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Enzymes produced by bacteria within biofilms of dairy origin and their effect on dairy products

**A thesis presented in partial fulfilment of the requirements for
the degree of Doctor of Philosophy**

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ABSTRACT

Prior to the current study, there was no scientific evidence that enzymes produced by bacteria within biofilms in milk transport tanker could have a detrimental effect on the quality of dairy products.

Bacteria attached to the internal surfaces of milk tankers were isolated, identified, and characterized in terms of their ability to produce heat-stable enzymes (protease and lipase) and to form biofilms. Twelve of the bacterial isolates were identified by 16s DNA sequencing as belonging to the genera *Bacillus*, *Staphylococcus*, *Streptococcus*, *Pseudomonas*, and *Serratia*.

Six of the dairy bacterial isolates were evaluated for their ability to produce proteolysis in milk when growing within either single culture or co-culture biofilms in an *in vitro* model system that simulated the upper part of a milk tanker during a typical summer's day of milk collection in New Zealand. Proteolysis per cfu decreased as the temperature of incubation increased (20–37 °C), and proteolysis per cfu was generally higher within biofilms compared with the corresponding planktonic cultures.

Lipolysis by bacteria within biofilms in the *in vitro* model was investigated using single or co-culture biofilms or planktonic cultures of four dairy bacteria and a known lipase-producing bacterium. The hydrolysis of *p*-nitrophenol palmitate was at least 10 times higher by bacteria within biofilms (0.01 to 8.35 nU/CFU) than in planktonic cultures (0.01 to 0.07 nU/CFU).

The effect of proteases on UHT skim milk was determined by exposing sterile skim milk to a multispecies biofilm formed on an *in vitro* model of a milk tanker. The amount of free peptides which indicated proteolysis in the UHT milk was monitored over five months of storage. Free peptides were higher in UHT milk that had been made from milk exposed to the multispecies biofilm, than in UHT milk that had been made from milk that had not been exposed to the biofilm. Enzymes that are secreted from biofilms into raw milk during transportation can potentially reduce the quality of dairy products. Improvements at this early stage of dairy manufacture may reduce economic loss in the dairy industry.

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2. Teh, K. H., Flint, S., Palmer, J., Andrewes, P., Bremer, P., Lindsay, D., 2012. Proteolysis produced within biofilms of bacterial isolates from raw milk tankers. *International Journal of Food Microbiology* 157, 28-34.
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ABBREVIATIONS

4-MU	4-methylumbelliferone
AHL	<i>N</i> -acylhomoserine lactone
BNA	Biofilm formation on the stainless steel surface under nutrient abundance
BNL	Biofilm formation on the stainless steel surface under nutrient limitation
CIP	Clean in place
cfu	Colony forming units
DNA	Deoxyribonucleic acid
eDNA	extracellular DNA
ELISA	Enzyme-linked immune-sorbent assay
EON	Biofilm formation on the stainless steel surface of an inadequately cleaned tanker that was left to stand empty overnight
EPS	Exopolysaccharides
GC	Gas chromatography
HPLC	High-performance liquid chromatography
MPCA	Milk plate count agar
MSI	Ministry of Science and Innovation
NB	Nutrient broth
NSLAB	Non-starter lactic acid bacteria
PAS	Planktonic growth in the absence of stainless steel surfaces
PCR	Polymerase chain reaction
pnpp	<i>p</i> -nitrophenol palmitate
PPS	Planktonic growth in the presence of stainless steel surfaces
PQQ-ADH	Pyroquinoline quinone-dependent alcohol dehydrogenase
rDNA	Ribosomal DNA
RI	Refractive Index
RPM	Revolutions per minute
RSM	Reconstituted skim milk
SEB	Staphylococcal Enterotoxin B
SEM	Scanning electron microscopy
SMC	Biofilm formation on the stainless steel surface of an inadequately cleaned tanker with subsequent milk collection

SS	Stainless steel
TMC	Biofilm formation on the stainless steel surface after a typical 9.25 h of milk collection
Tris-HCl	Tris-hydrochloride
UV	Ultraviolet

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Chapter 1. Introduction

1.a Background

The international growth in the dairy industry has seen the development of large manufacturing plants and associated issues related to the handling of large volume of milk and milk products. Milk is a perishable product and maintaining quality can be difficult. The quality of the milk can be compromised by operational factors (handling, transportation), and natural factors (bacterial or indigenous enzymes, somatic cell counts). The poor quality of raw milk can have an economic impact on the dairy industry including reduced yields, poor product functionality, and the occurrence of sensory defects such as bitterness or rancidity (Celestino et al., 1997a, Chen et al., 2003; Guinot-Thomas et al., 1995; Shah 1997). Starting with a quality raw material is important and a challenge when having to transport raw milk long distances to a manufacturing plant.

The presence of biofilms within milk tankers contributes to microbial contamination and may be a source of microbial enzymes during the transportation of raw milk. The formation of biofilms can occur because of:-

- High temperature ($>7^{\circ}\text{C}$) in bulk raw milk collect from the farm
- The long distance bulk raw milk is transported
- The absence of refrigeration systems in milk tankers
- Inadequate and sanitation of milk tankers

If the temperature of bulk raw milk exceed 7°C , it can promote bacterial growth (Flint et al., 2005), and the formation of biofilms during raw milk transportation. Biofilms are usually composed of a variety of bacteria including pathogenic and spoilage organisms, all interacting in a microbial community (Sutherland, 2001). It has been suggested that microbial cells within biofilms are the predominant form in nature and that they may function at a level that is similar to multicellular organisms (Costerton et al., 1995, Davey and O'Toole, 2000, Gilbert et al., 1993). Biofilm formation may enhance the survivability of bacteria, for example, *Pseudomonas fluorescens* is more resistant to chlorine dioxide based sanitizers when grown as biofilms in co-culture with *Bacillus cereus* (Lindsay et al., 2002). Once biofilms are formed, they are difficult to remove possibility due to the exopolysaccharides and heterogeneity of cells which occur within the biofilm matrix (Hood and Zottola, 1997).

Biofilms may provide bacteria with a niche for both extracellular and cell-associated enzyme production. Enzyme production is known to occur within medical and industrial biofilms such as waste water treatment and fine chemical production (Bartowsky and Henschke, 2008, Dagher et al., 2010, Nucleo et al., 2010, Rosche et al., 2009, Wang and Chen, 2009). However, the production of spoilage enzymes within biofilms, and their effects on product quality has not been investigated within the dairy industry.

1.b *Motivation*

The undesirable effects of bacterial enzymes on dairy products have been reported (Celestino et al., 1997b, Chen et al., 2003, Schroeder et al., 2008, Shah, 1994), however, most of these studies examined the enzymes produced in planktonic cells, not by bacteria within biofilms (Marchand et al., 2009b, Nemeckova et al., 2009, Sørhaug and Stepaniak, 1997). For example, the metabolic activities and physiological state of microbial cells within biofilms are different from the microbial cells in planktonic form, and the amount of enzymes produced is greater within the sludge biofilm community than in the planktonic community (Frølund et al., 1995, Oosthuizen et al., 2001). The activity of enzymes is likely to occur at various stages of dairy manufacture, starting with raw milk at the dairy farm, during transportation, the various stages of dairy product manufacture and finally the dairy products on the commercial market shelves.

Enzyme production from biofilms forming during the transportation of raw milk are likely to have the biggest impact on manufactured dairy products as they are generated at the start of the manufacturing process. There is however, to date no information to support this hypothesis. The aim of the thesis is to establish scientific evidence to determine if spoilage enzymes produced by bacteria within biofilms during the transportation of raw milk can have detrimental effect on dairy products.

1.c Hypothesis and objectives

The hypothesis of this study was that spoilage enzymes produced by bacteria within biofilms during the transportation of raw milk can have detrimental effects on dairy products. In order to establish the scientific evidence for the hypothesis, the following objectives were set:-

1. To determine the internal surface temperatures of a milk tanker during its daily operation (**Chapter 3.a**)
2. To isolate and identify the bacteria found on the internal surfaces of milk tankers after milk collection, and characterize the bacteria in terms of enzyme production and ability to form biofilm (**Chapter 3.b**)
3. To measure the production and accumulation of protease from biofilms formed in an *in vitro* model that simulates the fluctuating surface temperatures of a milk tanker during its typical daily operation (**Chapter 4.a**)
4. To measure the lipolysis from single and co-culture biofilms in three different scenarios, lipolysis within biofilms in the presence or absence of liquid medium and lipolysis within the planktonic population (**Chapter 4.b**)
5. To measure the free peptides and pH change in UHT product previously exposed to a multispecies biofilm under conditions that model the transportation of raw milk in a milk tanker (**Chapter 5.a**)

Note: The findings from objective 2, 3 and 4 have been published in peer-reviewed journals, while the findings from object 5 have been submitted to peer-reviewed journal. Most of main findings have been presented at national or international conferences as poster or oral presentations.

Chapter 2. Literature Review

2.a ***Biofilms: an unrecognised source of dairy spoilage enzymes?***

2.a.1 ABSTRACT

Enzymes play an important role in food processing, where they either increase or decrease the value of food commodities. Within the dairy context, undesirable enzymes include indigenous enzymes originating from the cow, or microbial enzymes produced by the natural bacteria associated with the cow and its environment. Some of the heat-stable enzymes can remain active after the heat treatments applied during processing and eventually reduce the quality of the final product. Biofilms may play a role in promoting enzyme production in microorganisms due to microenvironments created within the biofilm. Many studies have been carried out on indigenous and bacterial enzymes that occur in milk, but few studies have looked at the relationship between spoilage enzymes and biofilms. We suggest that bacterial biofilms in dairy manufacture may be an unrecognised source of dairy spoilage enzymes.

2.a.2 INTRODUCTION

Biofilms are communities of microbial cells, and are enclosed in a matrix of primarily polysaccharide materials and other non-cellular materials which can be found virtually on all surfaces (Donlan, 2002).

Biofilms are usually composed of a wide range of bacteria, and this may promote interaction among the bacteria within the biofilms (Sutherland, 2001). It has been suggested that microbial cells within biofilms are the predominant form in nature and that they may function at a level that is similar to multicellular organisms (Costerton et al., 1995, Davey and O'Toole, 2000, Hood and Zottola, 1997).

This review will discuss the structure and the formation of biofilms in general, and explore the evidence for enzyme production within medical and industrial biofilms and look at how enzyme production from biofilms may affect the dairy industry. This review will also discuss the role of spoilage enzymes in the dairy industry, their method of detection, and the possibility of spoilage enzyme production within dairy biofilms.

2.a.3 BACTERIAL BIOFILM

2.a.3.1 Structure of biofilms

An integral part of a biofilm is the extracellular matrix consisting primarily of expolysaccharides (EPS) which account for 50% to 90% of the total organic carbon in the extracellular polymeric substances. Most of the EPS possess backbone structures that have 1, 3- or 1, 4- β -linked hexose residues, which are rigid in structure, and either poorly soluble or insoluble (Donlan, 2002, Sutherland, 2001). These EPS molecules may be present as a gel due to the entanglements of long chains of stiff macromolecules and also, for some polymers, to the ionic environments in which they occur (Sutherland, 2001). Even though most of EPS are rigid in structure, some EPS structures can be flexible due to 1, 2- α - or 1, 6- α -linkages in their polysaccharides. The overall structures of the polysaccharides determine the primary conformation of the EPS within biofilms.

The biofilm extracellular matrix also contains material of cellular origin, including extracellular DNA (eDNA). The presence of eDNA in biofilms is due to an number of mechanisms including cell lyses, and it is accumulated within biofilms in a filamentous

network of grid-like structures (Allesen-Holm et al., 2006, Böckelmann et al., 2006, Flemming et al., 2007, Palmgren and Nielsen, 1996, Turnbull and Whitchurch, 2012). This filamentous network is postulated to act as a means of communication or intercellular connections between the cells within the biofilm (Allesen-Holm et al., 2006, Flemming et al., 2007, Yang et al., 2007). Biofilms may also contain non-cellular materials, such as protein particles, fats, mineral, and corrosion materials in the matrix. These non-cellular materials are incorporated into the biofilms from the environment surrounding where the biofilms are formed (Denyer et al., 1993, Donlan, 2002, Sutherland, 2001).

The physicochemical properties of the extracellular matrix vary between biofilms due to the environment and the types of microbial communities that form the biofilms. Single species biofilms tend to have less EPS compared with multi species biofilms. For example, single species biofilms of either *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* are thinner compared with the corresponding mixed species biofilms (James et al., 1995). Furthermore, older biofilms of acidophilic microbial biofilms tend to have higher amounts of EPS than younger biofilms (Jiao et al., 2010).

2.a.3.2 Formation of biofilms

The formation of biofilms involves several factors which influence the interaction between the microbial cell and the surfaces. These can be classified into three major influences; the substratum surfaces, environment and microbial cells which are discussed in the following sections.

2.a.3.2.1 Influence of substratum surfaces

The attachment of microbial cells onto substratum surfaces can be influenced by the charge on the substratum surfaces (Palmer et al., 2007). An electrostatic barrier can occur between the substratum surface and microbial cells due to the similarity of electrostatic charge. This electrostatic barrier needs to be overcome by an attractive force before the microbial cells can attach onto the surfaces (Denyer et al., 1993). Similarly, attractive forces can occur when the electrostatic charge between the microbial cells and the substratum surfaces are different.

Besides the electrostatic property of the substratum surfaces, the hydrophobicity of the surfaces may also affect the microbial attachment where microbial cells more readily to

attach to hydrophobic, non-polar surfaces such as Teflon and other plastics, than to hydrophilic materials such as glass or stainless steel (Araujo et al., 2010 , Bendinger et al., 1993 , Donlan, 2002, Sinde and Carballo, 2000). For example, *Salmonella* and *Listeria monocytogenes* have better attachment on plastic surfaces (hydrophobic materials) compared with stainless steel surfaces (hydrophilic materials) (Veluz et al., 2012).

The composition of materials such as copper, iron, magnesium, titanium or calcium may affect the microbial attachment (Arnold and Silvers, 2000, Whitehead and Verran, 2007, Wilks et al., 2006). Some metals such as molybdenum and silver may have antimicrobial properties which may reduce the initial attachment of bacterial cells. A pure molybdenum surface was shown to support lower bacterial cell counts compared with a stainless steel surface (Percival, 1999). An example of the use of these antimicrobial surfaces are catheters that are coated with silver where the attached population of *Staphylococcus aureus* is lower compared with the untreated catheters, even after 30 days of incubation (Paladini et al., 2012).

The effect of surface roughness on microbial attachment is inconclusive. There are studies that demonstrate that the surface roughness correlates with microbial attachment, while others show no correlation (Coquet et al., 2002, Flint et al., 2000, Goulter-Thorsen et al., 2011, Nguyen et al., 2012b, Rodriguez et al., 2008). For example, the attachment of two bacterial strains of *Yersina* correlates with the roughness amplitude of the surface, while the attachment of thermo-resistant streptococci has a low correlation with the roughness amplitude (Coquet et al., 2002, Flint et al., 2000). The inconclusive effects of the surface roughness on attachment are most possibility due to the different bacterial strains and the methods used in those studies.

Surface conditioning of the substratum surface can result in physicochemical modification of the material surface which may affect the rate and the extent of microbial attachment (Denyer et al., 1993). These effects can be either advantageous or detrimental to the initial attachment. Surfaces that were previously conditioned with cooked mussel juice have a higher initial microbial attachment than those that have not been exposed to the juice (Saa et al., 2009). However, in another study, where organic material were present on surfaces, the attachment of *Salmonella* and *Listeria monocytogenes* was reduced compared with attachment to clean surfaces (Veluz et al., 2012). This observation is also supported by

another study where bacterial attachment on stainless steel surfaces was reduced due to the soiling of the surfaces by a variety of animal and plant extracts (Bernbom et al., 2009). With prolonged incubation, the number of bacterial cells in the biofilms will increase due to growth on surface.

2.a.3.2.2 *Environmental influences*

The flow velocity of the aqueous medium in the environment may influence the rate of attachment. Linear velocity is dependent on the hydrodynamic boundary layer where the region of flow immediately adjacent to the substratum/liquid is slow. The region outside the boundary layer is characterized by substantial mixing or turbulence (Donlan, 2002). Microbial attachment can only occur after the microbial cells traverse between the boundary layer and the surface. The boundary layer decreases, as the velocity increases, and the turbulence outside the boundary layer increases. Therefore, high flow velocity can increase the rate of attachment by bringing the microbial cells and the substrate in close proximity. However, high velocity can result in dispersion of the microorganisms from the surface due to the exertion of substantial shear force on the biofilm.

The nutrient availability in the environment will also affect biofilm growth. For example, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* can form biofilms under almost any nutrient concentration that allows growth. *Escherichia coli* O157:H7 can form biofilms in a low level nutrient environment but *E. coli* K-12 and *Vibrio cholerae* require a nutrient-rich environment (O'Toole et al., 2000). In addition, some studies have reported that bacteria form biofilms when they are grown in a limited nutrient environment due to a stress response (Spector and Kenyon, 2012, Thomason et al., 2012). The one ecological advantage of bacterial attachment to surfaces is to gain nutrients that are also attracted to the surfaces (Palmer et al., 2007).

The formation of biofilms can occur over a range of temperatures from high temperature environments such as terrestrial geothermal settings and hydrothermal vents to low temperature environments such as in arctic streams (Clarke et al., 2010, Pysz et al., 2004, Reysenbach and Shock, 2002). Some bacteria can form biofilms at temperatures below their optimum growth temperature. *E. coli* are able to form biofilms at 4°C although

their optimum growth temperature is 37°C (Dourou et al., 2011). *L. monocytocens* is found to have better attachment after undergoing cold-stress at -20°C (Slama et al., 2012).

The pH of the surrounding environment also plays a role in bacterial attachment where highly acidic (pH 3) or alkaline (pH 12) conditions may reduce bacterial attachment (Zmantar et al., 2010). For example, the attachment of *S. aureus* and *S. epidermis* is reduced in a high alkaline environment, compared with a neutral or slightly acidic environment, resulting in a poorly formed biofilm (Nostro et al., 2012). In another example, the initial attachment of lactic acid bacteria is higher when the pH of the milk is either neutral or slightly alkaline compared with a slightly acidic environment (Nguyen et al., 2010). However, the thickness of biofilms is greater at a lower pH due to the coagulation of the milk protein, and possible physiological changes within the bacterial cells (Nguyen et al., 2012a).

2.a.3.2.3 *Influence of microbial cells*

The presence of fimbriae or flagella can influence the rate and extent of attachment of microbial cells (Katsikogianni and Missirlis, 2004). Most fimbriae contain a high proportion of hydrophobic amino acid residues, which contribute to cell surface hydrophobicity and attachment (Donlan, 2002). Both fimbriae and flagella play an important role in overcoming the repulsive forces associated with the substratum (Donlan, 2002, O'Toole et al., 2000). For example, motile *Campylobacter jejuni* have a higher attachment compared with non-motile *C. jejuni* (Reeser et al., 2007).

Quorum sensing or cell-to-cell signalling plays a role in the formation of biofilms (Donlan, 2002, Reeser et al., 2007). For example, two different cell-to-cell signalling systems, the lasR-lasI and the rhlR-rhlI (vsmR-vsml) found in *P. aeruginosa*, are involved in biofilm formation (Davies et al., 1998). These cell-to-cell signalling systems are not initially involved in the initial attachment of *P. aeruginosa* but are involved with biofilm differentiation, when there is sufficient population density (Davies et al., 1998). In another example, the induction of genetic competence (enabling the uptake and incorporation of exogenous DNA by transformation) was mediated by quorum sensing in *Streptococcus mutans* where transformational frequencies were 10-600 times higher in biofilms compared with their corresponding planktonic counterparts (Li et al., 2001). Furthermore, the living

cells are also able to acquire chromosomal DNA from dead cells of the same microorganisms within biofilms (Li et al., 2001).

Phase variation or phenotypic variation is a process of reversible, high-frequency phenotypic switching that is used by bacteria to generate population diversity which increases bacterial fitness under certain circumstance and appears to be important in niche adaptation (Van Den Broek et al., 2005). The phase variation may be influenced by the temperature, medium composition, and stress conditions (Marchand et al., 2009b). Phase variation in *P. aeruginosa* regulates the expression of pili, which affects swimming, swarming and twitching motility, and thus, influences biofilm formation (Weiser et al., 1998). Phase variation can be divided into two groups; (i) programmed variation characterised by a family of genes encoding proteins with similar function which is combined with the ability to express only one of the gene family members at a time, altering the expression of these members from time to time, (ii) un-programmed variation, characterised by DNA alteration through the accumulation of errors during DNA replication, imperfect DNA repair, the recombination between two non-identical genes, or re-assortment of gene segments (Van Den Broek et al., 2005).

2.a.4 ENZYMES WITHIN THE CONTEXT OF A BIOFILM

Within the biofilm, enzymes within the periplasmic space or within the matrix of the biofilm, bound by lectins, electrostatic interactions or diffusion limitation, are referred to as cell-associated enzymes (Wang and Chen, 2009). Enzymes in the surrounding environment are considered to be cell-free or extracellular enzymes (Wang and Chen, 2009). Many species of bacteria within biofilms have the ability to produce cell-associated or cell-free enzymes either by excretion or autolysis.

Biofilms can act as reservoirs for microbial produced enzymes either through the entrapment of enzymes produced by the microorganisms comprising the biofilm or enhanced production of enzymes by those microorganisms in the biofilm (Bagge et al., 2004, Khiyami et al., 2006, Rajendran et al., 2010). For example, the amount of enzyme produced in a sludge biofilm has been reported to be higher compared with that produced by corresponding planktonic cultures (Frølund et al., 1995). Similarly in fungal biofilms, Gamarra et al., (2010) reported that even though the biomass of *Aspergillus niger* from a

biofilm fermentation was lower than that of a submerged and solid-state fermentation, the yield of cellulase was significantly higher in the biofilm than in the planktonic form. Biofilm cultures may produce more enzyme than the submerged forms due to differential expression in enzyme-encoding genes (Gamarra et al., 2010, Wang and Chen, 2009).

Enzymes within biofilms have been reported to occupy particular niches (Iwashita et al., 2001). For example, β -glucosidases within *Aspergillus* biofilms tend to be cell-associated when the biofilm is submerged, while in solid-state fermentation, the β -glucosidases tend to be extracellular (Iwashita et al., 1998). The authors suggested that the extracellular soluble polysaccharide from *Aspergillus kawachii* influenced the stability and the localization of the β -glucosidases within the biofilm (Iwashita et al., 2001). In another study, proteases were found to be tightly bound to cell walls within sludge biofilms while α -amylase and α -glucosidase were immobilized in the matrix as cell-free enzymes (Yu et al., 2007).

Both enzymes and biofilm production by bacteria can be influenced by factors such as the flow velocity of the aqueous medium, presence of flagella, quorum sensing and phase variations (Khiyami et al., 2006, O'Toole et al., 2000, Reeser et al., 2007, Van Den Broek et al., 2005). Quorum sensing signal molecules, such as *N*-acylhomoserine lactone (AHL), or other AHL-related products by *Pseudomonas* spp., have been shown to increase the activity of the *aprX* promoter, a gene encoding an alkaline metalloprotease, which may increase the amount of proteases produced (Liu et al., 2007). The AHL molecule has been shown to increase protease production and biofilm formation by *Aeromonas hydrophila* (Khajanchi et al., 2009, Swift et al., 1999). While quorum sensing may influence the production of enzymes within biofilms, correlations between the amount of AHL molecule present, and biofilm formation or enzyme production, have not been reported (Khajanchi et al., 2009, Marchand et al., 2009b).

2.a.4.1 Evidence of enzymes from biofilms in the medical world

Enzymes within biofilms in medical settings may protect the bacteria from antibiotics, a process which could impact upon human health, especially for immuno-compromised patients. For example, the production β -lactamase produced by biofilm associated cells of *P. aeruginosa*, may accumulate within the biofilm matrix, which in turn may inactivate the

antibiotic and increase the resistance of the bacteria, and thus reduce the effectiveness of antimicrobial treatment (Bagge et al., 2000, Bagge et al., 2004, Nucleo et al., 2010). Enzymes produced within a biofilm by one species may benefit other species within that biofilm. For example, the production of β -lactamase by *Moraxella catarrhalis* within a mixed biofilm enhanced the survival of *Streptococcus pneumoniae* in the presence of benzylpenicillin and amoxicillin (Budhani and Struthers, 1998).

2.a.4.2 Evidence of enzymes from biofilms in industry

The beneficial uses of bacterial enzymes within biofilms include waste water treatment, or biofuel, vinegar and fine chemical production (Dagher et al., 2010, Hahn-Hagerdal et al., 2006, Hartmann and Ahring, 2005, Li et al., 2006, Rosche et al., 2009, Wang and Chen, 2009). Optimization of enzyme activities within biofilms can reduce costs and increase production yields, while reducing waste and energy requirements. The physiochemical properties of a biofilm make it suitable for use in biotechnology industries as biofilms protect the attaching microbial cells from the toxicity of by-products, and provide a microenvironment for enzyme activity (Li et al., 2006, Licitra et al., 2007, Rosche et al., 2009, Wang and Chen, 2009).

Biofilms are similar to immobilised cell reactors as in both instances, microbial cells need to be immobilised before the substrates can be used (Dagher et al., 2010). Immobilised cell reactors have successfully been used in the food industry, especially in the production of vinegar where acetic acid bacteria oxidise alcohol to acetic acid. The alcohol is trickled onto a fixed bed microbial film reactor containing acetic acid bacteria adhered to the surfaces of pumice stone, ceramic chips or wood shavings. The rate of vinegar production is influenced by the oxygen transfer rate, biomass, and the direct contact of alcohol with the bacterial cells (Lee, 2003). The oxidation of the alcohol is accomplished by two enzymes, membrane-bound, pyrroquinoline quinone-dependent alcohol dehydrogenase (PQQ-ADH) and membrane bound aldehyde dehydrogenase (Bartowsky and Henschke, 2008, Yakushi and Matsushita, 2010). The alcohol dehydrogenase oxidises the ethanol to acetaldehyde, which in turn undergoes further oxidation to acetic acid.

Undesirable effects on products, such as wine, can occur due to enzymes produced by bacteria, many of which are likely to be in biofilms (Yakushi and Matsushita, 2010) .

The enzymes such as alcohol dehydrogenase and aldehyde dehydrogenase produced by acetic acid bacteria are undesirable in wine production as they can result in sourness and a reduction in fruity characteristics. The spoilage of bottled red wine has recently been associated with the development of biofilms of acetic acid bacteria in the necks of bottles where air is present due to a faulty cork seal (Yakushi and Matsushita, 2010). The development of biofilm on the wine bottles' surfaces tends to occur slowly over time after packaging (Joyeux et al., 1984).

2.a.5 THE IMPORTANCE OF ENZYMES IN DAIRY MANUFACTURE

Enzymes may have both positive and negative effects in the dairy industry. Enzymes have been used extensively in dairy manufacture, increasing the value and the quality of the dairy products by generating desirable sensory qualities including texture, flavour, aroma, and by increasing nutritional value. One example of an enzyme used in the dairy industry is rennet, a protease of animal origin which is important in cheese manufacture. Enzymes produced by lactic acid-producing bacteria are used to produce the distinctive flavour and texture in dairy products such as cheese and yoghurts. For example, the hydrolysis of milk fat to free fatty acids by either esterase or lipases have been associated with the development of flavour of cheese (Holland et al., 2005). However, undesirable enzymes produced by bacteria are also responsible for producing off-flavours (bitterness and rancidity), reduced yield, and shortening the shelf-life of dairy products. There are two types of enzymes found in milk, indigenous enzymes and bacterial enzymes, which can have detrimental effects on dairy products.

2.a.5.1 Indigenous enzymes in milk

The effects of indigenous proteases and lipases on dairy products are well known (Chen et al., 2003, Deeth, 2006, Fox and Kelly, 2006, Kelly et al., 2006, Schroeder et al., 2008, Sousa et al., 2001). These enzymes are produced in the bloodstream of the lactating cow, and are naturally present in raw milk (Fox and Kelly, 2006). There are principally two groups of indigenous enzymes in milk: proteases (plasmin and lysosomal proteases) and lipases (lipoprotein lipase, bile salts-stimulated lipases, phospholipase and esterase) (Fox and Kelly, 2006, Kelly et al., 2006). Some of these indigenous proteases are heat-stable,

remaining active after dairy processing and, hence, reducing product shelf-life (Celestino et al., 1997a).

