A study of the effects of temperature cycling on thermophilic biofilms

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Dedicated to my love Peter Xin

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 (except where explicitly defined in the acknowledgments), 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.

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ABSTRACT

The objective of this research was to determine whether the behaviour of thermophilic bacteria growing as biofilms would change after being subjected to temperature cycling. It was used to test the practicality of a temperature cycling method for use in the heat transfer sections (milk pre-heaters and evaporators) of dairy plants for reducing the biofilm growth of thermophilic bacteria. Biofilms may protect bacterial cells from cleaning-in-place operations (CIP). CIP is the standard technique for cleaning dairy plant in New Zealand (AS/NZS 2541, 1998). If the temperature cycling method is used in the heat transfer sections of dairy plants, surviving thermophilic bacteria in the form of biofilms may be continuously exposed to the temperature cycling environment; thus a sub-population which is resistant to temperature cycling may adhere in greater numbers or grow more efficiently under the selective conditions.

Wild-type strains of *Geobacillus stearothermophilus* and *Anoxybacillus flavithermus* were isolated from a commercial milk powder sample obtained from a milk powder production plant in New Zealand. These two wild-type strains were subjected to a temperature cycling regime consisting of 55 ℃ for 15 minutes then 35 ℃ for 35 minutes for 192 hours using a modified Polymerase Chain Reaction (PCR) thermocycler and hexagonal flat stainless steel reactors. At the end of each of four serial experiments, sub-population isolates for both species were obtained from the effluent milk. Wild-type strains and the sub-population strains of these two organisms were tested for the ability to

adhere and grow as biofilms on stainless steel surfaces by using a Centre for Disease Control (CDC) biofilm reactor. The cell counts per unit area of adhesion, maximum specific growth rates (μ_{max}) of the biofilms, and cells released in out-flowing milk were measured. The analysed data of the wild-type strain and the sub-population strain of each organism were compared by using p-value of 2 samples t-test statistical analysis to check whether the strains had adapted to the temperature cycling treatment. The results showed that the ability of planktonic cells of the wild-type *A. flavithermus* strain to adhere to stainless steel surfaces was significantly changed with p-value = 0.001 (< 0.05) after being subjected to 192 hours temperature cycling, but the sub-population growth as biofilms on stainless steel surfaces was no different from the wild-type strain with p-value = 0.235 (> 0.05). In contrast, the sub-population of *G. stearothermophilus* was significantly different from the wild-type after being subjected to 192 hours temperature cycling. The *G. stearothermophilus* sub-population strain was more resistant to temperature cycling, having a greater ability to adhere (p-value = 0.000 < 0.05) and grow as a biofilm (p-value = 0.008 < 0.05) than the wild-type strain. Since *G. stearothermophilus* is a common contaminant in dairy plants, the temperature cycling method may not be a viable long-term solution for dairy plants for reducing the growth rates of thermophilic bacteria, and further studies, such as different temperature profiles both in terms of magnitude and duration, are required.

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Chapter 1:

INTRODUCTION

Dairy products are very popular with more than 6 billion people throughout the world consuming dairy products as daily food (Food and Agriculture Organization of the United Nations, 2014). Infant formula milk powder is the only nutrient supply for some babies, so its quality and safety is very important. Thermophiles (organisms capable of growth in the temperature range 40 - 70℃) grow as biofilms in milk evaporators and heat exchangers in milk powder production plants (Stadhouders *et al.*, 1982; Warnecke, 2001; Scott *et al.*, 2007). The presence of vegetative cells and spores in the final product can have significant negative effects on the quality, pricing and acceptability of the milk powder products in the marketplace (Kirtley & McGuire, 1989). Fortunately, thermophiles are generally not pathogenic. *Geobacillus* and *Anoxybacillus* species are common thermophilic bacteria found in dairy plants (Flint *et al.*, 2001; Ronimus *et al.*, 2003) owing to the high temperature environment, which is optimal for the growth of these thermophiles. The milk powder industry cleans the pre-heaters and evaporators every 18 to 20 hours to remove the thermophilic bacteria and spores to ensure the numbers of bacteria in milk powder are below the specifications set for the product being produced (Hinton *et al.*, 2003). If the growth rates of these thermophilic bacteria could be reduced, the run time of the pre-heaters and evaporators could be extended, resulting in greater productivity of milk powder (Somerton*et al.*, 2013). To achieve this, a more efficient method is required in order to control the growth of thermophilic bacteria as biofilms in milk powder production plants.

Temperature cycling has been proposed as a disruptive technology to reduce the problems

caused by growth of thermophilic bacteria in milk evaporators. In a previous study by Knight *et al.*, temperature cycling (temperature spiking) was used to control the formation of biofilms of *Streptococcus thermophilus* in a pilot plant-scale cheese-milk pasteurisation plate heat exchanger. The object was to achieve a goal of no growth of *S. thermophilus* in a 20 hours production run by using a cycling temperature-time regime of 55 ℃ 10 minutes then 35 ℃ 60 minutes in the regeneration stage (Knight *et al.*, 2004). Recently, a researcher from our laboratory at AUT analysed the effects of temperature cycling on the growth of thermophilic bacilli. She investigated the optimum temperature-time regime for reducing the growth of *G. stearothermophilus* as a biofilm. The optimum temperature-time regime was 55 ℃ for 15 minutes then 35 ℃ for 35 minutes for this particular *G. stearothermophilus* strain (Kaur, 2014).

Along with the study of temperature cycling as a disruptive technology for the thermophilic bacteria growing as biofilms in dairy plants, some potential problems of this approach, especially the economics and practical application aspects, need to be discussed. In the present research project, the microbiological consequences of the application of temperature cycling to the milk heaters of dairy plants were investigated.

Cleaning-in-place (CIP) is the standard technique for cleaning dairy plant in New Zealand and Australia (AS/NZS 2541, 1998). Biofilms are resistant to a number of adverse conditions and can remain attached to dairy plant surfaces even after a CIP (Austin & Bergeron, 1995; Flint *et al.*, 1999; Hinton *et al.*, 2003). Thus, if the temperature cycling method is used in milk evaporators or heat exchangers for extended periods of

time, a sub-population of these thermophilic bacteria which is more resistant and has a greater ability to survive temperature cycling may be selected. Therefore, the technique may not be an effective method in the long run to reduce the number of thermophilic bacteria present as biofilms in pre-heaters and milk evaporators of dairy plants.

The test organisms for this research were *Geobacillus stearothermophilus* and *Anoxybacillus flavithermus* strains isolated from a commercial milk powder sample. The hypothesis of this research was that temperature cycling selects for a sub-population strain which has a greater ability to survive than the wild-type strain. The characteristics of both the wild-type and sub-population strains of each thermophilic bacteria were examined in order to test the hypothesis, and the practical application of temperature cycling technique for reducing the thermophilic bacteria problem in milk evaporators of dairy plants was investigated.

Chapter 2:

LITERATURE REVIEW

2.1 Biofilm background

2.1.1 Biofilm definition

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

2.1.2 Biofilm structures and characteristics

Bacteria grow preferentially as biofilms on a surface in oligotrophic environments (Hall-Stoodley & Stoodley, 2005; Brooks & Flint, 2008). The structure of a biofilm can stabilize the environment around the vegetative cells (Hall-Stoodley & Stoodley, 2005). The cells of the biofilm are adherent and embedded in the self-produced extracellular polymeric substance (EPS), which is generally composed of polysaccharides, water, extracellular DNA, glycoproteins and glycolipids (Sutherland, 2001). Biofilms contain 90–97% water (Zhang *et al.*, 1998). The thickness of biofilms may be a few micrometres to several millimetres (Brooks & Flint, 2008). A biofilm can be formed by either a single species or mixed species of micro-organisms. Mixed-species biofilms may be more stable than mono-species biofilms (Mosteller & Bishop, 1993).

In some cases, biofilms resemble mushroom-type structures when examined by Confocal Scanning Laser Microscopes (CSLM) and are different from other micro colonies (Sutherland, 2001; Antony, 2011). In other situations, the biofilms may be monolayers of

cells. The exact structures of biofilms are determined by the specific environment in which they develop (Stoodley *et al.*, 1999). Wimpenny & Colasanti (1997) also stated that biofilm structure is mainly determined by the concentration of nutrients. The thickness of the film may also be determined by the fluid shear. The biofilm structure can be changed when the bacteria species have a different physiological need to survive (Antony, 2011).

A primitive communication system is present in biofilms. The name for the phenomenon is called Quorum Sensing (QS) and can be defined as a cell density-dependent bacterial intercellular communication (Xie, 2000). QS is involved in gene expression and the behavior change of the biofilm cells, such as the resistance to stress conditions (Xie, 2000). QS supports a unified response of the bacterial community, which benefits the population (Davies, 1998). QS occurs in both mono-species biofilms and mixed-species biofilms, and it essentially serves as a single communication network (Shiner *et al.*, 2005). Parker, *et al.* have shown what might be a quorum sensing effect in biofilms of *G. flavithermus* (*Bacillus flavothermus*) growing on stainless steel coupons in a laboratory reactor (Parkar *et al.*, 2003).

2.1.3 Biofilm life cycle

The biofilm life cycle consists of attachment, aggregation, maturation and detachment (Sauer et al., 2002; Davey and O'Toole, 2000). Ecosystems such as waster systems

where biofilms develop are generally environments consisting of limited nutrients. The attachment of planktonic cells onto a surface is an important first step of biofilm formation (Mittelman, 1998; Chmielewski & Frank, 2003).

2.1.3.1 Attachment

The initial adhesion of bacterial cells to an abiotic surface is governed by non-specific interactions consisting of electrostatic, van der Waals' and acid-base forces (Stoodley *et al.*, 2002). At this stage, cells are considered to be reversibly bound to the surface and can be removed.

If the cells remain attached to the surface, they can produce multiple adhesins to anchor themselves on the surface (Wang *et al.*, 1995). The gene expression of the colonial microbes is modified, and the microbes produce complex EPS and biofilm formation begins (Zottola, 1994; Palmer *et al.*, 2007). From this stage onwards, the attachment is irreversible and the structures are extremely difficult to remove (Dunne, 2002).

2.1.3.2 Aggregation and Maturation

After attachment, and if conditions are favourable, the micro-organisms actively replicate on the surface (Davies *et al.*, 1998). The basic structural unit of the biofilm is the microcolony. Depending on the bacterial species, the microcolony may be composed of 10–25% cells and 75–90% self-produced EPS matrix, and the most dense matrix

material often appears in the central area of the microcolony (Costerton, 1999). These micro-organisms and EPS interact with each other, resulting in the generation and maturation of the biofilm architecture (Sauer *et al.*, 2002). According to a study by Whiteley *et al.* (2001), the sessile bacteria in a biofilm showed different levels of genetic and protein expression compared with the planktonic cells of the same species of bacteria.

2.1.3.3 Detachment

When biofilms reach their maturity, the cells in the peripheral layer detach and are released as planktonic organisms (Allison *et al.*, 1998). Detachment (dispersal) of cells from the peripheral layer of a biofilm is an essential stage of the biofilm lifecycle, which results in cells that can attach and colonise new surfaces. Enzymes that degrade the biofilm extracellular matrix within play an important role in dispersal of cells from a biofilm (Davies & Geesey, 1995; Davies *et al.*, 1998). The dispersed cells from biofilm can contaminate product downstream and increase the numbers of bacteria in the final product (Flint & Brooks, 2001).

2.1.4 Biofilm growth factors

Some external stimuli such as temperature, pH value, nutrient availability, water activity, hydrodynamics or presence of other microbial species may influence the biofilm growth

by changing gene expression and EPS production (Adams & Mclean, 1999; Cramton *et al.*, 1999; Dalton & March, 1998; Gilbert *et al.*, 1991; Kim & Frank, 1994; Mclean *et al.*, 1997; Pratt & Kolter, 1998; Trachoo & Brooks, 2005).

2.1.4.1 Temperature

Biofilm growth is reduced at the periphery of the growth temperature range (personal communication, Li, 2013). Temperature cycling and temperature spiking may influence biofilm growth. In this research and in previous work (Li, 2013) "Temperature cycling" refers to sine wave variation of temperature, which "Temperature spiking" refers to a square wave pattern with variable dwell times at maximum and minimum temperatures. A previous study by Knight *et al.* (2004) demonstrated the effects of temperature cycling by using it to control the formation of biofilms of *Streptococcus thermophilus* in a pilot plant-scale cheese-milk pasteurisation plate heat exchanger. Another study by Kaur (2014) investigated temperature cycling for control of *G. stearothermophilus* growth as a biofilm on the stainless steel surface of a small flat reactor. The concentrations of *G. stearothermophilus* in the out-flowing milk were significantly reduced in the temperature cycling trials (Kaur, 2014). After a recent study, Li (2013) suggested that temperature cycling interfered with the biofilm development stage by disrupting the exponential growth phase of these micro-organisms in the biofilm (personal communication, Li, 2013). The rate of temperature change, range of temperature and dwell time at the extremes are all likely to influence microbial growth.

2.4.1.2 pH value

Bacteria have many chances to develop on milk residues in dairy farms. For cleaning those milk residues in dairy farms or production plants without dismantling, a Clean-in-Place procedure which uses of a mix of chemicals, heat and water is using. Due to Caustic Soda, Phosphoric and Nitric acids, Sodium Hypochlorite (Hypo) and Peracetic Acid (PAA) are used as the chemicals of CIP, the pH value of machinery surfaces which are the living environment of bacteria changes. The relationship between decrease of pH value and biofilm formation was studied by Nguyen *et al.* (2012) when the bacteria formed as biofilms in this environment after a CIP. This study involved the control of milk at pH 7.0 by adding sterile sodium hydroxide during long-term biofilm formation of *Bacillus licheniformis* NBRC 12195 and *Lactobacillus paracasei* subsp. *paracasei* NBRC 15889 on stainless steel coupons in different dilutions of skim milk (0.1%, 1.0% and 5.0%). The results showed that when the environmental pH value was kept at a neutral range, milk coagulation was prevented and the biofilm formation was reduced long-term (Nguyen *et al.*, 2012). A reconcilable result was given by Lindsay (2005) who found that most *Bacillus* species isolates from alkaline wash solutions used for a CIP in South African dairy factories attached better at pH 4 and 10 compared with pH 7. Moreover, the higher the concentration of the skim milk used in the study by Nguyen *et al.*, the better the growth of biofilm achieved (Nguyen *et al.*, 2012).

2.1.4.3 Nutrient availability

Nutrient availability has a large influence on biofilm structure. High concentrations of nutrients tend to produce thicker and denser biofilms compared with environments with low nutrient concentrations (Stoodley *et al.*, 2002). Stoodley demonstrated that when the nutrient concentration was increased ten-fold, a mature mixed-species biofilm may change its morphology from ripples and streamers to densely packed mound-like structures (Stoodley *et al.*, 2002). On the contrary, when nutrient was limited, the rate of detachment of *Aeromonas hydrophila* biofilms was increased (Stoodley *et al.*, 2002).

2.1.4.4 Water activity

In dairy plants, the concentration of milk solids, and hence water activity, can affect the growth of biofilms. If the concentration is too low, as in clean tanks and pipes, there will be insufficient nutrients for biofilms to grow; on the hand, if the concentration is too high, as in the concentrate being fed to the spray drier, the biofilms would not grow due to the low water activity (Brooks & Flint, 2008). Li (2013) found the optimum concentration of skim milk solids for *Geobacillus stearothermophilus* biofilm growth is 10% to 14%. When the concentration of milk solids was 18%, the biofilm did not grow at all.

2.1.4.5 Hydrodynamics

Hydrodynamics influence biofilm structure. After bacterial attachment, flow rate or shear force of laminar or turbulent flows lead to distinct differences in biofilm structure and content (Bryers, 1987; Davey & O'Toole, 2000). Biofilms growing in turbulent flow consist of structures of cell clusters or ripples that migrate downstream along solid surfaces. When biofilms are exposed to shear stresses, they may be structurally deformed owing to the biofilm viscoelasticity. For long time grown under high shear conditions, the biofilm structure usually becomes monolayer, compared to large 'pillar-like' structures in laminar flow. When biofilms were living in laminar flow, they were patchy and consisted of cell clusters separated by interstitial voids (Davey & O'Toole, 2000; Stoodley *et al.*, 2002).

2.1.5 Plating techniques for measuring biofilm growth

Swabbing, rinsing, scraping and vortexing are the common conventional methods to remove bacteria for enumerating bacteria in biofilms (Flemming *et al.*, 1992; Kumar & Anand, 1998; Wehr & Frank, 2004; Frank & Koffi, 1990; Mustapha & Liewen, 1989).

Bacteria removed from a surface, by one of the methods above are traditionally enumerated using standard spread plating techniques. A flame-sterilized glass rod is used to spread 0.1 ml of a liquid sample onto an agar plate (Herigstad, 2001). After an appropriate incubation period, the colony forming units (CFU) are counted.

The Whitley automated spiral plater (WASP) is an automated plater that has been used to

test the levels of bacteria in the food industry and in non-food sectors such as the pharmaceuticals industry (Raugel, 1999). WASP has a microprocessor for controlling all aspects of the deposition of liquid samples onto the surface of a rotating agar plate. A stylus arm dispenses liquid samples with an Archimedes Spiral, either uniformly across the agar plate or as a continuous decreasing volume. 10 µl to 400 µl dispensed volumes provide flexibility and repeatability. Loading a sample, inoculating a plate and cleaning the stylus are finished with a single keystroke (Raugel, 1999). Plate incubation is the same as for the spread plate method, and then counting may be performed manually or by an image analyser counting system.

2.1.6 Laboratory systems for biofilm study

2.1.6.1 Plate reactor system

An hexagonal stainless steel plate reactor was developed by Aplin & Flint in Fonterra Co-operative Group for temperature cycling experiments in order to simulate the stainless steel surfaces of a plate heat exchanger of a dairy plant which is exposed to varying temperatures (Aplin & Flint, 2007). The reactor consists of a stainless steel hexagonal chamber with tubes on either end for feeding nutrient medium into and out of the reactor. Silicon tubes are used to connect the plate reactor with the stainless steel heating tube in order to create a single pass flow-through system. Milk is pre-heated by submerging the stainless steel heating tube in a water bath. The temperature of the reactor can be controlled by placing it on a modified PCR thermocycler programmed with a continuous

temperature cycling regime. Thermocouples are inserted into the inlets or outlets of the reactor to monitor the exact temperature profile. At the end of the experiment, the top plate of the reactor can be opened and bacteria on the inner surfaces of the reactor can be enumerated for study of the biofilm growth after several hours of temperature cycling (Aplin & Flint, 2007).

2.1.6.2 Centre for Disease Control (CDC) reactor system

The CDC reactor is supplied by Bio-Surface Technologies Corporation for growing biofilms in laboratories under high shear conditions which can be set up for simulating factory conditions by adjusting temperature and stirring rate (CDC, 2009). It has a one litre vessel with an effluent spout set to give an approximate 400ml working volume, a magnetically driven paddle for continuous mixing, and a top for supporting eight independent coupon holders with three removable biofilm growing coupons each, providing a total of 24 sampling opportunities. The removable holders allow for intermittent aseptic removal for coupon sampling at different time points (CDC, 2009). For ensuring bacteria grow only on the coupons as biofilms but not planktonically, the flow rate of the media can be adjusted to ensure that the dilution rate (D h^{-1}) is greater than the planktonic growth rate of the bacteria (μh^{-1}).

2.1.7 Biofilm cleaning and sanitising

Biofilm control efforts most often focus on effective cleaning of potential growth sites

rather than nutrient and water limitation, equipment design or temperature control methods, since it is not usually possible to change the nutrients in the product, nor to modify the equipment (Frank, 2000; Chmielewski & Frank, 2003), though bacterial growth will be inhibited under some conditions, such as where the water activity of milk concentrate is too low, or where the process temperature is too high. The presence of EPS makes it more difficult to remove established biofilms using a cleaning regime. Cleaning procedures should be effective to remove food debris and other soils which may contain micro-organisms or promote microbial growth. The steps for most cleaning regimes are application of chemical agents, rinsing with cold or warm water, and sanitation. Cleaning can be accomplished by using chemicals or a combination of chemical and physical force. Chemical cleaners decrease surface tension, emulsify fats, and peptize proteins to dissolve food residues to clean. High temperatures may reduce, but not remove, the need for physical force (Frank, 2000). As a general rule, chemicals, elevated temperatures, physical work and suitable contact time are all required for successful cleaning.

2.1.7.1 Cleaning-in-Place (CIP)

Cleaning-in-Place has been used to clean the interior surfaces of pipes, vessels, process equipment and associated fittings without disassembly since the 1950s (Tamime, 2008). Many industries, including dairy, beverage, brewing, processed foods, pharmaceutical and cosmetics industries, rely on CIP for frequent cleaning owing to the reduced labour

and shorter cleaning time (Tamime, 2008). The current CIP regime in the dairy industry is the standard caustic (2%, 75 ℃, 30min) and nitric acid (1.8%, 75 ℃, 30min) treatment at Reynolds number of fluid greater than 2000, which totally inactivated and removed biofilms of *A. flavithermus* from cold-rolled stainless steel surfaces in a laboratory trial (Parkar *et al.*, 2004). However, research by Flint (1998) showed that after the routine CIP regime in dairy plants, not all micro-organisms were removed. The surviving cells in the remaining film may cause rapid recolonization of the plant by seeding, and providing surface for new cell attachment (Flint, 1998).

