The Microflora of Raw Milk and the Impact of Milk on their Survival at Low pH

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Piet de Vegt

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Abstract

Milk is an excellent food source as it contains a plentiful supply of nutrients and minerals. Although normally consumed after pasteurisation, there is growing evidence that raw milk provides health benefits beyond nutrition alone. Epidemiological studies of children have shown that those who regularly consume raw milk appear to have a lower incidence of asthma and non-specific allergy than those who consume processed commercial milk. The gastrointestinal tract is a key location for immune development as interaction with microflora can occur at the mucosal surface. Milk may have a role to play in the early stages of this process either due to the microbes it harbours or to the physical and chemical properties of milk itself.

The aim of this study was to identify bacterial isolates unique to raw milk, that would not survive pasteurisation; and to determine whether milk allowed for a greater survival of these bacteria during ingestion. Bacterial isolates were cultured from either raw or pasteurised milk and tested for their ability to survive pasteurisation. A subset of thermosensitive isolates were identified for further analysis representing those species likely to be present in raw milk but not processed. This thermosensitive subset was challenged for their ability to tolerate acid conditions (pH 2.5) both in the presence and absence of milk to determine the likelihood of their survival during ingestion. A high throughput acid tolerance test was developed to screen raw milk bacteria for acid tolerance. Data supports the hypothesis that milk significantly increased the survival of raw milk bacteria exposed to pH 2.5 and that specific components found specifically in milk were, at least in part responsible for this effect. In conclusion, a unique subset of bacteria found only in raw bovine milk, and not in processed milk, has been identified that when ingested with milk are able to come through an acid challenge not dissimilar to that of the stomach and survive. This opens the possibility that these bacteria present in raw milk are able to enter the lower GI tract and interact with the immune system via Peyers patches.

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Abbreviations

aOR	adjusted Odds Ratio
APC	Antigen Presenting Cell
APT	All Purpose Tween
CFU	Colony Forming Units
CI	Confidence Interval
CM	Casein Micelle
DC	Dendritic Cell
DGGE	Denaturing Gradient Gel Electrophoresis
EDTA	Ethylenediaminetetraacetic acid
GAD	glutamic acid decarboxylate
GI	Gastrointestinal
GLMM	Generalised Linear Mixed Model
Ig	immunoglobulin
IL	Interleukin
IFN-α	Interferon-alpha
LB	Luria Bertani
LSD	Least Significant Difference
M	Molar (mol / L)
M-PCA	Milk-Plate Count Agar
mM	milli-Molar
MRD	Maximum Recovery Diluent
OR	Odds Ratio
PCA	Plate Count Agar
r16S	ribosomal 16S
SD	Standard Deviation
SSCP	Single Strand Conformational Polymorphism
T_H1	T-Helper type 1
T_H2	T-Helper type 2
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UHT	Ultra-high Temperature
WPC	Whey Protein Concentrate

1. Introduction

It is often said that 'you are what you eat'. This is most apt from a microbial point of view as 90 % of cells within our bodies are microbial and most bacterial species are introduced to our bodies via ingestion (Adlerberth *et al.*, 2009). The food we eat and the microbes it contains can change the composition of the gut microflora at the lower gastrointestinal tract, which in turn has a major effect on our health (Guarner *et al.*, 2003; Neish, 2009; Round *et al.*, 2009). From an early age, milk is a key food for a growing child. At this time, a delicate balance between the microflora and the immune system is reached. Immune disorders such as allergy and asthma have increased, with the recent estimates suggesting that up to 30-40 % of the world population suffers from allergic disease in 2011 (Pawankar *et al.*, 2011). Modern clean living conditions have changed the way that the immune system responds to common antigens. Recent studies have concluded that children who consume raw milk from a young age are less likely to develop such immune disorders (Bieli *et al.*, 2007; Loss *et al.*, 2011; Riedler *et al.*, 2001; Waser *et al.*, 2007). Raw bovine milk contains a wide variety of microorganisms, and these have the potential colonise the lower GI tract permanently or transiently (Manson *et al.*, 2008). However, to enter the lower gastrointestinal tract, bacteria must survive acid exposure in the stomach. Although many bacteria possess strategies to survive exposure to acid conditions, the acid tolerance of raw milk microflora as a whole has not been well characterised. This is important, as these bacteria may be involved in the development of a healthy immune system. This thesis contains a review of the mechanisms of allergic disease, and the modulation of the immune system by bacteria at the lower GI tract. This forms the rationale for carrying out the research done in this work, to show that a significant and varied proportion of the raw bovine milk flora are able to survive significant acid exposure and thus reach a position where they may be able to modulate the immune system at the lower GI tract.

1.1 Overview of Allergic Disease

The immune system is a protective mechanism that functions to protect the body from both infectious diseases and cancer. However, immune disorders can lead to disease, such as autoimmune disease, graft rejection, or allergies and asthma (Goldsby *et al.*, 2000). The single most common form of immune dysfunction comes in the form of allergies, affecting up to 20 % of the global population (Johansson *et al.*, 2004). Allergy is defined as an over-stimulated immune response to a variety of common and often harmless environmental antigens. This type of immune response can lead to a variety of disorders including hay fever, eczema, and allergic asthma.

1.1.1 The Background of Immune Dysfunction Leading to Allergic Disorder

The adaptive immune response is characterised by the ability to recognise billions of foreign antigens, then mount and memorise either a humoral or cell mediated response. The symptoms that cause allergic reactions are the manifestations of a dysfunctional humoral response, termed 'type 1 hypersensitivity' or atopy. Atopy is driven by an excessive Immunoglobulin-E (IgE) response that ultimately results in local or systemic inflammatory responses (Goldsby *et al.*, 2000; Jarvis *et al.*, 1998; Yazdanbakhsh *et al.*, 2001) (Figure 1).

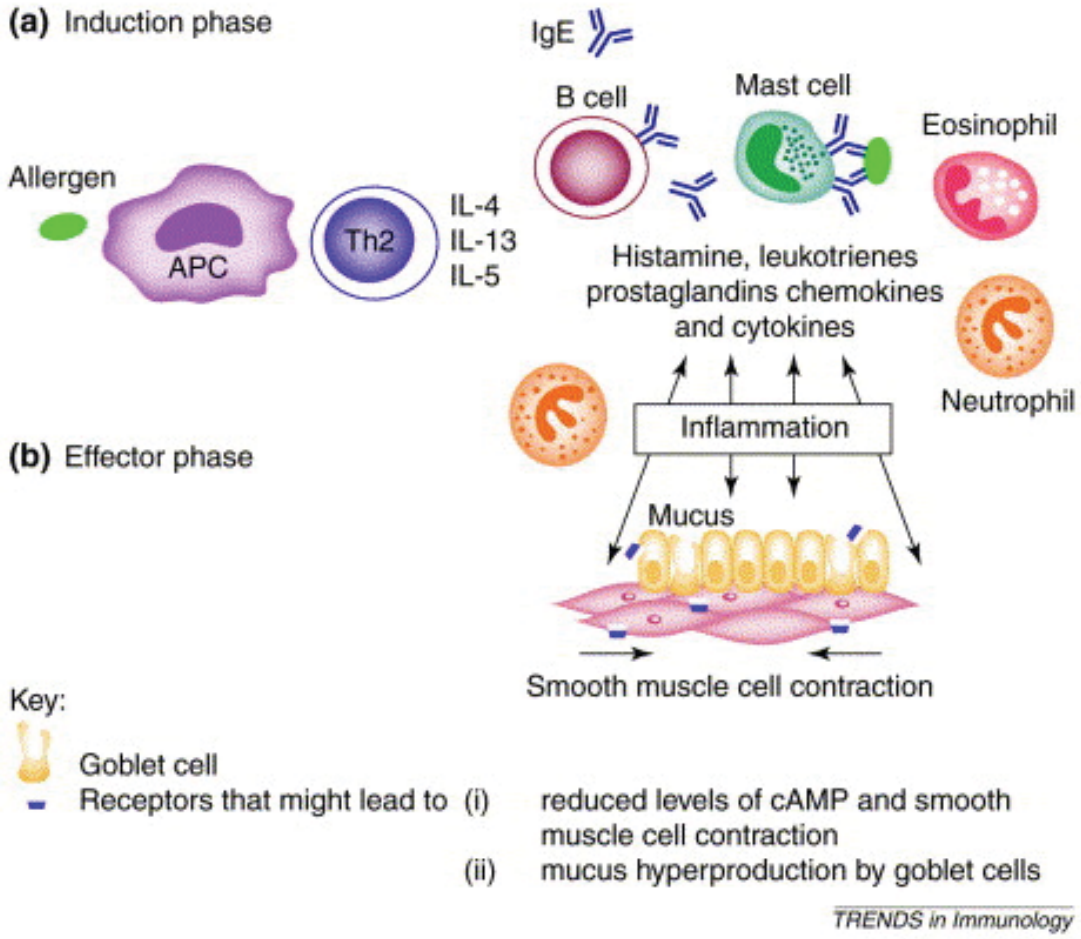


Figure 1 - The development of atopy. Atopy is initiated in the induction phase (a) where an allergen is presented to an Antigen-Presenting Cell (APC). The APC interacts with and stimulates a T_H2 lymphocyte to release signalling cytokines (interleukins) IL-4, IL-13 and IL-5, which in turn trigger B-cell proliferation and the production of allergen specific IgE antibodies. These antibodies bind to the B cells, Mast Cells, Eosinophils and Neutrophils and subsequently release inflammatory molecules such as histamine, leukotrienes, prostaglandins, chemokines and cytokines. These signalling factors initiate the effector phase (b) that cause inflammation, smooth muscle contraction and mucus production that result in the symptoms of asthma and/or allergy. Figure from Yazdanbakhsh (2001).

1.1.2 Regulation of the Immune Response: The T_H1/T_H2 Balance

While the biological pathway that leads to the symptoms of allergic disease is known, the regulation of the immune system that leads to this response is less well understood. The exposure of an antigen by an antigen-presenting cell to a T-lymphocyte can trigger either a humoral (antibody mediated) or cell mediated immune response. This depends largely on the cytokine environment in which T-lymphocytes are spawned. If T-helper type 1 (T_H1) cells predominate, immune reactions tend towards a cell-mediated response. If T-helper type 2 (T_H2) cells predominate, immune reactions tend towards a humoral response. This discovery led to research investigating the T_H1/T_H2 balance, as this is a major determinant in the development of allergic disease (Kidd, 2003).

A number of potential factors have been identified that result in one type of T-helper cell dominating over the other, however many details remain to be elucidated. Firstly, dendritic cells may play a role in shifting the balance towards a T_H2 response. Dendritic cells of patients with allergic rhinitis displayed reduced T_H1 signals such as IL-12 and IFN-α (Interferon-alpha), suggesting that the T_H2 response may be predisposed for at the antigen-presenting stage (Pilette *et al.*, 2013). Secondly, IL-10 is a key regulatory cytokine that is involved in shifting an allergen specific IgE to an IgG response, thereby reducing the level of harmful chemokines that cause the symptoms of allergy (Akdis *et al.*, 2004; Meiler *et al.*, 2008; Punnonen *et al.*, 1993). However, IL-10 is also involved in inhibiting the T_H1 response (Kidd, 2003), and a dysfunction at either step of these pathways may have a role in the elevated T_H2 responses.

Although it is likely that there is a genetic explanation for some of the immune regulation that results in a T_H1/T_H2 imbalance, evidence suggested that environmental conditions through previous infectious disease was also able to alter T and B cell repertoires (Macpherson *et al.*, 2004). However, no single underlying cause has been identified for all diseases. Furthermore, the increase in type-2 disorders is not countered by a similar decrease in type-1 immune disorders such as type-1 diabetes (Yazdanbakhsh *et al.*, 2002). However, it is possible that T_H2 factors are also involved in the pathogenesis of major T_H-type-1 immune disorders, such as rheumatoid arthritis, multiple sclerosis, and Type-1 diabetes (Kidd, 2003).

1.1.3 The Impact of the Hygiene Hypothesis on Immune Stimulation

The steady rise of allergic disease is due to a complex interaction of both genetic and environmental factors (Holgate, 1999). One possible explanation for the importance of environmental factors is the hygiene hypothesis, which suggested that the periodic exposure to infectious disease, especially at an early age, can account for a decrease in immune disorders (Frei *et al.*, 2012; Martinez, 2001; Yazdanbakhsh *et al.*, 2002). Inversely, lower rates of interaction with livestock, cleaner households, smaller family sizes, improved sanitation, increased antibiotic use, and low helminth & orofaecal burdens are all thought to contribute to increased rates of immune disorders (Suen *et al.*, 2003). In a pioneering study, a significant inverse relationship was discovered between the incidence of allergic rhinitis and family size. In particular, a lower position in the household was strongly inversely related to developing allergic disease as infections may be introduced by older siblings (Strachan, 1989). Further, a farming lifestyle was also identified as a preventative factor, as it may increase the chance of acquiring infection through contact with animals, or via consumption of raw milk (Kilpelainen *et al.*, 2000; Perkin *et al.*, 2006). Furthermore, not all infectious diseases prevent allergic disease. Certain respiratory diseases, such as whooping cough, or croup, are significant predictors of asthma development (Farooqi *et al.*, 1998; Illi *et al.*, 2001), and rhinovirus (common cold) infection is also a predictor of childhood wheezing (Lemanske Jr *et al.*, 2005). In addition, many gastrointestinal bacterial exposures, pathogenic or commensal have been shown to have a positive impact on the reduction or prevention of allergic disease (see section 1.2).

1.2 The Impact of Bacterial Exposure at the Gastrointestinal (GI) Tract on Immune Development

The GI tract is the largest mucosal surface of the body, and is an important interface between the body and the external environment (Kato *et al.*, 2005; Shanahan, 2002). The gut is a complex microbial environment containing at up to 1000 different bacterial species (Hooper *et al.*, 2002), which is established within the first years of life. The bacterial flora in the GI tract is numerous, diverse and dynamic. The microflora inhabiting the human gastrointestinal tract may be either permanent or transient colonisers (Manson *et al.*, 2008). Factors such as diet, lifestyle, bacterial exposure and antibiotic use can affect the composition of the bacteria in the GI tract. Studies comparing germ-free animals with those containing gastrointestinal flora show that this microflora is critical in the maturation of a functional immune system (Kelly *et al.*, 2007). There is constant interplay between the immune system and the bacteria that reside in the GI tract

1.2.1 Influence of Lower GI Tract Microflora on Allergic Disease

The composition of the microflora of the GI tract can influence the immune system of the host, which is thought to occur by 'priming' the immune system to react appropriately to future immune challenges (Clarke *et al.*, 2010; Macpherson *et al.*, 2004). Immune cells actively sample the GI microflora, influencing the ongoing development of the immune system (Neutra *et al.*, 1996). It was proposed that there might be differences in the gut micro-biota of allergic and non-allergic children, and that these differences may influence the outcome of allergic disease. A number of different studies have investigated this hypothesis.

Impact of Commensal Microflora on Allergic Disease

Multiple studies have indicated a role for the gut microbiome in improper immune development, as reviewed by Ly (2011). Children in healthy control groups had a greater diversity in gut flora than those displaying allergic symptoms (Forno *et al.*, 2008). Furthermore, children with allergies were less often colonized with Lactobacilli or other anaerobic bacteria (Björkstén *et al.*, 1999), and had a higher incidence of Clostridia (Kalliomäki *et al.*, 2001a), particularly *C. difficile* (Böttcher *et al.*, 2000; Penders *et al.*, 2007). Colonisation by a group of Lactobacilli, notably *L. casei*, *L. paracasei* and *L. rhamnosus,* was recently found to lower the risk of allergy at five years of age despite having an hereditary predisposition (Johansson *et al.*, 2011). The role of Clostridia in

development of allergic disorders has been disputed, as Verhulst (2008) found an association between anaerobic bacteria and wheezing, but not with *Clostridium*. In another study investigating the effects of gut flora on childhood wheezing no statistically significant difference was found in the presence of lactic acid bacteria or *Bifidobacterium*; however, Bifidobacteria were less prevalent in those that suffered from eczema (Murray *et al.*, 2005).

Gastrointestinal Infection

The development of the immune system may not only be influenced by the commensal microflora of the gut, but also by transient organisms, such as those that cause GI infections. Young children consuming raw milk are at risk of common bacterial infections including *Campylobacter*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Yersinia enterocolitica* (Gibson *et al.*, 2005). Infectious burden was used as a determinant of atopy in a study comparing the health of children in Russia and Finland. A strong inverse relationship was found between *H. pylori* infection and atopy (Von Hertzen *et al.*, 2006). The prevalence of *S. aureus* in allergic infants was higher compared with non-allergic (61 % compared with 23 % respectively) (Björkstén *et al.*, 2001). The link between infectious disease and the development of allergic disease has not been confirmed conclusively by all studies. When investigating GI infections, Illi (2001) found that the chances of developing asthma or wheeze was lower in children who had had more than four GI tract infections, compared with those that had three or less. However this was not statistically significant. Further, Kramer (2004) could not substantiate that there was a significant correlation between respiratory or gastrointestinal infection and the onset of eczema and recurrent wheezing in the first 12 months of life.

Probiotics

Given that differences in GI microflora have been noted in children suffering from allergic disease, it was proposed that administrating probiotics in early childhood might help prevent or treat allergic disease. Strains of *Lactobacillus*; *Bifidobacterium*, *Lactococcos, Proprionibacterium,* or a combination of these have been used (Prescott *et al.*, 2007). Kalliomäki *et al.* (2001b) showed, in a double blind trial, that there was a 50 % reduction in children developing eczema with the perinatal consumption of *Lactobacillus* GG compared with a placebo. However, there was no difference in atopic sensitisation in the two groups, suggesting that the protective effect was not IgE mediated (Kalliomäki *et al.*, 2007). A similar study examined the effect of *L. reuteri* in infants with a family history of allergic disease, and found that while rates of eczema were similar, IgE-associated eczema was significantly lower in those children taking probiotics (Abrahamsson *et al.*, 2007). In a

New Zealand study, the perinatal administration of *L. rhamnosus* reduced the prevalence of eczema, but did not significantly affect atopy after two years of age (Wickens *et al.*, 2008), mirroring the earlier results of Kalliomaki. Based on these results, it appeared that the impact of probiotics may be primarily to reduce the severity of allergic disease, rather than preventing them from occurring. Furthermore, it was suggested that diversity in general, rather than specific bacterial exposures were more important in developing an effective immune system (Abrahamsson *et al.*, 2012).

Antibiotics

While bacterial exposure in the gastrointestinal tract has been shown to have a beneficial impact on the onset of allergic disease, the use of oral antibiotics has been found to be a significant risk factor. Administration of broad-spectrum oral antibiotics within the first two years of life increases the chance of atopy by more than 2-fold (Hurwitz *et al.*, 2000). Droste (2000) found that antibiotic use in infancy was a significant risk factor for asthma, hay fever and eczema, especially when stratified for parental hay fever. Interestingly, in children where there was no history of parental hay fever, antibiotics did not have a significant impact on the development of allergic disease, suggesting that there may be a complex interaction between genetic and bacterial exposure.

Antibiotics are administered in response to bacterial infection and often prophylactically against secondary infections associated with viral infections (Droste *et al.*, 2000), making it difficult to isolate the effects of either factor on the onset of allergic disease as the two variables are almost always connected. No data suggested that the use of prophylactic antibiotics was a risk factor. Furthermore, it was accepted that the bacterial flora of the gut was resilient to antibiotic treatment, with almost complete recovery occurring after oral treatment (Dethlefsen *et al.*, 2008). However, this may not be the case when antibiotics are administered in early infancy when the gut flora is not fully established (Adlerberth *et al.*, 2009).

1.2.2 Mechanisms of Bacterial Immune Stimulation

It is difficult to prove the causality of bacterial exposures in the GI tract on the incidence of allergic disorders without considering the mechanisms that might explain how this can occur. There are at least three different mechanisms that enable bacteria present in the gut to stimulate the immune system. Firstly, immune cells at the lower GI tract can sample the contents of the lumen, either through interaction with M-cells at the Peyers patches (Owen *et al.*, 1986), or direct sampling of the lumen by projections of the dendritic cells

through tight junctions between epithelial cells (Rescigno *et al.*, 2001). This sampling may be important as dendritic cells can influence the T_H1/T_H2 balance (Pilette *et al.*, 2013). Secondly, bacterial products may traverse the epithelial barrier and stimulate the immune system. Bacterial polysaccharide is able to help the maturation of the developing immune system and correct the T_H1/T_H2 balance in mice mono-colonised with *Bacteriodes fragilis* (Ivanov *et al.*, 2011). Thirdly, chronic bacterial infection may help reduce immune dysfunction by stimulating the gastric immune system, resulting in T_H1/T_H2 deviation, antigenic competition/homeostasis, immunoregulation, non-antigenic ligands, or gene-environment interactions (Okada *et al.*, 2010).

1.3 Bacterial Survival at Low pH

Bacteria have the ability to survive many adverse conditions; including extremes in heat, salinity, pressure and pH (Horikoshi *et al.*, 1998). Species that survive and grow at extremely low pH are termed acidophiles and grow optimally at pH 4.0 or lower (Cobley *et al.*, 1983). Acidophilic bacteria are rare. Almost all bacteria humans encounter are neutrophilic, as they grow optimally near pH 7 (Cobley *et al.*, 1983). Neutrophilic bacteria with acid tolerance must be able to resist excessive proton stress, which can result in an excessive proton motive force that damages the cytoplasmic membrane (Foster, 2004), and can damage acid sensitive proteins, or DNA (Cotter *et al.*, 2003). Bacteria have developed several strategies to survive or grow at a low pH. Hydrochloric acid is the primary acid secreted in the stomach. The pH of the stomach can reach pH 2.0 in a healthy individual (Audia *et al.*, 2001). Acid tolerance mechanisms allow neutrophilic bacteria to survive temporary exposure to extreme acid stress when ingested. These acid tolerance mechanisms have been reviewed extensively in recent years (Audia *et al.*, 2001; Bearson *et al.*, 1997; Cotter *et al.*, 2003; Foster *et al.*, 1999; Merrell *et al.*, 2002).

1.3.1 Regulation of the Acid Tolerance Response in Bacteria

To survive acid exposure, a bacterium must be able to sense changes in the external pH. The cell constantly monitors changes in the external and cytoplasmic pH using membrane proteins, pH-dependent proteolysis of regulatory proteins, or other markers of stress; that in turn activate acid stress proteins that mitigate the effects of acid shock (Booth *et al.*, 2002; Foster *et al.*, 1999; Rowbury, 1997).

Sigma Factors

Bacteria have a range of sigma factors that act as a global regulatory mechanism of transcription. Under certain conditions, alternative sigma factors may be expressed that result in the increased expression of subsets of genes required for a specific response. In Gram-negative bacteria, the sigma factor most associated with acid stress is RpoS (Hengge-Aronis, 1993). In Gram-positive bacteria, this function is carried out by the alternative sigma factor, RpoB (Cotter *et al.*, 2003) (Völker *et al.*, 1999). RpoS is controlled at transcription, translation and post-translational levels (Lange *et al.*, 1994). *rpoS* is constitutively expressed under normal conditions, but the mRNA is poorly translated due to a stable secondary structure (Muffler *et al.*, 1997). The exact regulatory mechanism resulting in the up-regulation of RpoS in acid stress scenarios is not completely

understood, but in response to acid stress, RpoS levels are up-regulated by increased translation (Hengge-Aronis, 2002; Muffler *et al.*, 1996) and reduced proteolysis (Zhou *et al.*, 1998).

Ferric Uptake Regulator

Fur is a major iron regulatory protein of bacteria (Wandersman, 2010). It is also found in *Salmonella* Typhimurium, and has a role in signal transduction and the initiation of acid tolerance mechanisms (Bearson *et al.*, 1997). Fur activates a group of RpoS independent acid shock proteins in response to low pH, independent of iron regulation (Hall *et al.*, 1996). A Fur homologue in *Helicobacter pylori,* a species of bacteria found in the mammalian stomach, was found to have the same function (Bijlsma *et al.*, 2002).

Two Component Regulatory Systems

In addition to regulatory proteins that control the expression of specific and global acid tolerance responses, a number of two-component regulatory systems both sense external pH and initiate acid shock responses in bacteria (Arnold *et al.*, 1995; Foster *et al.*, 1999). PhoPQ and OmpR/EnvZ in *Salmonella* Typhimurium each stimulate a distinct set of acid tolerance mechanisms in exponential phase and stationary phase respectively (Audia *et al.*, 2001; Foster, 2001). PhoPQ consists of membrane-bound PhoQ which activates the cytoplasmic PhoP, an acid shock protein (Foster, 1999). PhoP in turn activates a cohort of acid shock proteins (Bearson *et al.*, 1997). Where PhoPQ is the prime RpoS-independent exponential phase regulator, the EnvZ/OmpR two-component regulatory system becomes dominant at stationary phase, as phosphorylated OmpR initiates the expression of genes encoding another independent acid tolerance response (Bang *et al.*, 2000).

1.3.2 Bacterial Acid Tolerance Responses

Regulatory pathways detect changes in external pH and initiate acid tolerance responses that mitigate the effect of proton stress on the bacterial cell. Bacteria in an acidic environment need to maintain a near-neutral cytoplasmic pH by buffering or expelling cytoplasmic protons and maintaining membrane integrity. There is a complex array of acid shock proteins that achieve this goal. The main mechanisms are proton pumps, amino acid decarboxylases, protein and DNA repair mechanisms, membrane alteration and alkali production (Table 1).

Proton Pumps

In aerobic bacteria, the proton gradient between the external environment and the cytoplasm is used to generate energy. At neutral pH, this proton motive force is generated by oxidative phosphorylation in the electron transport chain, which results in the extrusion of protons. In acidic conditions, *E. coli* increases the expression of respiratory proteins resulting in a net outflow of protons (Slonczewski *et al.*, 2009).

The F_0F_1 ATPase is the main enzyme involved in converting the energy stored as the proton motive force into ATP. However, protons may also be expelled through the catalysis of ATP into ADP + P_i. In acid conditions, many bacteria, including those that lack an electron transport chain express this enzyme for the purpose of pH homeostasis (Cotter *et al.*, 2003; Harold *et al.*, 1970; Muntyan *et al.*, 1990). In many Gram-positive organisms, the ability to survive at acidic pH ultimately depends on the efficiency of this enzyme. For example, the acid tolerance of three oral Streptococci correlates to a more acidic pH optima of their respective F_0F_1 complexes (Sturr *et al.*, 1992).

Amino-Acid Decarboxylases

As well as removing protons directly from the cytoplasm, bacteria are able to absorb protons using amino acid decarboxylases. These systems are well described in *E. coli*, where Glutamic Acid Decarboxylase (GAD) and arginine decarboxylase are known to increase acid tolerance during stationary phase (Richard *et al.*, 2003). The enzymes deplete the cytoplasm of protons by catalysing the reaction between protons and either glutamate or arginine to form carbon dioxide and γ-aminobutyrate or agmatine respectively. These are then expelled by the cell by glutamate/γ-aminobutyrate and arginine/agmatine antiporters. These systems have been detected in other Gram-negative species, but only the GAD system has been found in Gram-positive bacteria (Cotter *et al.*, 2003). In *E. coli* this system can be so effective that it can result in the charge reversal of the proton motive force when stationary phase *E. coli* are exposed to arginine or glutamate at pH 2.5. This is a strategy also used by true acidophiles (Richard *et al.*, 2004). This is the mechanism hypothesized to prevent excessive proton motive force from disrupting the cytoplasmic membrane during exposure to extreme pH (Foster, 2004).

Protein Repair Mechanisms

To survive at low pH, bacteria must do more than simply manage proton concentrations. They must manage the effects that protons have on the bacterial superstructure by inducing chaperones. Although considered heat-shock proteins under the control of a different sigma factor (RpoH in Gram-negatives, and RpoA for Gram-positive bacteria) the

chaperones, such as DnaK and GroEL, can also be expressed in acid shock conditions in *Lactococcus lactis* subsp. lactis (Hartke *et al.*, 1996) and *S. typhimurium* (Foster, 1991). In addition, the effects of periplasmic chaperones may be critical in helping Gram-negative bacteria survive extreme acid exposure (Booth *et al.*, 2002).

DNA Repair and Protection

Acid stress can lead to damage not just to the structural components of the cell but also to the genetic material. RecA is one of the main bacterial DNA repair proteins, responsible for repairing double strand breaks through homologous recombination (Chen *et al.*, 2008). RecA has been shown to be required for acid tolerance in *Streptococcus mutans*, but in RecA deficient mutants acid tolerance was almost completely restored by mild acid exposure prior to acidification suggesting the presence of a RecA-independent repair system (Quivey *et al.*, 1995). A second protein, Dps, may be important in protecting DNA from acid stress. Dps is a non-specific DNA binding protein normally expressed at high concentrations during stationary phase. Dps binds to DNA, protecting it from acid/alkaline stress, oxidative stress, heat stress and UV radiation (Nair *et al.*, 2004). An *E. coli* mutant deficient in Dps was less acid tolerant (Jeong *et al.*, 2008).

Membrane Alteration

A number of Gram-positive species had increased acid tolerance when genes responsible for the assembly and maintenance of the cell membranes were mutated (Cotter *et al.*, 2003). *S. mutans* can change membrane structure by increasing the length of the fatty acids of the cytoplasmic membrane in response to acid pH resulting in lower proton permeability. An acid sensitive relative, *Streptococcus sobrinus*, is unable to make this adjustment in fatty acid chain length (Quivey *et al.*, 2000).

Alkaline Production

In addition to removing protons from the cell, some bacteria can produce alkaline substances that neutralise the acid in the environment. This strategy is used by the gastric pathogen, *H. pylori*, which can survive for several hours at pH 1.0 in the presence of urea, while maintaining a cytoplasmic pH close to neutral (Stingl *et al.*, 2002). Urease is produced which converts urea into carbon dioxide and ammonia; the latter is protonated by H^+ that leaks in from outside the cell (McCallum *et al.*, 1990; Scott *et al.*, 1998). Urease activity is important both inside, and outside the cell, as *H. pylori* that only have the cytoplasmic urease were susceptible to acid (Krishnamurthy *et al.*, 1998). Urease activity has also been detected in other bacterial species. Screening of over 1000 bacteria from

sheep lumen showed ureolitic activity in *Staphylococcus*, *Lactobacillus*, *Klebsiella* and *Streptococcus* species (Cook, 1976).

Table 1 - Compilation of known bacterial acid tolerance mechanisms. Some bacteria where these mechanisms have been identified are listed (Cotter *et al.*, 2003), (Bearson *et al.*, 1997).

Mechanism	Bacteria	References
	Proton Pumps	
Electron Transport Chain	*E. coli*	Slonczewski *et al.* (2009)
F_0F_1 ATPase	*Enterococcus hirae*	Harold *et al.* (1970)
	Amino Acid Decarboxylases	
Glutamate Decarboxylase	*E. coli*	Hersh *et al.* (1996)
	Shigella flexneri	Waterman *et al.* (2003)
	L. lactis	Sanders *et al.* (1998)
	L. monocytogenes	Cotter *et al.* (2001)
	Clostridium perfringens	Cozzani *et al.* (1970)
	Bacteroides sp.	Banks *et al.* (1989)
	Lactobacillus brevis	Ueno *et al.* (1997)
Arginine Decarboxylase	*E. coli*	Iyer *et al.* (2003)
	Salmonella Typhimurium	Foster *et al.* (1991)
Lysine Decarboxylase	*Vibrio cholerae*	Merrell *et al.* (2000)
	Salmonella Typhimurium	Park *et al.* (1996)
	E. coli	Sabo *et al.* (1974)
	Protein Repair Mechanisms	
DnaK	*Salmonella* Typhimurium	Foster (1991)
	S. mutans	Len *et al.* (2004)
	E. coli	
GroEL/GroES	*Salmonella* Typhimurium	Foster (1991)
	Streptococcus sp.	Takahashi *et al.* (1999)
	E. coli	Len *et al.* (2004)
	DNA Repair and Protection	
RecA	*Salmonella* Typhimurium	(Foster, 1995)
	S. mutans	Quivey *et al.* (1995)
Dps	*E. coli*	Choi *et al.* (2000)
	Membrane Alteration	
Fatty Acid profiles	*S. mutans*	Quivey *et al.* (2000)
Cell Surface Hydrophobicity	*Salmonella* Typhimurium	Leyer *et al.* (1993)
	Alkali production	
Urease	*H. pylori*	McCallum *et al.* (1990)
	Streptococcus salivarius	Sissons *et al.* (1990)
	Staphylococcus sp.	Cook (1976)
	L. casei	
	Klebsiella sp.	
Arginine deiminase	*L. monocytogenes*	Ryan *et al.* (2009)
	Streptococcus sp.	Curran *et al.* (1995)

1.3.3 Bacterial Survival in the Stomach and the Role of Food

Survival during ingestion is not only dependent on the intrinsic acid tolerance of bacteria, but also on other factors that alter conditions within the stomach. There are a number of factors that influence whether or not a bacterium can survive gastric transit, particularly the effect of food. After ingestion of food, the pH of the stomach will increase to around pH 4-6, depending on the buffering capacity of the food ingested, and will stabilise to around 2.5 to 3.5 (Holzapfel *et al.*, 1998). To more accurately gauge bacterial survival, many studies on probiotic survival during gastric transit have employed the use of dynamic artificial gastric systems, first published by (Minekus *et al.*, 1995). These systems allowed the addition of food and were shown to be comparable to the *in vivo* situation for the survival of lactic acid bacteria. The development of *in vitro* digestion trials allowed more detailed studies on the survival of probiotics and their interaction with food. When a kefir, a fermented milk food was included in survival assays, probiotic survival was higher than using batch assays (Mainville *et al.*, 2005). However, the initial pH was as high as pH 4 at the initiation of the experiment. Other investigations found that the survival of three common Lactobacilli was greater in a milk than in water at acid pH, possibly due to the lower buffering capacity of water compared with milk (Lo Curto *et al.*, 2011).

Experiments where bacteria were grown in fermented milk and subsequently exposed to acid showed that the fermented milk matrix offered protection against exposure to low pH. Under these growth conditions, the bacteria may have adapted or become tolerant to lower pHs through exposure to weak acids within the fermented milk (Faye *et al.*, 2012). Binding of bacteria to cheese particles may also facilitate survival (Pitino *et al.*, 2012). It is likely that food proteins binding to the surface of bacteria, or the burial of bacteria within food will provide some protection against the low pH of the stomach. However, the extent of this protection will depend on the properties of the foods consumed.

1.4 Milk Components That May Affect the Incidence of Allergic Disease

Since the early 20th century, the vast majority of the western population have consumed pasteurised milk. This was primarily to safeguard against microbial diseases, such as tuberculosis or scarlet fever (Atkins, 1992). There are some people that choose to consume raw milk, either because it is readily available to them in a rural environment, or due to their anthroposophic beliefs, which promote natural food types (Waser *et al.*, 2007). Despite the risk of food-borne illness, there may be some health benefits in consuming raw milk, as those that regularly consume raw milk from a young age suffer lower rates of allergic disease than those who consume pasteurised milk (Loss *et al.*, 2011).

1.4.1 The Impact of Raw Milk on Reducing Rates of Asthma and Atopic Allergy

The increase in the rates of allergic disease in the past century has been attributed in part to the change from a more rural lifestyle to a more urban lifestyle. This hypothesis may be corroborated by the farm effect, where children who live on farms have significantly lower rates of allergic disease than those that live in the same areas but do not live on farms (Ehrenstein *et al.*, 2000; Riedler *et al.*, 2001; Riedler *et al.*, 2000; Von Mutius *et al.*, 2010). Together with allergy protective farm exposure, the consumption of raw milk in childhood was found to reduce the incidence of asthma and allergic disease (Table 2). Interestingly, Wickens (2002) found there was a greater prevalence of allergic disease on farms in general, although this may be an artefact of a smaller sample size made up of only 293 children. This is much smaller than the GABRIELA study where 8334 children were included (Loss *et al.*, 2011).

Table 2 - Studies that link raw milk consumption to reduced rates of asthma and allergic disease. (OR) = odds ratio, (aOR) - Covariate adjusted odds ratio. An odds ratio below 1.0 denotes an inverse association.

Leading Author	Study Location	Cohort size	Reductions observed		Reference
Loss	Germany, Austria Switzerland	8334 children	Asthma Atopy Hay fever	aOR[a], 0.59; 95 % CI, 0.46 - 0.74 aOR[a], 0.74; 95 % CI, 0.61 - 0.90 aOR[a], 0.74; 95 % CI, 0.61 - 0.90	Loss *et al.* (2011)
Ege	Europe	8263 children	Asthma	OR[b], 0.77; 95 % CI, 0.60 - 0.99	Ege *et al.* (2007)
Waser	Europe	14,893 children	Asthma	OR[b], 0.74; 95 % CI 0.61 – 0.88	Waser *et al.* (2007)
Perkin	England	4767 children	Asthma	OR[b], 0.67; 95 % CI 0.49 - 0.91	Perkin *et al.* (2006)
Radon	Germany	921 children	Atopy	OR[b], 0.65; 95 % CI 0.36 – 1.18	Radon *et al.* (2004)
Wickens	New Zealand	293 children	Atopic Eczema	OR[b], 0.20; 95 % CI 0.10 – 0.80	Wickens *et al.* (2002)
Barnes	Greece	929 children	Atopy	OR[b], 0.58; 95 % CI 0.34 – 0.98	Barnes *et al.* (2001)
Riedler	Germany, Austria, Switzerland	3054 children	Asthma Hay fever Atopic sensitisation	OR[b], 0·48; 95 % CI 0·21 – 1·10 OR[b], 0·24; 95 % CI 0·10 – 0·56 OR[b], 0·43; 95 % CI 0·24 – 0·77	Riedler *et al.* (2001)

a - aOR – adjusted Odds ratio, b – odds ratio

1.4.2 Components of Milk Capable of Immune Modulation

The mechanism of this effect by raw milk against allergic disease has not been elucidated. Hypotheses focus either on a biochemical mechanism by the proteins or fatty acids in milk (Van Neerven *et al.*, 2012), or through interaction with the milk microflora (Braun Fahrländer *et al.*, 2011). Although, a direct causal relationship has not been proven, the consensus is that this is a property of raw milk that is lost during processing (Braun Fahrländer *et al.*, 2011; Perkin *et al.*, 2006). Notably, the protective effect was lost when raw farm milk was boiled before consumption (Loss *et al.*, 2011), suggesting that the factor responsible must be heat sensitive. Two components that may modulate the immune system differently in raw milk as opposed to pasteurised milk are therefore the milk proteins, or the milk microflora (Von Mutius *et al.*, 2010).

Milk Proteins

Milk proteins form approximately 3.4 % of bovine milk, and are broadly divided into caseins and whey proteins (Jenness, 1974). Caseins are the most common group of milk proteins, comprising approximately 80 % of milk protein (Schmidt, 1982). However, caseins are heat stable (Tuinier *et al.*, 2002) and are unlikely to be altered significantly by pasteurisation. The remaining 20 % of milk protein consists of the whey proteins, which are more heat labile as they denature at temperatures above 70 °C (De Wit *et al.*, 1984).

The immunoglobulins are the most heat-sensitive of the major whey protein groups (De Wit *et al.*, 1984). Bovine milk contains four key immunoglobulins, IgG1 IgG2 IgA and IgM at levels of approximately 0.8 mg / ml combined during normal lactation (Butler, 1973). Immunoglobulins in milk can cross the species barrier and instil protection against pathogenic bacteria via passive immunity (Korhonen *et al.*, 2000). Immunoglobulins are moderately stable at low temperature treatments, but are impaired by UHT processing (Kummer et al. 1992; Li-Chan et al. 1995). Immunoglobulins are stable during refrigeration, suggesting that some activity may be retained in commercial milks.

The immunostimulatory activity of other whey proteins has been investigated. Lactoferrin, has been shown to stimulate the immune system of mice by binding to the intestinal mucosa (Tome, 1998).

Many of the proteins in milk appear to have biological functions as short peptides as opposed to full-length proteins (Shah, 2000). A variety of 'bioactive' peptides have been identified in milk. These short peptides are released when the milk is hydrolysed, or digested (Meisel *et al.*, 1999). Milk proteins that release peptides with physiological activity include α-lactalbumin, β-lactoglobulin, casein (α, β, and κ), and lactoferrin (Clare *et al.*, 2000). These peptide fragments have a range of effects on the body once they are released, including antimicrobial activity, antihypertensive, antithrombotic, immune-stimulatory, opoid and antagonistic properties (Clare *et al.*, 2000; Shah, 2000). Due to the varied and diverse roles that bioactive peptides have on the body, it was hypothesised that bioactive peptides may be involved in the stimulation or modulation of the immune system. Pasteurisation is not thought to adversely affect the viability of these bioactive peptides (Korhonen *et al.*, 1998), suggesting that many of their properties are conserved in processed milk.

Microflora

Bacteria can have an impact on the incidence or severity of allergic disease (see Section 1.2). Raw bovine milk is known to contain many different microbial genera and species (Quigley *et al.*, 2013b). The microflora of milk is affected by pasteurisation, as most bacterial species are killed when milk is heated. Some thermotolerant or spore-forming species may survive. Furthermore some species may be reintroduced after pasteurisation (Eneroth *et al.*, 1998). The microflora that are ingested along with milk may directly influence the immune system by the colonisation of the gut in a permanent or transient manner, or stimulate immune activity through infection. It has been shown that there is a significant reduction in the incidence of allergic disease in those that drink raw milk as opposed to processed milk. It is not thought that heat-killed bacteria as a result of pasteurisation will have a significant effect on immune activity. This is consistent with observations made in the literature where comparisons made with viable and non-viable probiotics show that viable bacteria have the greatest health benefit, and in comparison the effects of non-viable bacteria are often limited (Ouwehand *et al.*, 1998).

