pilot plant, between 50 and 35°C (temperature of bulk milk) and was detected in the end product flow after 8-10 hours of production start, with levels reaching to 10^6 CFU.ml^{-1} after 16 hours. These thermoduric bacteria and other non-thermoduric

bacterial species originating from the raw milk grew in the heating regions of the plant also but to a comparatively lesser extent.

The pilot plant was operated in two configurations i.e., partial or full regenerative configurations. An attempt at controlling the growth of *S. Thermophilus* involved periodic alteration of the temperature profile of the regenerative section in both partial and full regenerative configurations during the complete production run. The introduction of step-changes was achieved by increasing the outlet temperature of the first heating section which resulted in a temperature rise of the inlet milk at the cold end of the regenerative section. This temperature ramping caused the increase in the temperature profile of the complete regenerative section, which in turn elevated the temperature of the *S. thermophilus* growth region (55-35°C) during the entire step-change. The step change was terminated by decreasing the temperature of the first heating section to its usual processing temperature which ultimately resulted in restoration of the normal temperature in the regenerative section to its normal range. A time period of 1-2 minutes was required to get the pilot plant to step-change the temperature and 3-5 minutes to restore to conventional processing conditions. The time taken from the start of the increase in the outlet temperature of the first heating section to the start of the restoration of the normal operating conditions was defined as one complete step-change (Knight et al., 2004).

During normal operation of the plant, the temperature of milk is reduced from 72°C to 35°C in eight passes in both the configurations. In the partial regenerative configuration, the milk cools down to 55°C from 72°C in the first four passes and then further down to 35°C in the next four passes. With the introduction of the step-change to 55°C, the temperature profile during the first four passes was not affected but the temperature during the entire eight passes in the regenerative section including the *S. thermophilus* growth region remained at 55°C. An increase in the cooling capacity of the second cooling section was used to decrease the temperature of the milk from 55°C to 35°C. When operating conventionally with the full regenerative configuration, the milk was cooled from 72°C to 35°C in eight passes within the regenerative section. When a step-change to 55°C was involved, the temperature profile of the cooling side was increased from 72-35°C to 72-57°C which resulted in the temperature of the *S. thermophilus* growth region rising from 50-35°C to 63-57°C. The cooling capacity of the second cooling section was used to cool the milk from 57°C to 35°C, the same as in the partial configuration (Knight et al., 2004).

Although it is difficult to decipher the exact mechanism through which step-changes are able to interfere with biofilm formation, the results from this study were used as a basis for the present research project, with the objective of controlling thermophilic biofilms during dairy manufacturing processes. Thus, the aim of this current study was to apply a similar principle of step-changes and evaluate the effect on *G. stearothermophilus* biofilms on stainless steel surfaces.

Chapter 3:

MATERIALS AND METHODS

3.1. Growth Medium:

Members of the genus *Bacillus* and the obligate thermophilic bacilli generally have simple nutritional needs; therefore they do not require specific amino acids for growth and are able to grow on simple media such as tryptone soya agar (TSA). The optimum growth temperature of the thermophilic bacilli is usually between 50 and 65°C, but varies between species and strains (Burgess et al., 2010).

3.1a. Tryptic Soy Broth:

Powdered TSB (30g) was dissolved in 1000ml of de-ionized distilled water and autoclaved at 121°C for 15 min. The prepared medium was stored at 4°C in sealed Schott Duran bottles for further use. The prepared medium was used for growing the inoculation cultures as well as for the dilution series before the plating step.

3.1b. Tryptic Soy Agar:

Powdered TSA (40g) was dissolved in 1000ml of de-ionized distilled water and autoclaved at 121°C for 15 min. The prepared medium was stored at 4°C in sealed Schott Duran bottles for further use.

3.2. Milk:

The milk was prepared by dissolving 10g skimmed powder in 100ml of de-ionized distilled water. A total of 10,000 ml of milk was used for each trial. The prepared milk was stored at 4°C until required. The skimmed milk powder was sourced from Fonterra. Before use, the milk powder was sent for irradiation to Scherling-Plough Animal Health at Lower Hutt, Wellington, New Zealand. This step was performed to eliminate all thermophilic spores from the milk powder.

3.3. Stock cultures:

The *Geobacillus stearothermophilus* strain used in the project was sourced from a fellow PhD student, who isolated the same strain from milk powder as a part of her research. The culture was designated as Milk Isolate 1 or geo1. The cultures were procured as vials of beads coated with the bacterial growth and stored at -20°C.

The stored bacterial cells were used to make further stock cultures specifically for this project. The stock cultures for each bacterium were prepared using freshly made 15% glycerol-BHI broth. The bacterial colonies from the source cultures were used to prepare overnight broth cultures. The overnight cultures of the bacteria were plated onto dried TSA

plates and incubated at 55°C for 24 hours. The grown cultures were swabbed from the plates using a sterile cotton swab and squeezed into a glass vial containing Brain-heart infusion (BHI)-15% glycerol. The resulting broth was suspended in screw-capped vials and stored at -20°C.

The inoculum culture for the experiment was grown in two stages. In the first part of the inoculum preparation, the frozen bacterial culture was thawed and spread onto two TSA plates. These plates were incubated overnight at 55°C. In the second part, 1cm^2 area of the plates was swabbed to inoculate a Schott Duran bottle containing 21ml TSB. The inoculated TSB was incubated in a water bath (no shaking) at 55°C for 6-8 hours before use.

3.4. Experimental Set-up:

All the experiments were carried out using the same apparatus, which included two water baths, a two-way solenoid valve, a thermocouple and thin silicon tubes. Below (Figure 1) is an illustration of the experimental set-up.
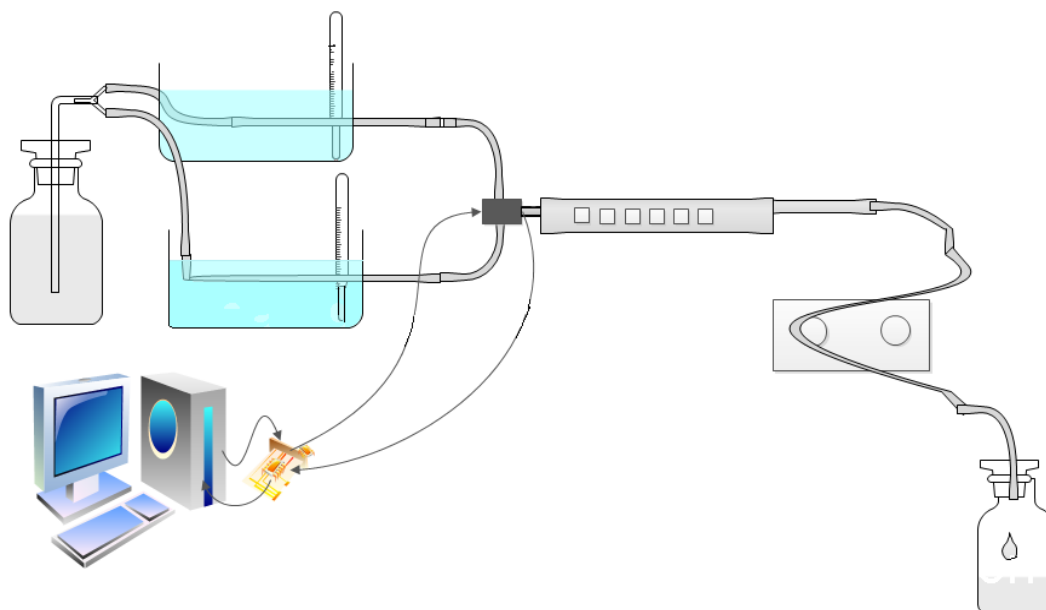
Figure 1: A process flow diagram depicting the experimental set-up. As mentioned before, this is merelyan illustration drawn with limitations using EDRAW© software. The diameter of the inlet tubes was the same throughout, unlike as seen in the diagram.

The milk reservoir was a 10,000 ml autoclavable plastic bottle. The outgoing milk was collected in a sealed 5000 ml Schott Duran bottle. The thin silicone tubes worked as the inlet tubes which remained immersed in either water bath during the experiment. First, the milk travelled from the reservoir to the inlet tubes in the water bath; it was then warmed up to a settemperature as it passed through the water bath and finally it entered the reactor at the

same temperature.The reactor was encased in a block of expanded polystyrene as a thermal insulator. Another silicone tube, also referred to as a pump tube was inserted into the peristaltic pump for the periodic pumping of milk from the attached reservoir. The pump tube was attached to the end of the reactor to carry the milk from the reactor to the collection vessel. The pump was placed after the reactor to ensure that the reactor did not become pressurised in the event of a blockage. Before the run, the ends of the thin silicon tubes and the pump tube were covered with aluminium foil and then sterilized in the autoclave at 121°C for 15 minutes.To ensure sterility, the bag was not opened until just prior to setting up all the required equipment necessary for a run.The reservoir bottle was plugged with a cotton plug having a glass tube going through the centre of the plug for the transfer of the milk to the inlet tubes. The cotton plug and the glass tube were covered with aluminium foil and autoclaved at 121°C for 15 minutes. The two-way solenoid valve was sterilized by pumping hypochorite solution (1000 ppm) at a high flowrate through both the inlets. To remove any milk foulant or blockage due to coagulated milk particles, the inlets and outlets of the valve were cleaned before each run by blowing compressed air through them. After each run, all the equipment was rinsed with water and then undiluted Trigene® before being autoclaved. After the autoclaving was finished, the equipment including all the glassware was washed in hot water with Pyroneg®.

3.4a. Biofilm Reactor:

A silicon tube of outside diameter 12mm and 1 mm wall thickness was used as a reactor for the growth of biofilms. Ten stainless steel coupons, each with an area of $1cm^2$ (total surface area $2cm^2$) were inserted into the tube prior to each experiment to provide a surface for the bacteria to form biofilms. The initial preparation of the reactor included covering it with aluminium foil and autoclaving after the coupons had been inserted at 121°C for 15 minutes in an autoclave bag.

3.4b. Introduction of Step-Change:

The water baths were used as heaters to bring the milk to different temperatures as it passed through the tubes lying submerged in the water. One of the water baths designated as being "low" was kept at the test temperature, while the other one, designated as "high", was always at the control temperature of 55°C.

The experiment was designed to incorporate a step-change cycle. The step-change cycle was introduced into the system through Arduino-based software specifically developed

by Dr. Chris Pookfor this project. The Arduino was programmed to switch the 2-way valve to bring preheated milk from one or the other of the water baths into the reactor. The program allowed the user to select the time for the milk flowing through the reactor to remain at the control temperature (high) and the test temperature (low). To determine the exact temperature of the milk entering the reactor, a thermocouple was inserted into the feed line just before the milk reached the reactor.

3.4c. Time and Temperature Regimes:

Initially it was decided to introduce a step-change at a temperature higher (65°C) than the optimum growth temperature (55°C). However, after some initial runs, it was decided to try temperatures less than the optimum growth temperature. This decision was based on a simple growth experiment conducted involving *G. stearothermophilus* culture inoculated into 10 ml of TSB and incubated at 60°C, 65°C and 70°C. The bacterial culture was able to grow substantially even at these high temperatures and therefore, the alternative option of testing step-changes to lower temperatures was selected. After carrying out some direct growth trials in incubators at 30°C, 35°C, 37°C; 30°C and 35°C were selected as the step-change temperatures. The time regimes were decided upon based on research being carried out by a fellow PhD student using a different approach to control the thermophiles. The time regimes used in that project were selected for this project, so that the results of the two projects might be compared.

The following are the various time and temperature combinations used during the course of the project:

Time	Temperature
5 min and 35 min	30°C and 55°C
5 min and 15min	30°C and 55°C
15 min and 15min	30°C and 55°C
15min and 35 min	30°C and 55°C
5 min and 35 min	35°C and 55°C
5 min and 15min	35°C and 55C
15 min and 15min	35°C and 55°C
15min and 35 min	35°C and 55°C

3.4d. Sampling:

The sampling was done by plating 2 coupons after every 4 hours up to 16 hours, and then 2 coupons at the end of the 24 hour run. Before the plating, each coupon was vortexed with glass beads and 10ml of TSB in a 20 ml plastic vial for 2 minutes. This medium was used as the 10^{-1} dilution (Brooks, 2013). Subsequent dilutions were made and then plated onto the TSA plates using the Whitley Automatic Spiro Plater (WASP)$^{©}$ in duplicates. The Spiroplater provides an option for the plating to be done either in a log mode or a linear mode. For the project, the milk samples were plated using the log mode while the coupon samples were done in linear mode. The selection of the modes was based on the convenience of counting the developed colonies. The milk samples were counted using an electronic plate reader, protoCOL3 from Symbiosis (Cambridge, UK) which recognises only log mode. The coupon sample plates were read manually.

The outgoing milk was also sampled at 2 hour intervals up to 16 hours, and the last sample was taken at the end of the 24 hour cycle. The sample was used to prepare subsequent dilutions and plated in duplicate onto the TSA plates using the WASP©.

3.4e. Inoculation Methods:

The starter culture of *G. stearothermophilus* was introduced into the experimental system via two different methods.

3.4e (i) Inoculation of the Milk:

In the first method, the prepared sterile milk was inoculated with 100ml of 10^{-5} dilution of the of starter culture to make the final concentration of 100 cells per ml. This was

done to mimic the conditions in the dairy processing plants where the raw milk is believed to contain the same concentration of thermophiles at the start of a production run. But this method caused coagulation of milk in the thin inlet tube carrying milk through the 55°C water bath, perhaps as a result of slow protein denaturation. The tube became blocked at around 12 hours of trial run. To overcome the problem, it was decided to change the inlettube every 12 hours, which proved effective. The sampling was carried out the same way as explained above. It was later realised that this method may also have permitted biofilm growth on the wall of the tube, thus giving a continuously increasing inoculum to the reactor during the run.

3.4e (ii) Inoculation of the Reactor:

In the second method, 7 ml of the undiluted starter bacterial culture was inserted into the reactor. The culture was allowed to stand for 30 minutes before the reactor was attached to the complete experimental set-up and the culture was removed by rapidly pumping approximately 20 ml of milk from the reservoir through the reactor and into the collection vessel. The reactor therefore mimicked the condition in the pre-heater of the milk processing plant where there is no contamination present initially, but the equipment becomes contaminated over time from the incoming raw milk. The biofilm that forms acts as a source of recontamination to the otherwise pasteurised milk. The sampling was carried out in the same way as explained above.

3.5. Statistical Analysis:

Dr. Robin Hankin, Senior Lecturer with the School of Computer and Mathematical Sciences was approached for guidance on the statistical analysis of the data. A lengthydiscussion with Dr. Hankin led to the conclusion that, although there is a clear difference between the two growth patterns (test and control) as seen in the scatterplots, providing a formal p-value, i.e. the statistical equivalent of a decision forming parameter for the null hypothesis of "no difference" is not straightforward, because we do not have access to a well-understood growth mechanism (Hankin, 2013). The statistical testing can be appliedonly when the growth curve reaches a stationary phase which was not achieved in these experiments.

Chapter 4:

RESULTS

4.1. Results for trials with First method of Inoculation (Inoculation in milk):

A total of 16 test trials in 2 sets of 4 trials and 4 replicates, were conducted using the first method of inoculation. Two control runs were carried out to compare the results. In the collation of the data, the control runs were normalized with each other and then the best fit values were chosen as the single standard control data. The experimental design was such that only one trial could be performed at a time which made it impossible to perform an individual control experiment for each trial. So, the one standard control derived from two individual control experiments was used as the control for all the trials with this method. The growth was recorded as colony forming units (CFU) per cm^2 of the coupons and per ml for the milk samples. The electronic plate reader was used occasionally to help with the colony counting procedure. Spiral plate with two-sector mode was the setting chosen for the plate reader in order to count the colonies.The $CFU.ml^{-1}$ and $CFU.cm^{-2}$ values were converted to their respective log-count values in order to get a better understanding of the results and plotted as a scatter-plot against the time of the run. All the values collected for the colony counts were normalized before being plotted on the scatter-plots for better comparison.

For every Run, as explained in Section 3.4d, samples were taken of the outgoing milk as well as the coupons in the biofilm reactor. As mentioned previously, in the methods section, each sample taken was plated in triplicates and 2 coupons were sampled at each sample point.

For every set, there is a discussion at the end of the results for the 4 comprising trials.

4.1a. SET I: In this set, the step-change temperature was 30°C.

a) TRIAL 1:

In the first trial, the following time and temperature regime was used:

Temperature	Time (minutes)	No. of Trials	Duration of the run
55°C	5	2	24 hours
30°C	35		

Table 1: Summary of the log-count readings observed of the outgoing milk during the step-change and the duplicate runs along with the control run i.e. run at 55°C

Time	Log-Count (Step-change)	Log-Count -Duplicate (Step-change)	Log-Count (Control)
0	2.72	2.72	2.72
2	0.79	0.38	3.77
4	2.62	0.21	4.18
6	2.38	1.08	4.47
8	1.31	1.66	4.71
10	1.87	1.99	4.8
12	2.54	3.04	4.97
14	2.97	3.11	5.03
16	4.34	3.61	6.27
24	4.88	4.44	6.35

(i)

Figure 2: Scatterplot demonstrating the growth patterns of the bacteria in the outflowing milk when operated under step-change conditions (30ºC for 35 minutes and 55ºC for 5 minutes) and control temperature regime.