2.2 Thermophilic bacilli in the dairy industry

Thermophilic bacilli are the most common biofilm forming microorganisms in dairy manufacture (Burgess *et al.*, 2010). This group of bacteria is able to grow on the stainless steel surfaces of preheating and some evaporation sections of milk powder plants, since these locations provide optimal temperatures for their growth (40–65 ℃; Murphy *et al.*,1999; Scott *et al.*, 2007). The thermophilic bacilli can be divided into the facultative thermophiles and the obligate thermophiles. In the dairy processing context, facultative thermophiles belong to the *Bacillus* genus, such as *B. coagulans*, *B. licheniformis* and *B. pumilus*, which can grow at both mesophilic and thermophilic temperatures. However, the obligate thermophiles, such as *G. stearothermophilus* and *A. flavithermus*, tend to grow primarily in milk powder manufacturing plants (Scott *et al.*, 2007). The growth of thermophilic bacilli as biofilms in a milk powder

manufacturing plant is an indicator of poor hygiene. Although these micro-organisms are not pathogenic, the presence of biofilms can produce enzymes and acid that may potentially spoil the products (Chopra & Mathur, 1984; Chen *et al.*, 2003). The spores of *G. stearothermophilus* and *A. flavithermus* are also often found in the final products and can survive through pasteurization and germinate when the growth conditions permit, potentially spoiling subsequent products in which they are used.

2.2.1 *Geobacillus stearothermophilus*

Geobacillus stearothermophilus has motile rod-shaped cells and is Gram-positive. Each cell has one ellipsoidal or cylindrical endospore located terminally or sub-terminally. It is a facultative anaerobe, and the growth temperature range is 37 ℃ – 75 ℃, with optimum temperature in the range 55 ℃ to 65 ℃. Its growth pH range is around 6 to 8.5 with optimum pH value at 6.2 to 7.5. The isolates of this strain form catalase and do not produce acid from lactose (Nazina, 2001). This is the main thermophilic species of concern for the dairy industry. *G. stearothermophilus* is able to form biofilms after approximately 6 hours of incubation using a laboratory continuous flow reactor (Flint *et al.*, 2001). After 12 hours, the biofilm contains up to 10^6cells cm^{-2}. Both vegetative cells and spores can be released into the milk (Flint *et al.*, 2001).

2.2.2 *Anoxybacillus flavithermus*

This strain was first isolated from manure and described as a facultatively anaerobic, catalase-positive, oxidase-positive and spore-forming bacillus (Pikuta, 2000). The cells are Gram-positive, straight, non-motile rods. Colonies are round, smooth and yellow. The growth temperature range is between 30 ℃ and 72 ℃ with optimum temperature around 60 ℃ (aerobic) and 65 ℃ (anaerobic). The growth pH range is between 6.0 and 9.0. The strain grows in 2.5% NaCl broth, but not in 3% NaCl. It does not hydrolyze starch, but does hydrolyse gelatine (Pikuta, 2000). The biofilm formation and sporulation of *A. flavithermus* can occur very rapidly, within 6 to 8 hours. The *A. flavithermus* biofilm could develop from spores and release vegetative cells at 8 hours and spores at 14 hours post-inoculation (Pikuta, 2000).

Chapter 3:

MATERIALS AND METHODS

3.1 Source of bacterial isolates

Wild-type strains of *Geobacillus stearothermophilus* and *Anoxybacillus flavithermus* were isolated and purified by a previous researcher, Isabel Li, from a commercial skim milk powder sample obtained from a milk powder production plant in New Zealand, and were stored in cyrovials *G. stearothermophilus* Geo1 and *A. flavithermus* Anoxy2 were identified using molecular biological methods, including polymerase chain reaction and MALDI-ToF (maxtrix-assisted laser desorption/ionisation time of flight) analyses.

3.2 Bacteriological methods

3.2.1 Media

Tryptic soy agar (TSA) (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and tryptic soy broth (TSB) (Becton Dickinson Labware) were prepared according to the instructions in 500 ml sealed Schott Duran bottles and autoclaved at 121 ℃ for 15 min before use.

Skim milk powder was provided by Fonterra New Zealand, and was sent to Schering-Plough Animal Health (Upper Hutt) for gamma irradiation to eliminate all the spores before use. 400g of irradiated milk powder was dissolved in 4L sterile deionised distilled water in a sterile reservoir on a clean bench for preparing a 10% concentration (w/v) of re-constituted milk which is the optimum concentration for these thermophilic bacilli (Li, personal communication). The prepared milk was stored at 4℃ for a maximum of one day before use to ensure that the powder had dissolved properly and

the protein denaturation caused by growth of contaminating bacteria had not occurred.

3.2.2 Culture preparation

For temperature cycling experiments, a sterilised swab was used to scrape a heavy inoculum (around 100 colonies) of *G. stearothermophilus* from a 24 hours 55 ℃ cultured plate, then transferred by immersing the swab in a small sterilised glass bottle containing 20ml TSB. This bottle was shaken to re-suspend the bacteria. The bottle was incubated at 55 ℃ in a water bath for 6 hours and used for the inoculation step for experiments involving *G. stearothermophilus*. For the *A. flavithermus* strain, the procedure was the same as for *G. stearothermophilus,* except the incubation time was 5 hours at 55 ℃. The determination of incubation times and temperatures for both strains were based on the growth curves to obtain cultures in the late logarithmic stage of growth.

For adhesion assays and biofilm development experiments of both the wild-type strains and the final sub-population strains (from the biofilm sample of temperature cycling run 4), a sterile swab was used to scrape heavy inocula (around 300 colonies) of the original strain or the ultimate sub-population of *G. stearothermophilus* from a 24 hours 55 ℃ incubator cultured plate; then transferred by immersing the swab in a 500ml sterilised Schott Duran bottle containing 300ml TSB. This bottle was sealed with a lid, and shaken to suspend the bacteria. The bottle was incubated at 55 ℃ in a water bath for 6.5 hours and used for the inoculation step of *G. stearothermophilus* runs. For *A. flavithermus* strain, the procedure was the same as for *G. stearothermophilus,* except that the incubation time was

5.5 hours instead of 6.5 hours.

3.2.3 Standard plate counts

All the bacterial samples were diluted as 1: 10 dilution series with TSB and plated onto TSA using the spiral plater. Dilutions were plated to achieve plates with countable colonies, 30 to 300 colonies, on the plate. For the inocula measurement, serial 10-fold dilutions to 10^{-6} were prepared (5 times 1:10 dilution manually and one additional 1:10 dilution by the spiral plater). For the samples of the first 12 hours of both the control runs and temperature cycling runs of both the micro-organisms, serial 10-fold dilutions to 10^{-4} were prepared; and for the samples of the 24-hour, 36-hour, 48-hour and the final biofilm for both the control runs and temperature cycling runs of these two micro-organisms, serial 10-fold dilutions to 10^{-6} were prepared. For the samples of adhesion assays, serial 10-fold dilutions to 10^{-2} were prepared. For the samples of both the out-flowing milk and coupons of the biofilm development experimental runs for these two micro-organisms, serial 10-fold dilutions to 10^{-2} were prepared.

For the control runs of the temperature cycling experiments and the first run (run 1) of each strain for the adhsion assay and biofilm development experiments, four sequential dilutions were plated. For the temperature cycling runs and the other runs (run 2 and run 3) of each strain of the adhsion assays and biofilm development experiments, three sequential dilutions were plated.

3.2.4 Spiral plating for bacterial enumeration

A Whitley automated spiral plater (WASP) was used to enumerate bacteria present in samples for all experiments during the course of this study (Figure 1). Samples were diluted as mentioned in section 3.2.3. The diluted samples were poured into disposable 5-ml cups and placed in the sample cup holder. A 100 µl sample was spiral plated onto triplicate plates of TSA. The log profile setting was chosen for plating samples resulting in a continuously decreasing volume mode across the plate in order to achieve single colonies at the edge of a cultured plate for storing isolates and monitoring contamination. The stylus was automatically sanitised between samples in order to eliminate vegetative bacteria and spores by rinsing with sodium hypochlorite (5000ppm) once and sterile distilled water twice from two 110ml bottles. The sterile distilled water was changed when the water level was below 85ml. The Whitley vacuum vessel was sterilised after every experiment.

Figure 1: Whitley automated spiral plater

3.2.5 Storage of bacterial isolates

After each temperature cycling run of both *G. stearothermophilus* and *A. flavithermus* strains, the isolates from the inoculated plates after 24 hours culturing of both the 48-hour out-flowing and biofilm samples were stored at -20 ℃. In preparation for storage, single colonies of each were scraped from an agar plate, re-suspended in 20 ml TSB, and incubated for either 4 hours (*A. flavithermus*) or 6 hours (*G. stearothermophilus*) at 55 ℃. Aliquots were added to glycerol (final concentration 15% w/v), placed in individual centrifuge tubes, and stored at -20 ℃. Each isolate package was labeled with the name of the bacterium, the experimental running time (i.e. Run 1), the type of sample (48-hour out-flowing sample or biofilm sample), the reactor of samples (U for Upper reactor or L for Lower reactor) and the date.

3.2.6 Cleaning and sterilising process

The main materials that were used in this research were glass, silicone and stainless steel. After each experimental run, all the sampling and culture bottles containing viable bacteria were autoclaved at 121 ℃ for 15 minutes. All the glassware and silicone tubing were washed with Pyroneg powder and hot water in a dishwasher. For washing the stainless steel materials like the hexagonal plate reactors or the coupons of the Centre for Disease Control (CDC) biofilm reactor, Parkar's cleaning sequence (Parkar, 2004) was used. The stainless steel materials were immersed in 2% sodium hydroxide at 75 ℃ for 30 minutes, then adequately rinsed with deionised distilled water. After that, the

stainless steel items were immersed in 1.8% nitric acid at 75 ℃ for 30 minutes and rinsed with deionised distilled water. All glassware, tubing and reactors were dried in a lab oven.

For temperature cycling runs, cleaned hexagonal plate stainless steel reactors were constructed by placing a black silicon gasket between the top and bottom stainless steel pieces (Figure 2). Two reactors were constructed for each experiment and named Upper (U) and Lower (L). The two hexagonal plate reactors, clean silicon tubing and some cotton wool were placed into separate autoclave bags and autoclaved at 121 ℃ for 15 minutes.

The CDC biofilm reactor was prepared by placing 24 clean coupons onto 8 coupon holders which were inserted into the top of the reactor vessel with the centre shaft end entering the stir blade (Figure 3). The head of the vessel and the outlet were covered by aluminum foil and sealed with autoclave tape. The apparatus was then autoclaved at 121 ℃ for 15 minutes.

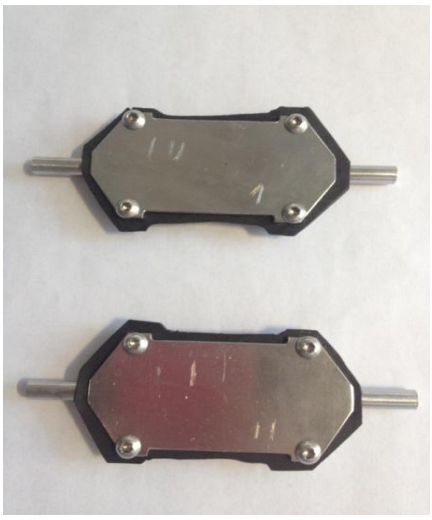

Figure 2: Hexagonal plate stainless steel reactors (U & L)

Figure 3: CDC biofilm reactor

3.3 Temperature cycling to select sub-population

A previous research student (Kaur, 2014) had shown that the alternating cycling of 55 ℃ for 15 minutes and 35 ℃ for 35 minutes is the best temperature cycling mode for disrupting the growth of thermophilic bacilli isolated from a NZ dairy plant. This procedure was used to select a sub-population of the original inocula. The hexagonal plate stainless steel reactors were operated in this mode for 48 hours. Four thermocycling runs were completed for each thermophilic bacillus strain, inoculating the subsequent run from the final biofilm population of the previous run. The intention was to select a sub-population that may have adapted to temperature cycling. In addition, control runs without thermocycling (55 ℃ for 48 hours) were conducted for both *G. stearothermophilus* and *A. flavithermus.*

3.3.1 Experimental system

A modified thermocycler (PCR machine) was used to disrupt the stable conditions in the reactors to select a sub-population strain that might be adapted to the temperature cycles and thus have a greater ability to survive than the wild-type strain. The experimental system is shown below (Figure 4).

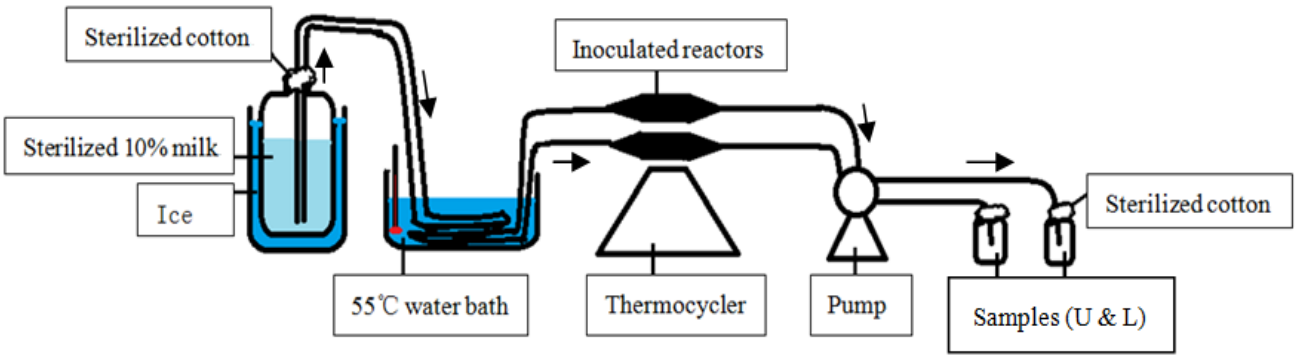

Figure 4: The process flow diagram of the temperature cycling experimental system

Both upper (U) and lower (L) sterilised hexagonal plate stainless steel reactors were inoculated by adding the prepared bacterial culture (section 3.2.2) and holding for 1 hour. Unattached bacteria were then removed by washing the reactors by pumping sterile peptone water. The inoculation was monitored by plating samples of the inoculum and the outflow of reactors after the 1 hour holding time. The sterile 10% milk reservoir, prepared one day earlier (section 3.2.1), was removed from the cool room and placed in a clean bucket, then covered with ice (as Figure 4). Milk was drawn from the reservoir through sterile glass tubing. The milk was preheated by passage through the silicone tubing immersed in a water bath held at 55 ℃. The reactors were set on the

thermocycler heating plate and glued by thermal conductive paste. Samples of the effluent from each reactor were collected in separate sterile 200ml specimen bottles, sealed with sterile cotton wool. The bottles were labeled appropriately. The peristaltic pump was placed after the reactor so that milk was sucked through the system rather than pumped into the system, thereby avoiding any rupture of the tubing due to pressure buildup caused by possible coagulation of milk in the tubing. The pump flow rate was set at 1.5ml/min (section 3.3.3). For control runs, the thermocycler was set at 55 ℃, and the running time set for 48 hours; when temperature cycling experiments were run, the temperature-time regime was set as 55 ℃ for 15 minutes then 35 ℃ for 35 minutes, with a running time of 48 hours. The thermocycler was started when the milk reached the inlets of the U and L reactors. Both *G. stearothermophilus* and *A. flavithermus* were tested in this system.

3.3.2 The actual temperature profile of the thermocycler

The actual temperature profile of the flowing milk inside hexagonal plate reactors (U & L) when the thermocycler was running in cycling mode (55 ℃ for 15 minutes, 35 ℃ for 35 minutes) was measured with thermocouples inserted into U and L reactor outlets. The system was set up in exactly the same way for all experimental runs. A laboratory computer connected to the thermocouples *via* an Arduino interface (constructed by Dr. Chris Pook, School of Applied Sciences, AUT) was able to log the data from each reactor.

3.3.3 The milk flow rate

If the residence time of the bulk fluid in the hexagonal plate reactor is less than the doubling time (t_d), all the released planktonic bacteria from the biofilm will be washed out before they have chance to grow within the bulk liquid phase.

According to the formula that maximum specific growth rate (μ_{max}) = 0.693/t_d (t_d is the bacterial doubling time) and the results exposed by a previous researcher Isabel Li that the μ_{max} of *G. stearothermophilus* is 1.46/h, and the μ_{max} of *A. flavithermus* is 1.75/h at 55 ℃ in 10% milk, the calculated results are that the t_d of *G. stearothermophilus* is 28.5 minutes and the t_d of *A. flavithermus* is 23.8 minutes at 55 ℃ in 10% milk. Since the volume of one hexagonal plate reactor is 4ml, when the slowest frowrate of this peristaltic pump (1.5ml/min) was used, the residence time of the bulk fluid in the reactor was 2.67 minutes (4ml divided by 1.5ml/min) which is much smaller than the doubling times of both microorganisms (28.5 minutes and 23.8 minutes). Therefore, the slowest flowrate of this peristaltic pump, 1.5ml/min, is practicable.

3.3.4 Sampling

To enumerate bacteria in the out-flowing milk, two 90ml samples were collected from the U and L reactor outlets, and placed in two 200ml sterile bottles each hour for the first 12 hours. Between 12 and 48 h, 20ml samples of out-flowing milk were collected at 12 hours intervals from both U and L reactors. To avoid confusion of bottles, the first hour sample from the U reactor was named 1U, and all the samples were named by this

regime. After each sample collected, the sample was shaken immediately for 2 minutes by a vortex mixer set at level 8, and then plated by the spiral plater after serial dilution (section 3.2.3 and 3.2.4).

In order to obtain the 48 hours biofilm samples, the outer surface of each reactor was sanitised with 5% hypochlorite solution, and washed with sterile peptone water. The top and bottom pieces were removed and immersed into a sterile 20ml TSB bottle with 70g glass beads, and the bacteria were suspended by a vortex mixer at level 8 for 20 minutes. The biofilm samples were spiral plated after serial 10-fold dilutions as described for the out-flowing samples.

After the spiral plating step, the plates of both out-flowing milk and biofilm samples were placed in a 55 ℃ incubator for 24 hours and counted manually.

3.4 The adhesion of the wild-type stains and the final sub-population strains

The ability of cells to adhere to a stainless steel surface was measured for the wild-type and the final sub-population (from the biofilm sample of temperature cycling run 4) strains. The measurements were completed on both test organisms. For each strain, three parallel adhesion assays were made. Thus, a total of twelve adhesion assays were performed. The adhesion experiment was the same as the inoculation step of the biofilm development experiment.

3.4.1 CDC biofilm reactor

The CDC biofilm reactor (BioSurface Technologies Corporation Company, Bozeman Montana USA; Figure 3) consisted of a one litre glass vessel with an effluent spout positioned to provide approximately 330ml operational fluid capacity. Continuous mixing of the reactor's bulk fluid is provided by a baffled stir bar that is magnetically driven. An ultra high molecular mass polyethylene top supports eight independent and removable polypropylene coupon holders. Each holder may hold 3 removable stainless steel coupons (biofilm growth surfaces) for a total of 24 sampling opportunities. One air filter vent, one nutrients injection port and one temperature probe vent are mounted in the top of the reactor. The CDC biofilm reactor operates as a continuous flow stirred tank reactor into which nutrients are continuously pumped and flow out of the reactor at the same rate.

3.4.2 Experimental method

Cultures of either *G. stearothermophilus* or *A. flavithermus* were grown as described previously in section 3.2.2. A 300ml aliquot of a culture was poured into a sterilised CDC biofilm reactor vessel (section 3.4.1) which contained 8 coupon holders each with three stainless steel coupons. The CDC reactor was held at the room temperature (around 25 ℃) for 1 hour for the thermophilic bacilli to adhere to the surfaces of the coupons.

3.4.3 Sampling

After 1 hour at room temperature, three stainless steel coupons were removed and washed adequately by gently rinsing in sterile peptone water. A slight modification was made to the Bead Beating Method (Teh *et al.*, 2012). After washing, each coupon was immersed in an individual bottle containing 5ml sterile TSB and 5g sterile glass beads (5-mm diameter). The bottles were vortexed for 5 minutes by a vortex mixer set at level 8 to remove the bacteria from the surface. The resulting suspensions were spiral plated (section 3.2.4) and incubated at 55 ℃ for 24 hours and then counted manually.

3.5 The biofilm development of the wild-type stains and the final sub-population strains

The biofilm growth experiment was used to check the abilities of the wild-type and the final sub-population strains of both *G. stearothermophilus* and *A. flavithermus* to grow as biofilms. Three parallel biofilm development experimental runs were done for each strain (e.g. the wild-type strain of *G. stearothermophilus*). Therefore, the data from a total of twelve experimental runs of these four strains were achieved.

3.5.1 Experimental system

The growth of a biofilm was monitored using a CDC biofilm reactor over 12 hours at the optimum temperature of 55 ℃.The experimental system is shown below in Figure 5.