1.5 The Microflora of Raw and Processed Milk

The microflora of raw milk may be involved in the prevention of allergic disorder by the stimulation the immune system. For this reason, the microflora of raw milk will be reviewed, including what is known about its' diversity. The likely sources of the raw milk microflora will be reviewed, and this flora will be compared to the microflora of processed milk.

1.5.1 The Microflora of Raw Milk

Microbial Species Commonly Associated with Milk

The bacterial species commonly associated with milk have extensively reviewed by Robinson (Robinson, 1990). A table of common bacterial species listed by Robinson is attached as an appendix (Appendix 1). Milk is an animal product and is produced in many different environments and methods. This results in the contamination of a wide variety of bacterial species (Robinson, 1990). As a result, one should not consider any bacteria to be normally present in milk. Rather that some bacterial species are more commonly found to contaminate milk than others. Some of the key milk contaminants are described.

Gram-negative aerobes of the Pseudomonaceae and the Neisseriaceae families are often found in raw milk (Robinson, 1990). Many of the Pseudomonads are capable of growth in the psychrotrophic temperature range, and can produce heat-stable enzymes that contribute to the spoilage of raw and pasteurised milk (Robinson, 1990). *Pseudomonas aeruginosa* can cause bovine mastitis (Watts, 1988). Of the bacteria in the Moraxellaceae family (formerly Neisseriaceae), the genera that are commonly associated with milk is limited to the *Acinetobacter* and *Moraxella*-like bacteria (Robinson, 1990).

With the exception of *Erwinia*, *Obesumbacterium*, *Xenohabdus*, *Rhanella*, *Cedecea*, and *Tatumella* genera, the Enterobacteriaceae are commonly associated with raw milk (Robinson, 1990). The most significant Enterobacteriaceae found in milk, according to this author, are the *Escherichia*, *Salmonella*, *Citrobacter*, *Enterobacter* and *Yersinia*. These bacteria are found in the lower intestine, and are considered indicators of faecal contamination (Tallon *et al.*, 2005). The Enterobacteriaceae can also cause coliform mastitis, which may become acute (Hogan *et al.*, 2003). Consumption of raw milk has been

implicated in the transmission of pathogenic Enterobacteriaceae such as *Salmonella, or E. coli* O157:H7 (Claeys *et al* 2013, Langer *et al*, 2012).

Gram-positive cocci that may be found in raw milk include the Micrococcaceae & Staphylococcaceae (formerly Micrococcaceae), Streptococcaceae and Enterococcaceae (Robinson, 1990). The *Staphylococcus* may cause serious food poisoning due to the production of enterotoxins (Balaban *et al.*, 2000). Key *Staphylococcus* species found in raw cows' milk include *S. aureus*, *S. hyicus*, *S. chromogenes* and *S. epidermis* (Robinson, 1990). The Streptococci, notably *S. uberis* are often associated with bovine mastitis (Leigh, 1999). The genus *Enterococcus* may also be isolated from milk, is indicative of faecal contamination, and may also cause mastitis (Robinson, 1990). The genera *Lactococcus* and *Leuconostoc* may be found in raw milk, and both have importance to the dairy industry as part of starter cultures in the production of dairy products such as butter or cheese (Corroler *et al.*, 1998; Robinson, 1990).

The spore-forming *Bacillus* and *Clostridium* genera are commonly isolated from raw milk (Robinson, 1990). *Bacillus and Clostridium* species may grow at psychrotrophic, mesophilic or thermophilic temperatures (Robinson, 1990). *Bacillus cereus* can cause food poisoning due to the production of enterotoxins (Granum *et al.*, 1997). *Bacillus* species are common spoilage organisms of pasteurised fluid milk due to the heat-resistant nature of their spores (Ternström *et al.*, 1993). *C. botulinum* has been found in silage and bovine intestinal tract of infected animals, and some outbreaks of botulism from contaminated dairy products have been reported (Lindstrom *et al.*, 2010). *Clostridium perfringens* also inhabits the bovine instestinal tract, is a foodborne pathogen, and can cause mastitis (Robinson, 1990).

Non-sporulating Gram-positive rods that may be found in raw milk include the *Lactobacillus*, *Listeria* and the coryeform bacteria (Robinson, 1990). The Lactobacilli are widely used as mesophilic or thermophilic starters and may cause spoilage of some cheeses (Robinson, 1990). The Lactobacilli also have probiotic properties (Spanhaak *et al.*, 1998). *Listeria monocytogenes* is a well-known pathogen and may be isolated throughout the farming environment, including soil, silage, sewage and milk (Farber *et al.*, 1991).

Some yeast, moulds and viruses may be found in raw milk. Yeasts belonging to the genera *Debaryomyces*, *Kluyveromyces*, *Saccharomyces* and *Candida* are associated with dairy products, and of these, only *Kluyveromyces* is likely to be isolated from milk. The remainder are more closely associated with cheese, or other fermented dairy products

such as Kefir (Robinson, 1990). Moulds such as *Geotrichum* sp., *Sporendoma* sp., or *Penicillium* sp. are also often found on cheeses (Robinson, 1990). The double-stranded DNA Cow-pox virus may sometimes be transmitted to the hands of dairy workers (Robinson, 1990). Other viruses that may be transmitted to humans via milk are poliovirus and Rubella (Robinson, 1990). Milk can also contain a range of bacteriophages that infect the lactic acid bacteria; i.e. members of the *Lactococcus*, thermophilic *Streptococcus, Lactobacillus*, and *Leuconostoc* genera (Robinson, 1990).

Diversity of Bacterial Populations in Milk

The collation of many different studies over time has given us an insight of the types of bacteria that may be present in raw milk. However it also important to investigate the raw milk microflora as a whole, to understand what one is exposed to when raw milk is consumed. Early studies used plating methods, and recently DNA based methods have been included to provide greater insights into the total microflora, not just those that can be grown on agar plates.

An early work on the diversity of bacteria in raw milk was published in 1962 (Thomas *et al.*, 1962). The authors found that the type of bacteria isolated changed with total concentration of bacteria in the milk sample. Milk that had low total counts on Yeastrel-Milk Agar (< 5 x 10^3 CFU / ml) contained predominantly *Micrococcus* sp. (68.7 % of isolates). The proportion of Micrococci decreased as the total plate count increased. However, Micrococci were still detected in 90 % of all samples taken. In comparison, milk that contained high total plate counts on Yeastrel-Milk Agar (> 1 x 10^6 CFU / ml) contained predominantly Gram-negative rods (51.2 % of isolates) (Thomas *et al.*, 1962).

Each farmers' milking practices may contribute to the diversity of bacteria found in the milk they produce (Verdier-Metz *et al.*, 2009). These authors found that after comparing milk from 67 different dairy farms and surveying farmers about milking habits, that certain methods resulted in a significantly different set of bacteria being prevalent in the milk. Distinct groups of bacteria were identified by Single Strand Conformational Polymorphism analysis. Bacteria of one group had a low diversity index and consisted mainly of skin bacteria. This was consistent with good hygiene practices such as washing of teats and good animal husbandry. A second group contained a much wider range of bacteria, and came from farms where udder hygiene was less strictly observed (Verdier-Metz *et al.*, 2009).

Driven by a desire to understand raw milk microflora for cheese production, Mallet *et al.* compared the microflora of raw milk produced in the French winter, when cows are kept indoors with spring where they are grazed outside. The counts of the Lactobacilli were two-fold higher in the spring, compared to the winter. This increase was attributed to seasonal variation. Also of note, this study reported a high variation in the Standard Plate Counts of individual farms, and that a high degree of diversity was observed (Mallet *et al.*, 2012). Mallet et al. also show that milking and farm management pratices such as pre-dipping of teats or herd size also affected the level of diversity seen in raw milk (Mallet *et al.*, 2012).

Several raw milk diversity studies have been performed in recent years. Quigley et al. have reviewed these in a recent publication (Quigley *et al.*, 2013b), and the key microbial species found in raw milk are summarised (Table 3). The most prevalent bacterial species reported in culture-dependent diversity studies belong to the genera *Microbacterium, Lactobacillus Lactococcus, Enterococcus* and *Chryseobacterium*. The *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Corynebacteria* and *Acinetobacter* are described as less prevalent. Many other bacterial species are only occasionally detected. The exact bacterial populations present in a sample are likely to be dependent on the environmental conditions and the milking practices employed at the farm from which milk is obtained.

Table 3 – Bacterial populations detected in raw cows' milk using culture-dependent* diversity studies.

Prevalent Populations	**Occasionally Detected Populations**
Microbacterium liquefaciens/oxydans lacticum	*Rhodococcus erythropolis*
Lactobacillus casei/curvatus/mindensis animalis/coryneformis/curvatus/delbrueckii johnsonii/paracasei/paraplantarum plantarum/rhamnosus/amylovorus	*Serratia liquefaciens/odorifera*
Lactococcus lactis/garvieae	*Enterobacter gergoviae*
Enterococcus faecalis/gallinarum saccharominimus	*Klebsiella ozaenae/oxytoca*
Chryseobacterium species	*Kocuria carniphila/kristinae/rhizophila*
	Frigoribacterium species
	Paracoccus species
	Micrococcus species
	Ochrobactrum anthropi/tritici
	Pantoea agglomerans
	Propionibacterium freudenreichii/jensenii
	Providencia stuartii
Less Prevalent Populations	*Psychrobacter maritimus*
Staphylococcus capitis/cohnii saprophuticus/equorum/xylosus/aureus haemolyticus/hominis/epidermis	*Pseudoclavibacter helvolus*
	Rahnella aquatilis
Streptococcus uberis/parauberis	*Renibacterium salmoninarum*
Pseudomonas alcalophila/stutzeri synxantha/fluorescens/putida	*Sphingomonas* species
	Achromobacter delicatulus
Corynebacterium ammoniagenes/freneyi glutamicum/variabilis/casei	*Aeromonas hydrophila*
	Arthrobacter arilaitensis/psychrolactophilus
	Brachybacterium nesterenkovii
Acinetobacter johnsonii/junii/haemolyticus lwoffii	*Deinococcus* species
	Dermacoccus species
	Leuconostoc mesenteroides
	Escherichia coli
	Aerococcus viridans
	Bacillus cereus
	Brevibacterium helvolum/linens

* Culture-dependent studies have been based on isolation of bacteria using agar-based methods followed by genotypic or phenotypic identification. The information in this table is based six studies that have been undertaken in four countries, as reviewed by Quigley *et al.,* (2013b).

New insight into the microbial diversity present in milk has occurred with the use of DNA technologies that do not require cultivation of microbial isolates. These culture-independent methods, such as Denaturing Gradient Gel electrophoresis (DGGE) (Giannino *et al.*, 2009), Single-Strand Conformational Polymorphism (SSCP) analysis (Verdier-Metz *et al.*, 2009) and 16S rRNA bacterial clone libraries (Delbes *et al.*, 2007; Rasolofo *et al.*, 2010), have in recent years been used to analyse the populations of bacteria that may be found in milk. While DNA based methods promise great insight on the bacterial populations present in a sample, there are some limitations that should be considered (Schloss *et al.*, 2011). Importantly, bias may be introduced when some 16S sequences amplify more readily than others (Klindworth *et al.*, 2013). Also, chimeric sequences may be produced after several rounds of replication where similar sequences anneal during the PCR cycles (Haas *et al.*, 2011; Smyth *et al.*, 2010).

The composition of two 16S clone libraries generated from raw milk in such manners is shown in Table 4. The milk used by Delbes (2007) was obtained from a single farm in France from which a clone library with 125 individual clones was constructed. Rasolofo (2010), conducted a 16S rRNA analysis of raw milk from a dairy processing plant in Canada, which contained raw milk from multiple farms. A clone library with 168 clones was constructed. Both studies revealed strikingly similar bacterial profiles in raw milk, despite being geographically separated. A high proportion of bacteria detected in both studies were Firmicutes. Rasolofo identified a higher proportion of *Staphylococcus* than Delbes (32.7 % and to 1.6 % respectively). Anaerobic clostridia made up 24 % and 9 % of the bacterial genera found, respectively, which would not have been detected using traditional aerobic culture methods. The Proteobacteria made up between 15 % and 20 % of bacterial species detected, and it appears as though there is some variation in the genera detected between the two studies. The Bacteroidetes were detected in both studies. This phylogenetic branch contains *Bacteroides*, a common faecal microbe (Mariat *et al.*, 2009). This demonstrated that anaerobic microflora might be present in raw milk.

Despite the strength of using DNA based bacterial identification, there is a limitation in that these methods may only identify dominant bacteria in a given sample. The bacterial populations present in milk are now being analysed using 454 pyrosequencing methods, which allows for the detection of rare species by analysing a high volume of sequences. Using 454-pyrosequencing, the microflora in Danish milk used for cheese making was analysed. In two samples 211 and 256 different species were identified (Masoud *et al.*, 2011). Only those comprising of at least 0.1 % of the total population were presented. Some of the rare isolates identified included the genera of *Alistipes*, *Caulobacter*,

Carnobacterium, *Kurthia* or *Ruminococcus*. Interestingly, other bacteria more commonly isolated in milk were found to be present in low numbers, including *Bacillus subtilis* and *Enterococcus faecalis* (Masoud *et al.*, 2011).

Table 4 - Microbial genera found in raw milk based on two independent 16S clone libraries.

Group	**Genus**	**Composition (%)** (Delbes *et al.*, 2007)	**Composition (%)** (Rasolofo *et al.*, 2010)
Bacilli	*Staphylococcus*	1.6	32.7
	Turicibacter	4.8	-
	Jeotgalicoccus	0.8	-
	Facklamia	2.4	5.4
	Lactobacillus	4	3.6
	Lactococcus	4	3.0
	Streptococcus	4.8	4.8
	Enterococcus	0.8	??
	Trichococcus	-	3.0
	Aerococcus	-	1.8
	Other Bacilli	-	8.3
Clostridia	*Clostridium*	24	9.5
Unaffiliated Firmicutes		10.4	-
Actinobacteria	*Corynebacterium*	5.6	6.0
	Arthrobacter	6.4	-
	Other *Actinobacter*	4.8	6.0
Proteobacteria	*Pseudomonas*	0	2.4
	Enterobacter	0.8	-
	Acinetobacter	1.6	6.5
	Ralstonia	12	-
	Other Proteobacteria	7.2	5.4
Bacteroidetes		4	1.2

Diversity Studies of Raw Milk in New Zealand

Given New Zealand's strong interest in the dairy industry and unique farming environment compared to the northern hemisphere, large-scale diversity studies of raw milk have been carried out, but have not been published (Hill, 2014). Farming conditions in NZ are different to those in most other countries, particularly in the northern hemisphere. Key differences are that New Zealand dairy cattle are mostly housed outside and fed mainly fresh grass. The herds are much larger than in many other countries and the industry is seasonal with cows being milked for only 8-9 months of the year (Hill *et al.*, 2012). These factors are likely to influence the microbiological profile of milk in New Zealand.

1.5.2 Sources of Bacteria Entering Raw Milk

A diverse range of bacterial species may be found in raw cows' milk. Although, milk produced in a healthy udder is considered sterile (Tolle, 1980), bacteria can be introduced into the milk, during and after milking (Gleeson *et al.*, 2013). Sources of contamination include, from within the udder, the teats and the exterior of the udder (Desmasures *et al.*, 1997), as well as the milking and storage equipment (Christiansson *et al.*, 1999; Gleeson *et al.*, 2013; Te Giffel *et al.*, 2002). The primary source of these bacteria is the commensal flora of the cow, or the environmental organisms present in the dairy farm environment.

Infection Within the Udder

Bovine milk from a healthy cow is considered sterile. However, some bacteria may enter and infect the udder, causing mastitis. Bacteria commonly associated with mastitis include the Streptococci, Enterococci, Aerococci and Lactococci (Devriese *et al.*, 1999), as well as *Klebsiella* sp. (Munoz *et al.*, 2007), *Escherichia* sp. (Burvenich *et al.*, 2003) and *Pseudomonas aeruginosa* (Robinson, 1990). Mastitis may be clinical or subclinical, the latter being where there is infection without the visible signs the udder or the milk is affected (Busato *et al.*, 2000). The bacterial concentration of an infected udder may be up to 10^7 bacterial cells per ml of milk (Zadoks *et al.*, 2004). It has been show in New Zealand that the incidence of clinical mastitis was 14 cows for every 100 cows per annum. Most of these incidences occurred around calving (McDougal, 2002). In comparison, the mean incidence rate of clinical mastitis in Canadian herds was 23 cows per 100 cows per annum (Olde Riekerink *et al.*, 2008). In New Zealand, milk from mastitic udders is excluded from

the supply to avoid penalties, and mastitic quarters are treated with antibiotics. However, subclinical mastitis is difficult to detect visually, and as a result milk from cows suffering from subclinical mastitis often does reach the bulk milk tank.

Bacteria Entering Milk from Teat Skin

In New Zealand, suction cups and mechanical pulsation facilitate the extraction of milk on most dairy farms. This physical action, often on wet teats, allows bacteria to be transferred from the udder surface, teat cistern and teat canal into raw milk (Tolle, 1980). Bacteria that normally reside on teat surface include *Micrococcus varians, Staphylococcus* spp. and *Streptococcus* spp. (Robinson, 1990). Culture-dependent methods have been applied to identify the bacterial diversity of the teat canal and skin specifically. Bacteria detected in the teat canal using these methods include both the bovine skin microflora and those likely to be of environmental origin (Gill *et al.*, 2006). The authors also found that many bacterial species detected inside the teat canal were normally associated with soil, water or the gastrointestinal tract (Gill *et al.*, 2006). A study investigating the bacterial populations on the teat exterior was published in 2012. The authors found a diverse range of bacteria present on the teat surface, which were also commonly found in raw milk. Bacteria detected were related to *Enterococcus*, *Pediococcus*, *Enterobacter*, *Pantoea*, *Aerococcus*, and *Staphylococcus* (Verdier-Metz *et al.*, 2012).

Contamination by Soil Bacteria

The bovine udder is often contaminated with dirt that may contain bacteria or their spores (Gleeson *et al.*, 2013). Many bacteria that typically live in soil environments may be associated with milk. Examples include the genera *Pseudomonas*, *Acinetobacter*, *Shewanella*, *Arthrobacter*, and *Aeromonas* (Robinson, 1990). Bacterial spores may also be present. These are thermotolerant and can survive pasteurisation (Christiansson *et al.*, 1999; McKinnon *et al.*, 1983). The *B. cereus* spore content of milk correlated strongly to amount of soil on the teat during milking (Christiansson *et al.*, 1999). Given that the vast majority of dairy cows in New Zealand are pasture fed throughout their lactation (Hill *et al.*, 2012), contamination by soil and water, i.e. mud, is likely to be greater than in countries where cows are housed indoors.

Contamination by Feed, Faecal or Bedding Bacteria

The type of feed cows are given can have a strong impact on the types of micro-organisms that are found in the milk they produce. Different types of feed may contain bacteria or their spores (Te Giffel *et al.*, 2002), or can affect the growth of different groups of bacteria in the bovine gastrointestinal tract (De Menezes *et al.*, 2011). There is a link between the

bacteria found in silage and their spores found in raw milk. RAPD-PCR typing has shown that the spores of *Bacillus* spp. and *Clostridia* spp. found in high numbers in silage were also found in raw milk (Te Giffel *et al.*, 2002). The authors write that these spores are ingested in the silage, pass through the bovine gastrointestinal tract, and are expelled in faeces. Spores then enter milk through faecal contamination of the udder.

In northern hemisphere countries, cows are often housed inside farm buildings and kept on bedding such as straw or sawdust. These materials can exacerbate the contamination of the udder when cows lie in bedding materials contaminated with faecal matter (Natzke *et al.*, 1976; Rendos *et al.*, 1975). The bacterial counts in bedding material were also positively associated with rates of clinical mastitis (Hogan *et al.*, 1989). *S. uberis,* a common mastitis organism, has been isolated from bedding material (Bramley, 1982). The gastroenteritic *Escherichia coli* is also known to cause mastitis when cows are housed in dirty bedding (Robinson, 1990). The bacteria that contaminate the udder enter the milk stream when cows are milked. Bedding materials are not typically used in New Zealand during the milking season.

Milking Equipment

Raw milk is passed through a network of rubber and stainless steel piping into storage tanks. Severe fouling, leading to the formation of bacterial biofilms can be found on interior surfaces of the milking plant and have been shown to be a source of contamination in milk (Lewis *et al.*, 1987; Mittelman, 1998). Thermotolerant bacteria may be readily found in raw milk, and significant numbers may re-contaminate milk when these bacteria build up on the milking equipment (Gleeson *et al.*, 2013). Milking equipment can act as reservoirs for highly heat-resistant spores once they become established in the plant (Scheldeman *et al.*, 2005).

The colonisation of bacteria in the milking plant may be compounded by bacteria attaching to and growing on deteriorated rubberware (Teixeria, 2005). Bacteria introduced to the plant in small numbers can multiply in this manner and become significant contaminants (Gleeson, 2013). It is most important that the milking plant is properly cleaned to prevent excessive counts of thermotolerant contaminants in raw milk (Chatelin *et al.*, 1981). Water used within the milking plant could also be a source of contamination. Although sanitisers are used, the wash is always completed using a fresh-water rinse (Flint, 1997). This may be an issue in New Zealand as milking plants are often cleaned with water drawn from natural streams or bores (J. Muwunganirwa, personal communication, 15 January 2015). While ultimately, bacteria contaminate the milking equipment via either water or milk, bacteria could multiply within the plant if left to grow

unchecked. This growth could eventually become so great that this in turn becomes a source contamination of new milk that flows through the plant at a later date.

1.5.3 The Processing and the Microflora of Processed Milk

Milk is pasteurised to inactivate bacteria found in raw milk, to improve the safety and shelf-life of milk sold on to consumers. To understand how bacteria may survive processing, this process will be addressed in the following section.

The Processing of Raw Milk

The processing of fluid raw milk can involve the separation of cream and skim milk fractions, pasteurisation, and homogenisation steps. In many dairy processing plants fluid raw milk is pasteurised by heating to 72 °C for 15 seconds (Grant *et al.*, 1998). Although this step is effective, it does not result in milk that is sterile. In England and Wales, the bacterial concentration of pasteurised milk considered unacceptable was 30,000 CFU / ml (determined by total plate count) (Robinson, 1990). Milk and cream may be recombined into a standardised product in a pre-defined ratio, depending on the level of milk-fat required in the final product. Processed milk may be homogenised to prevent, or extend the separation time of milk-fat in the commercial product. Homogenisation is the reduction of fat globule size by mechanical action under pressure. This may be achieved by a needle and seat mechanism (Michalski *et al.*, 2006; Pouliot *et al.*, 1991), or by forcing milk through an annulus (Phipps, 2009).

Bacteria Found in Processed Milk

Research into the microflora present in processed milk has generally focused on the identification and control of spoilage organisms or the survival of pathogens. Bacteria that caused the spoilage of pasteurised milk may be categorised into two categories; those that are thermotolerant and those that contaminate milk post-pasteurisation. Both of these may, together or in isolation, contribute to the spoilage of a given pasteurised milk sample (Ternström *et al.*, 1993).

Bacteria capable of surviving pasteurisation are considered thermoduric or thermotolerant. They can withstand periods of high temperature yet are not necessarily thermophiles (Egdell *et al.*, 1950). Thermotolerant bacteria found in milk that may survive pasteurisation include *Micrococcus, Microbacterium, Streptococcus, Lactobacillus*, *Bacillus,*

Clostridium, the Coryneform bacteria and some Gram-negative rods (Thomas *et al.*, 1967). Some of these, e.g. the *Streptococcus*, *Micrococcus* and the Coryneform bacteria grow slowly at refrigeration temperatures (Robinson, 1990; Seiler *et al.*, 1984). Others, such as the psychrotrophic spore formers can survive pasteurisation and also proliferate at low temperatures (Griffiths *et al.*, 1990). The predominant genera that comprise this group are the *Bacillus* spp. (Meer *et al.*, 1991).The *Bacillus* sp. are commonly found in pasteurised milk. This is because their spores can withstand high temperatures and can grow at low temperatures (Meer *et al.*, 1991). *B. cereus* can reach spoilage levels of 1×10^6 CFU / ml in extended shelf-life (UHT) milk (Schmidt *et al.*, 2012). *B. cereus* can also contribute to enzymatic spoilage of milk, and produce foodborne toxins (Schmidt *et al.*, 2012).

Despite being pasteurised, a significant amount of spoilage occurs from thermosensitive bacterial species. Investigating the spoilage of pasteurised milk, Schroeder found that all bacteria he isolated were heat labile Gram-negative rods, and postulated that they must have entered as post-pasteurisation contaminants (Schröder *et al.*, 1982). In a more modern study, the microflora that were found to contaminate milk after thermal treatment were mainly *Pseudomonas fluorescens*, *P. putida* and *Janthinobacterium levidium* (Eneroth *et al.*, 2000). This is mirrored by work of Dogan, who also found that *Pseudomonas* species often spoil pasteurised milk (Dogan *et al.*, 2003). It is not known how these bacteria are re-introduced, but one key area where this may occur is during bottle filling in the milk plant (Eneroth *et al.*, 1998).

Commercially sold milk may contain pathogenic bacteria even though it has been pasteurised. In France, pathogenic Enterobacteriaceae were found in 7 % of pasteurised milk or cream after storage at 7 °C (Lindberg *et al.*, 1998). The most dominant species found in milk or cream were *Seratia liquefaciens*, *Hafnia alvei* and *Rahnella aquatilis*. Milk may also contain spores of the *Clostridium* and *Bacillus* genera. The *Clostridia* do not generally sporulate in fluid milk due to the high redox potential (Robinson, 1990). However, they may be found to grow in mascarpone cheese made from pasteurised milk (Franciosa *et al.*, 1999). This may be because it is stored under oil, creating an anaerobic environment.

Mycobacterium avium subsp. *paratuberculosis* is the bacteriological agent responsible for Johne's disease in cattle, and can be found in milk from infected cows (Taylor *et al.*, 1981). It may also be associated with Crohn's disease in humans (Feller *et al.*, 2007). There was strong debate over whether this bacterium is capable of surviving pasteurisation (Ellingson *et al.*, 2005). However, this has largely been refuted by the work of Pearce *et al.*

(2001). However, the live bacterium has been isolated from commercial milk in the United Kingdom (Grant *et al.*, 2002) and the United States of America (Ellingson *et al.*, 2005), most likely due to inadequate pasteurisation.

Studies on the Bacterial Diversity in Processed Milk

To date, only one study has made an attempt to perform a diversity study on pasteurised milk (Quigley *et al.*, 2013a). The authors used culture-dependent sampling together with ethidium monoazide to differentiate live from dead cells. A key finding was that non-thermotolerant bacterial species were detected in a damaged and non-culturable form (Quigley *et al.*, 2013a). The authors argue that these bacteria are often present in pasteurised milk but may be overlooked due to their inability to be cultured.

1.6 Aims and Hypothesis

The link between the consumption of raw milk and lower rates of allergic disease in young children has been well documented in a number of studies. It has been hypothesized that the exposure to the microflora contained within raw milk, lost during processing, may modulate the immune system through host-microbe interaction at the mucosal interface of the gut. The aim of this thesis was to compare the microflora of raw and commercially processed New Zealand milks to determine the difference in microflora, and to determine which of those organisms present solely in raw milk would be able to survive acid exposure and would therefore be able to enter in the lower GI tract and have the potential to affect the immune system through host-microbe interactions.

The hypotheses that were tested:

(a) There is greater bacterial diversity in raw milk than in processed milk.

(b) Milk significantly improves bacterial tolerance and survival during acid challenge.

(c) Bacterial survival in milk is facilitated through direct or indirect interaction with specific milk components such as milk fat globules or casein micelles.

2. Methods

2.1 Bacterial Isolation, Growth, and Characterisation

2.1.1 Bacteria and Growth Conditions

For bacterial cloning, One Shot® TOP10 Chemically Competent *E. coli* (Life Technologies) were used. *E. coli* was grown in Tryptic Soy Broth (TSB) and on Agar (TSA) (Difco), or Luria-Bertani broth (LB) and Agar (LA) (Difco) containing 50 μg / ml kanamycin where appropriate. *E. coli* was incubated at 37 °C for 18 h, and shaken at 200 rpm where appropriate.

Bacteria in milk were enumerated by serial dilution of broth culture in Maximum Recovery Diluent (MRD, Difco) before being spread-plated onto agar plates. Cultures plated onto Plate Count Agar (PCA), MacConkey agar and TSA were grown at 30 °C for 3 days unless specified; while those plated on All Purpose Tween (APT) agar were grown at 37 °C, 5.0 % CO_2 for 3 days unless specified.

Bacteria isolated from milk either raw milk or processed milk were maintained on one of two agar types. Bacteria originally isolated on MacConkey Agar or PCA agar were maintained on Tryptic Soy Agar or Broth. Bacteria originally isolated from APT agar were maintained using APT broth or agar. Milk isolates grown on TSB or TSA were incubated at 30 °C for 3 days unless specified; while those plated on APT agar were grown at 37 °C, 5.0 % CO_2 for 3 days unless specified.

2.1.2 Sources of Milk Used in this Study

Raw milk was obtained from one of three New Zealand dairy farms. One is located in the Waikato region, and two are located in the Manawatu. The milk from these farms were used for the purposes is outlined (Table 5).

Table 5 – Raw Milk Sources used in this study.

Raw Milk Source	**Location**	**Purpose**
Waikato Farm A*	Morrinsville.	Isolation of milk flora.
		Pasteurisation tolerance assays.
		Acid tolerance assays.
Massey University Research farm No 1	Batchelar Rd, Palmerston North.	Isolation of milk flora.
		High-throughput acid tolerance.
		Identifying acid tolerant strains.
Massey University Research farm No 4	Tennent Drive, Palmerston North.	Isolation of milk flora.
		High-throughput acid tolerance.
		Identifying acid tolerant strains.

* The name and address of the farm owners have not been disclosed.

2.1.3 Storage Trial and Bacterial Isolation

Milk was transferred to sterile containers in 100 ml aliquots, and incubated at 10 °C for a period of 10 days. Aerobic plate counts were conducted at 0, 3 and 10 days and enumerated by spread-plating on All Purpose Tween (APT) agar, PCA agar and MacConkey agar using the method described in section 2.1.1.

The agar plates used in the enumeration of bacteria in milk were used to isolate a range of bacterial species present in milk. A selection of colonies was chosen at random, and individual colonies were streaked onto fresh agar plates and incubated as appropriate. This step was repeated another two times to ensure that pure cultures were attained. Isolates were stored at -80 °C in TSB containing 25 % glycerol until required.

2.1.4 Bacterial Characterisation

Bacteria isolated from milk were characterised both by colony and cell morphology. Cell morphology was described using Gram staining (Gram, 1884). Oxidase reactivity was tested using Dryslide^{tm} test kit (BD biosciences). Catalase reactivity was determined by adding a loop of culture to a single drop of hydrogen peroxide (3 %). The presence of catalase enzyme was indicated by effervescence.

2.2 Molecular Biology

2.2.1 DNA Extraction

Bacterial DNA was extracted from bacteria grown in broth using the Highpure® DNA extraction kit for genomic DNA (Roche) according to the manufacturers' instructions. To increase the concentration of bacterial DNA, the elution volume was decreased to 50 µl. The DNA primers used in PCR reactions are shown (Table 6).

Table 6 - Primer names and sequences used in this study.

Primer Name	Purpose	Sequence	References
M13 forward (-40)	Sequencing r16S gene within plasmid	5'-GTTTTCCCAGTCACGAC-3'	Chen *et al.* (1985)
M13 reverse (-26)	Sequencing r16S gene within plasmid	5'- CAGGAAACAGCTATGAC-3'	Chen *et al.* (1985)
PA	Amplification and Sequencing r16S gene	5'-AGAGTTTGATCCTGGCTCAG-3'	Hutson *et al.* (1993)
PH*	Amplification and Sequencing r16S gene	5'-AAGGAGGTGATCCAGCCGCA-3'	Hutson *et al.* (1993)
533-F	Internal Primer for Sequencing r16S gene	5'-GTGCCAGCAGCCGCGGTAA-3'	Weisburg *et al.* (1991)
981-R	Internal Primer for Sequencing r16S gene	5'-GGGTTGCGCTCGTTGCGGG-3'	Saul *et al.* (1993)

2.2.2 Polymerase Chain Reactions

All Polymerase Chain Reactions (PCR) were performed in a thermal cycler (PTC-100, MJ Research, Inc.).

***Taq* polymerase:** PCR reactions using *Taq* polymerase were performed using the Platinum® *Taq* polymerase (Life Technologies). Each 50 µl reaction contained: 1 × buffer, 200 µmol of each dNTP, 3 mM $MgCl_2$, 50 pmol forward primer, 50 pmol reverse primer, 1 unit of polymerase, template (0.5-5 µl) and ultrapure water up to 50 µl. Initial denaturation was at 93 °C for 5 min, followed by 30 cycles of denaturation (92 °C for 1 min), annealing (55 °C for 1 min) and elongation (72 °C for 2 minutes). A final elongation step was performed at 72 °C for 7 minutes. Samples were then held at 4 °C.

PFX polymerase: PCR reactions using PFX polymerase were performed using the AccuPrime™ Pfx SuperMix kit (Life Technologies). Each 50 µl reaction contained: 1 × buffer; 50 pmol forward primer; 50 pmol reverse primer; PFX polymerase (1 U); template (5 µl) and ultrapure water (up to 50 µl). The initial denaturation was at 93 °C for 5 min, followed by another 29 cycles of denaturation (95 °C for 1 min), annealing (55 °C for 1 min) and elongation (68 °C for 2 minutes). A final elongation step was performed at 68 °C for 7 minutes. Samples were then held at 4 °C.

AmpliTaq polymerase: Reactions using amplitaq polymerase were performed using the AmpliTaq Gold® DNA Polymerase kit (Life Technologies). Each 50 µl reaction contained: 1 × reaction buffer, 50 pmol forward primer; 50 pmol reverse primer; 0.5-5 µl template DNA and ultrapure water. An initial denaturation step was performed at 93 °C for 3 min, followed by 30 cycles of denaturation (92 °C for 1 min), annealing (55 °C for 1 min) and elongation (72 °C for 2 minutes) cycle. A final elongation step was performed at 72 °C for 5 minutes before being held at 4 °C. All AmpliTaq Gold® DNA Polymerase PCR reactions contained PA and PH* primer pairs (Table 6).

Completed PCR reactions were analysed by agarose gel electrophoresis. An agarose gel was formed containing 1 % agarose (Gibco BRL) in 0.5 × Tris/Borate/EDTA buffer and 25 µg / ml ethidium bromide (Biorad). The cast gel was placed in a gel tank containing 0.5 × Tris/Borate/EDTA buffer, and each well contained 5 µl of PCR product was loaded with 1 µl of loading dye (0.25 % bromophenol blue, 0.25 % xylene cyanol and 40 % w/v sucrose). The electrophoresis was performed at 100 mV for 45 minutes. Gel electrophoresis was

alternatively performed using the E-gel® electrophoresis system with pre-cast 0.8 % gel cartridges containing CYBR safe dye (Life Technologies) according to manufacturers' instructions. PCR products separated by electrophoresis were observed and photographed using a Molecular Imager® Gel Doc™ XR+ System.

2.2.3 Extraction of Total Bacterial Genomic DNA from Milk

Total bacterial DNA was extracted from milk using one of two methods.

Method 1:

100 ml of milk was combined with 100 ml of 20 % ethanol (v/v), and mixed thoroughly at RT. The mixture was centrifuged at 4600 × g for 10 minutes and the supernatant discarded. Pellets were resuspended in 1 ml MRD. The suspended pellet was transferred to a 1.5 ml tube, and the genomic DNA extracted using the Highpure® DNA extraction kit (Roche), according to the manufacturers' instructions. The elution volume was decreased to 50 µl to increase the concentration of DNA.

Method 2:

A milk sample (10 ml) was placed in a sterile 15 ml centrifuge tube and incubated in a water-bath at 40 °C for ten minutes. Samples were then centrifuged at low speed (350 × g) for ten minutes (Hereaus Multifuge, rotor ID 6435). 2 ml of supernatant was added to 8 ml of 125 mM EDTA and incubated overnight (18 h at 37 °C). The EDTA/milk mixture was centrifuged at high speed (10,000 × g) for ten minutes and the supernatant discarded. Bacterial DNA was extracted using Faecal DNA Extraction kit (Zymo). DNA was eluted from the final column using 30 µl of elution buffer.

2.2.4 Culture Independent 16S rDNA Analysis Using a Plasmid Cloning Kit

Template DNA was amplified using the PFX polymerase and PA and PH* primers (Table 6). PCR products were separated by agarose gel electrophoresis and the 1500 bp band excised from the gel, purified using a gel extraction kit (Qiaquick, Qiagen) and ligated into a pCR®II-blunt-TOPO® vector (Zero-blunt® TOPO® PCR cloning kit, version N) according to manufacturers' instructions. The ligated DNA was transformed into chemically competent

E. coli (Top10, Invitrogen) as per manufacturer's instructions. Transformed cells were spread onto LB agar containing 50 µg / ml kanamycin. Single colonies were isolated and purified by streaking onto LB agar containing kanamycin. Plasmid DNA was isolated using a plasmid extraction kit (ISOLATE plasmid mini kit, Bio-line) according to the manufacturer's instructions. Purified plasmid DNA was sequenced using M13F and M13R primers by the Waikato Sequencing Unit (Table 6). Usable sequence reads of between 400 and 800 nucleotides were obtained. DNA chromatographs were reviewed using 4Peaks software (Softonics®), which included the trimming of junk sequence data from the beginning and end of the sequence reads, and validating the sequence data. DNA sequences were compared to the nucleotide nr database using BLAST[1] (Zhang *et al.*, 2000). DNA sequences were also analysed using the Seqmatch tool of the Ribosomal Database Project (RDP)[2] (Wang *et al.*, 2007).

1 http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome

2 http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp

2.2.5 Culture Independent 16S rDNA Analysis by Sequencing of PCR products

Bacterial isolates were inoculated into TSB broth or APT broth from pure culture and incubated as described in section 2.1.1. DNA extraction was performed as described in section 2.1.2. The bacterial 16S rDNA gene was amplified using the Amplitaq Polymerase Chain Reaction kit described in section 2.2.2. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using PA and PH* primers by the Waikato Sequencing Unit (Table 6). DNA chromatographs were reviewed using 4Peaks software (Softonics®), which included the trimming of junk sequence data from the beginning and end of the sequence reads, and validating the sequence data. DNA sequences were analysed using two methods. The first was to compare the nucleotide nr database using BLAST[1] (Zhang *et al.*, 2000). DNA sequences were also analysed using the Seqmatch tool of the Ribosomal Database Project (RDP)[2] (Wang *et al.*, 2007).

[1] http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome

[2] http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp

2.3 Thermotolerance Assays

2.3.1 Heat Transfer Modelling

Heat transfer modelling was performed to estimate the heat transfer times in bench top pasteurisation experiments. This was performed using Food Product Modeller (version 3.00, MIRINZ) with default settings unless specified. The product shape was specified as a finite cylinder and the product composition was entered as water, at an initial temperature of 4 °C. All surfaces were treated as being identical, and the external temperature was set at 63 °C. The heat transfer was calculated, and the temperature of the surface, subsurface, centre and mean were modelled at intervals of 1 minute.

2.3.2 Validation and Calibration of Probes

A temperature probe was constructed by affixing three copper-constantine thermocouples to a thin plastic support using heat-wrap, and placed inside a 20 ml Hungate tube containing milk (Figure 2). The temperatures were validated by immersion in boiling water (100 °C) or ice water (0 °C) and comparing these to a reference thermometer. Thermocouple readings during experiments were recorded using a Squirrel 1200 series data logger.

2.3.3 Alkaline Phosphatase Assay

The alkaline phosphatase assay was used as described in the IDF standards methodology (IDF, 1987). Milk samples were tested in triplicate. 5 ml p-nitro phenyl phosphate was combined with 1 ml milk and incubated at 37 °C for 30 minutes. The samples were photographed at the completion of the assay.

2.3.4 Thermotolerance Assays

Purified bacterial isolates were inoculated into individual tubes containing reconstituted skim milk powder (R-SMP, 10 % w/v) to a final concentration of 10^7 CFU / ml (± 1 log). A volume of 10 ml inoculated R-SMP was dispensed into 20 ml Hungate tubes. One control tube contained the thermocouple probe (Figure 2). A rack containing all Hungate tubes was placed inside a water-bath with the level of water exceeding the level of R-SMP by at least 1 cm. Samples were held at 62.7 °C, for 30 minutes (International Dairy Federation, 1994). After 30 minutes incubation milk samples were cooled in an ice water-bath. Samples were removed aseptically using a Pasteur pipette and transferred to sterile 1.2 ml microtitre tubes, being careful to not contaminate the sample with a part of the tube not submerged in the water bath. The bacterial concentration was measured by plate counts.