Table 2: Summary of the log-count readings observed of the coupon samples taken every 2 hours in duplicates during the step-change and the duplicate runs, together with the control run

Time	Log-Count (Step-change)	Log-Count -Duplicate (Step-change)	Log-Count (Control)
4	-	-	2.02
8	2.65	2.65	2.65
12	4.3	4.32	3.34
16	4.26	4.35	5.77
24	5.19	5.15	6.75

(ii)

Figure 3: Scatterplot demonstrating the growth patterns of the bacteria for coupon sampleswhen operated under step-change conditions (30ºC for 35 minutes and 55ºC for 5 minutes) and control temperature regime

b) TRIAL 2:

In the second trial, the following time and temperature regime was used:

Temperature	Time (minutes)	No. of Trials	Duration of the run
55°C	5	2	24 hours
30°C	15		

For the log-counts observed for milk samples and coupon samples, refer to table 3 and table 4 respectively in Appendix I (Section 1), after the end of the reference section.

Below are the scatterplots for milk samples (i) and coupon samples (ii) count results during the step-change and control conditions:

(i)

Figure 4: Scatterplot demonstrating the growth patterns of the bacteria in the outflowing milk when operated under step-change conditions (30ºC for 15 minutes and 55ºC for 5 minutes) and control temperatureregime

(ii)

Figure 5: Scatterplot demonstrating the growth patterns of the bacteria for the coupon samples when operated under step-change conditions (30°C for 15 minutes and 55°C for 5 minutes) and control temperature regime.

c) TRIAL 3:

In the third trial, the following time and temperature regime was used:

Temperature	Time (minutes)	No. of Trials	Duration of the run
55°C	15	2	24 hours
30°C	35		

For the log-counts observed for milk samples and coupon samples refer to Table 5 and 6 respectively in Appendix I (Section 1) after the end of the reference section.

Below are the scatterplots for milk samples (i) and coupon samples (ii) count results during the step-change and control conditions:

(i)

Figure 6: Scatterplot demonstrating the growth patterns of the bacteria in the outflowing milk when operated under step-change conditions (30ºC for 35 minutes and 55ºC for 15 minutes) and control temperature regime.

(ii)

Figure 7: Scatterplot demonstrating the growth patterns of the bacteria for the coupon samples when operated under step-change conditions(30ºC for 35 minutes and 55ºC for 15 minutes) and control temperature regime.

d) TRIAL 4:

In the fourth trial, the following time and temperature regime was used:

Temperature	Time (minutes)	No. of Trials	Duration of the run
55°C	15	2	24 hours
30°C	15		

For the log-counts observed for milk samples and coupon samples refer to Table 7 and 8 respectively in Appendix I (Section 1) after the end of the reference section.

Below are the scatterplots for milk samples (i) and coupon samples (ii) count results during the step-change and control conditions:

(i)

Figure 8: Scatterplot demonstrating the growth patterns of the bacteria in the outflowing milk when operated under step-change conditions (30ºC for 15 minutes and 55ºC for 15 minutes) and control temperature regime

(ii)

Figure 9: Scatterplot demonstrating the growth patterns of the bacteria for the coupon samples when operated under step-change conditions (30ºC for 15 minutes and 55ºC for 15 minutes) and control temperature regime.

e) Discussion For Set I:

These data were further used to calculate maximum specific growth rates (μ_{max}) for the test experiment and the control experiment.

Table 9: Summary of growth rate for the bacteria in the outflowing milk:

Time at 55°C (minutes)	Time at 30°C (minutes)	μ_{max} (control)	μ_{max}(Step-Change)	μ_{max}(Step-Change-duplicate run)	Average decrease in μ_{max}
5	35	4.185	1.645	1.416	2.655
5	15	4.289	2.631	2.881	1.533
15	35	3.386	2.058	2.167	1.274
15	15	3.129	2.995	2.838	0.213

These data clearly show that there is significant effect on growth potential of the bacteria when it is subjected to thermal cycling. The best results, in terms of growth rate reduction were obtained using the test temperature of 30°C for 35 min and 55°C for 5 min;5 minutes on 55°C withjust 15 min on 30°C also resulted in reduction of growth rate. The differences in the growth rates for the rest of the two experiments were less satisfactory.

Table 10: Summary of growth rate of the bacteria for coupon samples:

Time at 55°C (minutes)	Time at 30°C (minutes)	μ_{max} (control)	μ_{max}(Step-Change)	μ_{max}(Step-Change-duplicate run)	Average decrease in μ_{max}
5	35	3.153	2.732	2.774	0.4
5	15	4.94	3.798	3.838	1.122
15	35	4.853	3.235	3.105	1.683
15	15	4.296	3.694	3.782	0.558

However, in the case of the coupon samples, the results were less substantial. Although all the experiments showed a decreased growth rate when thermal cycling was applied, there was less effect on biofilm growth than those observed from the outflowing milk samples. From the table, the best treatment methodfor coupons, and thus for biofilm control,observed in these experimentswas the trial with 15 minutes at 55°C and 35 minutes at 30°C run over24 hours.

4.1b.SET II: The step-change temperature used here was 35°C.

a) TRIAL 5:

In the fifth trial, the following time and temperature regime was used:

Temperature	Time (minutes)	No. of Trials	Duration of the run
55°C	5	2	24 hours
35°C	35		

For the log-counts observed for milk samples and coupon samples refer to Table 11 and 12 respectively in Appendix I (Section 1) after the end of the reference section.

Below are the scatterplots for milk samples (i) and coupon samples (ii) count results during the step-change and control conditions:

(i)

Figure 10: Scatterplot demonstrating the growth patterns of the bacteria in the outflowing milk when operated under step-change conditions (35°C for 35 minutes and 55°C for 5 minutes) and control temperature regime

(ii)

Figure 11: Scatterplot demonstrating the growth patterns of the bacteria for the coupon
samples when operated under step-change conditions (35°C for 35 minutes and
55°C for 5 minutes) and control temperature regime.

b) TRIAL 6:

In this trial, the following time and temperature regime was used:

Temperature	Time (minutes)	No. of Trials	Duration of the run
55°C	5	2	24 hours
35°C	15		

For the log-counts observed for milk samples and coupon samples refer to Table 13 and 14 respectively in Appendix I (Section 1) after the end of the reference section.

Below are the scatterplots for milk samples (i) and coupon samples (ii) count results during the step-change and control conditions:
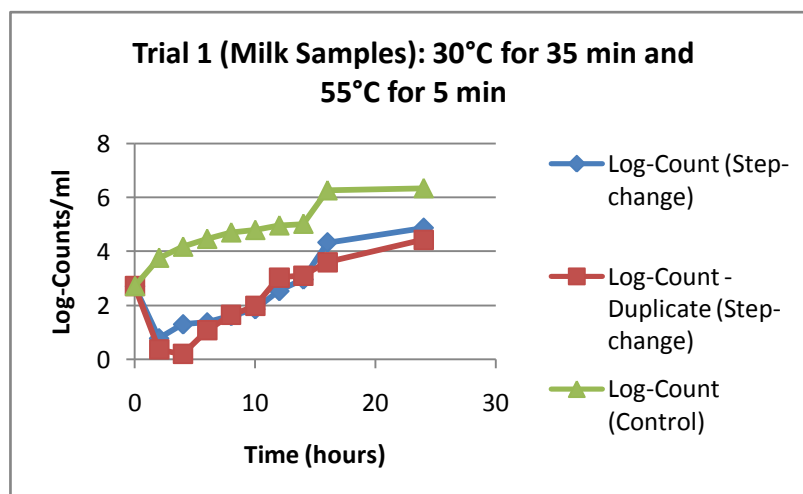
(i)

Figure 12: Scatterplot demonstrating the growth patterns of the bacteria in the outflowing milk when operated under step-change conditions (35ºC for 15 minutes and 55ºC for 5 minutes) and control temperature regime

(ii)

Figure 13: Scatterplot demonstrating the growth patterns of the bacteria for the coupon
samples when operated under step-change conditions (35ºC for 15 minutes and
55ºC for 5 minutes) and control temperature regime.

c) TRIAL 7:

In the next trial, the following time and temperature regime was used:

Temperature	Time (minutes)	No. of Trials	Duration of the run
55°C	15	2	24 hours
35°C	35		

For the log-counts observed for milk samples and coupon samples refer to Table 15 and 16 respectively in Appendix I (Section 1) after the end of the reference section.

Below are the scatterplots for milk samples (i) and coupon samples (ii) count results during the step-change and control conditions:

(i)

Figure 14: Scatterplot demonstrating the growth patterns of the bacteria in the outflowing milk when operated under step-change conditions (35°C for 35 minutes and 55°C for 15 minutes) and control temperature regime.
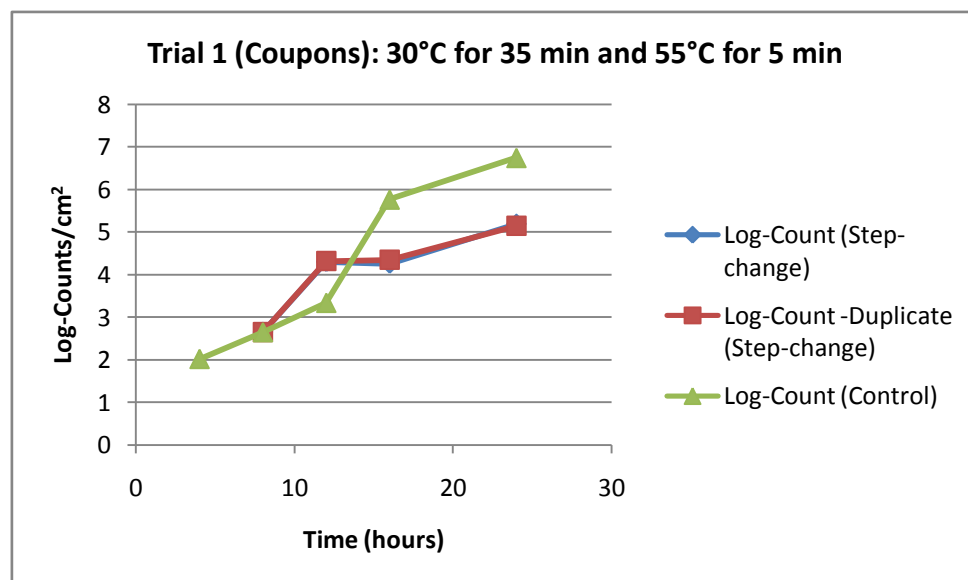
(ii)

Figure 15: Scatterplot demonstrating the growth patterns of the bacteria for the coupon samples when operated under step-change conditions (35ºC for 35 minutes and 55ºC for 15 minutes) and control temperature regime.

d) TRIAL 8:

In the last trial, the following time and temperature regime was used:

Temperature	Time (minutes)	No. of Trials	Duration of the run
55°C	15	2	24 hours
35°C	15		

For the log-counts observed for milk samples and coupon samples refer to Table 17 and 18 respectively in Appendix I (Section 1) after the end of the reference section.

Below are the scatterplots for milk samples (i) and coupon samples (ii) count results during the step-change and control conditions.
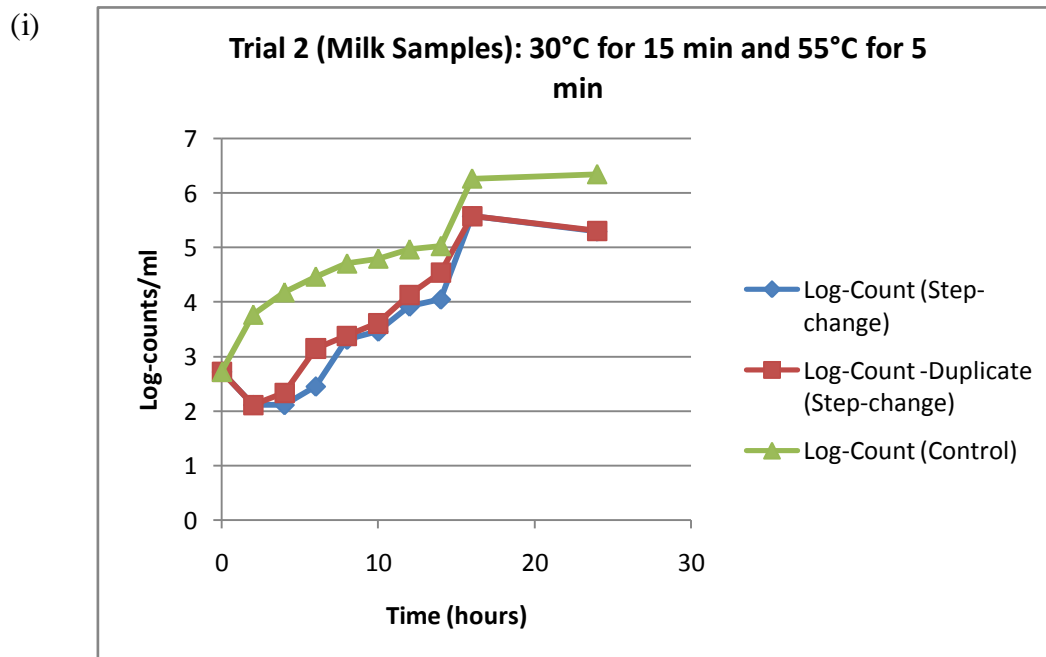
(i)