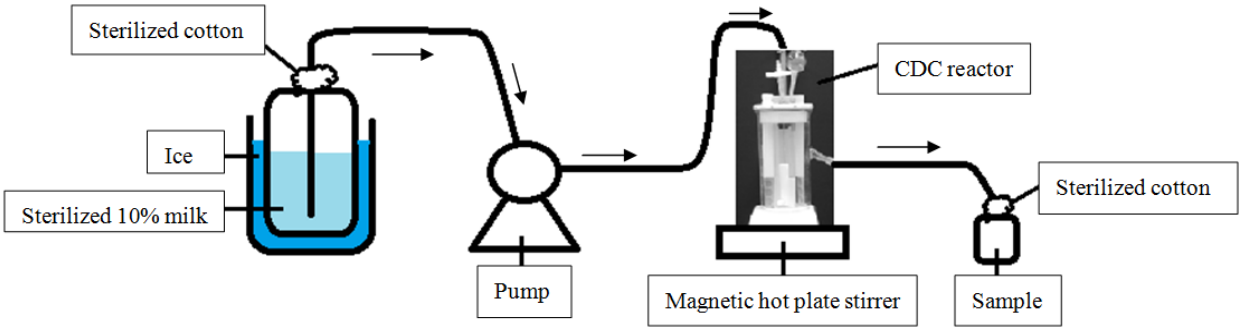

Figure 5: The process flow diagram of biofilm growth experimental system

The inoculation procedure was the same as the adhesion experiment (section 3.4.2). The 300ml prepared culture was inoculated into a sealed sterilised CDC biofilm reactor containing 24 sterilised stainless steel coupons, which were held in 8 coupon holders. The CDC reactor was held at room temperature (around 25 ℃) for 1 hour for the thermophilic bacilli to attach to the surfaces of the coupons. The reactor head was then removed from the vessel and the coupons were washed with sterile peptone water to remove unattached bacteria. The head was then fitted to a new sterilised CDC biofilm reactor vessel, the assembly was placed on a magnetic hot plate stirrer and the temperature probe in the reactor was connected to the heating stirrer. Samples of the original prepared culture and the culture after inoculation were taken for counting (section 3.2.3 and 3.2.4). The sterile 10% milk reservoir, prepared one day earlier (section 3.2.1), was placed in a clean bucket and covered with ice (Figure 5). Sections of sterilised silicon tubing (section 3.2.6) were connected aseptically. The sterilised silicone outlet tube was placed in a sterilised waste reservoir (later changed to sampling bottles), and sealed with sterilised cotton wool. Finally, an air feed pump was connected to the sterile air filter

vent on the reactor top.

Previously prepared sterile 10% milk (330 ml) was poured aseptically into the CDC reactor. Then the magnetic hot plate stirrer was turned on, and the temperature set at 55 ℃ and the stir rate at 120 rpm. When the actual temperature was around 50 ℃, the peristaltic pump was turned on, and set to deliver 13.2ml/min for the wild-type and the final sub-population (from the biofilm sample of temperature cycling run 4) strains of *G. stearothermophilus*, while for the wild-type and the final sub-population strains of *A. flavithermus*, the flowrate was set at 15ml/min (section 3.5.2). Timing of the run began when the first drop of the milk was running out of the outlet.

3.5.2 The milk flow rate

The flow rate of the milk was calculated as previously described in section 3.3.3. Since the volume of the CDC biofilm reactor is 330ml, when the flowrate of the peristaltic pump was 13.2ml/min (level 7), the residence time of the bulk fluid in the reactor was 25 minutes (330ml divided by 13.2ml/min) which is smaller than the doubling times of *G. stearothermophilus* (28.5 minutes under these conditions); and when the flowrate of the peristaltic pump was 15ml/min (level 8), the residence time of the bulk fluid in the reactor was 22 minutes (330ml divided by 15ml/min) which is smaller than the doubling times of *A. flavithermus* (23.8 minutes). Therefore, the flowrates, 13.2ml/min and 15ml/min respectively, were used for the biofilm growth ability experimental runs of *G. stearothermophilus* and *A. flavithermus* strains.

3.5.3 Sampling

Samples were taken at 1.5 hour intervals. For the out-flowing milk samples, only the last half an hour of effluent was collected in a labeled sterile 500ml Schott Duran bottle. A total of eight out-flowing milk samples were obtained in each run. Each out-flowing sample was shaken for 2 minutes by a vortex mixer level 8, then immediately plated using the spiral plater after serial dilution (section 3.2.3 and 3.2.4).

At each sampling period, one coupon rod was aseptically removed from the reactor and gently rinsed with sterile peptone water. The three coupons were separately immersed in glass bottles containing sterilised 5ml TSB and 5g glass beads. The bottles were labeled with the position (U for the top coupon, M for the middle one and L for the lowest one) and running time (e.g. 1.5hU) and shaken for 5 minutes by a vortex mixer set at level 8 to suspend the bacteria. The suspensions were then spiral plated onto TSA after serial dilution (section 3.2.3 and 3.2.4).

After spiral plating, the plates of both out-flowing milk and coupon biofilm samples were incubated at 55 ℃ for 24 hours, then counted manually and recorded.

3.6 Statistical analysis

The statistical comparison of the number of bacteria in the final biofilm samples from the internal surfaces of the hexagonal plate stainless steel reactor of the four serial temperature cycling runs and one control run for each isolate was determined by Tukey's

grouping of Minitab Version 16.1.0.0 (developed by Barbara Ryan in 1972). Furthermore, the statistical comparison of the three parallel experimental runs on the adhesion of vegetative cells from each isolate to stainless steel was also determined by Tukey's grouping.

The 2-sample t-test of Minitab Version 16.1.0.0 was used to calculate the p-value to compare the attachment of wild-type and final sub-population cells to stainless steel. In the biofilm development experimental runs, the 2-sample t-test was used to determine significant differences between maximum specific growth rates (μ_{max}) of the wild-type and final sub-population cells exiting the CDC reactor.

All tests were performed with a confidence level of 95%.

Chapter 4:

RESULTS

4.1 Results of temperature cycling experiments

For each thermophilic bacillus strain (*G. stearothermophilus* or *A. flavithermus*), four temperature cycling runs (55 ℃ for 15 minutes, 35 ℃ for 35 minutes) were conducted to select a sub-population, and one control run (55 ℃ for 48 hours) was carried out as a baseline to compare with the four temperature cycling runs. As mentioned, in the methods section, two plate reactors (U and L) were used as duplicates for each run. So, two out-flowing milk samples were collected at each sampling time, and two final biofilm samples were obtained after the run finished. The number of micro-organisms from each sample was enumerated in triplicate using a spiral plater.

Firstly, the actual temperature profiles of the flowing milk inside the hexagonal plate reactors (U & L) were measured to ensure that the thermocycler was providing the correct temperature for both U and L reactors during temperature cycling runs.

For the experimental runs, the numbers of colony forming units (CFU) on the plates of samples were counted manually. The numbers of bacteria were recorded as CFU per ml against time for the out-flowing samples, and CFU per cm^2 for the final biofilm samples of the two reactors. For purpose of analysis, the CFU/ml and CFU/cm^2 values were transformed to log-count values.

The log-count values of the two out-flowing milk samples from the U and L reactors of the same sample time point of each run were averaged to obtain the mean log-count values. Then, these mean log-count values of the out-flowing samples of the four

temperature cycling runs and one control run of each thermophilic bacillus strain were plotted against time as five scatter-plots in one graph for comparison and discussion.

4.1.1 The actual temperature profiles of the thermocycler

When the thermocycler was running with a continuous regime (55℃ for 15 minutes, 35℃ for 35 minutes), the actual temperature profiles of the flowing milk inside the hexagonal plate reactors (U & L) were measured by thermocouples to ensure that the temperature of the milk inside the reactor was changing as intended. The thermocouple which was implanted in U reactor was named A; the other was named B. The actual temperature profiles are shown below (Figure 6).

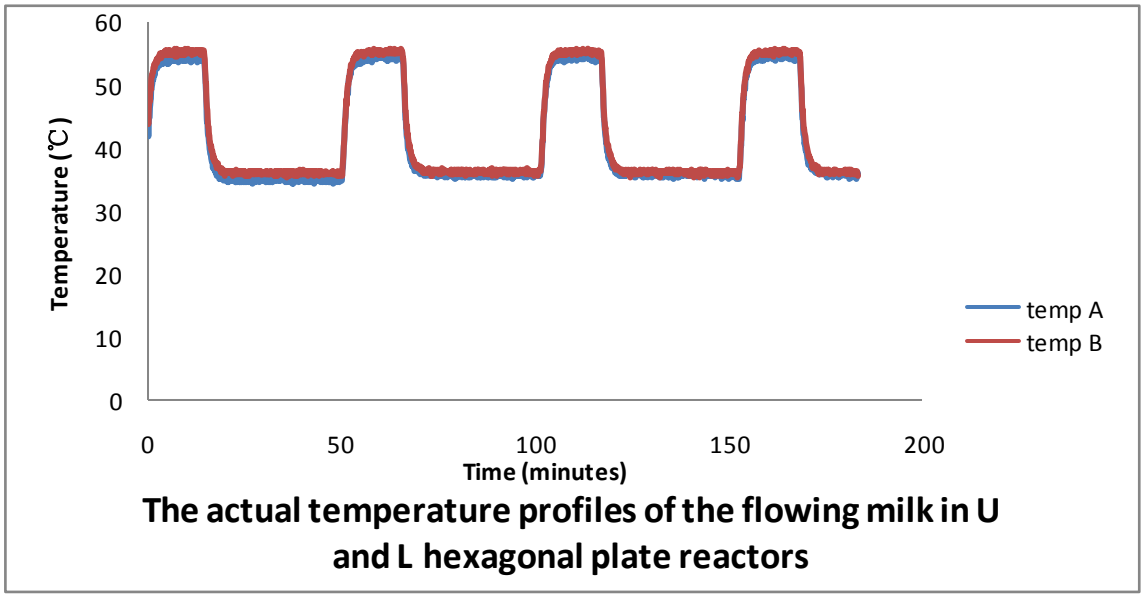

Figure 6: The actual temperature profiles of the flowing milk in U an L hexagonal plate reactorswhen the thermocycler was running with continuous regime (55℃ 15 minutes, 35℃ 35 minutes)

The lines are very thick owing to the high frequency of sampling by the thermocouples. These two actual temperature profiles are very close to each other; moreover, the curve is close to a square curve. Therefore, this method of thermal cycling the milk in the laboratory set-up worked well with U and L hexagonal plate stainless steel reactors. The temperature of the milk changed very fast, ensuring that the suspended bacteria in milk were not able to grow or adapt during the cool down stage (55 ℃ to 35 ℃). The results show that both of the two reactors were exposed to the same heating regime during the run.

4.1.2 Results of temperature cycling runs of the *G. stearothermophilus* strain

The effects of temperature cycling on out-flowing cells and biofilm development of *G. stearothermophilus* in serial runs (each run inoculated by the final population from the biofilm sample of the hexagonal reactor internal surface of the previous run) was determined by using the thermocycler to control the temperature of U and L hexagonal plate stainless steel reactors (section 3.3)

a) Comparison of the cell count profiles of out-flowing milk samples in the control run and four temperature cycling runs

The mean log-counts, averaged over the counts of the out-flowing milk samples from the Upper and Lower reactors of the four temperature cycling runs (55 ℃ for 15 minutes,

35 ℃ for 35 minutes) and one control run (55 ℃ for 48 hours), are listed in Table 1 in the Appendix. The comparative plot of the cell count profiles (Figure 7) of out-flowing milk samples of the four serial temperature cycling runs and one control run is shown below.

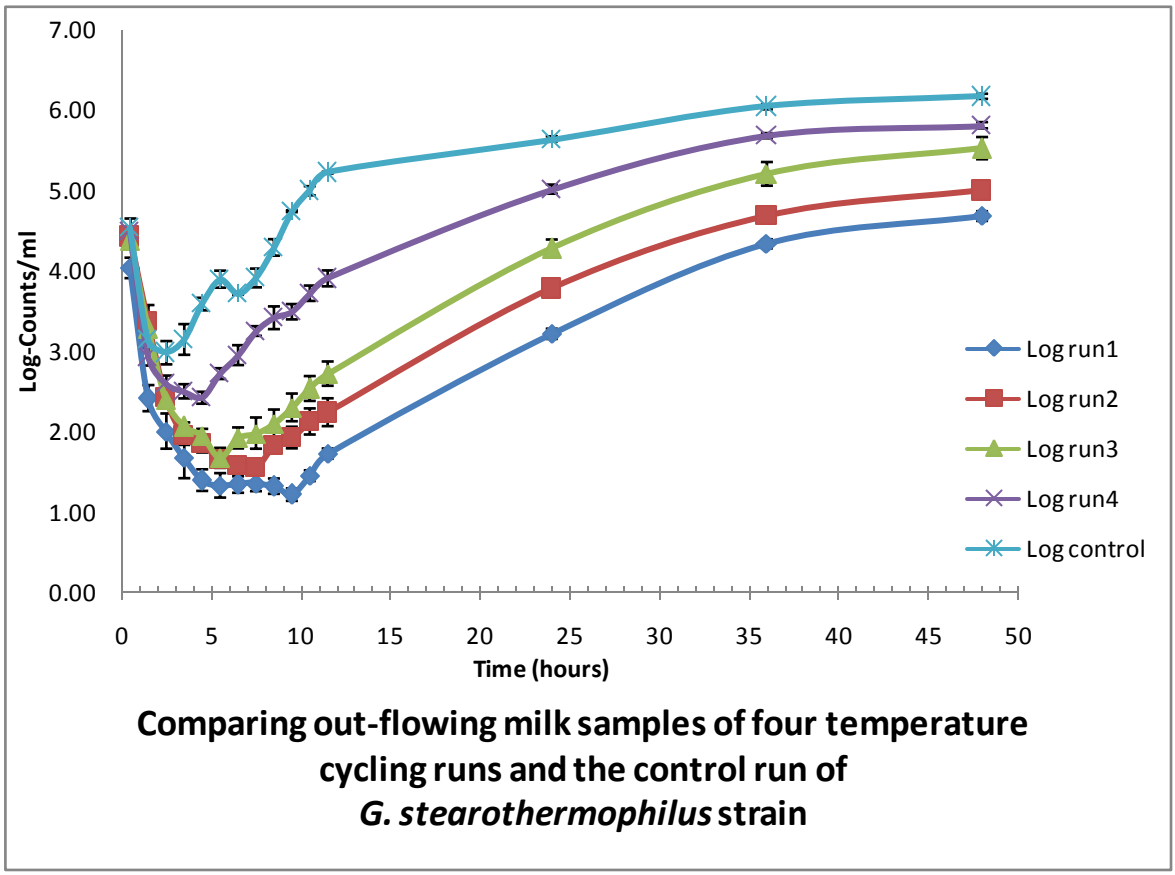

Figure 7: Five plots demonstrating the mean out-flowing log cell counts profiles of the *G. stearothermophilus* strain during the four serial temperature cycling runs (55 ℃ for 15 minutes, 35 ℃ for 35 minutes) and one control run (55 ℃ for 48 hours). Values represent the means of the log of out-flowing cell counts of Upper and Lower reactors path ways' replicates while error bars represent the standard deviation of means.

According to Figure 7, all the five out-flowing log cell count profiles of the

G. stearothermophilus strain started at a similar level in the range from 4.03 to 4.54 log-counts/ml, decreased in the first 3 to 10 hours to a varying extent, then increased rapidly, with some fluctuations, for several hours, eventually stabilising in the range 4.68 to 6.17 log-counts/ml at around 35 hours. The control run showed the highest effluent cell numbers. The four temperature cycled runs showed the first run had the biggest decrease in cell numbers; successive serial runs showed the effluent cell count to be increasing at each sampling point, the final run approaching more closely the profile of the control run. Moreover, the initial decrease in effluent cell numbers of the control run lasted for 3 hours with an overall 1.56 log-counts/ml fall. The initial decrease in effluent cell numbers of temperature cycling run 1 was extended 10 hours with an overall decrease of 2.82 log-counts/ml. The trend continued, until the initial decrease in temperature cycling run 4 extended for only 5 hours, with a decrease of 2.06 log-counts/ml.

b) Comparison of the cell counts of final biofilm samples of the control run and four temperature cycling runs

The mean log-counts, averaged over the counts of the final biofilm samples from the Upper and Lower reactors of the four serial temperature cycling runs (55 ℃ for 15 minutes, 35 ℃ for 35 minutes) and one control run (55 ℃ for 48 hours), are listed in Table 2 in the Appendix. The comparative bar graph (Figure 8) of the mean log cell counts of the final biofilm is shown below. According to the Tukey's groupings, the five runs were classified into different groups depending on the concentration of the log cell counts of

the final biofilm samples.

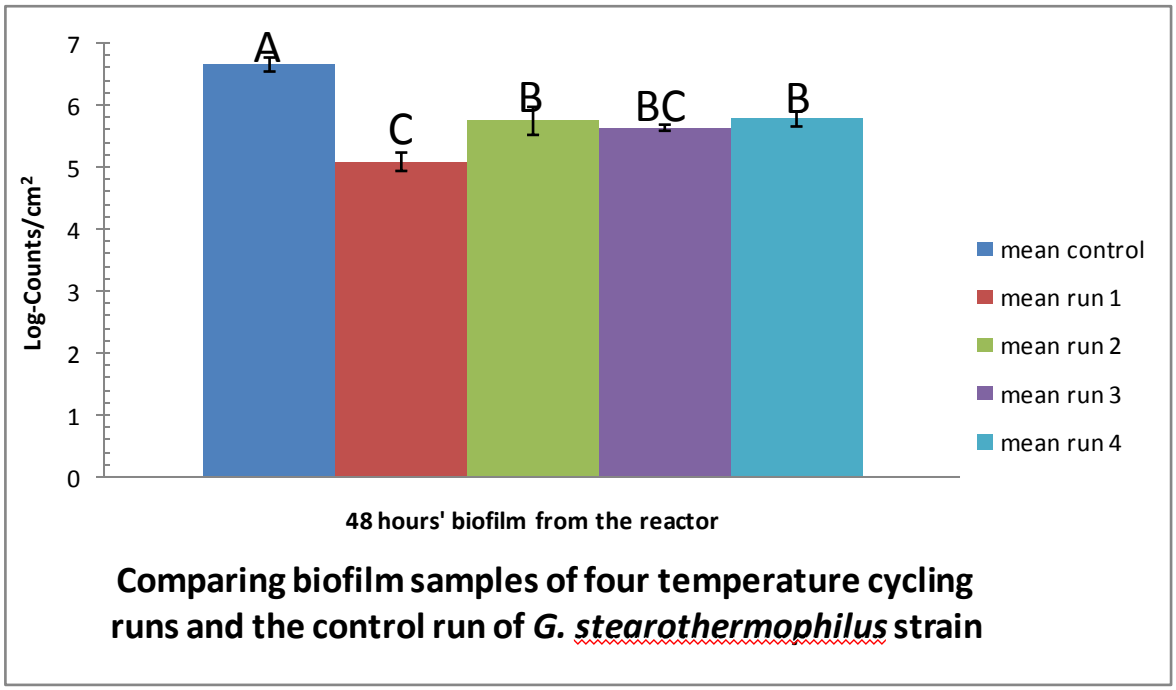

Figure 8: Five-bar graph demonstrating the mean log cell counts of the 48 hour biofilm samples of *G. stearothermophilus* strains from U and L reactors in the four temperature cycling runs (55 ℃ for 15 minutes, 35 ℃ for 35 minutes) and one control run (55 ℃ for 48 hours). Values represent the means of the log of unit biofilm cell counts of Upper and Lower reactors' replicates while error bars represent the standard deviation of means.

According to Figure 8, the mean log cell count of the final biofilm samples of the control run of the *G. stearothermophilus* strain (6.67 log-counts/cm^2) was significantly higher than the temperature cycling runs results. The first temperature cycling run showed a significantly lower cell count per unit area than the control. Runs 2 to 4 were not significantly different from each other, but were still different from the control.

4.1.3 Results of temperature cycling runs of the *A. flavithermus* strain

The effects of temperature cycling on out-flowing cells and biofilm development of *A. flavithermus* was determined in the same way as for *G. stearothermophilus* by using the thermocycler to control the temperature of U and L hexagonal plate stainless steel reactors (section 3.3)

a) Comparison of the cell counts profiles of out-flowing milk samples in the control run and four temperature cycling runs

The mean log-counts, averaged over the counts of the out-flowing milk samples from the Upper and Lower reactors of the four serial temperature cycling runs (55 ℃ for 15 minutes, 35 ℃ for 35 minutes) and one control run (55 ℃ for 48 hours), are listed in Table 3 in the Appendix. The comparative plot of the cell count profiles of out-flowing milk samples of the four serial temperature cycling runs and one control run is shown below (Figure 9).