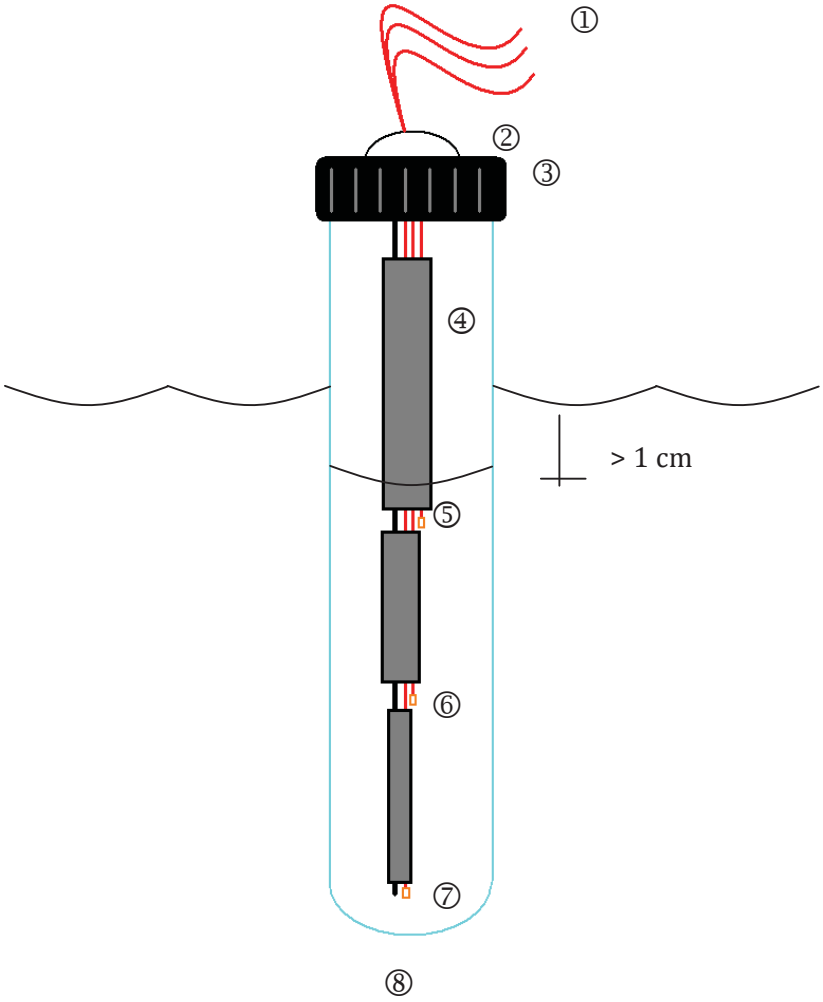


Figure 2 - Experimental design of Hungate tube fitted with thermocouple temperature sensors. Cables leading to data-logger (①), silicon sealant (②), Hungate lid with hole (③) shrink-wrap over plastic spine (④) top sensor (⑤) middle sensor (⑥) Bottom sensor (⑦); Hungate tube (⑧). The level of the water-bath exceeded the level of the milk by at least 1 cm.

2.4 Acid Tolerance Assays

2.4.1 Quantification of Acid Tolerance in TSB Broth

Acid tolerance of bacterial isolates in pure culture was performed in TSB broth at pH 2.5. Sterile acidified TSB broth was prepared by adding 950 µl concentrated hydrochloric acid to 100 ml TSB broth. Samples of TSB broth were taken aseptically and the pH measured. Adjustments were made by adding minute volumes of HCl to attain pH 2.5. Acidified TSB broth (pH 2.5) was stored at 4 °C for up to 1 week.

Bacterial cultures were grown in TSB or APT broths as previously described. 10 µl of broth was added to 990 µl acidified TSB broth at pH 2.5 to attain a bacterial concentration of 1×10^7 CFU / ml (± 1 log). A second volume of 10 µl was diluted in 990 µl TSB (pH 7.2) and the bacterial concentration determined by plate counts on TSA or APT as appropriate. Both acidified and standard broths were incubated at 37 °C for 2 h and the bacterial concentration determined by plate counts on APT or TSA. Plate counts were performed in triplicate unless indicated.

2.4.2 Quantifying Acid Tolerance in TSB and Milk Component Mixtures

Acid tolerance assays containing milk (or milk components) were performed by incubating bacterial cultures in a mixture containing one part acidified broth with one part milk. To achieve a final pH of 2.5 when these parts are combined, the volume of acid required was determined combining 10 ml milk with 10 ml acidified broth at pH 2.5. The pH was monitored and the volume of acid required to reduce the pH of the mixture back to 2.5 was determined for each batch of milk or milk component used in acid tolerance assays. This volume was used to produce a second acidified TSB broth (pH << 2.5). The pH of this broth was undefined, but much less than pH 2.5. When combined with an equal volume of milk, the acidity was confirmed at pH 2.5 prior to use. Acidified TSB broth (pH << 2.5) was stored at 4 °C for up to 1 week.

Bacterial cultures were grown in TSB or APT broths as previously stated. 10 µl of broth was added to 490 µl milk, or milk component solution. The inoculated milk was then

combined with 500 µl acidified broth (pH << 2.5) to attain a bacterial concentration of 1 × 10^7 CFU / ml (± 1 log) at pH 2.5. 10 µl of bacterial culture was also diluted in a volume of 490 µl milk and mixed with 500 µl TSB (pH 7.2) and the bacterial concentration was determined by plate counts on TSA or APT as appropriate. Both acidified and control broths were incubated at 37 °C for 2 h and the final bacterial concentration determined by plate counts.

2.4.3 Milk and Milk Components Used in Acid Tolerance Assays

Commercial Cows' Milk and Soy Milk

Two types of commercial cows' milk were used during acid tolerance assays. These were UHT treated 'Blue', and UHT treated 'Trim' milk (Anchor). The commercial soy milk used during acid tolerance assays was Sanitarium's 'So Good – Lite'. All commercial milks were sourced from a local supermarket. After opening, milk was stored chilled at 4 °C and used within 7 days.

Sodium Caseinate

Sodium caseinate (SC) was sourced from the Institute of Food, Nutrition and Human Health, Massey University. Dehydrated SC was stirred until dissolved in sterile deionised water at a concentration of 3.7 g / 100 ml. To completely dissolve the sodium caseinate, the mixture was heated to 60 °C for 1 h. The final solution was stored at 4 °C.

Whey Protein Concentrate

Whey Protein Concentrate (WPC) was sourced from the Institute of Food, Nutrition and Human Health, Massey University. Dehydrated WPC was added to sterile deionised water at a concentration of 3.7 g / 100 ml and stirred until dissolved. The final solution was stored at 4 °C.

Hydrolysed Casein

A solution of hydrolysed protein derived from casein was prepared using hydrolysed casein (BD Bacto™). Hydrolysed casein was dissolved in sterile deionised water at a final concentration of 3.7 g / 100 ml. The final solution was stored at 4 °C.

Casein Micelles

Casein micelles (CM) were prepared using a method adapted from two published methods (Knoop *et al.*, 1979; Semo *et al.*, 2007). Casein micelles were prepared using 200 ml 5 % (w/v) reconstituted SC powder in sterile deionised water (Semo *et al.*, 2007). The

reconstituted SC was stirred (400 rpm) in a water-bath at 37 °C and pH was adjusted to 6.7 - 7. Initial volumes of 4 ml 1 M tri-potassium citrate; 24 ml 0.2 M K_2HPO_4 and 20 ml 0.2 M $CaCl_2$ were added at the beginning of the experiment. At eight consecutive 15 min intervals, 2.5 ml 0.2 M K_2HPO_4 and 5 ml 0.2 M $CaCl_2$ were added. Sterile de-ionised water was added to a final volume of 400 ml and the mixture stirred for a further 2 h, before being stored at 4 °C.

Salts

A colloidal salt solution was prepared identical to the salts used in the preparation of casein micelles. 4 ml tri-potassium citrate (1M), with 44 ml K_2HPO_4 (0.2 M) and 60 ml $CaCl_2$ (0.2 M), was added to 200 ml deionised water. The colloidal salt mixture was autoclaved and stored at 4 °C prior to use.

2.4.4 Bacterial Isolation from Acidified Raw Milk

Raw milk was collected from bulk tank milk as previously described. Milk was acidified by stepwise addition of concentrated HCl to reach a pH of 2.5 ± 0.05. Acidified milk was incubated in a water-bath at 37 °C for a period of 2 h. Volumes of 50 ml were aseptically removed from the acidified milk at intervals of 15, 30, 60, and 120 minutes and were neutralised by the addition of 900 µl NaOH (5 M). Neutralised acidified milk was stored at -20 °CC until required for DNA extraction. Bacterial concentration was enumerated using plate counts on PCA agar, MacConkey agar, and APT agar as described in section 2.1.1. Bacterial isolates were randomly selected from the agar plates used for enumeration, and were plated onto new agar plates. Individual colonies were streaked onto new agar plates three times to ensure they were in pure culture.

2.4.5 High-Throughput Acid Tolerance Assays

Bacterial cultures were grown in micro-titre plates in either TSB or APT in a humid environment to prevent drying. The time taken to reach OD_{600} of 0.5 was determined by daily measurements using a 96-well plate reader (Molecular Devices, VERSAmax Tunable Microplate Reader). An inoculation schedule was prepared for each of the isolates tested and these were inoculated at 72 h, 48h or 24h prior to the acid screen experiment as appropriate, so that all strains are grown to a an OD_{600} of approximately 0.5.

The survival of each bacterial isolate was tested in acid broth, and acid broth with UHT Skim milk. 10 µl of bacterial culture was added to 1.2 ml microtitre tubes and 1 ml of acidified broth (pH 2.5) was added. A 10 µl volume of bacterial broth was added into a second 1.2 ml microtitre tube. Then 500 µl of milk was added, followed by 500 µl of acidified TSB (pH << 2.5) resulting in a final pH of 2.5. Plates were incubated at 37 °C for 2 h.

Following this incubation the contents of the tubes were mixed well. 200 µl of each sample was added into 800 µl TSB containing 10 mM NaOH to neutralise the acid in the sample. A sterile swab was used to spread the cells onto labelled quarters of TSA or APT plates and incubated as previously stated. Survival was determined qualitatively and quarters with bacterial growth were scored '1' and quarters without bacterial growth, were scored '0'.

2.5 Microscopy

2.5.1 Light Microscopy

Light microscopy was performed using an Olympus BX51 fluorescence microscope. The microscope was fitted with Nikon Digital Sight DS-5Mc Camera, and images were digitally recorded with NIS-Elements F v 2.20 software.

Gram Stain

Gram stain (Gram, 1884) of microbial cultures were performed according to (Madigan *et al.*, 1997) using reagents supplied by BD Difco.

2.5.2 Electron Microscopy

Bacterial cells were harvested by centrifugation (17,000 × g, 1 minute) and suspended in a 500 µl solution of milk components to be tested, and combined with 500 µl of acidified TSB resulting in a final pH of 2.5 ± 0.1. Samples were prepared for transmission electron microscopy by the Manawatu Microscopic Imaging Centre, Massey University, Palmerston North, New Zealand.

2.6 Statistics

Significance was calculated using the students' T-test. Where direction is known, such as bacterial concentration before and after treatment, the one-tailed P-values were calculated.

Raw data from the high-throughput assay was analysed by a Generalised Linear Mixed Model (GLMM). A GLMM is a based on the binomial distribution, and was used to determine the significance of binary data. The analysis included two fixed effect variables. First 'treatment', described whether the isolate was tested in TSB or TSB with milk. Second treatment was 'milk type', which accounted for whether the isolate was originally isolated from raw, or acidified raw milk. The remaining variables, isolate-replicate (individual replicates of each isolate), growth time, growth media, farm of origin and date of collection were treated as random effect variables.

Statistical analysis was performed to determine if the proportion surviving acid treatment in a TSB/milk mixture is statistically significantly different compared with those that survived in TSB alone. To make this comparison, those bacteria isolated from raw milk directly were tested separately from those that were isolated from acidified raw milk. The survival of each replicate was scored as 0 (no survival observed) or 1 (survival observed). A chi-squared test was performed to determine if the data are independent. To enable the statistical comparison between the sample sets, a transformation was required so that the sample distribution was symmetrical. An inverse Logit transformation was applied and the mean proportions of acid tolerance were predicted.

3. Microbial Characterisation of Raw and Processed Milk

The composition of raw milk microflora is affected by pasteurisation, as steps are taken to intentionally inactivate them to improve both the food safety and shelf life of dairy products. The microflora that are inactivated during pasteurisation may account for the differences observed in the health status of young, regular consumers of raw milk (Waser *et al.*, 2007). The aim of this chapter was to identify bacterial species present in raw and pasteurised milk. The thermotolerance of the bacterial strains uniquely found in raw milk was tested. This approach highlighted bacterial species that would be lost when milk was processed, and would therefore only found in unpasteurised milk.

3.1 Isolation and Characterisation of Milk Bacteria

Raw and pasteurised (homogenised and standardised whole) milk was obtained from the bulk tank milk from a local dairy farm and purchased from a local supermarket respectively (Anchor® 3.3 % w/v) fat, homogenised & pasteurised milk). Bacterial concentrations were determined, and bacterial isolates were retained for further characterisation.

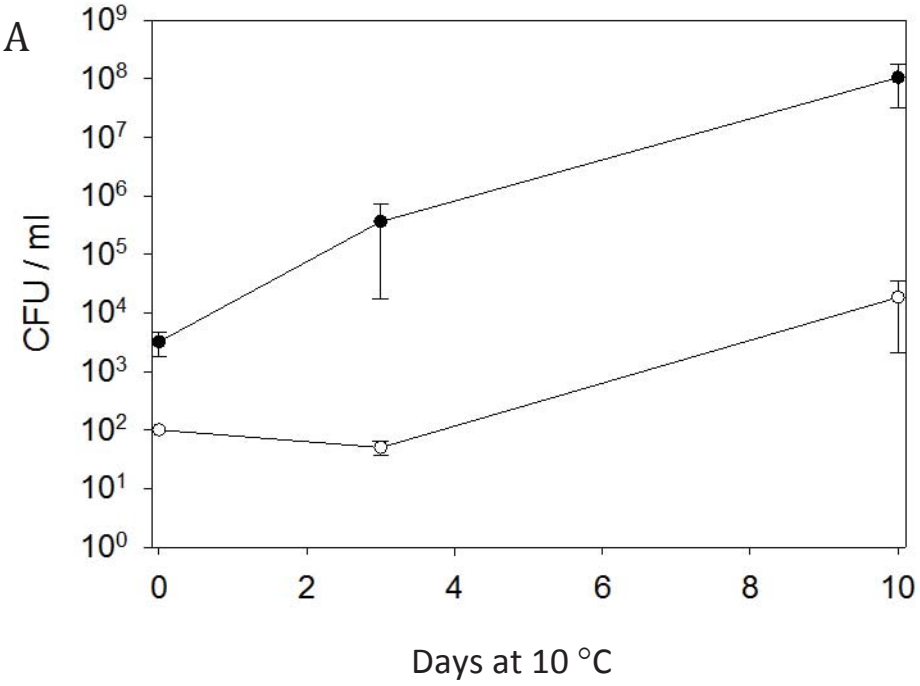
3.1.1 Quantification of Microbial Flora of Raw and Processed Milk

Bacterial concentrations of raw and pasteurised milk were compared during a ten-day storage trial at 10 °C to isolate bacterial species that might be found in milk during storage and thus consumed in the home. Aerobic bacteria present in plate counts of milk were enumerated by culturing serial dilutions of milk onto three different agar types: Plate Count Agar (PCA) as a general purpose agar, All Purpose Tween (APT) agar to select for thiamine requiring lactic acid bacteria, and MacConkey agar to select for bile tolerant microorganisms. Plate counts were performed at day 0, day 3 and day 10. The aerobic plate count from raw milk on PCA agar at day 0 was $3.2 \pm 1.4 \times 10^3$ CFU / ml (Figure 3A). The bacterial concentration of raw milk was significantly different to the bacterial concentration of pasteurised milk, which was $1.0 \pm 0.0 \times 10^2$ CFU / ml ($p = 0.045$). During the storage trial, the bacterial concentration present in raw milk increased to $1.1 \pm 0.73 \times 10^8$ CFU / ml, and the concentration of processed milk increased to $1.8 \pm 1.6 \times 10^4$ CFU / ml. The bacterial concentration of raw milk was higher than the concentration of pasteurised milk at both day 3 and day 10. These differences had p values of 0.142 and 0.083 respectively.

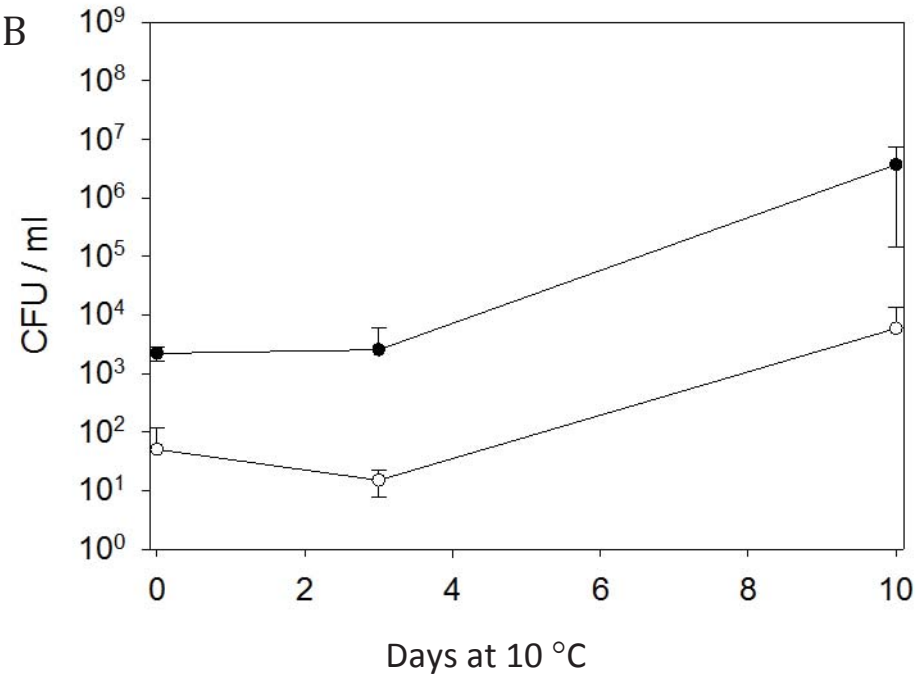
The aerobic plate counts on APT agar at day 0 were significantly different ($p = 0.027$) for raw and pasteurised milk with $2.2 \pm 0.57 \times 10^3$ CFU / ml × and $1.0 \pm 0.0 \times 10^2$ CFU / ml respectively (Figure 3B). During the storage trial, the bacterial concentration of raw milk increased to a concentration of 3.7×10^6 CFU / ml $\pm 3.6 \times 10^6$, compared with pasteurised milk which was $5.9 \pm 7.3 \times 10^3$. Although the bacterial concentration of raw milk was higher than pasteurised milk at day three and day ten, this difference was not statistically significant.

MacConkey agar supports the growth of enteric bacteria, which often appear in milk from faecal contamination. Counts for raw milk on day 0 were $9.5 \pm 0.71 \times 10^1$ CFU / ml. No bacteria were detected in the pasteurised milk (Figure 3C). These were significantly different ($p = 0.0028$). During the storage trial, the bacterial concentration of raw milk enumerated on MacConkey agar increased to a final concentration of $5.4 \pm 0.70 \times 10^7$ CFU / ml, compared with pasteurised milk at $9.0 \pm 0.57 \times 10^2$ CFU / ml. The bacterial concentration of raw milk was significantly higher at day three and day ten, ($p = 0.044$ and 0.0042 respectively).

A


CFU / ml
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
2
4
6
8
10
Days at 10 °C

B


CFU / ml
10^9
10^8
10^7
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10^2
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10^0
0
2
4
6
8
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Days at 10 °C

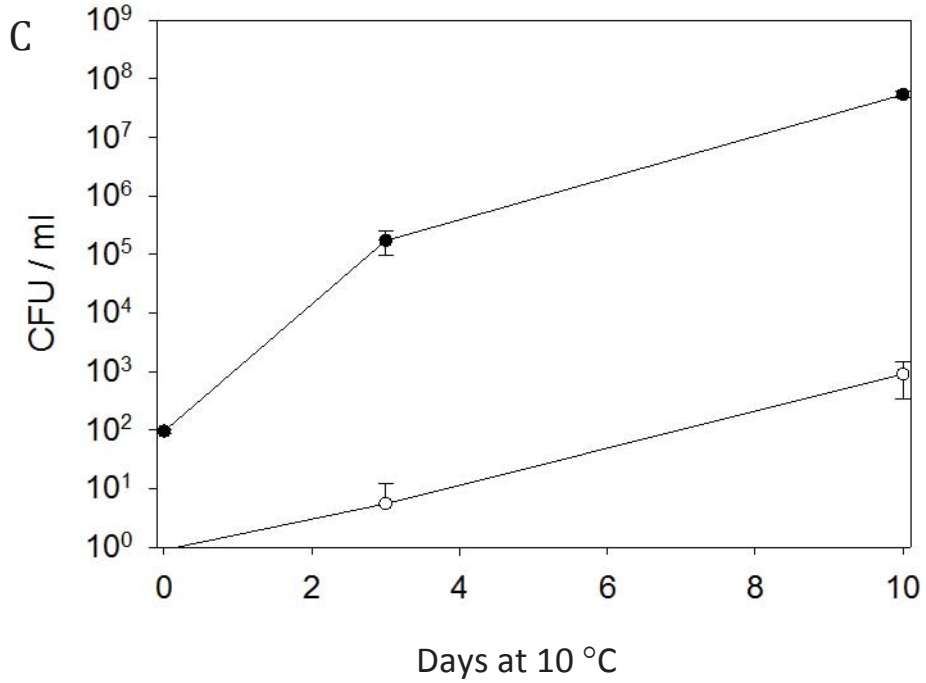

Figure 3 - Aerobic plate counts of raw milk (●) and processed milk (○) during incubation for ten days at 10 °C enumerated on PCA agar (A), APT agar (B) and MacConkey agar (C). The error bars denote one standard deviation from the mean of duplicate plates. The limit of detection was 10 CFU / ml.

3.1.2 Characterisation of Bacterial Isolates

A total of 98 isolates were selected at random from agar plates used to enumerate bacteria from both raw and pasteurised milk and re-streaked onto fresh agar. A total of 79 and 19 colonies were selected from raw and pasteurised milk respectively and formed the set of bacteria used in subsequent work. All selected bacterial isolates were characterised by colony morphology, cell morphology, and the presence of catalase and oxidase enzyme activity.

Colony Morphology and Ecological Diversity Indices

Several colony phenotypes were identified within the selected isolates selected from raw (Table 7) and pasteurised milk (Table 8). A total of 28 different phenotypes were obtained from 79 different raw milk isolates, while 13 different phenotypes were obtained from the 19 pasteurised milk isolates. The number of unique colonies and their relative frequencies were entered into an online calculator[1] to calculate ecological diversity indices (Table 9).

The Simpson Index was developed by Simpson in 1949. It gives the probability that two members of a population, chosen at random are the same species (Equation 1), where D is the Simpson Index, n_i is the number of individuals of a particular species, and N is the total number of any species in the population. The Simpson index ranges from 0 (infinite diversity) to 1.00 (only one species present) (Simpson, 1949).

$$D_{Simpson} = \frac{\sum_i n_i(n_i - 1)}{N(N-1)} \qquad \text{(Equation 1)}$$

The Simpson Index gives a counter-intuitive indication of diversity, as an increased diversity results in a lower index. For this reason, the Simpson Index of Diversity may be

[1] http://www.alyoung.com/labs/biodiversity_calculator.html

used. This index is the Simpson Index result subtracted from 1 (Equation 2). This describes the probability that two members randomly selected from a given population are different species.

$$D_{Simpsonindexofdiversity} = 1 - \left(\frac{\sum_i n_i (n_i - 1)}{N(N-1)} \right) \qquad \text{(Equation 2)}$$

The Shannon index (Equation 3) was also used to describe the diversity of the two bacterial populations isolated (Hill, 1973). The value of the index increases where the numbers of species increase or the evenness of those species increases (De Jong, 1975). Using a natural logarithm (ln), this index showed that raw milk was more diverse than processed milk with indices of 2.93 and 2.48 respectively.

$$D_{Shannon} = -\sum_i \left(\frac{n_i}{N} . \ln \left(\frac{n_i}{N} \right) \right) \qquad \text{(Equation 3)}$$

A third index, the Margalef Richness index was also used to describe the ecological diversity of the raw and processed milk samples (Margalef, 1958). This index, which represents the richness of species in a population showed that the richness of the isolates obtained from raw milk, with an index of 4.23 was greater than those isolated from processed milk, with an index of 3.58.

$$R = \frac{S-1}{\ln N} \qquad \text{(Equation 4)}$$

Table 7 – The relative frequencies of colony morphologies of bacterial isolates from raw milk (n = 79).

Media	Size*	Shape	White	Cream	Yellow	Brown	Orange	Pink
PCA	Large	Round	1	5		1		
		Irregular	1					
		Mucoid	1		1			
	Medium	Round	6	4	1	1		
	Small	Round	11	1	1		2	
		Mucoid			1			
APT	Large	Round		2				
		Mucoid		1				
	Medium	Round	1	1				
		Mucoid		1				
	Small	Round	6	8	1			
		Mucoid				1		
MacConkey	Large	Round						5
	Medium	Round						8
		Mucoid						1
	Small	Round	5					

* Size criteria - small (<1 mm); medium (1-2 mm); large (>2 mm).

Table 8 - The relative frequencies of colony morphologies of bacterial isolates from processed milk (n = 19).

Media	Size*	Shape	White	Cream	Yellow	Brown	Orange	Pink
PCA	Large	Mucoid						
		Irregular	2					
		Filamentous	1					
	Medium	Round	2	2				
		Irregular	1					
	Small	Round		1				
APT	Large	Round	1	1		2		
		Mucoid				1		
	Medium	Round		1		1		
		Irregular						
MacConkey	Medium	Irregular						3

*Size criteria – small (<1 mm); medium (1-2 mm); large (>2 mm).

Table 9 – Ecological diversity indices of bacteria isolated raw milk and processed milk.

	Raw Milk (n = 79)	Processed Milk (n = 19)
Unique isolates	28	13
Simpson Index of Diversity	0.884	0.918
Shannon Diversity Index	2.93	2.48
Margalef Richness Index	4.23	3.58

Cellular Morphology

The cellular morphology of bacterial isolates was determined by Gram stain and cell shape. Of the 79 raw milk isolates tested, both Gram-positive and Gram-negative cocci and rods were present (Table 10). The Gram-negative rods were the dominant group with 41 strains identified followed by the Gram-positive cocci with 26. Gram-positive organisms were dominant in processed milk, with 6 cocci and 8 bacilli isolated, and only 5 Gram-negative rods were identified. No Gram-negative cocci were isolated from processed milk.

Table 10 - Cell morphologies of bacteria isolated from raw and processed milk.

Raw milk (n = 79)		
	Gram-positive	Gram-negative
Cocci	26 (32.9 %)	5 (6.3 %)
Rods	7 (8.9 %)	41 (59.9 %)
Processed milk (n = 19)		
	Gram-positive	Gram-negative
Cocci	6 (31.6)	0 (0.0 %)
Rods	8 (42.1 %)	5 (26.3 %)

Catalase & Oxidase

Catalase and oxidase tests were used to further differentiate the microorganisms that were obtained from raw and pasteurised milk. Most microorganisms isolated from raw milk (n = 57) were catalase-positive (Table 11). Both oxidase-positive and oxidase-negative representatives were obtained. A total of 22 isolates were found to be catalase-negative, including 20 that were also oxidase negative. Of the isolates obtained from pasteurised milk, 16 isolates were identified as catalase positive, and included a mixture of oxidase positive and oxidase negative phenotypes. Three isolates were identified as both catalase and oxidase negative.

Table 11 – Catalase and oxidase reactivity of bacterial isolates from raw and processed milk.

Raw milk (n = 79)		
	Catalase positive	Catalase Negative
Oxidase positive	19 (24.1 %)	2 (2.5 %)
Oxidase Negative	38 (48.1 %)	20 (25.3 %)
Processed milk (n = 19)		
	Catalase positive	Catalase Negative
Oxidase positive	4 (21.0 %)	0 (0.0 %)
Oxidase Negative	12 (63.2 %)	3 (15.8 %)

Microbial Groupings

Using cell morphology and enzyme reactivity of the bacterial isolates, six distinct microbial groupings were identified (Table 12). Raw milk was found to contain representatives of all six groupings, while pasteurised milk contained only three. A total of 34.2 % of isolates in raw milk were classed as group B, which notably contained the Enterobacteriaceae. A further 21.5 % of isolates were classed as group D, and 19 % group C. Of the processed milk isolates, group F dominated, comprising 42.1 % of bacterial isolates. Group E made up 31.6 % of bacterial isolates. There were no representatives of groups A, C or D found in processed milk.

Table 12 – Microbial groups isolated from raw milk and processed milk based on cell morphology and catalase/oxidase reactivity.

Raw Milk			
Group	Phenotype	n	%
A	Gram-negative cocci	5	6.3
B	Gram-negative rod, oxidase negative	27	34.2
C	Gram-negative rod, oxidase positive	15	19.0
D	Gram-positive cocci, catalase negative	17	21.5
E	Gram-positive cocci, catalase positive	9	11.4
F	Gram-positive rods	6	7.6
		79	100
Processed Milk			
Group	Phenotype	n	%
A	Gram-negative cocci	0	0
B	Gram-negative rod, oxidase negative, catalase varied	5	26.3
C	Gram-negative rod, oxidase positive	0	0
D	Gram-positive cocci, catalase negative	0	0
E	Gram-positive cocci, catalase positive	6	31.6
F	Gram-positive rods	8	42.1
		19	100

3.2. Thermotolerance of Raw and Pasteurised Milk Isolates

Microflora of raw milk differs both in composition and concentration to that of pasteurised milk. Most bacteria found in raw milk are killed when milk is pasteurised. However, pasteurisation does not eliminate all bacteria, as thermotolerant organisms are able to survive. A laboratory pasteurisation test was used to determine whether the bacterial isolates from raw and processed milk were able to survive pasteurisation.

3.2.1 Thermotolerance of Raw Milk Isolates

The thermotolerance of raw and pasteurised milk isolates was determined using a bench top pasteurisation assay. This was achieved by inoculating pure cultures into Reconstituted Skim Milk Powder (R-SMP) and subjecting them to a pasteurisation trial. Bacterial survival was determined by plate counts, before and after exposure to pasteurisation at 62.8 °C for 30 minutes. The temperature was measured and recorded using the thermocouple assembly (Figure 2).

Reproducibility of the Temperature Profile

The temperature profiles for three independent pasteurisation experiments were recorded using a thermocouple probe placed in a Hungate tube containing 10 ml UHT milk. The actual temperatures of milk correlated well with the temperatures predicted by heat transfer modelling of water (Figure 4). The time taken to attain a pasteurisation temperature of 62.8 °C was 5 minutes, compared with 7 minutes calculated by heat transfer modelling (Appendix 2). The temperature probes were validated using ice-water and boiling water and compared to a reference thermometer (appendix 3). The pasteurisation of milk was shown to be effective as determined by the alkaline phosphatase test on a batch of raw milk (Appendix 4).

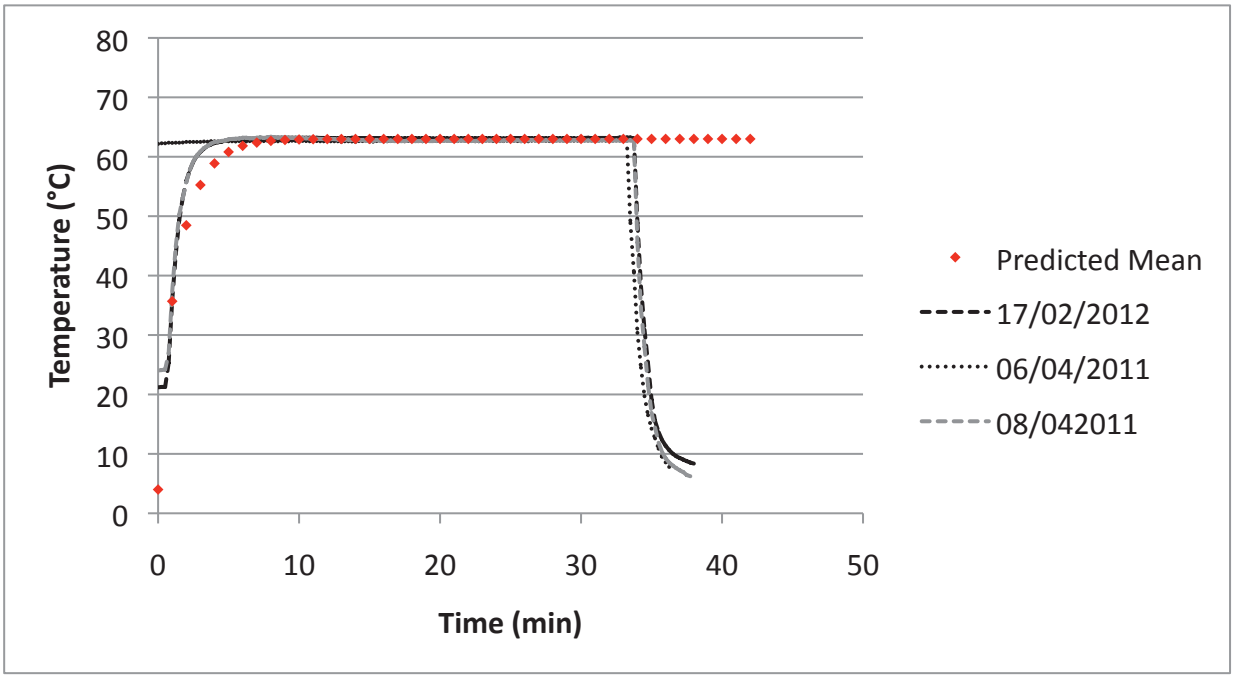

Figure 4 - Temperature profiles of pasteurisation experiments comparing the mean temperature predicted by heat transfer modelling with the average temperature of the top, middle and bottom thermocouple probes of three independent pasteurisation experiments. The average temperatures of three pasteurisation experiments were compared to the temperatures predicted by heat-transfer modelling (Predicted Mean).

Quantification of Thermotolerance

The thermotolerance raw milk and processed milk bacterial isolates was defined as an inability to survive exposure to 63 °C for a period of 30 minutes. If there was no evidence of bacterial growth on agar plates following pasteurisation, strains were defined as thermosensitive. If there were one or more colonies present on agar plates, that isolate was defined as thermotolerant. In total 79 raw and 19 pasteurised milk isolates were assessed (Table 13). Of the 79 raw milk isolates tested, two isolates were thermotolerant. Of the 19 pasteurised milk isolates tested, five were found to be thermotolerant. In total, 77 isolates from raw milk were thermosensitive; seven isolates from either raw milk or pasteurised milk were thermotolerant; and 14 isolates from pasteurised milk were thermo-sensitive. In total, seven thermotolerant isolates were identified that may be found in both raw and pasteurised milk, based on the thermotolerance alone. Isolates showing thermotolerance came from a range of bacterial groups. One thermotolerant raw milk isolate was identified from group B and one from Group D. Three thermotolerant processed milk isolates came from group E, as well as one from group B and one from group F.

Table 13 – Thermotolerance of raw and processed milk bacterial isolates.

	Thermosensitive	Thermotolerant
Raw milk (n = 79)	77 (78.6 %)	2† (2.0 %)
Processed milk (n = 19)	14 (14.3 %)	5‡ (5.1 %)

† Thermotolerant isolates from raw milk are from groups B and D.
‡ Thermotolerant isolates from processed milk are from groups B, E and F.

3.3 Identification of Raw and Processed Milk Isolates

In this study, DNA was extracted from both the bacterial isolates and, or from milk directly. From this DNA, the 16S rRNA gene was amplified using *Taq* polymerase, sequenced and compared those of known species using BLAST nucleotide collection nr/nt database and the Ribosomal Database Project using Seqmatch. This allowed a culture dependent, or culture independent analysis of the bacterial species present in raw and processed milk.

3.3.1 Partial r16S Ribosomal DNA Sequencing of Milk Isolates

Eighteen bacterial isolates were selected from raw and processed milk for identification using partial sequencing of the 16S rRNA gene. At least one isolate was included from each grouping. Sequence data was obtained with read lengths of between 474 and 808 bases, with similarity scores of between 99 % and 100 % to the closest known relatives in the BLAST nucleotide collection nr/nt database (Tables 14 & 15). DNA sequence data was also analysed with Seqmatch, and similarity scores of between 0.930 and 1.000 were attained. Gram-negative genera identified include *Rahnella, Pseudomonas* and *Enterobacter*. Gram-positive isolates identified included *Enterococcus*, *Streptococcus*, and *Staphylococcus*. There was good agreement between the closest known relative as given by sequence analysis and the characterisation based on cell morphology and enzyme reactivity. However, there were four discrepancies. An isolate resembling a Gram-negative coccus was most closely related to an *Acinetobacter* by DNA sequencing. An isolate from group B corresponded to a *Pseudomonas*, despite testing oxidase negative. Conversely, an isolate from group C with a positive oxidase test was found to relate most closely to an *Enterobacter* sp. Finally an isolate was found to relate to the genus *Oerskovia* (which is typically coccoid), was categorised as group D as it exhibited rod-shaped cell morphology.

Four isolates obtained from pasteurised milk were sequenced. The closest known relatives of these isolates were *Acinetobacter* sp., *Kocuria* sp., and *Bacillus* sp. from groups B, E and F respectively (Tables 14 & 15).

Bacterial 16S sequences were compared using BLAST and the Seqmatch search tools. These tools employed the use of the n/r nucleotide database (NCBI) and the Ribosome Genome Project Databases. There was generally good agreement between the closest known relatives in both databases. However, where BLAST searches would return several members of a genus as having identical similarity, the RGP database would generally have

one closest match. The Seqmatch scores, used to judge similarity, were lower than the BLAST scores. This may be due to a different method used to calculate similarity, but also due to the more limited database, which only included 'type' bacterial species.

Table 14 - Closest known relatives of strains isolated from raw and pasteurised milk identified by partial 16S rRNA gene sequencing using PA forward primer.

Raw Milk				
Grouping (*ex* Table 12)	Closest known relative	Query Length[a]	Percent Identity[b]	Seqmatch Score[c]
A	*Acinetobacter guilloieae*	798	99	0.954
B	*Rahnella aquatilis*	605	99	0.968
B	*Rhodococcus corynebacterioides*	717	100	0.991
B	*Rhodococcus corynebacterioides*	604	100	0.991
B	*Enterobacter amnigenus*	730	99	0.970
B	*Pseudomonas psychrophila*	690	99	0.989
C	*Escherichia/Shigella flexneri*	802	99	0.961
C	*Enterobacter amnigenus*	802	100	0.991
D	*Enterococcus faecalis*	803	100	0.992
D	*Streptococcus uberis*	603	99	0.978
E	*S. aureus* subsp*. aureus*	792	100	1.000
E	*S. aureus* subsp*. aureus*	791	100	1.000
F	*Oerskovia enterophila*	831	99	0.984
Processed Milk				
Grouping (*ex* Table 12)	Closest known relative	Query Length[a]	Percent Identity[b]	Seqmatch Score[c]
B	Acinetobacter	778	99	0.982
E	*Kocuria varians*	827	99	0.967
F	*Bacillus subtilis*	790	99	0.992
F	*Bacillus licheniformis*	634	99	0.977

[a] Query length represented as number of nucleotides. [b] Maximum identity as a result of BLAST nucleotide search. [c] Seqmatch Score as a result of Seqmatch search, Ribosomal Database Project. Sequences obtained from purified PCR fragments using PA forward primer.

Table 15 - Closest known relatives of strains isolated from raw and pasteurised milk identified by partial 16S rRNA gene sequencing using PH* reverse primer.

Raw Milk				
Grouping (*ex* Table 12)	Closest known relative	Query Length[a]	Percent Identity[b]	Seqmatch Score[c]
A	*Acinetobacter guilloieae*	787	99	0.992
B	*Rahnella aquatilis*	820	99	0.982
B	*Rhodococcus corynebacterioides*	816	99	0.966
B	*Rhodococcus corynebacterioides*	576	99	0.948
B	*Enterobacter amnigenus*	405	99	0.952
B	*Pseudomonas psychrophila*	695	99	0.957
C	*Escherichia/Shigella flexneri*	720	99	0.975
C	*Enterobacter amnigenus*	749	99	0.974
D	*Enterococcus faecalis*	778	100	0.960
D	*Streptococcus uberis*	684	99	0.975
E	*S. aureus* subsp*. aureus*	615	100	0.983
E	*S. aureus* subsp*. aureus*	691	100	0.985
F	*Oerskovia enterophila*	781	99	0.989
Processed Milk				
Grouping (*ex* Table 12)	Closest known relative	Query Length[a]	Percent Identity[b]	Seqmatch Score[c]
B	Acinetobacter	533	99	0.977
E	*Kocuria varians*	760	99	0.985
F	*Bacillus subtilis*	490	99	0.925
F	*Bacillus licheniformis*	801	100	1.000

[a] Query length represented as number of nucleotides. [b] Maximum identity as a result of BLAST nucleotide search. [c] Seqmatch Score as a result of Seqmatch search, Ribosomal Database Project. Sequences obtained from purified PCR fragments using PH* reverse primer.