2.a.5.2 Bacterial enzymes in milk

Bacterial enzymes (protease or lipase) can be produced by a range of bacteria species found in the dairy environment including *Bacillus*, *Pseudomonas* and *Serratia* (Cleto et al., 2012, Fairbairn and Law, 1986, Marchand et al., 2012). Different bacterial species can produce different types of enzymes with different molecular weights. For example, *Pseudomonas* species can produce neutral zinc metallo-proteinase, with molecular weights ranging from 39.2 ± 0.7 to 45.3 ± 1.3 kDa, in raw milk (Marchand et al., 2009b). Other bacterial isolates, like *Bacillus* spp., can produce more than one type of protease with, different molecular weights (Chopra and Mathur, 1985, Fairbairn and Law, 1986).

The production of enzymes such as proteases and lipases by dairy bacteria usually occurs in the mid to late exponential or early stationary phase of bacterial growth, and it is a complex process, influenced by quorum sensing, temperature, iron content and phase variation (Chen et al., 2003, Haddadi et al., 2005, Liu et al., 2007, Marchand et al., 2009b, Nicodème et al., 2005, Van Den Broek et al., 2005, Woods et al., 2001). The production of proteases by bacteria is temperature dependent where temperature may either enhance or inhibit the production of proteases (Buchon et al., 2000, Nicodème et al., 2005). There is also some variation in protease production between different species of the same genus at the same temperature. For example *P. chlororaphis* CIP 103295 was reported to have higher protease production compared with *P. fluorescens* CIP 69.13 and *P. chlororaphis* CIP 75.23 when incubated at 30°C (Nicodème et al., 2005).

Bacterial enzymes may have a detrimental effect on dairy products during prolonged storage (Shah 1994). Their activity reduces or alters the physico-chemical properties of the finished product, thus, causing defects in functionality and sensory properties (Celestino et al., 1997a, Chen et al., 2003, Guinot-Thomas et al., 1995, Martins et al., 2006). Many bacterial proteases and lipases are heat-stable, remaining active over a broad range of temperatures and in low water activities after heat treatment. For example, heat-stable lipases produced by both *Pseudomonas* spp., and *Bacillus* spp., can remain active after thermal processing during milk powder manufacture and retain their highest

catalytic activities at temperatures ranging from 60 to 75°C (Dharmsthiti and Luchai, 1999, Law et al., 1976). In addition, the heat stability of heat-stable lipases was shown to increase when multiple heat-stable lipases were present (Chopra and Mathur, 1985).

A study by Celestino et al., (1997a) showed that whole milk powder, manufactured from bulk milk which had been stored at 4°C for 2 days, had a higher free fatty acid content than milk powder manufactured from fresh raw milk. Physical and chemical changes also occurred during storage of this powder (Celestino et al., 1997a, b). Another undesirable property, caused by heat-stable lipases in milk, is the lipolysis of fat causing instability in the foaming of milk, used in beverages such as cappuccino (Huppertz, 2010).

Heat-stable enzymes have also been responsible for limiting the shelf-life of Ultra High Treatment (UHT) milk where proteolysis was detected after six months of storage at 25°C (Barbano et al., 2006, Celestino et al., 1997b, Sørhaug and Stepaniak, 1997). This may be due to the degradation of casein, mainly κ -casein, by different types of bacterial proteases present in the milk (Akerstedt et al., 2012, Fairbairn and Law, 1986, Grufferty and Fox, 1998). The degradation of casein may weaken the casein micelle structure, which in turn results in the coagulation of milk (Fairbairn and Law, 1986). Proteolysis can also be influenced by the structure of the milk protein fraction where α_{s2} -caseins are more readily degraded by bacterial protease due to its random coil structure (Akerstedt et al., 2012, Farrell et al., 2009). Therefore, bacterial enzymes are of concern to the dairy industry as they can remain active in dairy products at low temperatures during prolonged storage and reduce the shelf-life of the dairy products which may lead to financial loss (Celestino et al., 1997b, Janzen et al., 1982, Kang and Frank, 1988).

2.a.5.3 Methods of detection of bacterial enzymes

2.a.5.3.1 Proteases

The degradation of milk protein by proteolytic enzymes can reduce the quality and quantity of dairy products as well as the functionality of the products; therefore, the detection of proteolytic activity in milk is important. The general detection of proteolytic activities includes the Kjeldahl method, electrophoresis, high-performance liquid chromatography, spectrophotometric, fluorimetric, and immunological methods (Chen et al., 2003, Nemeckova et al., 2009). Each assay has advantages and disadvantages. Either the assay is

simple to use but has a low level of detection or it is complex, using radioactive materials or specialist instruments, but has a higher detection level.

The Kjeldahl method has been used to measure protein in a wide range of food products and ingredients, and is recognized internationally (Moore et al., 2010). Kjeldahl methods can be divided into two variants, the total protein content, and true protein. For the total protein content, the total amount of nitrogen is measured and converted into protein content by multiplication with a conversion factor, 6.25. True protein is determined by measuring the nitrogen content of the precipitated protein and multiplying by the conversion factor of 6.38. The limitation of this method is that it is time consuming, non-selective, and there is the possibility of a false estimation of protein content due to interference from other sources of nitrogen (Moore et al., 2010).

Electrophoresis has been used to identify the molecular weights of bacterial protease and the products of proteolysis (Marchand et al., 2009b, Recio et al., 1997). The quantification of proteolysis is determined by the intensity of the clearing of milk protein and/or large polypeptides (Chove et al., 2011). In addition, the by-products of the proteolysis such as γ_1 -, γ_2 -, and γ_3 - caseins can be detected using electrophoresis (Recio et al., 1997). However, the limitation of this method is the use of hazardous chemicals, poor resolution of low molecular weight peptides and it is time-consuming (Chove et al., 2011). High-performance liquid chromatography has been used to differentiate proteolysis by plasmin and bacterial protease in UHT milk and it is regarded as simple, reproducible, accurate, and sensitive for low levels of proteases. However, the cost of the equipment and finding suitable standards for quantification limits its use for routine testing (Chen et al., 2003, Chove et al., 2011).

Spectrophotometric and fluorimetric methods have been used to measure proteolysis by using modified substrates such as synthetic chromogenic (azo-caseins) or fluorogenic substrates (fluorescein-thiocarbamoyl- β -casein) (Chen et al., 2003, Recio et al., 1997). These methods are less sensitive at low levels of proteolysis, and may compete with the natural occurring substrates in the assay. Fluorescamine has been used to quantify the amount of free peptides due to proteolysis where the fluorogenic compound reacts with free peptides to form a highly fluorescent product (Le et al., 2006). The advantage of fluorescamine is that it is simple, rapid, and sensitive to low levels of protease (Chove et

al., 2011). Immunological methods such, as the enzyme-linked immune-sorbent assay (ELISA), are very sensitive; however, ELISA may overestimate the amount of active enzyme as it cannot differentiate between active and inactive enzymes. It has been suggested that a combination of ELISA and spectrophotometric assays could produce a new method that can be used for quality control during processing (Chen et al., 2003). This will be a useful tool to detect the early stage of spoilage in dairy products.

2.a.5.3.2 Lipases

At present, there are few methods for detecting lipolysis in milk or milk products, and the accuracy of these methods are compromised due to interference from naturally occurring lipids in the milk. The methods for detection include measuring changes in the level of free fatty acids either by titration, chromatography or flourometric methods (Chen et al., 2003, Hasan et al., 2009).

The titration methods are simple, straightforward and cheap compared to the other methods; however, they are time-consuming and are not sensitive enough to detect levels of lipolysis lower than 0.1 μmol per min (Hasan et al., 2009). Lipolysis is measured by neutralising the free fatty acids, released from the substrate with an alkali solution such as sodium hydroxide, to a constant pH end point value. Properly cleaned electrodes have been found to enhance titration performance between assays (Ballot et al., 1982).

The hydrolytic products of lipolysis such as free fatty acids, mono- and diacylglycerols, during the incubation of lipase with an ester substrate, can be quantified by gas chromatography (GC) or high-performance liquid chromatography (HPLC). GC is generally preferred over HPLC as GC is more sensitive. GC may be used to separate and quantify the hydrolytic products of lipase (Louwrier et al., 1996, Patel et al., 1996). The advantage of HPLC over GC analysis is that GC analysis requires the fatty acids to be derivitised before chromatographic separation, but this can be simplified with the use of kits designed specifically for this purpose (Thomason et al., 1999). Sample preparation for HPLC is simple, requiring incubation of the lipase with a substrate emulsion. A chloroform-methanol mixture is used to stop the reaction and to extract the reaction products. Normal phase separation using silica columns enables free fatty acids from mixed triacylglycerols to be eluted in one peak which adds to the sensitivity of the method. By

modifying the analytical column conditions, the substrates and products of the lipase reaction can be monitored. The quantification of lipase activity can be measured by Ultraviolet (UV) absorption (Veeraragavan, 1990) or refractive index (RI) (Ergan and Andre, 1998). RI is most suitable for complex substrates, as UV gives different responses for different fatty acids. Even though HPLC is not as sensitive as GC, both of these methods have been used extensively in analytical laboratories. Furthermore, HPLC analyses are more sensitive than titration methods.

Fluorometric methods can be used to detect low levels of lipolysis. The acyl esters of the fluorescent compound 4-methylumbelliferone (4-MU) can be used as a substrate in detecting lipolysis in skim milk, skim milk powder, whey powder and whey protein concentrate (Fitzgerald and Deeth, 1983). The activity can be expressed as the amount of 4-MU released per unit time, using a standard curve of the fluorescence of 4-MU, as the release of 4-MU accompanies lipolysis, and the resulting fluorescence increases directly with 4-MU concentration.

2.a.6 IMPORTANCE OF BIOFILMS IN THE DAIRY INDUSTRY

The occurrence of biofilms on the dairy farm or dairy manufacturing plants may result in economic losses due to lower quality, yields, and food spoilage. As mentioned previously, various conditions are believed to promote the attachment of bacteria, and initiate the formation of biofilms. Bacterial attachment and biofilm formation can occur at any stage in the dairy industry, from the milking cups on dairy farms to the stainless steel pipeline in dairy manufacturing plants (Vlková et al., 2008). For example, the formation of biofilms within milking equipment on the farm has been identified as one of the reservoirs or entry points for heat-resistant spores to contaminate raw milk (Scheldeman et al., 2005).

Bacteria have been shown to transit from the dairy farm to the dairy manufacturing plant (Huck et al., 2008). This may be due to the dispersion of biofilm-derived cells into milk during milking or processing, which subsequently contaminates pipeline further down-stream (Flint et al., 1997, Latorre et al., 2010, Wijman et al., 2007). For example, *Pseudomonas* cells released from biofilm attach more readily compared with planktonic cells (Rollet et al., 2009). Strains of non-starter lactic acid bacteria (NSLAB) within biofilms in a cheese manufacturing plant, have been found to be the same as those found in

the final cheese product, suggesting that the contamination of cheese by NSLAB may be from the biofilms in the manufacturing plant (Somers et al., 2001). The presence of NSLAB is commonly associated with poor hygiene in the cheese manufacturing plant.

Even with established clean-in-place (CIP) systems, numerous bacteria can be found in the scratches on the milking equipment (Latorre et al., 2010, Wirtanen et al., 1995). Bacteria, such as *Streptococcus thermophilus* or *L. monocytogenes*, have a greater resistance to heat and sanitizers when growing as biofilms compared with their planktonic counterparts (Flint et al., 2002, Frank and Koffi, 1990). The increased resistance is associated with the amount of growth and possibility a change in the physiology of the cell as well as EPS protection (Dhir and Dodd, 1995, Frank and Koffi, 1990, Steward et al., 2006). The efficiency of CIP is influenced by several factors such as the type of cleaning agents, concentration of cleaning agents, the hardness of water, temperatures, and duration of the CIP. All these factors are important to ensure a proper CIP. For example, the hardness of water may hinder the effectiveness of caustic cleaning agents as a high concentration of ions can react with the caustic cleaning agents causing precipitation (Cords et al., 2001). Water softeners are used to reduce the water hardness during CIP, and this is found to reduce the bacterial count due to the prevention of precipitation of the cleaning agents (Elmoslemany et al., 2009).

The efficacy of CIP is also dependent on the washing temperatures used. For example, a study by Latorre et al., 2010 showed that high bacterial cell counts were detected on dairy farms with low washing temperatures (47-53°C). This is possibility due to old or incorrect setting of the heating systems (Bava et al., 2011). This was also observed in the study by Elmoslemany et al., (2009) where bacterial spores, were found attached to stainless steel surfaces in dairy manufacturing plants following cleaning (Elmoslemany et al., 2009, Flint et al., 1997). The efficacy of CIP on biofilms were also found to be reduced with the reduction of caustic concentration and temperatures (Bremer et al., 2006).

2.a.7 EVIDENCE FOR ENZYMES WITHIN BIOFILM ASSOCIATED WITH DAIRY MANUFACTURE OR PRODUCTS

Evidence for enzyme production by biofilm-bound cells related to dairy processing is found in the production of traditional, cheese such as Ragusano (a Sicilian Cheese) or Salers (a French cheese). Ragusano and Salers are made from raw milk using traditional methods, where the raw milk is curdled in wooden vats known as “Tina” and “gerle” respectively. Commercial starter cultures are not used in the manufacture of these cheeses. The naturally occurring bacteria present in the raw milk, and on the surfaces of the Tina, provide the natural starter cultures needed for cheese manufacture (Licitra et al., 2007). The natural starter cultures found on Tina include *Lactococcus lactis*, *L. delbrueckii lactis* and *L. acidophilus*, and the predominant and stable species of lactic acid bacteria is *Streptococcus thermophilus*, while the dominant species in gerle are *Lactobacillus casei*, *L. lactis*, *L. pseudomesenteroides*, *L. garvieae*, *L. mesenteroides*, and *L. plantarum* (Didienne et al., 2012, Licitra et al., 2007, Lortal et al., 2009). Only *L. lactis* is found in both types of wooden vat (Didienne et al., 2012, Lortal et al., 2009). These natural starter cultures possess a wide range of hydrolytic enzymes, which may contribute toward the formation of short peptides and amino acids in the cheese, thus, developing the cheese flavour during ripening (Bouton et al., 1998, Sousa et al., 2001, Williams and Banks, 1997).

In a recent study, psychrotrophic bacteria isolated from refrigerated raw milk were found to produce protease and had the ability to adhere to stainless steel surfaces (Nörnberg et al., 2011). Given sufficient time, these bacteria may form biofilms on the stainless steel surface and produce protease in dairy manufacturing plants. However, there are no published studies on the production of undesirable enzymes by dairy bacteria within biofilms.

Given the widespread occurrence of biofilms on surfaces associated with the production and processing of milk and the realisation that biofilms are a significant source of enzymes, it seems reasonable to speculate that biofilms of spoilage populations associated with dairy processing surfaces may also produce enzymes which may affect the shelf-life of manufactured products.

2.a.8 CONCLUSION

Biofilms within dairy processing equipment may be an unrecognized source of spoilage enzymes that may have important economic consequences for dairy manufacturers. Biofilm development in the dairy industry has been well studied and enzyme spoilage of milk is also well reported. However, the potential link between biofilms and the production of enzymes that may be responsible for spoilage in the dairy or any other food industry has not been proven. If biofilms are indeed a significant source of enzymes that result in degraded dairy products, controlling biofilms in dairy manufacture may improve the quality of the final products.

Chapter 3. Preliminary Studies On Milk Tankers

3.a ***Internal surface temperature of milk tankers***

3.a.1 ABSTRACT

In this study, the surface temperatures of milk tankers based in the Manawatu region of New Zealand, were monitored during winter and summer, 2009-2010 using data loggers over 23 h on five separate occasions for each season. The average internal surface temperatures of empty milk tankers during winter and summer were 10°C and 20°C, respectively, while the average internal surface temperatures of full milk tankers during winter and summer were 7°C and 10°C, respectively. The upper part of the milk tanker was the warmest compared with the other parts of the milk tanker during winter and summer with an average of 10°C and 19°C, respectively. This study showed that the surface temperature of a milk tanker may promote the proliferation of psychrotrophic bacteria on the internal surfaces of a milk tanker and subsequently the formation of biofilms.

3.a.2 INTRODUCTION

Raw milk is transported from the dairy farm to the dairy processing plant by milk tankers. Most of the milk tankers in New Zealand are single-skinned and without refrigeration systems. The temperature of the bulk milk is expected to be maintained by the large volume of chilled milk picked-up from the dairy farm. Chilled milk is collected from several dairy farms before being transported back to the dairy processing plants for pasteurization. Milk collection schedules are seasonal, with more frequent milk collection in summer compared with winter. Clean-in-place (CIP) of milk tankers is only performed every 12 h, and if CIP is not performed on the milk tanker after 12 h, the milk tanker undergoes CIP before the start of a new milk run.

The surface area of a milk tanker is estimated to be $2.7 \times 10^5 \text{ cm}^2$, and the thickness of the tanker wall is 3 mm. During the transportation of milk, the bulk milk closest to the tanker wall is generally accepted to be warmer than the core of the bulk milk due to heat transfer between the tanker wall and the surrounding environment, or sun radiation. The upper part of milk tanker is more likely to have a higher surface temperature compared with the other parts of the milk tanker. This is due to the presence of an air headspace between the chilled milk and the upper part of the milk tanker walls where the chilled milk is not in contact with the surfaces. The surface temperatures of a milk tanker may be different during winter and summer as the atmospheric temperature during winter is generally colder, therefore, the heat transfer is lower. The temperature on the milk tanker wall surfaces, if warm enough, may promote bacterial growth. Furthermore, the splashing of the milk on the upper part of the milk tanker may promote pre-conditioning of the surface for bacterial attachment (Palmer et al., 2007, Shi and Zhu, 2009), which can occur after a short contact period (Vanhaecke et al., 1990) and provide the start of a biofilm.

Data loggers have been used extensively in monitoring temperature over time in many applications ranging from monitoring the temperature of food products during shipment to the internal body temperature measurement of animals (Abad et al., 2009, Lovegrove, 2009, Raab et al., 2011). Data loggers can be used for monitoring the surface temperatures of a milk tanker during its milk run by affixing them onto the external surface of the tanker. The data loggers used in this study were DS1921G ThermoChron® iButtons.

These were selected due to their light weight and size, about 3 g and 6 (h) x 17(d) mm respectively. The electronic components of the iButtons are protected by a robust stainless steel capsule, which is resistant to environmental stress such as dirt and moisture (Maxim ThermoChron iButton data sheet). The data logger can measure time, date, and temperatures, all of which are user-defined. The temperature data that is stored in the data logger can be uploaded and analysed with 1-Wire®, computer software.

There are no studies on the effects of the fluctuating surface temperatures of milk tankers in regards to biofilm formation. This study aimed to monitor and measure the surface temperatures of the different parts of a milk tanker during its typical milk collection. The surface temperatures of the milk tankers were analysed and evaluated for subsequent studies on biofilm formation and enzyme production.

3.a.3 MATERIALS AND METHODS

3.a.3.1 Monitoring the surface temperatures of an *in vitro* model simulating a milk tanker while stationary and during milk collection

A 10 L stainless steel vessel was filled with cold water (7°C) and was set-up to simulate a milk tanker while stationary (Figure 3.a-1A and 3.a-1C). Data loggers (DS1921G ThermoChron® iButtons, San Jose, United States of America) were affixed onto the surfaces with polystyrene and duct tape.

For monitoring the surface temperatures of an *in vitro* model simulating a milk tanker during milk run, the set-up was described as above with slight modification. A fan was placed in front of the stainless steel vessel to simulate the air flow going over the milk tanker as the milk tanker moves (Figure 3.a-1B).

The internal and external surface temperatures were monitored by the data loggers. The temperatures were recorded at one minute interval for a period of 240 minutes. The experiment was performed on three separate occasions. The internal and external surface temperatures from the data loggers were analysed and compared.

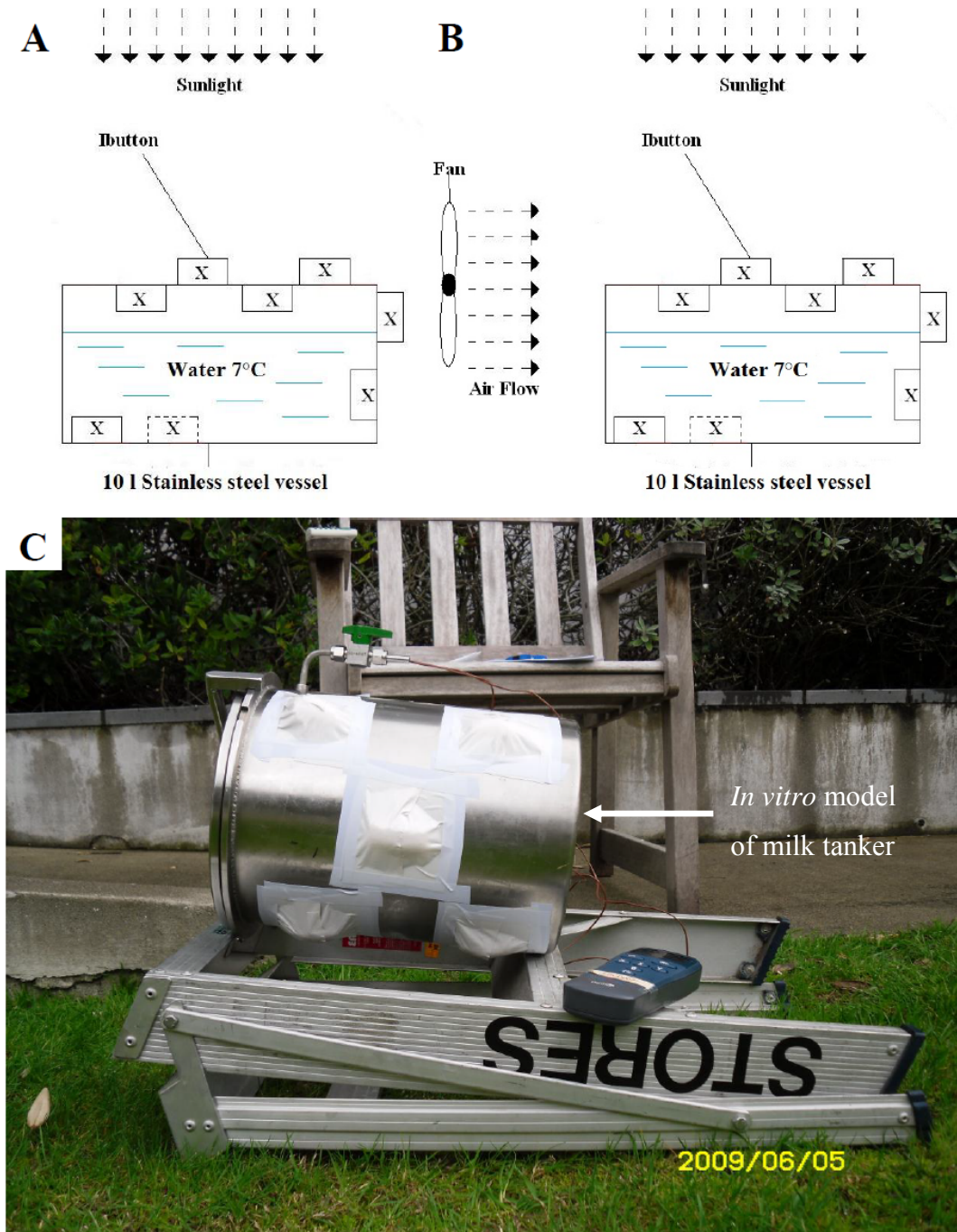


Figure 3.a-1 The schematic diagram of the *in vitro* model of milk tanker while stationary (A), the schematic diagram of the *in vitro* model of milk tanker during its milk run (B), and the affixation of the data loggers on the *in vitro* model of a milk tanker while stationary (C).

3.a.3.2 Surface temperature monitoring

In this study, the surface temperatures of milk tankers based in the Manawatu region of New Zealand were monitored during the summer and winter of 2009-2010. In total, ten milk tankers were monitored, five from each season. The surface temperatures were monitored by affixing data loggers on the outer layer of the milk tanker.

In total, ten data loggers were affixed on the outer layer of the milk tanker, (two of the data loggers on the upper part of milk tanker, three of the data loggers on each side of the milk tanker, one of the data logger on each end of the milk tanker; Figure 3.a-2).

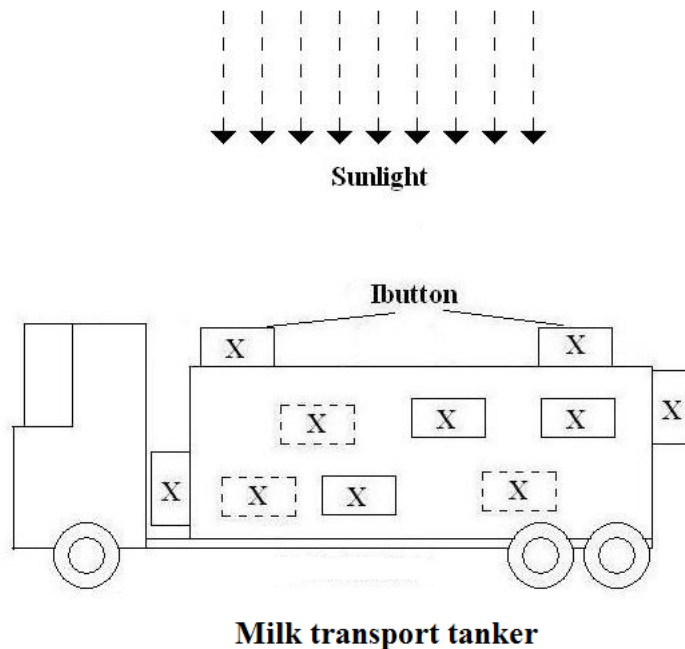


Figure 3.a-2 The schematic diagram of data loggers' placed on a milk tanker.

Polystyrene and duct tape were used to insulate the data loggers and prevent the data loggers from dislodging from the outer surface of the milk tanker during its typical milk collection (Figure 3.a-3). The insulation of the data loggers by polystyrene was to prevent the data loggers from measuring environmental temperatures. By affixing the data loggers on the outer layer of the tank, and insulating with polystyrene and taping with duct tape, the data loggers were able to estimate the internal surface of the milk tanker. The data

loggers were programmed to store temperatures at minute intervals for a period of 23 h. At the end of the 23 h period, the data loggers were retrieved from the surfaces of the milk tanker and the data were downloaded to the computer software, 1-Wire®. The raw data were then exported to Microsoft excel 2010 for analysis.



Figure 3.a-3 The affixation of the data loggers on the outer surface of a milk tanker.

3.a.3.3 Statistical analysis

The average surface temperatures of the milk tanker were analysed with SAS 9.2 software using analysis of variance, Tukey's test with a critical probability of $P \leq 0.05$.

3.a.4 RESULTS

3.a.4.1 Monitoring the surface temperatures of an *in vitro* model simulating a milk tanker while stationary and during milk collection

The internal and external surface temperatures of the *in vitro* model of a milk tanker while stationary were not significantly different (Table 3.a-1). Although there was a significant difference on the surface temperature in the *in vitro* model of the moving milk tanker, the average temperature difference between the internal and external surface temperature was less than 1°C.

Table 3.a-1 Comparison of internal and external surface temperatures of the *in vitro* model of a milk tanker.

	Average internal surface temperature (°C)	Average external surface temperature (°C)
" Milk tanker at stationary"	10.4 ± 1.1 ^a	10.6 ± 1.3 ^a
" Milk tanker during milk collection"	15.4 ± 1.4 ^a	14.6 ± 1.8 ^b

Means with the same letter in the same row are not significantly different at P≤0.05.

3.a.4.2 Internal surface temperatures of milk tankers over a period of 23 h

The data loggers were able to record the internal surface temperatures of milk tankers during the summer and winter of 2009 -2010 (Figure 3.a-4 and Figure 3.a-5). The internal surface temperatures of the milk tankers varied from day to day and between the two seasons. The internal surface temperatures of the milk tankers for summer and winter ranged from 4 to 46°C and from -6 to 28°C, respectively. Temperature reading above 50°C in the graphs indicated when the milk tanker underwent CIP. Most of the milk tankers underwent at least one full CIP during the period of study except one milk tanker during the winter period (Figure 3.a-5A). The data loggers showed that there was a CIP failure in this particular tanker as the observed temperatures were below the recommended 65-75°C. In addition, the contact time for the hot cleaning solution was estimated to be 5 minutes or less.