Figure 16: Scatterplot demonstrating the growth patterns of the bacteria in the outflowing milk when operated under step-change conditions (35°C for 15 minutes and 55°C for 15 minutes) and control temperature regime
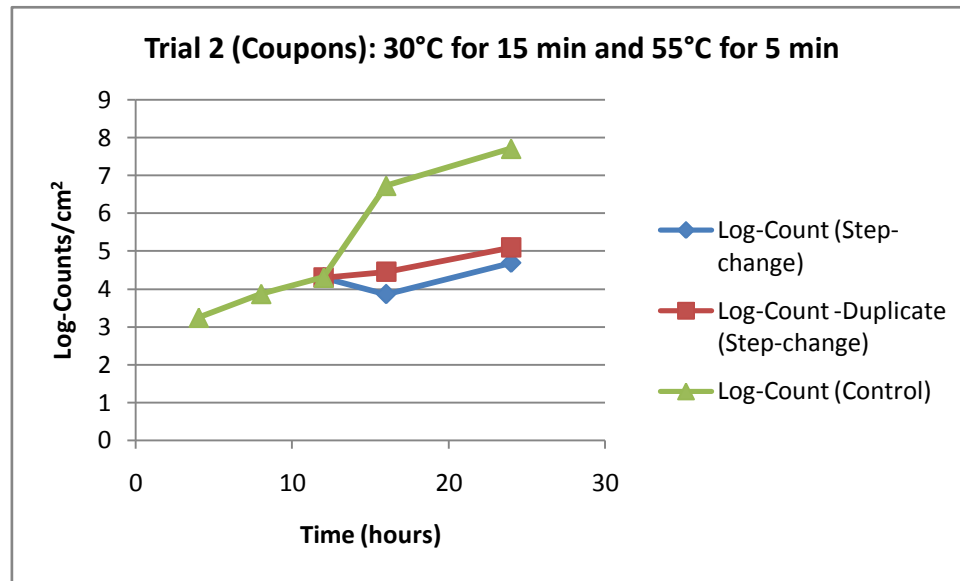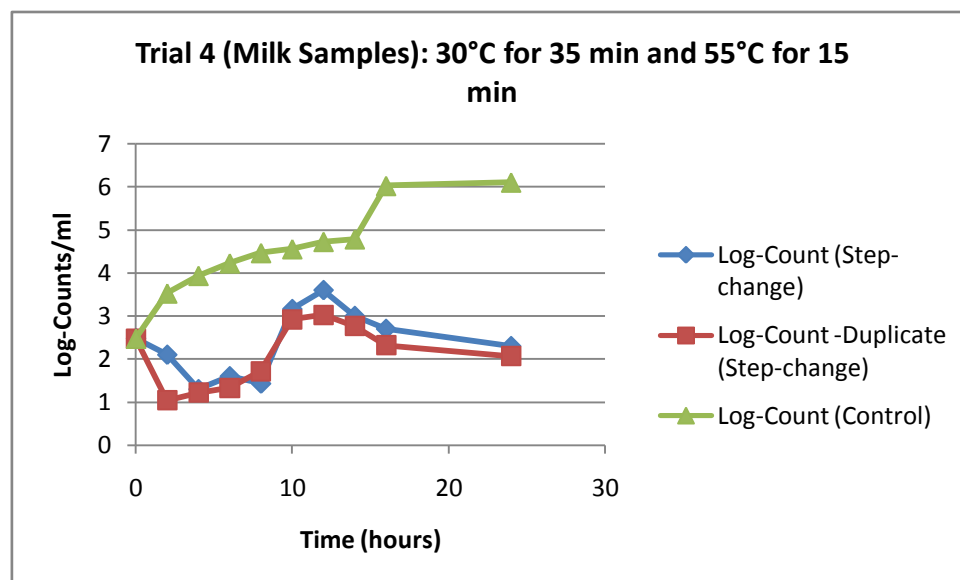
(ii)
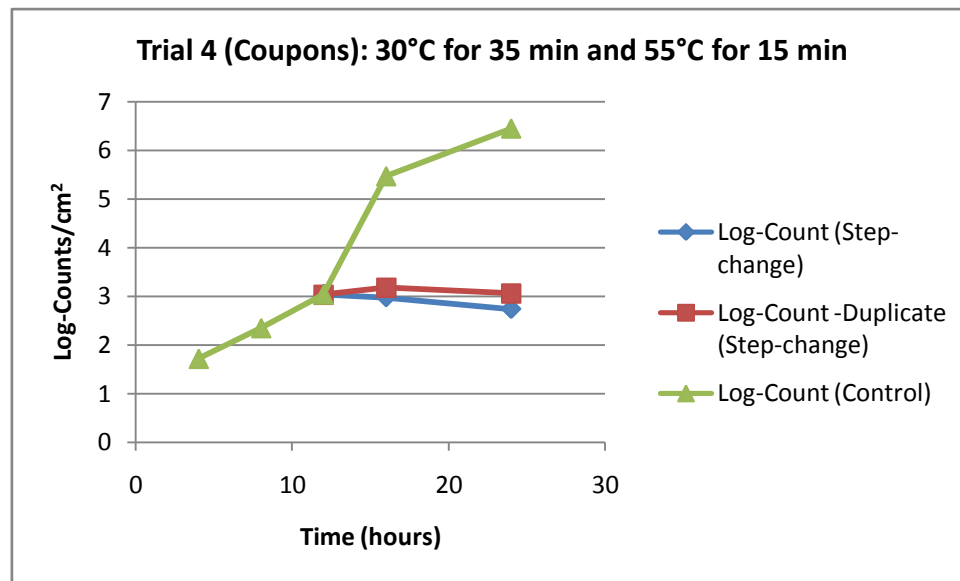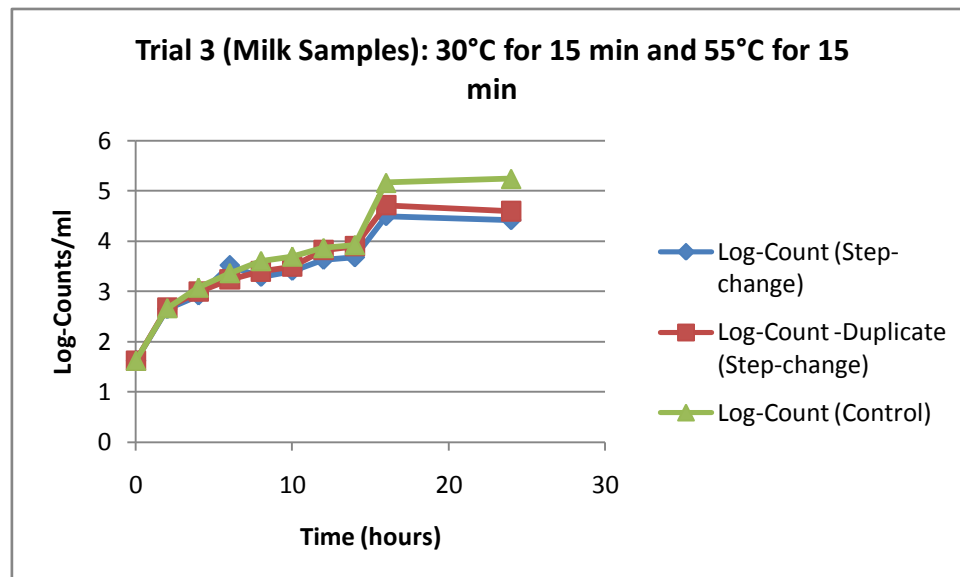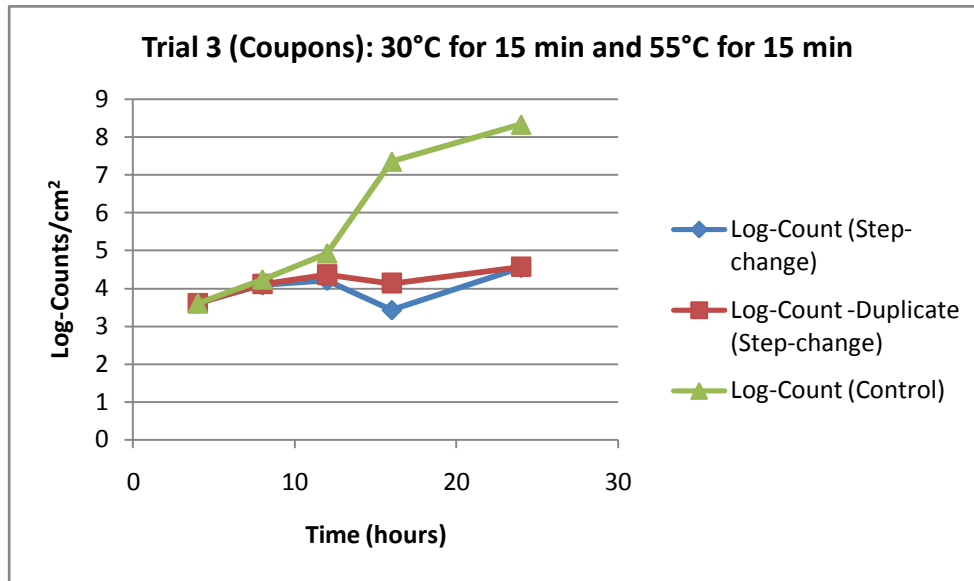

Figure 17: Scatterplot demonstrating the growth patterns of the bacteria for the coupon samples when operated under step-change conditions (35ºC for 15 minutes and 55ºC for 15 minutes) and control temperature regime.

e) Discussion for Set II:

These experiments conclude the set of trials in which the inflowing milk was inoculated with the bacterial culture. As before, maximum specific growth rate(μ_{max}) for the 4 trials was calculated to determine, if any, difference from the control run.

Table 19: Maximum specific growth rates (μ_{max}) of *G.stearothermophilus* recorded from the outflowing milk samples

Time at 55°C (minutes)	Time at35°C (minutes)	μ_{max}(control)	μ_{max}(Step-Change)	μ_{max}(Step-Change-duplicate run)	Average decrease in μ_{max}
5	35	3.988	2.581	2.441	1.477
5	15	3.988	3.465	3.212	0.65
15	35	2.437	2.016	1.207	0.826
15	15	3.505	2.832	2.703	1.611

Table 20: Maximum specific growth rates (μ_{max}) of *G. stearothermophilus* observed from coupon samples

Time at 55°C (minutes)	Time at 35°C (minutes)	μ_{max} (control)	μ_{max}(Step-Change)	μ_{max}(Step-Change-duplicate run)	Average decease in μ_{max}
5	35	3.599	2.095	2.182	1.461
5	15	3.449	2.86	2.829	0.605
15	35	4.339	2.896	2.861	1.461
15	15	3.559	3.312	3.192	0.307

It is evident from these data that although thermal cycling is capable of reducing the growth rate of the bacteria, the temperature of the treatment plays an important role in the effect produced. The growth rates observed in the experiments with the test temperatures show a decline in the growing capacity of the bacteria which indicates some physiological or environmental pressure. Although, the differences in the growth rates between the control experiments and test experiments is not as striking as those achieved with 30°C as test temperature, they are still positive values. From these data, the best time regime would be maintaining the milk at 35°C for 35 min and 55°C for 5 min, which resulted in the greatest reduction in μ_{max}observed in both outflowing milk samples and the coupon samples.

The second set of experiments was conducted using irradiated milk and inoculation was done in the reactor. This was carried out to nullify any part played by the pre-heater tubes (inlet tubes) submerged in the water bath at 55°C. It was discovered during the course of another similar research project that the pre-heater was proving to be a growth surface for the bacteria owing to the optimum growth temperature therein. However, after conducting an experiment with only the inlet tube as the reactor and collecting the outflowing milk samples, it became clear that there was not much significant growth contributed by the inlet tube. This was attributed to the decision to change the tube after every 12 hours to counter the problem of milk coagulation in the beginning of the project only. Nevertheless, the second set of experiments was continued in order to introduce a second set of conditions. The results for the experiments with sterile milk will be discussed in the next section.

4.2. Results for Trials with Sterile Inflowing Milk:

A total of 8 test trials were conducted in 2 sets of 4 trials each using the second method of inoculation. One control run was carried out to compare the results. The irradiated milk being used was also plated to check for any bacterial contamination but the milk was found to be sterile.The growth was recorded as colony forming units (CFU) per cm^2 of the coupons and per ml for the milk samples. The electronic plate reader was used occasionally to help with the colony counting procedure. The plate reader was set to spiral plate with two-sector mode to count the colonies. The $CFU.ml^{-1}$ and $CFU.cm^{-2}$ values were converted to their respective log-count values as with the previous data. The data here have also been normalized before plotting on the scatter-plot. The sampling was carried out in the same way as has been described for the previous experiments.

4.2a.SET I: For these experiments, the first set was tested with a thermal cycling down to35°C.

a) TRIAL 1:

In the first trial, the following time and temperature regime was used:

Temperature	Time (minutes)	No. of Trials	Duration of the run
55°C	5	1	24 hours
35°C	35		

For the log-counts observed for milk samples and coupon samples refer to Table 21 and 22 respectively in Appendix I (Section 2) after the end of the reference section.

Below are the scatterplots for milk samples (i) and coupon samples (ii) count results during the step-change and control conditions.
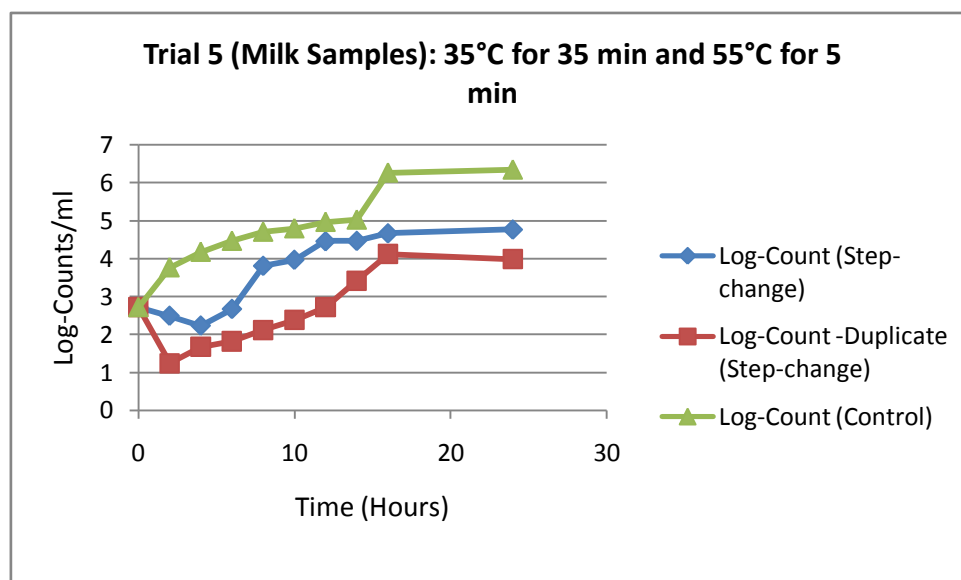
(i)

Figure 18: Scatterplot demonstrating the growth patterns of the bacteria in the outflowing milk when operated under step-change conditions (35ºC for 35 minutes and 55ºC for 5 minutes) and control temperature regime
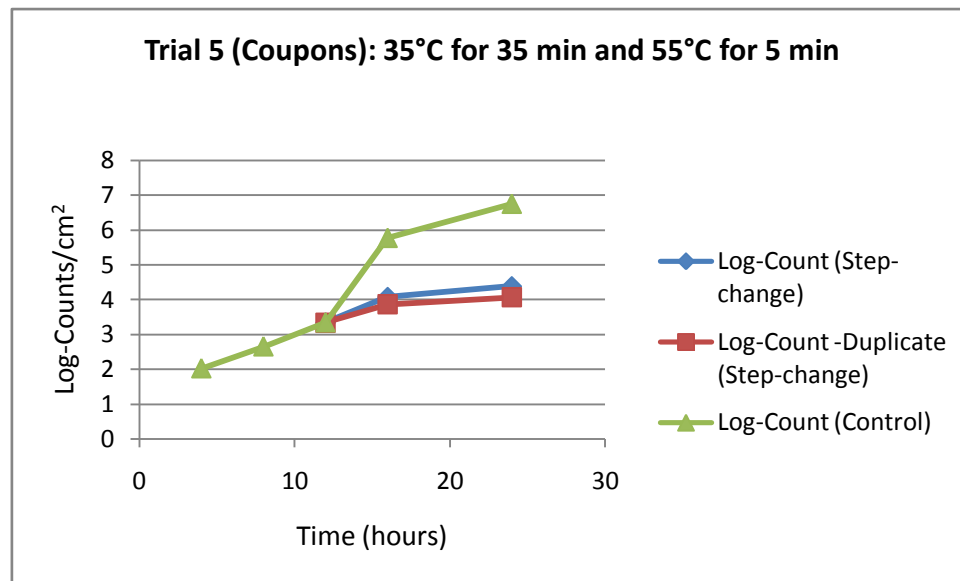
(ii)

Figure 19: Scatterplot demonstrating the growth patterns of the bacteria for the coupon
samples when operated under step-change conditions (35ºC for 35 minutes and
55ºC for 5 minutes) and control temperature regime.

b) TRIAL 2:

In the second trial, the following time and temperature regime was used:

Temperature	Time (minutes)	No. of Trials	Duration of the run
55°C	5	1	24 hours
35°C	15		

For the log-counts observed for milk samples and coupon samples refer to Table 23 and 24 respectively in Appendix I (Section 2) after the end of the reference section.

Below are the scatterplots for milk samples (i) and coupon samples (ii) count results during the step-change and control conditions:
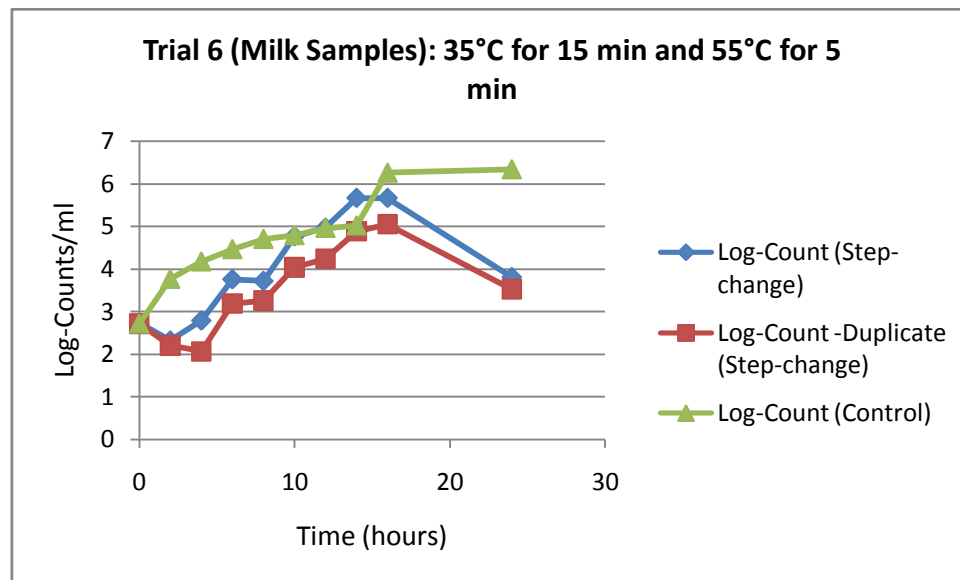
(i)

Trial 2 (Milk Samples): 35°C for 15 min and 55°C for 5 min

Figure 20: Scatterplot demonstrating the growth patterns of the bacteria in the outflowing milk when operated under step-change conditions (35ºC for 15 minutes and 55ºC for 5 minutes) and control temperature regime
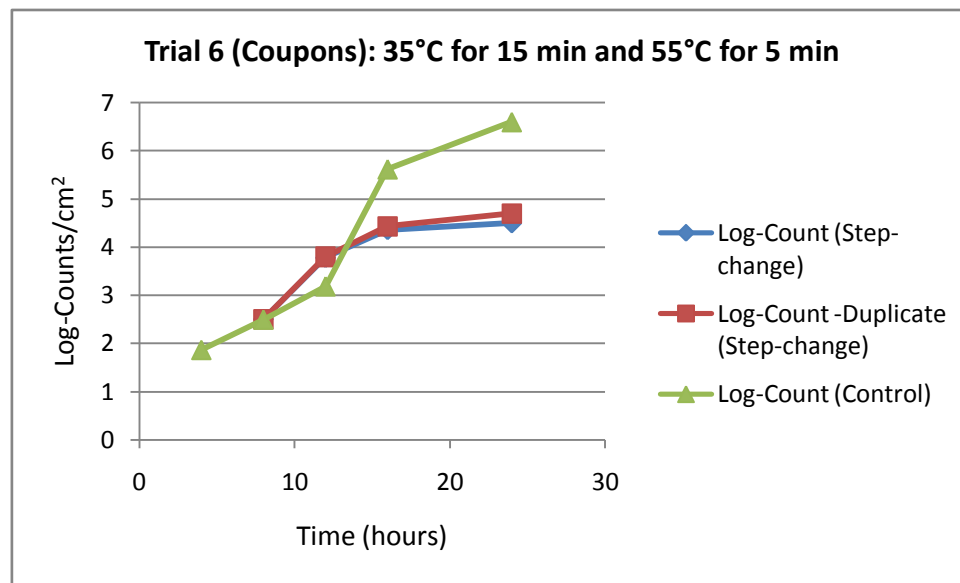
Figure 21: Scatterplot demonstrating the growth patterns of the bacteria for the coupon samples when operated under step-change conditions (35ºC for 15 minutes and 55ºC for 5 minutes) and control temperature regime.

c) TRIAL 3:

In the next trial, the following time and temperature regime was used:

Temperature	Time (minutes)	No. of Trials	Duration of the run
55°C	15	1	24 hours
35°C	35		

For the log-counts observed for milk samples and coupon samples refer to Table 25 and 26 respectively in Appendix I (Section 2) after the end of the reference section.