3.3.2 Culture-Independent Analysis of Milk

In addition to the identification of isolates that were cultured from milk, bacterial content of milk was also analysed using a culture independent method. Raw and pasteurised milk samples were obtained from a local dairy farm and the supermarket respectively, and bacterial genomic DNA extracted. The ribosomal 16S DNA sequence was amplified using PCR and cloned into a vector for DNA sequencing.

Pasteurised milk was split into two batches. From the first, DNA was extracted directly. The second batch was abused at room temperature for 18 h, prior to DNA extraction. The 16S genes in each sample were amplified and cloned into a plasmid vector before being sequenced, and analysed by BLAST using the nr/nt database. From the untreated processed milk sample, six 16S sequences were sequenced in both the forward and reverse directions. The closest known relative to all six sequences was the thermophile *Anoxybacillus flavithermus* (F). No other organisms were detected in non-abused processed milk.

Eight 16S rRNA genes from abused pasteurised milk were sequenced in both the forward and reverse directions. The closest known relatives of seven genes were *Bacillus cereus*, *B. thuringiensis*, *B. mycoides*, or *B. weihenstephanensis* (Table 17). Many of these microorganisms showed identical sequence homologies, making it difficult to identify these organisms individually to the species level. The remaining gene sequence was most closely related to *Anoxybacillus flavithermus*, although this was not confirmed by the reverse sequence.

Table 16 - Microbial strains detected in pasteurised milk.

M13 Forward primer

	Closest Relative	Query Coverage[a]	Percent Identity[b]	Seqmatch Score[c]
A1	*Anoxybacillus flavithermus*	100	99	0.887
A2	*A. flavithermus*	100	99	0.836
A3	*A. flavithermus*	100	99	0.814
A4	*A. flavithermus*	100	99	0.874
A5	*A. flavithermus*	100	99	0.832
A6	*A. flavithermus*	100	99	0.698

M13 Reverse primer

	Closest Relative	Query Coverage[a]	Percent Identity[b]	Seqmatch Score[c]
A1	n/a	-	-	-
A2	n/a	-	-	-
A3	*A. flavithermus*	100	100	0.927
A4	*A. flavithermus*	100	99	0.869
A5	*A. flavithermus*	100	99	0.919
A6	*A. flavithermus*	100	100	0.919

[a] Query Coverage represented as percentage of the DNA sequence matched to the database. [b] Maximum identity as a result of BLAST nucleotide search. [c] Seqmatch score as a result of Seqmatch search, Ribosomal Database Project. DNA sequences were obtained by sequencing of inserted DNA in pCR®II-blunt-TOPO® plasmid using M13 forward and reverse primers.

Table 17 - Microbial strains detected in abused pasteurised milk incubated at room temperature for 24 h.

	M13 Forward primer			
	Closest Relative	Query Length[a]	Percent Identity[b]	Seqmatch Score[c]
B1	*Bacillus cereus*	679	100	0.967
B2	*Bacillus cereus*	736	100	0.973
B3	*Bacillus weihenstephanensis*	679	100	0.939
B4	*Bacillus cereus*	125	100	0.890
B5	*Bacillus weihenstephanensis*	717	100	0.960
B6	*Anoxybacillus flavithermus*	724	100	0.894
B7	*Bacillus cereus*	658	100	0.952
B8	*Bacillus weihenstephanensis*	664	100	0.967

	M13 Reverse primer			
	Closest Relative	Query Length[a]	Percent Identity[b]	Seqmatch Score[c]
B1	*Bacillus weihenstephanensis*	745	100	0.972
B2	*Bacillus cereus*	705	100	.0971
B3	*Bacillus weihenstephanensis*	745	100	0.960
B4	*Bacillus cereus*	751	100	0.973
B5	*Bacillus weihenstephanensis*	734	100	0.985
B6	*Bacillus mycoides*	759	100	0.831
B7	*Bacillus cereus*	703	100	0.971
B8	*Bacillus weihenstephanensis*	683	100	0.973

[a] Query length represented as number of nucleotides. [b] Maximum identity as a result of BLAST nucleotide search. [c] Seqmatch score as a result of Seqmatch search, Ribosomal Database Project. Sequences obtained from purified PCR fragments using PH* reverse primer. DNA sequences were obtained by sequencing of inserted DNA in a pCR®II-blunt-TOPO® plasmid using M13 forward and reverse primers.

3.4 Discussion

The aim of this chapter was to isolate a diverse cross-section of bacteria from raw and pasteurised milk and identify bacteria that are only found in raw milk, and not in pasteurised milk. This was achieved through microbial characterisation of raw and pasteurised milk isolates obtained from a time-course storage trial, and then tested for their thermotolerance. Using this approach, raw milk bacteria that were lost when milk was pasteurised could be identified.

Milk is not immediately consumed when it is brought into the home, but may be consumed over a period of many days. For this reason, a storage trial was designed to isolate bacteria that were found in milk initially, as well as those that proliferate over time. A temperature of 10 °C was chosen to store milk during the trial. Although this is above the recommended temperature inside home refrigerators (see Food Hygiene Regulations 1974), in the home refrigerators temperatures can vary significantly. In a New Zealand study it was found that temperatures ranged between 0 and 11 °C (O'Brien, 1997). The selected temperature of 10 °C lies within this range. A period of ten days was chosen as this marked the end of the expiration date of the pasteurised milk. No expiration date is available for raw milk, but was tested for the same period in the interest of uniformity.

To determine if organisms were uniquely found in raw milk, a pasteurisation trial was developed to test thermotolerance. To accurately test the thermotolerance of bacterial isolates, the temperature of the pilot tube was compared to the increase that was predicted using heat transfer modelling (Figure 4). The performance of the thermocouple probe was compared to a reference thermometer in ice water (0 °C) and boiling water (100 °C). There was good agreement between the reference thermometer and the probe reading. The entire volume of the Hungate tube was shown to reach pasteurisation temperatures within 5 minutes. The de-activation of alkaline phosphatase was used to validate the pasteurisation test used in this study. A sample of raw milk was pasteurised and compared with the alkaline phosphatase activity of raw milk, and UHT pasteurised milk. The alkaline phosphatase activity of the pasteurised raw milk matched the UHT milk control, and showed that the pasteurisation was successful.

Isolating and characterising bacterial organisms using a culture-dependent approach has limitations in that only the bacterial species that grow at the specified conditions will be found. Three different bacterial media were used under aerobic conditions to enumerate

and isolate generic, hetero-fermentative and bile tolerant organisms. Using this approach, some important organisms likely to be present in raw milk would be excluded; for example those bacteria that grow only anaerobically, or on another media, or may not be culturable on any media. For this reason, culture-dependent, and culture-independent methods were used, or attempted to identify bacterial species present in raw milk. The amplification of 16S rDNA from raw milk was not successful. It is unclear why this was the case, but an over-abundance of mammalian DNA could cause the PCR amplification to fail, as this may sequester Mg^{2+} ions required by the polymerase. An excess of contaminants present in the raw milk that could inhibit the polymerase also could not be ruled out.

In this study, the bacterial concentrations in raw milk and pasteurised milk were compared during a ten-day storage trial. The concentration of bacteria in raw milk was found to be 2-3 log higher than that of pasteurised milk for the duration of the trial, as enumerated on PCA agar, APT agar and MacConkey agar. The initial counts of raw milk flora on PCA agar were 3.2×10^3 CFU / ml, which is within the threshold of A-grade milk in the United States of America (Barbano *et al.*, 2006). The threshold of A grade milk at Fonterra, New Zealand's largest milk processor, is aerobic plate counts of 2.0×10^4 CFU / ml, and penalties are given when bacterial levels are found to be above 5×10^4 CFU / ml (Suppliers Services Team, 2013).

The initial bacterial concentration of pasteurised milk, in contrast, was below 1×10^2 CFU / ml, which was consistent with the effects of pasteurisation (Gruetzmacher *et al.*, 1999). During storage, the bacterial concentration of both raw and pasteurised milk increased. The concentration of raw milk was consistently greater than the concentration of pasteurised milk by 2-3 $\log_{10}$. This means that at all times, the bacterial exposure of people that consume raw milk must be greater than that of people that consume pasteurised milk.

The thermotolerance of 79 raw and 19 pasteurised milk isolates were assessed using laboratory pasteurisation, exposing pure cultures of bacteria inoculated into milk to 62.8 °C for 30 minutes. It is not possible to determine if thermotolerant organisms in a batch of processed milk are introduced at the farm, or at the milk plant and can persist until the final product. Therefore, those thermotolerant organisms in raw milk could be considered equivalent to pasteurised milk isolates, as their presence could not be considered unique to raw milk. In total, seven thermotolerant isolates from either raw or processed milk were found. A proportion of bacterial isolates obtained from pasteurised milk are heat sensitive, suggesting that they may have been introduced into milk post-pasteurisation. One study investigating post-pasteurisation contamination found that bacteria were often

introduced when containers were filled (Eneroth *et al.*, 1998). The heat-sensitive population of raw milk therefore differs from the combination of thermotolerant and thermosensitive bacteria found in processed milk.

The diversity of microbial species present in milk was assessed based on colony morphology. There are many different diversity indices that may be used to compare the diversity of different ecological populations (Hill, 1973). Each have their positive and negative attributes, and it has been proposed that a comparison of several different kinds may elicit the most useful comparison. In this study, three different diversity indices were used. These three indices did not agree as to which population was more diverse. However, the interpretation of these results depends on an understanding of what each index represents. The Simpson Index of Diversity provides the probability that two random isolates in a population are different species, and is used most aptly to describe the evenness of the population. Due to the spikes of the relative frequencies in raw milk, the probability of selecting two different species is reduced. The Shannon index may be more appropriate, as "it gives equal weight to rare and common species" (Gering *et al.*, 2003). The Shannon index however, can be misrepresented if the sample size and number of unique 'species' is different, which is the case in this study. A third index, the Margalef Richness index, may be the most appropriate measure, as it compares the number of unique 'species' relative to sample size. With scores of 4.23 compared with 3.58, this index shows that the 'richness' of the species found raw milk is greater than that of the pasteurised milk despite its greater sample size. This conclusion can also be drawn from the biochemical analysis of the raw and pasteurised milk bacterial isolates, where representatives of all six microbial groupings were detected in raw milk, while only three were detected in pasteurised milk. The implication of this greater diversity means that not only are there more bacteria in raw milk, there are also more types of bacteria. The probability that a consumer has significant amounts of immunostimulatory bacteria is therefore greater when raw milk is consumed compared with the same quantity of pasteurised milk.

Many of the thermosensitive raw milk flora identified belonged to the γ-Proteobacteria, including Enterobacterial genera such as *Rahnella* and *Enterobacter*; as well as aerobic Proteobacteria such as *Pseudomonas*. These microorganisms may have entered milk via contamination with either soil or faecal matter. A number of Gram-positive isolates were identified. These included *Streptococcus*, and *Staphylococcus* species, which may be parasitic or commensal organisms living in, or on the skin of, the udder (Bramley *et al.*, 1984; Tolle, 1980). A Rhodococcus belonging to the Actinobacteria was identified, and an

Oerskovia sp. was identified from group F. This bacteria is also from the class Actinobacteria (Stackebrandt *et al.*, 2002), and has the potential to be pathogenic in humans (Rihs *et al.*, 1990). *Oerskovia*, are 'extensively distributed' soil bacteria (Cruikshank *et al.*, 1979), and may have entered the milk via contamination of the udder (Robinson, 1990). Despite exhibiting coccoid cell morphology, an isolate characterised as group A was related to the *Acinetobacter*. The *Acinetobacter* are Gram-negative bacilli that can appear as cocci and it can be difficult to differentiate these cell morphologies (Gilardi, 1969).

The composition of the microbial population identified in this study was similar to other studies performed outside of New Zealand. The *Staphylococcus*, *Streptococcus*, *Pseudomonas* and *Acinetobacter* genera are well-known members of the milk microflora (Robinson, 1990). The *Rahnella,* while listed as part of the Enterobacteriaceae, is not often found in raw milk (Robinson, 1990). However, it has been found in raw milk in a recent study (Quigley *et al.*, 2013b). Two *Rhodococcus* isolates were identified (Tables 14 & 15). This genus may also be occasionally detected in raw milk (Quigley *et al.*, 2013b).

The bacterial profile of pasteurised milk was determined using both culture-dependent and culture-independent techniques. Of the isolates that were identified, four thermosensitive processed milk isolates were sequenced. Two isolates, an *Acinetobacter* sp. and *Kocuria* sp., are not known to be thermotolerant (Baumann, 1968; Stackebrandt *et al.*, 1995) and were most likely post-pasteurisation contaminants. Two isolates showed sequence similarity to *Bacillus* sp. Members of the genus *Bacillus* are generally thermotolerant through their ability to sporulate; however neither of these isolates survived pasteurisation assays. This suggested that there were only vegetative cells present and no spores were present in the sample that was tested. The conditions present in the growth media prior to inoculation into milk was most likely not suitable to induce these isolates to sporulate before the pasteurisation assay.

Anoxybacillus flavithermus do not grow readily under the conditions used in this study (Pikuta *et al.*, 2000). However, the analysis of microbial DNA present in pasteurised milk showed that *A. flavithermus* was the dominant organism in pasteurised milk. *A. flavithermus* is a thermophilic organism capable of growing in heat exchangers within the dairy plant (Burgess *et al.*, 2009). It is particularly problematic in the manufacture of milk powders (Scott *et al.*, 2007). It is likely that this species is introduced into milk in high numbers during the heat-treatment of milk in processing. The number of bacteria introduced appears to be sufficient to mask any mesophilic bacterial populations present

in the sample. Only one bacterial isolate was related to the *Anoxybacillus* genus. However, these organisms may be under-represented in the culture dependent sampling performed in this study as plates were grown at mesophilic temperatures.

To distinguish between live and dead microorganisms, and increase the concentration of bacterial DNA, milk was abused at RT for 24 hours. In abused milk *Bacillus* sp. were dominant. It is possible that the temperature abuse may have allowed any *Bacillus* spores to germinate and increase in abundance to become the dominant genera, as these organisms may grow efficiently in milk at this temperature.

This analysis shows that raw milk contains both greater numbers and greater richness in diversity than processed milk. Of the six groups identified in this study, three were found exclusively in raw milk. These include bacteria from the Gram-negative cocci; the Gram-negative oxidase-positive rods, and the Gram-positive catalase negative cocci (Groups A, C and D). These isolates were subjected to a pasteurisation test, and all but one isolate was found to be thermosensitive. One of the raw milk isolates tested was able to survive a laboratory pasteurisation assay. This isolate, found in group D, may belong to the thermotolerant Streptococci (Ordonez *et al.*, 1984) or Enterococci (McAuley *et al.*, 2012).

The remaining group (B, E and F) included bacteria that were found in both raw milk and processed milk. These groups included the, Gram-negative oxidase-negative rods; the Gram-positive catalase-positive cocci; and the Gram-positive rods. Thermotolerant bacteria have been identified from these three groups, indicating that there may be a link between the groups of bacteria isolated from pasteurised milk and thermotolerance. However, not all bacteria isolated from processed milk showed thermotolerant properties, suggesting that there may be a level of post-pasteurisation contamination in these samples.

There are some bacteria that are present in raw milk as well as processed milk. These may be bacteria that are able to survive heat treatment or survive as spores. Loss (2009) and other publications (Table 2) showed that the rates of allergic disease between those that regularly consumed processed milk were significantly different to those that consumed processed milk. It is therefore unlikely that the bacteria that are present both in raw milk and processed milk are likely to be a factor in the reduction of the allergic disease.

4. Acid Tolerance of Bacteria Isolated from Raw Milk

To interact with the immune system in the lower gastro-intestinal tract, bacteria must first survive exposure to the acidic environment of the stomach. All organisms that survive gastric passage must have either some degree of acid tolerance, or be protected from the low pH by the food matrix. This chapter aimed to test two linked hypotheses:

- Raw milk contains a variety of microorganisms that are capable of surviving exposure to acid; and
- Milk can protect microorganisms to improve their chances of survival at low pH.

This study is unique because it examines the survival of the wider milk flora, rather than specific probiotic organisms. This group of bacteria has the potential to interact with the mucosal interface of the lower gastrointestinal tract and thereby the immune system.

4.1 Acid tolerance of the Waikato Bacteria Subset

To determine whether selected bacteria from raw milk were able to survive exposure to low pH, a subset of thermosensitive bacterial isolates was randomly selected from those isolated from raw milk obtained from a Waikato dairy farm (Table 18). The subset consisted of 11 Gram-negative (groups B and C) and 9 Gram-positive bacteria (groups D-F). After the selection of this subset, many isolates were identified by 16S DNA sequencing (Table 14).

Table 18 - Subset of thermosensitive bacteria originating from raw milk obtained from a Waikato dairy farm.

Grouping *(ex* Table 12)	**Isolate Number**
B	32, 67, 69, 73, 75, 76, 89
C	64, 66, 72, 82
D	20, 24, 45, 55
E	28, 30, 48, 52
F	54

The acid tolerance of the bacteria was determined by comparing the survival of the isolates by plate count before and after acid exposure in TSB broth at pH 2.5 for two hours. Isolates 67, 32 and 52 survived to within 1 log of the initial inoculum while isolate 54 survived at pH 2.5 despite a four-log reduction in concentration. None of the remaining 16 isolates demonstrated acid tolerance or survival at pH 2.5 in TSB broth (Figure 5).

To determine whether milk was able to enhance bacterial survival, bacteria were subjected to an acid challenge at pH 2.5 in a 1:1 mixture of TSB and UHT skim milk. UHT pasteurised milk was chosen for this assay because it contained very low levels of background microflora. The concentration of HCl was adjusted to counter the buffering effect of the milk to attain a final pH of 2.5. The addition of milk increased the number of bacterial isolates that survived exposure to pH 2.5 for 2 hours, but also the magnitude of their survival (Figure 6). With the addition of milk, a total of 16 isolates survived acid exposure at pH 2.5. Isolate 54, which demonstrated a low level of survival in the absence of milk, survived to within 1 log of the initial concentration when milk was included. All bacterial isolates survived incubation at pH 7.2 in broth, or milk and broth mixtures (Figures 5 & 6). Eleven isolates survived only when milk was present during incubation. Of these, eight were Gram-negative and three were Gram-positive.

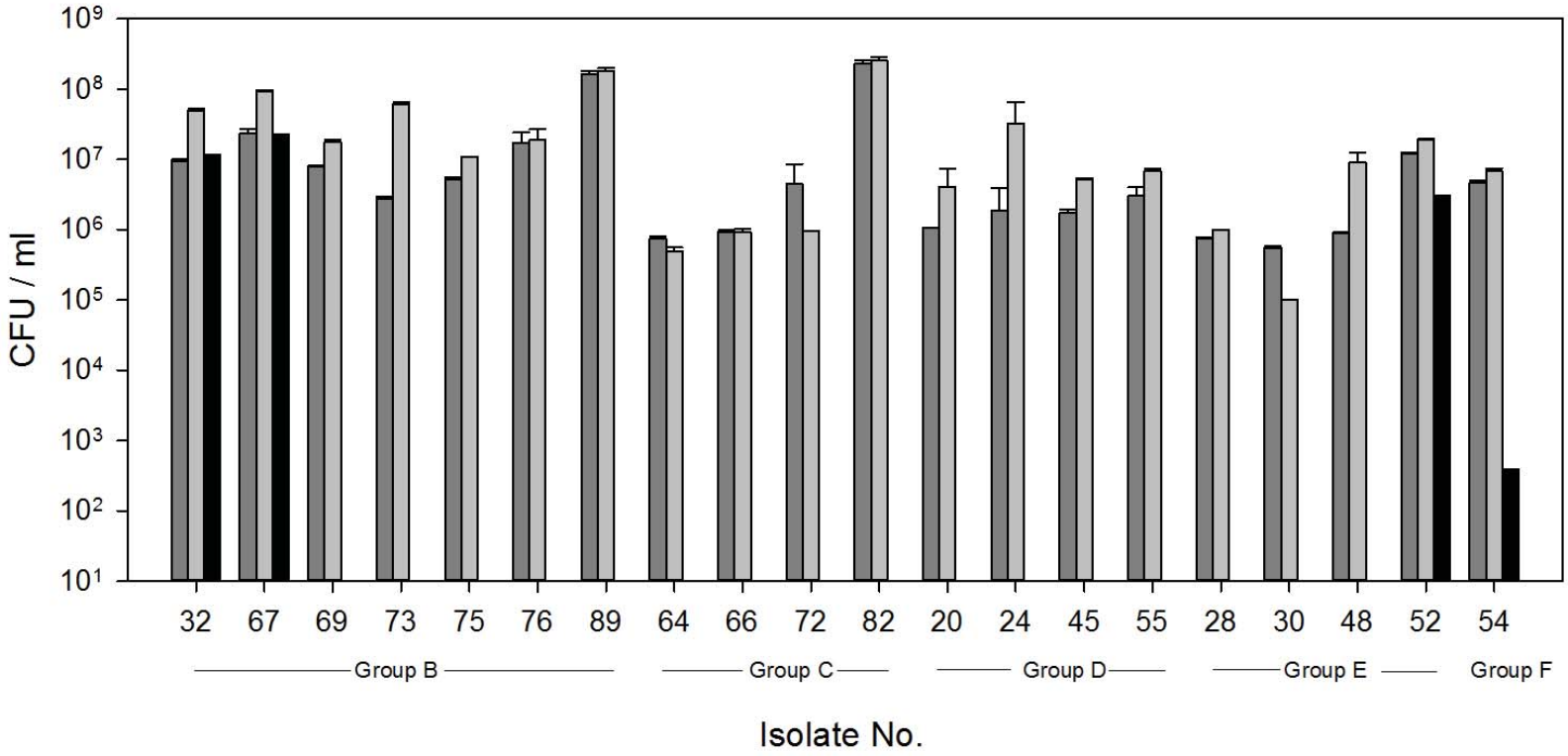

Figure 5 - Survival of 20 raw milk bacterial isolates exposed to pH 2.5 for 2 h in TSB. The bars indicate the initial bacterial concentration (■), survival after incubation for 2 h at pH 7 (■) and pH 2.5 (■). The limit of detection was 10 CFU / ml. Error bars denote one standard deviation.

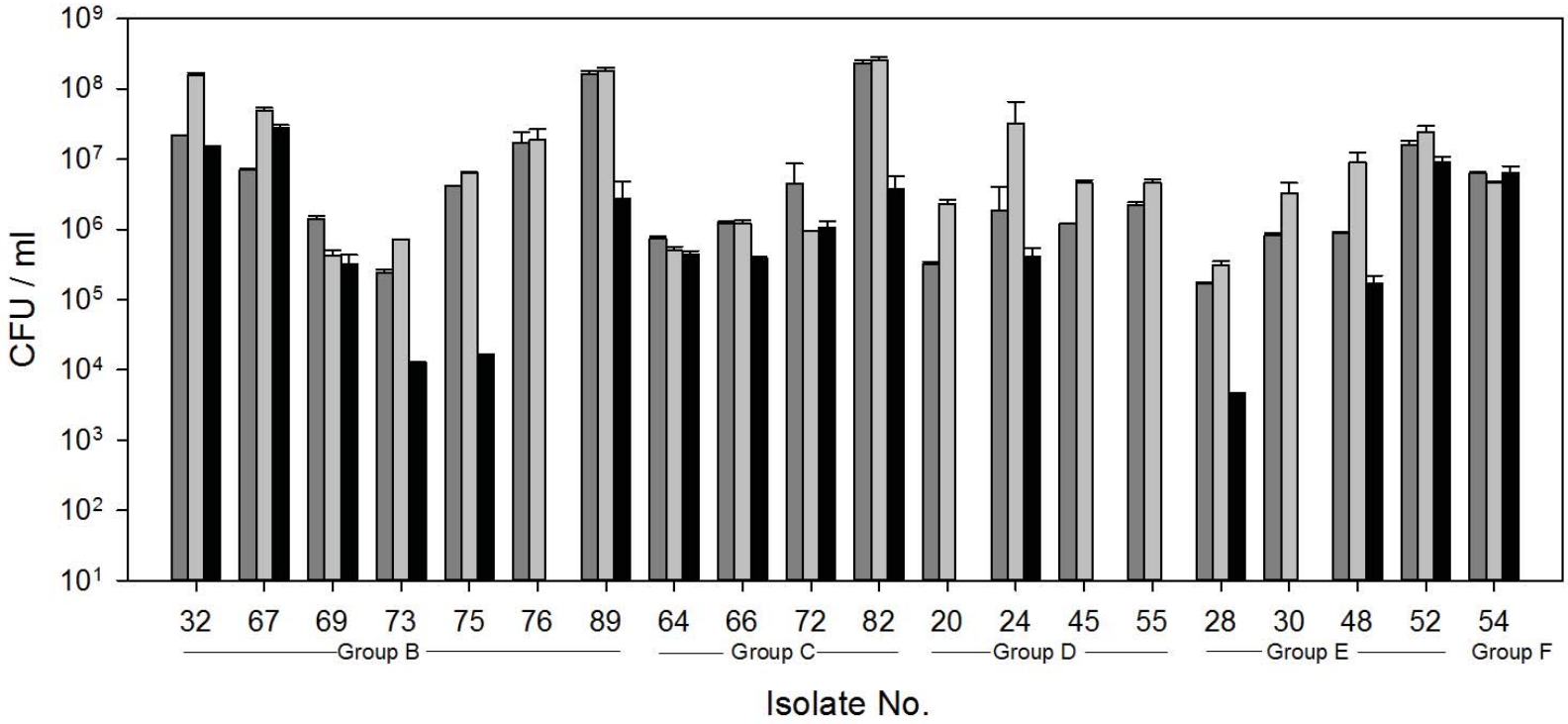

Figure 6 - Survival of 20 raw milk bacterial isolates exposed to pH 2.5 for 2 h in TSB with UHT skim milk. The bars indicate the initial concentration (■), survival after incubation for 2 h at pH 7 (■) and pH 2.5 (■). The limit of detection was 10 CFU / ml. The error bars denote one standard deviation.

4.2 Isolation and Characterisation of Manawatu Bacterial Isolates

To investigate whether bacteria present in raw milk from a geographically distinct region showed similar levels of acid tolerance, bacteria were isolated from two Massey University research farms over three summer months to isolate and compare bacteria over three time-points from two different farms.

4.2.1 Isolation of Bacteria from Raw Milk Obtained from Massey University Research Farms

Bacteria were isolated from raw milk collected from bulk storage tanks on two Massey University research farms located in the Manawatu. Milk was collected at three time-points in December 2011, January 2012 and February 2012. Milk samples were split into two; one half remained untreated, while the other was acidified to pH 2.5 for 2 h by the addition of HCl. The bacterial concentration in both raw and acidified milk was enumerated on M-PCA, MacConkey and APT agars.

In December 2011, the bacterial concentration of milk from farm No. 4 was 1×10^4 CFU / ml on M-PCA and APT agar, and 1×10^1 CFU / ml on MacConkey agar. These values were approximately 1 log higher than that from milk from farm No. 1 across all three media (Figure 7). After acidification, the bacterial concentration was reduced 100-fold to 1×10^2 CFU / ml on M-PCA agar for milk from both farms. Only bacteria in raw milk from farm No. 4 showed growth on APT agar after acidification.

In January, the bacterial concentration in raw milk from both farms was 1×10^4 CFU / ml on M-PCA agar (Figure 7). Similarly the bacterial concentration was 1×10^4 CFU / ml on APT for raw milk from farm No. 1, but was 1 log lower in raw milk from farm No. 4. The concentration of bacteria enumerated on MacConkey agar was 1×10^2 CFU / ml for milk from both farm No. 1 and farm No. 4. Bacterial concentration of acidified samples was comparable on both farms with 1×10^2 CFU / ml recovered on both M-PCA and APT agar. A concentration of 15 CFU / ml was seen on MacConkey agar on farm No. 1, which is at the limit of detection.

In February, the bacterial concentration in raw milk collected from farm No. 1 was 5.8 × 10^3 and 1.5 × 10^3 CFU / ml on M-PCA and APT respectively (Figure 7). An increase in bacterial concentration was observed in raw milk from farm No. 4, with 6.2 × 10^5 CFU / ml and 5.3 × 10^5 CFU / ml recovered on M-PCA and APT agar respectively. The concentration of bacteria capable of growing on MacConkey agar was comparable in January and February in both farms.

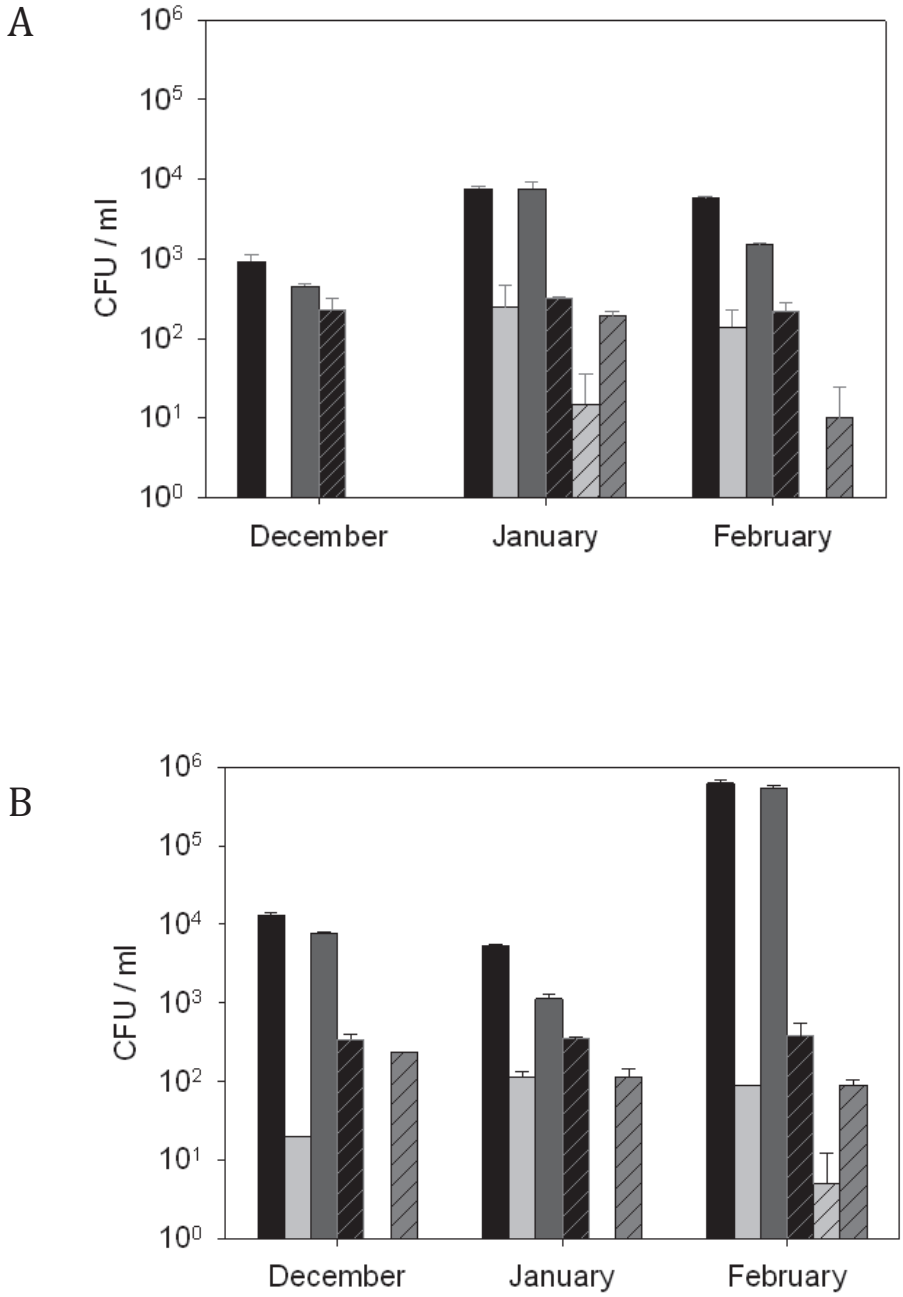

Figure 7 - Bacterial concentration in untreated raw milk (solid) and acidified raw milk (striped) at pH 2.5. Raw milk was obtained from Massey University Research Farm No. 1 (A) and Farm No. 4 (B) sampled in December 2011, January 2012, and February 2012. Bacterial concentrations were enumerated using M-PCA agar (■); MacConkey agar (■); and APT agar (■). The limit of detection was 10^1 CFU / ml. Error bars denote one standard deviation from three replicates.

4.2.2 Determination of Growth Times

To standardise the bacterial concentration used in the high throughput acid tolerance assays, growth times of all isolates were assessed using a 96-well plate reader, where incubation time required to reach an optical density of 0.500 at OD_{590} was recorded (Figure 8). Bacterial isolates were clustered into three categories based on time for growth to 0.500 at OD_{590}: 24, 48 or 72 hours. Bacteria originally isolated on APT agar were grown in APT broth, while those isolated on PCA or MacConkey agars were grown in TSB. The most common growth time for the Manawatu group of bacterial isolates was 72 h. The numbers in each growth-time category were similar for each month tested. The most common growth-time for the Waikato isolates was 24 h, with 51 out of 96 isolates reaching an OD_{590} of 0.500 within this period.

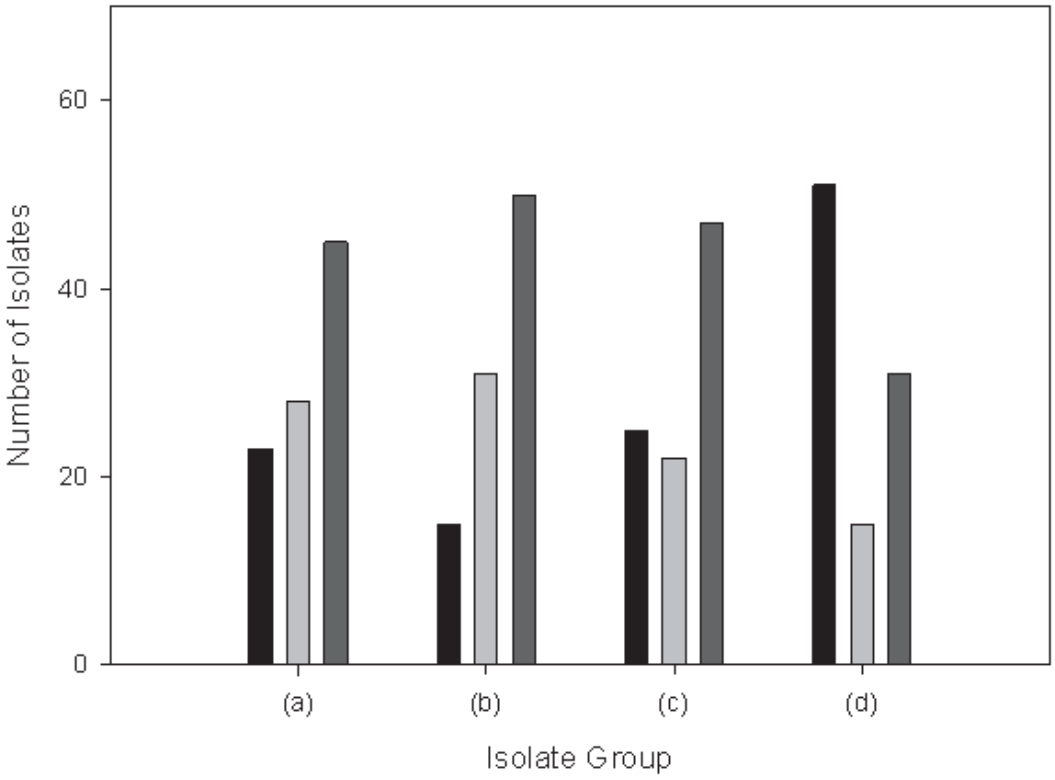

Figure 8 – The number of bacterial isolates sorted into sets according to the time required to reach an OD_{590} of 0.500. Four bacterial groups are - the Massey group - December 2011 (a); the Massey group - January 2012 (b); Massey group - February 2012 (c); and the Waikato group, - September 2009 (d). Bacteria were allocated to either 24 h (■), 48 h (■), and 72 h (■) depending on time to OD_{590} of 0.500.

4.3 High-Throughput Acid Tolerance Assays

The acid-tolerance capability of all the bacterial isolates obtained in this study was tested using a high-throughput acid tolerance assay. Bacteria were challenged at pH 2.5 for two hours in TSB and TSB with UHT milk.

4.3.1 High-Throughput Analysis

A total of 363 isolates were tested for acid tolerance using the high throughput assay. Overall, 70 isolates were acid tolerant, with all replicates surviving acid treatment in TSB (Table 19). 173 isolates displayed partial acid tolerance, where at least one replicate was acid tolerant in TSB. When the same set of isolates were assayed in TSB and milk, the number of isolates that were totally acid tolerant increased to 186, a significant increase of 116 (Table 19). The number of isolates that were partially acid-tolerant decreased to 144. 33 isolates were not acid tolerant under any conditions tested (Table 19).

Table 19 - Number of bacterial isolates that survived exposure at pH 2.5 for two hours TSB and TSB with Milk. Bacterial isolates were assayed in at least duplicate experiments.

Replicate	TSB	TSB with Milk
Total Acid Tolerance (All replicates survived)	70	186
Partial Acid Tolerance (at least one replicates survived)	173	144
No Acid Tolerance (No replicates survived)	120	33
Total	363	363

4.3.2 Statistical Analysis

Statistical analysis was performed using a Generalised Linear Mixed Model for binary data. The proportions of acid tolerant and acid sensitive bacteria were transformed using the model and the variances determined. Back-transformed mean acid tolerance of raw milk isolates were then determined and compared with the back-transformed mean acid tolerance in the presence of milk (Figure 9). The predicted means of both raw milk, and acidified raw milk isolates in TSB were significantly lower than in TSB and milk, compared to the average Least Significant Difference (LSD). After the means, standard error of the means, and the least significant difference were calculated, the data was back-transformed to reflect the true proportions. The mean survival of raw milk isolates in the acid challenge in TSB was 32.25 % which increased to 78.76 % with the addition of UHT milk and was statistically significant as this falls outside the 95 % confidence intervals. Of the isolates obtained from acidified milk, 64.0 % were acid tolerant and this increased to 83.48 % with the addition of milk. Again, this result was statistically significant as it falls outside 95 % confidence intervals.

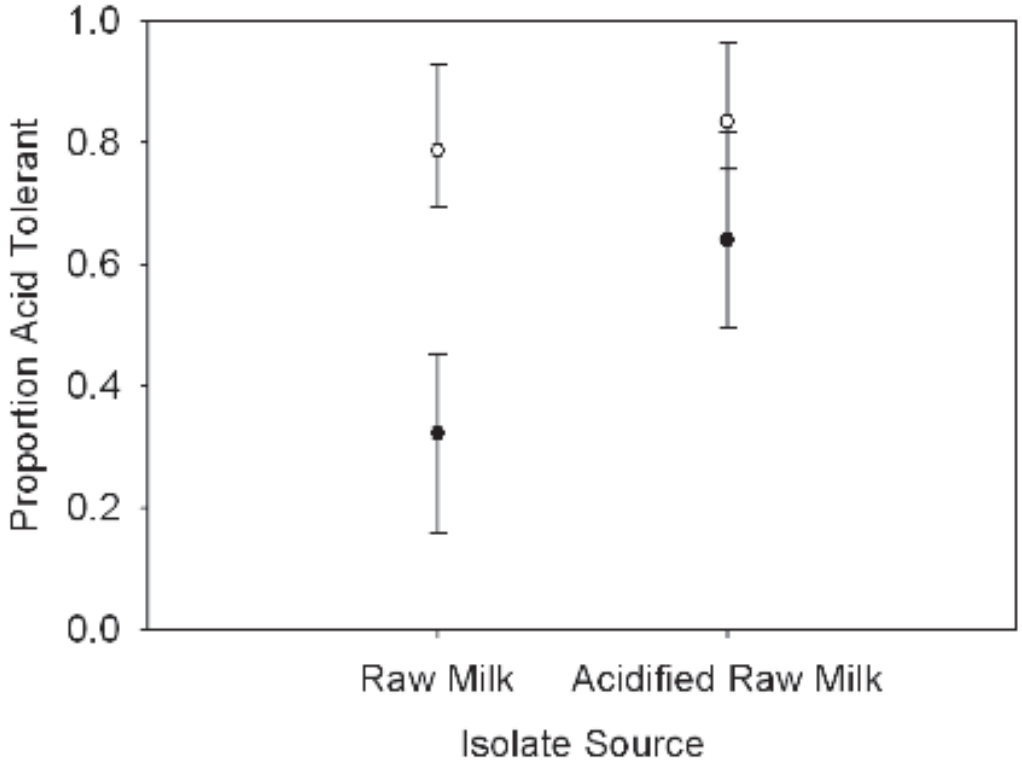

Figure 9 - Mean proportion of isolates from raw milk or acidified raw milk that are acid tolerant at pH 2.5 for 2 h in TSB (■), or TSB with milk (□). The error bars denote 95 % confidence intervals.

4.3.3 Identification of Strains Protected by Milk at Low pH

A number of bacterial isolates survived acid exposure at pH 2.5 in the presence of milk. 62 of these were identified by partial 16S rRNA sequencing. The 16S rRNA gene was sequenced using both forward and reverse primers. 53 of the 62 isolates sequenced were Gram-positive (Table 20). Of these, 19 isolates were *Staphylococcus* sp*.,* with 7 species identified. Of these, *S. aureus* belonging to the *S. haemolyticus* group (which include *S. haemolyticus* and *S. devriesei*) were the most abundant. In addition, isolates that belong to the Lactic Acid Bacteria clade were identified, including *Leuconostoc*, *Lactobacillus*, *Lactococcus*, and *Streptococcus.* Nine Gram-negative isolates were sequenced, and identified as *Enterobacter*, *Pantoea* and *Pseudomonas* (Table 21). Bacterial 16S sequences were analysed using both BLAST and Seqmatch search tools (Appendix 7).