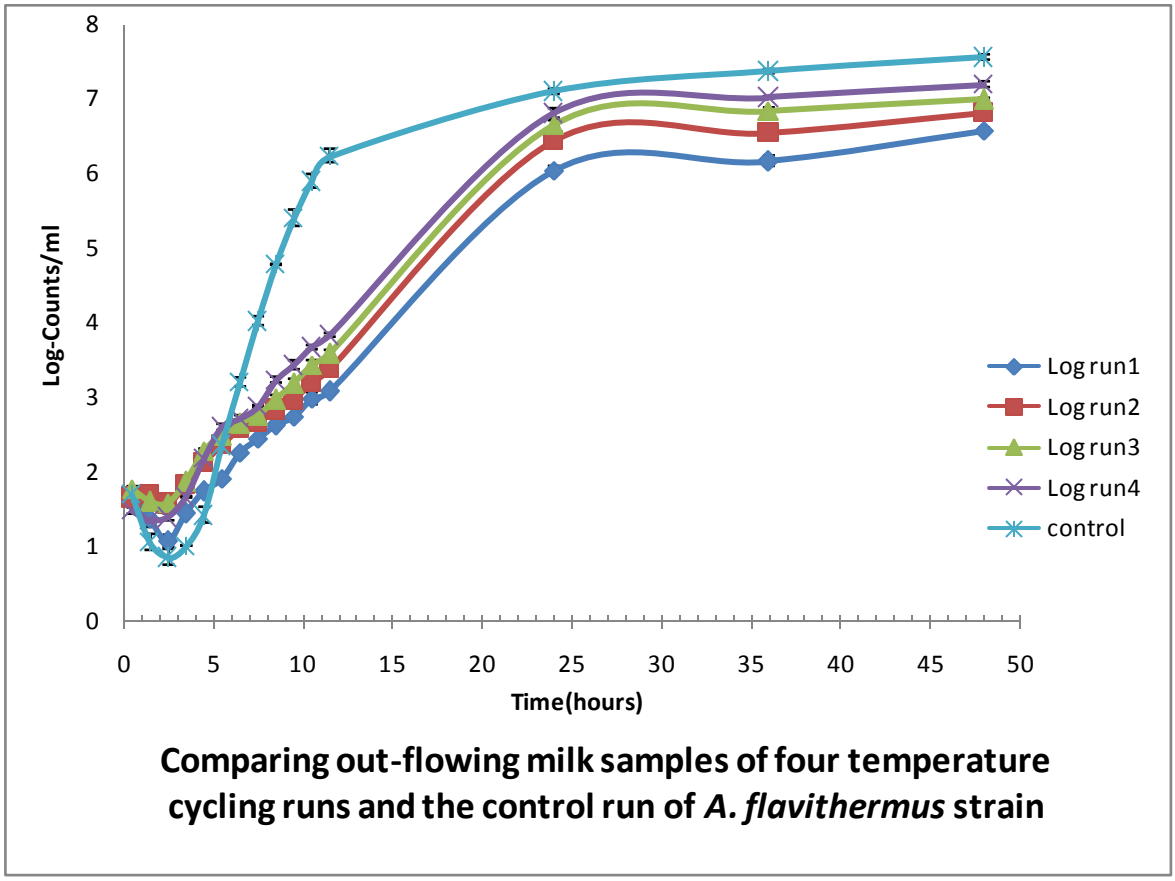

Figure 9: Five plots demonstrating the mean out-flowing log cell count profiles of the *A. flavithermus* strain during the four temperature cycling runs (55 ℃ for 15 minutes, 35 ℃ for 35 minutes) and one control run (55 ℃ for 48 hours). Values represent the means of the log of out-flowing cell counts of Upper and Lower reactors path ways' replicates while error bars represent the standard deviation of means.

According to Figure 9, all the four out-flowing log cell count profiles of the temperature cycling runs of the *A. flavithermus* strain presented similar patterns, with a start in the range of 1.47 to 1.77 log-counts/ml, a small amplitude drop over the first 3 hours, then a rise with minor fluctuations, stabilising at around 24 hours, with the final concentration ranging from 6.58 to 7.19 log-counts/ml. The out-flowing log cell count profile of the control run was significantly different from the four serial temperature cycling runs, with

the final concentration 7.55 log-count/ml. In all cases, temperature cycled cultures showed reduced growth rate, compared with the control run, for the first 24 hours, with specific growth rate in the four temperature cycling runs approximately the same. However, the final count in the outflowing milk approached that of the control, the best case being the first temperature cycled run, with a reduction of approximately 1 log cell count/ml.

b) Comparison of the cell counts of final biofilm samples of the control run and four temperature cycling runs

The mean log-counts, averaged over the counts of the final biofilm samples from the Upper and Lower reactors of the four serial temperature cycling runs (55 ℃ for 15 minutes, 35 ℃ for 35 minutes) and one control run (55 ℃ for 48 hours), are given in Table 4 in the Appendix. The comparative bar graphs are shown below (Figure 10). The five runs were classified into different groups by the Tukey's test, depending on the concentration of the log-cell-counts of the final biofilm samples.

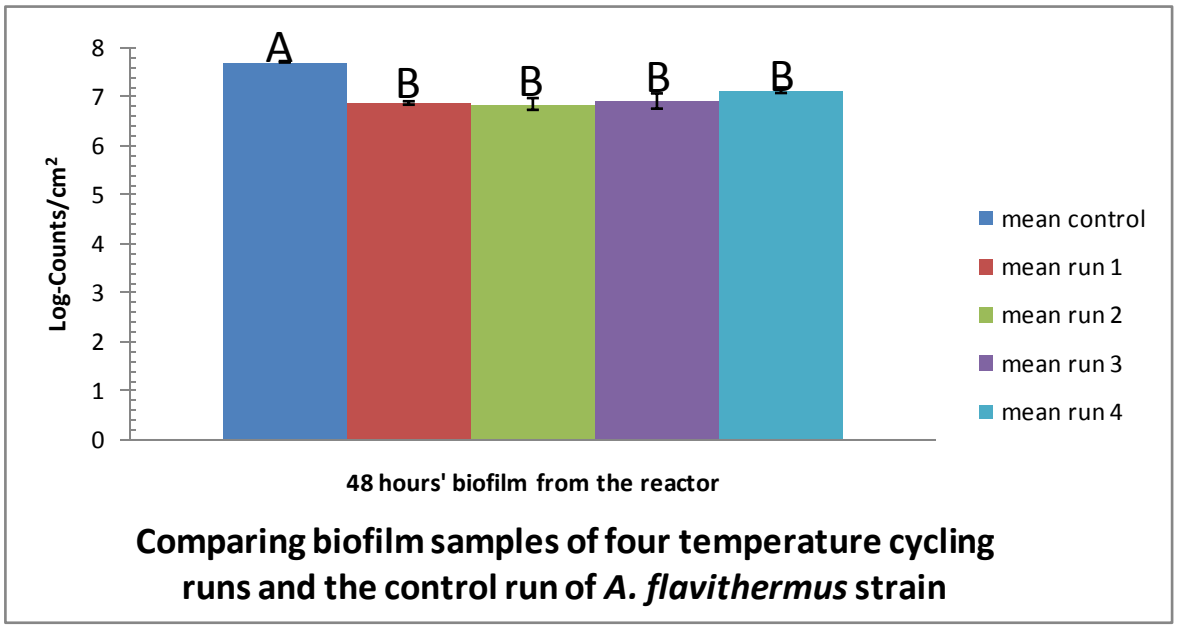

Figure 10: Five-bar graph demonstrating the mean log cell counts of the 48 hour biofilm samples of *A. flavithermus* strain from U and L reactors in the four serial temperature cycling runs (55 ℃ for 15 minutes, 35 ℃ for 35 minutes) and one control run (55 ℃ for 48 hours). Values represent the means of the log of unit biofilm cell counts of Upper and Lower reactors' replicates while error bars represent the standard deviation of means.

According to Figure 10, the mean log cell count of the final biofilm samples of the control run of the *A. flavithermus* strain (7.70 log-counts/cm^2) was significantly higher than the temperature cycling run results. The mean log cell counts of the final biofilm samples of the four temperature cycling runs were not significantly different.

4.2 Results of adhesion to stainless steel surfaces

Since temperature cycling apparently selected for a sub-population of the wild-type strains, it might be expected that other characteristics of the bacteria could also be different in the final sub-population strains. The characteristics of each strain were

therefore examined in more detail.

For each thermophilic bacillus strain of *G. stearothermophilus* or *A. flavithermus*, the ability of cells to adhere to a stainless steel surface was measured and compared with the wild-type strain and the final sub-population strain from the biofilm sample of temperature cycling run 4. For each strain, three parallel adhesion assays were made using a CDC reactor with stainless steel coupons (section 3.4). Thus, a total of twelve attachment assays were performed. Moreover, three coupons were taken out of the CDC reactor for each assay. The average number of adhered bacteria on each coupon was determined by triplicate spiral plates (section 3.2.3 and 3.2.4).

The number of bacteria attached to the coupon was recorded as CFU/cm^2 and converted to log-count/cm^2 values. The log-count values of the coupon samples from the parallel assays (run 1, run 2 and run 3) were plotted as three bars in one graph for each strain. All the log-count values of nine coupon samples of one strain were averaged to obtain the mean log-count value. The mean log-count values per unit area of adhesion of the wild-type strain and the final sub-population strain of *G. stearothermophilus* were plotted as two bar graphs for comparison.

The 2-sample t-test of Minitab 16 software was used to calculate the p-value with confidence interval 95% to check whether the unit count amount of cells of the final sub-population strain of *G. stearothermophilus* adhering to the stainless steel surfaces was significantly higher than the wild-type stain.

The method for comparing the cells adhesion ability of the wild-type and the final sub-population strains of *A. flavithermus* was the same as for *G. stearothermophilus*.

4.2.1 Results of the adhesion experiment of *G. stearothermophilus* wild-type and sub-population strains on stainless steel surfaces

The cell counts per one square centimetre on a coupon of the wild-type and the final sub-population strains of *G. stearothermophilus* adhering to stainless steel were measured in three parallel assays by using the CDC reactor with three stainless steel coupons in each assay (section 3.4).

a) Three parallel assays of the adhesion of the wild-type strain of *G. stearothermophilus*

The mean log-counts of the adhesion results from the three coupon samples of each assay are found in Table 5 in the Appendix, and presented as a comparative bar graph (Figure 11). According to the Tukey's groupings, the three runs were classified into the same group depending on the mean concentration of the cells adhering to the three coupon samples from each assay.

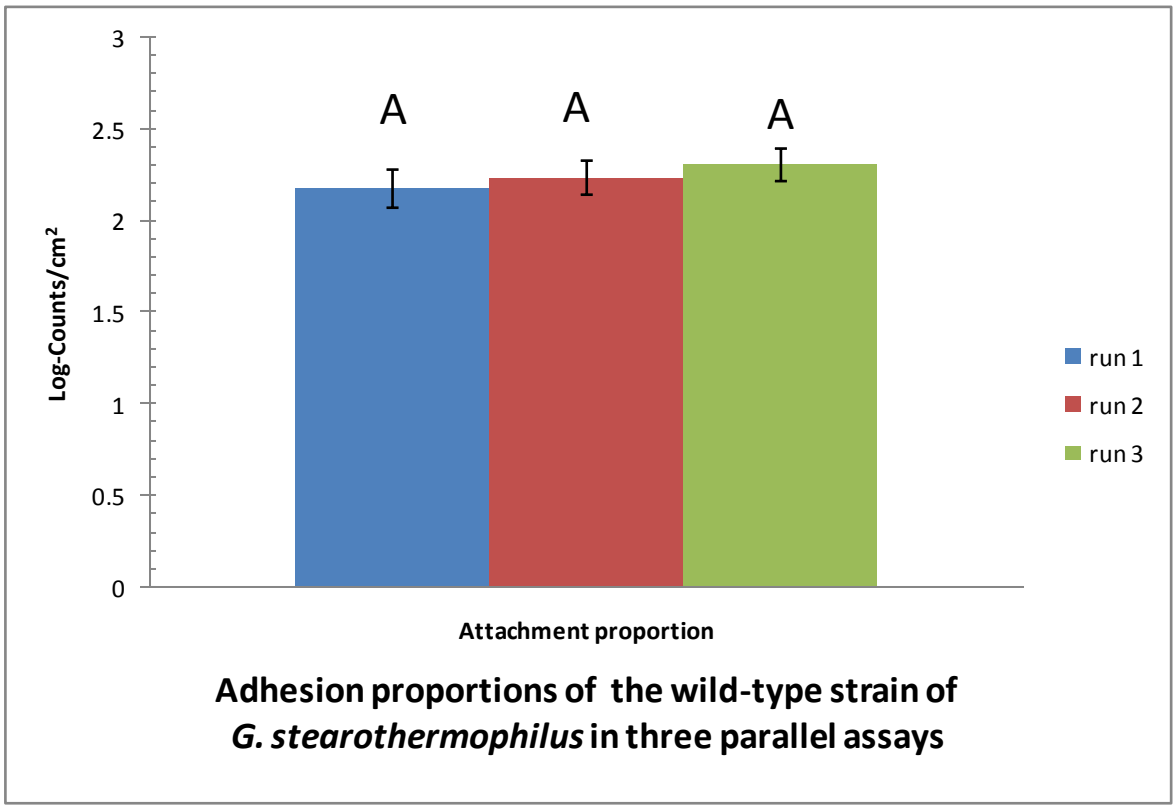

Figure 11: Three-bar graph demonstrating the mean values of the adhesion of the three coupon samples (Upper, Middle and Lower) from each assay of the wild-type strain of *G. stearothermophilus*. Error bars represent the standard deviations of the means of the triplicate coupon samples.

According to Figure 11, the log counts of adhered cells were statistically the same. It means that the three parallel adhesion assays on the wild-type strain of *G. stearothermophilus* had negligible differences and thus the assay method is credible.

b) Three parallel assays of the adhesion of the final sub-population strain of *G. stearothermophilus*

The mean log-counts from the three adhesion assays are found in Table 6 in the Appendix, and are presented as a comparative bar graph in Figure 12. According to the Tukey's

groupings, the three runs were classified by the mean concentration of the adhesion cell log-counts into the different groups.

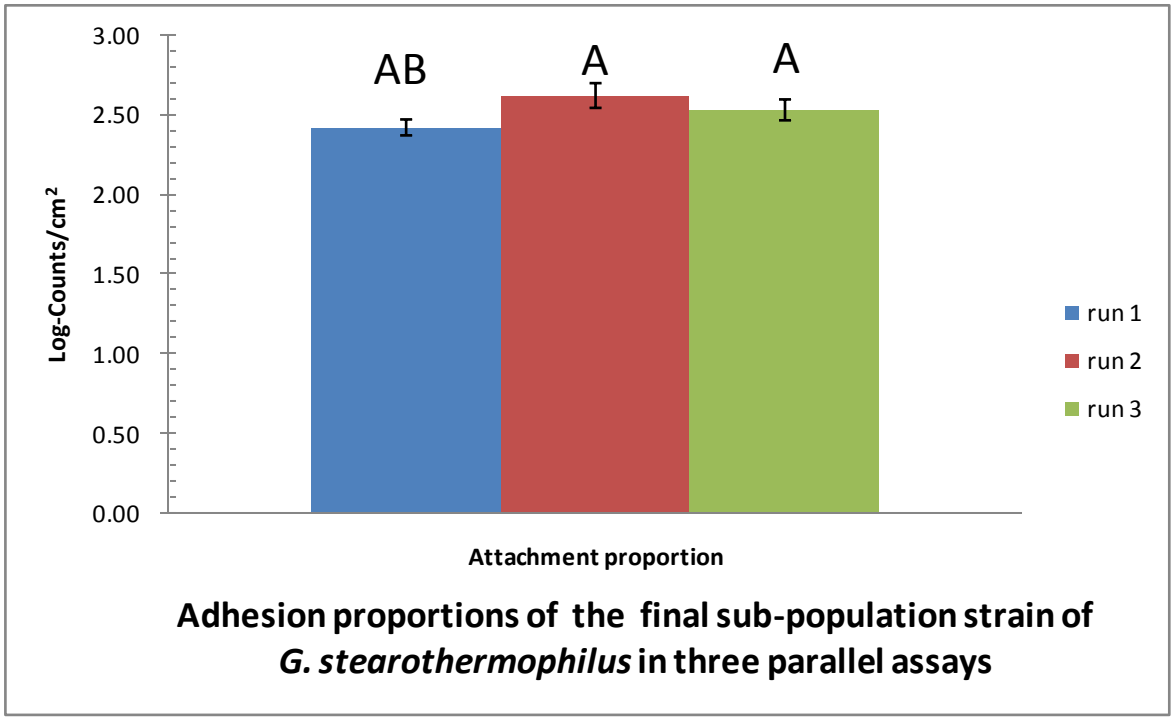

Figure 12: Three-bar graph demonstrating the mean values of the adhesion of the three coupon samples (Upper, Middle and Lower) from each assay of the final sub-population strain of *G. stearothermophilus*. Error bars represent the standard deviations of the means of the triplicate coupon samples.

According to Figure 12, the mean of the adhered cell log-counts of run1 (2.42 log-counts/cm^2) for the final sub-population strain of *G. stearothermophilus* was slightly smaller than the mean result of run 2 (2.62 log-counts/cm^2) and run 3 (2.53 log-counts/cm^2). These three parallel assays of adhesion of the final sub-population strain of *G. stearothermophilus* were similar to each other.

c) Comparison of the adhesion of *G. stearothermophilus* wild-type and final sub-population strains on stainless steel surfaces

In a similar manner to the comparison between parallel determinations of adhesion of the various strains, the adhesion of the final sub-population was compared with the initial wild type of *G. stearothermophilus*. The mean log-counts appear in Table 7 in the Appendix, and are presented as a comparative bar graph (Figure 13).

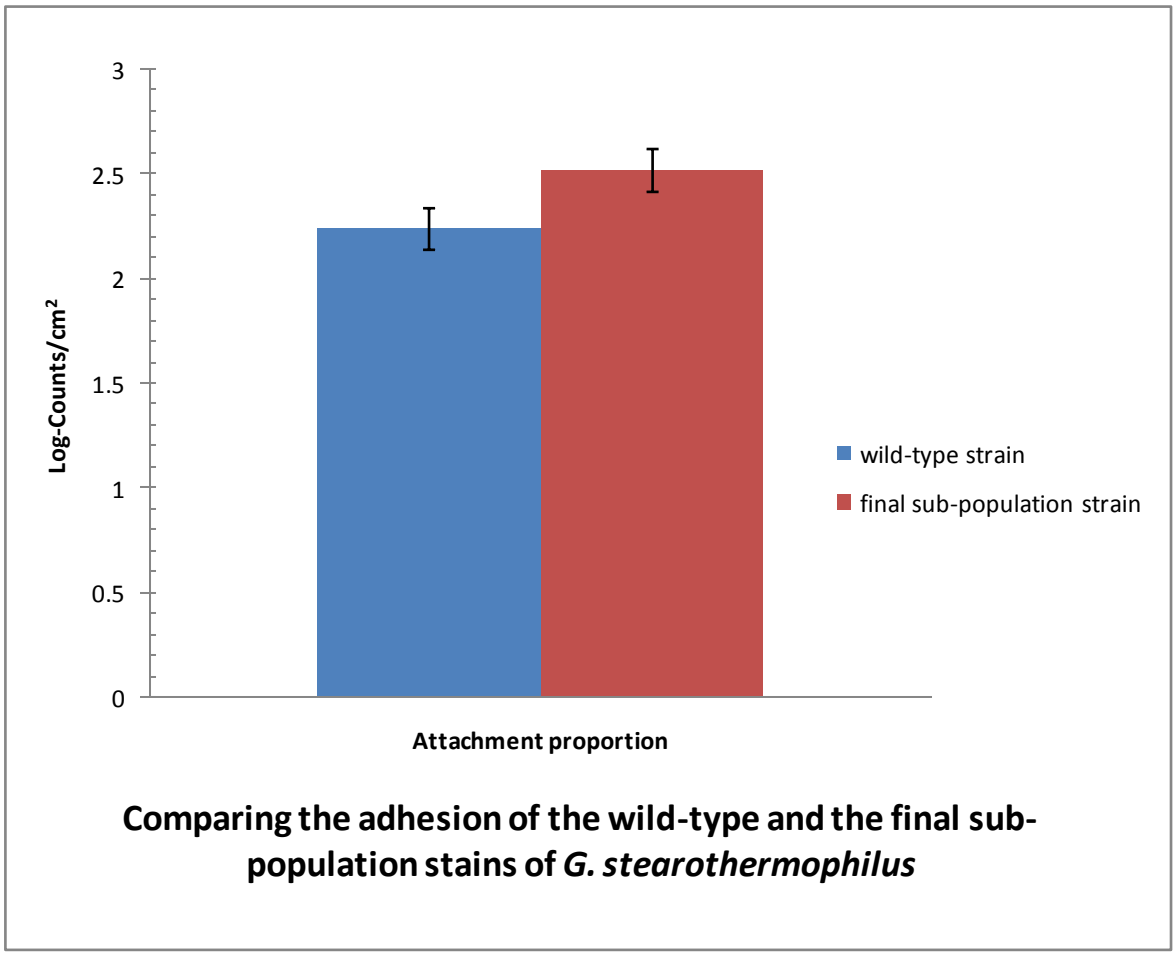

Figure 13: Two-bar graph demonstrating the total mean values of the adhesion results of the wild-type and final sub-population strains of *G. stearothermophilus*. Error bars represent the standard deviations of the means of nine coupon samples from three parallel assays.

According to the 2 samples t-test result which was calculated by the 18 coupon samples results (9 coupon samples for each strain), p-value = 0.000 < 0.05, therefore, the adhesion on stainless steel surfaces of final sub-population strain of *G. stearothermophilus* was significantly higher than the wild-type strain.

4.2.2 Results of the adhesion experiment of *A. flavithermus* wild-type and sub-population strains on stainless steel surfaces

The adhesion of the wild-type and the final sub-population strains of *A. flavithermus* were measured as before in three parallel assays by using the CDC reactor with stainless steel coupons (section 3.4)

a) Three parallel assays of the adhesion of the wild-type strain of *A. flavithermus*

The mean log-counts from the three adhesion assays are found in Table 8 in the Appendix, and are presented as a comparative bar graph in Figure 14. According to the Tukey's groupings, the three runs were classified into the same group based on the mean concentration of the adhered cell log-counts.