Figure 3.a-4 In total, five different milk tankers were monitored during summer of 2010. Each graph represents the surface temperature of a milk tanker over a period of 23 h.

The (---) red line represents the top surface area of the milk tanker

The (---) blue line represents the right surface area of the milk tanker

The (---) black line represents the left surface area of the milk tanker

The (---) yellow line represents the back surface area of the milk tanker

The (---) green line represents the front surface area of the milk tanker

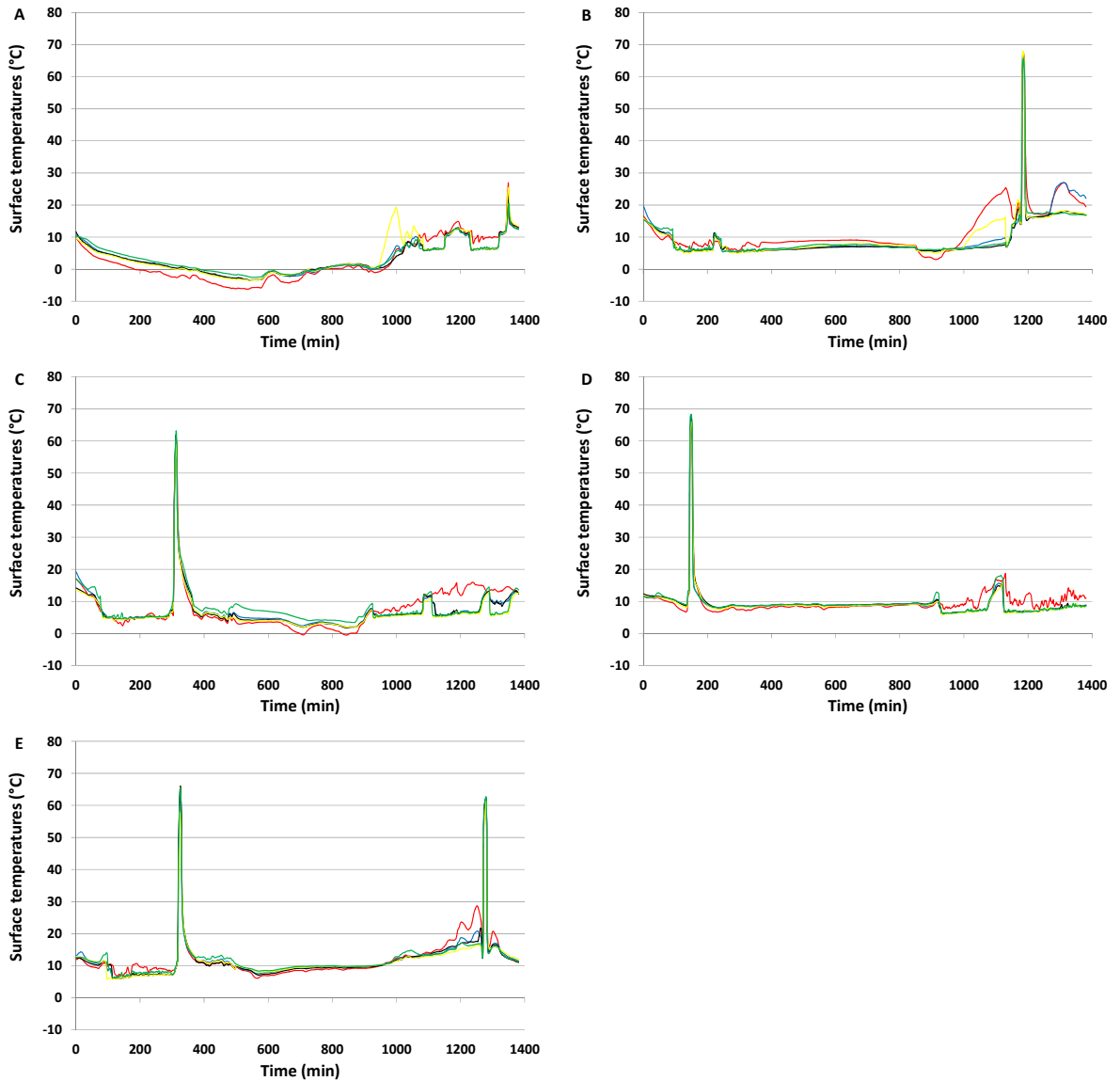


Figure 3.a-5 In total, five different milk tankers were monitored during winter of 2009. Each graph represents the surface temperature of a milk tanker over a period of 23 h.

The (---) red line represents the top surface area of the milk tanker

The (---) blue line represents the right surface area of the milk tanker

The (---) black line represents the left surface area of the milk tanker

The (---) yellow line represents the back surface area of the milk tanker

The (---) green line represents the front surface area of the milk tanker

The temperature of the upper surfaces of the milk tankers was found to be higher compared with the other parts of the milk tanker when the milk tankers were filled with raw milk. However, there was no difference between different parts of the milk tankers when the milk tankers were empty.

3.a.4.3 Average internal surface temperatures of milk tankers

The average internal surface temperatures of different parts of milk tankers were further analysed when the milk tankers were either full, empty during a milk run or stationary (Figure 3.a-6). The average surface temperatures found on the upper part of the milk tankers were significantly different from the other parts of the milk tankers when the milk tankers were full during milk runs in both seasons ($P \leq 0.05$). However, when the milk tankers were empty during a milk run or stationary, there was no significant difference among the different parts of the milk tanker ($P \leq 0.05$).

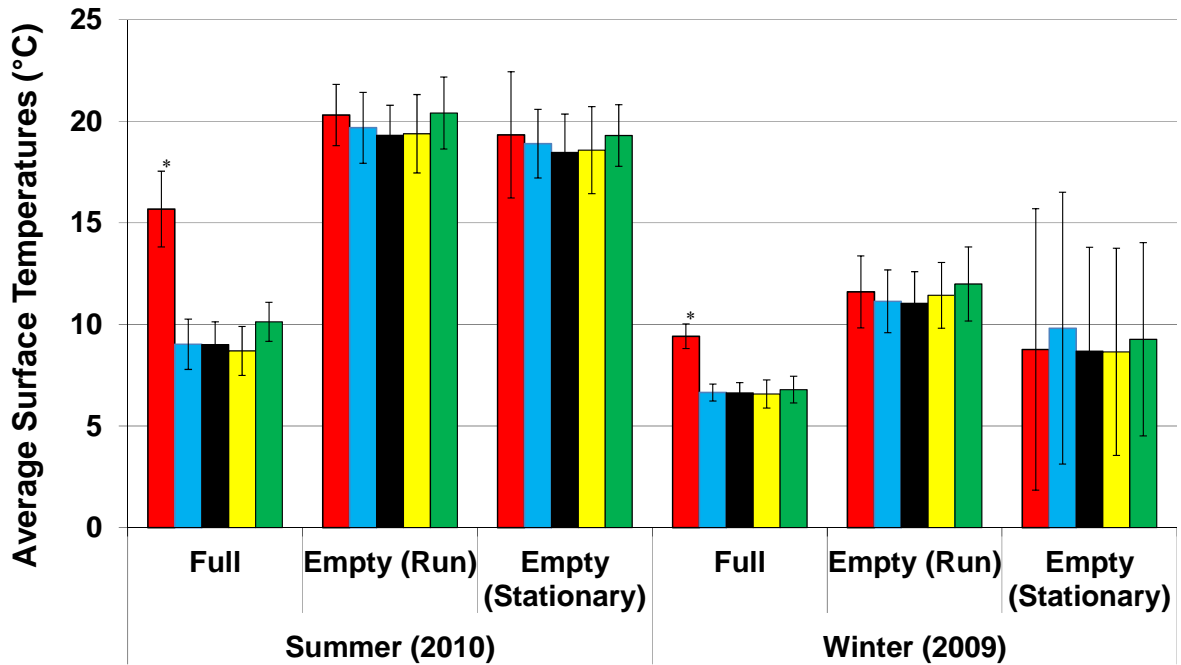


Figure 3.a-6 The average surface temperatures of different parts of milk tankers when they were full, empty on a milk run or while stationary during the summer and winter of 2009-2010. Trials were performed with five milk tankers from two seasons, and error bars represent standard deviations from the means.

* indicates statistically different, $P \leq 0.05$ (Tukey's test).

The ■ red bar represents the top surface area of the milk tanker

The ■ blue bar represents the right surface area of the milk tanker

The ■ black bar represents the left surface area of the milk tanker

The ■ yellow bar represents the back surface area of the milk tanker

The ■ green bar represents the front surface area of the milk tanker

3.a.4.4 Internal surface temperatures of milk tankers during a milk run

The average internal surface temperatures of the milk tankers when they were full during a milk run were 11 and 7°C during summer and winter, respectively (Figure 3.a-7). There was no significant difference between these two seasons when they were full during a milk run ($P \leq 0.05$). The average internal surface temperatures of the milk tankers when they were empty during milk run or stationary were found to be significantly different between the two seasons ($P \leq 0.05$). The average surface temperatures of the empty milk tanker during summer were approximately 20°C whereas the average surface temperatures of the empty milk tanker during winter were approximately 10°C.

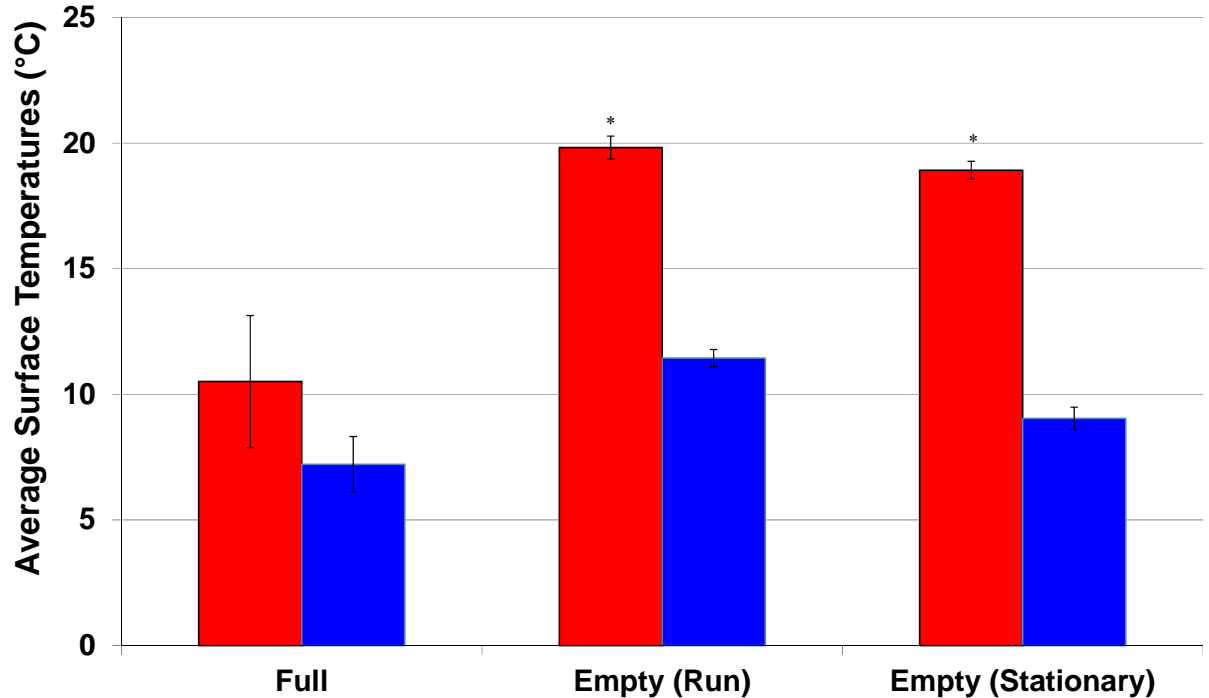


Figure 3.a-7 The average surface temperatures of milk tankers when they were full, empty on a milk run or while stationary during the summer and winter of 2009-2010. Trials were performed with five milk tankers from two seasons, and error bars represent standard deviations from the means.

* indicates statistically different, $P \leq 0.05$ (Tukey's test).

The ■ red bar represents summer of 2010

The ■ blue bar represents winter of 2009

3.a.5 DISCUSSION

The internal temperatures of a single skinned stainless steel milk tanker during its typical milk run can be monitored by adhering data loggers onto the outer surface of the milk tanker. The average temperature difference between the external and internal surface temperature was less than 1°C.

The surface temperatures of the stainless steel surfaces of milk tankers varied from day to day, and were season dependent. The variability of the surface temperatures was influenced by several factors such as the time of the day, the number of milk collections, the amount of bulk milk in the milk tankers, the temperature of the bulk milk, and the weather. These factors also influenced the surface temperatures of the different parts of the milk tankers.

The upper part of the milk tankers were found have the highest temperatures compared to the other parts of milk tankers in both seasons. This may promote biofilm growth as the surface temperatures were found to be in the growth range for psychrotrophic and mesophilic bacteria (Champagne et al., 1994, Lafarge et al., 2004, Martins et al., 2006). The bacteria found in the bulk milk may adhere to the upper part of the milk tanker during transportation due to the splashing effect of the bulk milk within the milk tanker. Furthermore, the headspace between the bulk milk and the upper part of milk tanker may allow biofilm formation on the air-liquid interface (Wijman et al., 2007). In addition, these surface temperatures may allow some of the bacteria found in raw milk to produce heat-stable enzymes during the transportation of raw milk, as bacteria can produce enzymes over a wide range of temperatures (Buchon et al., 2000).

The temperature used in CIP is important as temperatures less than 70°C are ineffective in removing biofilm from the stainless steel surfaces (Antoniou and Frank, 2005, Chmielewski and Frank, 2004, Elmoslemany et al., 2009). Temperatures less than the recommended temperatures may result from inadequate heating systems caused by either incorrect settings or old equipment (Bava et al., 2011). In addition, the contact time for the hot wash on surfaces should be approximately for 10 minutes (Giesen, 2012), however, in this study the contact time for the hot wash was approximately 5 minutes. This showed that the current CIP of milk tankers may need improvement for removing the milk soils or

biofilms as the contact time was short. Further studies are needed to investigate and improve the CIP of milk tankers to ensure they are thoroughly cleaned.

3.a.6 CONCLUSION

The surface temperatures on different parts of milk tankers vary from day to day. The surface temperatures found on the internal surfaces of milk tankers were suitable for the growth of both psychrotrophic and mesophilic bacteria. Naturally occurring bacteria in raw milk or dairy environments are most likely to attach to and grow on the internal surfaces of the milk tankers during transportation if the milk tankers are not properly cleaned. Given sufficient time, these bacteria may form biofilms and produce heat-stable enzymes within the biofilms on the internal surfaces of the milk tankers. Future studies were therefore undertaken to look at the development of biofilms in an *in vitro* model of a milk tanker simulating the fluctuating surface temperatures during its typical milk run, and the effects of enzyme production within these biofilms on the dairy products.

**3.b *Thermo-resistant enzyme-producing bacteria isolated
from the internal surfaces of raw milk tankers***

Published as

Teh, K. H., Flint, S., Palmer, J., Lindsay, D., Andrewes, P., Bremer, P., 2011. Thermo-resistant enzyme-producing bacteria isolated from the internal surfaces of raw milk tankers. *International Dairy Journal* 21, 742–747.



MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

**STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS**

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Koon Hoong Teh

Name/Title of Principal Supervisor: Steve Flint

Name of Published Research Output and full reference:

Teh, K. H., Flint, S., Palmer, J., Lindsay, D., Andrewes, P., Bremer, P., 2011.
Thermo-resistant enzyme-producing bacteria isolated from the internal surfaces of raw milk tankers. *International Dairy Journal* 21, 742–747.

In which Chapter is the Published Work: Chapter 3.b

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:
and / or
- Describe the contribution that the candidate has made to the Published Work:
The candidate did the necessary laboratory work, and prepared the manuscript for publication

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23/10/2012

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3.b.1 ABSTRACT

In this study, bacteria were isolated from the internal surfaces of raw milk road tankers by swabbing the surfaces during two seasons, winter and summer. Bacteria producing thermo-resistant enzymes were selected for further characterization and their ability to form biofilms *in vitro*. Of the 153 isolates able to produce enzymes, 52 produced thermo-resistant enzymes. The number of bacteria recovered from stainless steel chips during a biofilm screening assay after 24 h of exposure ranged from 2.7 to 7.6 Log₁₀ cfu/cm². Twelve of these bacteria, identified by 16S rDNA sequence analysis, belonged to the genera *Bacillus*, *Staphylococcus*, *Streptococcus*, *Pseudomonas*, and *Serratia*. In addition, some of these isolates were able to grow at low temperatures (7°C). This study showed that bacteria present on the internal surfaces of raw milk tankers may be a previously unreported source of thermo-resistant enzymes in raw milk.

3.b.2 INTRODUCTION

Dairy farms that produce good quality milk with low bacterial counts help to ensure the quality of the final dairy product. However, the quality of the milk can be reduced because of contamination or temperature abuse during transportation, processing, and packing. Raw milk is generally transported to the dairy manufacturing plant by road tankers.

The final quality of food products may be reduced because of the growth of a variety of different types of bacteria, resulting in unacceptable microbial contamination or spoilage, caused by enzymes produced by the bacteria. In addition, many of these bacteria have the ability to attach to food processing surfaces and form biofilms. Biofilms are an assemblage of microbial cells that are firmly bound to a surface and are enclosed in a matrix of primarily polysaccharide materials (Donlan, 2002). These biofilms not only harbor microbial populations but also act as sources of microbial contamination in the food industry (Wilks et al., 2006). Food spoilage microorganisms entrapped in biofilms may secrete by-products from the main body of the biofilm into the processing stream, thus contaminating the final product.

The majority of the bacteria in raw milk are inactivated during the pasteurization process or by other treatments involved in the manufacture of dairy products. However, some bacterial enzymes are heat-resistant and retain their activity over a broad range of temperatures and water activities (Chen et al., 2003, Marchand et al., 2009a, Nörnberg et al., 2011). Even though enzymes present in processed milk and milk products can be present at low concentrations, over time these enzymes will reduce or alter the physico-chemical properties of the finished dairy product, which can alter the product's functionality and sensory properties (Celestino et al., 1997b, Guinot-Thomas et al., 1995). Proteolytic enzymes are associated with bitterness in milk because of hydrolysis of the peptide bonds, whereas lipolytic enzymes hydrolyze milk fats and are associated with rancidity. Both of these enzymes have been responsible for limiting the shelf-life of UHT milk, with microbial enzymes remaining active in the UHT milk after 6 months of storage at 25°C (Celestino et al., 1997a, Licitra et al., 2007, Sørhaug and Stepaniak, 1997). Currently, little is known about the potential for bacteria to produce enzymes during the transportation of raw milk to the processing facility. For this reason, this study aimed to identify and characterise bacteria isolated from the internal stainless steel surfaces of raw

milk tankers. The growth temperature profiles of the isolates, as well as their ability to produce thermo-resistant enzymes and biofilms on stainless steel surfaces, were evaluated.

3.b.3 MATERIALS AND METHODS

3.b.3.1 Isolation of bacteria

In this study, bacteria present on the internal stainless steel surfaces of two raw milk tankers based in the Manawatu region of New Zealand were isolated during the summer and winter of 2009–2010 after emptying and before cleaning-in-place (CIP). These tankers were non-insulated and non-refrigerated. Approximately 1 m² of five randomly chosen sections of the internal surfaces of each raw milk tanker were swabbed using 3M sponges (3M, Global Science, Auckland, New Zealand). In addition, a raw milk sample from each tanker was taken before the milk was transferred to storage silos at the dairy manufacturing plant. The sponges and the milk samples were transported to the laboratory within 1 h, under refrigeration, and were analyzed immediately.

The sponges were transferred into 10 mL of 0.1% peptone solution and mixed by vortex mixing for 1 min. The suspensions were further diluted in serial 10-fold volumes of 0.1% peptone, and 0.1 mL aliquots from each dilution were plated in triplicate on to a selection of media and incubated as described in Table 3.b-1.

Table 3.b-1 Media used for the isolation of bacteria from the internal stainless steel surfaces of raw milk tankers

Media	Target bacteria	Incubation temperature (°C)	Incubation period (d)
Milk Plate Count agar (MPCA; Oxoid, Auckland, New Zealand)	Psychrotrophic bacteria	7	7
MPCA	Mesophilic bacteria	30	2
De Man, Rogosa and Sharpe agar (MRS, Merck, Darmstadt, Germany)	<i>Lactococcus</i> spp.	37	2
Baird-Parker agar with EY Tellurite Enrichment (Oxoid, Auckland, New Zealand)	<i>Staphylococcus</i> spp.	37	2

To determine the predominant microorganisms in the various samples, at least 10 typical colonies of the microorganisms representing 50% of each colony type were isolated from each sample at the highest dilution which had counts between 30 to 300 colonies (Von Holy and Holzapfel, 1988). All the colonies were purified on milk plate count agar (MPCA) (Oxoid, Basingstoke, UK). A total of 210 cultures were isolated and were further analyzed for their ability to produce biofilms and thermo-resistant enzymes.

3.b.3.2 Thermo-resistant enzyme screening

To determine the enzyme activity of the selected isolates, a pure colony was streak-plated on to spirit blue agar supplemented with lipase reagent (Difco, Becton, Dickinson and Company, Sparks, Nevada, USA) for lipolytic activity. The appearance of royal blue zones surrounding the streak indicates the occurrence of lipolytic activity (Starr and Burkholder, 1942). Calcium caseinate agar (Condalab, Laboratorios Conda, S.A., Spain) was similarly used to determine proteolytic activity. The appearance of clearing zones surrounding the streak indicates the occurrence of proteolytic activity (Frazier and Rupp, 1928). A total of 153 cultures were able to produce enzymes, which were further screened for thermo-resistance.

The enzyme-producing bacterial isolates were grown in 50 mL of sterile reconstituted skim milk (RSM) (100 g skim milk powder in 910 mL distilled water) for 72 h at 30°C and on an orbital shaker (Multitron version 2, Infors HT, Bottmingen Switzerland) set at 200 rpm. A volume (15 mL) of the culture was centrifuged at 1900 X g for 5 min, for separation of cell biomass and supernatant. A volume (9 mL) of the resulting supernatant was used as the crude enzyme to screen for thermo-resistance. The supernatant was heat treated at 63.5°C for 30 min, followed by the addition of 1 mL of 1% sodium azide (Scharlau, Scharlau Chemie, S.A., Spain) to ensure the inactivation of bacterial cells.

3.b.3.3 Milk coagulation assay – Test for thermo-resistant protease

A 1 mL aliquot of the heat-treated supernatant from each sample was mixed with 2 mL of commercial UHT whole milk in sterile tubes. The tubes were incubated at 25°C for up to 5 d. Unheated supernatant was used as a positive control and un-inoculated UHT whole milk was used as a negative control. Milk coagulation indicating positive protease activity (Nörnberg et al., 2010) was observed by visual inspection at different time intervals.

3.b.3.4 Lipolytic diffusion agar assay – Test for thermo-resistant lipase

An aliquot (50 µL) of the previously heated supernatant was drop-plated on to spirit blue agar. Lipase F-Ap15 (from *Rhizopus oryzae*, 150,000 U/g, Amano Enzyme Inc., Nagoya, Japan) was used as a positive control and un-inoculated RSM was used as a negative control. The plates were incubated at 25°C for up to 5 d. The appearance of royal blue zones surrounding the drop indicates the occurrence of lipolytic activity.

3.b.3.5 Biofilm screening assay

Aliquots (1 mL) containing 10^5 cfu/mL of each bacterial isolate were inoculated into 100 mL of sterile RSM containing 3 stainless steel chips (size 1 cm², grade 316) placed on the bottom of each 100 mL schott bottle, and incubated at 25°C for 24 h on an orbital shaker (Multitron) set at 100 rpm. Stainless steel grade 316 was used in the biofilm screening assay as it is thermodynamically favourable for the adhesion of bacteria, and most surfaces used in dairy manufacture are made from this grade of stainless steel (Simoes et al., 2007, Teixeira et al., 2005).

After incubation, the chips were rinsed by dipping in sterile distilled water three consecutive times to remove cells that were not firmly attached. Each stainless steel chip was then vortex mixed for 2 min with 10 mL of peptone diluent containing 15 g of glass beads (5 mm diameter). The peptone diluents containing the cells removed from the chips were diluted in 0.1% peptone and plated using the droplet plate technique on to MPCA plates (Lindsay and Von Holy, 1999). The MPCA plates were incubated at 30°C for 24 to 48 h, and the colonies were counted.

3.b.3.6 Identification of selected isolates

The bacterial isolates were grown on MPCA at 30°C for 24 h. The Gram-positive cultures were identified using the BD BBL Crystal Gram-Positive kit (Becton Dickinson and Company, Sparks, Nevada, USA), while the Gram-negative cultures were identified using the API 20 E kit for oxidase-negative isolates, or the API 20 NE kit for oxidase-positive isolates (bioMerieux, Marcy l'Etoile, France). The kits were incubated at 30°C for 24 h and read according to the manufacturers' instructions.

3.b.3.7 Identification of selected isolates by 16s rDNA sequence analysis

Twelve of the bacterial isolates that had the ability to produce thermo-resistant enzymes and form biofilms on test chips to various degrees were further identified by 16S rDNA sequence analysis. Their selection was based on their ability to produce thermo-resistant enzymes. In addition, a representative bacterial isolate from each genus identified using the biochemical data was selected. DNA was extracted from each isolate by a modified boiling method as described by Christison et al., 2007. A loopful of an overnight culture of each isolate was transferred into an Eppendorf tube containing 40 µL of sterile distilled and filtered water and 20 µL of chloroform. The mixture was centrifuged at 6440 X g for 5 min after boiling at 100°C for 20 min. The supernatant was used as the DNA template for the PCR.

Two different primer sets were used for the amplification of 16S rDNA of the bacterial isolates. The first primer sets were U1392R (5'-ACG GGC GGT GTG TRC-3') and Bac27F (5'-AGA GTT TGA TCM TGG CTG AG-3') (Christison et al., 2007) and the second primer sets were PAGES-F (5'-GAC GGG TGA GTA ATG CCT A-3') and PAGES-R (5'-CAC TGG TGT TCC TTC CTA TA-3') (Spilker et al., 2004). The second primer set was used to amplify the DNA of two atypical *Pseudomonas* bacterial isolates that were preliminarily identified using the API 20 NE kits. The product yields of the first and second primer sets were approximately 1.3 and 0.6 kbp, respectively. The resulting PCR products were purified and sequenced, and the sequences were analyzed by BLAST (<http://www.ncbi.nlm.nih.gov/blast/>) against 16S rDNA sequences from GenBank (GenBank database of the National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/Genbank/>). A genetic tree highlighting the genetic similarities of the isolates was constructed using DNAMAN version 4 (Lynnon Biosoft, Montreal, Quebec, Canada).

3.b.3.8 Determination of optimum growth temperatures for selected isolates

The optimum growth temperatures of twelve of the selected isolates were determined in a microtiter plate assay (for isolates that grew at 25°C) or using a temperature gradient incubator (Scientific Industries, Mineola, N.Y. USA) (for isolates that grew below 25°C).

For the microtiter plate assay, triplicate wells containing 200 μ L of nutrient broth (NB; Merck) were inoculated with 1% (v/v) overnight culture (in NB) of each isolate and were incubated at various temperatures (4, 7, 10, 20, 25, 30, 37, 44, and 55°C). Optical density measurements at 595 nm were performed every hour and were plotted against time. Experiments were carried out on two separate occasions.

For the temperature gradient incubator, tubes containing 15 mL of NB were inoculated with 1% (v/v) overnight culture (in NB) of each isolate and placed in an incubator set at between 7 and 47°C. Optical density measurements at 595 nm were determined every hour and were plotted against time.

Maximum, minimum, and optimum growth temperatures of the bacterial isolates were determined (Mohr and Krawiec, 1980).

3.b.4 RESULTS AND DISCUSSION

Fifty-two of the 153 isolates able to produce spoilage enzymes produced thermo-resistant enzymes (Table 3.b-2). Twenty-nine isolates produced only thermo-resistant proteolytic enzymes, 9 isolates produced only thermo-resistant lipolytic enzymes, and fourteen isolates produced both types of thermo-resistant enzymes. Most of the thermo-resistant enzymes produced by the bacteria isolated in summer were proteolytic (Table 3.b-2). By contrast, winter isolates largely produced both types of enzymes (Table 3.b-2). Results from our surface isolates study was in contrast with previous studies which have focused mainly on psychrotrophic enzyme producers in bulk liquid milk, where the majority of isolates tend to be proteolytic enzyme producers (Marchand et al., 2009a, Martins et al., 2006).