Table 20 – The closest known match of Gram-positive bacteria isolated from the Manawatu dairy farms that show an improved acid tolerance in the presence of milk.

Genera	Species	Frequency
Arthrobacter	*citreus*	2
	gandavensis	1
	agilis	1
Bacillus	*licheniformis*	3
Curtobacterium	*flaccumfaciens*	2
Kocuria	*rhizophila*	2
	varians	2
Lactobacillus	*paracasei*	1
Lactococcus	*lactis*	7
Leuconostoc	*lactis*	6
Microbacterium	*phylosphereae*	1
Micrococcus	*yunnanenensis*	1
Plantibacter	*flavus*	1
Rathayibacter	*festucae*	1
Staphylococcus	*aureus*	1
	auricularis	1
	chromogenes	2
	devriesei	3
	epidermis	2
	haemolyticus	9
	saprophyticus	1
Streptococcus	*parauberis*	1
	uberis	2
Total		53

Table 21 - The closest known match of Gram-negative bacteria isolated from the Manawatu dairy farms that show an improved acid tolerance in the presence of milk.

Genera	Species	Frequency
Enterobacter	*aerogenes*	2
Pantoea	*agglomerans*	3
Pseudomonas	*psychophila*	1
	rhizosphaerae	1
	moraviensis	2

4.4 Discussion

Several key cohort studies have demonstrated that raw milk may help improve immune stimulation (Table 2). One way that this may occur is by bacteria present in milk contributing to the bacterial flora present in the lower gastrointestinal tract, where they may be involved in immune stimulation. However to gain access to the intestinal tract, bacteria must survive exposure to the acid in the stomach. The aim of this study was to determine the impact of milk on the survival of bacteria at pH 2.5, and to identify bacteria found in raw milk that would survive these conditions. To achieve this, a high throughput acid tolerance assay was designed and validated to test the acid tolerance of bacterial isolates in TSB and TSB with milk incubated at pH 2.5 for two hours. This study showed that milk offers protection to a wide range of bacterial types, and importantly that this protection remains even when the buffering effect of the milk is negated.

The impact of milk on the survival of some probiotic species has been previously demonstrated (Hansen *et al.*, 2002). However, the impact of milk on the survival of the other milk flora has not been shown. In this study two groups of bacteria were isolated; those normally found in raw milk, and those that survived an acid challenge in the raw milk sample. This approach was used so that the acid tolerance of the general milk flora could be determined *in situ*, and a set of bacteria that survived acid exposure in milk could be isolated. This selection process increased the likelihood of isolating and identifying those bacteria that have the potential to survive gastric transit.

The acidity in the stomach can vary. The mean gastric pH of a fasting adult was 1.7, increasing to 6.7 after a meal. After a period of two hours, the pH returns back to normal (Dressman *et al.*, 1990). The consumption of a meal or milk increases the gastric pH to higher levels due to the buffering effect of the food. A pH of 2.5 has been as used in other acid tolerance studies and is a reasonable pH on which to assess gastric survival (Benjamin *et al.*, 1995; Uljas *et al.*, 1998; Waterman *et al.*, 1998). This was also selected for this study. Although milk was found to significantly buffer the acid, HCl was added to milk/broth mixture to maintain the pH at 2.5 and override the buffering capacity of milk. The pH was maintained at 2.5 for two reasons: Firstly, any buffering effect by food during digestion is temporary, due to additional stomach acid being produced shortly after a meal, and secondly, to test whether milk was able to afford any protection to the bacteria at a constant pH of 2.5 over and above normal buffering.

The survival of raw milk flora at low pH in TSB, and TSB in the presence of milk was tested using both a qualitative and a quantitative method. The qualitative acid tolerance assays showed that the acid tolerance of the 20-isolate bacterial subset was significantly improved with the addition of milk at pH 2.5. This was corroborated by the qualitative high-throughput acid tolerance assays where the proportion of organisms that were acid tolerant at pH 2.5 increased from 32.3 % to 78.8 % in the presence of milk. These results suggested that the impact of milk on bacterial survival at low pH is not limited to probiotics, but the other milk flora also.

Due to differences in the time and pH that bacteria are exposed to when ingested varies both within an individual and between individuals, it is difficult to come up with an assay that covers all eventualities. Stomach contents do not remain there for a fixed amount of time but are slowly pushed towards the small intestine and thus acid exposure will not be uniform. To accurately and reliably determine the acid tolerance of a multitude of isolates for comparison, the conditions that isolates are exposed to must be standardised. Survival at pH 2.5 for a period of two hours is a standard test, commonly used to determine bacterial acid-tolerance (Benjamin *et al.*, 1995; Conway *et al.*, 1987; Waterman *et al.*, 1998). Acid exposure did not include the addition of digestive enzymes. The effect of digestive enzymes was not tested. Although the presence of digestive enzymes will affect bacterial survival, exposure to acid was considered the primary stress to which bacteria are exposed during gastric passage.

As expected, many of the isolates obtained from acid-treated milk were acid tolerant at pH 2.5 in TSB, and only a minor increase in survival was observed when the same isolates were incubated in TSB and milk. This increase was not statistically significant, indicating that acid treatment of raw milk selected organisms that were intrinsically acid tolerant, and less so those that were acid tolerant in the presence of milk. It is interesting to note that some isolates, despite being isolated from acid-treated raw milk, were found to be acid-sensitive when tested in pure culture. It is not known why this occurred, but this may be due to the differences between raw milk, and the UHT milk used in the subsequent analysis.

Many of the raw milk isolates that survived acid exposure in the presence of milk, did so exclusively in the presence of milk. One interpretation is that milk may have specific properties that enhances bacterial survival, and may indicate that these bacteria would not survive gastric transit if consumed in another food type.

Many of the organisms that survived acid exposure in the presence of milk were Gram-positive. However, this does not necessarily mean that Gram-positive organisms are more protected by milk, as they may simply be over-represented in the isolate collection. Given that the majority of the Waikato isolates surviving in the 20-isolate subset were Gram-negative, this is most likely to be the case. Because a mixture of Gram-positive and Gram-negative bacterial species survived acid exposure due to the presence of milk, it is not thought that survival in milk is dependent on the bacterial cell wall morphology.

Several Lactic Acid Bacteria were able to survive pH 2.5 exclusively in the presence of milk. This included representatives of the *Lactococcus, Lactobacillus* and *Leuconostoc* genera. This observation is consistent with other studies that showed improved gastric survival of these LABs in the presence of milk (Mainville *et al.*, 2005). A number of *Staphylococcus* isolates survived after the addition of milk. *Staphylococcus* have been shown to bind to the human gastrointestinal lining and therefore must be able to survive ingestion (Vesterlund *et al.*, 2006), and thus may act to stimulate the immune system.

A number of *Pseudomonas* sp. survived acid exposure at pH 2.5 in milk. This is surprising, as they are not generally known for their acid tolerance. Some species can survive for short periods at pH 3.0 (Jørgensen *et al.*, 1999), it may therefore be plausible that milk allows the organism to tolerate a slightly lower pH for a longer time than normal. One explanation that may account for this observation is that the factors dictating bacterial survival may be a physical property of the milk, rather than a physiological change in the bacterium itself. This is addressed further in chapter five.

There are no major similarities between the organisms that are positively affected by milk during acid stress, with a mixture of different genera present. A potential requirement is that bacteria have some degree of intrinsic acid tolerance, which is enhanced when ingested in the presence of milk. There have been inferences made as to how milk protects at low pH, but a true mechanism of protection has not been proposed. Milk may protect bacteria by providing micro-niches of milder pH within the curd particles when milk denatures that allow the cells to survive lower pH for an extended time.

5. Mechanisms of Increased Bacterial Acid Tolerance in Milk

Data presented in chapter four showed that milk offers protection from acid exposure to a number of different bacteria present in raw milk. The mechanism by which milk protected these organisms at low pH has not been established. The aim of this chapter is to elucidate how milk protects bacteria at low pH. One hypothesis is that casein that forms the milk curd protects bacteria from direct acid exposure.

5.1 – Does Milk Extend the Range of Acid Tolerance Exhibited by Raw Milk Microflora?

Eleven out of 16 isolates demonstrated survival at pH 2.5 in a mixture of TSB and UHT milk, but not in TSB alone (Figures 5 & 6). These observations suggested that milk protected some organisms at low pH but not others. It was hypothesised that milk can enhance the tolerance of bacteria to lower pH, increasing the survival of bacteria beyond pHs that they can normally survive in TSB alone. To test this hypothesis, acid tolerance was tested at a range of different acidic pH values using six isolates in TSB for a period of two hours to determine the minimum pH that these isolates can tolerate. Once the level of acid tolerance was determined, bacteria were challenged at pH levels just below their level of survival in TSB to determine whether the addition of milk could increase their acid tolerance. The acid tolerance of six isolates was tested including four isolates that only demonstrated survival at pH 2.5 in the presence of milk (isolates 28, 64, 66 and 89); and two isolates (30 and 55) that did not survive at pH 2.5 in any of the conditions thus far tested. The minimum pH tolerated in TSB alone ranged between pH 3.0 and pH 4.0 (Figure 10). Bacteria were then exposed to pH values 0.5 units below their minima, in both TSB and TSB with milk. All isolates tested survived in TSB and milk, but not in TSB alone at these pH values (Figure 11). Notably, Isolates 30 & 55 were previously acid sensitive at pH 3.5 but were protected by milk at this pH, with recoveries of 2.0×10^3 CFU / ml and 8.0×10^4 CFU / ml respectively.

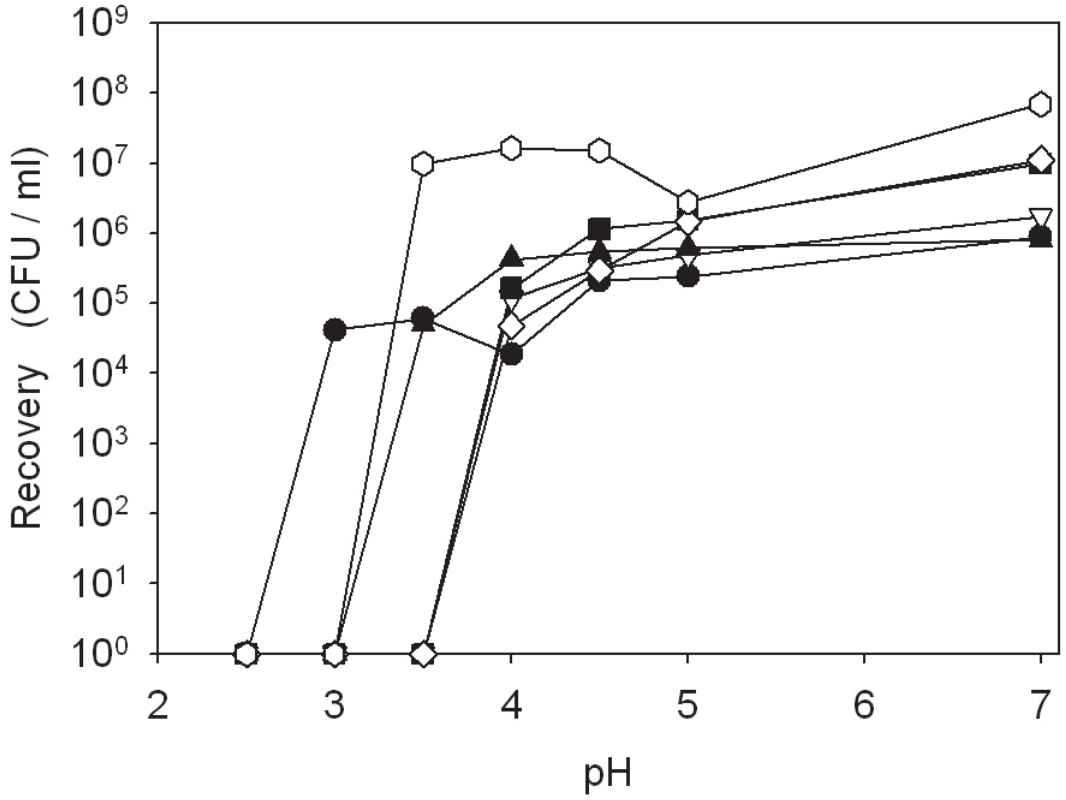

Figure 10 – Recovery of six raw milk isolates after incubation in TSB in a range of pH values for 2 h. The symbols denote isolates '28' (●), '30' (▽), '55' (■), '64' (▲), '66' (◇), and '89' (⬡).

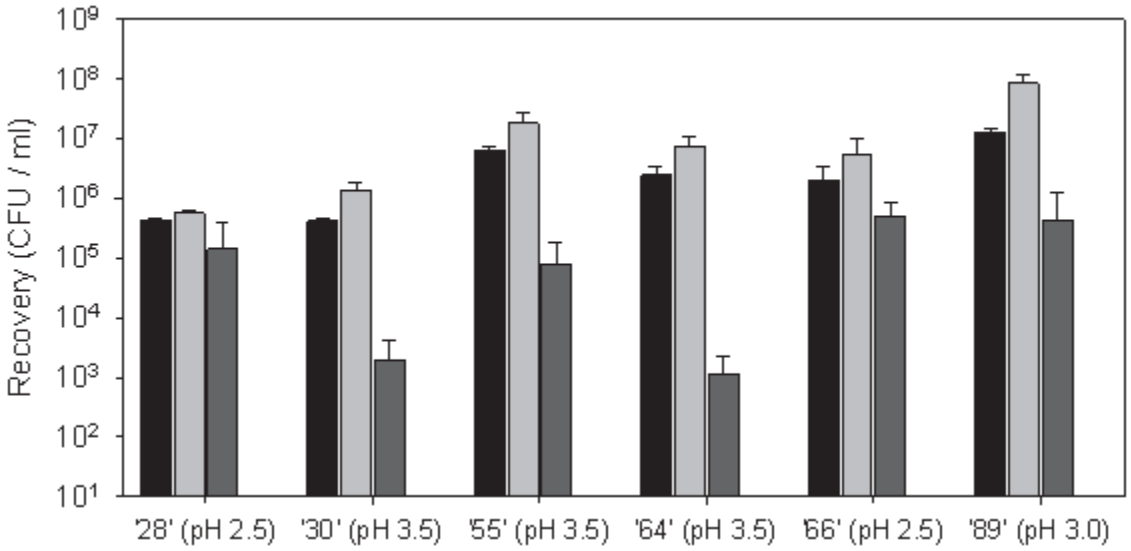

Figure 11 - Survival of six raw milk isolates in TSB and TSB with milk at a pH beyond their maximum. The concentration is presented at the initiation of the experiment (■) and after exposure to TSB and milk at pH 7.0 (■), TSB and milk at the specified pH (■). There was no survival in TSB alone at any of the specified pH values (not shown). The error bars denote the standard deviation. The detection limit is 10 CFU / ml.

5.2 – How Milk Protects Bacteria in Acidic Conditions

5.2.1 Survival of Two Bacterial Isolates in Standard, Skim and Soymilk at pH 2.5

Milk is a colloidal mixture that contains dissolved and particulate matter at a concentration of between 129 and 135 g / kg (Heck *et al.*, 2009). In order to determine which component of milk offers protection to bacteria in acidic environments, two bacterial isolates were selected for further analysis.

The two isolates selected for further study were Isolate 28 and Isolate 66. These isolates were selected because they are Gram-positive and Gram-negative representatives of the eleven isolates that, during acid tolerance assays, survived exposure to pH 2.5 exclusively in the presence of milk (Figures 5 & 6). To identify these isolates more comprehensively, the 16S gene was sequenced using internal and terminal primers. Bacteria were named using both their molecular and biochemical characterisations. The closest known relative of isolate 28 was *Staphylococcus aureus*. The closest known relative of isolate 66 was *Shigella flexneri*. The molecular identification does not agree with biochemical data obtained for this isolate, which shows that isolate 66 as being oxidase positive (Appendix 2). It is possible for biochemical tests to give false results, and the molecular result was taken as true. *S. aureus* and *S. flexneri* are not typically renowned for their probiotic properties, or their ability to survive well at extremely low pH. However they were investigated purely so that the mechanism by which they are protected at low pH could be determined.

To determine if other milk types were able to protect bacteria at low pH, survival was tested in standard UHT and soymilk. After exposure of *S. aureus* (28) to pH 2.5 in the presence of standard UHT milk, plate counts of 6.0×10^4 CFU / ml were observed (Figure 12A). This was less than 1 $\log_{10}$ lower than the initial count of 2.6×10^5 CFU / ml. A similar reduction of less than 1 $\log_{10}$ was also observed for *S. flexneri* (66) (initial inoculum 3.9×10^5 CFU / ml) (Figure 12B). Survival rates in skim milk were comparable to those observed in Figure 6. In trim soymilk however, no survival was observed for either isolate. This suggested that the observed protection was milk specific.

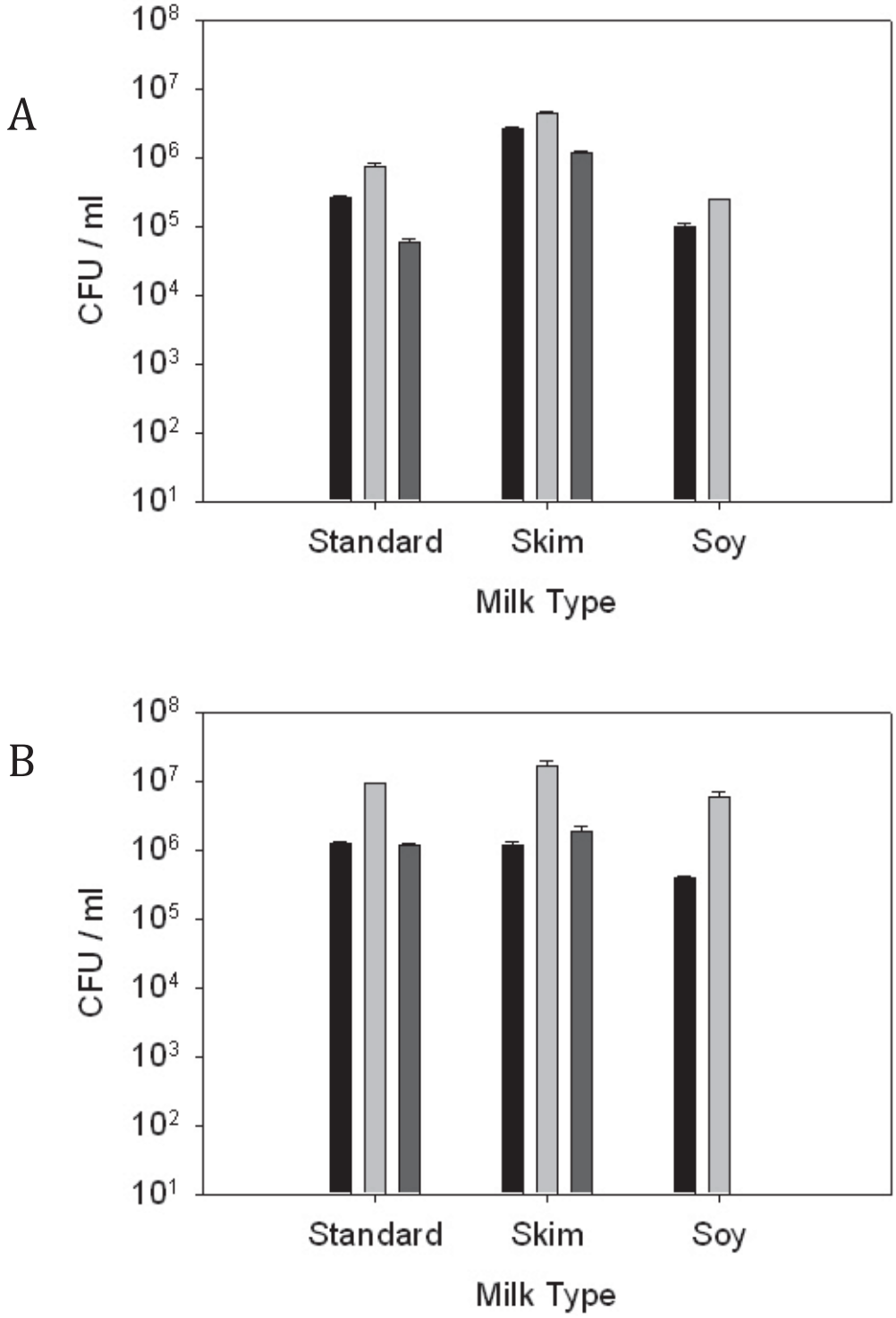

Figure 12 – Survival of *S. aureus* (28) (A) and *S. flexneri* (66) (B) in TSB mixed with standard UHT milk, skim UHT milk and trim soymilk at the initiation of the experiment (■), and after incubation for 2 h at pH 7 (■) and pH 2.5 (■). The error bars denote the standard deviation. The limit of detection was 10 CFU / ml.

Casein vs. Hydrolysed Casein

Since the survival of *S. aureus* (28) and *S. flexneri* (66) in both skim and standard UHT milk was comparable, this suggested that the fat component of milk was not the major determinant that protected bacteria at pH 2.5. Protein of which 80 % are caseins comprises a significant proportion of milk solids (Jenness, 1974). To determine whether casein was important for the protection of bacteria at pH 2.5, both isolates were incubated at pH 2.5 in TSB and sodium caseinate or hydrolysed casein.

The survival of *S. aureus* (28) and *S. flexneri* (66) in acidic conditions was tested using casein at 3.7 % w/v, the concentration of protein found in UHT skim milk (Figure 14). Casein, in the form of sodium caseinate did not provide any protection to either strain as no colonies were recovered after incubation at pH 2.5 for 2 hours. Hydrolysed casein at the same concentration was able to confer some protection to both *S. aureus* (28) and *S. flexneri* (66). *S. aureus* (28) survived with 1.5×10^3 CFU / ml, approximately a three $\log_{10}$ reduction from the initial concentration, with only a low level of growth observed for *S. flexneri* (66) at the limit of detection of 10 CFU / ml. This level of survival is lower than what is observed in skim milk alone (Figure 13).

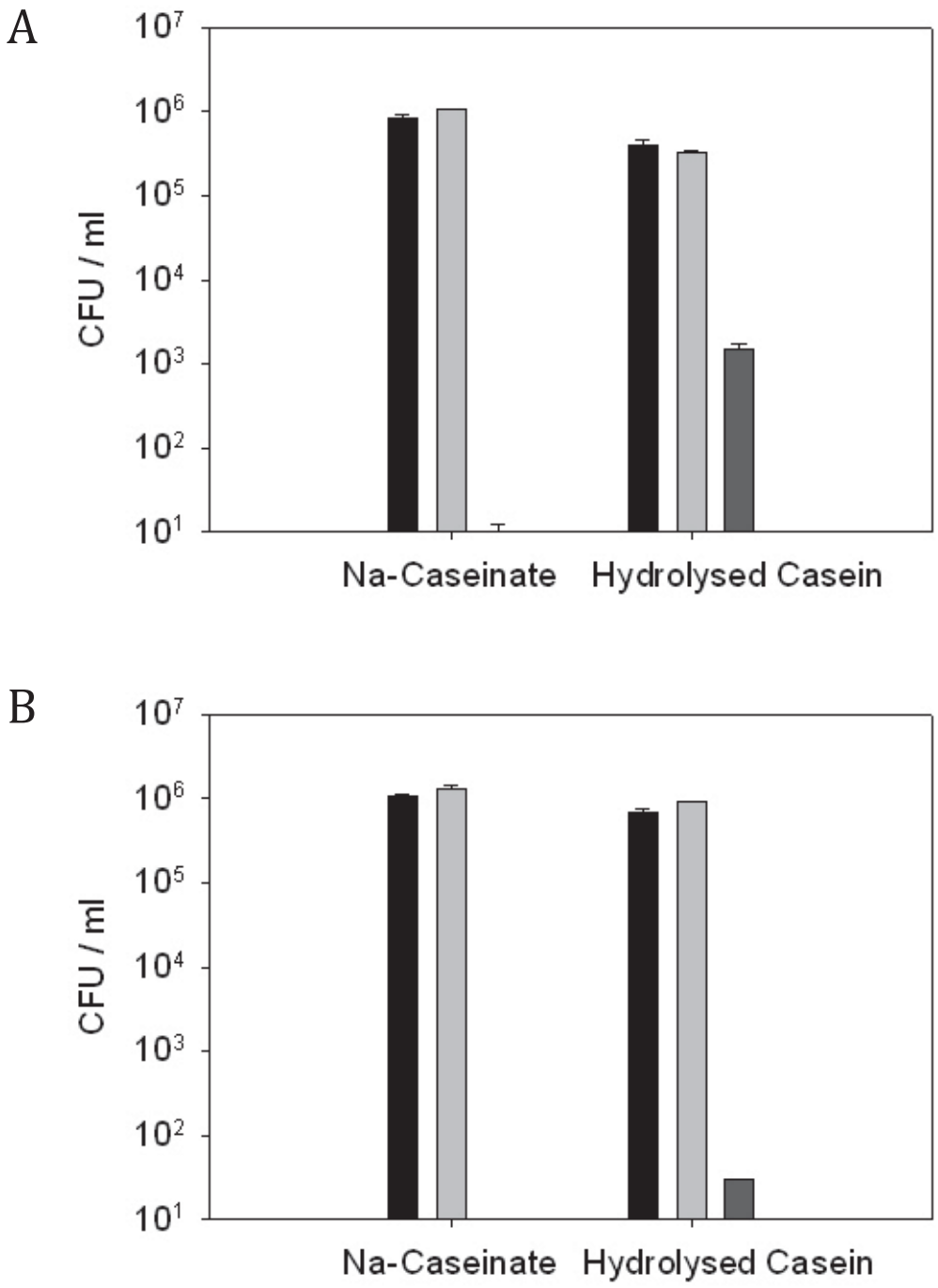

Figure 13 – Survival of *S. aureus* (28) (A) and *S. flexneri* (66) (B) after acid challenge in TSB mixed with equal proportions of Na-caseinate (37 g / L) or hydrolysed casein (37 g / L) after 2 hour acid challenge at pH 2.5. Plate counts are presented for the initiation of the experiment (■), and after incubation for 2 h at pH 7 (■) and pH 2.5 (■). The error bars denote the standard deviation. The limit of detection was10 CFU / ml.

Survival in Whey Protein Concentrate

Whey proteins form the second most abundant protein in milk, comprising approximately 0.6 % of raw milk (Jenness, 1974; Quigley *et al.*, 2013b). The survival of *S. aureus* (28) and *S. flexneri* (66) at pH 2.5 was determined in Whey Protein Concentrate (WPC) at 3.7 % w/v, the equivalent concentration of protein in skim UHT milk and 0.6 % w/v, the concentration of whey in raw milk (Figure 14). As a control, *S. aureus* (28) and *S. flexneri* (66) were inoculated into skim milk at approximately 1×10^5 CFU / ml and $1x10^6$ CFU / ml respectively. Survival of both isolates was observed at the high WPC concentration (3.7 % w/v), where the recovery of *S. aureus* (28) and *S. flexneri* (66) was 1.9×10^3 CFU / ml and 7.2×10^3 CFU / ml respectively. However, at the physiological whey concentrations (0.6 % w/v), *S. aureus* did not survive acid challenge and was significantly reduced for *S. flexneri* with 1.1×10^2 CFU / ml recovered. Based on these results, whey protein was not the main protective component of skim milk.

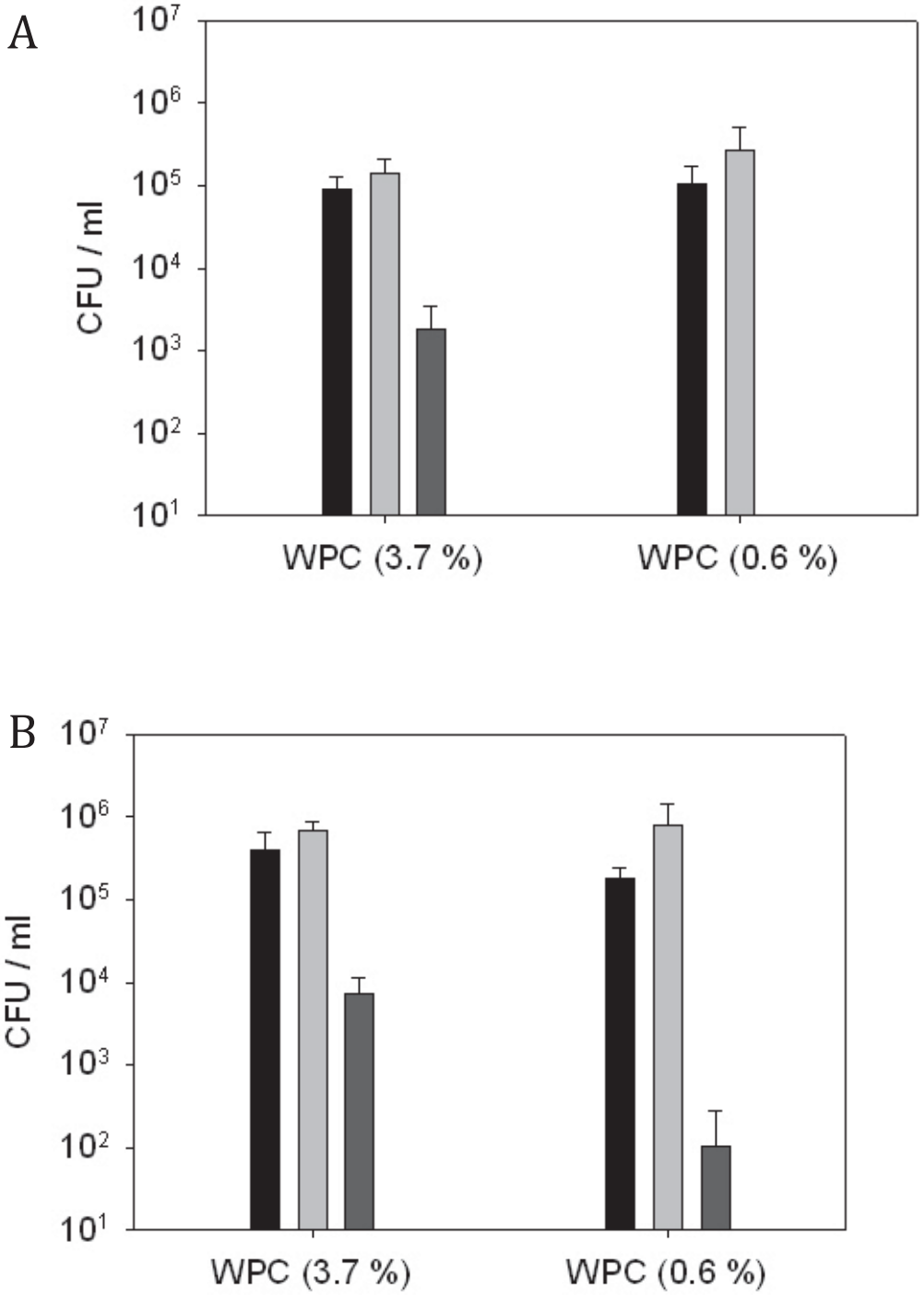

Figure 14 – Survival of *S. aureus* (28) (A) and *S. flexneri* (66) (B) after acid challenge in TSB mixed with equal proportions of 3.7 % and 0.6 % Whey Protein Concentrate (WPC) at the initiation of the experiment (■), and after incubation for 2 h at pH 7 (■) and pH 2.5 (■). The error bars denote the standard deviation. The limit of detection was 10 CFU / ml.

Survival in Casein Micelles

In milk, casein is structured into micelles, where casein is bound by colloidal calcium phosphate to form particles of between 5 and 50 nm in diameter (Knoop *et al.*, 1979; Shimmin *et al.*, 1964). For this study, casein micelles were reconstituted from sodium caseinate and used in acid tolerance experiments in TSB at pH 2.5. Survival of bacterial isolates was also measured in a salts-only control consisting of calcium, phosphate and citrate.

Both the *S. aureus* (28) and *S. flexneri* (66) isolates survived acid challenge in the presence of casein micelles with recoveries of 8.7×10^4 CFU / ml and 5.0×10^3 CFU / ml respectively (Figure 15). *S. aureus* (28) survival in the presence of micelles was comparable to that observed for UHT skim milk control, where 2.6×10^4 CFU / ml were recovered. However, the recovery of *S. flexneri* (66) was two $\log_{10}$ lower than for the UHT milk control. Both isolates survived acid challenge in the presence of the salts mixture, albeit at a reduced level, where TSB was supplemented with calcium chloride (15 mM), potassium orthophosphate (11 mM) and potassium citrate (5 mM). *S. aureus* (28) was recovered at 5.9×10^2 CFU / ml and *S. flexneri* (66) at 1.1×10^2 CFU / ml.

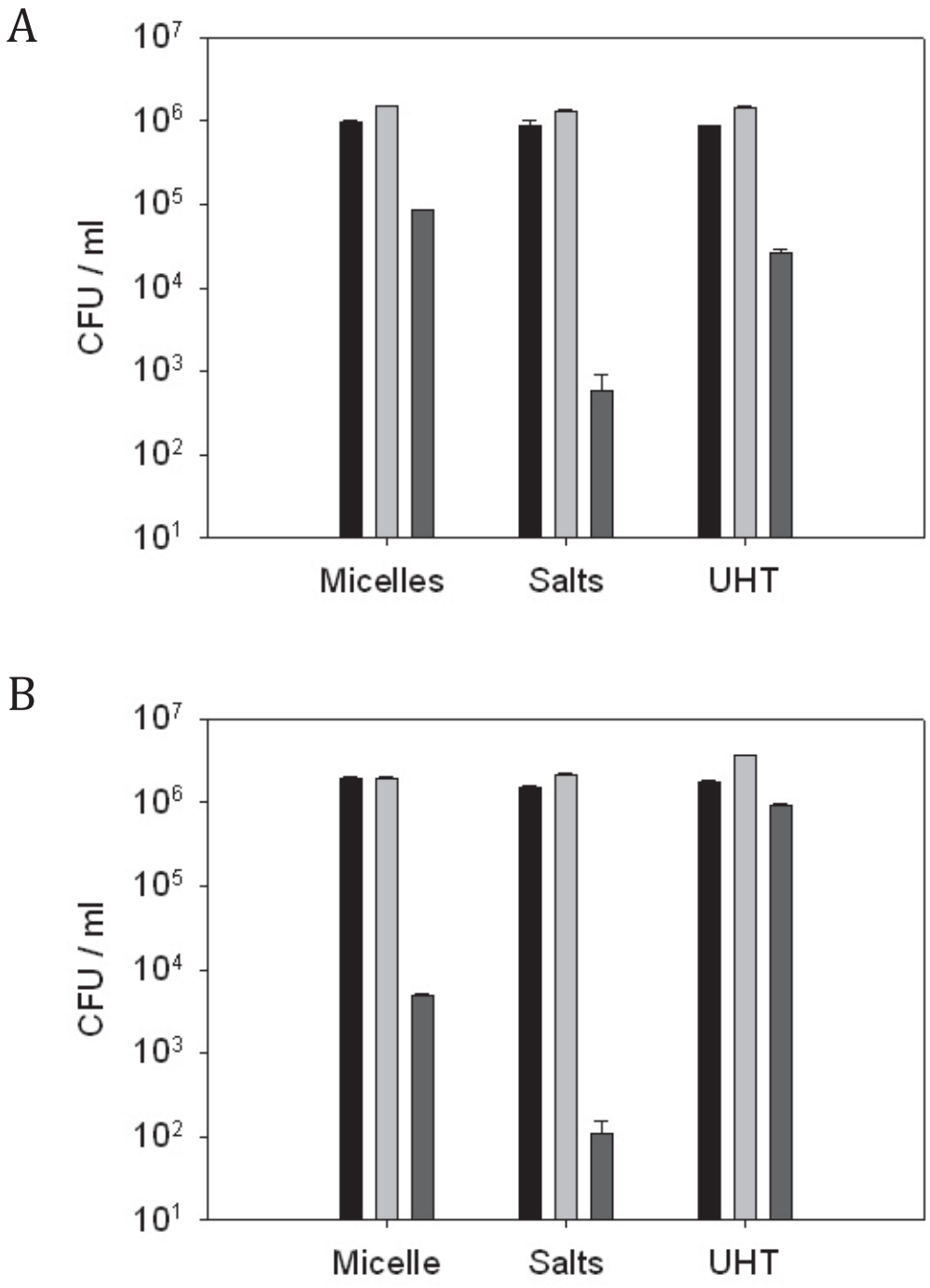

Figure 15 – Survival of *S. aureus* (28) (A) and *S. flexneri* (66) (B) during an acid challenge at pH 2.5 in the presence of UHT milk, an artificial casein micelle preparation and mixture of salts used to create the casein micelle preparation. Plate counts are presented for the initiation of the experiment (■), and after incubation for 2 h at pH 7 (■) and pH 2.5 (■). The error bars denote one standard deviation. The limit of detection was 10 CFU / ml.

5.2.2 Interaction between the Bacterial Cells and the Casein Micelles

Transmission electron microscopy was used to visualise the interaction between the casein micelles and *E. coli* (32). At neutral pH, casein micelles formed a layer at the surface of the bacterium, suggesting that they may be bound or interacting with the bacterial surface (Figure 16A). However at pH 2.5, the acidic environment caused the casein of the micelle to curdle. *E. coli* (32) was observed within a casein curd particle (Figure 16B).

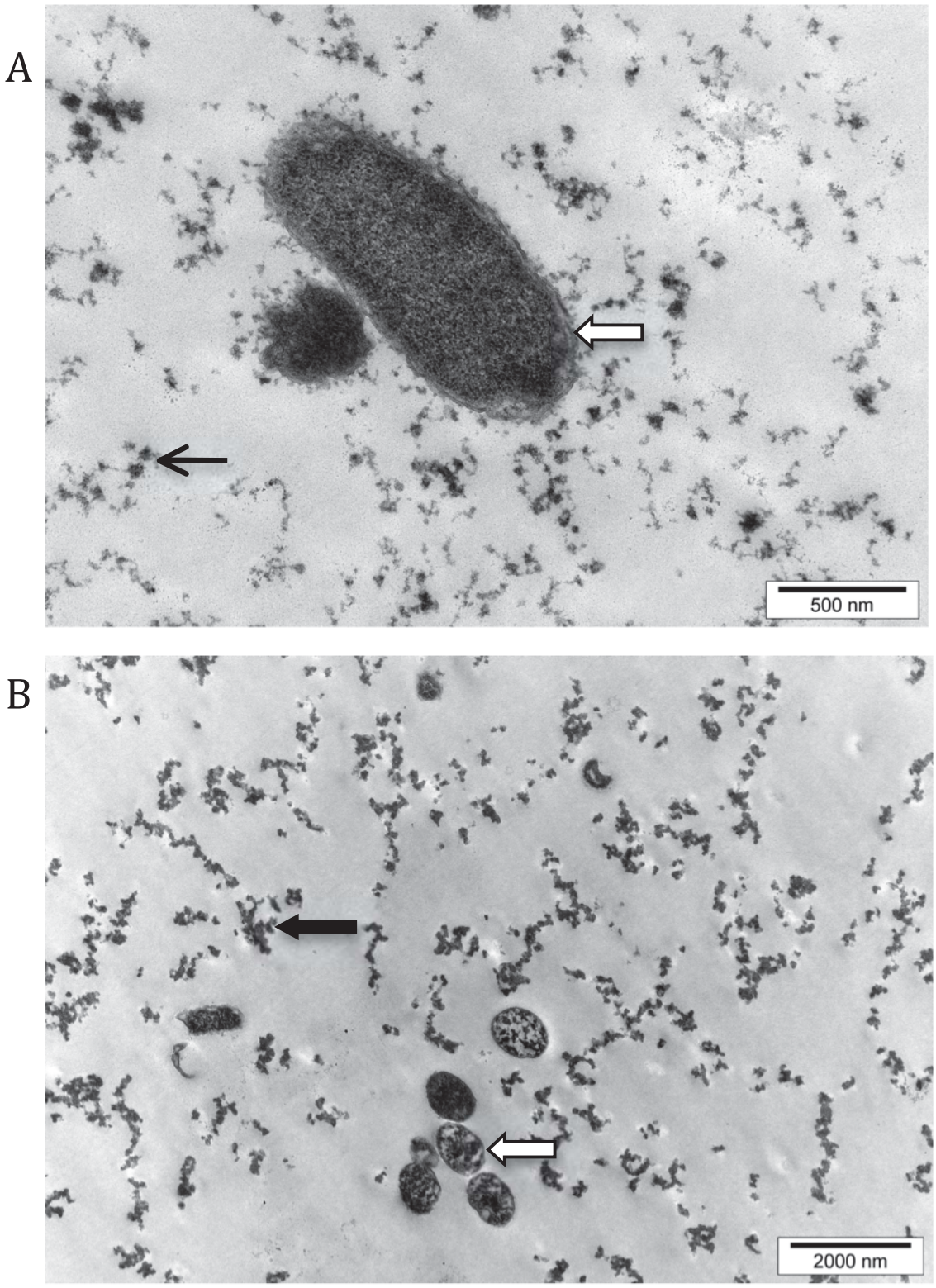

Figure 16 – *E. coli* (32) in reconstituted casein micelles at pH 7 (A) and in a cross-section of a curdled casein particle at pH 2.5 (B), imaged by transmission electron microscopy. The arrows denote the bacterial cell (⇦), casein micelles at pH 7 (⟵) and the casein curd at pH 2.5 (⬅).