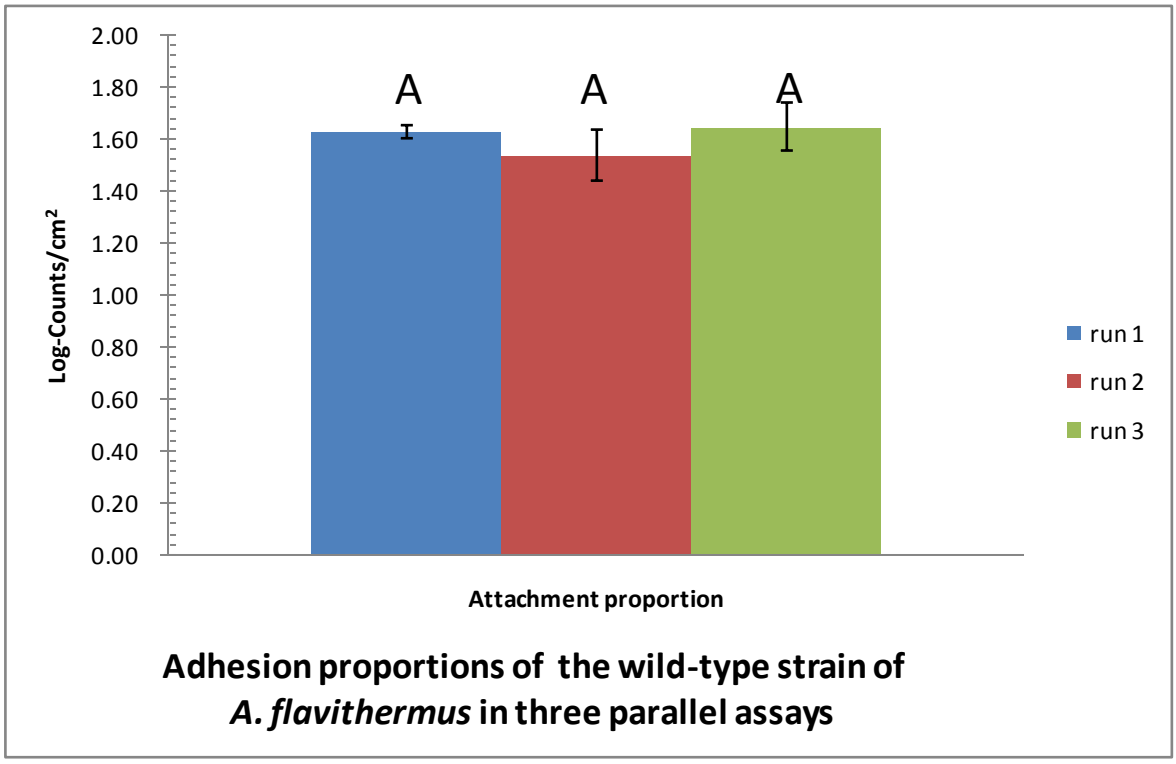

Figure 14: Three-bar graph demonstrating the mean values of the adhesion results of the three coupon samples (Upper, Middle and Lower) from each assay of the wild-type strain of *A. flavithermus*. Error bars represent the standard deviations of the means of the triplicate coupon samples.

According to Figure 14, the three mean log counts were statistically the same and all grouped in A.

b) Three parallel assays of the adhesion of the final sub-population strain of *A. flavithermus*

The mean log-counts from the three adhesion assays are found in Table 9 in the Appendix, and are presented as a comparative bar graph in Figure 15. According to the Tukey's groupings, the three runs were classified into the different groups depending on the mean concentration of the adhered cell log-counts from each assay.

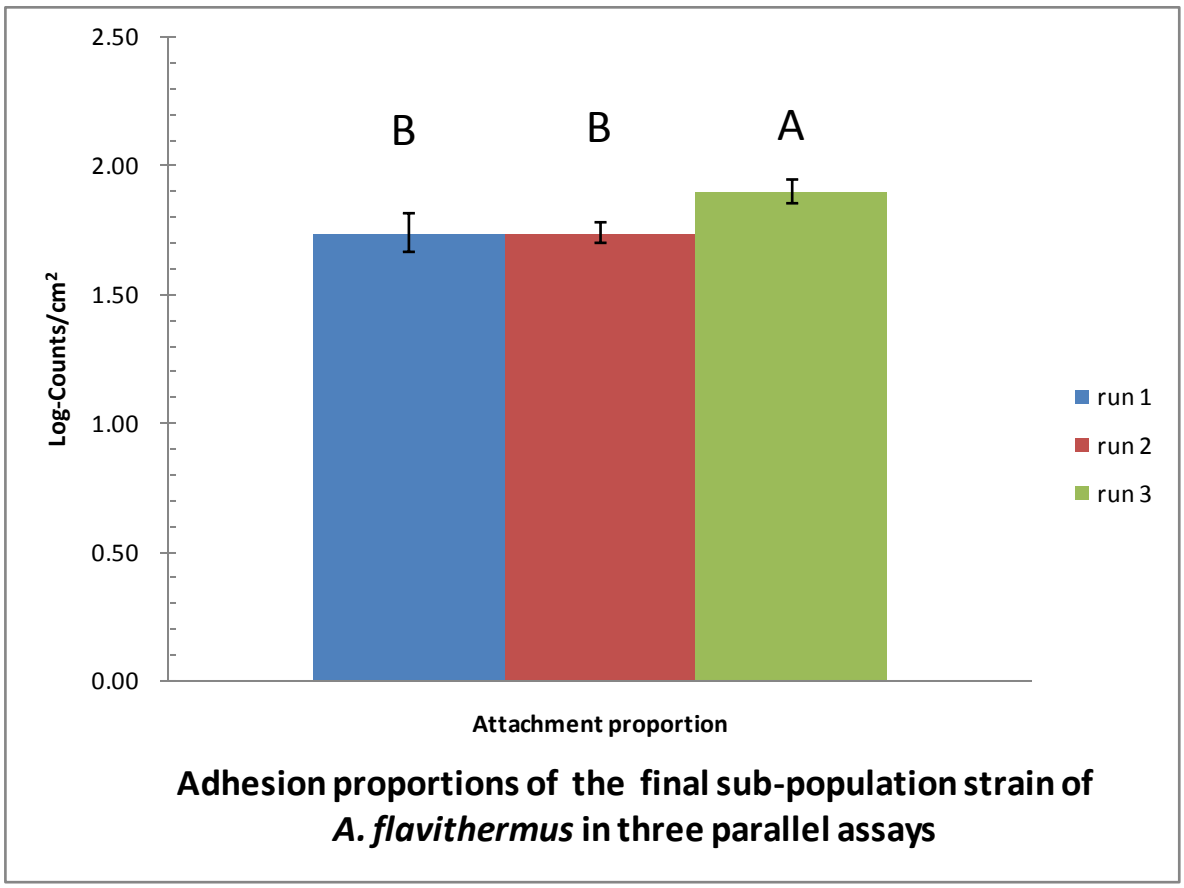

Figure 15: Three-bar graph demonstrating the mean values of the adhesion results of the three coupon samples (Upper, Middle and Lower) from each assay of the final sub-population strain of *A. flavithermus*. Error bars represent the standard deviations of the means of the triplicate coupon samples.

According to Figure 15, the mean adhesion cell log-counts result of run1 and run 2 (both 1.74 log-counts/cm^2) were significantly smaller than the mean result of run 3 (1.90 log-counts/cm^2).

c) Comparison of the adhesion of *A. flavithermus* wild-type and final sub-population strains on stainless steel surfaces

The mean log-counts of each strain of *A. flavithermus* are found in Table 10 in the Appendix, and presented as a comparative bar graph in Figure 16.

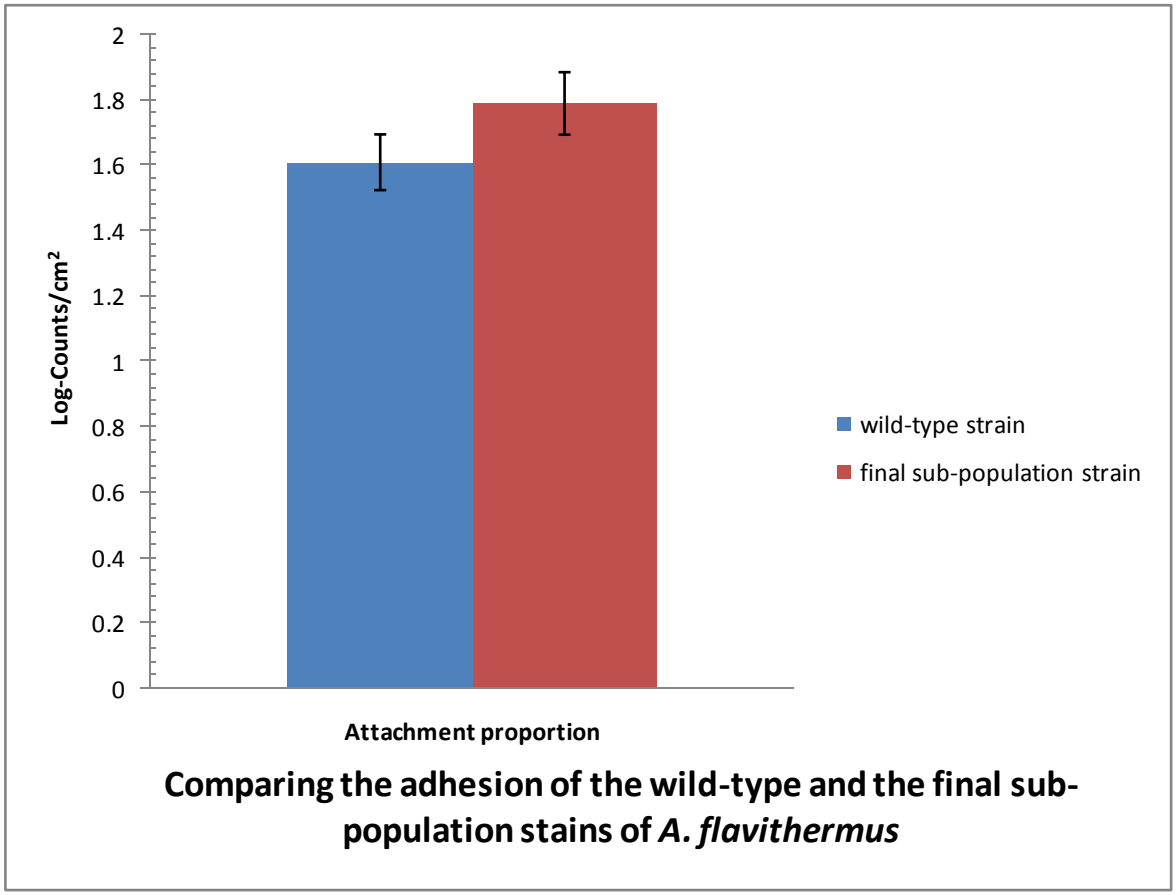

Figure 16: Two-bar graph demonstrating the total mean values of the adhesion results of the wild-type and final sub-population strains of *A. flavithermus*. Error bars represent the standard deviations of the means of nine coupon samples from three parallel assays.

According to the 2 samples t-test result which was calculated by the 18 coupon samples results (9 coupon samples for each strain), p-value = 0.001 < 0.05, therefore, the adhesion on stainless steel surfaces of final sub-population strain of *A. flavithermus* was

significantly higher than the wild-type strain of *A. flavithermus*.

4.3 Results of biofilm development experiment

For each thermophilic bacillus strain (*G. stearothermophilus* strain or *A. flavithermus* strain), the growth patterns of cells in out-flowing milk and the biofilms on coupons were measured and compared for the wild-type and the final sub-population strains. For each strain, three parallel experiments were performed. Moreover, three coupon samples and one out-flowing milk sample were measured at each sampling time. Each sample was plated in triplicate using the spiral plater.

All the colonies were counted manually. The numbers of bacteria were recorded as CFU/ml for the out-flowing samples and CFU/cm^2 for the coupon samples. The CFU/ml and CFU/cm^2 values were converted to the log-count values for analysis purposes. Then, the log-count values of the out-flowing milk samples and the coupon samples from the parallel experiments (run 1, run 2 and run 3) were respectively plotted against time in two graphs for each strain. All the curves on each scatter plot were normalised to the same start level for better comparison.

At the same sampling time, the three log-count values of cells in out-flowing milk from the three parallel experiments were averaged. The mean log-count values of the out-flowing milk samples of the wild-type and the final sub-population strains of *G. stearothermophilus* were plotted against time in one graph for comparison.

The mean maximum specific growth rates (μ_{max}) of the wild-type and the final sub-population were calculated by using the formula $\mu t = 2.303 * (\log X - \log X0)$. The 2-sample t-test of Minitab 16 software was used to calculate the p-value with confidence interval 95% to check whether the mean μ_{max} of the growth patterns of cells in out-flowing milk and cells from coupon biofilm samples of the final sub-population strain of *G. stearothermophilus* is significantly higher than the wild-type .

4.3.1 Results of biofilm development experiment of wild-type and final sub-population of *G. stearothermophilus*

Biofilm development of both the wild-type and final sub-population strains of *G. stearothermophilus* and cells present in the out-flowing milk were monitored in three parallel experimental runs by using the CDC reactor with stainless steel coupons (section 3.5).

a) Results of three parallel biofilm development experimental runs of the wild-type strain of *G. stearothermophilus*

At each sampling time, one out-flowing milk sample and three coupon samples were obtained from the CDC reactor and enumerated using a Whitley automated spiral plater.

(i) The mean log-counts of the cells in the out-flowing milk of the wild-type strain of *G. stearothermophilus* from the triplicate spiral plates for each biofilm development experimental run (55 ℃ for 12 hours) are found in Table 11 in the Appendix. The comparative plot of the $\log_{10}$ cell count numbers (Figure 17) of out-flowing milk samples

is shown below.

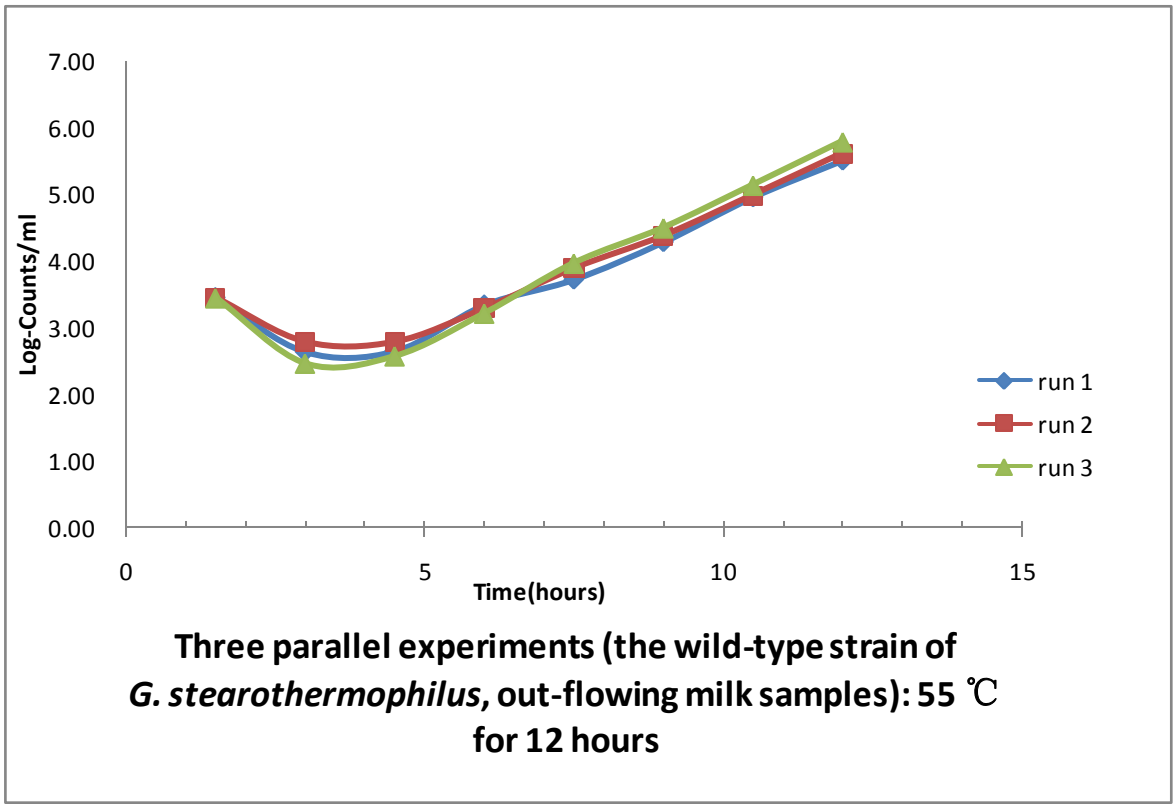

Figure 17: Three plots demonstrating the growth patterns of the wild-type strain of *G. stearothermophilus* in out-flowing milk in the three parallel experimental runs (55 ℃ for 12 hours). Error bars which are too small to see represent the standard deviations of the means of the triplicate spiral plates.

According to Figure 17, the three determinations of growth rate of the wild-type strain of *G. stearothermophilus* in out-flowing milk were similar over 12 hours. The final concentration of cells in out-flowing samples was around 5.63 log-counts/ml.

(ii) The mean log-counts of biofilm development on coupons that averaged from the triplicate coupon samples for each sampling time of each biofilm development experimental run (55 ℃ for 12 hours) of the wild-type strain of *G. stearothermophilus* are found in Table 12 in the Appendix, and presented as a comparative plot (Figure 18).

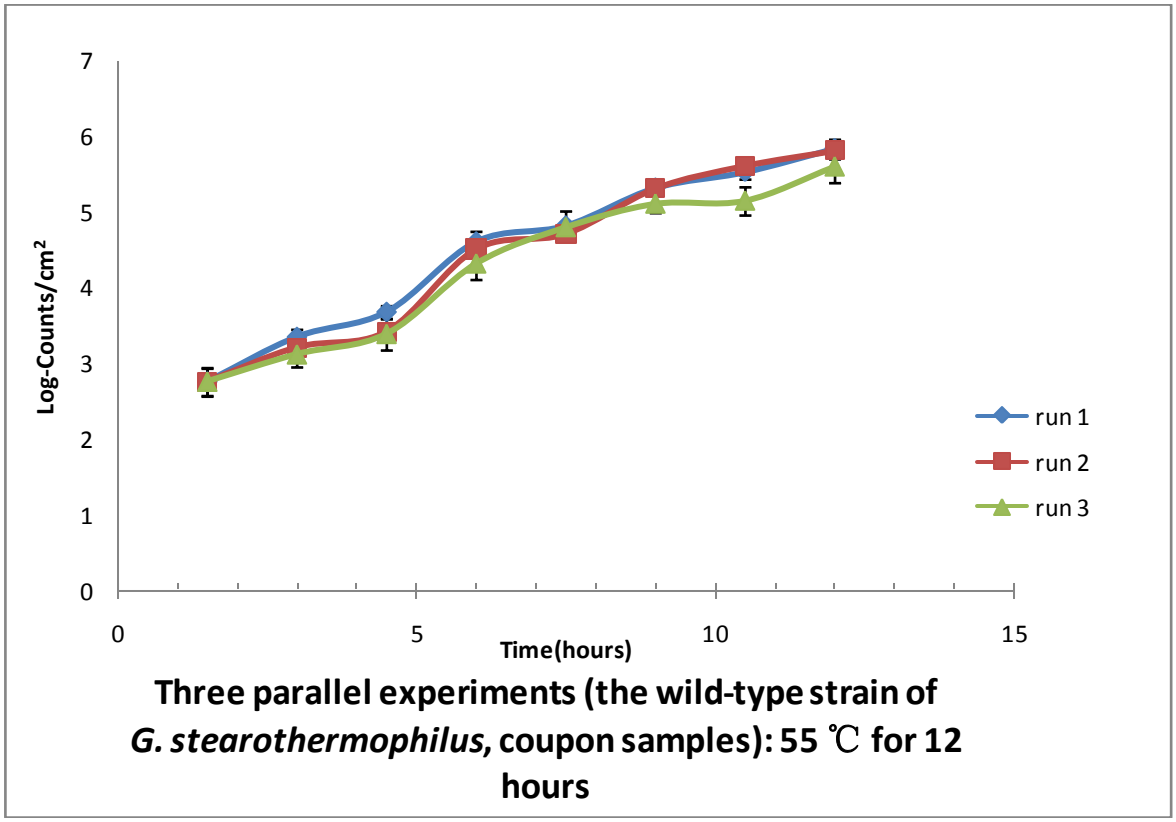

Figure 18: Three plots demonstrating the growth patterns of the wild-type strain of *G. stearothermophilus* on coupons in the three parallel experimental runs (55 ℃ for 12 hours). Error bars represent the standard deviations of the means of triplicate coupon samples.

According to Figure 18, the three biofilm growth patterns of the wild-type were similar, with the final cells concentration from the coupon samples at around 5.74 log-counts/ cm^2.

b) Results of three parallel biofilm development experimental runs of final sub-population strain of *G. stearothermophilus*

(i) The mean log-counts of the cells present in the out-flowing milk of the final sub-population strain of *G. stearothermophilus* averaged from triplicate spiral plates for each biofilm development experimental run (55 ℃ for 12 hours) are found in Table 13 in

the Appendix. Figure 19 shows the cell count profiles of out-flowing milk samples of the three parallel biofilm development experimental runs.