Table 3.b-2 Identities of the thermo-resistant enzyme-producing bacteria isolated from the internal surfaces of raw milk tankers during the winter and summer months of 2009–2010, and their colonisation of stainless steel chips *in vitro*.

Code	Bacterial identification *	Thermo-resistant enzyme production	Colonisation to stainless steel (Log ₁₀ cfu/cm ²)
Summer Isolates			
SF01	<i>Staphylococcus intermedius</i>	Lipase and Protease	4.8 ± 0.3
SC03	<i>Staphylococcus aureus</i>	Lipase	5.3 ± 0.5
C05	<i>Streptococcus uberis</i>	Lipase	4.0 ± 0.5
BC5	<i>Pseudomonas fluorescens</i>	Protease	5.7 ± 0.3
FC5	<i>Pseudomonas putida</i>	Protease	4.8 ± 0.5
F03	<i>Serratia liquefaciens</i>	Protease	6.0 ± 0.2
F09	<i>Serratia liquefaciens</i>	Protease	7.0 ± 0.4
CC6	<i>Serratia liquefaciens</i>	Protease	5.8 ± 0.3
DC4	<i>Serratia liquefaciens</i>	Protease	7.3 ± 0.3
FC1	<i>Serratia liquefaciens</i>	Protease	6.1 ± 0.4
FC2	<i>Serratia liquefaciens</i>	Protease	6.7 ± 0.9
F04	<i>Serratia liquefaciens</i>	Protease	7.6 ± 0.6
CC5	<i>Serratia liquefaciens</i>	Protease	6.1 ± 0.5
DC1	<i>Serratia liquefaciens</i>	Protease	7.3 ± 0.2
DC2	<i>Serratia liquefaciens</i>	Protease	6.4 ± 0.6
DC3	<i>Serratia liquefaciens</i>	Protease	6.0 ± 0.5
DC5	<i>Serratia liquefaciens</i>	Protease	7.1 ± 0.5
DC6	<i>Serratia liquefaciens</i>	Protease	6.9 ± 0.4
EC5	<i>Serratia liquefaciens</i>	Protease	6.4 ± 0.5
CC3	<i>Serratia liquefaciens</i>	Protease	7.0 ± 0.9
CC4	<i>Serratia liquefaciens</i>	Protease	6.1 ± 0.5
FC4	<i>Serratia liquefaciens</i>	Protease	5.9 ± 0.2
B09	<i>Staphylococcus aureus</i>	Protease	4.5 ± 0.4
A06	<i>Streptococcus uberis</i>	Protease	6.8 ± 0.4
AC4	<i>Streptococcus uberis</i>	Protease	7.3 ± 0.7

Winter isolates			
LL3	<i>Staphylococcus aureus</i>	Lipase and Protease	4.2 ± 0.4
SP1	<i>Staphylococcus aureus</i>	Lipase and Protease	3.8 ± 0.8
T1	<i>Staphylococcus aureus</i>	Lipase and Protease	3.8 ± 0.5
L5	<i>Staphylococcus intermedius</i>	Lipase and Protease	4.6 ± 0.2
P7	<i>Staphylococcus intermedius</i>	Lipase and Protease	4.0 ± 0.4
SB2	<i>Staphylococcus intermedius</i>	Lipase and Protease	3.7 ± 0.5
SB3	<i>Staphylococcus intermedius</i>	Lipase and Protease	4.2 ± 0.5
T4	<i>Staphylococcus intermedius</i>	Lipase and Protease	3.6 ± 0.5
T5	<i>Staphylococcus intermedius</i>	Lipase and Protease	3.5 ± 0.3
T8	<i>Staphylococcus intermedius</i>	Lipase and Protease	2.7 ± 0.5
LL2	<i>Staphylococcus saprophyticus</i>	Lipase and Protease	4.2 ± 0.4
SL3	<i>Staphylococcus saprophyticus</i>	Lipase and Protease	3.9 ± 0.2
SB1	<i>Staphylococcus scheiferi</i>	Lipase and Protease	4.9 ± 0.3
A34	<i>Staphylococcus aureus</i>	Lipase	3.7 ± 0.8
A32	<i>Staphylococcus aureus</i>	Lipase	4.8 ± 0.8
M2	<i>Staphylococcus intermedius</i>	Lipase	4.9 ± 0.4
M5	<i>Staphylococcus intermedius</i>	Lipase	3.8 ± 0.4
SR2W	<i>Staphylococcus intermedius</i>	Lipase	4.4 ± 0.3
SR2Y	<i>Staphylococcus intermedius</i>	Lipase	5.1 ± 0.3
B2	<i>Staphylococcus saprophyticus</i>	Lipase	4.5 ± 0.3
R4	<i>Bacillus coagulans</i>	Protease	3.4 ± 0.2
C12	<i>Pseudomonas fluorescens</i>	Protease	5.2 ± 0.3
C224	<i>Pseudomonas fluorescens</i>	Protease	7.1 ± 0.8
C221	<i>Pseudomonas fluorescens</i>	Protease	5.4 ± 0.2
LM2	<i>Staphylococcus aureus</i>	Protease	3.4 ± 0.1
ST2	<i>Staphylococcus intermedius</i>	Protease	4.8 ± 0.3
P10	<i>Staphylococcus warneri</i>	Protease	4.5 ± 0.2

* Bacterial isolates were identified by identification kits, BBL Crystal, API 20E and API 20 NE

Enzymes produced by these bacteria may reduce the quality of the final product as they can remain active after pasteurization (Chopra and Mathur, 1985, Dharmsthiti and Luchai, 1999, Griffiths et al., 1981, Marchand et al., 2009b). The production of enzymes usually occurs in the mid to late exponential phase or the early stationary phase of growth of the bacteria (Haddadi et al., 2005). However, the production of enzymes by bacteria appears to be complex, influenced by quorum sensing, temperature, iron content, and phase variation of the bacteria (Chen et al., 2003, Liu et al., 2007, Marchand et al., 2009a, Nicodème et al., 2005, Van Den Broek et al., 2005, Woods et al., 2001).

In addition, these isolates were also able to colonise stainless steel chips. The numbers of colonised cells recovered following the biofilm screening assay ranged from 2.7 to 7.6 Log₁₀ cfu/cm² (Table 3.b-2). Of the winter and summer isolates, C224 and F04 produced the highest numbers of colonised cells recovered (7.0 and 7.6 Log₁₀ cfu/cm² respectively). In general, the bacteria isolated during the summer were predominantly Gram-negative and grew to higher numbers on the stainless steel chips, compared to those isolated during winter, which were predominantly Gram-positive.

In this study, thermo-resistant enzyme-producing bacteria that were present on the internal surfaces of raw milk tankers, and which were able to colonise the stainless steel surfaces were identified by biochemical identification kits. Most of the thermo-resistant enzyme producers isolated during winter belonged to the *Staphylococcus* (85%) genus, while a mixture of bacteria were isolated during summer including *Serratia* spp. (68%), *Streptococcus* spp. (12%), *Staphylococcus* spp. (12%) and *Pseudomonas* spp. (8%). The warmer temperatures encountered during summer may have encouraged a greater diversity of bacteria in the raw milk for surface colonisation.

There are some limitations of identification of bacterial isolates by biochemical kits as they cannot differentiate to specific species level and may be misidentified (Alexopoulou et al., 2006, Croci et al., 2007). Therefore, twelve of the isolates selected for further study were identified as *S. uberis*., *S. liquefaciens*., *S. aureus*, *P. fluorescens*, *P. fragi* and *Bacillus licheniformis* (Figure 3.b-1) by 16S rDNA sequence analysis. These isolates are commonly found in raw milk (Lafarge et al., 2004). In this study, bacterial isolates that were initially identified as *S. intermedius*, *P. putida* and *B. coagulans* by biochemical kits were found to be *S. aureus*, *P. fragi* and *B. licheniformis* by 16S rDNA sequence analysis. The misidentified bacterial isolates may be due to their similarity in their

biochemical and genetic properties (Debartolomeo et al., 1991, Ercolini et al., 2007, Hajek, 1976).

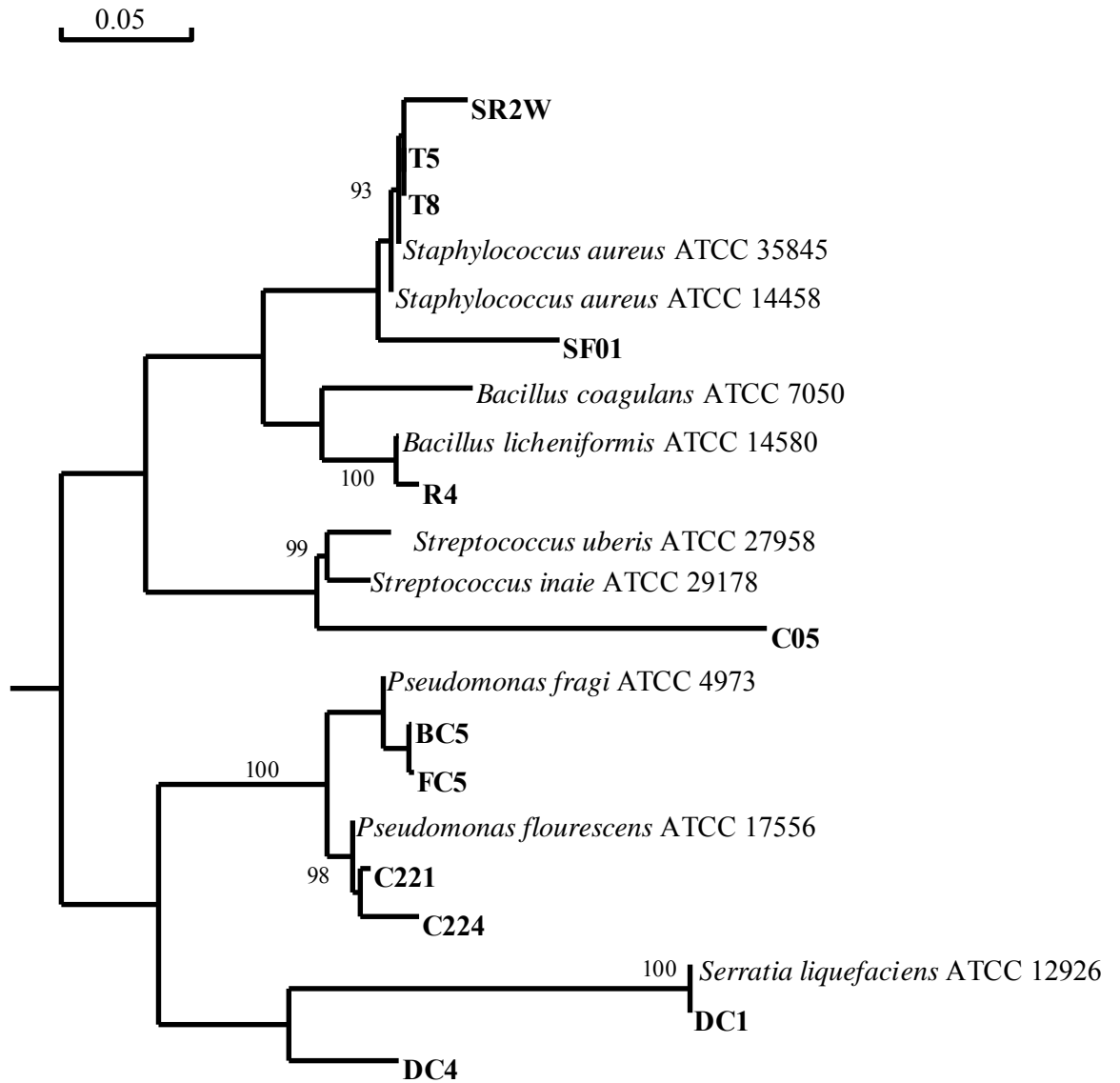


Figure 3.b-1 Phylogenetic tree showing clustering of bacterial strains isolated from the raw milk tanker. Bootstrapping values of 90 and above are indicated for the bacteria of interest.

Pseudomonas spp. reportedly produce a single type of protease – a neutral zinc metallo-proteinase with a molecular weight ranging from 39.2 ± 0.7 to 45.3 ± 1.3 kDa (Chopra and Mathur, 1985, Fairbairn and Law, 1986, Marchand et al., 2009a). Furthermore, bacterial proteases have the ability to hydrolyze casein to para- κ -casein, and destabilize the casein micelle, which in turn causes coagulation of the milk (Fairbairn and Law, 1986). In the raw milk, κ -casein and β -casein are the most susceptible protein components to psychrotrophic bacterial proteolysis, reducing the yield and the quality of the milk-derived protein products (Barnes et al., 1999). Bacterial species such as *Streptococcus* spp., *Staphylococcus* spp., and *Bacillus* spp. are known lipase producers (Dharmsthiti and Luchai, 1999, Georgalaki et al., 2000, Jung et al., 2002, Meyers et al., 1996, Smeltzer et al., 1992) and have the potential to produce thermo-resistant lipase, which can remain active in dairy products (Celestino et al., 1997b). Bacterial lipases can remain active after thermal processing during milk powder manufacture and retain the highest catalytic activities at temperatures ranging from 60 to 75°C (Dharmsthiti and Luchai, 1999, Law et al., 1976). More than one type of lipase can be produced by one species and, as with other lipases, vary in terms of properties and substrate specificities.

In previous studies, bacteria such as *Bacillus* spp., *Pseudomonas* spp., *Staphylococcus* spp., and *Streptococcus* spp. were also reported to produce biofilms in dairy processing plants (Flint et al., 1997, Teixeira et al., 2005). Biofilms can create a microenvironment that enhances microbial survival on the internal surfaces of raw milk tankers. Milk residues on the internal surfaces may provide the nutrients for the bacteria to survive and proliferate especially when growth conditions, such as temperature, are suitable. Bacterial cells that are enclosed within a biofilm matrix have been shown to produce enzymes, either by excretion or by autolysis, in other studies. These enzymes are either present in the biofilm matrix or dissolved into the surrounding medium (Wang and Chen, 2009, Zhang et al., 2007). Frølund et al., (1995) also reported that the amount of enzymes produced by cells within biofilms is generally greater than that produced by cells in planktonic cultures. For example, the enzymatic activities of mixed bacterial populations in a sludge biofilm have been reported to be 18 to 32 times higher than that of the individual bacterial cells in planktonic cultures (Frølund et al., 1995). Biofilms present on the internal surfaces of raw milk tankers could be an unrecognized source of enzymes contributing to the spoilage of finished dairy products.

Raw milk contains a wide variety of psychrotrophic and mesophilic bacteria from the dairy farm environment, which could potentially colonize the internal surfaces of raw milk tankers. In the present investigation, a total of 12 bacterial isolates from the internal surfaces of raw milk tankers grew over a wide range of temperature, in some cases as low as 7°C, with the optimum growth temperature varying from 25 to 44°C (Table 3.b-3).

Table 3.b-3 Growth temperature profiles of selected bacterial isolates

Bacterial isolates *	Minimum (°C)	Maximum (°C)	Optimum (°C)
Winter			
<i>Pseudomonas fluorescens</i> C221	10	36	25
<i>Pseudomonas fluorescens</i> C224	10	38	25
<i>Staphylococcus aureus</i> SR2W	10	55	37
<i>Staphylococcus aureus</i> T5	10	55	44
<i>Staphylococcus aureus</i> T8	10	55	44
<i>Bacillus licheniformis</i> R4	20	55	44
Summer			
<i>Serratia liquefaciens</i> DC1	10	38	25
<i>Serratia liquefaciens</i> DC4	10	44	30
<i>Pseudomonas fluorescens</i> BC5	7	37	30
<i>Pseudomonas fluorescens</i> FC5	7	37	30
<i>Streptococcus uberis</i> C05	10	55	37
<i>Staphylococcus aureus</i> SF01	10	55	37

* Bacterial isolates were identified by 16s rDNA sequence analysis

During the transportation of milk, it is likely that the growth of the more psychrophilic Gram-negative populations will be favoured in the bulk fluid as milk is usually held between 4 and 10°C (Lafarge et al., 2004, Martins et al., 2006). Bacteria, such as psychrotrophic *Pseudomonas* spp., are known to be prolific enzyme producers, which may play a major role in the spoilage of dairy products (Champagne et al., 1994, Chen et al., 2003, Marchand et al., 2009b, Nicodème et al., 2005). But in this study, a mixture of mesophilic and cold tolerant bacterial isolates were found on the internal surface of raw milk tanker. These bacterial isolates were able to produce biofilm to various degrees when they were incubated at 25°C. The internal temperature surfaces of raw milk tankers are generally higher than the temperature in bulk milk, especially, the upper part of the raw milk tanker. Furthermore, the temperature in top layer of milk was higher compared with the temperature in the bulk milk (Crawford, 1967). This may favour biofilm formation as biofilms can be found in the air-liquid interface in the internal surfaces of raw milk tankers (Wijman et al., 2007).

The significance of the presence of these bacteria on the surfaces of the raw milk tanker is their potential to play a role in spoilage during transportation of milk or of dairy products at various stages of manufacture or distribution.

3.b.5 CONCLUSION

The results of this study demonstrated that a variety of bacteria were present, presumably as biofilms, on the internal surfaces of raw milk tankers. These bacteria, likely to originate from the dairy farm or the dairy herd, attach on to the internal surfaces of raw milk tankers during transportation. The bacterial species present could grow over a range of temperatures, including as low as 7°C. Of the bacteria isolated, 14% produced thermo-resistant proteases, 4% produced thermo-resistant lipases and 7% produced both thermo-resistant enzymes. It is likely that, if conditions, such as time and temperature, are suitable, biofilms on the internal surfaces of milk tankers will develop and bacteria within these biofilms may produce enzymes with the potential to spoil dairy products. This could be a previously unreported source of thermo-resistant enzymes contaminating dairy products.

**Chapter 4. *In Vitro* Studies On
Enzyme Production Within Biofilm**

4.a *Proteolysis produced within biofilms of bacterial isolates from raw milk tankers*

Published as:

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(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Koon Hoong Teh

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In which Chapter is the Published Work: Chapter 4.a

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4.a.1 ABSTRACT

In this study, six bacterial isolates that produced thermo-resistant enzymes isolated from the internal surfaces of raw milk tankers were evaluated for their ability to produce proteolysis within either single culture biofilms or co-culture biofilms. Biofilms were formed in an *in vitro* model system that simulated the upper internal surface of a raw milk tanker during a typical summer's day of milk collection in New Zealand. The bacterial isolates were further evaluated for their ability to form biofilms at 25, 30 and 37 °C. Mutual and competitive effects were observed in some of the co-culture biofilms, with all isolates being able to form biofilms in either single culture or co-culture at the three temperatures. The proteolysis was also evaluated in both biofilms and corresponding planktonic cultures. The proteolysis per cell decreased as the temperature of incubation (20–37 °C) increased. Furthermore, mutualistic interactions in terms of proteolysis were observed when cultures were grown as co-culture biofilms. This is the first study to show that proteolytic enzymes can be produced in biofilms on the internal surfaces of raw milk tankers. This has important implications for the cleaning and the temperature control of raw milk transport tankers.

4.a.2 INTRODUCTION

Most of the raw milk in New Zealand is transported to dairy processing plants by raw milk tankers. During transportation, the milk is splashed about, coating the internal stainless steel surfaces of the raw milk tankers, including those not directly in contact with the bulk raw milk. This splashing effect may pre-condition the internal surfaces of the tankers for bacterial adhesion, and can result in bacteria naturally present in raw milk becoming attached to the tanker surfaces (Vanhaecke et al., 1990).

Bacteria that are capable of producing thermo-resistant proteolytic enzymes have been isolated from the internal surfaces of raw milk tankers (Teh et al., 2011). The proteolytic enzymes that are produced by such bacteria may be a problem in the dairy industry, as they may remain active after pasteurization and may cause defects in the final dairy products, because of hydrolysis of the peptide bonds, such as bitter flavour in milk and gelatinization of UHT milk during prolonged storage. Even small amounts of proteolytic enzyme can actively hydrolyse peptide bonds and can alter the physico-chemical, functional and sensory properties of dairy products (Celestino et al., 1997b, Chen et al., 2003, Guinot-Thomas et al., 1995, Martins et al., 2006). In addition to producing enzymes, these bacteria may also form biofilms on the internal surfaces of raw milk tankers, adding to the enzymatic load produced by planktonic microorganisms in the milk. The biofilms may detach from milk tanker surfaces into the milk and may subsequently contaminate the processing line (Flint et al., 1997, Wilks et al., 2006).

The temperature of the internal surface of raw milk tankers tends to fluctuate because of the temperature of the bulk milk and environmental factors, such as the ambient temperature and sun radiation. Warmer temperatures may promote biofilm growth on the inner stainless steel surfaces, which are contaminated by splashes from the raw milk. The air space between the bulk milk and the upper internal surface of the tanker may also influence the surface temperature of the upper part of the milk tanker. The top layer of milk may also be at a higher temperature than the bulk milk, which may lead to the production of enzymes in the biofilms forming at the air-liquid interface (Buchon et al., 2000, Crawford, 1967, Nicodème et al., 2005, Wijman et al., 2007). We have previously determined that different regions of raw milk tankers have different surface temperatures during milk

transportation, with the upper part of the milk tanker tending to be hottest during summer because of sun radiation. The surface temperature of an empty raw milk tanker can be as high as 20 °C during summer (Chapter 3.a).

Whereas many studies have focused on the impact of the temperature of the bulk milk on its quality, the effect of surface temperatures and/or fluctuating temperatures on the growth of biofilms and the production of enzymes has received little attention. The aim of this work was to evaluate the production and accumulation of proteolytic enzymes within biofilms formed in an *in vitro* model system that simulated the fluctuating surface temperatures of a raw milk tanker during a typical summer's day. This study also compared proteolysis by cells within biofilms with that of their planktonic counterparts.

4.a.3 MATERIALS AND METHODS

4.a.3.1 Bacterial strains

Six enzyme-producing bacterial strains isolated from the internal stainless steel surfaces of raw milk tankers based in the Manawatu region of New Zealand were used in this study (Teh et al., 2011). Gram-positive strains (*Bacillus licheniformis* R4, *Staphylococcus aureus* SF01, *Streptococcus uberis* C05) and Gram-negative strains (*Pseudomonas fluorescens* C224, *Pseudomonas fragi* BC5, *Serratia liquefaciens* DC1) were used. They were maintained in tryptic soy broth (Merck, Darmstadt, Germany) containing 20% (v/v) glycerol at -80 °C. The bacterial strains were pre-incubated in sterile reconstituted skim milk (RSM; 100 g of skim milk powder in 910 mL of distilled water) for 1 h at 30 °C before experiments.

4.a.3.2 Attachment and biofilm formation assay

A biofilm assay was set up using an *in vitro* model to simulate the upper internal surface of a raw milk tanker. Three scenarios were simulated:

Scenario i (TMC) – Bacterial attachment onto a stainless steel surface after a typical 9.25 h of milk collection (TMC).

Scenario ii (SMC) - Biofilm formation onto a stainless steel surface of an inadequately cleaned tanker with subsequent milk collection (SMC).

Scenario iii (EON) - Biofilm formation onto a stainless steel surface of an inadequately cleaned tanker that was left to stand empty overnight (EON).

A volume (1 mL) of the pre-incubated culture was inoculated into 4 mL of RSM in a 15 mL centrifuge tube (Greiner Bio-one, Auckland, New Zealand) containing 1 stainless steel coupon (grade 316 with 2B finish; 50 mm x 13 mm; ETECH, Palmerston North, New Zealand). The initial cell concentration was 10^3 cfu/mL. A set of three centrifuge tubes was placed horizontally in a rotating tube holder (KH-2011; Massey University, Palmerston North, New Zealand) set at 4 rpm. The coupons were semi-submerged in RSM, as the reactor rotated, the RSM washed onto the coupons. The rotating tube holder was incubated at temperatures that simulated the fluctuating temperatures of the upper internal surface of a raw milk tanker during milk collection on a typical warm summer's day (Table 4.a-1). The total initial incubation period was 9.25 h. Three sets of centrifuge tubes were prepared.

Table 4.a-1 The average temperatures of the upper internal surface of a raw milk tanker during milk collection on a typical warm summer's day.

Incubation period (h)	Average temperature (°C)
1.50	16
3.00	20
1.00	35
1.00	20
1.00	30
1.75	20

Note: The temperature of the upper internal surface of a raw milk tanker was determined previously by placing data loggers on a raw milk tanker during its milk collection (chapter 3.a).

Enumeration of attached cells recovered from the surfaces was performed on the first set of stainless steel coupons, which represented scenario i (TMC). For the enumeration procedure, the coupons were rinsed by dipping in sterile distilled water three consecutive times to remove any loosely attached cells from the coupons. The rinsed coupons were transferred into 10 mL of peptone diluent (Ford Richard, Auckland, New Zealand) and were vortex mixed with 15 g of glass beads (5 mm diameter) for 2 min; then serial 10-fold dilutions in peptone prepared and were plated on to milk plate count agar (MPCA; Oxoid, Auckland, New Zealand) using the droplet plate method (Lindsay and Von Holy, 1999). The MPCA plates were incubated at 30 °C for 24 h, and the colonies were counted and were expressed as Log₁₀ cfu/cm².

The stainless steel coupons from the second set were transferred into 15 mL centrifuge tubes containing fresh sterile RSM and were incubated at 20 °C for a further 14.75 h after the initial incubation period of 9.25 h. This was to simulate scenario ii (SMC), i.e. an inadequately cleaned milk tanker with subsequent milk collection. The stainless steel coupons from the third set were transferred into empty 15 mL centrifuge tubes and were incubated at 20 °C for a further 14.75 h after initial incubation period of 9.25 h. This was to simulate scenario iii (EON), i.e. an inadequately cleaned milk tanker that was left to stand empty overnight. Counts of the biofilm populations recovered from the surfaces were performed as described previously.

4.a.3.3 Proteolysis within single culture and co-culture biofilms, and corresponding planktonic cultures

4.a.3.3.1 Growth of biofilms and planktonic cultures

Single cultures and co-cultures of planktonic and biofilm cells were prepared. For single culture biofilms, 4.5 mL of RSM was inoculated with 0.5 mL of a pre-incubated culture of each of the six cultures; for co-culture biofilms, 4.5 mL of RSM was inoculated with 0.5 mL of each of two different pre-incubated cultures. The co-cultures were *S. aureus* SF01 and *P. fluorescens* C224, *B. licheniformis* R4 and *P. fragi* BC5, and *S. uberis* C05 and *S. liquefaciens* DC1. The initial concentration of cells was 10⁷ cfu/mL. Biofilm populations were prepared in a similar manner to the *in vitro* biofilm assay described for scenario iii (EON) except that the coupons were incubated at 20, 30 or 37 °C for 14.75 h after the

initial incubation of 9.25 h. Total biofilm populations recovered and Gram-positive biofilm populations recovered were counted using the droplet plate method, as described previously, on MPCA and were incubated at 30 °C and 42 °C, respectively, for 24 h. Gram-negative biofilm populations were counted on MPCA plates supplemented with 0.001 g crystal violet/L (BDH Prolab, UK) and were incubated at 30 °C for 24 h.

For the corresponding planktonic populations, 0.1 mL of the liquid culture in which the cultures were initially grown for 9.25 h was inoculated into 4 mL of fresh RSM and was incubated at 20, 30 or 37 °C for 14.75 h. After incubation, the planktonic cells were counted on MPCA, as described previously.

The results were expressed as $\text{Log}_{10} \text{cfu/cm}^2$ and $\text{Log}_{10} \text{cfu/mL}$ for biofilms and planktonic cultures respectively.

4.a.3.3.2 *Proteolysis assay*

A proteolysis assay using azocasein as the substrate was performed with slight modifications (Bussamara et al., 2010). A 3% azocasein solution (Sigma-Aldrich, Auckland, New Zealand) was prepared in 5 mM phosphate buffer solution, pH 7.5 with 0.1% sodium azide (Scharlau, Scharlau Chemie, Spain) and 0.1 mg chloramphenicol/mL (Sigma-Aldrich, Auckland, New Zealand). Both the biofilms and the planktonic cultures were prepared as described above. After incubation, the stainless steel coupons were transferred into 9 mL of the 3% azocasein solution, whereas the planktonic cultures were centrifuged at 10 000 g for 5 min and 0.1 mL of the supernatant was transferred into 0.9 mL of the 3% azocasein solution. An un-inoculated stainless steel coupon and RSM were used as blank controls. The azocasein solutions were incubated at 40 °C for 24 h.