Below are the scatterplots for milk samples (i) and coupon samples (ii) count results during the step-change and control conditions.
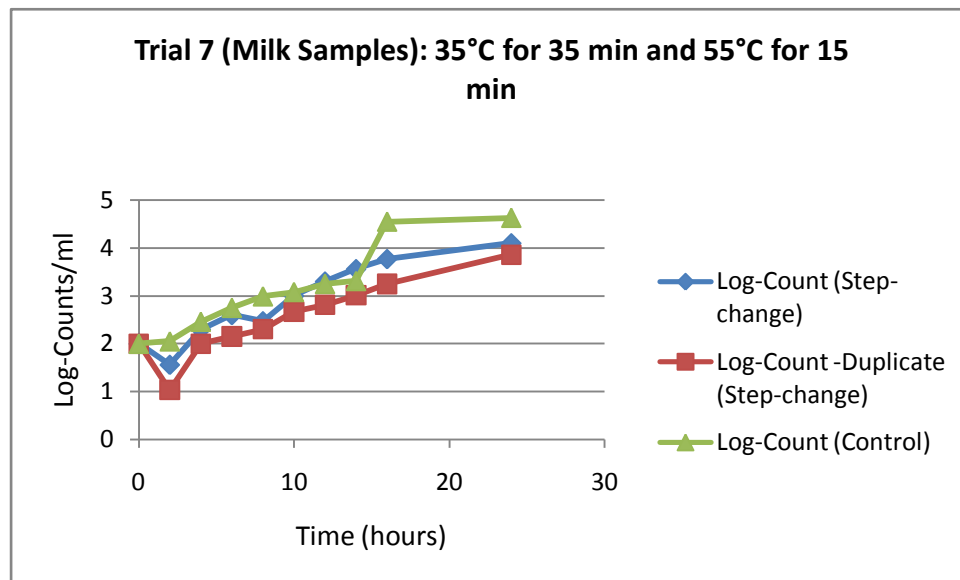
(i)

Figure 22: Scatterplot demonstrating the growth patterns of the bacteria in the outflowing milk when operated under step-change conditions (33ºC for 35 minutes and 55ºC for 15 minutes) and control temperature regime
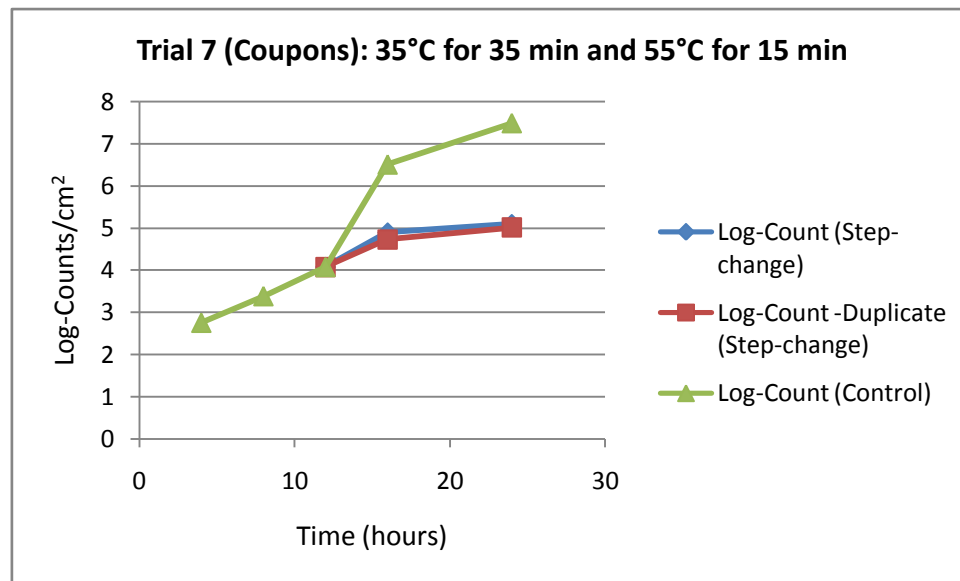
(ii)

Figure 23: Scatterplot demonstrating the growth patterns of the bacteria for the coupon samples when operated under step-change conditions (35ºC for 35 minutes and 55ºC for 15 minutes) and control temperature regime.

d) TRIAL 4:

In this trial, the following time and temperature regime was used:

Temperature	Time (minutes)	No. of Trials	Duration of the run
55°C	15	1	24 hours
35°C	15		

For the log-counts observed for milk samples and coupon samples refer to Table 27 and 28 respectively in Appendix I (Section 2) after the end of the reference section.

Below are the scatterplots for milk samples (i) and coupon samples (ii) count results during the step-change and control conditions.
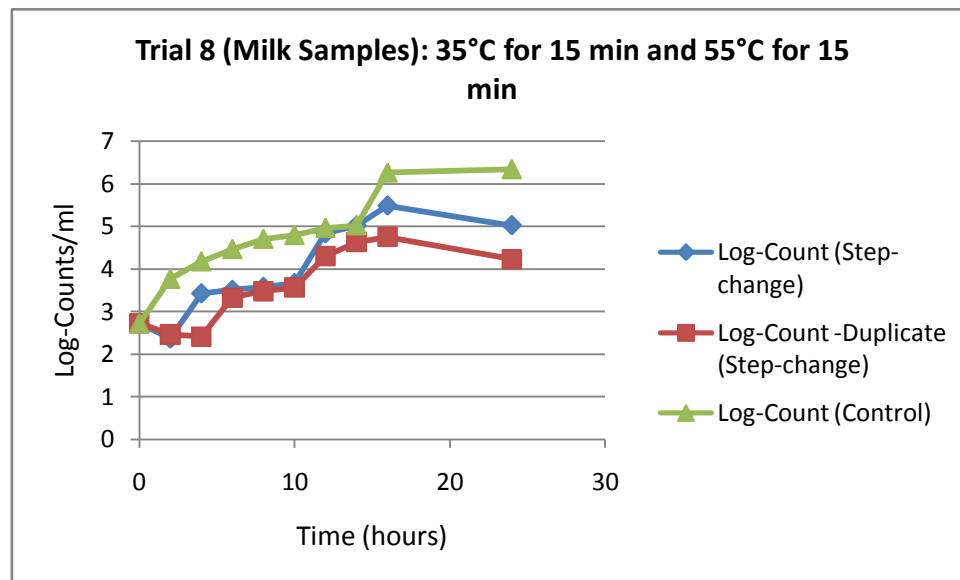
(i)

Figure 24: Scatterplot demonstrating the growth patterns of the bacteria in the outflowing milk when operated under step-change conditions (35ºC for 15 minutes and 55ºC for 15 minutes) and control temperature regime
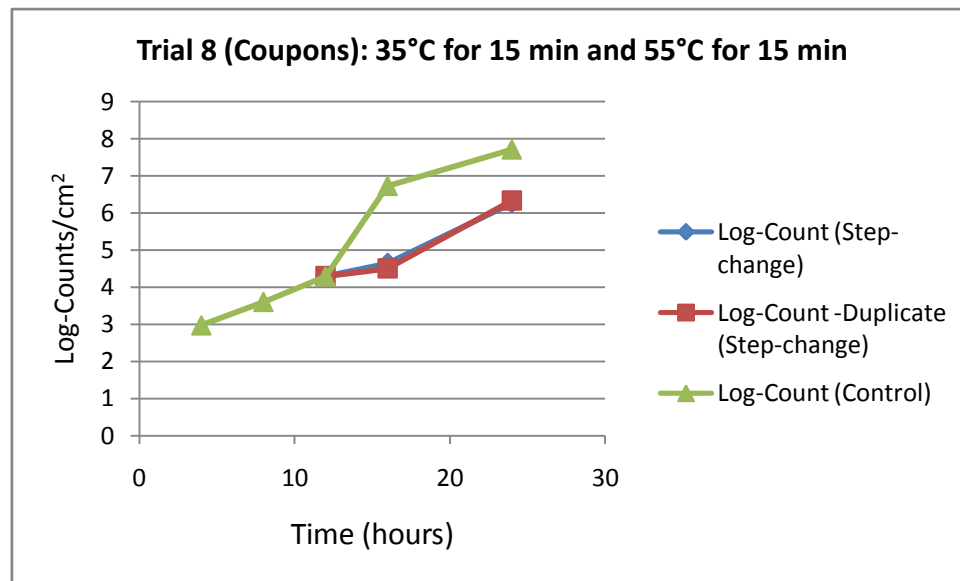
(ii)
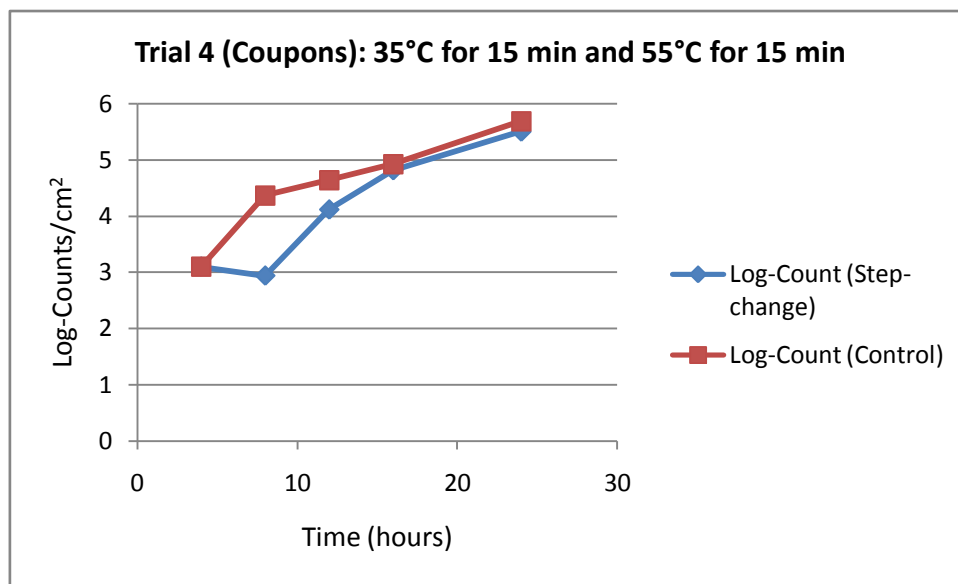
Figure 25: Scatterplot demonstrating the growth patterns of the bacteria for the coupon
samples when operated under step-change conditions (35ºC for 15 minutes and
55ºC for 15 minutes) and control temperature regime.

e) Discussion for Set I:

As with the preceding data, maximum growth rates have been calculated for these experiments also.

Table 29: Maximum specific growth rates observed from the outflowing milk:

Time at 55°C (minutes)	Time at 35°C (minutes)	μ_{max}(control)	μ_{max} (Step-Change)	Decrease in μ_{max}
5	35	3.453	2.936	0.517
5	15	3.453	1.74	1.713
15	35	3.453	1.111	2.342
15	15	3.453	2.726	0.727

Table 30: Maximum specific growth rates recorded from the coupon samples:

Time at 55°C (minutes)	Time at 35°C (minutes)	μ_{max} (control)	μ_{max}(Step-Change)	Decrease in μ_{max}
5	35	4.083	2.697	1.386
5	15	3.342	2.629	0.713
15	35	2.802	1.898	0.904
15	15	3.295	3.062	0.233

From these data, the best regime would be the step change temperature of 35°C with the duration of step-change of 35 minutes and difference between two step-changes of 15 minutes during which the milk remains at a control temperature. The 5 minute and 15 minute regime also appears to make some difference at the same step change temperature of 35°C.

4.2b.SET II: A temperature of 30°C was used to provide a step-change in growth temperature to the bacteria

TRIAL 5:

In the fifth trial, the following time and temperature regime was used:

Temperature	Time (minutes)	No. of Trials	Duration of the run
55°C	5	1	24 hours
30°C	35		

For the log-counts observed for milk samples and coupon samples refer to Table 31 and 32 respectively in Appendix I (Section 2) after the end of the reference section.

Below are the scatterplots for milk samples (i) and coupon samples (ii) count results during the step-change and control conditions.

(i)
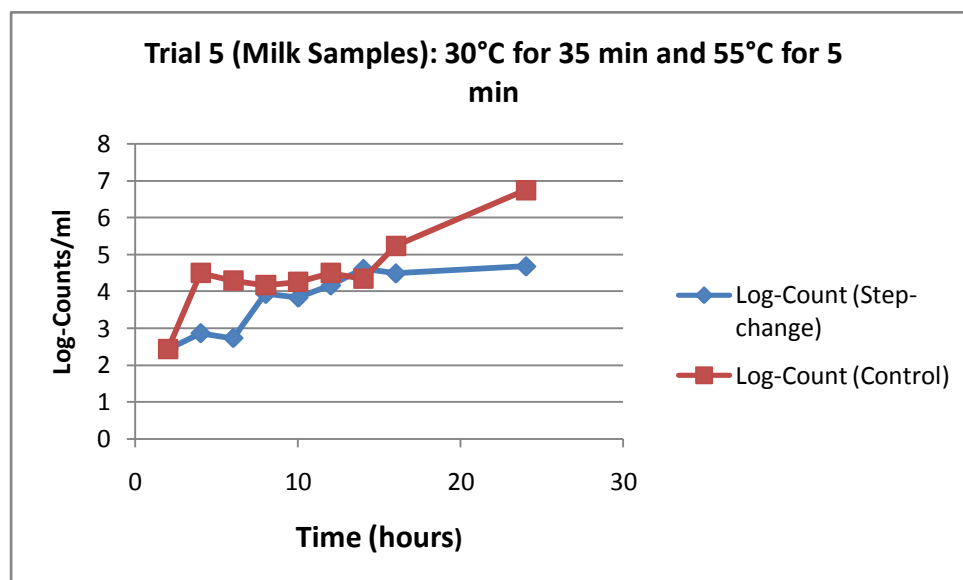
Figure 26: Scatterplot demonstrating the growth patterns of the bacteria in the outflowing milk when operated under step-change conditions (30°C for 35 minutes and 55°C for 5 minutes) and control temperature regime

(ii)
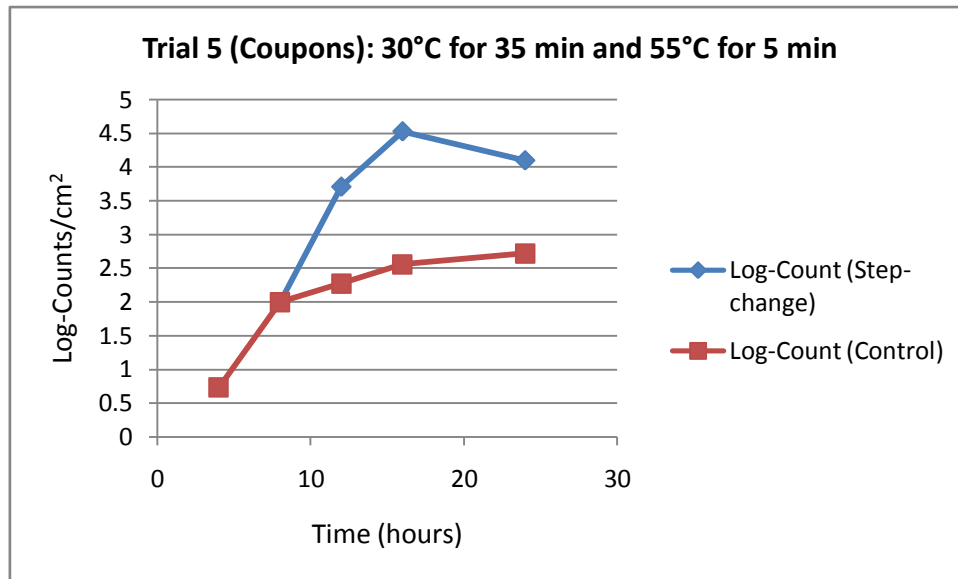
Figure 27: Scatterplot demonstrating the growth patterns of the bacteria for the coupon samples when operated under step-change conditions (30ºC for 35 minutes and 55ºC for 5 minutes) and control temperature regime.

b) TRIAL 6:

In this trial, the following time and temperature regime was used:

Temperature	Time (minutes)	No. of Trials	Duration of the run
55°C	5	1	24 hours
30°C	15		

For the log-counts observed for milk samples and coupon samples refer to Table 33 and 34 respectively in Appendix I (Section 2) after the end of the reference section.