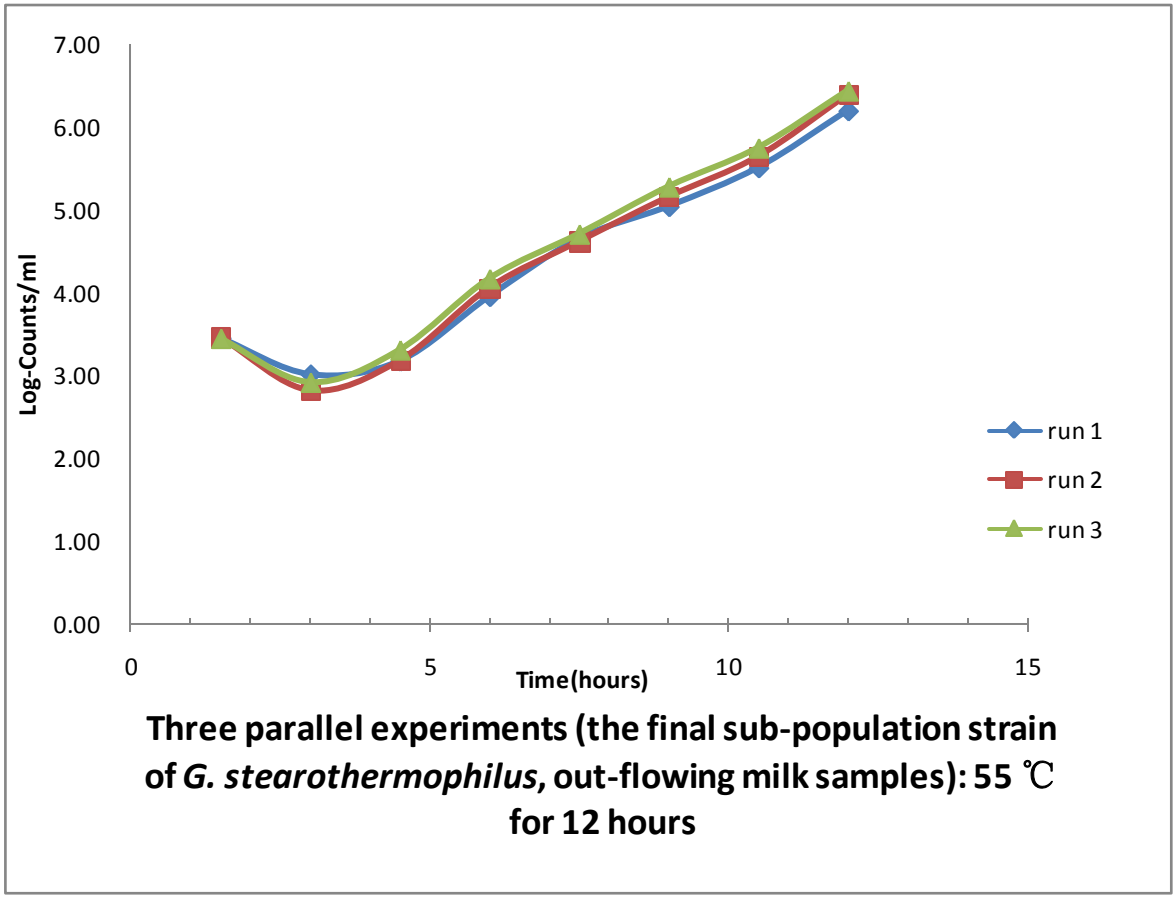

Figure 19: Three plots demonstrating the growth patterns of the final sub-population strain of *G. stearothermophilus* in out-flowing milk in the three parallel experimental runs (55 ℃ for 12 hours). Error bars which are too small to see represent the standard deviations of the means of triplicate spiral plates.

According to Figure 19, the three runs showed similar growth patterns of the final sub-population strain of *G. stearothermophilus* in out-flowing milk, with the final concentration of cells in out-flowing samples at around 6.34 log-counts/ml.

(ii) The mean log-counts of biofilm development of the final sub-population strain of

G. stearothermophilus on stainless steel coupons are found in Table 14 in the Appendix, and presented in Figure 20.

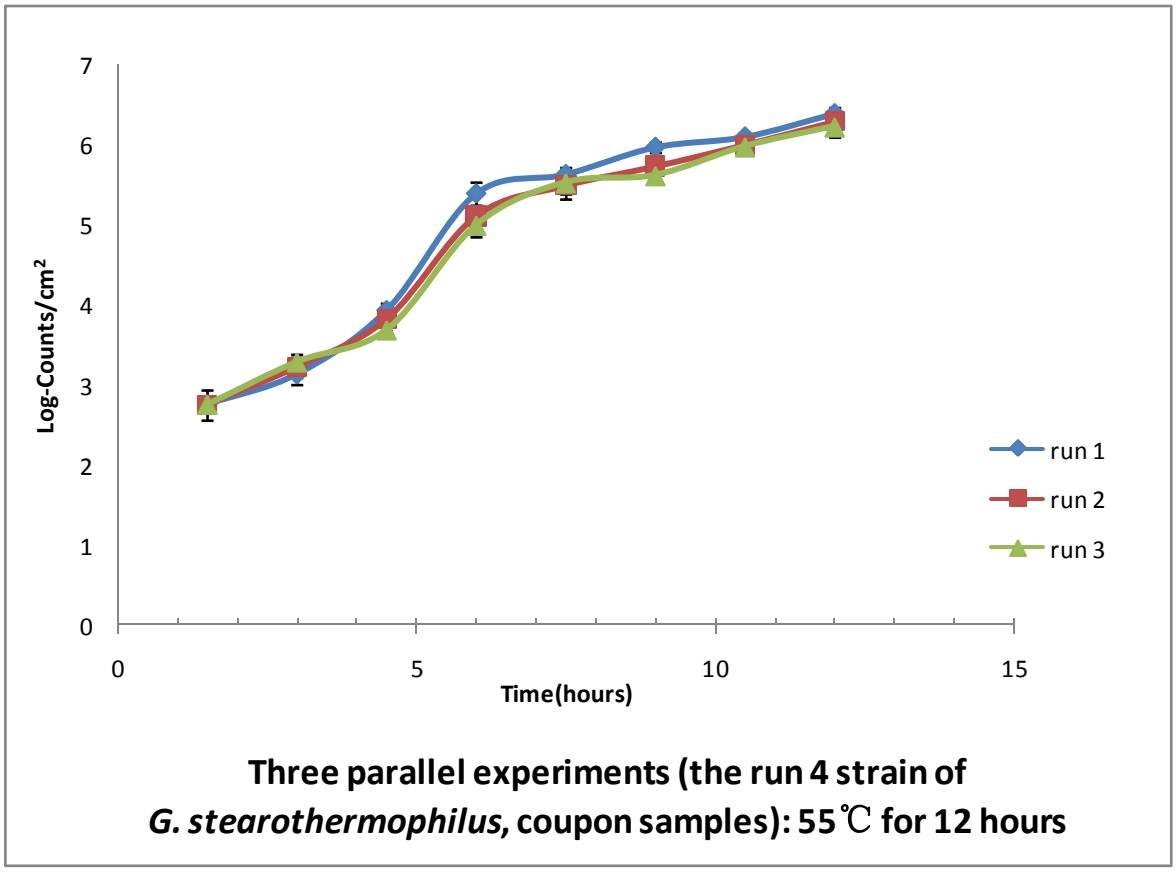

Figure 20: Three plots demonstrating the growth patterns of the final sub-population strain of *G. stearothermophilus* on coupons in the three parallel experimental runs (55℃ for 12 hours). Error bars represent the standard deviations of the means of triplicate coupon samples.

According to Figure 20, the three biofilm growth patterns of the final sub-population strain of *G. stearothermophilus* of the coupon samples showed similar curves, with rapid growth in the first 6 hours, then a more gradual increase to 12 hours, with the final cell concentration from the coupon samples at around 6.3 log-counts/ cm^2.

c) Comparison of the concentration of cells in out-flowing milk and the biofilms on coupons of the wild-type and the final sub-population strains of *G. stearothermophilus*

(i) The mean log-counts of the cells in the out-flowing milk of *G. stearothermophilus* strains are found in Table 15 in the Appendix and presented as a comparative plot (Figure 21) below.

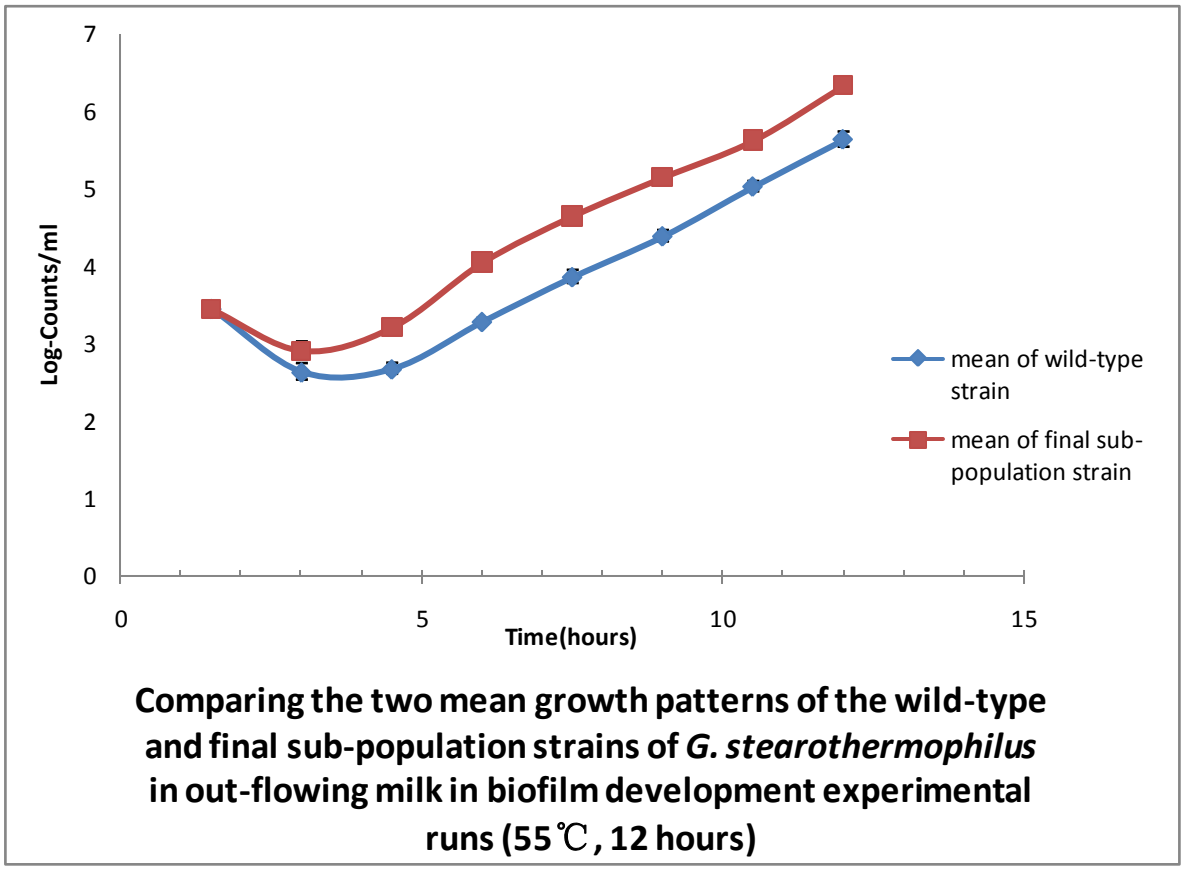

Figure 21: Two plots demonstrating the mean growth patterns of the wild-type and final sub-population strains of *G. stearothermophilus* in out-flowing milk during the biofilm development experimental runs (55℃, 12 hours). Error bars represent the standard deviations of the means of three out-flowing samples in three parallel runs.

According to Figure 21, during the biofilm development experimental runs (55℃, 12 hours), the wild-type strain of *G. stearothermophilus* produced fewer cells in

out-flowing milk than the final sub-population. The maximum specific growth rates (μ_{max}) were calculated for comparison of the cells releasing in out-flowing milk of the wild-type strain and final sub-population strain of *G. stearothermophilus* in the biofilm development experimental runs, and the μ_{max} are presented in Table 16.

Table 16: Maximum specific growth rates (μ_{max}, with the units /hour) of the wild-type and final sub-population strains of *G. stearothermophilus* recorded from out-flowing milk samples during the biofilm development experimental runs (55 ℃, 12 hours) along with the overall mean and standard deviation

	μ_{max}(a run)	**μ_{max}(b run)**	**μ_{max}(c run)**	**meanμ_{max}**	**Standard deviation**
Wild-type strain	0.46	0.40	0.50	0.45	0.05
Final sub-population strain	0.52	0.59	0.57	0.56	0.04

According to Table 16, for the out-flowing milk samples of the biofilm development experimental runs (55 ℃, 12 hours), the mean μ_{max} of the wild-type strain of *G. stearothermophilus* was 0.45 h^{-1}, and the meanμ_{max} of the final sub-population strain was 0.56 h^{-1}.

According to the 2 samples t-test result, p-value = 0.039 < 0.05, therefore, for the out-flowing milk samples of the biofilm development experimental runs (55 ℃, 12 hours), the mean μ_{max} of the final sub-population strain of *G. stearothermophilus* was significantly higher than the mean μ_{max} of the wild-type stain of *G. stearothermophilus*.

(ii) The mean log-counts of biofilm development on coupons of each strain of *G. stearothermophilus* were averaged from the results of three parallel biofilm development experimental runs (55℃ for 12 hours) and are found in Table 17 in the Appendix. Figure 22 shows the cell count profiles from the coupon samples of the wild-type and final sub-population strains of *G. stearothermophilus*.

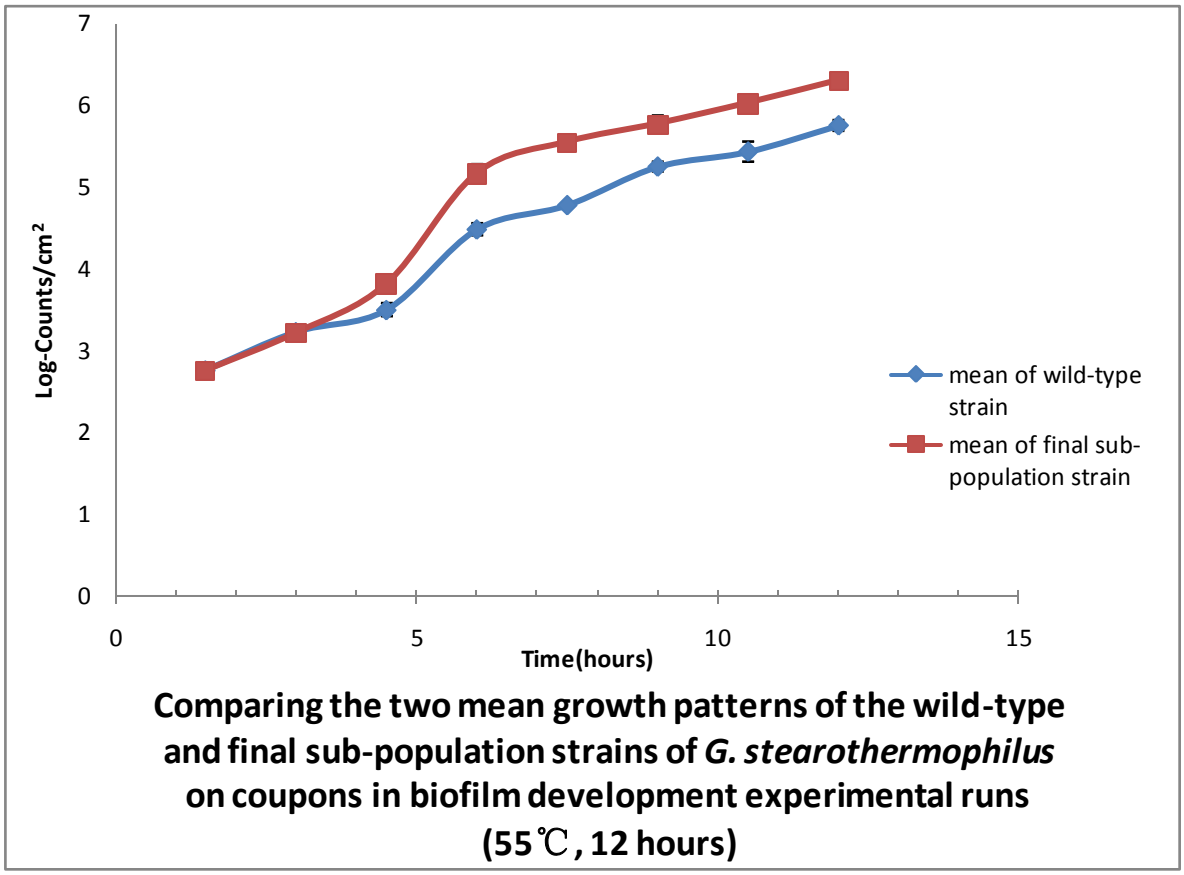

Figure 22: Two plots demonstrating the mean growth patterns of the wild-type and final sub-population strains of *G. stearothermophilus* on coupons during the biofilm development experimental runs (55℃, 12 hours). Error bars represent the standard deviations of the means of three mean values from the three parallel runs.

According to Figure 22, the wild-type strain produced fewer cells in the biofilm, compared with the final sub-population strain, both starting from the same concentration

2.75 log-counts/cm^2, and finished with the result of the wild-type strain at 5.74 log-counts/cm^2 and the result of the final sub-population strain at 6.3 log-counts/cm^2. The μ_{max} values were calculated and are shown in Table 18.

Table 18: Maximum specific growth rates (μ_{max}, with the units /hour) of the wild-type and final sub-population strains of *G. stearothermophilus* recorded from coupon samples during the biofilm development experimental runs (55 ℃, 12 hours) along with the total mean and standard deviation

	μ_{max}(a run)	**μ_{max}(b run)**	**μ_{max}(c run)**	**meanμ_{max}**	**Standard deviation**
Wild-type strain	0.62	0.73	0.62	0.66	0.06
Final sub-population strain	0.97	0.86	0.87	0.90	0.06

According to Table 18, for the coupon samples of the biofilm development experimental runs (55 ℃, 12 hours), the mean μ_{max} of the wild-type strain of *G. stearothermophilus* was 0.66 h^{-1}, and the mean μ_{max} of the final sub-population strain of *G. stearothermophilus* was 0.90 h^{-1}.

According to the 2 samples t-teat result, p-value = 0.008 < 0.05, therefore, for the coupon samples of the biofilm development experimental runs (55 ℃, 12 hours), the mean μ_{max} of the final sub-population strain of *G. stearothermophilus* was significantly higher than the mean μ_{max} of the wild-type stain of *G. stearothermophilus*.

4.3.2 Results of biofilm development experiment of wild-type and final sub-population of *A. flavithermus*

Biofilm development and cells present in the out-flowing milk were monitored for both the wild-type and final sub-population strains of *A. flavithermus* in three parallel experimental runs by using the CDC reactor with stainless steel coupons (section 3.5).

a) Results of three parallel biofilm development experimental runs of the wild-type strain of *A. flavithermus*

(i) The mean log-counts of the cells in the out-flowing milk of the wild-type strain of *A. flavithermus* from the triplicate spiral plates for each biofilm development experimental run (55 ℃ for 12 hours) are found in Table 19 in Appendix. The comparative plot of the cell count profiles (Figure 23) of out-flowing milk samples is shown below.

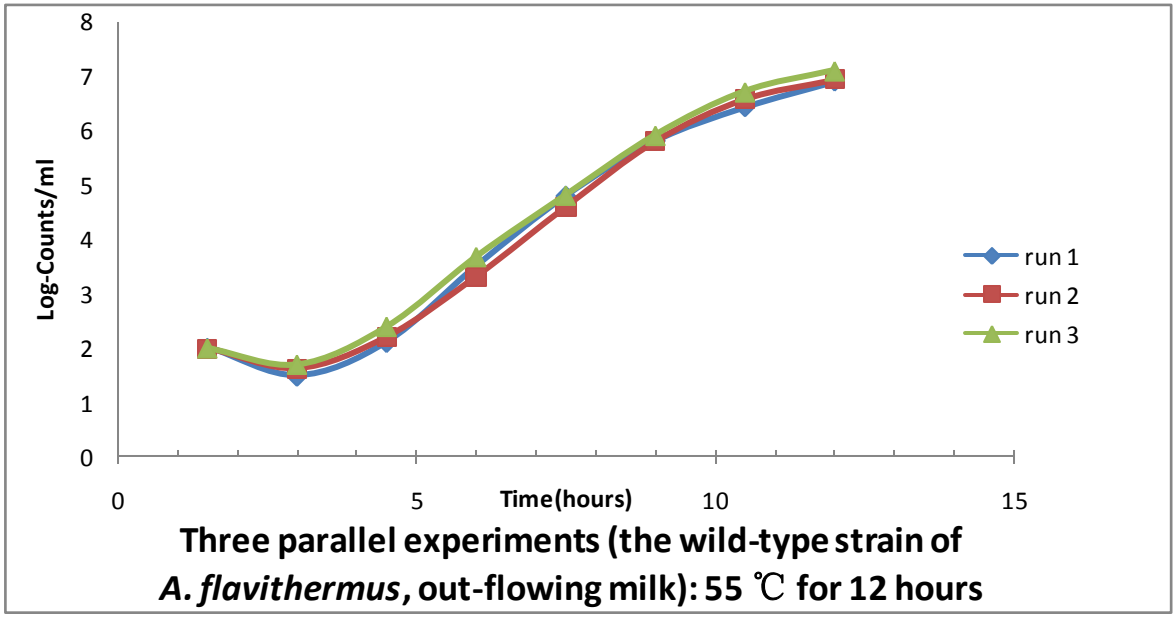

Figure 23: Three plots demonstrating the growth patterns of the wild-type strain of *A. flavithermus* in out-flowing milk in the three parallel experimental runs (55 ℃ for 12 hours). Error bars which are too small to see represent the standard deviations of the means of triplicate spiral plates.