5.2.3 The Effect of Pre-Incubation of Bacteria in Casein Micelle Preparations on Acid Tolerance

Electron microscopy revealed that casein micelles might bind or interact with the surface of *E. coli* (32) prior to acidification. For all previous acid tolerance experiments, bacterial isolates were suspended in the casein micelle preparation only minutes before acidification. However, an increased exposure time prior to acidification may allow increased association between the casein micelle and the bacteria. To test this hypothesis, *S. aureus* (28) and *S. flexneri* (66) were suspended in a casein micelle preparation 60 minutes and five minutes before acidification, to a final pH of 2.5. Bacterial concentration was determined by plate count at 0, 5, 30, 60 and 120 min during the incubation at pH 2.5. The bacterial inoculum was normalised to an initial count of 1.0×10^5 and 1.0×10^6 for *S. aureus* (28) and *S. flexneri* (66) respectively. The concentration of bacteria recovered from the sample pre-incubated in casein micelle for five minutes or 60 minutes were comparable (Figure 17). During the incubation, a steady decrease in recovery of both strains was observed, with an average reduction in bacteria recovered of 0.98 $\log_{10}$ and 1.2 $\log_{10}$ for *S. aureus* (28) and *S. flexneri* (66) respectively at each time point.

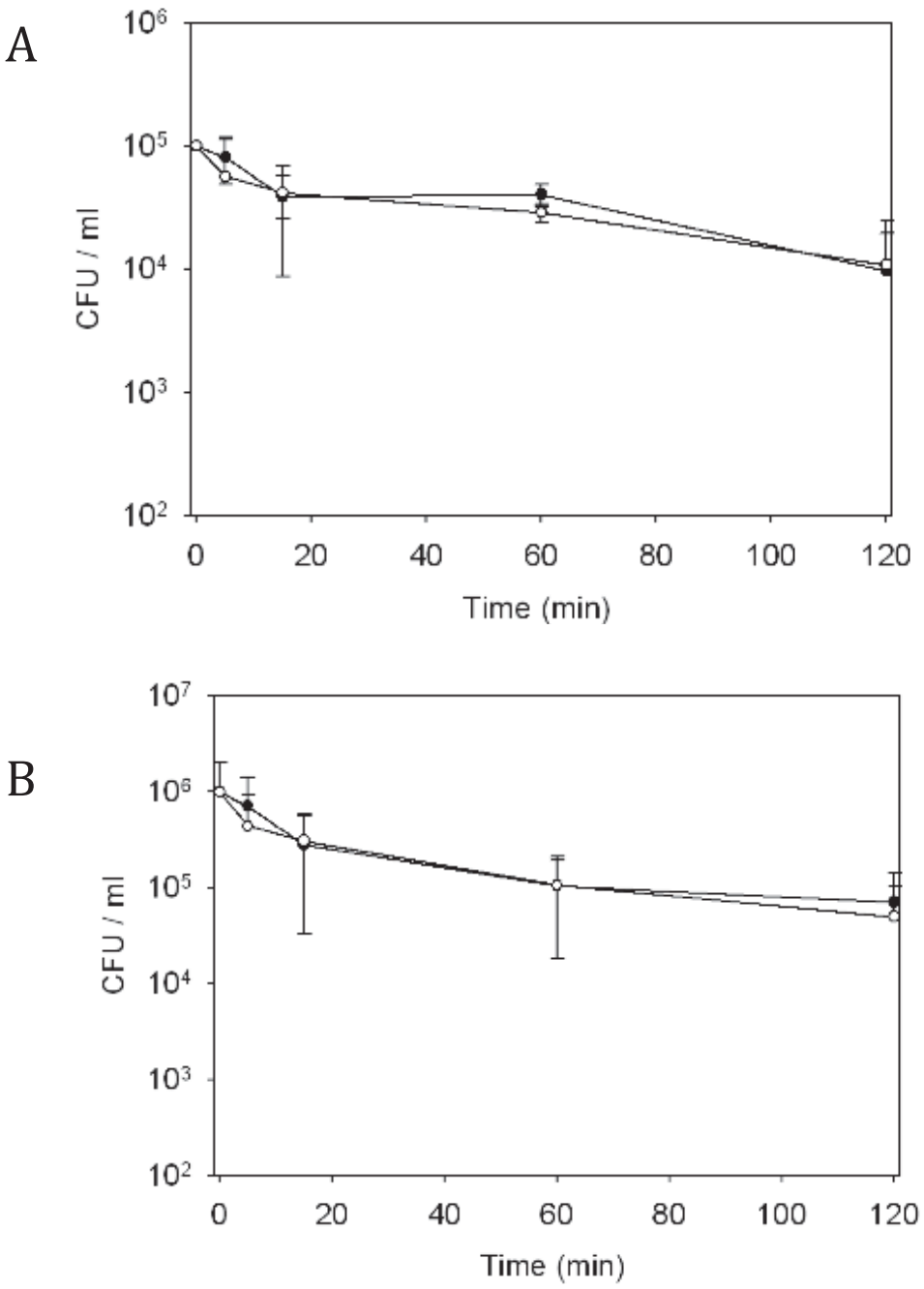

Figure 17 – Survival of *S. aureus* (28) (A) and *S. flexneri* (66) (B) pre-incubated in the micelle preparation for 5 minutes prior to acidification (○) and pre-incubated in the micelle prep for 60 minutes prior to acidification (●). Data normalised to initial concentration of 1×10^5 (A) and 1×10^6 (B). The error bars denote one standard deviation.

5.3 Protective Mechanisms of Ionic Milk Constituents

The salts used to reconstitute the casein micelles improved the survival of both *S. aureus* (28) and *S. flexneri* (66) during an acid challenge. These salts were investigated further to determine which salts provided this protection, and how their interaction increased the acid tolerance of *S. aureus* (28) and *S. flexneri* (66).

5.3.1 Acid Tolerance of Individual Salts that Comprise Casein Micelles

To determine which of the salts that were present in the casein micelle preparation afforded acid tolerance to *S. aureus* (28) and *S. flexneri* (66), both strains were suspended in 0.5 × strength TSB at a concentration of 1×10^5 CFU / ml. The cultures were incubated at pH 2.5 containing either 20 mM $CaCl_2$, 11 mM K_2HPO_4, or 5 mM K_3citrate, the same final concentrations used in the preparation of the casein micelles. *S. aureus* (28) survived in 20 mM $CaCl_2$ where 1.9×10^2 CFU / ml were recovered and K_2HPO_4 with 1.3×10^2 CFU / ml recovered after two hours at pH 2.5 (Figure 19). No survival was observed in TSB supplemented with K_3citrate at pH 2.5. *S. flexneri* (66) survived exposure to pH 2.5 in the presence of K_2HPO_4, with 2.2×10^2 CFU / ml recovered. A recovery of 11 CFU / ml was observed in $CaCl_2$ but *S. flexneri* (66) was not recovered from pH 2.5 TSB supplemented with K_3citrate (Figure 18).

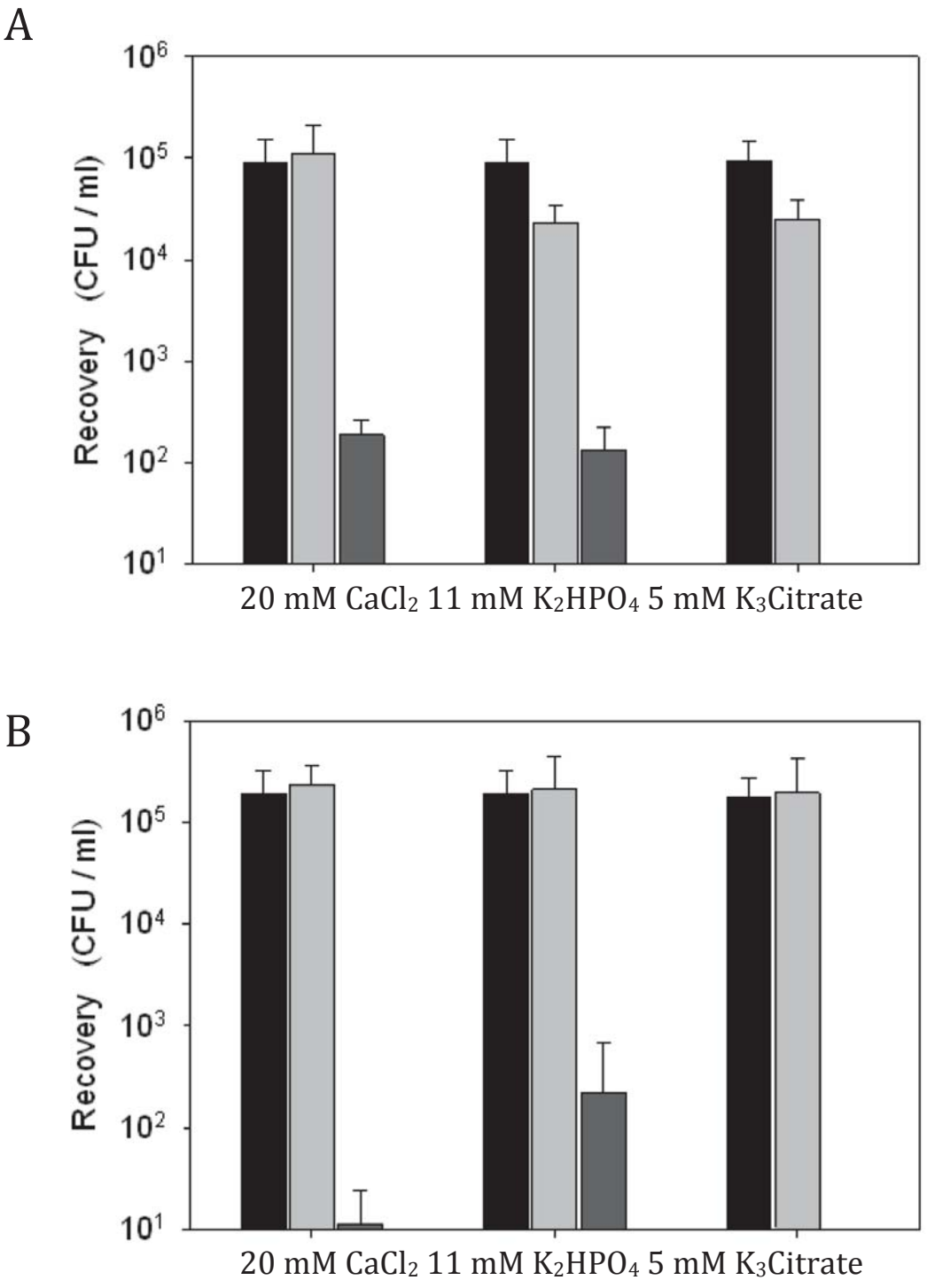

Figure 18 – Survival of *S. aureus* (28) (A) and *S. flexneri* (66) (B) in 0.5X TSB broth supplemented with 20 mM $CaCl_2$, 11 mM K_2HPO_4 or 5 mM K_3citrate after incubation at pH 2.5 for 2 h. Plate counts are presented at the initiation of the experiment (■), and after incubation for 2 h at pH 7 (■) and pH 2.5 (■). The error bars denote one standard deviation of triplicate experiments. The limit of detection was 10 CFU / ml.

5.3.2 Acid Tolerance with $CaCl_2$ and Dose Response of K_2HPO_4

Colloidal calcium phosphate is the main salt present in the casein micelle and structurally holds the particle together (Knoop *et al.*, 1979; Semo *et al.*, 2007). In the reconstituted casein micelle preparation, colloidal calcium phosphate was formed by the addition of $CaCl_2$ and K_2HPO_4 to a sodium caseinate solution. It was hypothesised that specific concentrations of K+ and Ca^{2+} ions were able to independently increase the tolerance of bacteria to acid stress. To test this hypothesis, the survival of *S. aureus* (28) and *S. flexneri* (66) was tested using a range of K^+ concentrations, both as chloride and phosphate salts, in both the presence and absence of 20 mM Ca^{2+}. The salts were dissolved in a 0.5 × TSB solution, comparable to the method using UHT milk and its components. To allow for the comparison of independent acid tolerance assays, bacterial concentrations recovered were normalised to an initial concentration of 1×10^5 CFU / ml for *S. aureus* (28) and 1×10^6 CFU / ml for *S. flexneri* (66).

S. aureus (28) did not survive well at K_2HPO_4 concentrations below 50 mM, except for a spike of survival at 15 mM. At 100 mM K^+ a recovery of 1×10^2 CFU / ml was observed after 2 h exposure to pH 2.5 (Figure 19). In the presence of 20 mM Ca^{2+}, survival increased to between 1.6×10^3 CFU / ml and 4.0×10^4 CFU / ml, irrespective of the K^+ concentration. At 50 mM K^+, however, no survival was observed either with or without Ca^{2+}. *S. flexneri* (66) did not survive exposure to pH 2.5 in the presence of between 7 mM and 25 mM K_2HPO_4 concentrations, but recovery was observed above 50 mM K^+. When 20 mM Ca^{2+} was added, a 1.5 - 3 $\log_{10}$ increase in recovery was observed at K^+ concentrations between 7 mM and 25 mM, but at 50 mM K^+ and above, the addition of Ca^{2+} did not improve the survival of *S. flexneri* (66).

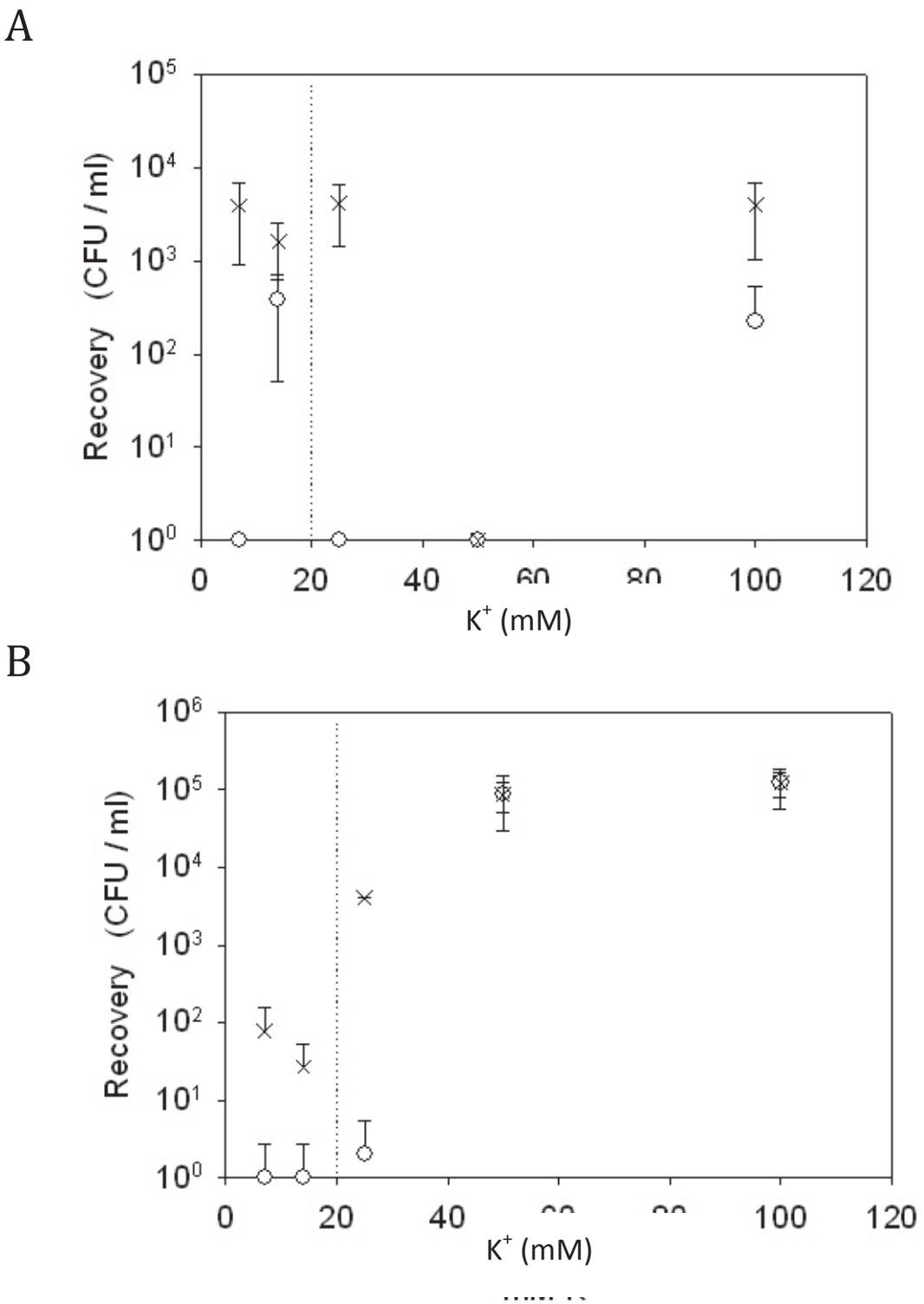

Figure 19 - Acid tolerance of *S. aureus* (28) (A) and *S. flexneri* (66) (B) as a function of K^+ from K_2HPO_4 (○) and K_2HPO_4 with 20 mM $CaCl_2$ (×). The K^+ concentration of a milk / TSB mixture is depicted by a dotted line (⋮). Data were normalised to an initial plate count of

10^5 CFU / ml for *S. aureus* (28) and 10^6 CFU / ml for *S. flexneri* (66). The error bars denote one standard deviation. The limit of detection was 10 CFU / ml.

5.3.3 Acid Tolerance with $CaCl_2$ and Dose Response of KCl

K_2HPO_4 appears to protect both *S. aureus* (28) and *S. flexneri* (66) at pH 2.5. However, phosphate is a key anion present in casein micelles that may be involved in bacterial survival at pH 2.5. To determine if phosphate had an effect on the bacterial survival during incubation at pH 2.5 for two hours, the experiment was repeated using KCl to determine the effect of the Ca^{2+} and K^+ salts. Initial bacterial concentrations were normalised to 1×10^5 CFU / ml for *S. aureus* (28) and 1×10^6 CFU / ml for *S. flexneri* (66). Both isolates showed little or no survival at K^+ concentrations below 50 mM, but at 100 mM, *S. aureus* (28) was recovered at 1.6×10^5 CFU / ml and *S. flexneri* (66) was recovered at 1.9×10^5 CFU / ml (Figure 20). An increase in survival was observed when 20 mM Ca^{2+} was added at low K^+ concentrations. However, at high K^+ concentration, addition of Ca^{2+} correlated with a decrease in recovery at pH 2.5, for both *S. aureus* (28) and *S. flexneri* (66) (two tailed $p = 0.059$ and $p = 0.002$ respectively). The survival of both isolates was comparable in both K_2HPO_4 and KCl at the equivalent K^+ concentrations, both when calcium was present or absent.

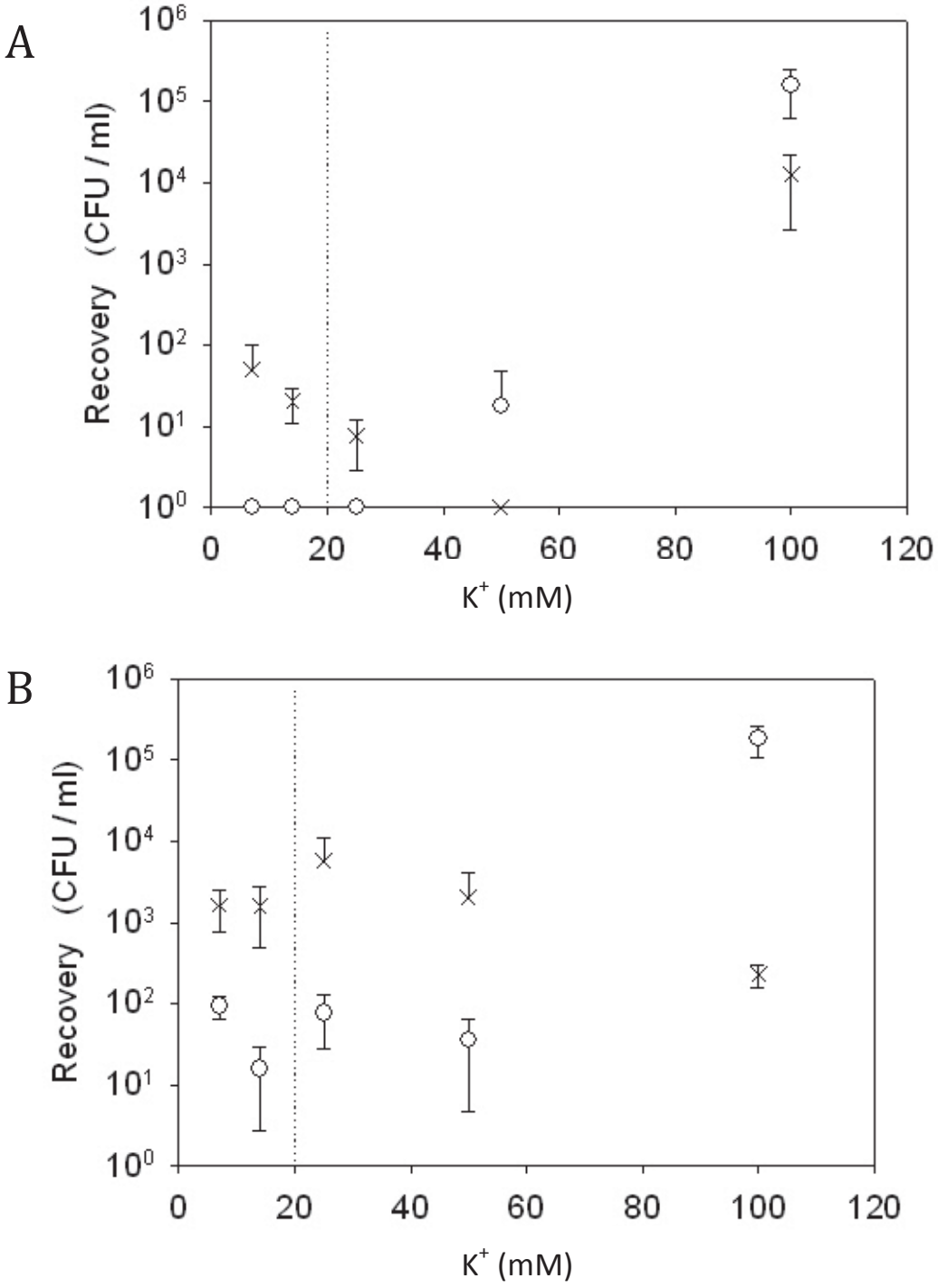

Figure 20 – Survival of *S. aureus* (28) (A) and *S. flexneri* (66) (B) as a function of K^+ from KCl (○) and KCl with 20 mM $CaCl_2$ (×) after incubation at pH 2.5 for 2 hours. The K^+ concentration of a milk / TSB mixture is depicted by a dotted line (⋮). Data values were normalised to an initial plate count of 10^5 CFU / ml for *S. aureus* (28) and 10^6 CFU / ml *S. flexneri* (66). The error bars denote one standard deviation. The limit of detection was 10 CFU / ml.

5.4 High-Throughput Acid Tolerance in Salts

The salt solution used to reconstitute casein micelles appeared to have an effect on the survival of a Gram-positive bacterium and a Gram-negative bacterium (Figure 15). These salts are naturally present in the milk at concentrations similar to those used in this study (Jenness, 1974). To determine if these salts have a wider impact on the survival of raw milk flora during acid challenge at pH 2.5, a high-throughput analysis was performed using a subset of 275 raw milk isolates comprised of isolates from both the Waikato and the Manawatu. This subset was considered, as there are data available under all conditions tested. The survival of this subset of Manawatu isolates was compared between a TSB/salt mixture and TSB alone.

Of the 275 isolates that were tested, the number of isolates that showed total survival, i.e. all replicates of an isolate that survived acid exposure, increased from 62 (22.5 %) to 122 (44.4 %) in the presence of the salt solution (Table 22). However, the number of isolates that showed a partial degree of survival was reduced, as 128 isolates (46.5 %) showed survival in TSB broth, and 160 (13.8 %) in TSB broth and salts. A GLMM statistical model was applied to the data to determine if this difference in survival was statistically significant. The proportion of isolates that survived was compared using the Logit transformation to ensure that any variation was symmetrical. For those isolates originally obtained from raw milk, 37.4 % were acid tolerant in TSB, which increased to 41.0 % when the salts solution was added. These data are obtained from the mean back transformed proportions of the GLMM model (Figure 21). This increase was not statistically significant. Bacterial isolates obtained from acidified raw milk were analysed separately. In TSB alone, 67.2 % of isolates were acid tolerant, compared with 72.6 % in TSB with salts. This increase was not statistically significant either.

Table 22 – Acid tolerance of 275 isolates in TSB, and TSB supplemented with 20 mM $CaCl_2$, 11 mM K_2HPO_4 and 5 mM K_3citrate. 'Total acid tolerance' means all replicates survived, 'partial acid tolerance' means at least one replicates survived; 'no acid tolerance' means no replicates survived exposure to pH 2.5 for 2 h (see appendix 5).

Replicate	**TSB**	**TSB with Salts**
Total Acid Tolerance	62	122
Partial Acid Tolerance	128	38
No Acid Tolerance	85	115
Total	275	275

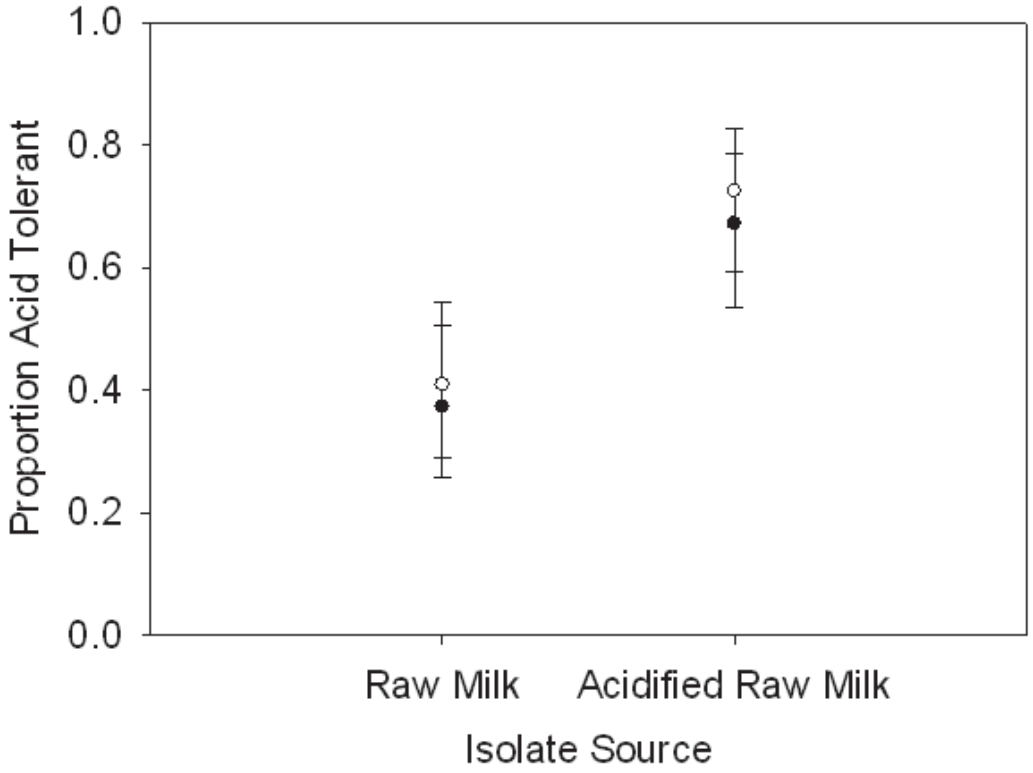

Figure 21 - Back-transformed mean proportion of isolates from raw milk or acidified raw milk that are acid tolerant at pH 2.5 for 2 h in TSB (●), or TSB with salts (○). The error bars denote the upper and lower confidence intervals (95 %). Data obtained using high-throughput acid tolerance survival data and the GLMM model (appendix Six).

5.5 Discussion

In chapter four, milk was shown to protect 12 out of 20 raw milk bacterial isolates exposed to acid conditions (pH 2.5). In this chapter, the mechanism for this protection was explored. The minimum pH tolerated by six different strains, was determined by assessing bacterial survival over a range of pH values (Figure 10). The survival of these same six isolates was then again tested at the highest pH not tolerated, but in the presence of UHT trim milk to determine if these isolates are able to survive a lower pH than previously possible. All isolates tested were able to survive at a lower pH in the presence of milk, than they could in TSB broth alone. These data show that milk is able to confer protection to a range of different organisms, and thus suggests that the mechanism by which milk protects bacteria is due to the milk itself, rather than a physiological change in the bacteria that the milk protects.

Isolate 66 was most closely related to *S. flexneri* when identified by 16S rDNA analysis. This is a surprising identification because *S. flexneri* is only found in human reservoirs (Murray, 2011). It is unusual to find this bacterium in a sample of raw cows' milk. Secondly, during biochemical characterisation this isolate was found to be oxidase positive. This test was performed after growth on TSA agar as opposed to a blood agar. The type of agar used may have interfered with the result. Given the doubt about the oxidase result, this isolate was identifed by molecular means only.

Isolate 32 was not conclusvely identified, but is most likely related to *E. coli*. It is able to ferment lactose on MacConkey agar, and was strongly acid tolerant. Based on this characterisation, isolate 32 was referred to as *E. coli* (32) in this chapter. It will be interesting to confirm these identifications using complete genome sequencing to remove any doubt about their identities. It would be an interesting research proposal to investigate the different acid tolerance mechanisms in these different but closely related bacteria.

This protection may be derived from several areas of the milk matrix, including amino acids (Figure 13), whey protein (Figure 14), casein micelles (Figure 15) and the mineral content (Figure 15). The mineral content, specifically the salts that form an integral part of the casein micelle, had an unexpected but noticeable effect on the survival of *S. aureus* and *S. flexneri*, compared with TSB broth alone, which did not allow these organisms to survive. Based on these data, the milk matrix provided protection to the milk flora by the

cumulative action of the protein component, as well as the mineral components that were present in the casein micelle.

No single dominant protective mechanism was identified, suggesting that the protective effect of the milk matrix was composed of a number of protective effects. However, several milk components were more effective than others, in particular the interaction with the casein micelle. In milk, this interaction may occur prior to ingestion and thereby to acidification, predisposing the bacteria to be trapped in the curdled casein particle where they may endure a milder pH than in the external environment outside the curd particle (see Figure 16). Such an interaction of bacteria with the casein micelle has been described for the probiotic, *Lactobacillus rhamnosus* using Atomic Force Microscopy adhesion force analysis (Burgain *et al.*, 2012). The pH of the interior of the curdled casein particle after acidification was not measured in this study. However, the open nature suggests that protons could move freely in and out of the structure. It may be hypothesised that protection from extreme pH is only a temporary effect that allows a bacterium to survive longer periods at the extreme pH because the pH is lowered less rapidly than in broth alone.

If the casein curd was not physically shielding bacteria for the entire two-hour incubation, another mechanism must aid their survival at low pH. The bacterium may be further protected by the mineral components of the micelle that are released when the micelle denatures. At acidic pH, the colloidal calcium phosphate particle dissolves (Dalgleish *et al.*, 1989). The exact concentration of dissolved ions inside the denatured casein particle was not measured; however, it is feasible that the concentration of ions that form the micelle, notably Ca^{2+}, or HPO_4^{2-} become available after the disintegration of the casein micelle. These minerals may provide additional protection to the bacteria during the early stages of exposure. Furthermore, the concentration of protons may initially be buffered inside the curd particle by the biomass and the release of ionic phosphate, such that the bacterium receives a delayed exposure to extreme pH outside the particle. However, such protection from proton stress is likely to be short-lived due to the porous nature of the curd matrix, and it is likely that equilibrium concentrations are met long before the end of the 2 h incubation period.

The mineral components of milk, notably Ca^{2+} and K^{+} have been shown to provide direct protection for the Gram-positive *S. aureus* (28) and a Gram-negative *S. flexneri* (66). There is no specific literature describing the effect of calcium on the survival of bacteria under acid conditions. However, Ca^{2+} is required for the growth of *Rhizobium meliloti* in acidic

soils (Howieson *et al.*, 1992). Furthermore, calcium has been shown to increase the survival of *Lactobacillus acidophilus* during freezing (Wright *et al.*, 1981). Despite the common use of dairy products in the delivery of probiotics, the potential protective effects of calcium ions against low pH has not previously been shown in this way. Calcium is a divalent cation known to have a structural role in prokaryotic cell walls (Smith, 1995). This was demonstrated through the action of chelators, such as EDTA, that can increase the permeability of cell membranes by stripping away the divalent ions (Vaara, 1992). The presence of Ca^{2+} ions may therefore help stabilise membrane proteins during stress imposed on the membrane by protons

The second mechanism by which the mineral components of milk may protect bacteria at low pH is via proton pumps that ultimately regulate the concentration of protons in the cytoplasm. Cation-proton antiporters are involved in the uptake of K^+ ions in exchange for H^+ and result in the net increase of cytoplasmic pH (Nakamura *et al.*, 1984). The survival of both *S. aureus* and *S. flexneri* was improved at high K^+ levels, suggesting that such protection may be important. However, the concentrations at which this effect was observed *in vitro* was greater than what is normally found in milk, therefore K^+/H^+ antiporters specifically are not likely to be the main driver for protection at low pH in milk.

Amino acids are known to both trigger and drive some bacterial acid tolerance mechanisms (Richard *et al.*, 2004). As milk is digested by gastric proteases, free amino acid concentrations will increase, and free arginine and glutamate would become available for bacterial cells to utilise for the protection at low pH. This protection is likely only to be available after gastric digestion of casein and other proteins present in milk. The concentration of free amino acids from the digestion of casein in the stomach may be low, as digestion of casein may not be complete until the digestate reaches the duodenum (Chabance *et al.*, 1998). In *E. coli* the arginine system is induced only be low pH and growth in complex media containing arginine (Castanie-Cornet, 1999). The minimum concentration required for the expression of the amino-acid dependent acid resistance mechanisms in *E. coli* has not been found in the literature. However, glutamate and arginine are both used catabolically during this process, so protection is directly proportional to concentration in the media. It is not known if trace levels of amino acid are able to trigger other acid tolerance responses, or influence bacterial survival later in gastric transit.

The protective effect observed when bacteria are exposed to pH 2.5 in the presence of milk, or milk fractions appears to be limited to milk, as there was no protection was observed when bacteria were incubated at pH 2.5 in soymilk. According to the manufacturers' specifications, Sanitarium® soymilk contains 3.1 g / 100 ml [1] protein, below that of UHT skim milk which contains 3.7 mg / 100 ml [2]. The calcium content is equivalent, as soymilk contains 30 mM Ca^{2+} compared to 33 mM Ca^{2+} in skim UHT milk. Perhaps calcium is not as readily bioavailable in soymilk compared to cows' milk, or bacteria may interact with the soy protein differently compared to bovine milk, thereby enabling bacteria to more readily survive in dairy products.

The milk matrix is a complex array of proteins and minerals that together provide protection to bacteria from the effects of proton stress, such as that which would occur when milk is ingested. Both standard and skim UHT milk exhibit this effect, but not a comparable soy based product. Casein, structured as micelles, was found to protect bacteria when exposed to pH 2.5. This protection was not observed in the presence of casein alone. The salts present in casein micelles, notably calcium, were shown to contribute to the protection of bacteria from proton stress at pH levels found in the stomach, but the salts alone did not account for the protection that is observed in casein micelles. Clearly this is a multi-factorial process, with both bacterial physiological changes combined with environmental factors enhancing survival.

1 (http://www.sanitarium.co.nz/products/beverages/so-good-chilled/so-good-lite (retrieved 15 August 2013)

2 (http://www.fonterrafoodservices.co.nz/component/ffpr/?view=product&id=224&manufacture=0, (retrieved 15 August 2013).

6. General Discussion

6.1 Discussion

Raw milk is often consumed by those who believe it to be superior to pasteurised product or who have raw milk readily available because of a rural lifestyle (Hegarty *et al.*, 2002). Despite the risk of serious food borne illness, cohort studies have shown those who drink raw milk from a young age have lower rates of asthma or allergy compared with their peers that drink processed milk (Table 2). There are many factors, both genetic and environmental, that can affect the onset of allergic disease in an individual (Holgate, 1999). The hygiene hypothesis was proposed after it was observed that those who were exposed to less hygienic environs tended to have lower incidences of allergic disorder, such as hay fever (Strachan, 1989). The consumption of raw milk may be linked with lower rates of allergic disease in this manner owing to the bacterial flora that raw milk contains. This flora is lost when milk is pasteurised, leading to the difference in the incidence of allergic disease in those that consume raw milk, with those that drink pasteurised milk bought commercially.

Bacterial diversity studies have shown that raw milk contains a wide range of microflora. This has been shown using culture-dependent methods (Mallet *et al.*, 2012; Quigley *et al.*, 2013b; Thomas *et al.*, 1962) and culture-independent methods (Delbes *et al.*, 2007; Rasolofo *et al.*, 2010). In summary, the key microbial groups found in milk are the *Microbacterium, Lactobacillus Lactococcus, Enterococcus* and *Chryseobacterium* genera. Less prevalent, genera include the *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Corynebacterium* and *Acinetobacter* (Table 3). This study also showed that milk contains a diverse flora including both Gram-positive and Gram-negative bacteria.

The bacteria found in raw milk are the same as those found in the broader farming environment. These can enter milk in a number of different ways, such as through contamination by mud or faeces, or skin bacteria released from the udder itself during milking (Christiansson *et al.*, 1999; Desmasures *et al.*, 1997; Gleeson *et al.*, 2013; Te Giffel *et al.*, 2002). Exposure to the farming environment from a young age is also a statistically significant factor in the reduction of allergic disease (Kilpelainen *et al.*, 2000; Riedler *et al.*, 2001). It has been shown that the protective effect of raw milk is maintained in those that

do not live on farms (Loss *et al.*, 2011). Raw milk, especially when drunk by those that do not live on farms, may be a carrier for the bacteria that are normally associated with farm exposure.

There can be little doubt that the foods we eat can impact our health. However, to reach the lower GI tract and stimulate the immune system, bacteria must survive gastric digestion. This study showed that a range of both Gram-positive and Gram-negative bacteria present in raw milk were able to survive the pH (pH 2.5) and would therefore be likely to transit the acidic environment of the stomach (Figure 6, Tables 20 & 21). This is in a agreement with other studies that demonstrated that a number of genera commonly found in raw milk, such as *Escherichia*, *Salmonella or Bacillus* were tolerant to pH as low as pH 2.5 (Cotter *et al.*, 2003; Gorden *et al.*, 1993).

The survival of milk microflora through the stomach depends on both the intrinsic ability of the bacterium to survive acid exposure and the extrinsic protective effect of the food matrix. Milk protects bacteria from the effects of stomach acid by buffering against the effects of hydrochloric acid (Mainville *et al.*, 2005). However, this is most likely a temporary effect, as the stomach produces acid soon after food is ingested, and the normal acidic pH of the stomach is soon restored. In this study, a number of raw milk isolates have been tested for their ability to survive exposure to pH 2.5 for two hours. It would be interesting to expand this research to capture a larger group of milk flora and to determine how these survive in a more realistic model incorporating digestive enzymes and accurate stomach emptying times.

When ingested, some of the Gram-positive and Gram-negative bacteria present in raw milk will survive acidic conditions such as that may be present in the stomach. This study has shown that the survival of these bacteria increased in the presence of the milk matrix (Figures 5 & 6). Milk proteins, specifically the intact casein micelle, in combination with the mineral content were able to protect *S. aureus* and *S. flexneri* at pH 2.5 even after the buffering effects of the milk was negated. This is the first time that this effect has been assayed on other microflora of milk that are not recognised as probiotic species.

This study has isolated and identified bacteria in both groups that are not only present in milk, but are also more readily able to survive acid exposure at pH 2.5 in the presence of milk. One *Lactobacillus paracasei* was identified (Table 20), an organism that has probiotic properties (Verdenelli *et al.*, 2009). However, the number of probiotic bacteria identified by high throughput acid tolerance assays was very low. Bacteria identified that could have

pathogenic properties include *Staphylococcus*, *Streptococcus*, and *Micrococcus* (Table 20). In total, 19 *Staphylococcus* isolates made up from 7 different species were identified. The Staphylococci are regarded as being important in establishing gut microbiota in early life, and may also be found in high number in human breast milk (Asquith *et al.*, 1979). *S. aureus* may adhere to the GI tract mucus lining (Vesterlund *et al.*, 2006), and therefore reach a position where it may interact with the immune system at the gastrointestinal tract.