Below are the scatterplots for milk samples (i) and coupon samples (ii) count results during the step-change and control conditions.

(i)
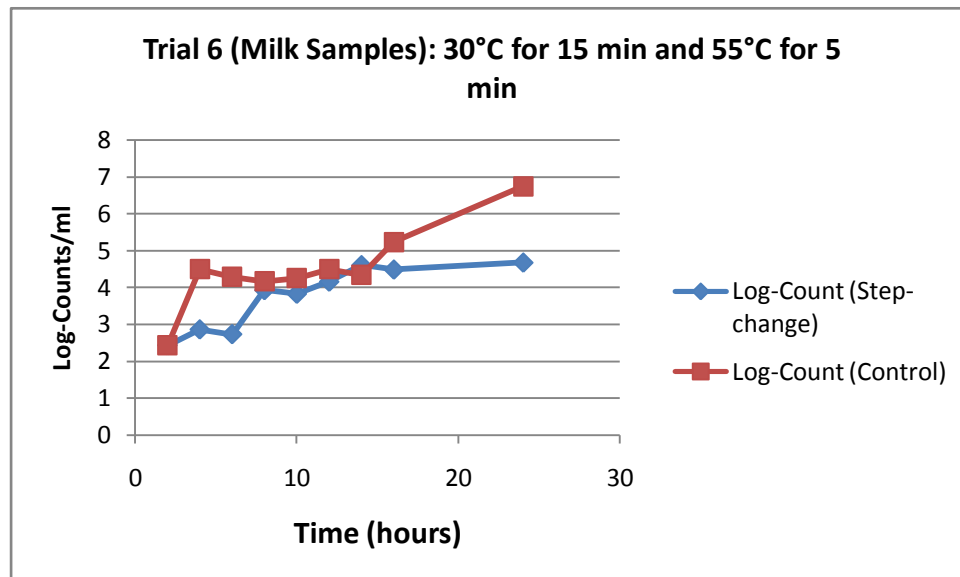
Figure 28: Scatterplot demonstrating the growth patterns of the bacteria in the outflowing milk when operated under step-change conditions (30ºC for 15 minutes and 55ºC for 5 minutes)and control temperature regime

(ii)
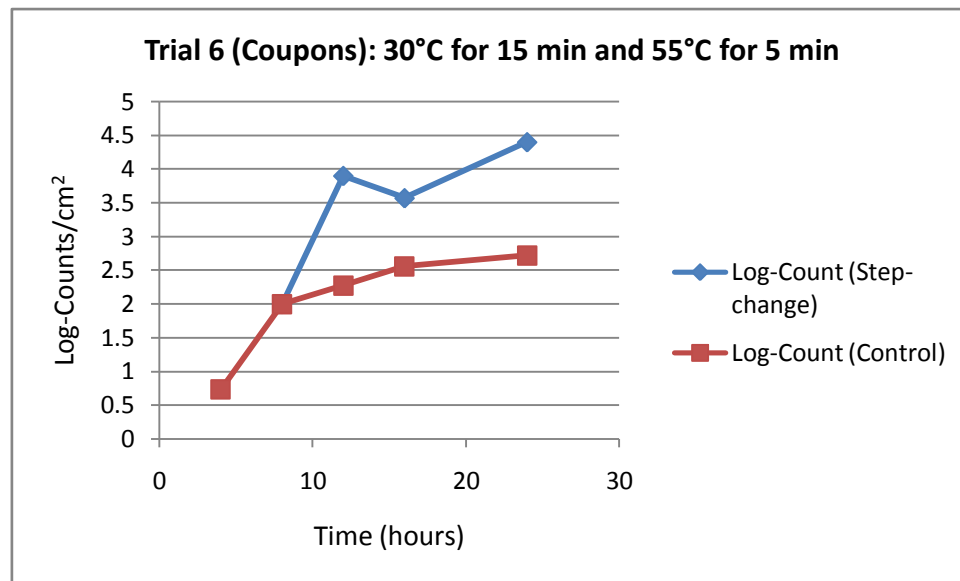
Figure 29: Scatterplot demonstrating the growth patterns of the bacteria for the coupon
samples when operated under step-change conditions (30ºC for 15 minutes and
55ºC for 5 minutes) and control temperature regime

c) TRIAL 7:

In the next trial, the following time and temperature regime was used:

Temperature	Time (minutes)	No. of Trials	Duration of the run
55°C	15	1	24 hours
30°C	35		

For the log-counts observed for milk samples and coupon samples refer to Table 35 and 36 respectively in Appendix I (Section 2) after the end of the reference section.

Below are the scatterplots for milk samples (i) and coupon samples (ii) count results during the step-change and control conditions.
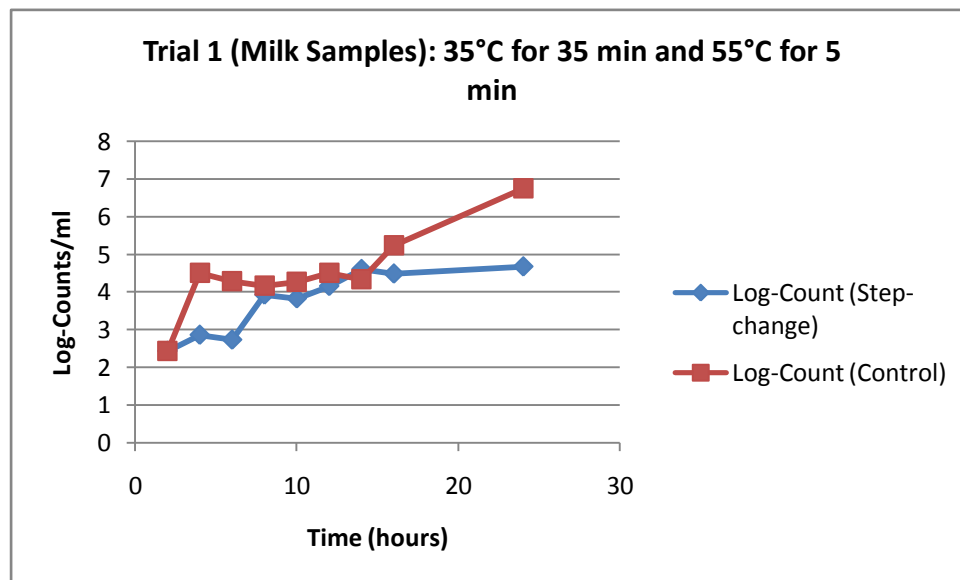
(i)

Figure 30: Scatterplot demonstrating the growth patterns of the bacteria in the outflowing milk when operated under step-change conditions (30ºC for 35 minutes and 55ºC for 15 minutes) and control temperature regime

(ii)

Trial 7 (Coupons): 30°C for 35 min and 55°C for 15 min
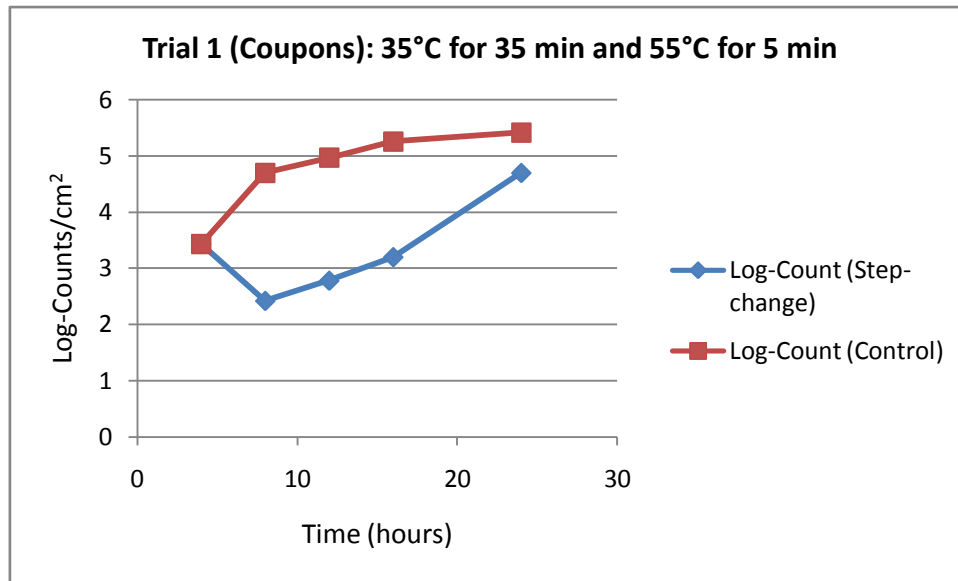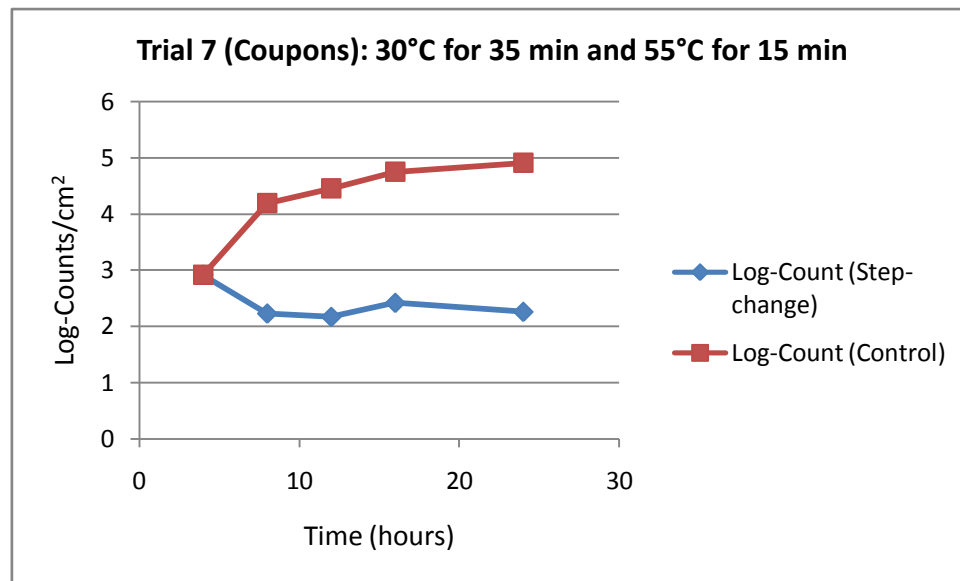
Figure 31: Scatterplot demonstrating the growth patterns of the bacteria for the coupon samples when operated under step-change conditions (30ºC for 35 minutes and 55ºC for 15 minutes) and control temperature regime

d) TRIAL 8:

In the last trial, the following time and temperature regime was used:

Temperature	Time (minutes)	No. of Trials	Duration of the run
55°C	15	1	24 hours
30°C	15		

For the log-counts observed for milk samples and coupon samples refer to Table 37 and 38 respectively in Appendix I (Section 2) after the end of the reference section.