According to Figure 23, the three runs show similar growth patterns of the wild-type strain of *A. flavithermus* in out-flowing milk with the final concentration of cells in out-flowing samples at around 6.97 log-counts/ml.

(ii) The mean log-counts of biofilm development on coupons of the wild-type strain of *A. flavithermus* are shown in Table 20 in the Appendix, and plotted in Figure 24.

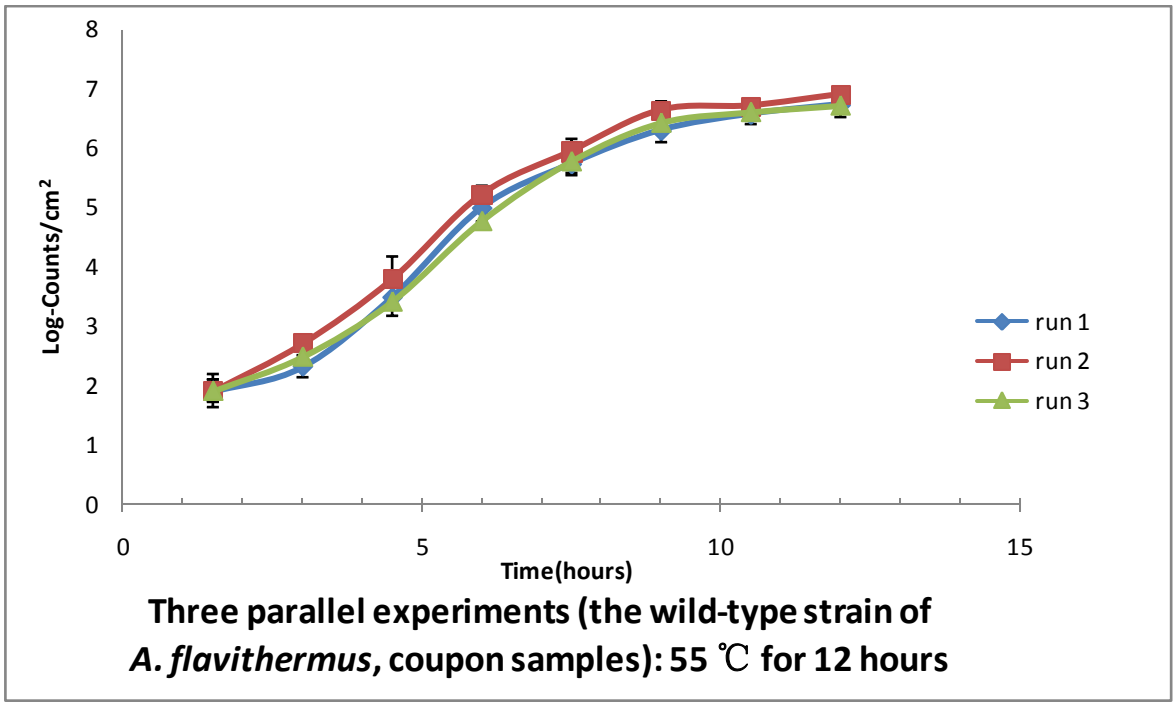

Figure 24: Three plots demonstrating the growth patterns of the wild-type strain of *A. flavithermus* on coupons in the three parallel experimental runs (55 ℃ for 12 hours). Error bars represent the standard deviations of the means of triplicate coupon samples.

As before, the three runs showed similar biofilm growth patterns of the wild-type strain with the final cell concentrations on the coupon samples at around 6.78 log-counts/ cm^2.

b) Results of three parallel biofilm development experimental runs of final sub-population strain of *A. flavithermus*

(i) The mean log-counts of the cells present in the out-flowing milk of the final sub-population strain of *A. flavithermus* are shown in Table 21 in the Appendix and plotted in Figure 25 below.

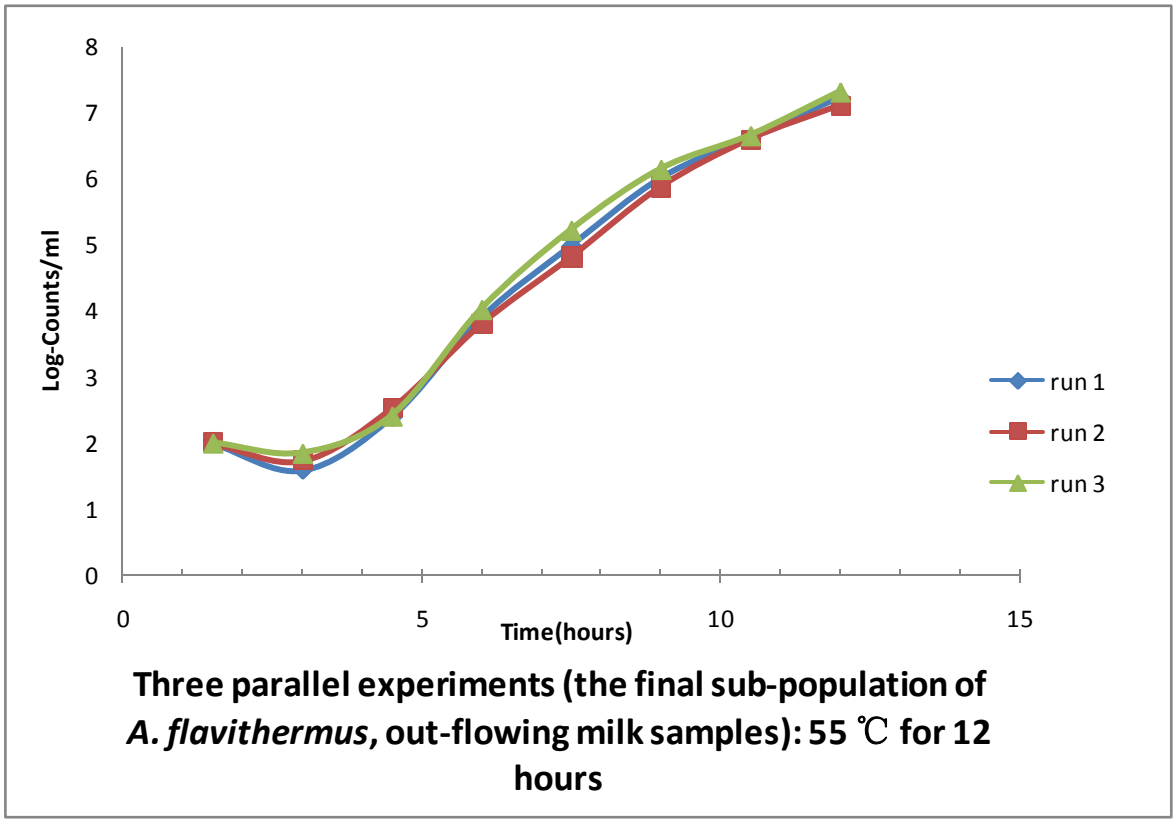

Figure 25: Three plots demonstrating the growth patterns of the final sub-population strain of *A. flavithermus* in out-flowing milk in the three parallel experimental runs (55 ℃ for 12 hours). Error bars which are too small to see represent the standard deviations of the means of triplicate spiral plates.

According to Figure 25, the three runs of the final sub-population strain of *A. flavithermus* produced similar growth curves, with the final concentration of cells in out-flowing samples at around 7.21 log-counts/ml.

(ii) The mean log-counts of biofilm development on coupons that averaged from the triplicate coupon samples for each sampling time of each biofilm development experimental run (55 ℃ for 12 hours) of the final sub-population strain of *A. flavithermus* are shown in Table 22 in the Appendix, and present as a comparative plot in Figure 26.

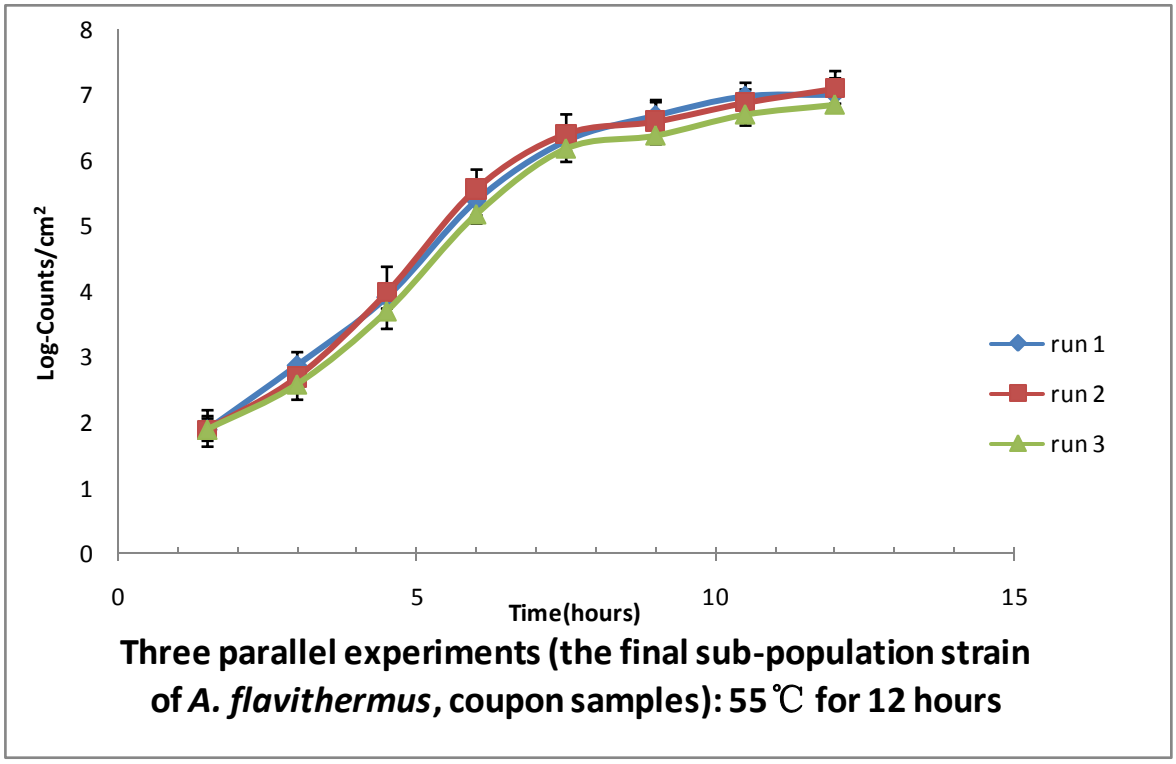

Figure 26: Three plots demonstrating the growth patterns of the final sub-population strain of *A. flavithermus* on coupons in the three parallel experimental runs (55 ℃ for 12 hours). Error bars represent the standard deviations of the means of triplicate coupon samples.

According to Figure 26, the three trials of the final sub-population strain of *A. flavithermus* grown on coupons produced similar growth curves with exponential growth slowing at around 7 hours. The final cell concentration from the coupon samples was around 6.99 log-counts/ cm^2.

c) Comparison of the concentration of cells in out-flowing milk and the biofilms on coupons of the wild-type and the final sub-population strains of *A. flavithermus*

(i) The mean log-counts of the cells present in the out-flowing milk of each strain are shown in Table 23 in the Appendix. The cell count profiles from the out-flowing milk samples of the wild-type and final sub-population strains of *A. flavithermus* shown in Figure 27.

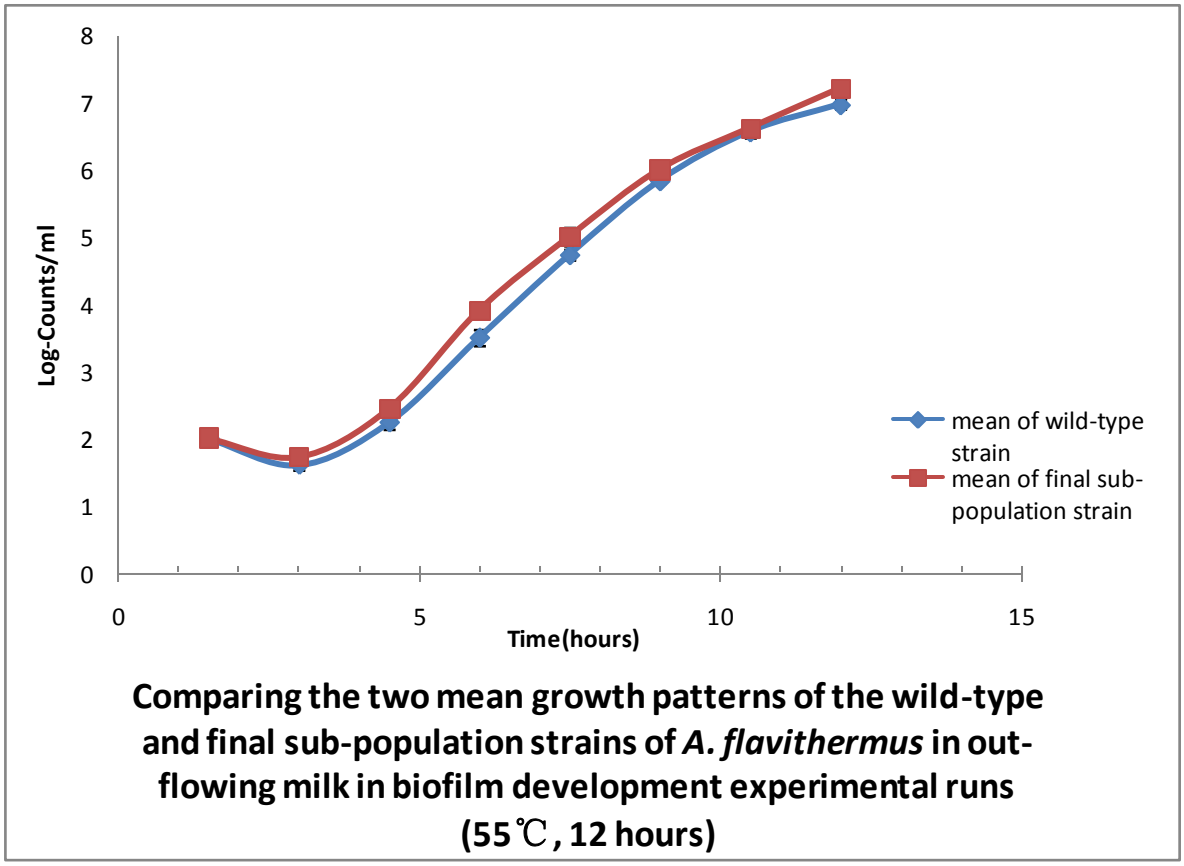

Figure 27: Two plots demonstrating the mean growth patterns of the wild-type and final sub-population strains of *A. flavithermus* in out-flowing milk during the biofilm development experimental runs (55 ℃, 12 hours). Error bars represent the standard deviations of the means of three out-flowing samples in three parallel runs.

From Figure 27, it is apparent the concentrations of the wild-type and final sub-population strains of *A. flavithermus* in out-flowing milk were essentially the same.

When the μ_{max} values were calculated, the final sub-population strain was found to have a maximum specific growth rate no different from the wild type (Table 24).

Table 24: Maximum specific growth rates (μ_{max}, with the units /hour) of the wild-type and final sub-population strains of *A. flavithermus* recorded from out-flowing milk samples during the biofilm development experimental runs (55℃, 12 hours) along with the total mean and standard deviation

	μ_{max}(a run)	μ_{max}(b run)	μ_{max}(c run)	meanμ_{max}	Standard deviation
Wild-type strain	0.93	0.85	0.86	0.88	0.05
Final sub-population strain	1.00	0.85	1.07	0.98	0.11

According to Table 24, for the out-flowing milk samples of the biofilm development experimental runs (55℃, 12 hours), the mean μ_{max} of the wild-type strain of *A. flavithermus* was 0.88 h^{-1}, and the mean μ_{max} of the final sub-population strain of *A. flavithermus* was 0.98 h^{-1}.

According to the 2 samples t-test result, p-value = 0.248 > 0.05, therefore, for the out-flowing milk samples of the biofilm development experimental runs (55℃, 12 hours), the mean μ_{max} of the final sub-population strain of *A. flavithermus* was not significantly higher than the mean μ_{max} of the wild-type stain of *A. flavithermus*.

(ii) The mean log-counts of biofilm development on coupons of each strain of *A. flavithermus* are found in Table 25 in the Appendix and plotted in Figure 28.

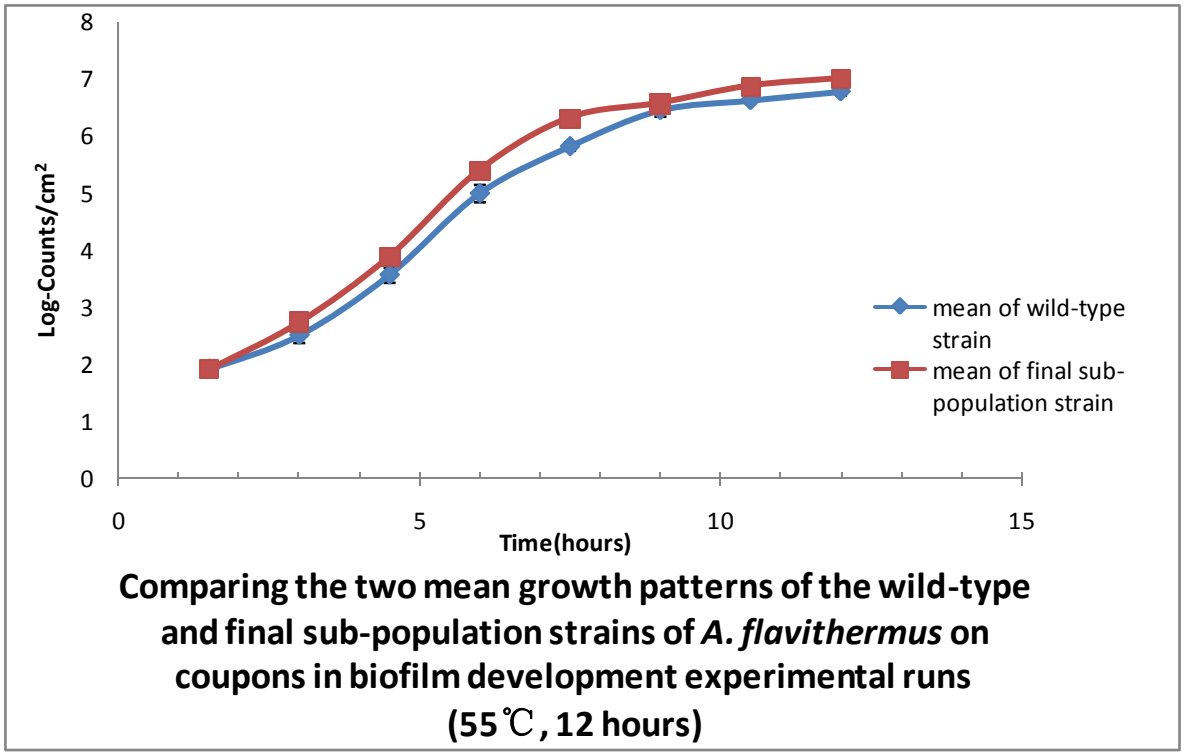

Figure 28: Two plots demonstrating the mean growth patterns of the wild-type and final sub-population strains of *A. flavithermus* on coupons during the biofilm development experimental runs (55℃, 12 hours). Error bars represent the standard deviations of the means of three mean values of the three parallel runs.

According to Figure 28, the mean growth pattern of the wild-type strain of *A. flavithermus* in the biofilm coupon samples was slightly below that of the final sub-population strain. The μ_{max} values were calculated to compare the growth on coupons of the wild-type and final sub-population strains of *A. flavithermus* (Table 26).

Table 26: Maximum specific growth rates (μ_{max}, with the units /hour) of the wild-type and final sub-population strains of *A. flavithermus* recorded from coupon samples during the biofilm development experimental runs (55 ℃, 12 hours) along with the total mean and standard deviation

	μ_{max}(a run)	μ_{max}(b run)	μ_{max}(c run)	meanμ_{max}	Standard deviation
Wild-type strain	1.01	0.95	0.90	0.95	0.05
Final sub-population strain	0.98	1.06	0.98	1.01	0.04

According to Table 26, for the coupon samples of the biofilm development experimental runs (55 ℃, 12 hours), the mean μ_{max} of the wild-type strain of *A. flavithermus* was 0.95 h^{-1}, and the meanμ_{max} of the final sub-population strain of *A. flavithermus* was 1.01 h^{-1}.

According to the 2 samples t-teat result, p-value = 0.235 > 0.05, therefore, for the coupon samples of the biofilm development experimental runs (55 ℃, 12 hours), the mean μ_{max} of the final sub-population strain of *A. flavithermus* is not significantly greater than the mean μ_{max} of the wild-type stain of *A. flavithermus*.

Chapter 5:

DISCUSSION

Some studies have shown that temperature cycling is an effective way to disrupt biofilm growth in dairy plants (Knight *et al.*, 2004; Kaur, 2014). Knight *et al.* demonstrated that the temperature cycling technique can reduce the biofilm growth of *Streptococcus thermophilus* in a pilot plant-scale cheese milk pasteurisation plate heat exchanger by using a temperature-time regime for 55 ℃ 10 minutes then 35 ℃ 60 minutes, which may extend the production cycle to 18 hours with a 6 hours extension (Knight *et al.*, 2004). However, few other studies have investigated the practicability of temperature cycling as a disruption technology for use in a dairy plant. One concern was that after being subjected to repeated temperature cycling, a sub-population would be selected, having a greater ability to survive the process and form biofilms in the plant. This would negate the value of the temperature cycling mode of operation.