A volume of 0.4 mL of the azocasein solution was mixed with 0.8 mL of 20% trichloroacetic acid (Sigma-Aldrich, Auckland, New Zealand) to stop the reaction and the resulting solution was centrifuged at 10 000 g for 5 min. A volume of 0.15 mL of the supernatant was transferred into microtitre plate wells (Difco, Becton, Dickinson and Company, Sparks, MD, USA) in six replicates and the absorbance was read at 400 nm using a microtitre plate reader (Spectrostar Nano, BMG Labtech, Auckland, New Zealand).

The proteolysis was measured by comparing the absorbance value of the samples with the absorbance value of the standard curve of the proteolysis by *Streptomyces griseus*

(3.9 units/mg; Sigma-Aldrich, Auckland, New Zealand) as a reference under the pH, temperature and incubation period used in this study. One unit of proteolysis was defined as the proteolysis produced by one milligram of the Sigma-Aldrich *S. griseus* protease standard under the assay conditions used in this study. The estimated concentration of the proteolysis produced was divided by the number of colony forming units (cfu) recovered from the stainless steel surfaces or in the planktonic cultures. The results were expressed as picoUnits of proteolysis per cfu ($\rho\text{U}/\text{cfu}$).

4.a.3.4 Morphology of single culture and co-culture biofilms

The morphologies of single culture and co-culture biofilms formed on stainless steel coupons (1 cm^2) in the *in vitro* model were observed using scanning electron microscopy (SEM). The stainless steel coupons were fixed overnight in 4% glutaraldehyde, dehydrated in ethanol (Lindsay and Von Holy, 1999), critically point dried, mounted, coated with gold and viewed using environmental SEM (FEI Quanta 200).

4.a.3.5 Statistical analysis

Three replicates were performed for each experiment, on two separate occasions. SAS 9.2 software was used for analysis of variance using Tukey's test with a critical probability of $P \leq 0.05$.

4.a.4 RESULTS

4.a.4.1 Attachment and the development of biofilms

The bacterial isolates used in the study were able to attach and form biofilms on the stainless steel coupons in the *in vitro* model, with the number of attached bacterial cells recovered in scenario i (TMC) ranging from about 1.5 to 3 $\text{Log}_{10}\text{ cfu}/\text{cm}^2$, and the number of biofilms bacterial cells recovered in scenario ii (SMC) and scenario iii (EON) ranging from about 0.5 to 5 $\text{Log}_{10}\text{ cfu}/\text{cm}^2$ and 1.5 to 4.5 $\text{Log}_{10}\text{ cfu}/\text{cm}^2$, respectively (Figure 4.a-1).

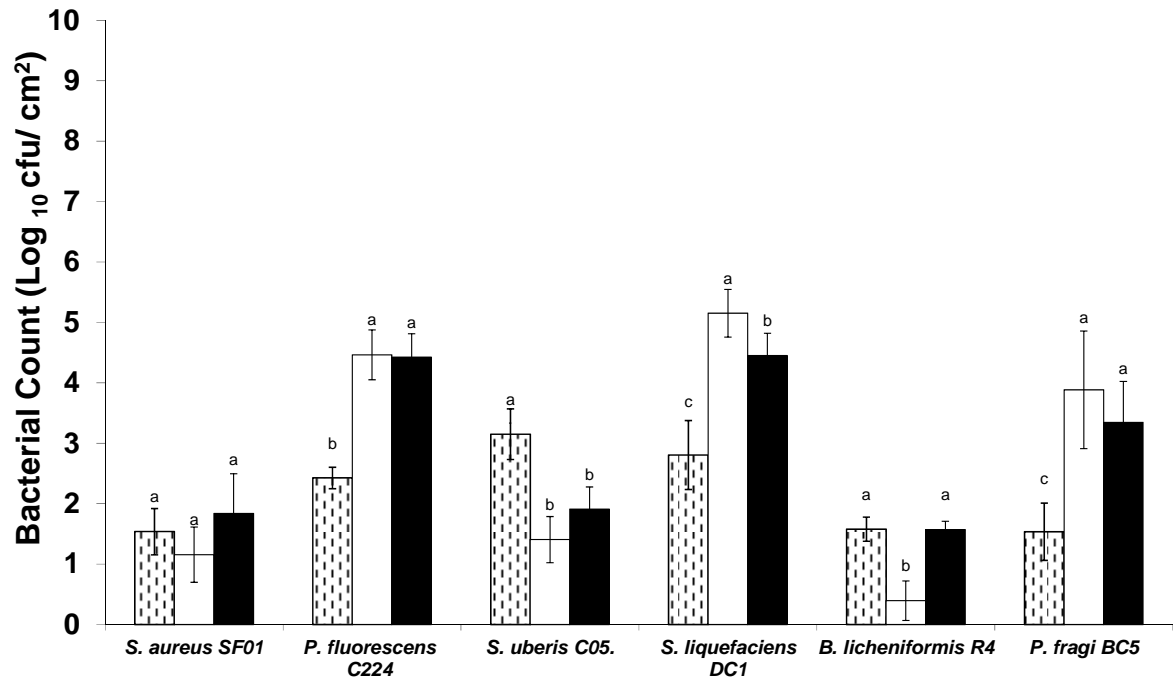


Figure 4.a-1 The number of cells of *B. licheniformis* R4, *P. fluorescens* C224, *P. fragi* BC5, *S. liquefaciens* DC1, *S. aureus* SF01 and *S. uberis* C05 recovered under three different scenarios: dotted bar, scenario (i), Bacterial attachment on the stainless steel surface after milk collection (TMC); white bar, scenario ii (SMC), biofilm formation on the stainless steel surface of an inadequately cleaned tanker with subsequent milk collection; black bar, scenario iii (EON), biofilm formation on the stainless steel surface of an inadequately cleaned tanker left to stand empty overnight. Experiments were performed with three replicates on two separate occasions, and error bars represent standard deviations from the mean. Different letters indicate statistically significant differences for each particular microorganisms, $P \leq 0.05$ (Tukey's test).

The number of *S. liquefaciens* DC1 cells recovered was higher in scenario ii (SMC) than in scenario iii (EON). There were no significant differences in the attached populations ($P < 0.05$) between scenario ii (SMC) and scenario iii (EON) for *P. fluorescens* C224 and *P. fragi* BC5. Fewer *S. uberis* C05 cells were recovered in both scenario ii (SMC) and scenario iii (EON) than in scenario i (TMC), whereas the number of *B. licheniformis* R4 cells recovered was significantly lower in scenario ii (SMC) than in scenario iii (EON). Overall, the number of cells recovered in scenario ii (SMC) and scenario iii (EON) was greater for the Gram-negative isolates than for the Gram-positive isolates.

4.a.4.2 Proteolysis within single culture and co-culture biofilms, and corresponding planktonic cultures

4.a.4.2.1 Growth of biofilms and planktonic cultures

All of the bacterial isolates used in the *in vitro* model were able to colonize the stainless steel surfaces to various levels at 20, 30 and 37 °C (Figure 4.a-2A). The number of bacterial cells recovered from the stainless steel surfaces for single culture and co-culture biofilms of *S. aureus* SF01 and *P. fluorescens* C224 ranged from about 5 to 6.5 Log₁₀ cfu/cm² and 6.5 to 7.5 Log₁₀ cfu/cm² respectively (Figure 4.a-2A). The number of *S. aureus* SF01 cells recovered was significantly higher ($P < 0.05$) in the presence of *P. fluorescens* C224 than in single culture biofilms at the three temperatures tested. There were no significant differences between the numbers of cells recovered from the single culture and co-culture biofilms of *P. fluorescens* C224 when they were grown at 20 or 30 °C; however, a significant reduction in *P. fluorescens* C224 cell numbers was observed when they were grown in co-culture with *S. aureus* SF01 at 37 °C ($P < 0.05$).

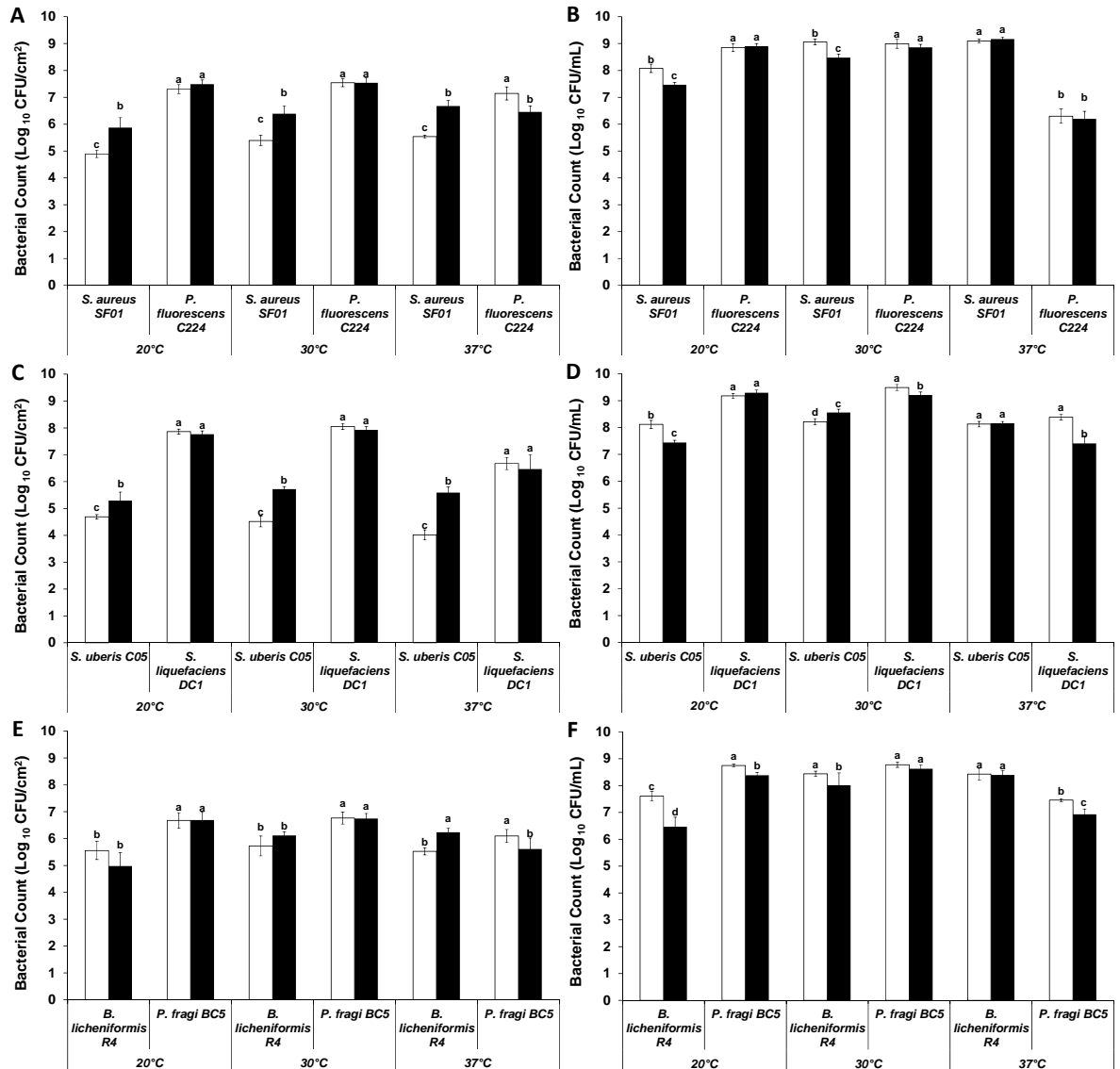


Figure 4.a-2 The number of cells recovered from single culture and co-culture biofilms (A, C, E) and their corresponding planktonic cultures (B, D, F) of *S. aureus* SF01 and *P. fluorescens* C224 (A, B), *S. uberis* C05 and *S. liquefaciens* DC1 (C, D), and *B. licheniformis* R4 and *P. fragi* BC5 (E, F) grown in the *in vitro* model that simulated the stainless steel surface of an inadequately cleaned tanker left overnight, after a typical 10 h milk collection, at three different temperatures, 20, 30 and 37 °C. The white bars represent the single cultures and the black bars represent co-cultures. Experiments were performed with three replicates on two separate occasions, and error bars represent standard deviations from the mean. Different letters indicate statistically significant differences, $P \leq 0.05$ (Tukey's test).

The number of bacterial cells recovered from the planktonic single cultures and co-cultures of *S. aureus* SF01 and *P. fluorescens* C224 ranged from about 7.5 to 9 Log₁₀ cfu/mL and 6 to 9 Log₁₀ cfu/mL respectively (Figure 4.a-2B). A reduction in the number of *S. aureus* SF01 cells recovered was observed when they were grown with *P. fluorescens* C224 at 20 or 30 °C. Even though the number of *P. fluorescens* C224 cells recovered was lower, both *S. aureus* SF01 and *P. fluorescens* C224 were found to be recovered in similar numbers when they were grown at 37 °C either in single culture or in co-culture.

A similar trend was observed in the single culture and co-culture biofilms of *S. uberis* C05 and *S. liquefaciens* DC1; the number of Gram-positive bacterial cells increased when they were grown in the presence of Gram-negative bacteria. The number of bacterial cells recovered from single culture and co-culture biofilms of *S. uberis* C05 and *S. liquefaciens* DC1 ranged from about 4 to 6 Log₁₀ cfu/cm² and 6.5 to 8 Log₁₀ cfu/cm² respectively (Figure 4.a-2C).

Lower numbers of *S. liquefaciens* DC1 cells were recovered in the presence of *S. uberis* C05 when they were grown as planktonic cultures at 30 or 37 °C. The number of bacterial cells in planktonic culture ranged from about 7 to 8.5 Log₁₀ cfu/mL and 7 to 9.5 Log₁₀ cfu/mL for *S. uberis* C05 and *S. liquefaciens* DC1 respectively (Figure 4.a-2D).

B. licheniformis R4 and *P. fragi* BC5 were able to form biofilms at 20, 30 and 37 °C in both single culture and co-culture. The number of bacterial cells recovered from the surfaces ranged from about 5 to 6 Log₁₀ cfu/cm² and 5.5 to 6.5 Log₁₀ cfu/cm² for *B. licheniformis* R4 and *P. fragi* BC5 respectively (Figure 4.a-2E). No interactions between these two bacterial species were observed at 20 or 30 °C; however, there was a significant increase ($P < 0.05$) in the number of *B. licheniformis* R4 cells recovered and a decrease in the number of *P. fragi* BC5 cells recovered when they were grown together at 37 °C.

Both *B. licheniformis* R4 cells and *P. fragi* BC5 cells were recovered in lower numbers when they were grown together at 20 °C. The number of bacterial cells in planktonic culture ranged from about 6.5 to 8 Log₁₀ cfu/mL and 7 to 9 Log₁₀ cfu/mL for *B. licheniformis* R4 and *P. fragi* BC5 respectively (Figure 4.a-2F).

4.a.4.2.2 *Proteolysis*

In general, the amount of proteolysis produced was higher in biofilm cells than in the corresponding planktonic cultures (Table 4.a-2). In addition, the estimated proteolytic enzyme activities of the biofilm and planktonic cultures appeared to be strain dependent under these assay conditions (Table 4.a-2).

P. fluorescens C224 produced proteolysis in single culture biofilms at 20, 30, and 37 °C, and in planktonic culture at 20 and 30 °C but not at 37 °C. The highest proteolysis for single culture biofilm was *Ps. fluorescens* C224 at 20°C (Table 4.a-2). In co-culture with *S. aureus* SF01, proteolysis was only produced at 20 and 30 °C in both biofilms and planktonic cultures (Table 4.a-2). The amount of proteolysis was found to be higher in the co-culture biofilms compared with their corresponding single culture biofilms.

The proteolysis of single culture biofilms of *S. liquefaciens* DC1 was estimated at 7–10 ρU /cfu. *S. liquefaciens* DC1 did not produce proteolysis at 37 °C. The amount of proteolysis produced in *S. liquefaciens* DC1 planktonic cultures decreased as the temperature was increased from 20 to 37 °C; however, the estimated amount of proteolysis produced per cfu in biofilms remained the same (Table 4.a-2). For co-cultures of *S. uberis* C05 and *S. liquefaciens* DC1, proteolysis was not observed at 37 °C either in biofilms or in planktonic cultures.

P. fragi BC5 was able to produce proteolysis in planktonic culture only at 20 °C, both as a single culture and as a co-culture with *B. licheniformis* R4 (Table 4.a-2). Neither *B. licheniformis* R4 nor *Ps. fragi* BC5 produced proteolysis in single culture biofilms; however, the overall highest proteolysis in the current study was detected in the co-culture biofilms of *B. licheniformis* R4 and *P. fragi* BC5 at 37°C (50 ρU /cfu).

Table 4.a-2 The estimated amount of proteolysis produced per colony forming units (pU /cfu) under the assay conditions within single culture and co-culture biofilms, and their corresponding planktonic cultures, of *S. aureus* SF01 and *P. fluorescens* C224, *S. uberis* C05 and *S. liquefaciens* DC1, and *B. licheniformis* R4 and *P. fragi* BC5, grown in the *in vitro* model that simulated the stainless steel surfaces of an inadequately cleaned tanker left overnight, after a typical 9.25 h milk collection, at three different temperatures, 20, 30 and 37 °C.

	Biofilm (pU /cfu)			Planktonic (pU /cfu)		
	20 °C	30 °C	37 °C	20 °C	30 °C	37 °C
Co-culture	16 ± 2 ^a	9 ± 2 ^b	< 1	9 ± 1 ^b	7 ± 1 ^b	< 1
<i>S. aureus</i> SF01	< 1	< 1	< 1	< 1	< 1	< 1
<i>P. fluorescens</i> C224	14 ± 1 ^a	7 ± 1 ^d	3 ± 1 ^e	11 ± 3 ^b	10 ± 1 ^c	< 1
Co-culture	13 ± 5 ^a	9 ± 4 ^a	< 1	8 ± 4 ^a	8 ± 1 ^a	< 1
<i>S. uberis</i> C05	< 1	< 1	< 1	< 1	< 1	< 1
<i>S. liquefaciens</i> DC1	10 ± 2 ^a	10 ± 3 ^a	< 1	9 ± 1 ^a	7 ± 1 ^a	< 1
Co-culture	< 1	< 1	50 ± 15 ^a	10 ± 3 ^b	< 1	< 1
<i>B. licheniformis</i> R4	< 1	< 1	< 1	< 1	< 1	< 1
<i>P. fragi</i> BC5	< 1	< 1	< 1	4 ± 1	< 1	< 1

Note: Experiments were performed with three replicates on two separate occasions, and values represent the means with standard deviations.

The different letters within each row indicate statistically significant differences, $P \leq 0.05$ (Tukey's test).

4.a.4.3 Morphology of single culture and co-culture biofilms

SEM of a single culture of an attached population of *S. aureus* SF01, suggested that the bacterial cells were spread out over the stainless steel surfaces (Figure 4.a-3A). *S. liquefaciens* DC1 appeared to form more tightly packed microcolonies and were surrounded by structures that resembled exopolysaccharide (EPS) (Figure 4.a-3B). In the co-culture biofilms of *S. aureus* SF01 and *P. fluorescens* C224, *S. aureus* SF01 was seen in separate microcolonies from *P. fluorescens* C224 (Figure 4.a-3C). Strands likely to represent EPS were also observed in the co-culture biofilms of *S. uberis* C05 and *S. liquefaciens* DC1 (Figure 4.a-3D).

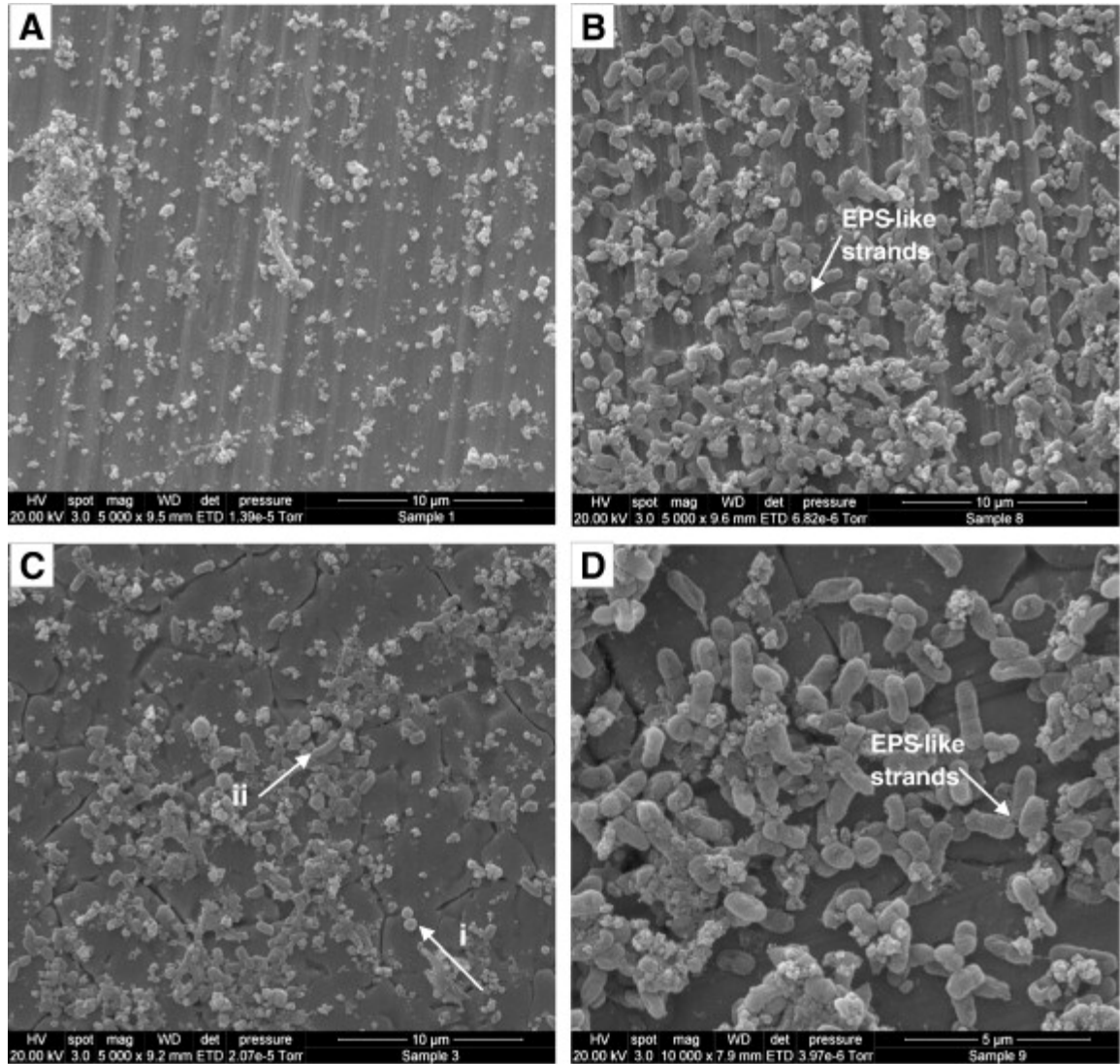


Figure 4.a-3 Scanning electron micrographs of attached population of *S. aureus* SF01 (A) and *S. liquefaciens* DC1 (B) and of co-culture biofilms of *S. aureus* SF01 (i) and *P. fluorescens* C224 (ii) (C) and *S. liquefaciens* DC1 and *S. uberis* C05 (D).

4.a.5 DISCUSSION

This study demonstrated proteolysis by bacterial cells of dairy origin growing in biofilms. Previous studies have evaluated either crude enzymes from the supernatant of planktonic cultures or from biofilms, but not enzymes produced by a complete biofilm (Frølund et al., 1995, Nørnberg et al., 2011). In this study, proteolysis was strain and temperature dependent under the assay conditions. In addition, in some cases, attachment to stainless steel appeared to enhance proteolysis. For example, *P. fluorescens* C224 was able to produce proteolysis at 20, 30, and 37 °C in biofilms but only at 20 and 30 °C in planktonic culture. In addition, *P. fluorescens* C224 was unable to grow at 37°C in planktonic culture, therefore proteolysis was not detected in that planktonic state. The growth of the *P. fluorescens* C224 cells appeared to be enhanced within biofilms compared with their corresponding planktonic cultures at higher temperatures. This phenomenon of growth of bacteria in biofilms at higher temperatures than their usual growth temperatures in planktonic state has been previously noted for other bacterial species (Flint et al., 2002, Nilsson et al., 2011, Rogers et al., 1994).

Most of the co-culture biofilms and planktonic cultures used in this study were able to produce proteolysis at 20 and 30 °C under the assay conditions. The exception was the co-culture of *B. licheniformis* R4 and *P. fragi* BC5 where both of these bacterial isolates were only able to produce proteolysis as co-culture biofilms at 37°C. Furthermore, some of the co-culture biofilms had higher proteolysis compared with their corresponding single culture biofilms. This may have been due to a mutualistic interaction. Previous studies have shown enhanced enzyme production by *Bacillus* species when in co-culture. For example, the production of amylolytic enzymes by *B. amyloliquefaciens* was increased when grown in co-culture with *Zymomonas mobilis* (Abate et al., 1999). Furthermore, quorum sensing may promote both biofilm growth and enzyme production as *N*-acylhomoserine lactone has been shown to increase both biofilm growth and protease production by *Aeromonas hydrophila* (Khajanchi et al., 2009, Swift et al., 1999).

In general, the amount of proteolysis produced per cfu by some of the bacterial isolates in this study was higher in biofilms than in their planktonic counterparts. Similar findings have been noted for biofilms used in biotechnology studies. Sludge biofilms and

fungal biofilms tend to have greater amounts of secreted enzymes than their planktonic counterparts (Frølund et al., 1995, Gamarra et al., 2010). It has also been suggested that bacterial cells within the biofilm population can accumulate enzymes that may aid in the survival of these populations (Budhani and Struthers, 1998).

In addition to enzyme production, the formation of biofilms by these bacterial isolates in the *in vitro* model that simulated the upper internal surface of a raw milk tanker was examined. The number of cells that colonized the stainless steel surfaces after a typical 9.25 h milk collection was different for each bacterial species, and may have been due to different cell surface zeta potentials, cell hydrophobicities, bacterial nanofibres and growth temperatures of the bacterial species (Hori and Matsumoto, 2010, Wang et al., 2011). The incubation temperature may have influenced the rate of attachment because it has been shown that the lag phase of mixed cultures of bacteria was increased when they were incubated at suboptimal temperatures (Patil et al., 2010). This may have favoured the growth of the Gram-negative isolates over the Gram-positive isolates, as the Gram-negative isolates used in the study of development of biofilms were psychrotrophic and the subsequent incubation temperature used was 20 °C after initial incubation at a series of fluctuating temperatures.

Scanning electron micrographs of the single culture and co-culture of attached populations showed that the Gram-negative bacterial isolates were able to form microcolonies and that the Gram-positive bacterial isolates tended to spread over the surfaces. In addition, the bacterial cells in co-culture biofilms were seen next to each other rather than being embedded in a single microcolony, as has been observed in other studies of mixed culture biofilms (Lindsay et al., 2002, Seo and Frank, 1999, Zameer et al., 2010).

This study demonstrated the formation of biofilms by these bacterial isolates in the *in vitro* model that simulated the upper part of a milk tanker with inadequate cleaning after milk collection at three different temperatures, 20, 30 and 37 °C. Co-culture biofilms appeared to enhance biofilm development even further, as two of the Gram-positive bacterial isolates were found to have higher cell counts in biofilms when they were grown as co-cultures with Gram-negative bacterial isolates. EPS production by Gram-negative bacterial isolates has been shown to enhance the attachment of Gram-positive bacterial isolates on stainless steel surfaces (Lindsay et al., 2002, Sasahara and Zottola, 1993). The

Gram-negative bacterial isolates used in this study, were hypothesized to produce EPS, as seen in the SEM images, possibly accounting for the observed enhancement of growth in co-culture biofilms.