Below are the scatterplots for milk samples (i) and coupon samples (ii) count results during the step-change and control conditions.
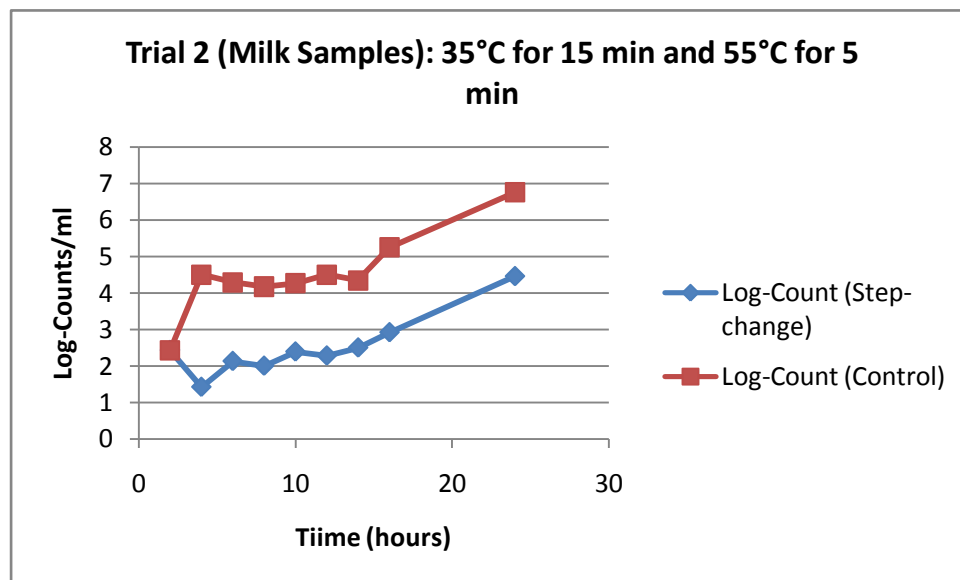
(i)
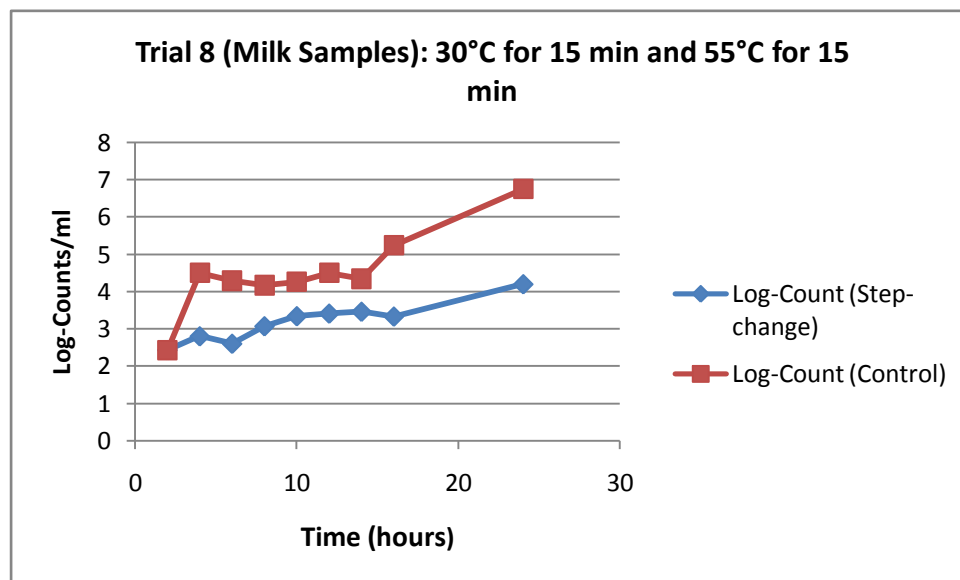
Figure 32: Scatterplot demonstrating the growth patterns of the bacteria in the outflowing milk when operated under step-change conditions (30ºC for 15 minutes and 55ºC for 15 min)and control temperature regime
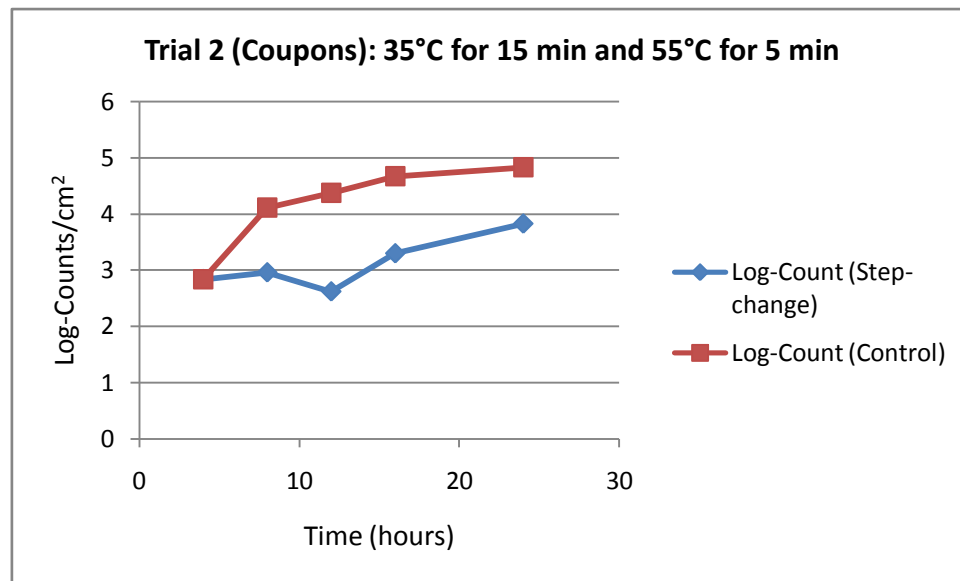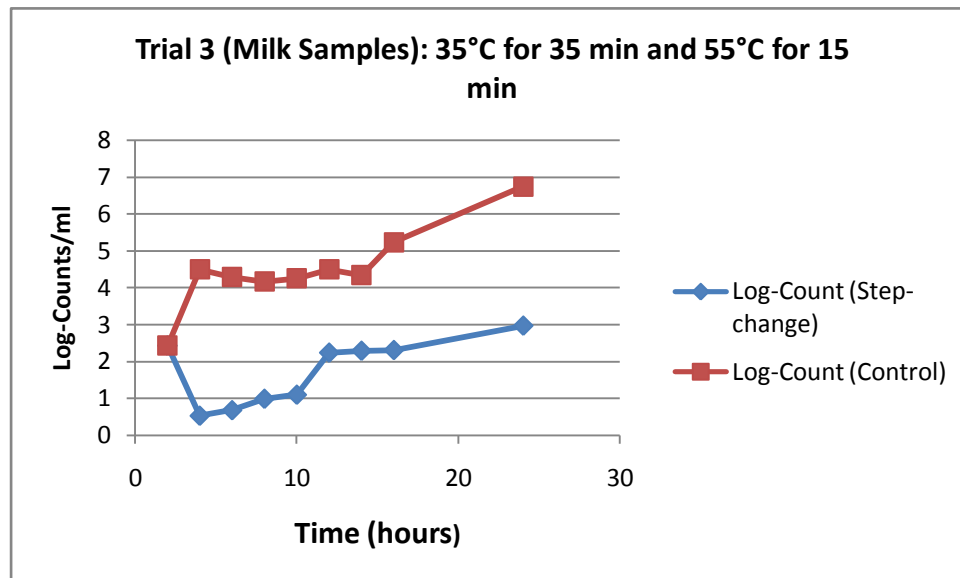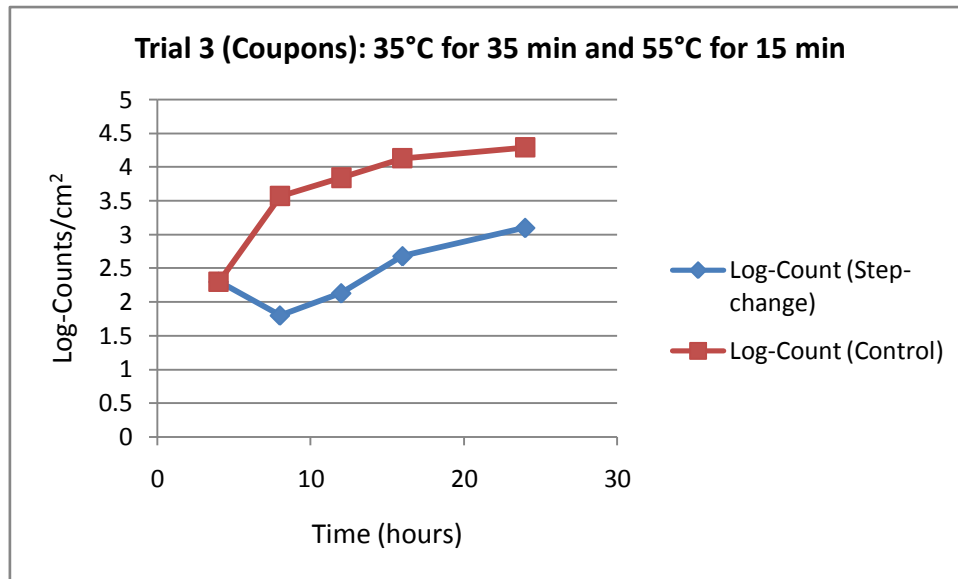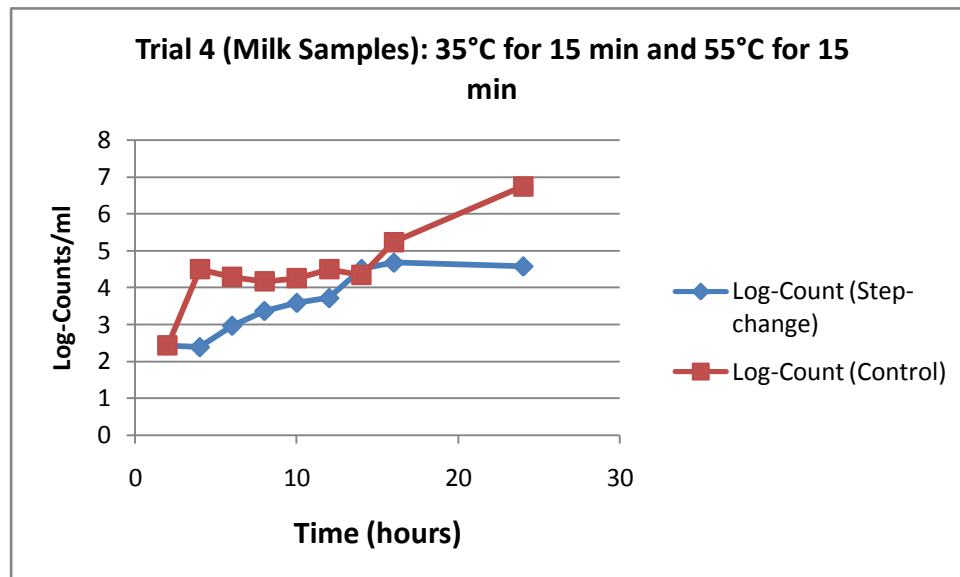
(ii)
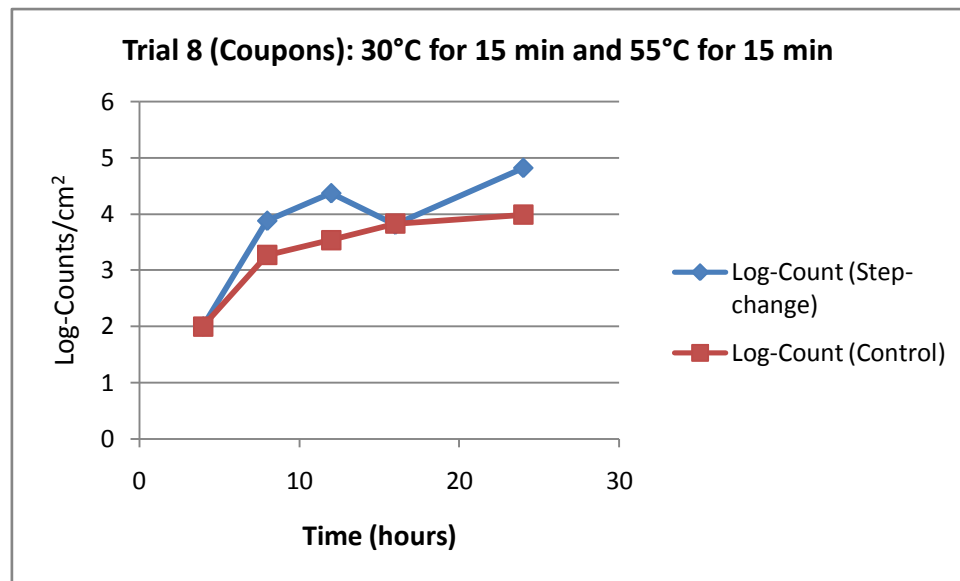
Figure 33: Scatterplot demonstrating the growth patterns of the bacteria for the coupon samples when operated under step-change conditions (30ºC for 15 minutes and 55ºC for 15 min) and control temperatureregime

e) Discussion for Set II:

This was the last set of experiments conducted. The μ_{max} calculated are tabulated in the tables below.

Table 39: Maximum specific growth rates (μ_{max}) recorded from the outflowing milk samples:

Time at 55°C (minutes)	Time at 30°C (minutes)	μ_{max}(control)	μ_{max}(Step-Change)	Decrease in μ_{max}
5	35	3.887	3.175	0.712
5	15	3.453	2.478	0.975
15	35	3.453	2.446	1.007
15	15	3.453	2.64	0.813

Table 40: Maximum specific growth rates (μ_{max}) observed in case of coupon samples:

Time at 55°C (minutes)	Time at 30°C (minutes)	μ_{max} (control)	μ_{max} (Step-Change)	Decrease in μ_{max}
5	35	1.534	2.643	-1.109
5	15	1.534	2.456	-0.922
15	35	2.71	3.271	-0.561
15	15	2.804	3.116	-0.312

In contrast to the data recorded from the first set of experiments with the non-sterile milk, these data show that the better step-change temperature would be 35°C rather than 30°C as was indicated by the previous set. However, the most noteworthy and unusual information provided by these data is the growth rates achieved during test runs for the step-change temperature of 30°C from the coupon samples. All the runs have higher growth rate than the control run. This may indicate that the step-change, instead of restricting the growth, encourages the bacteria to attach more strongly to the coupons, resulting in less sloughing in the output milk as is evident from the data. It must be remembered that the observed μ_{max} is actually the arithmetic sum of cell growth and cell biotransfer to the bulk milk.

These data suggest that the productivity i.e. cells sloughing off the surface of the coupons is decreased by the step-change treatment. In other words, the growth of the bacteria on the coupons might be the same as before but the capability or the preference of the bacterial cells to detach from the surface and enter the milk (product) stream has decreased. We might speculate this behaviour of the bacteria indicates that a step-change temperature of

30°C induces an environmental or biological change in or around the bacterial colonies that increases theircapacity to remain attached to the coupons and hence the planktonic counts in the outflowing milk are lower.

Chapter 5:

DISCUSSION

This project was started with the intention of determining if temperature spikes introduced within the growth range of the thermophilic bacterium, *Geobacillus stearothermophilus*can retard the growth as a biofilm. The various trials conducted using specified time and temperature regimes, in fact, show that the thermal cycling does make a difference. Although the concept of thermal cycling, first experimented by Knight et al., in 2004 involved thermoduric bacteria (*Streptococcus thermophilus*) and were designed for a cheese pasteurizer rather than a milk powder production line(Knight et al., 2004), those experiments served as a basis for this project.

The experiments were conducted using two different methods of inoculation. In the first method, the milk reservoir was inoculated with the bacterial culture, whereas in the second method, the inoculation was done in the reactor itself, with sterile milk passing through the reactor. In both scenarios, the growth rate of the biofilm on the stainless steel coupons and the concentration of bacteria in the output milk was measured. The former method represents the potential situation in an actual processingplant, but complicates the model by allowing growth in the pre-heater and consequent deposition of bacteria onto the coupons in the reactor. Both methods indicated a significant effect of step-changes in temperature on the growth rate of the bacteria, in agreement with the results achieved by Knight et al., in their research. By both the methods, it seems the step-change lowering of temperature from 55°C to 35°C produces the best result for the thermal cycling. The most significant results were achieved when the milk was maintained at 35°C for 35 minutes and at 55°C for 15 minutes. The results also indicated that reducing the time at the high temperature (i.e. 55°C) may decrease the effect of thermal cycling, as seen with the experiments wherein the milk was maintained at 55°C for 5 minutes with 35 minutes or 15 minutes at the lower temperature. The 35 minutes at 35°C and 15 minutes at 55°C regime proved useful in case of both the outflowing milk samples and the coupon samples which indicate that the microbial count of the outflow from the heated section can be reduced, and the growth of biofilms on the heated surfaces can also be limited by the spiking treatment. However, there is some disadvantage, in that the average temperature of the outflow is less than in the non-spiked process.

Some interesting results were obtained for the trials where the reactor was inoculated.It appeared that the growth rate of the biofilm was actually greater than in the control experiment, while the outflowing milk samples produced the now expected resultof more growth in the control trial as compared to the step-change trials. After careful

discussion and verification of the data, it was concluded that in these experiments the introduction of step changes seemed to encourage the retention of bacteria on the stainless steel surface; consequently, there is less release or sloughing of bacteria from the surface into the bulk milk stream. This was indeed an unexpected outcome and could definitely be studied further in future research.

A possible explanation for the decrease in the growth of the bacteria in their study was given by Knight et al. According to them, the introduction of step-change in the growth regions of the bacteria can possibly interfere with the development of biofilms in the pilot plant. This can occur either via disrupting the exponential growth phase of the target micro-organism through causing cesation of growth, inducing a lag phase or leading to cell death and/or disrupting the various stages of biofilm formation, including prevention of cell attachment and promotion of detachment (Knight et al., 2004).

Knight et al concluded from further examination of the three step-change variables i.e., the step-change temperature, duration of the step-change and the time interval between two consecutive step-changes were of equal importance in achieving optimum and desirable results from a step-change cycle. Their study also provided substantial evidence of step-change cycles causing damage within the adhering cells and/or detachment from the attachment surfaces along with limiting the exponential growth.

In the present study, it appears that detatchment was not promoted by the step change, but that the net result was a decrease in the number of cells being released into the bulk milk. Further study of this phenomenon, and the physiological effects of step changes, is warranted.

5.1. Statistical Analysis:

One of the objectives of the project was to attempt to extend the time of thecustomary production runs in the evaporator during the milk powder manufacturing process. To comply with customer specifications for maximum acceptable levels of thermophilic spores in milk powder, plants are shut down for cleaning earlier than would be the case if fouling of heat exchange surfaces were the criterion (Hinton et al., 2002). The milk powder industry usually shuts down the evaporators after 18-20 hours, primarily because of the growth of thermophiles as biofilms that result in contamination of the bulk milk stream. If this could be avoided, the run time could be extended until it were necessary to shut down for cleaning to remove the burned on protein (Brooks, 2013). This premature shutdown causes increased cost of production and labour and decreased product output (Brooks & Flint, 2008). So, the

project aimed to reduce the growth of the bacteria and thus produce powder within permissible limits until the evaporators are turned offline for the routine cleaning. Hence, the experiments were designed to run for 24 hours, during which the bacteria failed to reach a stationary phase.

Techniques such as ANOVA were inapplicable, because the observations were not independent, one of the preliminary assumption for various statistical hypothesis testing mechanisms (Hankin, 2013).

5.2. Future Research Potential:

Further work might involve carrying the growth runs beyond 24 hours and achieving a stationary phase suitable to fit a logistic growth curve. The fitted logistic growth curve could then be useful in important statistical hypothesis testing. The work could also include performing multiple replicates for each experiment to obtain an independent value for each sample taken throughout the experiment to enable us to conduct statistical tests like ANOVA and factorial design to prove there is a statistically significant difference between the treatments.

5.3. Industrial Application:

Initially, the aim of this project was to investigate the effect of thermal cycling on the thermophilic bacterium, *G. stearothermophilus*. The data collected has given a firm indication that, if applied in a specific time and temperature regime, the step-change phenomenon can help to deal with the higher than permissible concentrations of thermophiles that exist in the milk powder. Within the limits of this project, the time and temperature regime of 35°C for 35 minutes and 15 minutes for 55°C seems to be the best option for a pilot-scale industrial experiment. The better operating combination of time and temperature was selected on the basis of minimum growth bothin terms of biofouling of coupon surfaces and planktonic count of the outflowing milk during step-change trials.

One possible application for the step-changes might be to introduce the technology in the separator section of the production line, where the temperatures are often maintained at 52°C-55°C. Hot separation is more efficient than cold separation, which can alsobe conducted at 8–15°C, but is vulnerable to thermophile growth. The use of temperature cycling in this stage could reduce biofilm formation and growth, while allowing more efficient separation. The time and temperature regime recommended here may also be

applied to the plate heat exchanger in the preheating section. If the technology were applied here, the average temperature of the milk leaving the PHE will, of course, be reduced, compared with a non-cycled process. This will result in a loss of efficiency, which must be balanced against the reduction in biofilm growth on the plates, and thus the number of thermophiles shed into the product stream.

Thermophilic bacilli are also potential contaminants in a variety of other industries where elevated temperatures (40–65 °C) predominate during the manufacturing process as well as when the fluid product is stored; these industries include paper mills, canning, juice pasteurisation, sugar refining, gelatin production and dehydrated vegetable manufacture (S. Chen et al., 2006; De Clerck, Rodriguez-Diaz, Forsyth, et al., 2004; De Clerck, Rodriguez-Diaz, Vanhoutte, et al., 2004; De Clerck, Vanhoutte, et al., 2004; Denny, 1981; Hayes, 1985; Jay, Loessner, & Golden, 2005; Scott et al., 2007; Splittstoesser, Lee, & Churey, 1998; Suihko et al., 2004; Tai, Lin, Kuo, & Liu, 2004). To control the growth of thermophiles to increase productivity and reduce potential losses, the thermal cycling process devised in this project can also be applied on an experimental basis in these aforementioned industries.

Chapter 6:

CONCLUSION

The objective of this project was to determine whether the technique of thermal cycling is effective in reducing the growth rates of thermophilic bacteria. The test bacterium selected for the project was *G. stearothermophilus,* which is a recurring contaminant in the milk powder manufacturing plant. The data collected during the course of the project strongly indicate that introduction of step-changes within the growth temperature range of *G. stearothermophilus* can decrease the growth rate of the bacteria. As a recommendation derived from the analysis of the data, a step-change temperature of 35°C applied for 35 min with 15 min interval (wherein the temperature rises back to 55°C) between two step changes is the best combination for conducting a pilot-scale experiment in the separator or preheater stagesin milk powder production.

REFERENCES

Ash, C., Farrow, J. A. E., Wallbanks, S., & Collins, M. D. (1991). Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. *Letters in Applied Microbiology, 13*(4), 202-206.

Austin, J. W., & Bergeron, G. (1995). Development of bacterial biofilms in dairy processing lines. *Journal of Dairy Research, 62*(3), 509-519.