The aim of this research was to investigate whether the behaviour of thermophilic bacilli, *G. stearothermophilus* and *A. flavithermus*, growing as biofilms would change after being subjected to repeated temperature cycling. The trends of the cell counts in the out-flowing milk over time, and the concentration of the biofilms formed on the reactors were followed over four serial temperature cycling runs that were compared with control runs in which the temperature remained constant. The abilities of the wild-type and the final sub-population strains to adhere to stainless steel surfaces and form biofilms were also compared. To this end, four temperature cycling runs were conducted at 55 ℃ for 15 minutes then 35 ℃ for 35 minutes over a total of 192 hours, when the sub-population was isolated for study. For the biofilm development experiment, the profiles of the cell counts

in out-flowing milk and from the biofilms on stainless steel coupons over time were measured in CDC reactors for the wild-type and final sub-population strains of each thermophilic bacillus to show the effects of extended temperature cycling runs.

In the case of *G. stearothermophilus,* temperature cycling did select a sub-population more resistant to temperature cycling and that successive sub-populations showed a shorter lag time before the counts of cells in the out-flowing milk began to increase, and the final cell concentration in the milk was greater with longer exposure to the temperature cycling regime. On the other hand, the density of the final biofilms from the hexagonal plate stainless steel reactors remained fairly constant over the four successive temperature cycling runs. Xie (2000) has stated that the gene expression and the behavior of the biofilm cells may change under stress conditions, such as in an environment in which the temperature is changing. Quorum Sensing (QS) may support a unified response to benefit the bacterial community of the biofilm (Davies, 1998). For the *G. stearothermophilus* strain, it may coordinate the adaptation the temperature cycling environment. Moreover, since the cells in the peripheral layer of a biofilm may detach and be released as planktonic organisms when the biofilm reaches its maturity (Allison *et al.*, 1998), this might explain why final biofilm concentration in temperature cycling runs did not show a bigger upward trend. Similar results were obtained for *A. flavithermus* under the same temperature cycling experiments. However, the effect on the out-flowing cell count profiles from Run 1 to Run 4 was less marked and adaptation appeared to be less discernible with successive sub-populations than with *G. stearothermophilus*. The

density of biofilms of successive sub-populations were also statistically indistinguishable. These results showed that this wild type *A. flavithermus* strain Anoxy2 is more resistant to temperature cycling under the current conditions (55 ℃ for 15 minutes, 35 ℃ for 35 minutes) than the wild type *G. stearothermophilus* strain Geo1.

For the adhesion assays, according to the 2-sample t-test result, the number of bacteria attached on stainless steel coupon surfaces of the final sub-population strain (from the final biofilm of temperature cycling run 4) of *G. stearothermophilus* was significantly higher than the wild-type. This means that the *G. stearothermophilus* strain from the NZ dairy plant adapted to temperature cycling after 192 hours running time, and the final sub-population has a greater ability to adhere to a stainless steel surface. A similar result was found for the *A. flavithermus* strain isolated from the same NZ dairy. These results were statistically significant at the 95% confidence level.

For the biofilm development experiments conducted in CDC reactors (55 ℃, 12 hours), all maximum specific growth rates (μ_{max}) of the log cell count profiles in out-flowing milk and the log cell count profiles of the biofilms that developed on stainless steel coupons were calculated and compared. According to the 2-sample t-test result, the mean μ_{max} of both the cell count profiles for out-flowing milk and for the biofilms over time of the final sub-population strain of *G. stearothermophilu* that developed on the stainless steel coupons was significantly higher than those of the wild-type strain. Thus the *G. stearothermophilus* strain from the NZ dairy plant adapted to temperature cycling after 192 hours running time and the final sub-population grew significantly faster on

stainless steel surfaces. However, the calculated μ_{max} results of the final sub-population strain of *A. flavithermus*, for both the cell counts in out-flowing milk and the cell counts of the biofilm that developed on the coupons, were not significantly higher than the measured μ_{max} of the wild-type strain. This means that the maximum specific growth rate in the biofilm and rate of shedding had not changed after exposure to temperature cycling, despite the fact that the strain showed increasing resistance to temperature cycling. Both species tested appear to be resistant to sequential temperature cycling treatments; *G. stearothermophilus* appears to be capable of adaptation, while *A. flavithermus* is initially more resistant. Since both of these species were isolated from milk powder produced in a New Zealand milk powder plant, rather than being drawn from a culture collection, this work suggests that the beneficial effects of temperature cycling may be short lived and that it remains critically important to conduct cleaning operations effectively, so that biofilms are totally removed from heated plant, thus preventing the selection of resistant sub-populations.

5.1 Future research

Because the *G. stearothermophilus* strain represents about 64.7% of the thermophilic isolates in some milk powders (Chopra & Mathur, 1984), the temperature cycling method is not a viable solution at this stage. Several factors need to be considered, such as the rate of variation of the thermophiles, competitive or synergistic interactions of the mixed culture, in addition to the financial aspects of temperature cycling (capital and running

costs). Especially, the mixed thermophilic bacilli of the *G. stearothermophilus* and *A. flavithermus* strains need to be studied further because of the competitive or synergistic interactions that could occur in mixed-species biofilms and the gap of understanding of the behaviour of thermophiles in industrial evaporation plants.

Because the shapes, sizes and colours of these two bacterial colonies are very close to each other, a new method of isolating pure strains from the mixed culture is needed, such as using different pH values of the growth media or using different temperatures to culture. A result from my work may help for the further study: at 70 ℃ only the *G. stearothermophilus* strain grew on the TSA, and the CFU on the plate were similar to the count at 55 ℃; the *A. flavithermus* strain did not grow at all. An ideal solution would be the development of chromogenic media to distinguish the two species. Molecular based methods can be used in future studies to confirm the identification of any isolates.

Chapter 6:

CONCLUSION

The objective of this research was to investigate the behaviour of sub-populations of thermophilic bacilli growing as biofilms on stainless steel surfaces after being subjected to temperature cycling. The bacteria tested in this project were *G. stearothermophilus* and *A. flavithermus* strains, which were isolated from a New Zealand milk powder manufacturing plant. The final sub-population strain of *G. stearothermophilus* displayed a significant increase in survival abilities, both in adhesion and biofilm development on stainless steel surfaces, compared with the wild-type strain after being subjected to temperature cycling for 192 hours. Temperature cycling did not result in a significant change in the ability of *A. flavithermus* to form biofilms.

The overall conclusion is that this work has shown that the temperature cycling method is not yet a viable solution for the control of thermophilic biofilms in dairy plants, and that further research is required into the optimum conditions for thermal cycling and the possible interactions of different species of bacteria under these conditions.

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APPENDIX

Table 1: Summary of the mean log-count values observed of the four serial temperature cycling runs (55 ℃ for 15 minutes, 35 ℃ for 35 minutes) and one control run (55 ℃ for 48 hours) of *G. stearothermophilus*

Time (hours)	Mean Log-Counts/ml (run 1)	Mean Log-Counts/ml (run 2)	Mean Log-Counts/ml (run 3)	Mean Log-Counts/ml (run 4)	Mean Log-Counts/ml (control run)
0.5	4.03	4.42	4.37	4.48	4.54
1.5	2.41	3.37	3.28	2.93	3.14
2.5	2.00	2.42	2.39	2.60	2.98
3.5	1.67	1.95	2.06	2.50	3.14
4.5	1.39	1.84	1.95	2.42	3.58
5.5	1.33	1.65	1.68	2.72	3.89
6.5	1.34	1.58	1.92	2.95	3.71
7.5	1.36	1.55	1.98	3.25	3.91
8.5	1.32	1.82	2.10	3.41	4.29
9.5	1.21	1.92	2.30	3.49	4.74
10.5	1.45	2.12	2.53	3.72	4.99
11.5	1.72	2.24	2.72	3.90	5.22
24	3.21	3.78	4.28	5.01	5.64
36	4.33	4.68	5.20	5.68	6.04
48	4.68	4.99	5.52	5.81	6.17

Table 2: Summary of the mean log-count values observed of the 48 hour biofilm samples from the four serial temperature cycling runs (55 ℃ for 15 minutes, 35 ℃ for 35 minutes) and one control run (55 ℃ for 48 hours) of *G. stearothermophilus*

	Mean Log-Counts/cm^2 (control run)	**Mean Log-Counts/cm^2 (run 1)**	**Mean Log-Counts/cm^2 (run 2)**	**Mean Log-Counts/cm^2 (run 3)**	**Mean Log-Counts/cm^2 (run 4)**
48 hours biofilms	6.67	5.1	5.76	5.65	5.79

Table 3: Summary of the mean log-count values observed of the four serial temperature cycling runs (55 ℃ for 15 minutes, 35 ℃ for 35 minutes) and one control run (55 ℃ for 48 hours) of *A. flavithermus*

Time (hours)	**Mean Log-Counts/ml (run 1)**	**Mean Log-Counts/ml (run 2)**	**Mean Log-Counts/ml (run 3)**	**Mean Log-Counts/ml (run 4)**	**Mean Log-Counts/ml (control run)**
0.5	1.71	1.65	1.77	1.47	1.70
1.5	1.37	1.69	1.60	1.35	1.05
2.5	1.07	1.58	1.59	1.39	0.85
3.5	1.44	1.83	1.87	1.66	1.00
4.5	1.73	2.13	2.28	2.18	1.42
5.5	1.90	2.36	2.49	2.59	2.35
6.5	2.26	2.59	2.65	2.72	3.20
7.5	2.44	2.66	2.76	2.87	4.02
8.5	2.62	2.84	2.97	3.23	4.77
9.5	2.73	2.97	3.18	3.43	5.40
10.5	2.98	3.19	3.43	3.66	5.89
11.5	3.07	3.38	3.59	3.83	6.23
24	6.04	6.45	6.66	6.80	7.09
36	6.16	6.55	6.84	7.01	7.36
48	6.58	6.82	6.99	7.19	7.55

Table 4: Summary of the mean log-count values observed of the 48 hour biofilm samples from the four serial temperature cycling runs (55 ℃ for 15 minutes, 35 ℃ for 35 minutes) and one control run (55 ℃ for 48 hours) of *A. flavithermus*

	Mean Log-Counts/cm^2 (control run)	**Mean Log-Counts/cm^2 (run 1)**	**Mean Log-Counts/cm^2 (run 2)**	**Mean Log-Counts/cm^2 (run 3)**	**Mean Log-Counts/cm^2 (run 4)**
48 hours biofilms	7.70	6.86	6.84	6.90	7.12

Table 5: Summary of the log-count values of adhesion observed from the coupon samples (Upper, Middle and Lower) from the three parallel assays of the wild-type strain of *G. stearothermophilus* and the means and standard deviations of each assay

	Log-Counts/cm^2 (Coupon sample U)	**Log-Counts/cm^2 (Coupon sample M)**	**Log-Counts/cm^2 (Coupon sample L)**	**mean**	**Standard deviation**
Run 1	2.15	2.10	2.30	2.18	0.10
Run 2	2.28	2.13	2.30	2.24	0.09
Run 3	2.41	2.24	2.28	2.31	0.09

Table 6: Summary of the log-count values of adhesion observed from the coupon samples (Upper, Middle and Lower) from the three parallel assays of the final sub-population strain of *G. stearothermophilus* and the means and standard deviations of each assay

	Log-Counts/cm^2 (Coupon sample U)	**Log-Counts/cm^2 (Coupon sample M)**	**Log-Counts/cm^2 (Coupon sample L)**	**mean**	**Standard deviation**
Run 1	2.42	2.37	2.47	2.42	0.05
Run 2	2.64	2.53	2.68	2.62	0.08
Run 3	2.54	2.46	2.59	2.53	0.07

Table 7: Summary of the mean log-count values of adhesion observed from the three parallel assays of the wild-type and final sub-population strains of *G. stearothermophilus* and the standard deviations of each strain

	Mean Log-Counts/cm^2	**Standard deviation**
wild-type strain	2.24	0.10
final sub-population strain	2.52	0.10

Table 8: Summary of the log-count values of adhesion observed from the coupon samples (Upper, Middle and Lower) from the three parallel assays of the wild-type strain of *A. flavithermus* and the means and standard deviations of each assay

	Log-Counts/cm^2 (Coupon sample U)	**Log-Counts/cm^2 (Coupon sample M)**	**Log-Counts/cm^2 (Coupon sample L)**	**mean**	**Standard deviation**
Run 1	1.65	1.60	1.63	1.63	0.03
Run 2	1.65	1.48	1.48	1.54	0.10
Run 3	1.70	1.54	1.70	1.65	0.09

Table 9: Summary of the log-count values of adhesion observed from the coupon samples (Upper, Middle and Lower) from the three parallel assays of the final sub-population strain of *A. flavithermus* and the means and standard deviations of each assay

	Log-Counts/cm^2 (Coupon sample U)	**Log-Counts/cm^2 (Coupon sample M)**	**Log-Counts/cm^2 (Coupon sample L)**	**mean**	**Standard deviation**
Run 1	1.74	1.81	1.66	1.74	0.08
Run 2	1.78	1.74	1.70	1.74	0.04
Run 3	1.85	1.93	1.93	1.90	0.05

Table 10: Summary of the mean log-count values of adhesion observed from the three parallel assays of the wild-type and final sub-population strains of *A. flavithermus* and the standard deviations of each strain

	Mean Log-Counts/cm^2	**Standard deviation**
wild-type strain	1.61	0.09
final sub-population strain	1.79	0.10

Table 11: Summary of the log-count values for each sampling time observed from the out-flowing milk samples of the wild-type strain of *G. stearothermophilus* from the three parallel biofilm development experimental runs

Time (hours)	**Run 1 (Log-Counts/ml)**	**Run 2 (Log-Counts/ml)**	**Run 3 (Log-Counts/ml)**
1.5	3.45	3.45	3.45
3	2.64	2.79	2.47
4.5	2.65	2.79	2.58
6	3.34	3.29	3.22
7.5	3.72	3.89	3.97
9	4.28	4.38	4.50
10.5	4.95	4.99	5.14
12	5.50	5.61	5.79

Table 12: Summary of the log-count values for each sampling time observed from the coupon samples of the wild-type strain of *G. stearothermophilus* from the three parallel biofilm development experimental runs

Time (hours)	**Run 1 (Log-Counts/ cm^2)**	**Run 2 (Log-Counts/ cm^2)**	**Run 3 (Log-Counts/ cm^2)**
1.5	2.75	2.75	2.75
3	3.34	3.20	3.11
4.5	3.67	3.41	3.38
6	4.60	4.50	4.31
7.5	4.81	4.70	4.79
9	5.31	5.30	5.10
10.5	5.52	5.60	5.14
12	5.83	5.80	5.59

Table 13: Summary of the log-count values for each sampling time observed from the out-flowing milk samples of the final sub-population strain of *G. stearothermophilus* from the three parallel biofilm development experimental runs

Time (hours)	**Run 1 (Log-Counts/ml)**	**Run 2 (Log-Counts/ml)**	**Run 3 (Log-Counts/ml)**
1.5	3.45	3.45	3.45
3	3.01	2.81	2.92
4.5	3.17	3.17	3.31
6	3.95	4.05	4.17
7.5	4.65	4.61	4.71
9	5.04	5.15	5.28
10.5	5.51	5.64	5.75
12	6.20	6.39	6.44

Table 14: Summary of the log-count values for each sampling time observed from the coupon samples of the final sub-population strain of *G. stearothermophilus* from the three parallel biofilm development experimental runs

Time (hours)	**Run 1 (Log-Counts/ cm^2)**	**Run 2 (Log-Counts/ cm^2)**	**Run 3 (Log-Counts/ cm^2)**
1.5	2.75	2.75	2.75
3	3.13	3.22	3.28
4.5	3.93	3.82	3.68
6	5.39	5.11	4.99
7.5	5.63	5.49	5.52
9	5.97	5.73	5.61
10.5	6.10	6.00	5.97
12	6.39	6.29	6.22

Table 15: Summary of the mean log-count values for each sampling time observed from the out-flowing milk samples of the wild-type and final sub-population strains of *G. stearothermophilus* from the biofilm development experimental runs and the standard deviations of each strain

Time (hours)	**Mean Log-Counts/ml (wild-type)**	**Mean Log-Counts/ml (sub-population)**	**Standard deviation (wild-type)**	**Standard deviation (sub-population)**
1.5	3.45	3.45	0.00	0.00
3	2.63	2.91	0.16	0.10
4.5	2.67	3.22	0.11	0.08
6	3.28	4.06	0.06	0.11
7.5	3.86	4.66	0.13	0.05
9	4.39	5.16	0.17	0.12
10.5	5.03	5.63	0.17	0.12
12	5.63	6.34	0.13	0.13

Table 17: Summary of the mean log-count values for each sampling time observed from the coupon samples of the wild-type and final sub-population strains of *G. stearothermophilus* from the biofilm development experimental runs and the standard deviations of each strain

Time (hours)	Mean Log-Counts/ cm^2 (wild-type)	Mean Log-Counts/ cm^2 (sub-population)	Standard deviation (wild-type)	Standard deviation (sub-population)
1.5	2.75	2.75	0.00	0.00
3	3.22	3.21	0.12	0.08
4.5	3.49	3.81	0.16	0.13
6	4.47	5.16	0.15	0.21
7.5	4.77	5.55	0.06	0.07
9	5.24	5.77	0.12	0.18
10.5	5.42	6.02	0.25	0.07
12	5.74	6.30	0.13	0.09

Table 19: Summary of the log-count values for each sampling time observed from the out-flowing milk samples of the wild-type strain of *A. flavithermus* from the three parallel biofilm development experimental runs

Time (hours)	Run 1 (Log-Counts/ml)	Run 2 (Log-Counts/ml)	Run 3 (Log-Counts/ml)
1.5	2.00	2.00	2.00
3	1.48	1.63	1.70
4.5	2.10	2.21	2.40
6	3.50	3.32	3.68
7.5	4.79	4.60	4.81
9	5.80	5.80	5.91
10.5	6.42	6.57	6.71
12	6.89	6.93	7.10

Table 20: Summary of the log-count values for each sampling time observed from the coupon samples of the wild-type strain of *A. flavithermus* from the three parallel biofilm development experimental runs

Time (hours)	**Run 1 (Log-Counts/ cm^2)**	**Run 2 (Log-Counts/ cm^2)**	**Run 3 (Log-Counts/ cm^2)**
1.5	1.90	1.90	1.90
3	2.31	2.70	2.48
4.5	3.48	3.78	3.40
6	4.99	5.21	4.76
7.5	5.73	5.94	5.76
9	6.30	6.63	6.41
10.5	6.57	6.71	6.59
12	6.73	6.89	6.70

Table 21: Summary of the log-count values for each sampling time observed from the out-flowing milk samples of the final sub-population strain of *A. flavithermus* from the three parallel biofilm development experimental runs

Time (hours)	**Run 1 (Log-Counts/ml)**	**Run 2 (Log-Counts/ml)**	**Run 3 (Log-Counts/ml)**
1.5	2.00	2.00	2.00
3	1.59	1.73	1.84
4.5	2.39	2.52	2.41
6	3.89	3.80	4.02
7.5	4.98	4.81	5.22
9	6.00	5.87	6.14
10.5	6.61	6.59	6.65
12	7.22	7.10	7.31

Table 22: Summary of the log-count values for each sampling time observed from the coupon samples of the final sub-population strain of *A. flavithermus* from the three parallel biofilm development experimental runs

Time (hours)	**Run 1 (Log-Counts/ cm^2)**	**Run 2 (Log-Counts/ cm^2)**	**Run 3 (Log-Counts/ cm^2)**
1.5	1.90	1.90	1.90
3	2.88	2.70	2.59
4.5	3.92	3.99	3.70
6	5.40	5.58	5.18
7.5	6.31	6.42	6.18
9	6.70	6.61	6.38
10.5	7.00	6.89	6.70
12	7.02	7.11	6.85

Table 23: Summary of the mean log-count values for each sampling time observed from the out-flowing milk samples of the wild-type and final sub-population strains of *A. flavithermus* from the biofilm development experimental runs and the standard deviations of each strain

Time (hours)	**Mean Log-Counts/ml (wild-type)**	**Mean Log-Counts/ml (sub-population)**	**Standard deviation (wild-type)**	**Standard deviation (sub-population)**
1.5	2.00	2.00	0.00	0.00
3	1.60	1.72	0.11	0.13
4.5	2.24	2.44	0.15	0.07
6	3.50	3.90	0.18	0.11
7.5	4.73	5.00	0.12	0.20
9	5.84	6.00	0.06	0.13
10.5	6.57	6.62	0.15	0.03
12	6.97	7.21	0.11	0.11

Table 25: Summary of the mean log-count values for each sampling time observed from the coupon samples of the wild-type and final sub-population strains of *A. flavithermus* from the biofilm development experimental runs and the standard deviations of each strain

Time (hours)	**Mean Log-Counts/ cm^2 (wild-type)**	**Mean Log-Counts/ cm^2 (sub-population)**	**Standard deviation (wild-type)**	**Standard deviation (sub-population)**
1.5	1.90	1.90	0.00	0.00
3	2.50	2.72	0.20	0.15
4.5	3.55	3.87	0.20	0.15
6	4.99	5.38	0.23	0.20
7.5	5.81	6.30	0.11	0.12
9	6.45	6.56	0.17	0.17
10.5	6.62	6.86	0.07	0.15
12	6.78	6.99	0.10	0.13