In the planktonic co-cultures, the Gram-positive bacterial isolates had lower cell counts when they were grown with Gram-negative bacterial isolates at 20 °C. The psychrophilic Gram-negative bacteria may have outcompeted and/or outgrown the Gram-positive bacteria at 20 °C as this is a suboptimal temperature for the mesophilic Gram-positive bacterial isolates used in the current study.

Overall, this study has indicated that microbial interactions affect both enzyme activity and the development of biofilms and their planktonic growth. When biofilms that originate from a raw milk tanker detach, they may contaminate the milk during processing. Proteolytic enzymes produced from biofilms and from planktonic cultures may have equally important roles in milk spoilage. According to Champagne et al. (1994), at least 10^6 cfu/mL are required to cause defects in milk products, and protease levels higher than 1 ng/mL can cause off-flavours in UHT milk (Champagne et al., 1994, Shah, 1994). The amount of proteolysis that was produced in the biofilms in this study may have an impact on the quality of the dairy products produced from the raw milk.

The number of bacterial cells found in the biofilms in this study ranged from approximately 10^4 to 10^8 over an area of 1 cm^2 . The internal surface area of a raw milk tanker is approximately $2.7 \times 10^5 \text{ cm}^2$. The number of bacteria within these biofilms on a single tanker could in theory be approximately 2.7×10^{13} cfu in a worst-case scenario. The estimated amount of proteolytic enzyme produced within these biofilms under the assay conditions tested ranged from 3 to 50 pU /cfu. In the worst-case scenario, this translates into 1.35 g of proteolytic enzyme produced within a milk tanker if the worst-case level of microbial contamination is reached. Under such conditions, and if these enzymes remain active, this could potentially spoil 1×10^6 L of milk. Such a scenario can be circumvented by the proper cleaning of milk tankers.

4.a.6 CONCLUSION

Bacteria that originated from the internal surfaces of a raw milk transport tanker were shown to produce biofilms and, most importantly, proteolytic enzymes in an *in vitro* model that simulated a milk tanker during the transport of raw milk. Because such proteolytic enzymes can remain active after pasteurization, these results highlight the importance of maintaining the quality of the raw milk during milk transportation and of having adequate cleaning and sanitation regimes for these tankers, to ensure good quality final dairy products.

4.a.7 ACKNOWLEDGEMENTS

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**4.b *Lipolysis within single culture and co-culture biofilms
of dairy origin***

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Journal of Food Microbiology*.



MASSEY UNIVERSITY
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**STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS**

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Koon Hoong Teh

Name/Title of Principal Supervisor: Steve Flint

Name of Published Research Output and full reference:

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In which Chapter is the Published Work: Chapter 4.b

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4.b.1 ABSTRACT

Bacteria in raw milk can produce heat-stable lipases, which survive pasteurisation and subsequently reduce the shelf life of dairy products because of their ability to break down the milk fat and increase rancidity. In this study, four bacteria, originating from the surfaces of raw milk transport tankers, and a known lipase-producing bacterium were evaluated for their ability to produce lipolysis in planktonic and biofilm cultures. The amount of butyric acid released per cell within biofilms and planktonic cultures ranged from 0.1 to 1110.3 and 0.1 to 0.3 ng/cfu, respectively. Lipolysis was at least 10 times higher within biofilms than within corresponding planktonic cultures. This is the first study to show that lipolysis occurs within biofilms of bacteria that were originally isolated from the surfaces of raw milk tankers. This is relevant to the dairy industry, highlighting the importance of eliminating biofilms on milk tanker surfaces as a source of heat-stable lipases.

4.b.2 INTRODUCTION

Biofilms in the dairy industry are a major concern, as they can be found on virtually all types of product contact surface from milk cups on the dairy farm to heat exchangers in the processing plant (Burgess et al., 2010, Flint et al., 1997, Scheldeman et al., 2005). Biofilms are communities of bacteria that are associated with surfaces and may promote either interspecies or intraspecies interaction. The occurrence of biofilms in the dairy industry may result in the deterioration of product quality because of the production of microbial enzymes (lipases and proteases). Whereas the production of proteases and their effect on dairy products have been studied extensively (Celestino et al., 1997a, Santos et al., 2003, Shah, 1994, Sørhaug and Stepaniak, 1997), there are limited studies on lipase production within biofilms in the dairy industry.

Lipases produced by bacteria, originating from the raw milk, can remain active after heat treatment, causing lipolysis, which can reduce the quality and the shelf life of dairy products. Some bacterial lipases are known to withstand commercial sterilisation at 130 °C for 15 s (Shah, 1994). These heat-stable lipases can cause rancidity, as a result of the hydrolysis of the milk fat to free fatty acids (Santos et al., 2003). As well as causing sensory defects, lipolysis can alter the physico-chemical properties of milk, causing instability in milk foam beverages (Celestino et al., 1997a, Huppertz, 2010).

The internal surface of the milk tanker may promote both biofilm formation and enzyme production. While a recent study, using an *in vitro* model of a milk tanker has shown that proteolysis can occur within biofilms after the biofilms were formed in the absence of milk (Teh et al., 2012), studies on lipase production within biofilms or planktonic populations, and during the transportation of the raw milk from the dairy farm to the dairy processing plant, have not previously been reported. Bacterial growth and lipase production within a raw milk tanker may occur in several ways: for example, (a) lipase production within biofilms in the presence of the bulk milk in the milk tanker; (b) lipase production within biofilms in the presence of milk residues in the milk tanker; (c) lipase production within the planktonic population of the bulk milk in the milk tanker. The aim of the current study was to investigate bacterial growth and lipase production in single

cultures and in co-cultures within the three scenarios described above, using an *in vitro* model system that simulated a milk tanker during a typical summer's day in New Zealand.

4.b.3 MATERIALS AND METHODS

4.b.3.1 Bacterial strains

Four bacterial strains were selected in this study. These bacteria were originally isolated from the internal stainless steel surfaces of raw milk tankers based in the Manawatu region of New Zealand (Teh et al., 2011). Two strains were lipase-producing bacteria (*Staphylococcus aureus* SF01 and *Streptococcus uberis* C05) and two were non-lipase-producing bacteria (*Pseudomonas fluorescens* C224 and *Serratia liquefaciens* DC1). In addition, a known lipase-producing bacterium (*Pseudomonas aeruginosa* ATCC 27853) was used as a positive control. The bacterial isolates were maintained in nutrient broth (Merck, Darmstadt, Germany) containing 20% (v/v) glycerol at $-80\text{ }^{\circ}\text{C}$. The bacterial strains were pre-incubated in nutrient broth for 1 h at $30\text{ }^{\circ}\text{C}$ before each experiment. Nutrient broth was used as the test medium because there was concern that the proteins and lipids in milk would interfere with the enzyme assays (Chen et al., 2003).

4.b.3.2 Growth of single culture and co-culture biofilms, and corresponding planktonic cultures

4.b.3.2.1 Preparation of single cultures and co-cultures

For single culture trials, a volume (0.5 mL) of each of the pre-incubated cultures was inoculated into nutrient broth (4.5 mL) in 15 mL centrifuge tubes (Greiner Bio-one, Auckland, New Zealand), each containing a stainless steel coupon (grade 316 with 2B finish; 50 mm x 13 mm; ETECH, Palmerston North, New Zealand); for co-culture trials, the nutrient broth was inoculated with 0.5 mL of each of two different pre-incubated cultures. The co-cultures were: *S. aureus* SF01 and *P. aeruginosa* ATCC 27853; *S. aureus* SF01 and *P. fluorescens* C224; *S. aureus* SF01 and *S. liquefaciens* DC1; *S. uberis* C05 and *P. aeruginosa* ATCC 27853; *S. uberis* C05 and *P. fluorescens* C224; and *S. uberis* C05 and *S. liquefaciens* DC1. The initial concentration of cells for each bacterial culture was 10^7 cfu/mL.

4.b.3.2.2 Growth of biofilms and planktonic cultures

A rotating biofilm reactor developed in our laboratory (KH-2011; Massey University, Palmerston North, New Zealand) was set up to simulate the growth of bacteria in milk tankers (Teh et al., 2012). Four growth scenarios were simulated: biofilm formation on the stainless steel surface under nutrient abundance (BNA); biofilm formation on the stainless steel surface under nutrient limitation (BNL); planktonic growth in the presence of stainless steel surfaces (PPS); and as a control planktonic growth in the absence of stainless steel surfaces (PAS).

Two sets of three inoculated centrifuge tubes were placed in the KH-2011 reactor, which was set at 4 rpm. The reactor was incubated over a fluctuating range of temperatures for 9.25 h. The temperatures chosen for incubation represented the average surface temperatures of the stainless steel surface in a milk tanker on a hot day when milk was collected. These included sequential incubation for 1.50 h at 16 °C, 3.00 h at 20 °C, 1.00 h at 35 °C, 1.00 h at 20 °C, 1.00 h at 30 °C and 1.75 h at 20 °C.

The stainless steel coupons were shaken gently to remove any excess medium prior to subsequent incubation. To simulate both scenarios (BNA) and (PPS), the first set of stainless steel coupons was transferred into 15 mL centrifuge tubes containing fresh nutrient broth after the initial incubation for 9.25 h. These cultures were incubated for a further 14.75 h at 30 °C. The second set of stainless steel coupons was transferred into empty 15 mL centrifuge tubes and incubated at 30 °C for a further 14.75 h to simulate scenario (BNL). For scenario (PAS), a volume (0.1 mL) of the liquid culture in which the biofilms were initially grown was inoculated into 4 mL of fresh nutrient broth and was incubated at 30 °C for 14.75 h.

4.b.3.2.3 Cell enumeration procedure

For the enumeration of total, Gram-negative and Gram-positive bacteria from single culture and co-culture biofilms and planktonic cultures, a droplet plating method was performed (Lindsay et al., 2002) and differentiated using different temperatures for incubation (Teh et al., 2012). The results were expressed as $\text{Log}_{10} \text{ cfu/cm}^2$ and $\text{Log}_{10} \text{ cfu/mL}$ for biofilms and planktonic cultures respectively.

4.b.3.3 Analysis of lipolysis

In enzymology, lipase (EC.3.1.1.3) is defined as a subclass of carboxylic ester hydrolase enzymes (EC 3.1.1). In this paper, we screened for carboxylic ester hydrolase activity with two substrates: an ester (*p*-nitrophenol palmitate) and a lipid (tributylin). Enzymes that have carboxylic ester hydrolase activity on these substrates are frequently also able to hydrolyse milk fat (Andrewes et al., 2007; Georgalaki et al., 2000; Holland et al., 2005). In this paper, we have assumed that such enzymes are lipases but, such enzymes could also be esterases, phospholipases, or other enzymes. Thus, in future work it will be important to verify these screening results using other substrates but, more importantly, actual dairy products.

4.b.3.3.1 *p*-Nitrophenol palmitate assay

Hydrolysis of *p*-nitrophenol palmitate (pnpp) substrates has been used previously to determine lipolysis (Bussamara et al., 2010; Gupta et al., 2003). The pnpp assay was carried out according to Bussamara et al. (2010) with slight modifications. A 0.05% pnpp solution (Sigma-Aldrich, Auckland, New Zealand) was prepared by dissolving 5 mg of pnpp in 1 mL of isopropanol and 9 mL of 50 mM Tris-HCl solution, pH 8 containing 40 mg of Triton X-100 and 0.2 mg of arabic gum with 0.1% sodium azide (Scharlau Chemie, Barcelona, Spain) and 0.1 mg chloramphenicol/mL (Sigma-Aldrich, Auckland, New Zealand). Sodium azide and chloramphenicol were added to inhibit bacterial growth (Andrewes et al., 2007, Teh et al., 2012).

Both the biofilms and the planktonic cultures were prepared as described above. After incubation, the stainless steel coupons were transferred into 9 mL of the 0.05% pnpp solution, whereas the planktonic cultures were centrifuged at 10 000 *g* for 5 min and 0.1 mL of the supernatant was transferred into 0.9 mL of the 0.05% pnpp solution. Each stainless steel coupon was shaken gently by hand, removing all excess liquid. An uninoculated stainless steel coupon and nutrient broth were used as blank controls. The 0.05% pnpp solutions were incubated at 40 °C for 8 h.

A volume (0.6 mL) of each 0.05% pnpp solution was mixed with 0.6 mL of 96% ethanol (Sigma-Aldrich, Auckland, New Zealand) to stop the reaction and the resulting solution was centrifuged at 10 000 *g* for 5 min. A 0.15 mL volume of the supernatant was

transferred into microtitre plate wells (Difco™, Becton, Dickinson and Company, Sparks, MD, USA) in six replicates and the absorbance was read at 405 nm using a microtitre plate reader (Spectrostar Nano, BMG Labtech, Auckland, New Zealand). The amount of hydrolysis was calculated by comparing the absorbance value of the samples with the absorbance value of the standard curve of *p*-nitrophenol. The estimated amount of hydrolysis produced was divided by the number of bacterial cells colonising the stainless steel surfaces or the number of bacterial cells in the planktonic cultures. The results were expressed as hydrolysis per colony forming units (nU/cfu).

4.b.3.3.2 Ion-exchange chromatography and butyric acid assay

In addition to the pnpp assay, the amount of butyric acid released during the hydrolysis of tributyrin was estimated using ion-exchange chromatography. Butyric acid is associated with rancidity, and tributyrin is often used as substrate for the detection of lipolysis (Andrewes et al., 2007; Chen et al., 2003; Saxena et al., 1999).

Biofilms (the whole stainless steel coupons) and supernatants (1 mL) from planktonic cultures were placed in centrifuge tubes containing 9.8 mL of 5 mM sodium phosphate buffer pH 7 and 0.2 mL of tributyrin (Sigma Chemical Co., St. Louis, MO, USA). The buffer contained 0.1% sodium azide and 0.1 mg chloramphenicol/mL with 0.5 mM pentonic acid as an internal standard. The centrifuge tubes were incubated at 40 °C for 24 h.

After incubation, the solutions were diluted ten-fold with milliQ water and filter sterilised. The concentrations of butyric acid released in the solutions during the lipolysis of tributyrin were measured using ion-exchange chromatography (ICS-2000 Ion Chromatography System, Dionex, Sunnyvale, CA, USA) and expressed as mg/mL. To determine the concentration of butyric acid per cell, the concentrations of butyric acid released in the solution were divided by the number of bacterial cells colonising the stainless steel surfaces or in the planktonic cultures. The final results were expressed as the amount of butyric acid released per colony forming units (ng/cfu).

4.b.3.4 Statistical analysis

Statistical analyses of cell enumeration, hydrolysis of pnpp and tributyrin per colony forming units (cfu) were performed with three replicates on two separate occasions. SAS 9.2 software was used for analysis of variance using Tukey's test with a critical probability of $P \leq 0.05$.

4.b.4 RESULTS

4.b.4.1 Planktonic growth in the presence or the absence of stainless steel surfaces

The number of planktonic cells recovered in this study ranged from approximately 8 to 9 Log₁₀ cfu/mL (Figure 4.b-1). High planktonic cell counts for single culture and co-culture populations were observed for *S. aureus* SF01, whereas low planktonic cell counts were observed for *S. uberis* C05. Overall, the planktonic cell counts of *P. aeruginosa* ATCC 27853, *P. fluorescens* C224 and *S. liquefaciens* DC1 were significantly lower when they were in co-culture with *S. aureus* SF01 ($P < 0.05$).

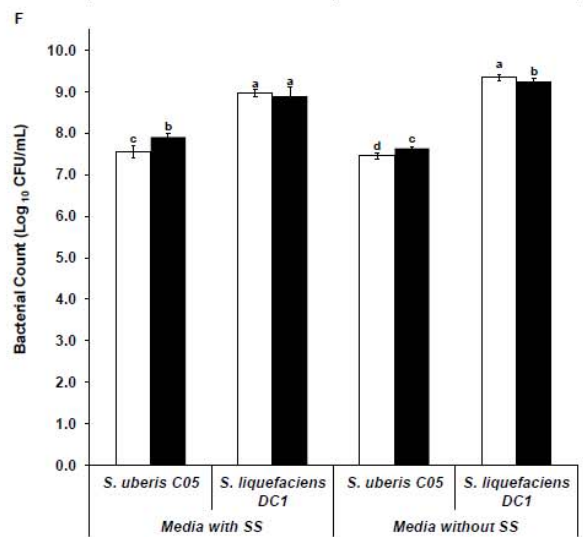
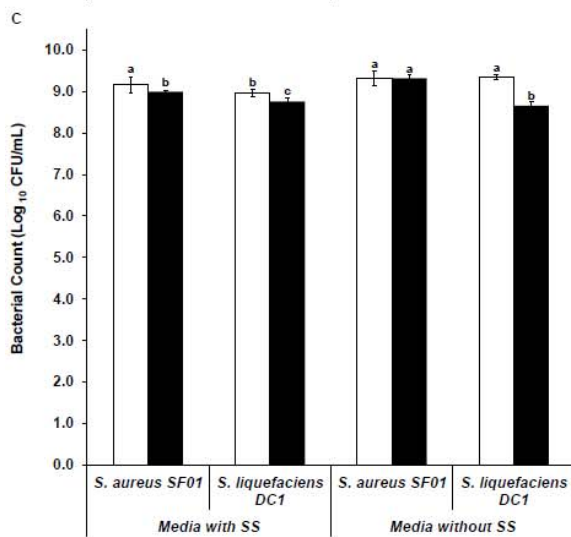
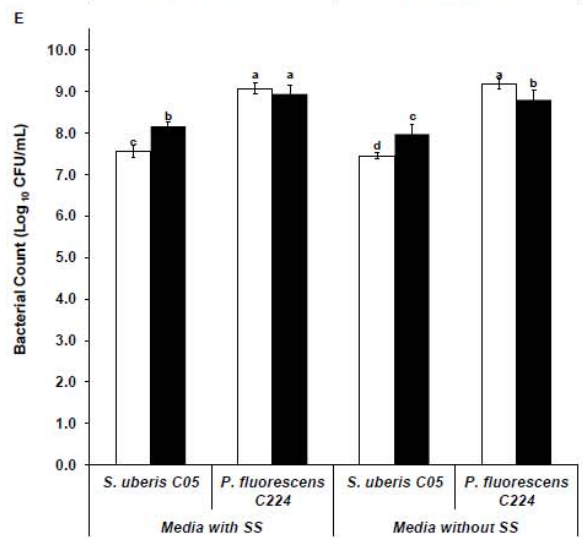
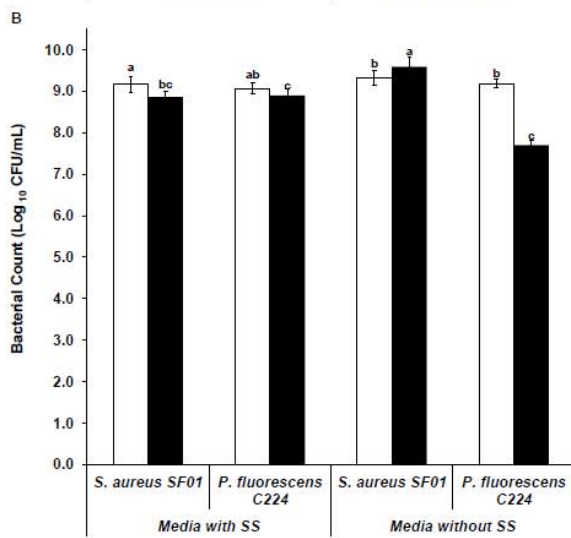
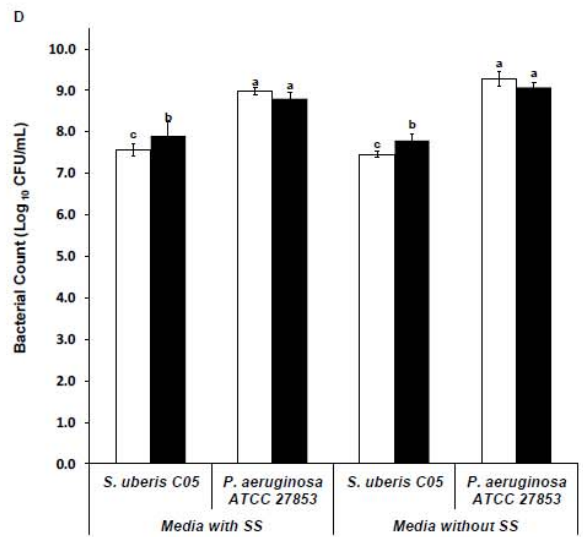
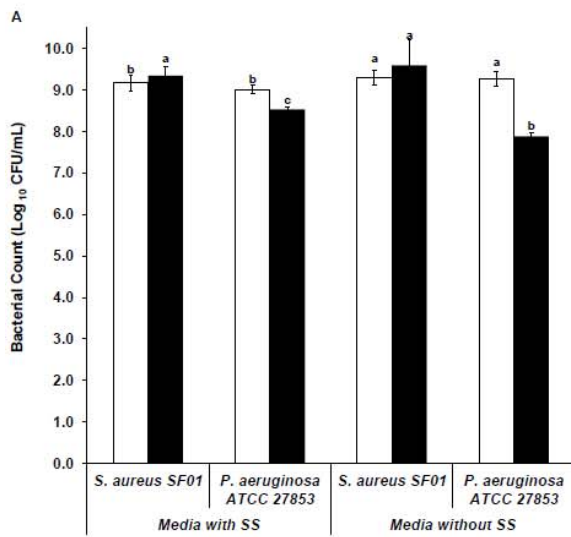


Figure 4.b-1The number of planktonic cells recovered from single cultures and co-cultures of *S. aureus* SF01 and *P. aeruginosa* ATCC 27853 (A), *S. aureus* SF01 and *P. fluorescens* C224 (B), *S. aureus* SF01 and *S. liquefaciens* DC1 (C), *S. uberis* C05 and *P. aeruginosa* ATCC 27853 (D), *S. uberis* C05 and *P. fluorescens* C224 (E) and *S. uberis* C05 and *S. liquefaciens* DC1 (F) grown in the presence or absence of stainless steel (SS) surfaces. The white bars represent the single cultures and the black bars represent co-cultures. Experiments were performed with three replicates on two separate occasions, and error bars represent standard deviations from the mean. Different letters within each condition indicate statistically significant differences, $P \leq 0.05$ (Tukey's test).

4.b.4.2 Biofilm growth under different nutrient availability

All of the bacterial isolates used in the current study were able to grow as both a single culture biofilm and as co-culture biofilms. The number of bacterial cells recovered from surfaces ranged from approximately 4 to 8 Log₁₀ cfu/cm² (Figure 4.b- 2). *S. liquefaciens* DC1 had the highest bacterial cell count and *S. uberis* C05 had the lowest bacterial cell count when grown in single culture biofilms, regardless of the nutrient availability.

For co-culture biofilms, under nutrient abundance, *P. fluorescens* C224 had the highest bacterial cell count when in co-culture with *S. uberis* C05, whereas, under nutrient limitation, *S. liquefaciens* DC1 had the highest bacterial cell count when in co-culture with *S. uberis* C05. Overall, *S. uberis* had the lowest bacterial cell count when in co-culture with *S. liquefaciens* DC1.

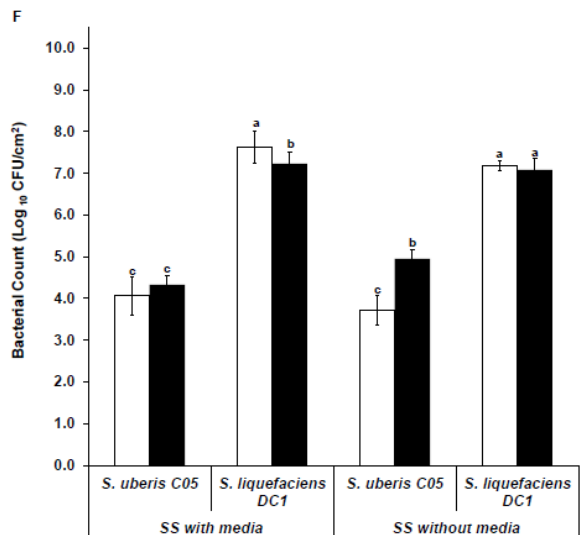
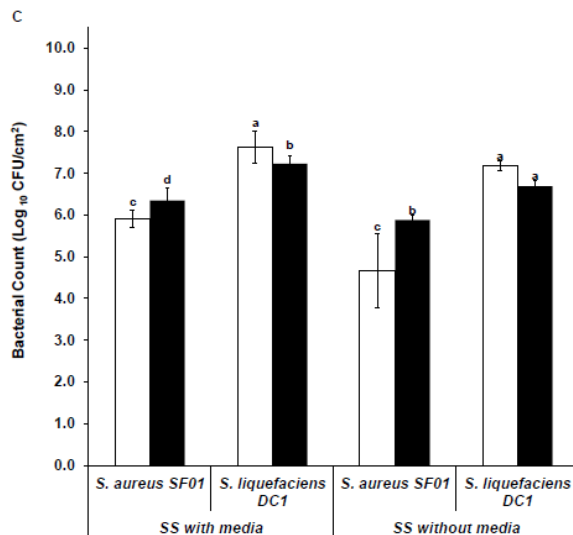
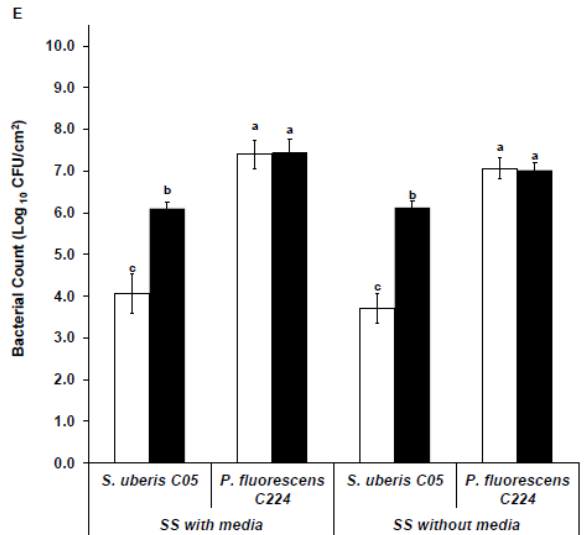
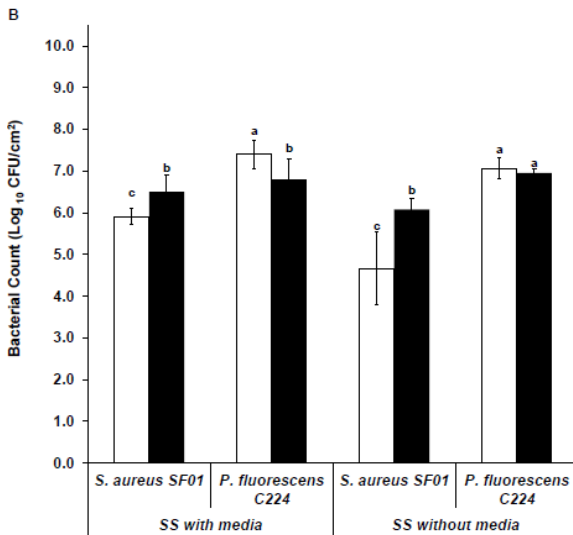
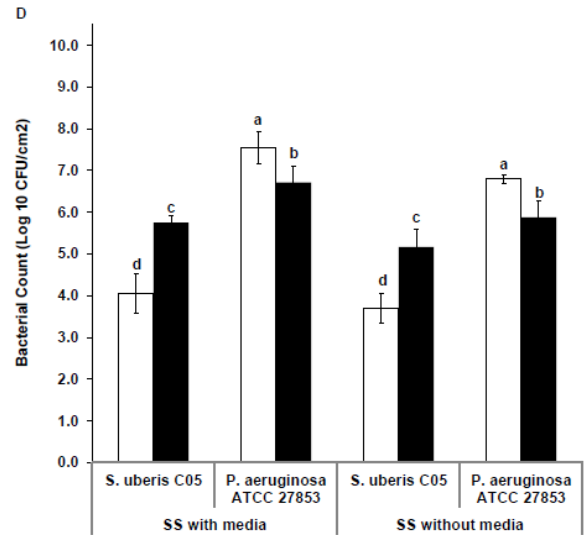
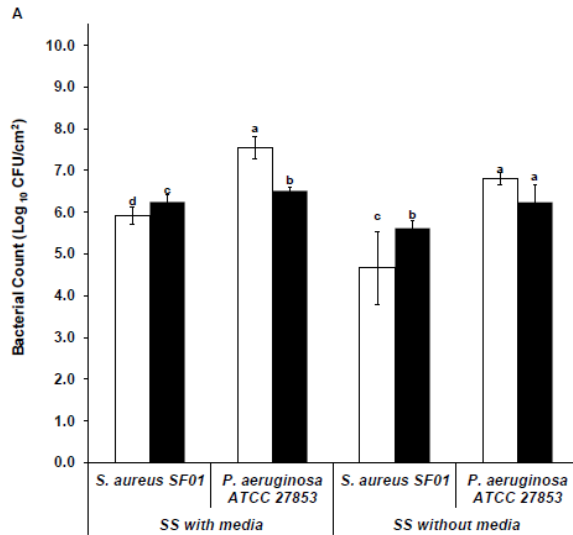


Figure 4.b-2 The number of cells recovered from single culture and co-culture biofilms of *S. aureus* SF01 and *P. aeruginosa* ATCC 27853 (A), *S. aureus* SF01 and *P. fluorescens* C224 (B), *S. aureus* SF01 and *S. liquefaciens* DC1 (C), *S. uberis* C05 and *P. aeruginosa* ATCC 27853 (D), *S. uberis* C05 and *P. fluorescens* C224 (E) and *S. uberis* C05 and *S. liquefaciens* DC1 (F) grown on a stainless steel (SS) surfaces in the presence or absence of medium. The white bars represent the single cultures and the black bars represent co-cultures. Experiments were performed with three replicates on two separate occasions, and error bars represent standard deviations from the mean. Different letters within each condition indicate statistically significant differences, $P \leq 0.05$ (Tukey's test).