Basappa, P., Shroff, D. N., & Srinivasan, R. A. (1974). Growth characteristics of thermophilic micro-organisms in milk. *Indian Journal of Dairy Science, 27*, 103-108.

Bouman, S., Lund, D. B., Driessen, F. M., & Schmidt, D. G. (1982). Growth of thermoresistant *Streptococci* and deposition of milk constituents on plates of heat exchangers during long operating times [*Streptococcus thermophilus*]. *Journal of Food Protection, 45*.

Brooks, J. D. (2013).

Brooks, J. D., & Flint, S. H. (2008). Biofilms in the food industry: problems and potential solutions. *International Journal of Food Science & Technology, 43*(12), 2163-2176. doi: 10.1111/j.1365-2621.2008.01839.x

Burgess, S. A., Brooks, J. D., Rakonjac, J., Walker, K. M., & Flint, S. H. (2009). The formation of spores in biofilms of *Anoxybacillus flavithermus*. *Journal of applied microbiology, 107*(3), 1012-1018.

Burgess, S. A., Lindsay, D., & Flint, S. H. (2010). Thermophilic bacilli and their importance in dairy processing. *International journal of food microbiology, 144*(2), 215-225.

Carpentier, B., Wong, A. C. L., & Cerf, O. (1998). Biofilms on dairy plant surfaces: What's new? *International Dairy Federation*.

Chen, Coolbear, T., & Daniel, R. M. (2004). Characteristics of proteinases and lipases produced by seven *Bacillus* sp. isolated from milk powder production lines. *International dairy journal, 14*(6), 495-504.

Chen, S., Tang, Q., Zhang, X., Zhao, G., Hu, X., Liao, X., . . . Xiang, H. (2006). Isolation and characterization of thermo-acidophilic endospore-forming bacteria from the concentrated apple juice-processing environment. *Food microbiology, 23*(5), 439-445.

Chopra, A. K., & Mathur, D. K. (1984). Isolation, screening and characterization of thermophilic Bacillus species isolated from dairy products. *Journal of applied microbiology, 57*(2), 263-271.

Cosentino, S., Mulargia, A. F., Pisano, B., Tuveri, P., & Palmas, F. (1997). Incidence and biochemical characteristics of *Bacillus* flora in Sardinian dairy products. *International journal of food microbiology, 38*(2), 235-238.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., & Lappin-Scott, H. M. (1995). Microbial biofilms. *Annual Reviews in Microbiology, 49*(1), 711-745.

Criado, M.-T., Suarez, B., & Ferreiros, C. M. (1994). The importance of bacterial adhesion in the dairy industry. *Food technology, 48*.

Czechowski, M. H., & Rapp, C. (1990). Cleaning and sanitation. *Australian journal of dairy technology, 45*(1), 38-39.

De Clerck, E., Rodriguez-Diaz, M., Forsyth, G., Lebbe, L., Logan, N. A., & DeVos, P. (2004). Polyphasic Characterization of *Bacillus coagulans* Strains, Illustrating Heterogeneity within this Species, and Emended Description of the Species. *Systematic and applied microbiology, 27*(1), 50-60.

De Clerck, E., Rodriguez-Diaz, M., Vanhoutte, T., Heyrman, J., Logan, N. A., & De Vos, P. (2004). *Anoxybacillus contaminans sp. nov*. and *Bacillus gelatini sp. nov*., isolated from contaminated gelatin batches. *International journal of systematic and evolutionary microbiology, 54*(3), 941-946.

De Clerck, E., Vanhoutte, T., Hebb, T., Geerinck, J., Devos, J., & De Vos, P. (2004). Isolation, characterization, and identification of bacterial contaminants in semifinal gelatin extracts. *Applied and environmental microbiology, 70*(6), 3664-3672.

Deak, T., & Timár, É. (1988). Simplified identification of aerobic spore-formers in the investigation of foods. *International journal of food microbiology, 6*(2), 115-125.

Denny, C. B. (1981). Thermophilic organisms involved in food spoilage: Introduction. *Journal of Food Protection, 44*, 144-145.

Driessen, F. M., & Bouman, S. (1979). Growth of thermoresistant streptococci in cheese milk pasteurizers—trials with a model pasteurizer. *Zuivelzicht, 71*, 1062-1064.

Escher, A., & Characklis, W. G. (1990). Modeling the initial events in biofilm accumulation. *Biofilms*, 445-486.

Flint, S. H., Bremer, P. J., & Brooks, J. D. (1997). Biofilms in dairy manufacturing plant-description, current concerns and methods of control. *Biofouling, 11*(1), 81-97.

Flint, S. H., & Brooks, J. D. (2001). Rapid detection of *Bacillus stearothermophilus* using impedance-splitting. *Journal of Microbiological Methods, 44*(3), 205-208.

Flint, S. H., Palmer, J., Bloemen, K., Brooks, J. D., & Crawford, R. (2001). The growth of *Bacillus stearothermophilus* on stainless steel. *Journal of applied microbiology, 90*(2), 151-157.

Goto, K., Fujita, R., Kato, Y., Asahara, M., & . (2004). Reclassification of *Brevibacillus brevis* strains NCIMB 13288 and DSM 6472 (= NRRL NRS-887) as *Aneurinibacillus danicus* sp. nov. and *Brevibacillus limnophilus* sp. nov. *International Journal of Systematic and Eolutionary Microbiology, 54*(2), 419-427.

Gundogan, N., & Arik, M. T. (2004). Comparison of the protease activity of psychrotrophic and thermophilic *Bacillus* spp. isolated from raw milk samples. *Indian veterinary journal, 81*(9), 1013-1015.

Hankin, R. (2013). [Further statistical analysis].

Hartley, N. J., & Flint, S. H. (1993). An investigation to reduce the contamination of whey protein concentrate with thermophilic bacteria. New Zealand: New Zealand Dairy Institute.

Hayes, P. R. (1985). Quality assurance and production control *Food Microbiology and Hygiene* (pp. 255). London: Elsevier Applied Science Publishers.

Heyndrickx, M., Lebbe, L., Kersters, K., De Vos, P., Forsyth, G., & Logan, N. A. (1998). *Virgibacillus*: a new genus to accommodate *Bacillus pantothenticus* (Proom and Knight 1950). Amended description of *Virgibacillus pantothenticus*. *International journal of systematic bacteriology, 48*(1), 99-106.

Heyndrickx, M., Lebbe, L., Vancanneyt, M., Kersters, K., De Vos, P., Logan, N. A., . . . Berkeley, R. C. W. (1997). A Polyphasic reassessment of the Genus *Aneurinibacillus*, reclassification of *Bacillus thermoaerophilus* (Meier-Stauffer et al. 1996) as *Aneurinibacillus thermoaerophilus* comb, nov., and amended descriptions of *A. aneurinilyticus* corrig., *A. migulanus*, and *A. thermoaerophilus*. *International journal of systematic bacteriology, 47*(3), 808-817.

Heyndrickx, M., Vandemeulebroecke, K., Hoste, B., Janssen, P., Kersters, K., De Vos, P., . . . Berkeley, R. C. W. (1996). Reclassification of *Paenibacillus* (formerly *Bacillus*)

pulvifaciens (Nakamura 1984) Ash et al. 1994, a later subjective synonym of *Paenibacillus* (formerly *Bacillus*) larvae (White 1906) Ash et al. 1994, as a subspecies of *P. larvae*, with amended descriptions of *P. larvae* as *P. larvae* subsp. larvae and *P. larvae* subsp. *pulvifaciens*. *International journal of systematic bacteriology, 46*(1), 270-279.

Heyrman, J., Logan, N. A., Busse, H.-J., Balcaen, A., Lebbe, L., Rodriguez-Diaz, M., . . . De Vos, P. (2003). *Virgibacillus carmonensis* sp. nov., *Virgibacillus necropolis* sp. nov. and *Virgibacillus picturae* sp. nov., three novel species isolated from deteriorated mural paintings, transfer of the species of the genus *Salibacillus* to *Virgibacillus*, as *Virgibacillus marismortui* comb. nov. and *Virgibacillus salexigens* comb. nov., and amended description of the genus *Virgibacillus*. *International journal of systematic and evolutionary microbiology, 53*(2), 501-511.

Hill, B. M. (2004). *Thermophilic spores: UHT and retort processing of recombined milks.* Paper presented at the Proceedings of the 4th International Symposium on Recombined Milk and Milk Products, US Dairy Export Council, Cancun, Mexico.

Hill, B. M., & Smythe, B. W. (1994). Progress in understanding the behaviour of thermophilic bacteria during milk powder manufacture. *Milk Powders for the Future II*, 19-26.

Hinton, A. R., Trinh, K. T., Brooks, J. D., & Manderson, G. J. (2002). Thermophile Survival in Milk Fouling and on Stainless Steel During Cleaning. *Food and Bioproducts Processing, 80*(4), 299-304. doi: http://dx.doi.org/10.1205/096030802321154817

Hoffman, W. (1983). *CIP of straight pipes as a function of surface roughness and other factors.* (Doctor of Philosphy), Christian-Abrechis University, Kiel.

Hup, G., & Stadhouders, J. (1979). Growth of thermoresistant S*treptococci* in cheese-milk pasteurizers. *Zuivelzieht, 19*(7), 9.

Ito, K. A. (1981). Thermophilic organisms in food spoilage: flat-sour aerobes. *Journal of Food Protection, 44*(2), 157.

Jay, J. M. (2003). A review of recent taxonomic changes in seven genera of bacteria commonly found in foods. *Journal of Food Protection, 66*(7), 1304-1309.

Jay, J. M., Loessner, M. J., & Golden, D. A. (2005). Food protection with high temperatures, and characteristics of thermophilic microorganisms. *Modern Food Microbiology*, 415-441.

Jonhstone, K., Ellar, D. J., & Appleton, T. C. (1980). Location of metal ions in *Bacillus megaterium* spores by high-resolution electron probe X-ray microanalysis. *FEMS Microbiology Letters, 7*(2), 97-101.

Kalogridou-Vassiliadou, D. (1992). Biochemical Activities of *Bacillus* Species Isolated from flat sour evaporated milk. *Journal of Dairy Science, 75*(10), 2681-2686.

Kirtley, S. A., & McGuire, J. (1989). On differences in surface constitution of dairy product contact materials. *Journal of Dairy Science, 72*(7), 1748-1753.

Knight, G. C., Nicol, R. S., & McMeekin, T. A. (2004). Temperature step changes: a novel approach to control biofilms of *Streptococcus thermophilus* in a pilot plant-scale cheese-milk pasteurisation plant. *International journal of food microbiology, 93*(3), 305-318.

Koutzayiotis, C. (1992). Bacterial biofilms in milk pipelines. *South African Journal of Dairy Science, 24*, 19.

Langeveld, L. P. M., Bolle, A. C., & Vegter, J. E. (1972). The cleanability of stainless steel with different degrees of surface roughness. *Netherland Milk Dairy Journal, 26*, 149-154.

Langeveld, L. P. M., van Montfort-Quasig, R. M. G. E., Waalewijn, R., & Wever, J. S. (1996). *Adherence, growth and release of bacteria in a tube heat exchanger for milk*: European Commission.

Lewis, S. (2003). *Optimal Separation Temperature.* (Master of Dairy Science and Technology), Massey University, Palmerston North, New Zealand.

Lin, H.-P. P., Tai, S. K., Kuo, J., & Liu, J.-K. (2004). Isolation and characterization of a cellulolytic *Geobacillus thermoleovorans* T4 strain from sugar refinery wastewater. *Extremophiles, 8*(5), 345-349.

Marchant, R., Banat, I. M., Rahman, T. J., & Berzano, M. (2002). The frequency and characteristics of highly thermophilic bacteria in cool soil environments. *Environmental microbiology, 4*(10), 595-602.

Maxcy, R. B. (1973). Nature and cause of yellow film occurring on dairy equipment. *Journal of Dairy Science, 56*(2), 164-167.

McGuiggan, J., McCleery, D. R., Hannan, A., & Gilmour, A. (2002). Aerobic spore-forming bacteria in bulk raw milk: factors influencing the numbers of psychrotrophic, mesophilic and thermophilic Bacillus spores. *International journal of dairy technology, 55*(2), 100-107.

Mosteller, T. M., & Bishop, J. R. (1993). Sanitizer efficacy against attached bacteria in a milk biofilm. *Journal of Food Protection, 56*.

Muir, D. D., Griffiths, M. W., Phillips, J. D., Sweetsur, A. W. M., & West, I. G. (1986). Effect of the bacterial quality of raw milk on the bacterial quality and some other properties of low-heat and high-heat dried milk. *International journal of dairy technology, 39*(4), 115-118.

Murphy, P. M., Lynch, D., & Kelly, P. M. (1999). Growth of thermophilic spore forming bacilli in milk during the manufacture of low heat powders. *International journal of dairy technology, 52*(2), 45-50.

Murugan, B., & Villi, R. A. (2009). Lipolytic activity of *Bacillus* species isolated from milk and dairy products. *Indian veterinary journal, 86*(1), 80-81.

Nazina, T. N., Tourova, T. P., Poltaraus, A. B., Novikova, E. V., Grigoryan, A. A., Ivanova, A. E., . . . Belyaev, S. S. (2001). Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus,Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus, G. thermocatenulatus, G. thermoleovorans, G. kaustophilus, G. thermodenitrificans. International journal of systematic and evolutionary microbiology, 51*(2), 433-446.

Notermans, S. (1994). The significance of biofouling to the food industry. *Food technology, 48*(7), 13-14.

Parkar, S. G., Flint, S. H., & Brooks, J. D. (2004). Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel. *Journal of applied microbiology, 96*(1), 110-116.

Parkar, S. G., Flint, S. H., Palmer, J. S., & Brooks, J. D. (2001). Factors influencing attachment of thermophilic bacilli to stainless steel. *Journal of applied microbiology, 90*(6), 901-908.

Rainey, F. A., Fritze, D., & Stackebrandt, E. (1994). The phylogenetic diversity of thermophilic members of the genus *Bacillus* as revealed by 16S rDNA analysis. *FEMS Microbiology Letters, 115*(2), 205-211.

Reddy, P. C., Atwal, J. S., & Srinivasan, R. A. (1975). Studies on the survival of thermophilic bacteria during manufacture and storage in dried milks. *Indian Journal of Dairy Science, 28*, 289-295.

Ren, P.-G., & Zhou, P.-J. (2005). *Tenuibacillus multivorans* gen. nov., sp. nov., a moderately halophilic bacterium isolated from saline soil in Xin-Jiang, China. *International journal of systematic and evolutionary microbiology, 55*(1), 95-99.

Romney, A. J. D. (1990). *CIP: cleaning in place*: Society of Dairy Technology.

Ronimus, R. S., Parker, L. E., Turner, N., Poudel, S., Rückert, A., & Morgan, H. W. (2003). A RAPD-based comparison of thermophilic bacilli from milk powders. *International journal of food microbiology, 85*(1), 45-61.

Rössler, D., Ludwig, W., Schleifer, K. H., Lin, C., Mcgill, T. J., Wisotzkey, J. D., . . . Fox, G. E. (1991). Phylogenetic diversity in the genus *Bacillus* as seen by 16s rRNA sequencing studies. *Systematic and applied microbiology, 14*(3), 266-269.

Scott, S. A., Brooks, J. D., Rakonjac, J., Walker, K. M. R., & Flint, S. H. (2007). The formation of thermophilic spores during the manufacture of whole milk powder. *International journal of dairy technology, 60*(2), 109-117.

Seale, R. B., Flint, S. H., McQuillan, A. J., & Bremer, P. J. (2008). Recovery of spores from thermophilic dairy bacilli and effects of their surface characteristics on attachment to different surfaces. *Applied and environmental microbiology, 74*(3), 731-737.

Shida, O., Takagi, H., Kadowaki, K., & Komagata, K. (1996). Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. *International journal of systematic bacteriology, 46*(4), 939-946.

Sneath, P. H. A. (1986). Endospore-forming Gram-positive rods and cocci. In P. H. A. e. a. Sneath (Ed.), *Bergey's Manual of Systematic Bacteriology* (pp. 1104-1207). Baltimore, United States of America: Williams & Wilkins.

Somers, E. B., Johnson, M. E., & Wong, A. C. L. (2001). Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in the dairy environment. *Journal of Dairy Science, 84*(9), 1926-1936.

Speers, J. G. S., & Gilmour, A. (1985). The influence of milk and milk components on the attachment of bacteria to farm dairy equipment surfaces. *Journal of applied microbiology, 59*(4), 325-332.

Splittstoesser, D. F., Lee, C. Y., & Churey, J. J. (1998). Control of *Alicyclobacillus* in the juice industry. *Dairy, food and environmental sanitation: a publication of the International Association of Milk, Food and Environmental Sanitarians, 18*.

Stadhouders, J., Hup, G., & Hassing, F. (1982). The conceptions index and indicator organisms discussed on the basis of the bacteriology of spray-dried milk powder. *Netherlands Milk and Dairy Journal, 36*.

Stoodley, P., Lewandowski, Z., Boyle, J. D., & Lappin-Scott, H. M. (1999). The formation of migratory ripples in a mixed species bacterial biofilm growing in turbulent flow. *Environmental microbiology, 1*(5), 447-455.