The exact composition of microorganisms that are to be able to withstand acid exposure and reach the gastrointestinal tract when raw milk is consumed is likely to be highly affected by environmental and working practices at farms where milk is obtained. What is clear is that the diversity of raw milk microflora is greater than that of processed milk (Magurran, 2004), a point also demonstrated in this work by the greater level of species richness identified in raw milk. It is therefore certain that the diversity, or richness of bacteria reaching the GI tract would be greater when raw milk is consumed. It is also plausible that these organisms are able to reach the gastro intestinal tract where they may be involved in immune stimulation. Bacterial species may not need to have specific probiotic or pathogenic properties. It may simply be enough for these environmental bacteria, with the capacity to become opportunistic pathogens, to test the immune system on a regular basis. This could readily be achieved through the regular consumption of unpasteurised cows milk. This means that research should not be limited to probiotic or pathogenic organisms, but the entire flora ingested should be considered as a whole.

Pasteurisation is a method that reduces the live bacterial counts in milk by thermal inactivation. The microflora that are killed when cows' milk is pasteurised may be responsible for this observed reduction in the rates of asthma and/or allergic disease. Bacterial exposure is known to affect the incidence or severity of allergic disease, as there is a correlation between rates of infectious disease and childhood asthma (Liu, 2007). In addition the administration of the probiotic *Lactobacillus rhamnosus* has been shown to reduce the impact of atopic eczema (Kalliomäki *et al.*, 2007). Loss (2011) demonstrated through the large cohort GABRIELA study that the protective effect of raw farm milk was lost when raw milk was boiled at home.

The correlation between consuming raw milk and reduced rates of allergic disease is most strong when raw milk is consumed in the first five years of life, and particularly at the younger end of this range (Loss *et al.*, 2011; Riedler *et al.*, 2001; Waser *et al.*, 2007). This is also a time when the gut microflora is still maturing, and the impact of raw milk microflora

on the development of the immune system is likely to be the strongest. Although the ages at which children typically begin to drink raw milk is not available, it is likely to vary from household to household.

Bacteria are not removed from milk when it is pasteurised, but remain as dead cells. These cells can potentially interact with the immune system in the gut when they are ingested. However, the protective effect of milk flora is either reduced or absent in pasteurised milk. Therefore, it is more likely that bacteria need to be alive to elicit a protective effect. This strengthens an argument that this protective effect is due to the interactions of probiotic or pathogenic bacteria with the immune system, rather than any antigenic constituents of these bacteria that remain. A similar trend is seen when live attenuated vaccines are used in comparison with killed vaccines (Belshe *et al.*, 2007; Sirard *et al.*, 1999). However, the interaction will be different as these are injected directly into the bloodstream, as opposed to interacting at the mucosal surface of the gut.

Exposure to the microflora of raw milk is likely to increase the diversity of bacteria that potentially can reside in the gut, either permanently or transiently and thus increase the diversity of bacterial interaction that can occur in the lower GI tract. Such interactions are likely to provide an appropriate immune stimulation of the T_H1/T_H2 pathway and reduce rates of allergic disorders (Kidd, 2003). This hypothesis is supported by the fact that diversity in gut flora has been linked to a reduced incidence of immune disorders (Forno *et al.*, 2008). By limiting the exposure to milk micro-flora in the gut by consuming pasteurised milk, the immune stimulation to 'foreign' microbes is greatly reduced, and is likely to have an impact, especially at a young age as the immune system is developing.

There have been many technological and sociological changes to the way we consume milk in the past two centuries. The advent of the industrial revolution saw the ability to centralise and standardise our foods, and production techniques meant that many of the foods we now consume have become cleaner from a microbiologically point of view. Milk is no exception, being routinely sold pasteurised since the early 1900s (Atkins, 1992). These changes in food production have greatly reduced exposure to food borne infectious diseases, but at the same time have decreased our exposure to non-pathogenic bacteria that would normally be present. These changes including the pasteurisation of milk, may have inadvertently contributed to the increase of immune dysfunction by limiting microbial exposure in the gut.

The incidence of allergic disease is a complex issue driven by both genetic and environmental factors (Holgate, 1999). The consumption of raw milk is one environmental factor that has been identified as being correlated with reducing the rates of allergic disease. However, the consumption of raw milk does not specifically exclude the development of allergic disease, but it may mitigate its effects. Such an effect is in accordance with the hygiene hypothesis, which states appropriate microbial exposure is able to stimulate the immune system in a manner not readily observed in modern lifestyles (Strachan, 1989).

6.2 Future Directions

Despite it being potentially beneficial to consume raw milk with the intent to stimulate the immune system, the risk of serious infectious disease, particularly in young children is of major concern. The next step in the elucidation of the impact that milk microflora may have on immune stimulation would be to identify those bacteria involved directly in immune stimulation. Such microorganisms could be cultured and added to milk and/or milk products in a safe manner, as novel probiotics. However, before we are able to take such steps, the exact interaction of the raw milk flora and the immune system needs to be understood. For instance, it is not implausible that a low but constant level of exposure to mild pathogens present in milk are able to stimulate the immune system, just as the presence of a select group of probiotic species could. The nature of these bacteria needs to be understood before they could be added to a commercial product.

Research in this thesis has shown that milk can protect a range of bacterial species against the effects of extremely low pH. However, some details about this protection require further research. In particular, the environment that bacteria within the casein micelle particle endure has not been completely investigated. It has been suggested in this work that the pH in these particles may be milder, but this has not been proved. It would be of interest to this work to investigate the pH inside curdled casein micelle particles so that the apparent mechanism of protection by the casein micelle particles can be clarified further.

A number of different raw milk bacteria have been identified as being able to survive gastric passage and reach a position to stimulate the immune system at the lower GI tract. The next step will be to identify which bacteria are able to stimulate the immune system, and what kind of responses they elicit. It may be helpful to focus on genera with probiotic or pathogenic properties. However, those that do not readily fit these categories cannot be ignored. It may be that the body's natural defences against a wide range of environmental organisms, such as those typically encountered in raw milk, is enough to stimulate the immune system, and help reduce the rates of allergy and asthma in those that regularly drink raw milk from a young age. There is still much scope for some interesting research to be conducted in this area.

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Appendices

Appendix 1 – Bacterial Species Commonly Associated with Milk

Genus	Species	Notes
Spirochetes		
Leptospira	*enterrogans*	Reservoir is wild and domestic animals. Shed in urine.
Gram-negative Aerobic Rods and Cocci		
Campylobacter	*jejuni*	Common cause of gastroenteritis, found in raw and improperly pasteurised milk.
Pseudomonas	*fluorescens* *fragi* *putida* *aeruginosa*	Found in soil and water, common in food spoilage. Psychotrophic, and can produce heat stable hydrolytic enzymes. P. aeruginosa may cause mastitis.
Xanthomonas	*maltophilia*	Isolated from water and milk.
Acinetobacter	spp.	Occur naturally in soil, water and sewage. Species may be psychrotrophic or mesophilic.
Moraxella	spp.	Capable of growth at phychrotrophic temperatures, and may be pathogenic.
*Shewanella**	*putrefaciens*	Commonly found in soil and water. Causes spoilage in milk and milk products, and may be an opporunistic pathogen.
Flavobacterium	spp.	Can cause psychrotrophic spoilage of milk or milk products.
Alcaligenes	spp.	Isolated from soil, water and dairy products. Some species may be thermotolerant
Brucella	*abortus* *melitensis*	Killed by pasteurisation, but may be an issue in post-pasteurisation contamination. May be pathogenic in humans.
Facultative Anaerobic Gram-negative Rods		

Escherichia	*coli*	Can cause severe mastitis, especially when the bedding material is heavily contaminated with faeces. Can spoil milk and other dairy products.
Salmonella	Typhimurium	May cause outbreaks of serious food poisoning.
Citrobacter	spp.	May be found in water, sewage or food.
Enterobacter	*aerogenes*	Occurs in the natural environment, water, soil and sewage.
Yersinia	*enterocolitica*	Psychrotrophic and can be isolated from raw and pasteurised milk.
Aeromonas	spp.	Found in drinking water, soil and food.
Chromobacterium	spp.	Common in soil and water, but less often found in milk.
Coxiella	*burnetii*	Infected animals may transmit this bacteria in their milk.
Gram-Positive Cocci		
Micrococcus	*varians*	Found on skin, water and milk. Strictly aerobic.
Staphylococcus	*aureus* *hyicus* *chromogenes* *epidermidis* *caprae*	*Staphylocuccus* Sp. may be found on skin or nasal membranes. May be involved in causing mastitis.
Streptococcus	*pyogenes* *agalactiae* *dysgalactiae* *uberis* *salivarius* *equisimilis* *zooepidemicus*	*Streptococcus* may be found on skin of cows. *S. uberis* is also found in raw milk and is a common cause of mastitis.
Enterococcus	*faecalis*	The presence of this bacterium is indicative of faecal contamination. May also cause mastitis.
Lactococcus	*lactis*	two subspecies, lactis or cremoris. May be used as starter cultures.
Leuconostoc	*mesenteroides* *paremesenteroides* *lactis*	Found in milk and milk products. These species may be used as starter cultures.
Spore-forming Gram-positive Rods and Cocci		
Bacillus	*cereus* *subtilis* *licheniformis*	Form spores in aerobic conditions. Bacillus spores can spoil UHT treated milk products. B. stearothermophilus is

	stearothermophilus *coagulans*	thermophilic. B. coagulans is aciduric, and can grow at a pH of pH 4.0.
Clostridium	*butyricum* *tyrobutyricum* *sporogenes* *perfringens*	Form spores in anaerobic conditions. Clostridia are found in sediment, and intestinal tract of humans and animals. Enter milk through faeces, soil and contamination by silage.
Non Spore-forming Gram-positive Rods		
Lactobacillus	*lactis** *helviticus* *acidophilus* *casei* *plantarum* *brevis* *fermentum*	Important bacteria for the dairy industry as these genera are used as thermophilic or mesophilic starter cultures for yoghurt and cheese.
Listeria	*monocytogenes*	L. monocytogenes is an important food bourne pathogen.
Kurthia	spp.	Occasionally isolated from milk, possibly due to contamination by animal faeces.
Corynebacterium	spp.	May cause urogenital tract infections or mastitis, and occasionally contaminate milk.
Arthrobacter	spp.	Very common in soil.
Brevibacterium	spp.	Most often found in cheese and human skin.
Caseobacter	spp.	Found on rind of soft cheeses.
Microbacterium	*lacticum*	May be thermotolerant.
Aureobacterium	*liquifaciens*	Found in milk, cheese, and dairy equipment.
Propionobacterium	*freundenrichii*	Found in raw milk, or cheeses.
Actinomyces	*pyogenes*	Possibly a commensal organisms to mucosal surfaces.
Mycobacterium	*tuberculosis* *bovis*	Mycobacterium cause tuberculosis in cattle and man. Grows slowly.

The bacteria listed in this table are reviewed extensively in (Robinson, 1990)

* Renamed since the publication of the original reference.

Appendix 2 – Characterisation of Waikato Isolates, September 2009

Isolate No	Media	Colony Morphology			Cell Morphology		Enzyme Reactivity				
		Size	Colour	Shape	Gram Stain	Cell Morphology	Catalase	Oxidase	Grouping	Heat Sensitivity[a]	Acid Tolerance
1	PCA	Medium	Cream	Round	Positive	Cocci	Positive	Negative	E	1	
2	PCA	Medium	Cream	Round	Positive	Cocci	Positive	Negative	E	0	
3	APT	Medium	Light Brown	Round	Positive	Rod	Positive	Positive	F	0	
4	APT	Large	Brown	Mucoid	Positive	Rod	Negative	Negative	F	0	
5	PCA	Small	Cream	Round	Positive	Rod	Positive	Negative	F	0	
6	PCA	Large	White	Irregular	Positive	Cocci	Positive	Positive	E	1	
7	PCA	Med	White	Irregular	Positive	Rod	Positive	Positive	F	0	
8	APT	Med	Cream	Round	Positive	Cocci	Positive	Negative	E	0	
9	Mac	Med	Pink	Irregular	Negative	Rod	Positive	Negative	B	0	
10	Mac	Med	Pink	Irregular	Negative	Rod	Positive	Negative	B	1	
11	Mac	Med	Pink	Irregular	Negative	Rod	Positive	Negative	B	0	
12	APT	Large	White	Round	Positive	Cocci	Positive	Negative	E	0	
13	APT	Large	Cream	Round	Positive	Rod	Positive	Negative	F	0	
14	APT	Large	Brown	Round	Positive	Rod	Negative	Negative	F	0	
15	APT	Large	Brown	Round	Positive	Rod	Negative	Negative	F	1	
16	PCA	Large	White	Filamentous	Positive	Cocci	Positive	Negative	E	1	
17	PCA	Large	White	Irregular	Positive	Rod	Positive	Positive	F	0	
18	PCA	Medium	White	Round	Negative	Rod	Positive	Negative	B	0	
19	PCA	Medium	White	Round	Negative	Rod	Positive	Negative	B	0	
20	APT	Small	White	Round	Positive	Cocci	Negative	Negative	D	0	0
21	APT	Small	White	Round	Positive	Cocci	Negative	Negative	D	0	0
22	APT	Small	White	Round	Positive	Cocci	Negative	Negative	D	0	0
23	APT	Small	White	Round	Positive	Cocci	Negative	Negative	D	0	0

24	APT	Small	Cream	Round	Positive	Cocci	Negative	Negative	D	0	0
25	APT	Small	White	Round	Positive	Cocci	Negative	Negative	D	0	0
26	APT	Small	Cream	Round	Positive	Cocci	Negative	Negative	D	0	0
27	APT	Small	Cream	Round	Negative	Cocci	Positive	Negative	A	0	0
28	APT	Small	Yellow	Round	Positive	Cocci	Positive	Negative	E	0	0
29	APT	Small	White	Round	Positive	Cocci	Negative	Negative	D	0	0
30	APT	Large	Cream	Round	Positive	Cocci	Positive	Positive	E	0	0
31	APT	Large	Cream	Round	Positive	Cocci	Negative	Negative	D	0	0
32	Mac	Medium	Pink	Round	Negative	Rod	Positive	Negative	B	0	1
33	Mac	Medium	Pink	Round	Negative	Rod	Positive	Negative	B	0	1
34	Mac	Medium	Pink	Round	Negative	Cocci	Positive	Negative	A	0	0
35	Mac	Medium	Pink	Round	Negative	Rod	Positive	Negative	B	0	0
36	Mac	Small	White	Round	Negative	Rod	Positive	Negative	B	0	0
37	Mac	Small	White	Round	Positive	Rod	Positive	Positive	F	0	0
38	Mac	Small	White	Round	Positive	Cocci	Positive	Negative	E	0	0
39	Mac	Small	White	Round	Negative	Rod	Positive	Positive	C	0	0
40	Mac	Large	Pink	Round	Negative	Rod	Positive	Negative	B	0	0
41	Mac	Medium	Pink	Round	Negative	Rod	Positive	Negative	B	0	0
42	PCA	Medium	White	Round	Negative	Rod	Positive	Positive	C	0	0
43	PCA	Small	White	Round	Negative	Rod	Positive	Positive	C	0	0
44	PCA	Small	White	Round	Negative	Rod	Negative	Negative	B	0	0
45	PCA	Small	White	Round	Positive	Cocci	Negative	Negative	D	0	0
46	PCA	Small	Orange	Round	Negative	Rod	Positive	Negative	B	0	-
47	PCA	Small	White	Round	Positive	Rod	Positive	Negative	F	0	-
48	PCA	Medium	White	Round	Positive	Cocci	Positive	Negative	E	0	0
49	PCA	Small	Cream	Round	Positive	Cocci	Positive	Positive	E	0	-
51	PCA	Small	White	Round	Positive	Cocci	Negative	Negative	D	0	-
52	PCA	Medium	Cream	Round	Positive	Cocci	Positive	Negative	E	0	1
53	PCA	Small	White	Round	Positive	Cocci	Negative	Positive	D	0	-
54	PCA	Small	White	Round	Positive	Rod	Negative	Positive	F	0	1
55	PCA	Medium	Brown	Round	Positive	Cocci	Negative	Negative	D	0	0
56	PCA	Medium	White	Round	Positive	Cocci	Negative	Negative	D	1	-

57	PCA	Medium	Yellow	Round	Positive	Cocci	Positive	Negative	E	0	1
59	PCA	Small	Yellow	Round	Positive	Rod	Positive	Negative	F	0	
60	Mac	Large	Pink	Round	Negative	Rod	Positive	Negative	B	0	
61	Mac	Med	Pink	Round	Negative	Rod	Positive	Negative	B	0	1
62	Mac	Large	Pink	Round	Negative	Rod	Positive	Negative	B	0	0
63	Mac	Med	Pink	Round	Negative	Rod	Positive	Negative	B	0	0
64	Mac	Large	Pink	Round	Negative	Rod	Positive	Positive	C	0	0
66	Mac	Small	White	Round	Negative	Rod	Positive	Positive	C	0	
67	APT	Med	White	Round	Negative	Rod	Positive	Negative	B	0	1
68	APT	Small	Cream	Round	Positive	Cocci	Negative	Negative	D	0	
69	APT	Small	Cream	Round	Negative	Rod	Positive	Negative	B	0	0
70	APT	Small	Cream	Round	Negative	Rod	Positive	Negative	B	0	0
71	PCA	Small	White	Round	Negative	Cocci	Positive	Negative	A	0	0
72	PCA	Large	Cream	Round	Negative	Rod	Positive	Positive	C	0	0
73	PCA	Small	Yellow	Mucoid	Negative	Rod	Positive	Negative	B	0	1
74	PCA	Small	White	Round	Positive	Cocci	Positive	Negative	E	0	1
75	PCA	Large	Yellow	Mucoid	Negative	Rod	Positive	Negative	B	0	0
76	PCA	Small	Orange	Round	Negative	Rod	Positive	Negative	B	0	0
77	PCA	Small	White	Round	Negative	Rod	Positive	Negative	B	0	
78	PCA	Medium	Cream	Round	Positive	Rod	Positive	Negative	F	0	
79	PCA	Medium	Cream	Round	Negative	Cocci	Positive	Positive	A	0	
80	PCA	Medium	White	Round	Negative	Cocci	Negative	Negative	A	0	0
81	PCA	Large	White	Mucoid	Negative	Rod	Positive	Positive	C	0	0
82	PCA	Medium	White	Round	Negative	Rod	Positive	Positive	C	0	1
83	PCA	Medium	White	Round	Negative	Rod	Positive	Negative	B	0	0
84	PCA	Large	Brown	Round	Positive	Cocci	Positive	Negative	E	0	1
85	PCA	Large	Cream	Round	Negative	Rod	Positive	Positive	C	0	-
86	PCA	Large	Cream	Round	Negative	Rod	Positive	Positive	C	0	1
87	PCA	Large	Cream	Round	Negative	Rod	Positive	Positive	C	0	1
88	PCA	Medium	Cream	Round	Negative	Rod	Positive	Positive	C	0	0
89	APT	Small	Cream	Round	Negative	Rod	Negative	Negative	B	0	
91	APT	Medium	Cream	Round	Positive	Cocci	Negative	Negative	D	0	

92	APT	Small	Brown	Mucoid	Negative	Rod	Positive	Negative	B	0	
94	APT	Large	Cream	Mucoid	Positive	Rod	Negative	Negative	F	0	
95	APT	Medium	Cream	Mucoid	Negative	Rod	Positive	Negative	B	0	
96	APT	Small	Cream	Round	Negative	Rod	Positive	Negative	B	0	
97	PCA	Large	White	Round	Negative	Rod	Positive	Negative	B	1	0
98	PCA	Large	White	Irregular	Negative	Rod	Positive	Positive	C	0	0
99	PCA	Large	Cream	Round	Negative	Rod	Positive	Positive	C	0	1
102	PCA	Small	White	Round	Positive	Cocci	Negative	Negative	D	0	
103	Mac	Medium	Pink	Mucoid	Negative	Rod	Positive	Positive	C	0	
105	Mac	Large	Pink	Round	Negative	Rod	Positive	Negative	B	0	
107	Mac	Medium	Pink	Round	Negative	Rod	Negative	Negative	B	0	

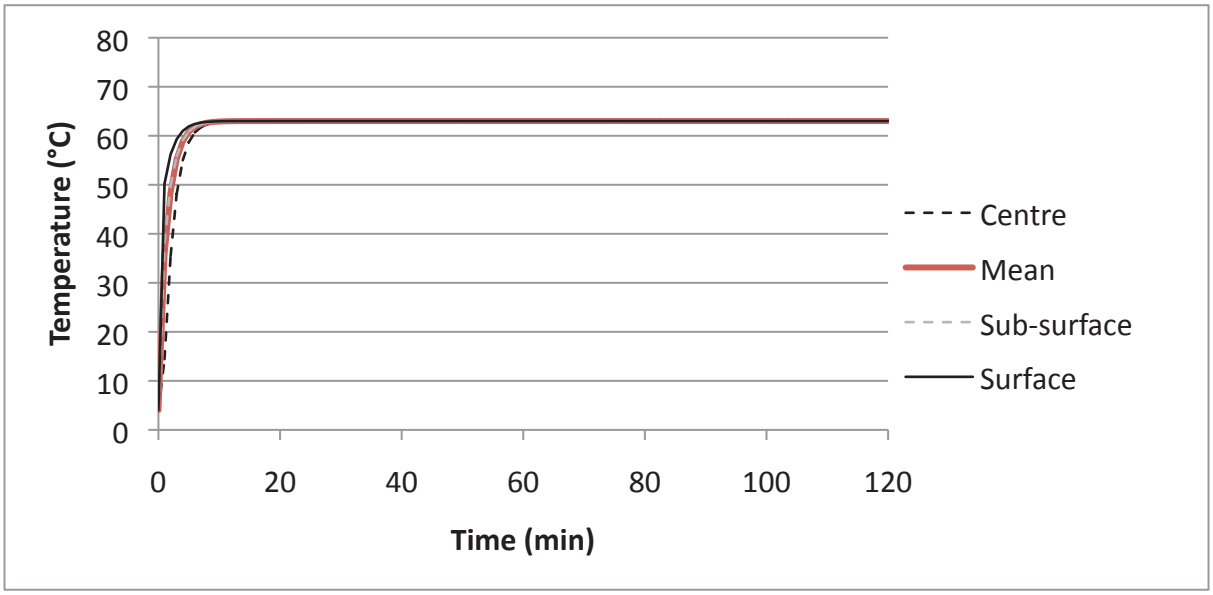
a – Scores of 1 denote that bacteria survived treatment. Scores of 0 denote that bacteria did not survive treatment.

Appendix 2 - Calibration and Validation of Pasteurisation Protocol

In order to determine which bacteria found in milk were able to survive pasteurisation conditions, a bench top pasteurisation apparatus was developed using a water bath held at the required temperature. Heat transfer modelling (Food Product Modeller, version 3.00, MIRINZ) was applied to predict the temperature that a body of liquid water is likely to attain over a given period of time.

Heat transfer was modelled using a volume of 20 ml (Figure 23). The temperature profile for tubes of this size was significantly quicker, as all parts of the cylinder modelled showed a uniform temperature increase, and reached the correct temperature in 7 minutes.

The Food Product Modeller software did not contain the heat-transfer co-efficient of milk. For this reason, the heat transfer was modelled using water.

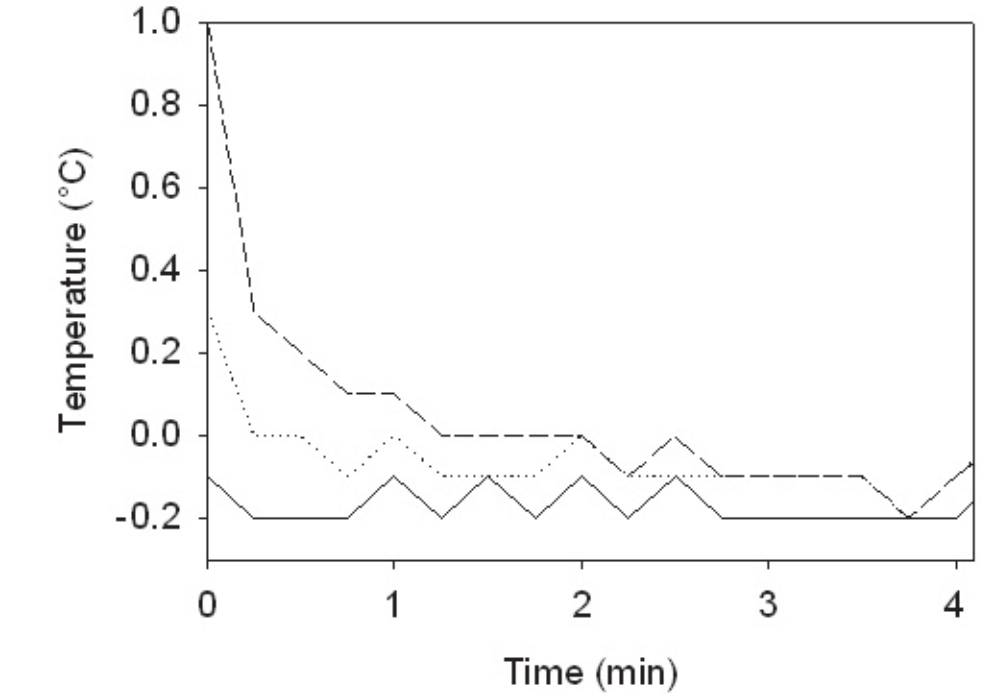


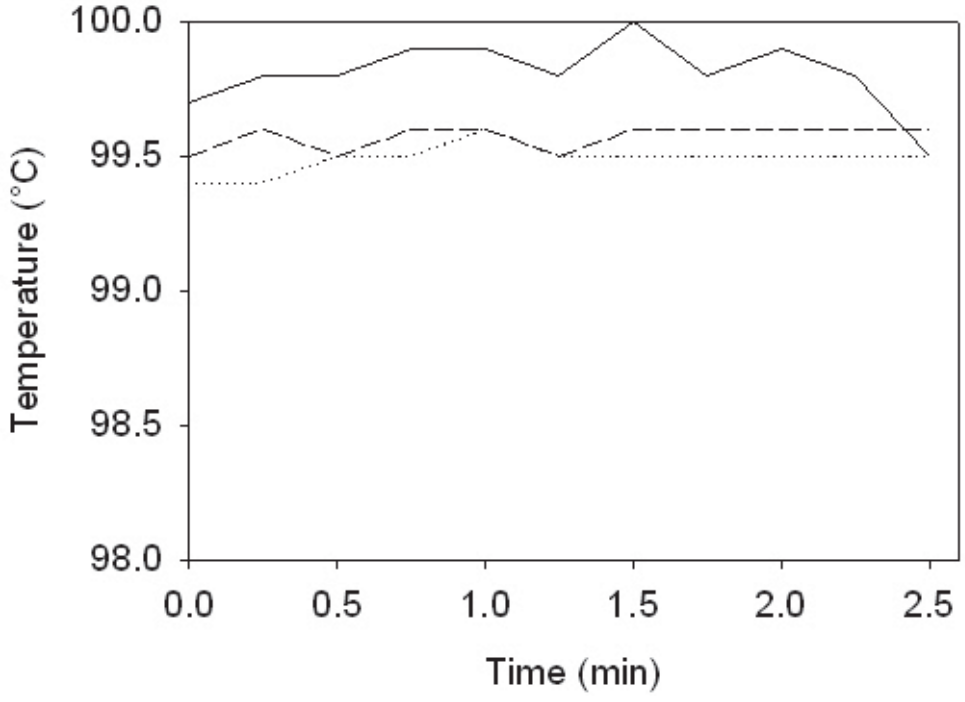
Appendix 2 - The predicted heat transfer of 20 ml water (4 °C) placed in a heated water bath. Parameters: Heat Transfer Co-efficient: 500 W m2 K; cylinder size: 110 × 80 mm (500 ml); material: water temperature profile: 63 °C (hold).

Appendix 3 - Validation of Thermocouple Probe

To analyse bacterial survival during the pasteurisation trial, a temperature probe was constructed with three thermocouples placed at the top, middle and bottom of the sample. The completed thermocouple was immersed in ice water at 0 °C and boiling water at 100 °C. When immersed in ice water, the temperature readings of all three thermocouples ranged between -0.2 °C and 0 °C. This reading was compared to a reference thermometer that displayed a temperature of 0 °C. This resulted in an error of 0.2 °C at freezing point.

When immersed in boiling water, the temperature readings of the three thermocouples ranged between 99.5 °C and 100°C. This was compared to a reference thermometer that displayed a temperature of 99.9 °C. The top thermocouple was reading close to this value, ranging between 99.8 °C and 100 °C. However, the middle and bottom thermocouple probes read between 99.5 and 99.6 °C. Based on these readings, the error of the probe at boiling point was 0.4 °C.

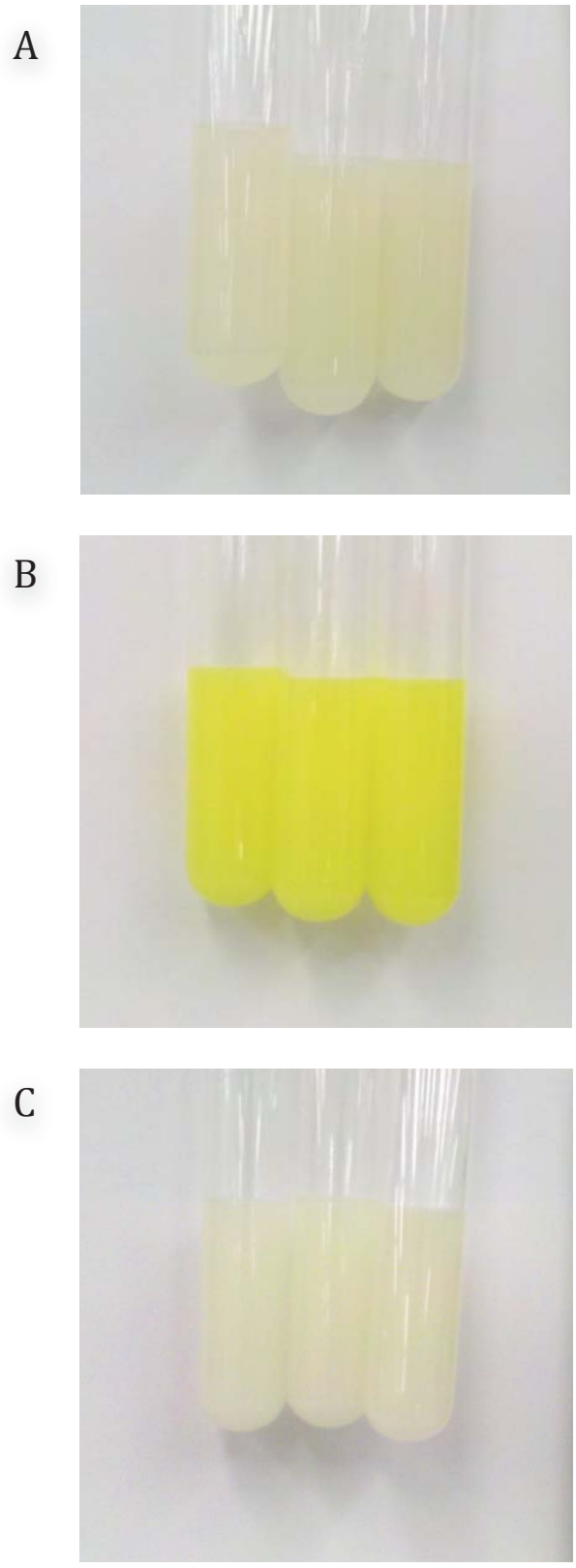




Appendix 3 - The temperature reading of the thermocouple probes held in 0 °C ice-water (A) and the temperature reading in boiling water (B). The thermocouples were located in the upper (—) middle (----) and lower (---) locations on the probe.

Appendix 4 - Alkaline Phosphatase Test

The phosphatase assay was used to test the efficiency of the pasteurisation process. Heat liable alkaline phosphatase should be inactivated if the sample of milk is pasteurised, and therefore remain white in this assay. A sample of raw milk was collected, and divided into positive control, and test samples. A sample of UHT treated milk was used as a negative control. Test samples were pasteurised by incubation at 62.8 °C for 30 minutes. Positive and negative controls were not pasteurised. Raw milk that was pasteurised using this bench top protocol did not result in the production of the yellow p-nitro phenyl product. This result matched the commercial UHT milk phosphatase negative control. The raw milk positive control showed a strong positive reaction (yellow) indicating the presence of active alkaline phosphatase.



Appendix 4 - Alkaline Phosphatase reaction in UHT milk (A), raw milk (B) and raw milk pasteurised in this study (C).

Appendix 5 – High Throughput Acid Tolerance Assay - Raw Data

Raw data obtained in high-throughput acid tolerance testing (sections 4.3 and 5.4). A score of 0 means that no survival was observed after exposure to pH 2.5 for two hours. A score of 1 means that survival was observed after exposure to pH 2.5 for two hours.

						pH 2.5 in TSB Broth only				pH 2.5 in TSB Broth and Milk				pH 2.5 in TSB Broth and Salts		
Identifier	Date	Farm	Media	Milk type	Growth time	1	2	3	4	1	2	3	4	5	6	7
A1	Dec-11	No 1	APT	Raw	48	0	0	1		1	1	1		0	1	1
A2	Dec-11	No 1	APT	Raw	48	0	0	0		1	1	0				
A3	Dec-11	No 1	APT	Raw	48	0	0	0		1	0	1				
A4	Dec-11	No 1	APT	Raw	48	0	0	0		1	1	0				
A5	Dec-11	No 1	APT	Raw	48	0	0	0		1	1	1				
A6	Dec-11	No 1	APT	Raw	48	1	0	1		1	1	1				
A7	Dec-11	No 1	APT	Raw	48	0	0	1		1	1	1				
A8	Dec-11	No 1	APT	Raw	48	0	0			1	0					
A9	Dec-11	No 1	APT	Raw	48	0	0	1		1	1	1				
A10	Dec-11	No 1	APT	Raw	48	0	1	0		1	1	1		0	0	0
A11	Dec-11	No 4	APT	Raw	24	0	0	0		1	0	0		1	1	1
A12	Dec-11	No 4	APT	Raw	72	0	0	0		0	0	0				
A13	Dec-11	No 4	APT	Raw	48	0	1	1		1	0	1		0	0	0
A14	Dec-11	No 4	APT	Raw	48	0	0	0		0	0	0				
A15	Dec-11	No 4	APT	Raw	48	0	1	0		0	0	1				
A16	Dec-11	No 4	APT	Raw	48	0	1	0		0	1	0				
A17	Dec-11	No 4	APT	Raw	24	1	0	1		1	0	1				
A18	Dec-11	No 4	APT	Raw	24	0	0			0	1					
A19	Dec-11	No 4	APT	Raw	48	0	1	1		0	1	1				
A20	Dec-11	No 4	APT	Raw	48	0	1	1		0	1	1				
A21	Dec-11	No 4	APT	Acid	24	1	0			1	1			0	0	0
A22	Dec-11	No 4	APT	Acid	48	1	1	0		1	0	0		1	1	1
A23	Dec-11	No 4	APT	Acid	48	1	1	1		1	0	1		1	1	1

A24	Dec-11	No 4	APT	Acid	24	0	1		0	1					
A25	Dec-11	No 4	APT	Acid	24	1	1		1	1					
A26	Dec-11	No 4	APT	Acid	24	0	0	1	0	0	1	1	0	1	
A27	Dec-11	No 4	APT	Acid	48	1	0	1	1	0	1	1	1	1	
A28	Dec-11	No 4	APT	Acid	24	1	1	1	1	1	1	1	1	1	
A29	Dec-11	No 4	APT	Acid	24	1	0	1	1	1	1				
A30	Dec-11	No 4	APT	Acid	24	0	0	1	0	1	1	0	0	0	
A31	Dec-11	No 1	MacConkey	Raw	48	0	0	1	0	1	1	0	0	0	
A32	Dec-11	No 1	MacConkey	Raw	24	0	0	0	0	0	1	0	0	0	
A33	Dec-11	No 1	MacConkey	Raw	24	1	0	0	1	1	1	0	0	0	
A34	Dec-11	No 4	MacConkey	Raw	24	1	1	0	1	1	1	0	0	0	
A35	Dec-11	No 4	MacConkey	Acid	72	0	1		0	1					
A36	Dec-11	No 4	MacConkey	Acid	72	0	1		0	0					
A37	Dec-11	No 1	M-PCA	Raw	72	1	1	0	1	0	1	0	0	0	
A38	Dec-11	No 1	M-PCA	Raw	72		0	1		1	1	1	1	1	
A39	Dec-11	No 1	M-PCA	Raw	72		0	0		0	1	0	0	0	
A40	Dec-11	No 1	M-PCA	Raw	72		1	0		0	1	0	0	0	
A41	Dec-11	No 1	M-PCA	Raw	24	1	1	1	1	1	1	1	1	1	
A42	Dec-11	No 1	M-PCA	Raw	72	1	1	1	1	0	1	1	1	1	
A43	Dec-11	No 1	M-PCA	Raw	48	0	0	1	1	1	1	0	1	1	
A44	Dec-11	No 1	M-PCA	Raw	72	0	0	0	0	0	1				
A45	Dec-11	No 1	M-PCA	Raw	72	1	0	1	1	1	1	1	1	1	
A46	Dec-11	No 1	M-PCA	Raw	72	0	1	0	1	0	0	0	0	0	
A47	Dec-11	No 1	M-PCA	Raw	72	1	0	1	1	1	1	1	1	1	
A48	Dec-11	No 1	M-PCA	Raw	48	1	1	0	1	1	1	0	1	1	
A49	Dec-11	No 1	M-PCA	Raw	72	0	0	0	0	1	1	0	0	0	
A50	Dec-11	No 1	M-PCA	Raw	72		1	0		0	1	0	0	0	
A51	Dec-11	No 1	M-PCA	Raw	48	0	1	1	1	1	1	1	1	1	
A52	Dec-11	No 4	M-PCA	Raw	72	0	1	0	1	1	1	0	0	0	
A53	Dec-11	No 4	M-PCA	Raw	72	1	1	1	1	1	1	1	1	1	
A54	Dec-11	No 4	M-PCA	Raw	72	0	0	1	1	1	1	1	1	1	
A55	Dec-11	No 4	M-PCA	Raw	72	0	1	1	1	1	1	0	0	0	
A56	Dec-11	No 4	M-PCA	Raw	72	1	0	1	1	1	1	1	1	1	
A57	Dec-11	No 4	M-PCA	Raw	72		0	0		1	1				

A58	Dec-11	No 4	M-PCA	Raw	72		0	0		1	1	1	0	0
A59	Dec-11	No 4	M-PCA	Raw	72	0	0		1	0				
A60	Dec-11	No 4	M-PCA	Raw	72	1	0	0	1	0	1	0	0	0
A61	Dec-11	No 4	M-PCA	Raw	72	0	0	0	1	1	1	0	0	0
A62	Dec-11	No 4	M-PCA	Raw	72	0	1	0	1	1	1			
A63	Dec-11	No 4	M-PCA	Raw	72	0	1	0	1	1	1			
A64	Dec-11	No 4	M-PCA	Raw	24	0	1	1	1	1	1	1	1	1
A65	Dec-11	No 4	M-PCA	Raw	72	0	0	0	1	1	1			
A66	Dec-11	No 4	M-PCA	Raw	72	0	1	0	1	1	0	0	0	0
A67	Dec-11	No 1	M-PCA	Acid	72	1	1	0	1	1	1	1	1	1
A68	Dec-11	No 1	M-PCA	Acid	24		0	0		0	1	1	1	1
A69	Dec-11	No 1	M-PCA	Acid	48	1	1	1	1	1	1	1	1	1
A70	Dec-11	No 1	M-PCA	Acid	24	0	1	0	1	0	1	0	1	0
A71	Dec-11	No 1	M-PCA	Acid	48	1	1	1	1	1	1	1	1	1
A72	Dec-11	No 1	M-PCA	Acid	48	1	1	1	1	1	1	1	1	1
A73	Dec-11	No 1	M-PCA	Acid	48		1	1		1	1	1	1	1
A74	Dec-11	No 1	M-PCA	Acid	24	1	0	0	1	0	1	1	1	1
A75	Dec-11	No 1	M-PCA	Acid	48		1	1		1	1	1	1	1
A76	Dec-11	No 1	M-PCA	Acid	24		1	0		0	1	0	0	0
A77	Dec-11	No 1	M-PCA	Acid	72	1	0	1	1	1	1	1	1	1
A78	Dec-11	No 1	M-PCA	Acid	24		1	1		0	1	1	1	1
A79	Dec-11	No 1	M-PCA	Acid	72	0	0	0	1	1	1	0	0	0
A80	Dec-11	No 1	M-PCA	Acid	72	0	1		0	1				
A81	Dec-11	No 1	M-PCA	Acid	72	1	1	1	1	1	1	1	1	1
A82	Dec-11	No 4	M-PCA	Acid	72	1	1	1	1	0	1	1	1	1
A83	Dec-11	No 4	M-PCA	Acid	72	1	1	1	1	1	1	1	1	1
A84	Dec-11	No 4	M-PCA	Acid	72	1	1	1	1	1	1	1	1	1
A85	Dec-11	No 4	M-PCA	Acid	24	0	1	1	1	0	1	1	1	1
A86	Dec-11	No 4	M-PCA	Acid	24		1	1		0	1	1	1	1
A87	Dec-11	No 4	M-PCA	Acid	72	1	1	1	1	1	1			
A88	Dec-11	No 4	M-PCA	Acid	72	1	1	1	1	1	1	1	1	0
A89	Dec-11	No 4	M-PCA	Acid	72	1	1	1	1	1	1	1	1	0
A90	Dec-11	No 4	M-PCA	Acid	72	0		1	1	1	1	0	0	1
A91	Dec-11	No 4	M-PCA	Acid	72	1	1	1	1	1	1	1	1	1