4.b.4.3 Hydrolysis of pnpp substrates

A known lipase-producing bacterium (*P. aeruginosa* ATCC 27853) was shown to hydrolyse pnpp in biofilm and planktonic cultures (Table 4.b-1), indicating that this assay could be used as a tool to screen for lipolysis in biofilms of other bacteria. All biofilms and planktonic populations hydrolysed pnpp, with the exception of *S. uberis* C05 and *P. fluorescens* C224. Hydrolysis per cfu within biofilms and planktonic cultures ranged from 0.01 to 8.35 and 0.01 to 0.07 nU/cfu respectively. Overall, biofilm populations of all isolates able to hydrolyse pnpp resulted in at least 10 times more hydrolysis than the corresponding planktonic populations (Table 4.b-1). In addition, biofilms formed under nutrient abundance, may also influenced the hydrolysis. For example, hydrolysis of pnpp per cfu of *S. aureus* SF01 within biofilms was found to be significantly higher under nutrient abundance (8.35 nU/cfu) than under nutrient limitation (1.03 nU/cfu) ($P < 0.05$).

Table 4.b-1 The amount of hydrolysis of the substrate pnpp per cfu within biofilms and planktonic cultures in two different growth modes.

	Within biofilms (nU/cfu)		Within planktonic cultures (nU/cfu)	
	Under nutrient abundance	Under nutrient limitation	In the presence of surfaces	In the absence of surfaces
<i>Single cultures</i>				
<i>S. aureus</i> SF01	8.35 ± 3.4 ^a	1.03 ± 0.58 ^b	0.02 ± 0.01 ^d	0.06 ± 0.03 ^c
<i>S. uberis</i> C05	< 0.01	< 0.01	< 0.01	< 0.01
<i>P. aeruginosa</i> ATCC 27853	0.31 ± 0.20 ^b	2.06 ± 0.67 ^a	0.02 ± 0.01 ^c	0.01 ± 0.03 ^d
<i>S. liquefaciens</i> DC1	0.01 ± 0.01	0.01 ± 0.01	< 0.01	< 0.01
<i>P. fluorescens</i> C224	< 0.01	< 0.01	< 0.01	< 0.01
<i>Co-cultures</i>				
<i>S. aureus</i> SF01				
<i>P. aeruginosa</i> ATCC 27853	2.30 ± 0.53 ^b	6.34 ± 1.97 ^a	0.04 ± 0.01 ^c	0.04 ± 0.01 ^d
<i>S. aureus</i> SF01				
<i>S. liquefaciens</i> DC1	0.54 ± 0.06 ^a	0.29 ± 0.08 ^b	0.07 ± 0.02 ^c	0.07 ± 0.02 ^d
<i>S. aureus</i> SF01				
<i>P. fluorescens</i> C224	0.79 ± 0.41 ^a	0.71 ± 0.41 ^b	0.04 ± 0.02 ^c	0.04 ± 0.00 ^d
<i>S. uberis</i> C05				
<i>P. aeruginosa</i> ATCC 27853	0.19 ± 0.08 ^b	2.27 ± 1.37 ^a	0.01 ± 0.01 ^b	0.02 ± 0.01 ^b
<i>S. uberis</i> C05				
<i>S. liquefaciens</i> DC1	0.02 ± 0.01	0.02 ± 0.01	< 0.01	< 0.01
<i>S. uberis</i> C05				
<i>P. fluorescens</i> C224	0.01 ± 0.00 ^b	0.01 ± 0.00 ^a	< 0.01	< 0.01

Note: Experiments were performed with three replicates on two separate occasions, and values represent the means with standard deviations.

The different letters within each row indicate statistically significant differences, $P \leq 0.05$ (Tukey's test).

4.b.4.4 Lipolysis of tributyrin substrates

The results from the lipolysis of tributyrin substrates corresponded with the results obtained for the hydrolysis of pnpp; the amount of butyric acid released per cfu was higher within biofilms than within corresponding planktonic cultures (Table 4.b-2). The amount of butyric acid released per cfu within biofilms and planktonic cultures ranged from 0.1 to 1110.3 and 0.1 to 0.3 ng/cfu respectively. In addition, most of the biofilms (with the exception of a single biofilm culture of *S. uberis* C05 formed under nutrient abundance) were shown to produce lipolysis. Overall, *S. uberis* C05 under nutrient limitation exhibited the highest level of butyric acid released per cfu (1110.3 ng/cfu).

Table 4.b-2 The amount of butyric acid released from hydrolysis of tributyrin per cfu within biofilms and planktonic cultures in two different growth modes.

	Within biofilms (ng/cfu)		Within planktonic cultures (ng/cfu)	
	Under nutrient abundance	Under nutrient limitation	In the presence of surfaces	In the absence of surfaces
<i>Single cultures</i>				
<i>S. aureus</i> SF01	52.6 ± 24.0 ^b	77.4 ± 50.8 ^a	0.2 ± 0.2 ^b	0.2 ± 0.2 ^b
<i>S. uberis</i> C05	< 0.1	1110.3 ± 579.1	< 0.1	< 0.1
<i>P. aeruginosa</i> ATCC 27853	0.5 ± 0.2 ^b	13.9 ± 3.3 ^a	0.1 ± 0.1 ^d	0.2 ± 0.1 ^c
<i>S. liquefaciens</i> DC1	0.2 ± 0.1 ^b	0.5 ± 0.3 ^a	0.2 ± 0.1 ^b	0.1 ± 0.0 ^b
<i>P. fluorescens</i> C224	0.1 ± 0.1	0.6 ± 0.5	< 0.1	< 0.1
<i>Co-cultures</i>				
<i>S. aureus</i> SF01				
<i>P. aeruginosa</i> ATCC 27853	8.3 ± 2.4 ^b	11.5 ± 6.3 ^a	0.1 ± 0.1 ^d	0.2 ± 0.1 ^c
<i>S. aureus</i> SF01				
<i>S. liquefaciens</i> DC1	2.9 ± 0.8 ^a	2.3 ± 0.5 ^b	0.2 ± 0.0 ^d	0.3 ± 0.1 ^{ce}
<i>S. aureus</i> SF01				
<i>P. fluorescens</i> C224	1.4 ± 0.2 ^a	1.1 ± 0.2 ^b	0.3 ± 0.1 ^d	0.3 ± 0.0 ^c
<i>S. uberis</i> C05				
<i>P. aeruginosa</i> ATCC 27853	2.1 ± 0.6 ^b	6.8 ± 3.0 ^a	0.1 ± 0.0 ^d	0.2 ± 0.1 ^c
<i>S. uberis</i> C05				
<i>S. liquefaciens</i> DC1	0.5 ± 0.1 ^a	0.2 ± 0.2 ^b	0.1 ± 0.0 ^c	0.1 ± 0.0 ^d
<i>S. uberis</i> C05				
<i>P. fluorescens</i> C224	0.1 ± 0.0 ^b	0.2 ± 0.1 ^a	< 0.1	< 0.1

Note: Experiments were performed with three replicates on two separate occasions, and values represent the means with standard deviations.

The different letters within each row indicate statistically significant differences, $P \leq 0.05$ (Tukey's test).

4.b.5 DISCUSSION

This study showed that several factors affected the production of lipolytic enzymes in bacteria of importance in dairy manufacture. Enzymatic activity is often higher within biofilms than within planktonic cultures (Frølund et al., 1995, Gamarra et al., 2010, Teh et al., 2012, Wang and Chen, 2009). In this study, lipolysis was found to be greater within biofilms than within their corresponding planktonic cultures grown under the experimental conditions.

The availability of growth nutrients may influence lipolysis within biofilms. Hydrolysis of pnpp within biofilms of *S. aureus* SF01 was found to be greater under nutrient-rich environment. This may have been because of the increased nutrient requirement to support biofilm growth in the presence of growth medium, whereas nutrient limitation may reduce the nutrient requirement by *S. aureus* SF01 due to a stress response. Studies have shown that nutrient availability may influence biofilm formation and cell survivability (Spector and Kenyon, 2012, Thomason et al., 2012). In contrast, hydrolysis of tributyrin by the single culture biofilm of *S. aureus* SF01 was found to be higher in the nutrient limited environment than in the nutrient-rich environment. This result differed from the results obtained from the hydrolysis of pnpp method, where hydrolysis of pnpp was found to be higher in the nutrient-rich environment. This difference may have been due to the different substrates used in the study. Staphylococcal esterases and lipases may have different preferred substrates (Casaburi et al., 2006, Rosenstein and Götz, 2000, Saggars and Stewart, 1968).

Lipolysis within planktonic cultures can also be influenced by the presence or the absence of stainless steel surfaces. Hydrolysis of pnpp was found to be greater within the planktonic cultures in the presence of stainless steel surfaces. Planktonic cultures in the presence of stainless steel surfaces may exhibit some biofilm-like characteristic, because biofilm cells were found to have higher hydrolysis than planktonic cultures in this study. Previous studies have shown that planktonic cultures may acquire biofilm-like characteristics in the presence of surfaces (Oosthuizen et al., 2001).

Furthermore, mutualistic interactions within the co-culture biofilms were observed in this study. For example, more hydrolysis was observed in co-culture biofilms of *S.*

aureus SF01 and *P. aeruginosa* ATCC 27853 under nutrient limitation than in single culture biofilms of *S. aureus* SF01 under nutrient limitation. Similarly, for *S. uberis* C05 and *P. fluorescens* C224, where hydrolysis was not detected in both of the individual bacterial strains, hydrolysis was detected when they were in co-culture biofilms. A similar phenomenon has been shown in co-cultures of different microorganisms. For example, a previous study has shown that proteolysis was increased in co-culture biofilms of *Bacillus licheniformis* and *Pseudomonas fragi* (Teh et al., 2012). This shows that enzyme production may be influenced by the physiological state and the growth conditions of the bacterial cells, which includes gene expression, phase variation and quorum-sensing (Gamarra et al., 2010, Hamon et al., 2004, Van Den Broek et al., 2005, Wang and Chen, 2009).

In addition to enzyme production, the growth of the bacteria was examined. In this study, both *S. aureus* SF01 and *S. uberis* C05 formed biofilms with *P. aeruginosa* ATCC 27853, *P. fluorescens* C224 and *S. liquefaciens* DC1 regardless of the conditions of growth and nutrient availability. In addition, the numbers of *S. aureus* SF01 and *S. uberis* C05 cells recovered were significantly greater from the co-culture biofilms than from the single cultures. This finding was in agreement with Sasahara and Zottola (1993) and Lindsay et al., (2002), who found an increase in Gram-positive cells in mixed species biofilms. The increase in Gram-positive cells may have been due to the production of exopolysaccharides by Gram-negative cells. This effect was more prominent when the co-cultures were grown under nutrient limitation in this study. The exopolysaccharides produced by the Gram-negative cells under nutrient limitation may aid in protecting the bacterial cells from desiccation. A previous study showed that exopolysaccharides can protect microorganisms in a desiccated environment; however, the effect was not observed in mixed populations (Ophir and Gutnick, 1994). This may have been due to different bacterial species used in the study. Furthermore, the numbers of *P. aeruginosa* ATCC 27853, *P. fluorescens* C224 and *S. liquefaciens* DC1 cells were similar or reduced when grown in co-cultures with *S. aureus* SF01 or *S. uberis* C05 in both biofilms and planktonic cultures. The effect was greater in the planktonic cultures than in the biofilms and is likely to represent competition between these species.

Overall, this study has shown that the conditions under which bacteria grow can influence the growth interaction and enzyme production between the bacteria either in biofilms or in planktonic cultures. This is a reasonable assumption, as it has been shown that genetic expression is different in bacteria within biofilms and the corresponding planktonic cell population (Davey and O'Toole, 2000, Oosthuizen et al., 2001, Schembri et al., 2003).

This study gives some insight into bacterial growth and enzyme production on the internal surfaces in an inadequately cleaned, non-refrigerated milk tanker on a hot day. We have previously reported that the internal surface temperature of an empty milk tanker during a New Zealand summer can reach 20 °C, which may promote the growth of psychrotrophic and mesophilic bacteria. We have already shown that bacteria isolated from these milk tankers form biofilms and are capable of producing thermo-resistant lipases (Teh et al., 2011). During transportation of the raw milk from the farm to the dairy processing plant, bacteria can grow either as biofilms on the internal surfaces of the milk tankers or in the milk as planktonic cells when conditions are suitable. Splashing of raw milk inside the tankers during transportation may promote bacterial attachment because only a short contact time between the bacteria and the surfaces is needed (Vanhaecke et al., 1990). In addition, the preconditioning of the surfaces with raw milk may promote bacterial attachment (Palmer et al., 2007, Shi and Zhu, 2009). Once biofilms have formed in the milk tanker, they may subsequently disperse into the bulk milk and colonise the processing line (Flint et al., 1997, Wilks et al., 2006). A recent study has shown that dispersed biofilm cells colonise surfaces more readily than planktonic populations (Rollet et al., 2009). The subsequent colonisation may also promote lipolysis within the dairy processing plant.

In conclusion, bacteria originating from the internal surfaces of raw milk tankers have the potential to produce lipases. Lipase production can occur within the biofilms on the inadequately cleaned milk tankers or within the planktonic population of the raw milk during its transportation. Lipolysis is generally higher within biofilms than within corresponding planktonic cultures. This has practical importance for the dairy industry because biofilms on milk tanker surfaces may be a source of heat-stable lipases. This once more highlights the ongoing need for thorough cleaning and sanitation of milk tankers.

**Chapter 5. The Spoilage Of Dairy
Products Due To Enzymes Produced
By Bacteria Growing Within Biofilm**

5.a *Proteolysis in ultra-heat-treated skim milk after exposure to multispecies biofilms under conditions modelling a milk tanker*

Teh, K. H., Lindsay, D., Palmer, J., Andrewes, P., Bremer., P., Flint, S., 2012. Submitted. Proteolysis in ultra-heat-treated skim milk after exposure to multispecies biofilms under conditions modeling a milk tanker. *International Journal of Dairy Technology*.



MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

**STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS**

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Koon Hoong Teh

Name/Title of Principal Supervisor: Steve Flint

Name of Published Research Output and full reference:

Teh, K. H., Lindsay, D., Palmer, J., Andrewes, P., Bremer., P., Flint, S., 2012. Submitted. Proteolysis in ultra-heat-treated skim milk after exposure to multispecies biofilms under conditions modeling a milk tanker. International Journal of Dairy Technology.

In which Chapter is the Published Work: Chapter 5.a

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:
and / or
- Describe the contribution that the candidate has made to the Published Work:
The candidate did the necessary laboratory work, and prepared the manuscript for publication

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5.a.1 ABSTRACT

Bacteria from milk tankers, can form multispecies biofilms and produce heat-stable enzymes. In this study, milk was exposed to multispecies biofilms in stainless steel vessels and was then used to produce ultra-heat-treated (UHT) milk, which was stored for 5 months. The UHT milks were assessed for microbial counts, free peptide concentration, and pH. The free peptide concentration, which indicated proteolysis, was higher in UHT milk that had been exposed to multispecies biofilms than in UHT milk that had not been exposed to biofilm. Biofilm formation may be promoted in milk tankers that are not properly cleaned, which may compromise the quality of dairy products.

5.a.2 INTRODUCTION

Defects in dairy products can be caused by heat-stable enzymes that have either originated from lactating cows or been produced by planktonic cells (Chen et al., 2003, Fox and Kelly, 2006, Sørhaug and Stepaniak, 1997). High bacterial numbers in raw milk tend to result in dairy products that have a lower yield, a shorter shelf life and undesirable flavours such as rancidity and bitterness (Chen et al., 2003, Shah, 1994). Psychrotrophic bacteria, present in raw milk, are known to produce heat-stable enzymes (De Jonghe et al., 2011, Nörnberg et al., 2010, Sørhaug and Stepaniak, 1997), which can remain active after pasteurization of milk (Shah, 1994).

As these heat-stable enzymes are often active over a broad range of conditions (temperature, water activity and pH), they can reduce the quality of a wide variety of dairy products during prolonged storage (Janzen et al., 1982, Kang and Frank, 1988, Wang and Chen, 2009). For example, only a small quantity (1 ng/mL) of heat-stable enzyme is required to reduce the quality of ultra-heat-treated (UHT) skim milk within 6 months of storage at 25 °C (Barbano et al., 2006, Celestino et al., 1997b, Shah, 1994, Sørhaug and Stepaniak, 1997).

Bacteria found in raw milk are able to form biofilms and produce enzymes at various temperatures (Cleto et al., 2012, Nörnberg et al., 2011, Teh et al., 2012). Biofilm may provide a niche for the activity of microbial enzymes due to the protective nature of the biofilm (Li et al., 2006, Licitra et al., 2007, Rosche et al., 2009, Wang et al., 2011)

Whereas biofilms in the dairy industry have been studied extensively, the formation of biofilms and the subsequent secretion of enzymes within a milk tanker during raw milk transportation have received little attention, even though this could comprise the quality of the final dairy product. The aim of the current study was to investigate the effect on the quality of UHT skim milk, measured by free peptide concentration, following of the exposure of skim milk to protease-producing bacterial biofilms under conditions that modelled transportation of raw milk in a milk tanker.

5.a.3 MATERIALS AND METHODS

5.a.3.1 Bacterial strains

Three bacterial strains, originally isolated from the internal stainless steel surfaces of milk tankers based in the Manawatu region of New Zealand, were used in this study (Teh et al., 2011). These bacterial strains – *Pseudomonas fluorescens* C224, *Serratia liquefaciens* DC1 and *Staphylococcus aureus* SF01 – were chosen because of their ability to produce heat-stable enzymes and form biofilms (Teh et al., 2011). They were maintained in nutrient broth (Merck, Darmstadt, Germany) containing 20% (v/v) glycerol at –80 °C, and were pre-incubated in sterile reconstituted skim milk (100 g of gamma-irradiated skim milk powder in 900 mL of distilled water) for 1 h at 30 °C prior to each experiment. Two batches of skim milk powder were used in this study. The first batch was used for the first six trials and the second batch was used for the final trial.

5.a.3.2 Treatment of stainless steel vessels to form biofilms

Skim milk (8 L, 10% w/v milk solids) was prepared from gamma-irradiated skim milk powder in a stainless steel vessel (dimensions: 23 cm diameter, 28 cm length). The three pre-incubated cultures (1 mL of each) were inoculated into the skim milk. Three different inoculum levels were used, with initial concentrations of cells in the skim milk at 10^3 , 10^5 and 10^7 colony forming units (cfu)/mL.

The stainless steel vessel was incubated (20 °C) horizontally on a bench shaker (Infors Ht Labotron, Basel, Switzerland) at 100 rpm for 24 h. After incubation, the vessel was emptied and the skim milk was discarded. The vessel was visually inspected to ensure that it was empty and did not contain any excess skim milk. The vessel was classified as slightly, moderately or heavily contaminated depending on the initial inoculum used (10^3 , 10^5 or 10^7 cfu/mL respectively). A clean vessel was used as an untreated control.

5.a.3.3 Preparation of UHT skim milk

Freshly reconstituted skim milk (8 L) was transferred into both the control vessel and the vessels containing the bacterial biofilms as described above. The vessels were further incubated horizontally at 20 °C on bench shakers for 10 h, simulating the time taken for a typical milk collection run. A sample of milk (1 mL) was taken to determine the final

concentration of planktonic cells in the milk, as described below, and the remaining milk was immediately indirectly UHT treated at 141 °C for 5 s in a small scale pilot plant (Massey University, Palmerston North, New Zealand).

The UHT skim milks were aseptically packed into sterile bottles (50 mL), which were stored at various temperatures (10, 20, 30 and 40 °C), and were assessed for microbial counts, concentration of free peptides and pH at weekly intervals for the first 12 weeks and then monthly for 2 months. All treatments, and the control, from all storage temperatures were assessed using a new bottle removed from storage at each time point. The UHT skim milk was prepared on three separate occasions for each initial inoculum (10^3 , 10^5 or 10^7 cfu/mL).

5.a.3.4 Microbial analysis

5.a.3.4.1 Microbial analysis of the surfaces of stainless steel vessels

Approximately 4 cm² of the surface of the stainless steel vessel was swabbed with a sterile cotton swab after the milk had been removed for UHT treatment. The cotton swab was transferred into 10 mL of 0.1% peptone solution with 15 g of glass beads (5 mm diameter), which was mixed by vortex for 2 min. The resulting suspension was further diluted in a series of ten-fold dilutions and a droplet plating method was used to determine the bacterial counts (Lindsay et al., 2002, Teh et al., 2012). The results were expressed as Log₁₀ cfu/cm².

5.a.3.4.2 Microbial analysis of milk samples

A 1 mL volume of each milk sample was diluted in a series of ten-fold dilutions and a droplet plating method was used, as above. The results were expressed as Log₁₀ cfu/mL.

5.a.3.5 Free peptides

The concentration of free peptides was measured using a fluorescamine method, with slight modification according to Le et al., (2006). A milk sample (5 mL) was mixed with an equal amount of 4% trichloroacetic acid (Sigma, Auckland, New Zealand) and mixed by vortex for 30 s. The mixture was left standing for 30 min at room temperature and was then centrifuged at 10 000 rpm for 5 min. The supernatant was filtered through a 0.45 µm

Millipore filter. The filtrate (0.1 mL) was mixed with 3 mL of 0.1 M sodium phosphate buffer at pH 8.0 and fluorescamine solution (0.1 mL, 0.2 mg/mL in acetone). The mixture was mixed by vortex, stored in the dark for 15 min and then 0.5 mL was further diluted in 4.5 mL of 0.1 M sodium phosphate buffer (pH 8.0). The fluorescence of the diluted mixture was measured with a spectrofluorophotometer RF-1501 (Shimadzu, Tokyo, Japan) at an excitation wavelength of 390 nm and an emission wavelength of 480 nm. The results were expressed as milligrams of free peptides per millilitre with reference to a standard curve of free peptides (Gly–Gly).

5.a.3.6 pH of UHT skim milk

The milk samples were equilibrated at room temperature for 1 h prior to pH measurement using a pH meter 420A (Orion Research Inc., Boston, MA, USA).

5.a.3.7 Statistical analysis

Statistical analyses of the number of cells, the concentration of free peptides and the pH were performed with three replicates on two separate occasions, with SAS 9.2 software using Tukey's test with a critical probability of $P \leq 0.05$.

5.a.4 RESULTS AND DISCUSSION

5.a.4.1 Microbial analysis

The formation of biofilms on stainless steel surfaces (such as in milk tankers during milk transport) can be influenced by the numbers of bacteria in the raw milk. In this study, the number of bacterial cells recovered from biofilms that formed on the surfaces of the treated stainless steel vessels after the second incubation (10 h) ranged from 5.6 to 8.8 Log₁₀ cfu cm² (Table 5.a-1). The high bacterial cell count (8.8 Log₁₀ cfu/cm²) on the heavily contaminated vessel was associated with the high initial microbial load (10⁷ cfu/mL) in the milk. The number of bacteria isolated from the stainless steel surface was also significantly higher in the moderately contaminated vessel ($P \leq 0.05$) than in the slightly contaminated vessel. The formation of multispecies biofilms in milk tankers can provide the bacteria with a microenvironment for enzyme production (Iwashita et al., 2001, Licitra et al., 2007), as

shown in a recent study in which bacteria from milk tankers grew and produced enzymes in an *in vitro* model that was incubated at fluctuating temperatures (Teh et al., 2012).

The numbers of bacterial cells recovered from the milk exposed to the biofilms, prior to UHT treatment, were 6.1, 6.5, and 8 Log₁₀ cfu/mL, for the slightly, moderately, and heavily contaminated vessels respectively (Table 5.a-1). The heavily contaminated trial was performed only once as the treated milk coagulated immediately after heat treatment, suggesting that extensive proteolysis had occurred. There was no significant difference in the final number of the bacterial cells in the treated milk when the vessel was either slightly or moderately contaminated ($P > 0.05$). Bacterial cells were not detected on the internal surface of, or in the milk added to the clean vessels (Table 5.a-1). Furthermore, bacterial growth was not detected in either the control UHT skim milk samples or the treated UHT skim milk samples during the storage trials (data not shown).

Table 5.a-1 Bacterial CFU analysis for the surfaces of and the milk from the treated and control stainless steel vessels after the second incubation

	Bacterial cell count on the surface (Log ₁₀ cfu/cm ²)	Bacterial cell count in the milk (Log ₁₀ cfu/mL)
<i>Heavily contaminated trial*</i>		
Treated vessels	8.8	8.0
Control vessels	< 1	< 1
<i>Moderately contaminated trials</i>		
Treated vessels	7.4 ± 0.62 ^a	6.5 ± 0.04
Control vessels	< 1	< 1
<i>Slightly contaminated trials</i>		
Treated vessels	5.6 ± 0.2 ^b	6.1 ± 0.3
Control vessels	< 1	< 1

Measurements were performed on three separate occasions, and values represent the means with standard deviations. The different letters within each column indicate statistically significant differences, $P \leq 0.05$ (Tukey's test).

* Measurement was performed on only a single occasion.

5.a.4.2 Free peptides

Previous studies have looked at spoilage caused by bacterial enzymes produced by bacteria in a planktonic culture, but studies on the spoilage effect of biofilm enzymes during the transportation of raw milk are limited (Janstova et al., 2006, Marchand et al., 2012). In the current study, the effects of proteases, produced within a multispecies biofilm, on a dairy product (UHT skim milk) were investigated. The UHT skim milk was previously exposed to the multispecies biofilms for up to 10 h to simulate milk being transported for a long distance between the dairy farm and the dairy processing plant. It was assumed that the effects observed in the UHT milk were caused by heat-stable enzymes produced within the multispecies biofilms because bacterial growth was not detected in the milk during storage. From the results, the free peptide concentration gradually increased over time in both the control UHT skim milk and the treated UHT skim milk (Figure 5.a-1).

UHT skim milk exposed to the slightly contaminated stainless steel vessel had a non-significant increase in its free peptide concentration compared with the control after 3 months of storage at 40 °C (Figure 5.a-1D). UHT skim milk exposed to the moderately contaminated stainless steel vessel generally had a higher free peptide concentration than the control UHT skim milk (Figure 5.a-1F, 5.a-1G and 5.a-1H). Although some variation was observed in the trials, there was a significant difference between the control UHT skim milk and one batch of treated UHT skim milk (from the moderately contaminated vessel) in samples taken after 2 weeks of storage at 30 and 40 °C. Although this result was not reproduced in two additional trials, the free peptide concentration in the treated UHT skim milk was observed to be higher at 30 and 40 °C after 3 and 2 months of storage respectively. The increase in the concentration of free peptides supports the hypothesis that proteolysis of milk exposed to either slightly or moderately contaminated stainless steel vessels can occur during prolonged storage at high temperatures.