Suárez, B., Ferreirós, C. M., & Criado, M.-T. (1992). Adherence of psychrotrophic bacteria to dairy equipment surfaces. *Journal of Dairy Research, 59*(03), 381-388.

Suihko, M. L., Sinkko, H., Partanen, L., Mattila-Sandholm, T., Salkinoja-Salonen, M., & Raaska, L. (2004). Description of heterotrophic bacteria occurring in paper mills and paper products. *Journal of applied microbiology, 97*(6), 1228-1235.

Sutherland, P. (1995). Bacterial biofilms - a sticky and expensive problem. *Australian Dairy Foods*.

Tai, S., Lin, H. P., Kuo, J., & Liu, J. (2004). Isolation and characterization of a cellulolytic *Geobacillus thermoleovorans* T4 strain from sugar refinery wastewater. *Extremophiles, 8*(5), 345-349.

Takami, H., Nishi, S., Lu, J., Shimamura, S., & Takaki, Y. (2004). Genomic characterization of thermophilic *Geobacillus* species isolated from the deepest sea mud of the Marina Trench. *Extremophiles, 8*(5), 351-356.

Van der Mei, H. C., De Vries, J., & Busscher, H. J. (1993). Hydrophobic and electrostatic cell surface properties of thermophilic dairy *Streptococci*. *Applied and environmental microbiology, 59*(12), 4305-4312.

Wainø, M., Tindall, B. J., Schumann, P., & Ingvorsen, K. (1999). *Gracilibacillus* gen. nov., with description of *Gracilibacillus halotolerans* gen. nov., sp. nov.; transfer of *Bacillus dipsosauri* to *Gracilibacillus dipsosauri* comb. nov., and *Bacillus salexigens* to the genus *Salibacillus* gen. nov., as *Salibacillus salexigens* comb. nov. *International journal of systematic bacteriology, 49*(2), 821-831.

Warnecke, F. (2001). *The ecology of thermophilic bacilli of milk powder processing plants.* University of Waikato.

Wirtanen, G., Husmark, U., & Mattila-Sandholm, T. (1996). Microbial evaluation of the biotransfer potential from surfaces with *Bacillus* biofilms after rinsing and cleaning procedures in closed food-processing systems. *Journal of Food Protection, 59*(7), 727-733.

Wirtanen, G., & Mattila-Sandholm, T. (1992). Removal of foodborne biofilms: comparison of surface and suspension tests. I. *Food Science and Technology, 25*(1), 43-49.

Wisotzkey, J. D., Jurtshuk, P., Fox, G. E., Deinhard, G., & Poralla, K. (1992). Comparative sequence analyses on the 16S rRNA (rDNA) of *Bacillus acidocaldarius,Bacillus acidoterrestris*, and *Bacillus cycloheptanicus* and proposal for creation of a new Genus, *Alicyclobacillus* gen. nov. *International journal of systematic bacteriology, 42*(2), 263-269.

Yoon, J.-H., Kang, K.-H., & Park, Y.-H. (2002). *Lentibacillus salicampi* gen. nov., sp. nov., a moderately halophilic bacterium isolated from a salt field in Korea. *International journal of systematic and evolutionary microbiology, 52*(6), 2043-2048.

Yoon, J.-H., Oh, T.-K., & Park, Y.-H. (2004). Transfer of *Bacillus halodenitrificans* (Denariaz et al. 1989) to the genus *Virgibacillus* as *Virgibacillus halodenitrificans* comb. nov. *International journal of systematic and evolutionary microbiology, 54*(6), 2163-2167.

Zarilla, K. A., & Perry, J. J. (1987). *Bacillus thermokovorans*, sp. nov., a Species of Obligately Thermophilic Hydrocarbon Utilizing Endospore-forming Bacteria. *Systematic and applied microbiology, 9*(3), 258-264.

Zottola, E. A., & Sasahara, K. C. (1994). Microbial biofilms in the food processing industry—Should they be a concern? *International journal of food microbiology, 23*(2), 125-148.

APPENDIX 1

8.1:Tabulated data for 1ˢᵗ method of inoculation (Inoculation in the Milk):

8.1a. Following are the tables for log-counts observed when the step-change temperature was 30°C. All the log-counts have been normalized for the ease of plotting scatterplots and deducing inferences. Symbol "-" has been used to indicate where no growth was detected on the medium plates.

Table 3: The normalized log-counts for milk samples when the experiment was run at 55°C for 5 minutes and 30°C for 15 minutes

Time (hours)	Log-Count (Step-change)	Log-Count -Duplicate (Step-change)	Log-Count (Control)
0	2.72	2.72	2.72
2	2.11	2.11	3.77
4	2.11	2.33	4.18
6	2.45	3.15	4.47
8	3.31	3.38	4.71
10	3.46	3.61	4.8
12	3.92	4.13	4.97
14	4.05	4.55	5.03
16	5.58	5.58	6.27
24	5.3	5.31	6.35

Table 4: The normalized log-counts for coupons when the experiment was run at 55°C for 5 minutes and 30°C for 15 minutes

Time (hours)	Log-Count (Step-change)	Log-Count -Duplicate (Step-change)	Log-Count (Control)
4	-	-	3.24
8	-	-	3.87
12	4.3	4.3	4.3
16	3.86	4.45	6.73
24	4.69	5.1	7.71

Table 5: The normalized log-counts for milk samples when the experiment was run at 55°C for 15 minutes and 30°C for 35 minutes

Time (hours)	Log-Count (Step-change)	Log-Count -Duplicate (Step-change)	Log-Count (Control)
0	2.48	2.48	2.48
2	2.1	1.04	3.53
4	1.3	1.22	3.94
6	1.6	1.33	4.23
8	1.43	1.72	4.47
10	3.16	2.92	4.56
12	3.6	3.03	4.73
14	3	2.77	4.79
16	2.7	2.32	6.03
24	2.3	2.07	6.11

Table 6: The normalized log-counts for coupons when the experiment was run at 55°C for 15 minutes and 30°C for 35 minutes

Time	Log-Count (Step-Change)	Log-Count Duplicate (Step-Change)	Log-Count (Control)
4	-	-	1.72
8	-	-	2.35
12	3.04	3.04	3.04
16	2.98	3.18	5.47
24	2.74	3.06	6.45

Table 7: The normalized log-counts for milk samples when the experiment was run at 55°C for 15 minutes and 30°C for 15 minutes

Time (hours)	Log-Count (Step-change)	Log-Count -Duplicate (Step-change)	Log-Count (Control)
0	1.62	1.62	1.62
2	2.65	2.68	2.67
4	2.92	3	3.08
6	3.52	3.24	3.37
8	3.29	3.4	3.61
10	3.41	3.5	3.7
12	3.63	3.83	3.87
14	3.68	3.9	3.93
16	4.5	4.72	5.17
24	4.42	4.6	5.25

Table 8: The normalized log-counts for coupons when the experiment was run at 55°C for 15 minutes and 30°C for 15 minutes

Time	Log-Count (Step-change)	Log-Count -Duplicate (Step-change)	Log-Count (Control)
4	3.61	3.61	3.61
8	4.09	4.11	4.24
12	4.21	4.36	4.93
16	3.43	4.14	7.36
24	4.55	4.57	8.34

8.1b. Below are the tables for log-counts observed when the step-change temperature was 35°C. These log-counts also have been normalized for the ease of plotting scatterplots and deducing inferences. Symbol "-" has been used to indicate where no growth was detected on the medium plates.

Table 11: The normalized log-counts for milk samples when the experiment was run at 55°C for 5 minutes and 35°C for 35 minutes

Time (hours)	Log-Count (Step-change)	Log-Count -Duplicate (Step-change)	Log-Count (Control)
0	2.72	2.72	2.72
2	2.49	1.24	3.77
4	2.23	1.67	4.18
6	2.67	1.82	4.47
8	3.81	2.12	4.71
10	3.97	2.38	4.8
12	4.46	2.72	4.97
14	4.47	3.42	5.03
16	4.67	4.12	6.27
24	4.77	3.99	6.35

Table 12: The normalized log-counts for coupons when the experiment was run at 55°C for 5 minutes and 35°C for 35 minutes

Time	Log-Count (Step-change)	Log-Count -Duplicate (Step-change)	Log-Count (Control)
4	-	-	2.02
8	-	-	2.65
12	3.34	3.34	3.34
16	4.08	3.86	5.77
24	4.39	4.06	6.75

Table 13: The normalized log-counts for milk samples when the experiment was run at 55°C for 5 minutes and 35°C for 15 minutes

Time (hours)	Log-Count (Step-change)	Log-Count -Duplicate (Step-change)	Log-Count (Control)
0	2.72	2.72	2.72
2	2.32	2.21	3.77
4	2.79	2.07	4.18
6	3.76	3.19	4.47
8	3.72	3.26	4.71
10	4.77	4.04	4.8
12	4.97	4.24	4.97
14	5.67	4.89	5.03
16	5.67	5.05	6.27
24	3.81	3.53	6.35

Table 14: The normalized log-counts for coupons when the experiment was run at 55°C for 5 minutes and 35°C for 15 minutes

Time	Log-Count (Step-change)	Log-Count -Duplicate (Step-change)	Log-Count (Control)
4	-	-	1.87
8	2.5	2.5	2.5
12	3.78	3.8	3.19
16	4.36	4.43	5.62
24	4.5	4.7	6.6

Table 15: The normalized log-counts for milk samples when the experiment was run at 55°C for 15 minutes and 35°C for 35 minutes

Time (hours)	Log-Count (Step-change)	Log-Count -Duplicate (Step-change)	Log-Count (Control)
0	2	2	2
2	1.56	1.03	2.05
4	2.3	2	2.46
6	2.6	2.15	2.75
8	2.47	2.3	2.99
10	3	2.67	3.08
12	3.3	2.81	3.25
14	3.56	3.01	3.31
16	3.77	3.25	4.55
24	4.1	3.86	4.63

Table 16: The normalized log-counts for coupons when the experiment was run at 55°C for 15 minutes and 35°C for 35 minutes

Time	Log-Count (Step-change)	Log-Count -Duplicate (Step-change)	Log-Count (Control)
4	-	-	2.76
8	-	-	3.39
12	4.08	4.08	4.08
16	4.9	4.73	6.51
24	5.1	5.01	7.49

Table 17: The normalized log-counts for milk samples when the experiment was run at 55°C for 15 minutes and 35°C for 15 minutes

Time (hours)	Log-Count (Step-change)	Log-Count -Duplicate (Step-change)	Log-Count (Control)
0	2.72	2.72	2.72
2	2.37	2.46	3.77
4	3.43	2.41	4.18
6	3.51	3.33	4.47
8	3.58	3.48	4.71
10	3.67	3.56	4.8
12	4.84	4.3	4.97
14	5.02	4.63	5.03
16	5.49	4.75	6.27
24	5.03	4.23	6.35

Table 18: The normalized log-counts for coupons when the experiment was run at 55°C for 15 minutes and 35°C for 15 minutes

Time	Log-Count (Step-change)	Log-Count -Duplicate (Step-change)	Log-Count (Control)
4	-	-	2.98
8	-	-	3.61
12	4.3	4.3	4.3
16	4.65	4.5	6.73
24	6.26	6.33	7.71

8.2. Tabulated data for 2nd method of inoculation (Inoculation in the Reactor):

8.2a. Followingare the tables for log-counts observed when the step-change temperature was 35°C. All the values have been normalized.

Table 21: The normalized log-counts for milk samples when the experiment was run at 55°C for 5 minutes and 35°C for 35 minutes

Time (hours)	Log-Count (Step-change)	Log-Count (Control)
2	2.43	2.43
4	2.86	4.5
6	2.73	4.29
8	3.93	4.17
10	3.83	4.26
12	4.16	4.5
14	4.61	4.34
16	4.49	5.24
24	4.68	6.75

Table 22: The normalized log-counts for coupons when the was run at 55°C for 5 minutes and 35°C for 35 minutes

Time (hours)	Log-Count (Step-change)	Log-Count (Control)
4	3.43	3.43
8	2.42	4.7
12	2.78	4.97
16	3.2	5.26
24	4.7	5.42

Table 23: The normalized log-counts for milk samples when the experiment was run at 55°C for 5 minutes and 35°C for 15 minutes

Time (hours)	Log-Count (Step-change)	Log-Count (Control)
2	2.43	2.43
4	1.42	4.5
6	2.13	4.29
8	2	4.17
10	2.39	4.26
12	2.28	4.5
14	2.5	4.34
16	2.92	5.24
24	4.46	6.75

Table 24: The normalized log-counts for coupons when the experiment was run at 55°C for 5 minutes and 35°C for 15 minutes

Time (hours)	Log-Count (Step-change)	Log-Count (Control)
4	2.84	2.84
8	2.96	4.11
12	2.62	4.38
16	3.3	4.67
24	3.83	4.83

Table 25: The normalized log-counts for milk samples when the experiment was run at 55°C for 15 minutes and 35°C for 35 minutes

Time (hours)	Log-Count (Step-change)	Log-Count (Control)
2	2.43	2.43
4	0.53	4.5
6	0.68	4.29
8	0.99	4.17
10	1.1	4.26
12	2.24	4.5
14	2.29	4.34
16	2.31	5.24
24	2.97	6.75

Table 26: The normalized log-counts for coupons when the experiment was run at 55°C for 15 minutes and 35°C for 35 minutes

Time (hours)	Log-Count (Step-change)	Log-Count (Control)
4	2.3	2.3
8	1.8	3.57
12	2.13	3.84
16	2.68	4.13
24	3.1	4.29

Table 27: The normalized log-counts for milk samples when the experiment was run at both 55°C and 35°C for 15 minutes

Time (hours)	Log-Count (Step-change)	Log-Count (Control)
2	2.43	2.43
4	2.39	4.5
6	2.97	4.29
8	3.37	4.17
10	3.59	4.26
12	3.72	4.5
14	4.51	4.34
16	4.68	5.24
24	4.58	6.75

Table 28: The normalized log-counts for coupons when the experiment was run at both 55°C and 35°C for 15 minutes

Time (hours)	Log-Count (Step-change)	Log-Count (Control)
4	3.1	3.1
8	2.94	4.37
12	4.12	4.64
16	4.82	4.93
24	5.51	5.69

8.2b. Below are the tables for log-counts observed when the step-change temperature was 30°C. All the values have been normalized here too. Symbol "-" has been used to indicate where no growth was detected on the medium plates.

Table 31: The normalized log-counts for milk samples when the experiment was run at 55°C for 5 minutes and 30°C for 35 minutes

Time	Log-Count (Step-change)	Log-Count (Control)
2	2.36	2.36
4	2.36	4.43
6	3.7	4.22
8	2.56	4.1
10	3.71	4.19
12	4.17	4.43
14	4.3	4.27
16	5.3	5.17
24	5.55	6.68

Table 32: The normalized log-counts for coupons when the experiment was run at 55°C for 5 minutes and 30°C for 35 minutes

Time (hours)	Log-Count (Step-change)	Log-Count (Control)
4	-	0.73
8	2	2
12	3.71	2.27
16	4.53	2.56
24	4.1	2.72

Table 33: The normalized log-counts for milk samples when the experiment was run at 55°C for 5 minutes and 30°C for 15 minutes

Time	Log-Count (Step-change)	Log-Count (Control)
2	2.43	2.43
4	2.54	4.5
6	3.43	4.29
8	2.95	4.17
10	3.51	4.26
12	3.59	4.5
14	4.61	4.34
16	4.92	5.24
24	6.52	6.75

Table 34: The normalized log-counts for coupons when the experiment was run at 55°C for 5 minutes and 30°C for 15 minutes

Time	Log-Count (Step-Change)	Log-Count (Control)
4	-	0.73
8	2	2
12	3.9	2.27
16	3.57	2.56
24	4.4	2.72

Table 35: The normalized log-counts for milk samples when the experiment was run at 55°C for 15 minutes and 30°C for 35 minutes

Time	Log-Count (Step-change)	Log-Count (Control)
2	2.43	2.43
4	2.35	4.5
6	2.51	4.29
8	2.73	4.17
10	3.03	4.26
12	2.99	4.5
14	3.68	4.34
16	3.73	5.24
24	3.87	6.75

Table 36: The normalized log-counts for coupons when the experiment was run at 55°C for 15 minutes and 30°C for 35 minutes

Time (hours)	Log-Count (Step-change)	Log-Count (Control)
4	2.92	2.92
8	2.23	4.19
12	2.17	4.46
16	2.42	4.75
24	2.26	4.91

Table 37: The normalized log-counts for milk samples when the experiment was run at 55°C for 15 minutes and 30°C for 15 minutes

Time	Log-Count (Step-change)	Log-Count (Control)
2	2.43	2.43
4	2.8	4.5
6	2.6	4.29
8	3.07	4.17
10	3.34	4.26
12	3.41	4.5
14	3.46	4.34
16	3.33	5.24
24	4.2	6.75

Table 38: The normalized log-counts for coupons when the experiment was run at 55°C for 15 minutes and 30°C for 15 minutes

Time (hours)	Log-Count (Step-change)	Log-Count (Control)
4	2	2
8	3.88	3.27
12	4.37	3.54
16	3.81	3.83
24	4.82	3.99