A92	Dec-11	No 4	M-PCA	Acid	72	1	1	1	1	0	1	1	1	1
A93	Dec-11	No 4	M-PCA	Acid	24	1	0	1	1	0	1	0	0	0
A94	Dec-11	No 4	M-PCA	Acid	72	1	1	1	1	1	1	1	1	1
A95	Dec-11	No 4	M-PCA	Acid	72	1	1	1	1	1	1	1	1	1
A96	Dec-11	No 4	M-PCA	Acid	72	1	0	1	1	1	1	1	1	1

Identifier	Date	Farm	Media	Milk Type	Growth Time	pH 2.5 in TSB Broth only				pH 2.5 in TSB Broth and Milk				pH 2.5 in TSB Broth and Salts		
						1	2	3	4	1	2	3	4	5	6	7
B1	Jan-12	No 1	APT	Raw	72	1	0	1		1	1	1		1	1	1
B2	Jan-12	No 1	APT	Raw	72	0	1	0		0	1	0		0	0	0
B3	Jan-12	No 1	APT	Raw	72	0	0	0		0	1	0		0	0	0
B4	Jan-12	No 1	APT	Raw	72	0	0	0		0	1	1				
B5	Jan-12	No 1	APT	Raw	24	0	0	0		0	0	1				
B6	Jan-12	No 1	APT	Raw	72	0	0	0		0	0	0		1	1	1
B7	Jan-12	No 1	APT	Raw	72	0	0	0		0	0	0				
B8	Jan-12	No 1	APT	Raw	72	0	0	0		0	1	0				
B9	Jan-12	No 1	APT	Raw	72	0	0	0		0	1	0				
B10	Jan-12	No 1	APT	Raw	72	0	0	0		0	1	0				
B11	Jan-12	No 4	APT	Raw	24	0	1	1		0	0	1				
B12	Jan-12	No 4	APT	Raw	24	0	0	1		0	1	1				
B13	Jan-12	No 4	APT	Raw	72	0	0			0	0					
B14	Jan-12	No 4	APT	Raw	24	0	0	0		0	1	1				
B15	Jan-12	No 4	APT	Raw	72	0	1	0		0	0	1		1	1	1
B16	Jan-12	No 4	APT	Raw	24	0	1	1		0	1	1		0	0	0
B17	Jan-12	No 4	APT	Raw	24	1	1	1		1	1	1		1	1	1
B18	Jan-12	No 4	APT	Raw	72	1	0			1	0					
B19	Jan-12	No 4	APT	Raw	72	0	1	1		0	1	1		0	1	0
B20	Jan-12	No 4	APT	Raw	24	0	1	0		0	0	0		0	0	0
B21	Jan-12	No 1	APT	Acid	24	1	0			1	0					
B22	Jan-12	No 1	APT	Acid	72	0	0	1		0	1	1				
B23	Jan-12	No 1	APT	Acid	24	0	0			0	0					
B24	Jan-12	No 1	APT	Acid	24	1	0			1	0					
B25	Jan-12	No 4	APT	Acid	24	1	0			1	0					
B26	Jan-12	No 4	APT	Acid	72	1	0	0		1	1	1		1	1	1
B27	Jan-12	No 4	APT	Acid	24	0	0			0	0					
B28	Jan-12	No 4	APT	Acid	24	1	0	0		1	0	0		1	1	1

B29	Jan-12	No 4	APT	Acid	24	1	0	0	1	0	0			
B30	Jan-12	No 4	APT	Acid	72	1	0		1	1		0	0	0
B31	Jan-12	No 1	Mac	Raw	72	0	0	0	1	1	1	0	1	0
B32	Jan-12	No 1	Mac	Raw	48	1	1	1	1	0	1	0	0	0
B33	Jan-12	No 1	Mac	Raw	72	0	0	0	1	1	1	0	0	0
B34	Jan-12	No 4	Mac	Raw	72	1	0	0	1	1	1	0	0	0
B35	Jan-12	No 4	Mac	Raw	72		1	1		1	1	0	0	0
B36	Jan-12	No 4	Mac	Raw	72		1	1		0	1	0	0	0
B37	Jan-12	No 4	Mac	Raw	72	1	0	0	1	1	0	1	1	1
B38	Jan-12	No 1	Mac	Raw	72	1	0	0	1	1	0	1	1	1
B39	Jan-12	No 1	Mac	Acid	72	0	1		0	1				
B40	Jan-12	No 4	Mac	Acid	72	0	1		0	1				
B41	Jan-12	No 1	PCA	Raw	72	0	0	0	0	1	1	0	1	0
B42	Jan-12	No 1	PCA	Raw	72	0	1	1	1	0	1	1	1	1
B43	Jan-12	No 1	PCA	Raw	48	1	1	1	1	0	1	0	1	1
B44	Jan-12	No 1	PCA	Raw	72	0	1	0	0	1	1	0	0	0
B45	Jan-12	No 1	PCA	Raw	72	0	1	1	1	1	1	1	1	1
B46	Jan-12	No 1	PCA	Raw	72	1	1	0	1	1	1	0	0	0
B47	Jan-12	No 1	PCA	Raw	72	1	1	0	1	0	1	1	1	1
B48	Jan-12	No 1	PCA	Raw	72	0	1	1	1	1	1	0	0	0
B49	Jan-12	No 1	PCA	Raw	72	0	1	1	1	1	1	0	0	0
B50	Jan-12	No 1	PCA	Raw	72	1	0	0	1	1	0	0	1	0
B51	Jan-12	No 1	PCA	Raw	72	1	0	0	1	1	1	1	1	1
B52	Jan-12	No 1	PCA	Raw	48	0	0	1	1	1	1	1	1	0
B53	Jan-12	No 1	PCA	Raw	72	0	0		1	1		0	1	0
B54	Jan-12	No 1	PCA	Raw	72	0	0	0	1	1	1	0	0	0
B55	Jan-12	No 1	PCA	Raw	48	1	1	0	1	1	0	1	1	1
B56	Jan-12	No 1	PCA	Raw	72	0	1	1	1	1	1	0	0	0
B57	Jan-12	No 1	PCA	Raw	24	0	0	0	0	0	0	0	1	1
B58	Jan-12	No 4	PCA	Raw	72	0	1	0	1	1	1	0	0	0
B59	Jan-12	No 4	PCA	Raw	48	0	1	1	1	1	1	1	1	1
B60	Jan-12	No 4	PCA	Raw	72	1	1	1	1	1	1	1	1	1
B61	Jan-12	No 4	PCA	Raw	72	0	1	1	1	1	1	0	0	0
B62	Jan-12	No 4	PCA	Raw	72	1	0	1	1	1	1	1	1	1

B63	Jan-12	No 4	PCA	Raw	48	0	1		0	1				
B64	Jan-12	No 4	PCA	Raw	48	1	1	1	1	1	1	1	1	1
B65	Jan-12	No 4	PCA	Raw	48	0	0	0	1	1	0	0	0	0
B66	Jan-12	No 4	PCA	Raw	48	0	1	1	1	1	1	0	1	1
B67	Jan-12	No 4	PCA	Raw	72	0	0	1	1	1	1	1	1	1
B68	Jan-12	No 4	PCA	Raw	48	0	1	1	1	1	1			
B69	Jan-12	No 4	PCA	Raw	48	0	1	1	1	1	1	1	1	0
B70	Jan-12	No 4	PCA	Raw	72	1	1	0	1	1	0	1	1	1
B71	Jan-12	No 4	PCA	Raw	72	1	0	1	1	1	1	1	1	1
B72	Jan-12	No 4	PCA	Raw	48	0	0	0	1	1	1	1	1	0
B73	Jan-12	No 4	PCA	Raw	48	1	0	1	1	1	1	1	1	1
B74	Jan-12	No 4	PCA	Raw	48	0	1	1	1	1	1	0	0	0
B75	Jan-12	No 4	PCA	Raw	72	0	1		0	1				
B76	Jan-12	No 4	PCA	Raw	48	0	0		0	1				
B77	Jan-12	No 1	PCA	Acid	48	1	1	1	1	1	1	0	1	1
B78	Jan-12	No 1	PCA	Acid	48	1	1	0	1	1	1	1	1	1
B79	Jan-12	No 1	PCA	Acid	48	1	1	1	1	1	1	0	0	1
B80	Jan-12	No 1	PCA	Acid	48		1	1		1	1	0	1	0
B81	Jan-12	No 1	PCA	Acid	72	1	1	0	0	1	0	0	0	1
B82	Jan-12	No 1	PCA	Acid	48	1	1	1	1	1	1	1	1	1
B83	Jan-12	No 1	PCA	Acid	48	1	1	1	1	1	1	1	0	1
B84	Jan-12	No 1	PCA	Acid	48	0	1	1	1	1	1	1	1	1
B85	Jan-12	No 1	PCA	Acid	48	0	1	0	1	1	0	0	1	1
B86	Jan-12	No 4	PCA	Acid	48	1	1	1	1	1	1	1	1	1
B87	Jan-12	No 4	PCA	Acid	48	1	0	0	1	0	0	1	1	1
B88	Jan-12	No 4	PCA	Acid	72	0	0	0	1	0	1	1	1	1
B89	Jan-12	No 4	PCA	Acid	48	1	1	1	0	0	1	1	1	1
B90	Jan-12	No 4	PCA	Acid	48	0	1	1	1	0	1	1	1	1
B91	Jan-12	No 4	PCA	Acid	48	0	1		1	1				
B92	Jan-12	No 4	PCA	Acid	72	1	0		1	0				
B93	Jan-12	No 4	PCA	Acid	48	1	0	1	1	1	1	1	1	1
B94	Jan-12	No 4	PCA	Acid	48	1	0	1	1	1	1	1	1	1
B95	Jan-12	No 4	PCA	Acid	72	0	0	0	1	1	1	0	0	1
B96	Jan-12	No 4	PCA	Acid	48	1	0	1	1	1	1	1	1	1

						pH 2.5 in TSB Broth only				pH 2.5 in broth and Milk				pH 2.5 in Broth and Salts		
Identifier	Date	Farm	Media	Milk Type	Growth Time	1	2	3	4	1	2	3	4	5	6	7
C1	Feb-12	No 1	APT	Raw	24	0	0	1	0	1	1	1	1	0	0	0
C2	Feb-12	No 1	APT	Raw	72	1	0	1	1	1	1	1	0			
C3	Feb-12	No 1	APT	Raw	24	0	0	0	0	1	1	1	1	0	0	0
C4	Feb-12	No 1	APT	Raw	72	0	0			1	0					
C5	Feb-12	No 1	APT	Raw	72	0	1	1	1	1	1	1	0	1	1	1
C6	Feb-12	No 1	APT	Raw	24	1	1	1	1	1	1	1	1	1	1	1
C7	Feb-12	No 1	APT	Raw	48	1	1	1	1	1	1	1	1	1	1	1
C8	Feb-12	No 1	APT	Raw	72	0	0	0	0	0	0	1	1	0	0	0
C9	Feb-12	No 1	APT	Raw	24	0	0	0	0	1	1	1	1			
C10	Feb-12	No 1	APT	Raw	72	0	0			0	0					
C11	Feb-12	No 4	APT	Raw	24	0	0	0	1	1	1	1	1	1	1	1
C12	Feb-12	No 4	APT	Raw	48	0	0			1	1					
C13	Feb-12	No 4	APT	Raw	24	0	0	0	0	1	1	1	1	0	1	0
C14	Feb-12	No 4	APT	Raw	72	1	1	1	1	1	1	1	1			
C15	Feb-12	No 4	APT	Raw	72	1	0	0	0	1	1	0	0			
C16	Feb-12	No 4	APT	Raw												
C17	Feb-12	No 4	APT	Raw												
C18	Feb-12	No 4	APT	Raw	72	1	1	0	0	1	1	0	0			
C19	Feb-12	No 4	APT	Raw	72	1	1	1	1	0	1	1	1			
C20	Feb-12	No 4	APT	Raw	72	1	1	1	1	1	1	1	1			
C21	Feb-12	No 4	APT	Acid	72	1	0	1	1	1	1	1	1	1	1	1
C22	Feb-12	No 4	APT	Acid	72	0	0	1	1	1	1	1	1	1	1	1
C23	Feb-12	No 4	APT	Acid	24	1	1	1	1	1	1	1	1	1	1	1
C24	Feb-12	No 4	APT	Acid	24	1	1	1	1	1	1	1	1	1	1	1
C25	Feb-12	No 4	APT	Acid	72	1	0	1	1	1	1	1	1	1	1	1
C26	Feb-12	No 4	APT	Acid	72	1	1	1	1	1	1	1	1			
C27	Feb-12	No 4	APT	Acid	24	1	1	1	1	1	1	1	1			
C28	Feb-12	No 4	APT	Acid	72	0	0	1	1	1	1	1	1	1	1	1

C29	Feb-12	No 4	APT	Acid	24	1	1	1	1	1	1	1	1	1	1	1
C30	Feb-12	No 4	APT	Acid	24	1	1	0	0	1	1	0	0	1	0	1
C31	Feb-12	No 1	APT	Raw	72	0	0	1	1	1	1	1	1	1	1	1
C32	Feb-12	No 1	APT	Raw	72	0	0	1	1	0	1	1	1	1	1	1
C33	Feb-12	No 1	MacConkey	Raw	72	0	0	0	0	1	1	0	0	0	0	0
C34	Feb-12	No 4	MacConkey	Raw	72	0	0	1	1	1	1	0	1	0	0	0
C35	Feb-12	No 4	MacConkey	Acid	72	0	0	1	1	1	1	1	1	0	0	0
C36	Feb-12	No 4	MacConkey	Acid	48	0	1	0	1	1	1	0	1	0	0	0
C37	Feb-12	No 1	M-PCA	Raw	48	1	1	0	1	1	1	0	1	0	0	0
C38	Feb-12	No 1	M-PCA	Raw	48	0	1	0	1	1	1	1	1	1	1	1
C39	Feb-12	No 1	M-PCA	Raw	72	0	0	1	1	1	1	1	1	1	1	1
C40	Feb-12	No 1	M-PCA	Raw	48	1	0	0	1	1	1	0	1	0	0	0
C41	Feb-12	No 1	M-PCA	Raw	72	0	0	0	1	1	1	1	1	0	0	0
C42	Feb-12	No 1	M-PCA	Raw	72	1	1	0	0	1	1	1	1	0	0	0
C43	Feb-12	No 1	M-PCA	Raw	72	0	0	0	0	1	1	0	1	0	0	0
C44	Feb-12	No 1	M-PCA	Raw	48			1	1			1	1	1	1	1
C45	Feb-12	No 1	M-PCA	Raw	72			0	0			0	1	0	0	0
C46	Feb-12	No 1	M-PCA	Raw	72	1	1			1	1					
C47	Feb-12	No 1	M-PCA	Raw	48	1	1			1	1					
C48	Feb-12	No 1	M-PCA	Raw	72	0	0	1	1	1	1	1	1	1	1	1
C49	Feb-12	No 1	M-PCA	Raw	24	0	0	1	1	1	1	1	1	1	1	1
C50	Feb-12	No 1	M-PCA	Raw	72	0	1	1	1	1	1	1	1	1	1	1
C51	Feb-12	No 1	M-PCA	Raw	24	0	0	0	0	1	1	0	0	0	0	0
C52	Feb-12	No 4	M-PCA	Raw	72	0	1	1	0	1	1	0	0	0	0	0
C53	Feb-12	No 4	M-PCA	Raw	72	0	0	0	0	1	1	0	0	0	0	0
C54	Feb-12	No 4	M-PCA	Raw	72			0	0			0	0	0	0	0
C55	Feb-12	No 4	M-PCA	Raw	72	0	0	0	0	1	1	0	0	0	0	0
C56	Feb-12	No 4	M-PCA	Raw	48	0	0	0	0	1	1	1	1			
C57	Feb-12	No 4	M-PCA	Raw	48			0	0			0	0	0	0	0
C58	Feb-12	No 4	M-PCA	Raw	24	0	0	0	0	1	1	1	1	0	0	0
C59	Feb-12	No 4	M-PCA	Raw	48	1	0	1	1	1	1	1	1	1	1	1
C60	Feb-12	No 4	M-PCA	Raw	24	1	1	0	0	1	1	1	1	1	1	1
C61	Feb-12	No 4	M-PCA	Raw	24	1	1	1	1	1	1	1	1	1	1	1
C62	Feb-12	No 4	M-PCA	Raw	48			0	0			0	0	1	1	1

C63	Feb-12	No 4	M-PCA	Raw	48	0	0	1	1	1	1	1	1	1	1	1
C64	Feb-12	No 4	M-PCA	Raw	48	0	0	1	1	1	1	1	1			
C65	Feb-12	No 4	M-PCA	Raw	72	1	0	0	1	1	1	1	1	0	0	0
C66	Feb-12	No 4	M-PCA	Raw	48	1	1	0	0	1	1	0	1	0	0	0
C67	Feb-12	No 1	M-PCA	Acid	72	0	1			1	1					
C68	Feb-12	No 1	M-PCA	Acid	24	0	0	1	1	1	1	1	1	1	1	1
C69	Feb-12	No 1	M-PCA	Acid	48	0	0	0	1	1	1	0	1	0	0	0
C70	Feb-12	No 1	M-PCA	Acid	72	0	0			1	1					
C71	Feb-12	No 1	M-PCA	Acid	48	0	1	0	0	1	1	0	1			
C72	Feb-12	No 1	M-PCA	Acid	72	1	1	0	0	0	1	1	0			
C73	Feb-12	No 1	M-PCA	Acid	72	1	1	0	1	1	1	1	1	1	0	0
C74	Feb-12	No 1	M-PCA	Acid	24	0	0	1	1	1	1	1	1			
C75	Feb-12	No 1	M-PCA	Acid	72	0	0	1	1	1	1	1	1	1	1	1
C76	Feb-12	No 1	M-PCA	Acid	72	1	1	0	0	1	1	0	0	0	0	0
C77	Feb-12	No 1	M-PCA	Acid	72	0	0	0	0	1	1	1	0	0	0	0
C78	Feb-12	No 1	M-PCA	Acid	72	1	1	1	1	1	1	1	1	1	1	1
C79	Feb-12	No 1	M-PCA	Acid	48	1	1	0	0	1	1	0	1	0	0	0
C80	Feb-12	No 1	M-PCA	Acid	24	1	1	1	1	1	1	1	1	1	0	1
C81	Feb-12	No 1	M-PCA	Acid	48	0	1	1	1	0	0	1	1	1	1	1
C82	Feb-12	No 4	M-PCA	Acid	72	0	0	1	0	1	1	1	1	0	1	1
C83	Feb-12	No 4	M-PCA	Acid	72	1	0	1	1	1	1	1	1			
C84	Feb-12	No 4	M-PCA	Acid	72	1	1	1	1	1	1	1	1			
C85	Feb-12	No 4	M-PCA	Acid	24	1	1	1	0	1	1	1	1	0	1	0
C86	Feb-12	No 4	M-PCA	Acid	24			1	1			1	0	1	1	1
C87	Feb-12	No 4	M-PCA	Acid	24	1	1	1	1	1	1	1	1	1	1	1
C88	Feb-12	No 4	M-PCA	Acid	24	1	1	0	0	1	1	0	0			
C89	Feb-12	No 4	M-PCA	Acid	24	1	1	0	0	1	1	1	1	0	1	1
C90	Feb-12	No 4	M-PCA	Acid	24	0	0	1	1	1	1	1	1	1	1	1
C91	Feb-12	No 4	M-PCA	Acid	48	1	1	1	1	1	1	1	1	1	1	1
C92	Feb-12	No 4	M-PCA	Acid	72	1	1	1	0	1	1	1	1	0	0	0
C93	Feb-12	No 4	M-PCA	Acid	72	1	1	1	1	1	1	1	1	1	1	1
C94	Feb-12	No 4	M-PCA	Acid	48	1	1	1	1	1	1	1	1	1	1	1
C95	Feb-12	No 4	M-PCA	Acid	48			0	0			0	1	0	0	0
C96	Feb-12	No 4	M-PCA	Acid	72			1	1			1	1	1	1	1

						pH 2.5 in TSB Broth only				pH 2.5 in broth and Milk				pH 2.5 in TSB broth and Salts		
Identifier	Date	Farm	Media	Milk Type	Growth Time	1	2	3	4	1	2	3	4	5	6	7
1	Sep-09	Waikato A	PCA	Processed	24	1	1			1	1			1	1	1
2	Sep-09	Waikato A	PCA	Processed	48	1	1			1	1			1	1	1
3	Sep-09	Waikato A	APT	Processed	24	0	0			0	0			1	1	1
4	Sep-09	Waikato A	APT	Processed	24	0	1			0	1			1	1	1
5	Sep-09	Waikato A	PCA	Processed	48	1	1			1	1			1	1	1
6	Sep-09	Waikato A	PCA	Processed	48	1	1			0	0			1	1	1
7	Sep-09	Waikato A	PCA	Processed	48	0	0			1	1			1	1	0
8	Sep-09	Waikato A	APT	Processed	48	1	1			1	1			1	1	1
9	Sep-09	Waikato A	MacConkey	Processed	24	0	0			1	0			0	0	0
10	Sep-09	Waikato A	MacConkey	Processed	24	0	0			1	0			0	0	0
11	Sep-09	Waikato A	MacConkey	Processed	48	0	0			1	1			0	0	0
12	Sep-09	Waikato A	APT	Processed	72	0	0			0	0					
13	Sep-09	Waikato A	APT	Processed	24	1	0			1	1			1	1	1
14	Sep-09	Waikato A	APT	Processed	48	0	0			0	0			1	1	1
15	Sep-09	Waikato A	APT	Processed	24	0	0			1	1			1	1	1
16	Sep-09	Waikato A	PCA	Processed	24	0	0			1	1			1	1	1
17	Sep-09	Waikato A	PCA	Processed	24	0	0			0	0			0	0	1
18	Sep-09	Waikato A	PCA	Processed	24	0	0			0	0					
19	Sep-09	Waikato A	PCA	Processed	24	0	0			1	1			0	0	0
20	Sep-09	Waikato A	APT	Raw	24	0	0			0	0			0	0	0
21	Sep-09	Waikato A	APT	Raw	72	0	0			0	0					
22	Sep-09	Waikato A	APT	Raw	72	0	0			0	0					
24	Sep-09	Waikato A	APT	Raw	24	0	0			0	1			0	0	0
25	Sep-09	Waikato A	APT	Raw	72	0	0			0	0					
26	Sep-09	Waikato A	APT	Raw	24	0	0			1	0			0	0	0
27	Sep-09	Waikato A	APT	Raw	72	0	0			0	0					
28	Sep-09	Waikato A	APT	Raw	72	1	1			1	1			1		1
30	Sep-09	Waikato A	APT	Raw	72	0	0			0	0					

31	Sep-09	Waikato A	APT	Raw	72	0	0	0	0				
32	Sep-09	Waikato A	MacConkey	Raw	24	1	1	1	1	1	1	1	
33	Sep-09	Waikato A	MacConkey	Raw	24	1	0	1	1	1	1	1	
34	Sep-09	Waikato A	MacConkey	Raw	24	0	0	0	1	0	0	0	
35	Sep-09	Waikato A	MacConkey	Raw	24	0	0	1	1	1	1	1	
36	Sep-09	Waikato A	MacConkey	Raw	48	0	1	1	1	0	0	0	
37	Sep-09	Waikato A	MacConkey	Raw	48	1	0	1	1	1	1	1	
38	Sep-09	Waikato A	MacConkey	Raw	72	0	0	0	0	0	0	0	
39	Sep-09	Waikato A	MacConkey	Raw	24	0	0	1	0	0	0	0	
40	Sep-09	Waikato A	MacConkey	Raw	24	0	0	1	1	0	0	0	
41	Sep-09	Waikato A	MacConkey	Raw	24	0	0	1	0	1	1	1	
42	Sep-09	Waikato A	PCA	Raw	24	0	0	1	0	0	0	0	
43	Sep-09	Waikato A	PCA	Raw	24	0	0	0	0	1	0	0	
44	Sep-09	Waikato A	PCA	Raw	72	0	1	1	0	0	0	0	
45	Sep-09	Waikato A	PCA	Raw	72	0	0	0	0	0	0	0	
46	Sep-09	Waikato A	PCA	Raw	72	0	1	1	1	0	0	0	
47	Sep-09	Waikato A	PCA	Raw	48	0	1	1	1	1	1	1	
48	Sep-09	Waikato A	PCA	Raw	72	0	1	1	0	0	0	0	
49	Sep-09	Waikato A	PCA	Raw	72	0	0	0	1	0	0	0	
51	Sep-09	Waikato A	PCA	Raw	72	0	0	0	0	0	0	0	
52	Sep-09	Waikato A	PCA	Raw	72	1	1	1	1	1	1	1	
53	Sep-09	Waikato A	PCA	Raw	48	0	1	1	0	0	0	0	
54	Sep-09	Waikato A	PCA	Raw	72	0	0	1	1	1	1	1	
55	Sep-09	Waikato A	PCA	Raw	72	0	0	0	0	0	0	0	
56	Sep-09	Waikato A	PCA	Raw	48	1	0	1	1	0	0	0	
59	Sep-09	Waikato A	PCA	Raw	72	0	0	0	1	0	0	0	
60	Sep-09	Waikato A	MacConkey	Raw	24	0	0	1	1	0	0	0	
61	Sep-09	Waikato A	MacConkey	Raw	48	0	0	1	1	0	0	0	
62	Sep-09	Waikato A	MacConkey	Raw	24	0	0	1	1	0	0	0	
63	Sep-09	Waikato A	MacConkey	Raw	48	0	0	1	1	0	0	0	
64	Sep-09	Waikato A	MacConkey	Raw	24	0	0	1	0	0	0	0	
66	Sep-09	Waikato A	MacConkey	Raw	72	1	1	0	1	1	1	1	
67	Sep-09	Waikato A	APT	Raw	24	1	1	1	1	1	1	1	
68	Sep-09	Waikato A	APT	Raw	24	0	0	0	0	0	0	0	

69	Sep-09	Waikato A	APT	Raw	24	0	0	1	0	0	0	0	
70	Sep-09	Waikato A	APT	Raw	24	0	0	1	1	0	0	0	
71	Sep-09	Waikato A	PCA	Raw	24	0	0	1	0	0	0	0	
72	Sep-09	Waikato A	PCA	Raw	72	0	0	0	1	0	0	0	
73	Sep-09	Waikato A	PCA	Raw	24	0	0	1	1	0	0	0	
74	Sep-09	Waikato A	PCA	Raw	24	1	1	1	1	1	1	1	
75	Sep-09	Waikato A	PCA	Raw	72	0	0	0	1	0	0	0	
76	Sep-09	Waikato A	PCA	Raw	72	0	0	0	1	0	0	0	
77	Sep-09	Waikato A	PCA	Raw	72	1	1	0	0	1	1	1	
78	Sep-09	Waikato A	PCA	Raw	72	0	0	0	1	0	0	0	
79	Sep-09	Waikato A	PCA	Raw	72	0	0	0	0	0	0	0	
80	Sep-09	Waikato A	PCA	Raw	24	0	0	0	0	0	0	0	
81	Sep-09	Waikato A	PCA	Raw	24	0	0	1	1	0	0	0	
82	Sep-09	Waikato A	PCA	Raw	24	1	0	1	1	1	1	1	
83	Sep-09	Waikato A	PCA	Raw	48	0	0	1	0	0	0	0	
84	Sep-09	Waikato A	PCA	Raw	24	1	1	1	1	1	1	1	
85	Sep-09	Waikato A	PCA	Raw	24	0	0	0	0	0	0	0	
86	Sep-09	Waikato A	PCA	Raw	24	0	0	1	1	1	1	1	
87	Sep-09	Waikato A	PCA	Raw	24	0	0	1	1	1	1	1	
88	Sep-09	Waikato A	PCA	Raw	24	1	1	1	1	0	0	0	
89	Sep-09	Waikato A	APT	Raw	24	0	0	1	0	0	0	0	
90	Sep-09	Waikato A	APT	Raw	24	1	0	1	0	0	0	0	
91	Sep-09	Waikato A	APT	Raw	24	0	0	1	1	0	0	0	
92	Sep-09	Waikato A	APT	Raw	24	0	0	1	1	0	0	1	
94	Sep-09	Waikato A	APT	Raw	24	0	0	0	1	0	0	1	
95	Sep-09	Waikato A	APT	Raw	24	0	0	1	1	0	0	1	
96	Sep-09	Waikato A	APT	Raw	72	0	0	0	0				
97	Sep-09	Waikato A	PCA	Raw	24	0	0	1	0	0	0	0	
98	Sep-09	Waikato A	PCA	Raw	24	0	1	1	1	1	1	1	
99	Sep-09	Waikato A	PCA	Raw	72	0	0	1	1	1	1	1	
102	Sep-09	Waikato A	PCA	Raw	24	0	0	1	1	0	0	0	
103	Sep-09	Waikato A	MacConkey	Raw	24	0	0	1	1	0	0	0	
105	Sep-09	Waikato A	MacConkey	Raw	72	0	0	1	0				
107	Sep-09	Waikato A	MacConkey	Raw	72	0	0	0	0				

Appendix 6 – Statistical Output for High Throughput Acid Tolerance Tests

The analysis was performed using packages lme4 (Bates *et al.*, 2012) and 'predictmeans' in R (R Development Core Team, 2005).

Chapter Four Statistical Analysis (n = 363)

```
> bact.glmer <- lmer(nScore ~
Trt*Milk_Type+(1|Date/Farm/Iso_rep)+(1|Media)+(1|Growth_Time),
family=binomial, data=bact)
> Anova(bact.glmer)
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: nScore
              Chisq          Df      Pr(>Chisq)
Trt         277.330          2       < 2.2e-16 ***
Milk_Type       81.013         1       < 2.2e-16 ***
Trt:Milk_Type   23.069         2       0.000009787 ***
---
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

> predictmeans(bact.glmer , "Trt:Milk_Type", Df=68.21, trans=invLogit)

$`Predicted Means`
 Milk_Type Acid Raw
Trt
pH 2.5 0.5752 -0.7424
pH 2.5+Milk 1.6202 1.3106

$`Standard Error of Means`
              Milk_Type       Acid    Raw
Trt
pH 2.5          0.36126 0.34735
pH 2.5+Milk     0.37343 0.34971

$`Standard Error of Differences`
 Max.SED Min.SED Aveg.SED
 0.23122 0.13025 0.18413
attr(,"For the Same Level of Factor")
                Trt     Milk_Type
Aveg.SED        0.1893 0.1758
Min.SED         0.1681 0.1303
Max.SED         0.2008 0.2312

$LSD
 Max.LSD Min.LSD Aveg.LSD
0.4613666 0.2598953 0.3674052
attr(,"Significant level")
[1] 0.05
attr(,"Degree of freedom")
[1] 68.21

$`Back Transformed Means`
 Trt Milk_Type Mean LL of 95% CI UL of 95% CI
1 pH 2.5        Acid    0.6400 0.4637 0.7852
2 pH 2.5        Raw     0.3225 0.1922 0.4877
3 pH 2.5+Milk   Acid    0.8348 0.7058 0.9141
```

```
4 pH 2.5+Milk Raw      0.7876 0.6486 0.8817
```

Chapter Five Statistical Analysis (n = 275)

```
> predictmeans(bact.glmer , "Trt:Milk_Type", Df=68.21, trans=invLogit)
$`Predicted Means`
 Milk_Type Acid Raw
Trt
pH 2.5 0.7191 -0.5151
pH 2.5+Milk 1.8135 1.4989
pH 2.5+Salt 0.9719 -0.3636

$`Standard Error of Means`
 Milk_Type Acid Raw
Trt
pH 2.5 0.29119 0.27059
pH 2.5+Milk 0.31600 0.27757
pH 2.5+Salt 0.29711 0.26874

$`Standard Error of Differences`
 Max.SED Min.SED Aveg.SED
 0.24416 0.14128 0.19527
attr(,"For the Same Level of Factor")
 Trt Milk_Type
Aveg.SED 0.2003 0.1871
Min.SED 0.1811 0.1413
Max.SED 0.2294 0.2442

$LSD
 Max.LSD Min.LSD Aveg.LSD
0.4871865 0.2819041 0.3896335
attr(,"Significant level")
[1] 0.05
attr(,"Degree of freedom")
[1] 68.21

$`Back Transformed Means`
 Trt Milk_Type Mean LL of 95% CI UL of 95% CI
1 pH 2.5 Acid 0.6724 0.5345 0.7859
2 pH 2.5 Raw 0.3740 0.2583 0.5062
3 pH 2.5+Milk Acid 0.8598 0.7655 0.9201
4 pH 2.5+Milk Raw 0.8174 0.7201 0.8862
5 pH 2.5+Salt Acid 0.7255 0.5936 0.8270
6 pH 2.5+Salt Raw 0.4101 0.2891 0.5431
```

Appendix 7 – BLAST and Seqmatch Results, Chapter Five.

BLAST and Seqmatch results of bacterial species isolated from milk that showed an increase in survivial at pH 2.5 when milk was present, as opposed to the same pH in TSB media alone.

Appendix 7(a) – Forward sequences using primer PA.

	Closest Match	length	coverage (%)	match (%)	S_ab
A1	*Staphylococcus haemolyticus*	717	100	100	0.969
A2	*Staphylococcus devriesei*	646	100	100	0.974
A3	*Staphylococcus devriesei*	678	100	100	0.974
A5	*Leuconostoc lactis*	697	100	100	1.000
A7	*Staphylococcus haemolyticus*	678	100	100	0.966
A9	*Staphylococcus chromogenes*	720	100	99	1.000
A10	*Leuconostoc lactis*	673	100	100	1.000
A29	*Staphylococcus aureus*	734	100	100	1.000
A31	*Pantoea agglomerans*	641	100	100	0.941
A33	*Pantoea agglomerans*	717	100	99	0.950
A34	*Pantoea agglomerans*	621	100	100	0.959
A38	*Staphylococcus devriesei*	766	100	100	0.968
A40	*Leuconostoc lactis*	627	99	100	0.976
A43	*Microbacterium phyllosphaerae or foliorum*	804	100	99	0.992
A45	*Staphylococcus haemolyticus*	750	100	99	0.957
A50	*Leuconostoc lactis*	753	100	100	0.997
A52	*Lactococcus lactis*	764	100	99	0.991
A54	*Staphylococcus haemolyticus*	739	99	100	0.996
A55	*Lactococcus lactis*	766	100	99	0.991
A57	*Lactobacillus paracasei*	733	100	100	0.989
A61	*Lactococcus lactis*	745	100	100	0.988
A62	*Lactococcus lactis*	747	100	100	0.988
A63	*Lactococcus lactis*	765	100	100	1.000
A70	*Curtobacterium flaccumfaciens*	730	100	100	1.000
A76	*Staphylococcus saprophyticus*	787	100	100	1.000
A79	*Micrococcus yunnanenensis*	642	100	100	0.990
B4	*Leuconostoc lactis*	765	100	100	1.000
B14	*Staphylococcus devriesei*	737	100	100	0.987
B26	*Bacillus licheniformis*	725	100	100	0.988
B31	*Pseudomonas moraviensis*	756	100	100	0.962
B33	*Pseudomonas moraviensis*	706	100	100	0.957
B34	*Pseudomonas rhizosphaerae*	696	100	100	0.979
B41	*Curtobacterium flaccumfaciens*	748	100	100	1.000
B51	*Kocuria varians*	685	100	99	0.943
B54	*Lactococcus lactis*	819	100	100	1.000
B58	*Lactococcus lactis*	803	100	100	0.996

B61	*Rathayibacter festucae*	677	97	94	0.733
B64	*Staphylococcus haemolyticus*	777	100	100	0.954
B65	*Plantibacter flavus*	811	100	100	1.000
B72	*Staphylococcus haemolyticus*	777	100	100	0.956
B82	*Bacillus licheniformis*	769	100	100	0.991
B88	No Data				
B95	*Arthrobacter citreus*	714	100	99	0.969
B96	*Kocuria varians*	597	100	99	0.973
C1	*Enterobacter aerogenes*	703	100	99	0.865
C8	*Leuconostoc lactis*	772	100	100	1.000
c9	*Staphylococcus epidermis*	727	100	100	1.000
c11	*staphylococcus chromogenes*	746	100	100	0.995
C13	*Staphylococcus epidermis*	748	100	100	0.996
c28	*bacillus licheniformis*	710	100	100	0.961
c38	*Staphylococcus auricularis*	783	99	100	0.991
C41	*Enterobacter aerogenes*	793	100	100	1.000
C49	*Pseudomonas psychrophila*	717	100	100	0.954
C50	*Staphylococcus haemolyticus*	765	100	100	0.957
C53	*Streptococcus parauberis*	711	100	100	0.805
C56	*Arthrobacter agilis*	778	100	100	0.974
C58	No Data				
C60	*Staphylococcus haemolyticus*	785	100	100	0.963
C61	*Streptococcus uberis*	569	99	98	0.908
C65	*Kocuria rhizophila*	789	99	99	0.974
C67	*Kocuria rhizophila*	737	100	99	0.980
C71	*Streptococcus uberis*	824	100	100	1.000
C77	*Arthrobacter gandavensis*	802	100	99	0.984
C82	*Arthrobacter citreus*	832	100	100	0.998

Appendix 7(b) - Reverse sequeces using primer PH*.

	Closest Match	Length	Coverage (%)	Match (%)	S_ab
A1	*Staphylococcus haemolyticus*	767	100	100	0.955
A2	*Staphylococcus devriesei*	674	100	100	1.000
A3	*Staphylococcus devriesei*	712	100	100	1.000
A5	*Leuconostoc lactis*	692	100	100	0.983
A7	*Staphtlococcus haemolyticus*	755	100	100	0.976
A9	*Staphylococcus chromogenes*	719	100	100	0.994
A10	*Leuconostoc lactis*	734	100	100	0.982
A29	*Staphylococcus aureus*	763	100	100	0.985
A31	*Pantoea agglomerans*	757	100	100	0.953
A33	*Pantoea agglomerans*	761	100	100	0.954
A34	*Pantoea agglomerans*	748	100	99	0.978
A38	*Staphylococcus haemolyticus*	759	100	100	0.976
A40	*Leuconostoc lactis*	826	100	100	0.983
A43	*Microbacterium phylosphereae*	759	100	100	1.000
A45	*Staphylococcus haemolyticus*	762	100	100	0.967
A50	*Leuconostoc lactis*	746	100	100	0.982
A52	*Lactococcus lactis*	781	100	100	0.994
A54	*Staphylococcus haemolyticus*	764	100	100	0.976
A55	*Lactococcus lactis*	808	100	100	1.000
A57	*Lactobacillus paracasei*	801	100	100	1.000
A61	*Lactococcus lactis*	770	100	100	1.000
A62	*Lactococcus lactis*	809	100	100	1.000
A63	*Lactococcus lactis*	728	100	100	1.000
A70	*Curtobacterium flaccumfaciens*	714	100	100	1.000
A76	*Staphylococcus saprophyticus*	781	100	100	1.000
A79	*Micrococcus yunnanenensis*	751	99	100	0.995
B4	*Leuconostoc lactis*	796	100	100	0.983
B14	*Staphylococcus devriesei*	813	100	100	0.992
B26	*Bacillus licheniformis*	806	100	100	1.000
B31	*Pseudomonas moraviensis*	759	100	100	0.989
B33	*Pseudomonas moraviensis*	764	100	100	0.980
B34	*Pseudomonas rhizosphaerae*	753	100	99	0.978
B41	*Curtobacterium flaccumfaciens*	798	100	100	1.000
B51	*Kocuria varians*	674	99	100	0.926
B54	*Lactococcus lactis*	816	100	100	1.000
B58	*Lactococcus lactis*	816	100	100	1.000
B61	*Rathayibacter festucae*	575	100	99	0.958
B64	*Staphylococcus haemolyticus*	798	100	100	0.975
B65	*Plantibacter flavus*	716	100	100	0.986
B72	*Staphylococcus haemolyticus*	807	100	100	0.976
B82	*Bacillus licheniformis*	806	100	100	1.000

B88	No Data				
B95	*Arthrobacter citreus*	851	100	99	0.975
B96	*Kocuria varians*	767	100	99	0.987
C1	*Enterobacter aerogenes*	479	100	100	0.994
C8	*leuconostoc lactis*	770	100	100	0.990
c9	*Staphylococcus epidermis*	780	100	100	0.993
c11	*Staphylococcus chromogenes*	753	100	100	0.997
C13	*Staphylococcus epidermis*	717	100	100	0.997
c28	*bacillus licheniformis*	703	100	100	1.000
c38	*Staphylococcus auricularis*	796	99	100	0.986
C41	*Enterobacter aerogenes*	693	100	100	0.955
C49	*Pseudomonas psychophila*	814	100	100	0.986
C50	*Staphylococcus haemolyticus*	796	100	100	0.969
C53	*Streptococcus parauberis*	756	100	100	0.950
C56	*Arthrobacter agilis*	828	100	100	0.996
C58	No Data				
C60	*Staphylococcus haemolyticus*	842	100	100	0.970
C61	*Streptococcus uberis*	663	100	99	0.822
C65	*Kocuria rhizophila*	847	100	100	0.989
C67	*Kocuria rhizophila*	800	100	100	0.989
C71	*Streptococcus uberis*	794	100	100	0.992
C77	*Arthrobacter gandavensis*	826	100	100	0.990
C82	*Arthrobacter citreus*	833	100	100	0.994