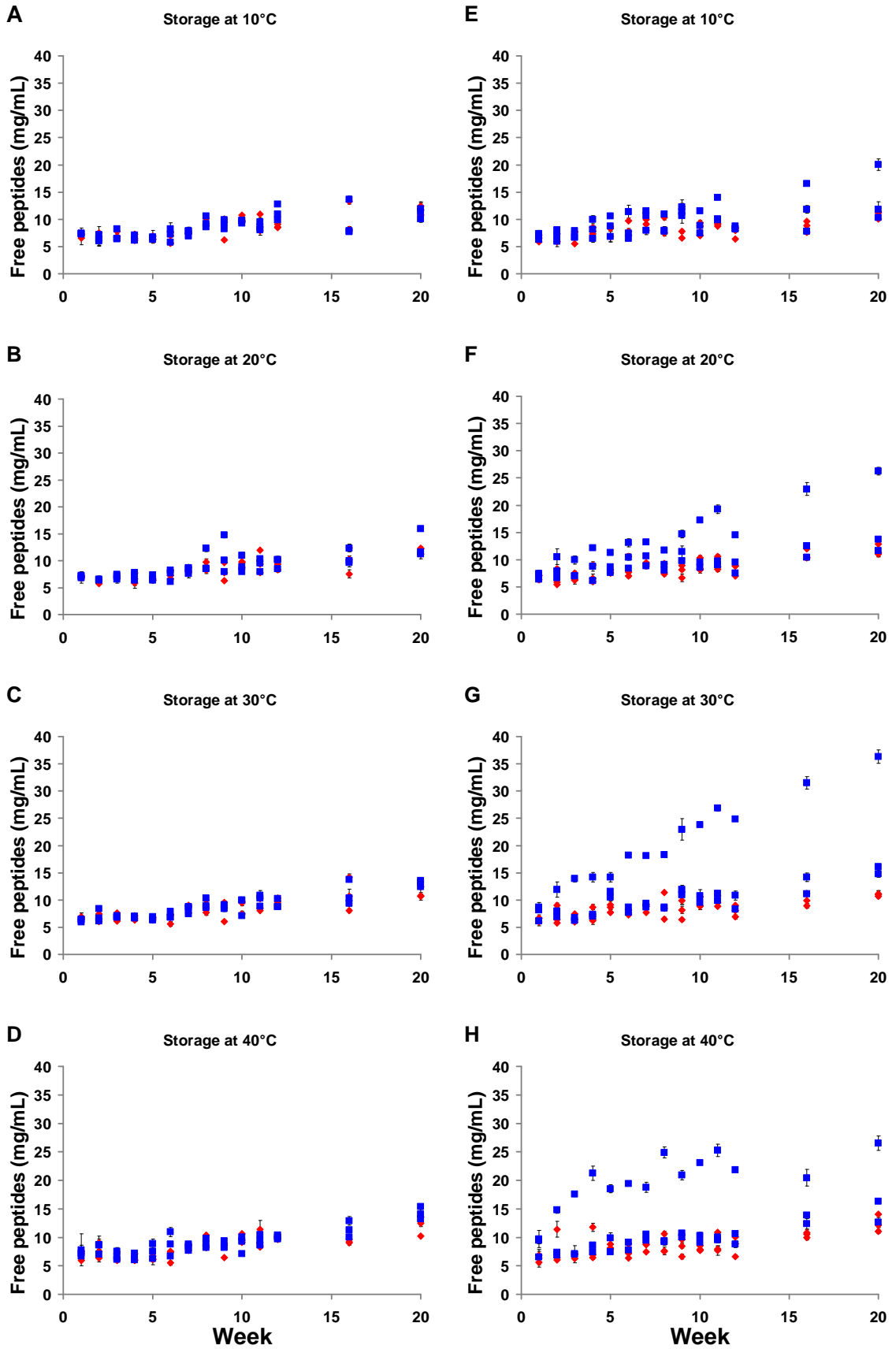


Figure 5.a-1 Average concentration of free peptides in control (◆) and treated (■) UHT skim milks processed from slightly contaminated stainless steel vessels (A, B, C, D) or moderately contaminated stainless steel vessels (E, F, G, H) over a period of 5 months. The UHT skim milk samples were stored at 10 °C (A, E), 20 °C (B, F), 30 °C (C, G) and 40 °C (D, H). Trials were performed with two replicates on three separate occasions, and error bars represent standard deviations from the means.

The increase in the free peptide concentration in the milk was probably due to heat-stable proteases in the milk from the multispecies biofilms, as the final concentrations of the planktonic cells in the milk were similar for both the slightly contaminated vessels and the moderately contaminated vessels. The large variation in the free peptide concentration observed in the UHT skim milk from the moderately contaminated stainless steel vessels may have been due to the variability in the amount or rate of activity of the enzymes produced during the 10 h of incubation. The enzymes produced by a single bacterial species are dependent on environmental factors (temperature, pH, substrates) and microbial factors (age, quorum sensing)(Dunstall et al., 2005, Membré and Burlot, 1994). The complexity of enzyme production and activity is likely to be increased in multispecies biofilms, compared with single-species biofilms, because of interspecies microbial interaction, growth factors, and environmental variables, which control the growth of multispecies biofilms (Azevedo et al., 2009, Hellweger and Bucci, 2009). Another factor that may have influenced the variability between trials is the milk powder substrate. Different batches of skim milk powder were used in the moderately contaminated vessel trials: one batch, which was used for two trials, showed slower enzyme activity; the other batch, which was used for the third trial, showed more rapid enzyme activity. The batch variability of the skim milk powder may have influenced either the production of bacterial enzymes or their activity. The variation in the skim milk powder may have been due to seasonal variation, handling of the raw milk or variations in the manufacturing process. For example, the transportation and the storage temperatures of milk prior to cheese manufacture can affect the ripening of cheese and its chemical composition (Franciosi et al., 2012).

From a practical perspective, milk from a heavily contaminated vessel can be less damaging to a manufacturing company than milk from either slightly or moderately contaminated vessels, because milk that is extensively degraded is usually downgraded to lower quality product before the escalation of spoilage defects can occur. In contrast, milk from either slightly or moderately contaminated vessels may be detected only during storage, when the commercial investment (e.g. distribution) and the potential losses (product recall) are at their greatest. The increase in the concentration of free peptides during storage of the UHT skim milk from the slightly and moderately contaminated vessels indicates that the milk may eventually have become unacceptable because of

coagulation or bitterness (Kilara and Panyam, 2003). In several instances in Australia, UHT products have been recalled because of spoilage; for example, in 2000, 1 L Blue Slime Brick Packs of Pura (a brand name of National Foods) were recalled because of the swelling of cartons, curdling and off-odours (www.recalls.gov.au/content/index.phtml/itemId/955901). The large variation in the proteolysis rate observed in this study is believed to reflect what happens in a commercial operation, in which spoilage defects can occur with high uncertainty because of raw material variability.

5.a.4.3 pH of UHT skim milk

In general, the pH of the UHT skim milk was dependent on the storage temperature, with the pH decreasing as the storage temperature increased (Table 5.a-2) and with significant differences ($P \leq 0.05$) among the storage temperatures (data not shown).

The pHs of the control and treated UHT skim milks processed from the slightly contaminated vessels were not significantly different ($P > 0.05$). However, the pH of the control UHT skim milk was significantly higher than that of the UHT skim milk processed from the moderately contaminated vessels at 10, 20, and 30 °C ($P \leq 0.05$).

Browning in the UHT skim milk also increased as the storage temperature increased. This observation was in agreement with Gaucher *et al.* (2008), who reported that the pH of UHT milk dropped to 6.21 because of browning during storage at 40 °C. In general, the reduction in pH is likely to be due to Maillard, and related, reactions that form acids and brown pigments (Gaucher *et al.*, 2008). There was no evidence of the proteolysis being sufficient to alter the pH. Likewise, there was no evidence that proteolysis (when observed) was effected by non-enzymatic reactions that changed the pH.

Table 5.a-2 Average pH of the control and treated UHT skim milks processed from contaminated stainless steel vessels at different storage temperatures for a period of 12 weeks

	<i>Control UHT milk</i>	<i>Treated UHT milk</i>
<i>Slightly contaminated vessels</i>		
10 °C	6.51 ± 0.02	6.50 ± 0.02
20 °C	6.48 ± 0.02	6.46 ± 0.02
30 °C	6.42 ± 0.03	6.41 ± 0.03
40 °C	6.29 ± 0.09	6.29 ± 0.09
<i>Moderately contaminated vessels</i>		
10 °C	6.54 ± 0.02 ^a	6.51 ± 0.03 ^b
20 °C	6.50 ± 0.02 ^a	6.47 ± 0.03 ^b
30 °C	6.44 ± 0.03 ^a	6.40 ± 0.03 ^b
40 °C	6.29 ± 0.09	6.27 ± 0.09

Measurements were performed on three separate occasions, and values represent the means with standard deviations. The different letters within each row indicate statistically significant differences, $P \leq 0.05$ (Tukey's test).

5.a.5 CONCLUSION

The internal surfaces of milk tankers may promote the formation of biofilms during raw milk transportation, especially if the tankers are not adequately cleaned. Such biofilms may become a source of microbial enzymes, which can be released into the raw milk during transportation, and their presence may lead to adverse effects on the qualities of both the raw material and the final product. To ensure the production of a high quality, long-shelf-life product, the cleanliness of the internal surfaces of milk transport tankers needs to be maintained by the implementation of an effective cleaning and sanitation regime.

What happens in a milk tanker can be modelled by simple *in vitro* experiments. However, even our simple model resulted in highly variable results, reflecting what has been observed in commercial manufacture. Clearly, new approaches that can model the complex behaviour of multispecies biofilms in the dairy industry without being confounded by large variation are needed.

5.a.6 ACKNOWLEDGEMENTS

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Chapter 6. Summarising discussion and conclusion

6.a **Highlights**

The highlights of this study supporting the hypothesis that “Enzymes produced by bacteria within biofilms during the transportation of raw milk by milk tankers can have detrimental effects on dairy products” are:-

- The internal surface temperature of milk tankers during raw milk transportation can be within the temperature range that is ideal for the proliferation of psychrotrophic bacteria (**Chapter 3.a**)
- Bacteria found on the internal surface of milk tankers after milk collection can form biofilms and can produce heat-stable enzymes (**Chapter 3.b**)
- Proteolysis or lipolysis was found to be higher by bacteria within biofilms compared with their corresponding planktonic cells when the bacterial cells were grown within an *in vitro* model of a milk tanker (**Chapter 4.a** and **Chapter 4.b**)
- Heat-stable enzymes produced by bacteria within multispecies biofilms produced in an *in vitro* model of a milk tanker degraded the protein content of UHT milk stored at 30 and 40°C (**Chapter 5.a**)

6.b **Summarising discussion**

There is evidence of enzymes from biofilms being indirectly used for manufacturing traditional cheeses such as Ragusano (Sicilian cheese) and Salers (French cheese) (Didienne et al., 2012, Lortal et al., 2009), however, enzymes from biofilms within the dairy environment have only recently been suggested as a possible cause of spoilage in dairy products (Marchand et al., 2012). It is highly likely that spoilage enzymes from biofilm will affect the quality of the dairy products at different stages of dairy manufacture as bacterial enzymes tend to be heat-stable, and can remain active after pasteurisation.

Prior to the current studies, the effects of biofilm enzymes, produced during raw milk transportation, on dairy products had not been reported. It was known that the population of psychrotrophic bacteria in raw milk increased during transportation (Lafarge et al., 2004, Martins et al., 2006), and that some of these bacteria had the ability to form biofilms and produce enzymes (Marchand et al., 2012, Nörnberg et al., 2011). This study investigated naturally occurring bacteria found on the internal surfaces of milk tanker after

raw milk transportation and determined their ability to produce enzymes within biofilms in an *in vitro* model that simulated the conditions within a milk tanker. In addition, the effect of these enzymes produced within biofilms on UHT milk during storage was investigated. To our knowledge, this is the first study to demonstrate the enzymes produced by bacteria within biofilm of dairy origin and their effect on UHT dairy products.

6.b.1 Preliminary investigation on milk tankers

The internal surface temperatures of milk tankers can potentially affect the formation of biofilms and enzymes produced by bacteria during the transportation of raw milk. In **Chapter 3.a**, the internal surface temperatures of milk tankers on five separate days for the summer and winter of 2009 – 2010 was reported. The internal surface temperatures of milk tankers for summer and winter ranged from 4 to 46°C and -6 to 28°C, respectively (**Figure 3.a-4** and **Figure 3.a-5**). The average internal surface temperature of a milk tanker was 19°C (summer) and 10°C (winter). This confirms that the internal surface temperatures of milk tankers tend to fluctuate during raw milk transportation, and were seasonal. The upper part of the milk tanker tended to be the hottest compared with the other parts of the milk tankers. This suggests that any bacteria exposed to the upper part of the milk tanker due to the splashing of raw milk, if they adhere to the surface, would have suitable temperatures to grow and form biofilm with the possibility of producing enzymes.

Bacteria from these biofilms and the enzymes they produce would be released into the raw milk during transportation. Most of the bacteria would be inactivated during heat treatment in a dairy manufacturing process; however, the heat-stable enzymes produced within the biofilms may retain their activity and contaminate the end-products. Therefore, in **Chapter 3.b**, the bacteria from the internal surfaces of milk tanker were isolated, identified, and characterized by their ability to form biofilms, and produce heat-stable enzymes (lipase and protease).

In total, only 52 out of the 153 bacterial isolates were able to form biofilms and produce either heat-stable protease (29), or lipase (9), or both enzymes (14) (**Table 3.b-2**). The bacterial isolates found on the surface of milk tankers varied between the two seasons. The predominant bacterial isolates during summer were Gram-negative, with 68% belonging to the genus *Serratia*, while during winter, the predominant bacterial isolates

were Gram-positive with 85% belongs to the genus *Staphylococcus*. Other studies have shown the bacterial populations in raw milk are seasonal or dominated by *Pseudomonas* species (Leriche and Fayolle, 2012, Marchand et al., 2009a). Twelve bacterial isolates were identified as *Bacillus licheniformis*, *Pseudomonas fluorescens*, *Pseudomonas fragi*, *Serratia liquefaciens*, *Staphylococcus aureus*, and *Streptococcus uberis* (**Figure 3.b-1**), and had a minimum growth range of 7 to 20°C (**Table 3.b-3**). It is interesting to note that *S. liquefaciens* is seldom associated with dairy spoilage, and may have been underrated as a potential spoilage organism compared with other psychrotrophic bacteria such as *Pseudomonas* species. The role of *S. liquefaciens* in dairy spoilage needs to be investigated as *S. liquefaciens* can be found in dairy environments, and has the ability to produce biofilms and enzymes (Cleto et al., 2012). In summary, the results from this part of the study showed that some naturally occurring bacteria found in raw milk may produce both biofilms and heat-stable enzymes.

S. aureus, isolated in this study, has the potential to cause food poisoning, however the toxin production by *S. aureus* was not examined in the current study. Pathogenic *S. aureus* are capable of forming biofilms, and producing toxin that causes food poisoning. We speculated that the secretion of enterotoxin from biofilm into the liquid product may be a potential source of food poisoning. Toxin production has been associated with *S. aureus* biofilm formation (Caiazza and O'Toole, 2003, Mizumachi et al., 2011). Staphylococcal strains with the Staphylococcal Enterotoxin B (SEB) gene are better at the formation of biofilms than strains lacking SEB (Mizumachi et al., 2011). The production of toxin within biofilm may not only be a problem for the dairy industry but also for other food industries.

From a practical perspective, the fluctuating internal surface temperatures of a milk tanker during raw milk transportation may promote the proliferation of biofilms of psychrotrophic bacteria capable of producing heat-stable enzymes. Bacterial cells embedded in alginate beads have been used as a model for dispersed biofilm cells (Xu et al., 1996), and immobilized cells within alginate beads have been shown to secrete lipases (Zakaria et al., 1992). Similarly, heat-stable enzymes may be embedded within dispersed biofilms. This may further enhance the heat stability of the enzymes due to the protective matrix of EPS. This phenomenon may result in milk spoilage, and subsequently a loss of revenue for the dairy industry due to a reduced yield, and a shorter product shelf-life.

6.b.2 *In vitro* studies of enzymes produced by bacteria within biofilms

An *in vitro* model that simulated the fluctuating temperatures in the upper part of an internal surface of a milk tanker was designed and run for approximately ten hours. This trial aimed to simulate raw milk collection in New Zealand, with an average tanker collection run of ten hours. The *in vitro* model only focused on the upper part of the internal surface of a milk tanker as the surface temperature here may be ideal for the formation of biofilms (**Chapter 3.a**).

Bacterial isolates from the milk tankers (**Chapter 3.b**) were used in the investigation of the production of proteases (**Chapter 4.a**) and lipases (**Chapter 4.b**), by bacteria within biofilms in the *in vitro* model. Although studies have shown most of these bacterial species are able to produce heat-stable enzymes within planktonic cultures, and have the ability to form biofilms (Marchand et al., 2012, Nörnberg et al., 2011), relatively little is known about the potential of these bacteria to produce enzymes within biofilms.

In **chapter 4.a**, proteolysis by biofilm cells was reported to be greater measured with corresponding populations of planktonic cells (**Table 4.a-2**). This finding is in agreement with sludge or fungal biofilms where a higher production of enzymes was observed from cells within the biofilms (Frølund et al., 1995, Gamarra et al., 2010). Proteolysis in this study was strain, temperature, and growth-mode dependent. For example, proteolysis and growth was only observed at 37°C by the biofilm cells of *P. fluorescens* C224 but not by the corresponding planktonic cells. This could be linked with the ability of cells in biofilms to grow at higher temperatures than their planktonic counterparts (Nilsson et al., 2011, Rogers et al., 1994). This may also be explained by the difference in metabolic activities and physiology of biofilms or planktonic cells (Oosthuizen et al., 2001). It has been suggested that the accumulation of enzymes within biofilms may aid in the survival of biofilm populations (Budhani and Struthers, 1998).

Some of the co-culture biofilms in this study, for example *B. licheniformis* R4 and *P. fragi* BC5, produced more proteolysis compared with their corresponding single culture biofilms. This observation was in agreement with another study where the production of amyolytic enzymes was increased in co-culture biofilms of *B. amyloliquefaciens* and *Zymomonas mobilis* compared with either single culture biofilm (Abate et al., 1999).

The production of lipase was examined with four dairy bacterial isolates and a lipase-producing bacterium as positive control (**Chapter 4.b**). The lipolysis in single culture or co-cultures, within biofilms and in planktonic cultures, was compared. The results were in agreement with the proteolysis studies, where lipolysis was ten times greater by bacteria within biofilms than by the planktonic cultures (**Table 4.b-2**). Lipolysis was found to be higher within the single culture biofilms under low nutrient availability compared with high nutrient availability. This may be due to a stress response, where an accumulation of enzymes within biofilms acts as a survival mechanism (Budhani and Struthers, 1998, Spector and Kenyon, 2012, Thomason et al., 2012). The effect of nutrient availability on lipolysis within co-culture biofilms was inconclusive, possibly due to the complications of microbial interaction. In general, mutualistic interactions were observed where the amount of lipolysis was increased in all of the co-culture biofilms of *S. uberis* C05 when grown in a nutrient rich environment. The lipolysis produced by biofilms can be influenced by the type of bacterial strain, the co-culture combinations, and nutrient availability.

From a practical perspective, the production of enzymes within biofilms is generally higher compared with the planktonic cells. This raises the possibility that these spoilage enzymes may be secreted into raw milk during transportation, and may end up in the final dairy product. In theory, the amount of spoilage enzymes produced within a highly contaminated milk tanker in a worst case scenario could spoil 1×10^6 L of milk. In reality, the amount of enzymes produced may be less than the theoretical amount of enzymes produced (1.35 g), however, only a trace amount of spoilage enzyme is required for the enzymatic spoilage in the dairy products (Shah, 1994). In addition, dispersed biofilms from the milk tanker may further colonize dairy processing surfaces, as dispersed biofilms cells readily attach to new surfaces (Rollet et al., 2009). This can be a problem in the dairy manufacturing plant as dispersed biofilms can contaminate other parts of dairy manufacturing plant and continue secreting heat-stable spoilage enzymes into the dairy product during processing.

Besides psychrotrophic bacteria, mesophilic and thermophilic bacteria found in raw milk may also be entrapped within these dispersed biofilms, and the populations within these biofilms may shift to favour the growth of mesophilic and thermophilic bacteria in the

dairy manufacturing plant. Bacterial populations within a multispecies biofilms can shift due to environmental factors and microbial interactions (Elias and Banin, 2012, Martiny et al., 2003). Some of the mesophilic and thermophilic bacteria, such as *Geobacillus stearothermophilus*, are known to produce heat-stable enzymes, and can be found in the dairy manufacturing environment (Burgess et al., 2010, Chopra and Mathur, 1985). During heat-treatment, the heat-stable enzymes may secrete from the biofilms into the heat-treated products, thus, shortening the shelf-life of these products. However, there are no reports confirming the hypothesis that the spoilage of dairy products can be affected by heat-stable enzymes produced within biofilms of psychrotrophic, mesophilic, or thermophilic bacteria in the dairy environment.

6.b.3 Practical study on the effects of biofilm enzymes produced during raw milk transportation on dairy products

The effect of bacterial enzymes on the spoilage of dairy products is well-known and usually associated with spoilage microorganisms growing planktonically in the milk. From **Chapters 4.a** and **4.b**, the production of enzymes by bacteria within biofilm was higher compared with planktonic cells, and are most likely to spoil milk and dairy products but this has not been confirmed. Therefore, **Chapter 5.a** extended the investigation to examine spoilage effects on heat-treated products by enzymes produced by bacteria within a multispecies biofilms during raw milk transportation. This is the first study, to our knowledge, that demonstrated the link between enzymes produced by bacteria within biofilms and their effect on UHT milk

The effects of the enzymes produced by bacteria within biofilm formed on an *in vitro* model of a milk tanker with three different microbial loads (10^3 , 10^5 , and 10^7 cfu/mL), comprising three bacterial isolates (*P. fluorescens* C224, *S. liquefaciens* DC1, *S. aureus* SF01) were examined, The results from this study showed that the enzymes produced by bacteria within biofilm could affect the quality of UHT milk. Milk exposed to a highly contaminated vessel was extensively degraded, and the effect was observed immediately when the milk was heat treated, resulting in coagulation of the milk.

The degradation of the UHT milk exposed to a moderately contaminated vessel was observed at 30 and 40°C during storage, while with UHT milk exposed to only a slightly

contaminated vessel degradation was only observed at 40°C (**Figure 5.a-1**). The degradation of milk was most likely due to the present of heat-stable proteases as the milk was previously subjected to heat treatment (141°C for 15 s). This is in agreement with other studies where heat-stable proteases are able to retain their activity, and affect the quality of UHT product during storage (Celestino et al., 1997b, Champagne et al., 1994, Shah, 1994). It appears that the proteolysis of the UHT milk during storage will occur with different levels of contamination with the time taken for spoilage dependent on the amount of contamination. This study has demonstrated that the presence of multispecies biofilms on the internal surfaces of a milk tanker during raw milk transportation may have detrimental effects on the quality of manufactured products due to enzyme secretion. This is the first study to show that enzymes produced within biofilm can cause spoilage in UHT dairy product during storage.

From a practical perspective, this highlights the link between enzymes produced by bacteria within biofilms present during raw milk handling and the spoilage of dairy products. Biofilms can form on the internal surface of a milk tanker regardless of the quality of the raw milk. If the milk tankers are not adequately cleaned, biofilm growth and enzyme secretion may damage even previously good quality milk. Raw milk that is extensively degraded is less damaging to the dairy company as the milk will be either rejected or diverted to less critical products. Milk that is contaminated with a low level of heat-stable enzymes is of more concern to a dairy company's products, their financial return, and reputation as commercial lost is at the greatest.

6.c Conclusion

This is the first study to show the link between the enzymes produced by bacteria within biofilms and their effect on UHT products. The fundamental knowledge obtained in this study relates to enzyme production in multi-species biofilm of dairy origin. Some of these dairy bacterial isolates are able to form biofilms and produce heat-stable enzymes in an *in vitro* model that simulates the fluctuating surface temperatures of a milk tanker during its daily operation. Proteolysis and lipolysis by bacteria within biofilms are generally greater compared with the planktonic cells, and also higher in co-cultures biofilms compared with single culture biofilms.

Heat-stable proteases produced by bacteria within multispecies biofilms can have a detrimental effect on the UHT product (skim milk). The quality of the UHT skim milk can be affected at various levels of contamination. This is the first report linking the production of enzymes by bacteria within biofilm to the quality of manufactured dairy products.

The practical contributions to knowledge made by this study are that the internal surface temperatures of milk tanker vary, and are dependent on environmental factors (season and weather) and operational factors (duration of milk run, temperature of the bulk milk, the volume of the bulk milk). The upper part of internal milk tanker has the potential to support both the production of biofilms and heat-stable enzymes by bacteria during raw milk transportation. The heat-stable enzymes can retain activity in manufactured dairy product that will result in economic loss. Even with a trace amount of heat-stable enzyme being produced within these multispecies biofilms, the quality of the UHT dairy product is affected during prolonged storage. The dispersed biofilm from the milk tanker may colonize other areas of the dairy processing plant, and may be a new source of microbial enzymes. This suggests that the role of a milk tanker in raw milk transportation is important, in order to ensure the production of good quality dairy products. This work confirmed that the enzymes produced by bacteria within biofilm during raw milk transportation can have a detrimental effect on the end dairy product.

For the first time, the effect of enzymes produced by bacteria within biofilms on UHT milk has been demonstrated, where the UHT milk underwent proteolysis due to the proteases produced by bacteria within biofilms. This is first time the link between the quality of dairy product and the enzymes produced by bacteria has been scientifically demonstrated and this could influence the quality of many dairy products. The dairy manufacturing companies should reevaluate the importance of raw milk transportation. This is to ensure the quality of the dairy products is not compromised by enzymes produced by bacteria within biofilm during the transportation of raw milk

6.d *Recommendations and future work*

This study has established an important link between the enzymes produced by bacteria within biofilms colonizing milk tankers and the quality of dairy products. This work has both a fundamental and practical perspective to the dairy industry and the wider food

industry. The recommendations and suggestions for future work are described in the following sections.

6.d.1 RECOMMENDATIONS

- The fluctuating surface temperature in milk tanker was found to be suitable for the proliferation of psychrotrophic bacteria during summer. A cost-benefit analysis of technical options for handling raw milk between dairy farms and the dairy processing plants is needed. Possible solutions may be the insulation or addition of refrigeration to milk tankers.
- This study emphasises the threat posed by inadequately cleaned milk tankers in the production of biofilms and heat-stable enzymes. The current duration of CIP for milk tankers may not be effective, as the duration of the hot wash on the internal surface of milk tanker of less than 10 min, may not be sufficient to remove attached bacteria. Therefore, an audit of tanker cleaning is recommended.
- The threat that these enzymes produced by bacteria within biofilm pose to dairy products needs to be further analysed. Such analysis may be assisted by the use of predictive modeling to account for the many variables that occur in the dairy manufacturing systems.

6.d.2 FUTURE WORK

- This work has provided evidence that enzymes produced by bacteria within biofilms colonising a milk tanker can have detrimental effects on UHT skim milk. However, this work was only based on proteolysis in UHT skim milk. The investigation of the effect of enzymes produced by bacteria within biofilms should be extended to other dairy products such as milk powder, yoghurt and cheese, manufactured from either skim or whole milk that has been previously exposed to biofilm.
- The types of enzymes produced by bacteria within biofilms may be different from the enzymes produced by their corresponding planktonic cultures. If the types of enzymes produced are different, the spoilage pathway may be different. This needs to be investigated.

- In this study, it was demonstrated that heat-stable enzymes are produced by psychrotrophic bacteria within biofilms in an *in vitro* model of milk tanker. However, there is no data on the production of heat-stable enzymes by mesophilic and thermophilic bacteria within biofilms in the dairy processing environment. It will be equally important to investigate the effects of these enzymes on dairy products.
- The production of enzymes by bacteria within biofilms in this work was investigated in an *in vitro* milk tanker model (a batch system), and may be different during milk processing (a continuous flow system). This should be investigated.
- The potential role of *S. liquefaciens* as a spoilage microorganism in raw milk needs to be investigated as *S. liquefaciens* is not currently recognized as a spoilage organism in raw milk.
- A possible pathway for toxin produced by *S. aureus* within biofilm should be investigated as a potential food safety hazard.

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